

# MicroRNA-21: From Cancer to Cardiovascular Disease

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**Abstract:** MicroRNA-21 (miR-21) expression is activated in multiple types of cancers, such as breast, liver, brain, prostate, myometrial cancers but also in cardiovascular disease. MiR-21 regulates a plethora of target proteins which are involved in cellular survival, apoptosis and cell invasiveness. MiR-21 regulation is complex due to an own promoter that is target for various transcription factors and hormones. The consistent miR-21 overexpression under pathophysiological conditions points to miR-21 as a valuable tool for new therapeutic strategies. In this review, we present and analyze current data about miR-21 expression in various pathologies ranging from cancer to cardiovascular disease. Further, miR-21 regulatory mechanisms and miR-21 downstream targets are discussed. Finally, we highlight the particular role of miR-21 as a therapeutic target in various diseases.

**Keywords:** micro-RNAs, miR-21, cancer, cardiovascular disease.

## miR-21 AND ITS ROLE IN THE MAMMALIAN PHYSIOLOGY

MicroRNAs (miRs) are small non-coding RNA molecules that occur naturally and downregulate protein expression by translational blockade of the target mRNA or by promoting mRNA decay. MicroRNAs are highly conserved among species and account approximately 1-5% of genes of worm, plant and vertebrate genomes. It is assumed that up to 50% of all genes are regulated by microRNAs [1, 2]. Molecular analyses of microRNAs showed that these small RNA molecules play a pivotal role in virtually all cellular functions, including apoptosis, cellular proliferation, migration and differentiation [3-7]. Since discovery of the first microRNAs, 6396 microRNAs have been identified so far (<http://microrna.sanger.ac.uk/>, release 11.0) including 678 in the human genome. For detailed information about general microRNA regulation see this issue [8].

The first evidence of miR-mediated protein regulation was presented in 1993 by Ambros and Rokun [9, 10] where the authors showed that small size RNA (22 nucleotides) coded by the *lin-4* gene negatively regulated the levels of Lin-14 protein during *C. elegans* development. This small RNA binds to 3'-UTR of *lin-14* messenger RNA and downregulate the protein expression at a posttranscriptional level without influencing *lin-14* gene transcription or RNA stability.

MicroRNA are coded as up to several thousand bases long pri-microRNAs in the introns of pre-mRNA or non-coding regions of the genome [11]. In the nucleus, pri-microRNAs are cleaved by RNase III endonuclease Drosha to 60 to 70 nucleotide stem-loop intermediates termed pre-microRNAs and are actively transported to the cytoplasm by Ran-GTP and Exportin-5 [12]. In the cytoplasm, pre-microRNAs are cleaved by another RNase III, Dicer, to 20-22 nt double-stranded fragment which is incorporated into

RNA-induced silencing complex (RISC) [13-15]. As part of the RISC complex, microRNAs fulfil their task as negative regulators of target genes. MicroRNA-dependent regulation of gene expression relies in part on miR-mRNA complementarity: in the case of perfect match, the messenger RNA is degraded whereas imperfect match leads to translational inhibition without influencing mRNA integrity [16, 17]. The residues 2 to 8 at the 5' region of microRNA play a pivotal role for the specificity of microRNA interaction with messenger RNA. This region is called "seed" and binds to the 3' untranslated region (3' UTR) of target mRNAs. MicroRNAs may have hundreds of putative mRNA targets which depend on the sequence complementarity; however their function depends on the target availability within a cell. The expression patterns of microRNAs vary from ubiquitous up to tissue- or cell-specific. For example, miR-1 and miR-133a are highly expressed in the heart and skeletal muscle whereas miR-122 and miR-7 are liver- and pituitary-specific, respectively [18]. Detailed information about expression patterns in various tissues and cell types have recently been summarized [19, 20]. MicroRNA expression is a dynamical process and certain miRs get highly changed during development and disease. Thus microRNAs which are deregulated during disease may be of particular interest. Several genome wide microRNA profiling studies have been performed on various cancer types, such as breast cancer, hepatocellular carcinoma, lung cancer colon cancer and others [21] (see Table 1). MicroRNA analysis showed abnormal expression pattern of several microRNAs in cancer cells compared to normal controls including miR-21, miR-17-5p and miR-191. MiR-17-5p and miR-191, which belong to different microRNA clusters, were upregulated in five cancer types, whereas miR-21 was overexpressed in six cancer types [22, 23]. Experimental data show that miR-21 functions in many cell types as an anti-apoptotic and pro-survival factor [24, 25]. Multiple studies have identified that miR-21 plays a significant role in cancer biology and diagnostics. Next to cancer, miR-21 appears to be strongly deregulated in cardiovascular disease [26, 27]. The role of miR-21 in cancer biology and in the development of cardiac disease will be discussed in the following sections.

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**Table 1. MiR-21 Expression in Various Cancer Types**

Cancer type	References
Breast cancer	[35, 49, 86, 104, 105]
Cervical, myometrial and ovarian cancers	[40, 41, 54, 55]
Colorectal cancer	[58-60, 106]
Hepatocellular cancer	[43, 63, 64, 85]
Glioma, glioblastoma	[66, 90, 107]
Esophageal cancer	[44]
Diffuse large B-cell lymphoma	[5, 45]
Prostate cancer	[23, 34, 108, 109]
Lung cancer	[46, 77, 110]
Gastric cancer	[93]
Pancreatic cancer	[23, 96, 97]
Laryngeal cancer	[111]
Cholangiocarcinoma	[80, 91]

## REGULATION OF miR-21 GENE EXPRESSION

Human miR-21 (hsa-miR-21) was cloned from HeLa cell total RNA and is highly conserved among species including human, rat, mouse, fish and frog [28]. The miR-21 gene is located on the chromosome 17, in the 10<sup>th</sup> intron of a protein-coding gene, TMEM49. The function of TMEM49 is currently unknown. Fujita and colleagues performed extensive miR-21 gene expression analysis and showed that miR-21 expression is driven by its own promoter and not by the promoter of the overlapping TMEM49 gene [29]. The authors demonstrated that miR-21 transcription was activated by activation protein 1 (AP-1) in conjugation with the SWI/SNF complex following phorbol 12-myristate 13-acetate (PMA) in human promyelocytic cell line, HL-10, whereas TMEM49 expression remained unchanged. The putative miR-21 promoter region additionally contains three AP-1 and one PU.1 binding site. Computation analysis predicted transcription repressor NFIB mRNA as a target for miR-21 and the miR-21 promoter itself contains a conserved binding site for the NFIB protein. Experimental data showed that NFIB protein expression was decreased in association with exogenous miR-21 overexpression whereas inhibition of miR-21 resulted in elevated NFIB protein levels [29]. These findings suggest conserved feedback regulation of miR-21 gene expression involving the miR-21 promoter, the miR-21 molecule and its target, NFIB. Further details about regulation of miR-21 expression was described by Wickramasinghe *et al.* who showed that female sex hormone estrogen (E2) efficiently downregulated miR-21 expression and thus increased expression of miR-21 target genes such as PDCD4, PTEN and Bcl2 in breast cancer cell line MCF7 [30]. Estrogen acts *via* its cognate receptors, estrogen receptor alpha and estrogen receptor beta (ER $\alpha$  and ER $\beta$ , respectively), which upon estrogen binding act as transcription factors. Liganded ERs bind to estrogen response elements (EREs) in the target promoter and regulate target gene expression [31-33]. ER $\alpha$  but not ER $\beta$  was involved in E2-

mediated miR-21 downregulation in MCF7 cells. Moreover, a non-consensus estrogen-response element (ERE) was identified in the promoter of miR-21 which was located 883 bp upstream of the TATA-binding site. Since miR-21 targets PTEN, PDCD4 and Bcl2 lack EREs, it is likely that E2-ER $\alpha$  acts by tethering transcription factors IKZF1 and PAX which are also located in the miR-21 promoter. Notably, the E2-ER $\alpha$  complex downregulated miR-21 expression but did not alter TMEM49 gene expression [29]. Another steroid hormone receptor, androgen receptor (AR), also directly interacted with miR-21 promoter and upregulated miR-21 expression in AR-positive prostate cancer cell lines C4-2 and CWR22Rv1 [34]. Although the molecular mechanisms of miR-21 expression regulation by ovarian steroids still need to be determined, this data provide an interesting link between steroid hormones and miR-21 expression control.

Bone morphogenic protein-6 (BMP-6) was shown to be a negative regulator of miR-21 promoter activity in breast cancer cell line MDA-MB-231. Transcription factor  $\delta$ EF1 activated the miR-21 promoter which was inhibited by BMP-6 in a dose-dependent manner [35]. BMP-6 did not interact directly with the miR-21 promoter but rather repressed promoter activity indirectly by downregulating protein expression of  $\delta$ EF1, c-Fos/c-Jun and TPA that bind to AP-1 binding sites on the miR-21 promoter.

The miR-21 promoter also contains two highly conserved binding sites for Signal transducer and activator of transcription 3 (Stat3) that were identified using computational approach and confirmed by chromatin immunoprecipitation and miR-21 expression changes following Stat3 modulation [36]. The transcription repressor Growth factor independent-1 (Gfi1) is a master regulator of miR expression and is a critical regulator of normal granulocytic differentiation. Gfi1 was shown to bind directly to the miR-21 promoter and downregulate miR-21 expression in bone marrow cells. Deregulation of miR-21 and miR-196b expression impaired primary myeloid cell differentiation to granulocyte-monocyte progenitor cells [37]. MiR expression profile and simultaneous transcription factor expression analysis in differentiated monocytes revealed several transcription factors, such as YY1, NFE2L2, ATF2, NFE2L1 and Sp1 which contain putative binding sites on miR-21 promoter [38]. This study was performed *in silico* and the data need to be confirmed experimentally.

Taken together, the current data demonstrate highly complex regulatory mechanisms of miR-21 expression which involve the unique miR-21 promoter, multiple transcription factors (see Table 2), hormones and signalling events that activate miR-21 transcription machinery. Further analysis of miR-21 expression regulation is needed to identify gene regulation networks and new molecular mechanisms involved in miR-21 regulation.

## EXPRESSION OF miR-21

MiR-21 is aberrantly expressed in multiple types of cancer including advanced human breast cancer [39], cervical and ovarian cancer [40, 41], colon carcinoma [42], hepatocellular cancer [43], gliomas [24], esophageal cancer [44] and B-cell lymphoma [45], prostate cancer [34], lung cancer [46] and other cancer types. More detailed

information about miR-21 expression in different cancers is presented in the following sections (see also Table 1).

**Table 2. Factors Involved in miR-21 Gene Regulation**

Factor	Interaction with miR-21 promoter	References
NFIB, AP-1, PU.1	direct interaction demonstrated in human promyelocytic cell line, HL-10	[29]
ER $\alpha$	direct interaction demonstrated in breast cancer cell line MCF7	[30]
AR	direct interaction demonstrated in AR- positive prostate cancer cell lines C4-2 and CWR22Rv1	[34]
BMP6	indirect interaction, miR-21 promoter regulation via transcription factors $\delta$ EF1, c-Fos/c-Jun and shown in TPA in breast cancer cell line MDA-MB-231	[35]
STAT3	direct interaction, two highly conserved STAT3 binding sites on miR-21 promoter in myeloma cells	[36]
Gfi1	direct interaction with miR-21 promoter in bone marrow cells	[37]
YY1, NFE2L2, ATF2, SP1	putative binding site on miR-21 promoter in differentiated monocytes; <i>in silico</i> analysis, no experimental evidence	[38]

## miR-21 AND CANCER

### The Role of miR-21 in Breast Cancer

Breast carcinoma is the second frequent cancer in women and affects 1.2 million women worldwide each year [47]. Breast carcinoma is often fatal, if not detected in the early stages. There are several major mammary carcinoma subtypes depending on gene/ protein expression status: basal-like, human epidermal growth factor receptor-2 positive (HER2-/ER+) /estrogen receptor negative (HER2-/ER-) and luminal-like breast cancers [48]. They differ in their patterns of mRNA gene expression, phenotypes, prognosis, and sensitivity to different treatments. One of the first results showing miR deregulation in breast cancer biopsies was published by Iorio *et al.* where they showed that miR-21 was one of the most consistently upregulated miRs in breast cancer and was progressively upregulated from normal breast to cancer with increasing tumor stage [49]. Investigations of miR expression profiles in normal tissue and in breast tumor specimens using miR array technology revealed that miR-21 was upregulated in virtually all cancer biopsies compared to normal tissue. Tissue localization studies showed that miR-21 was mainly localized in the cytosol of luminal epithelial cells and in fibroblasts of normal breast tissue. In tumor tissue, miR-21 was highly upregulated in carcinoma cells of epithelial origin compared to matching normal tissue. In some cases, increase of miR-21 expression was observed in tumor-associated fibroblasts but not in

carcinoma cells. Additionally, miR expression changes were investigated in the epithelial cell lines undergoing malignant transformation (MCF-7, BT-474, MDA-MB-231 and others). Again, miR-21 expression was elevated in tumorigenic cell lines (compared to non-tumorigenic ones, IMECs and MCF10A) [50]. *In vitro* experiments showed that miR-21 knockdown in the breast tumor cell line MCF-7 using sequence-specific and chemically modified oligonucleotide termed anti-miR-21 led to the reduction of cell growth in a dose dependent manner. To investigate the role of miR-21 in tumor formation *in vivo*, anti-miR-21 transfected MCF-7 cells were injected into mammary pads of female nude mice. Injected cancer cells where miR-21 expression was inhibited grew substantially slower compared to controls. Reduced tumor growth is likely due to lower proliferation or increased apoptosis caused by selective miR-21 downregulation. As already discussed in previous section, BMP-6 could efficiently suppress miR-21 expression in breast cancer cell line MDA-MB-231. Moreover, BMP-6 treatment reversed PDCD4 suppression and inhibited miR-21-mediated MDA-MB-231 cell invasion under *in vitro* conditions [35].

### miR-21 in Ovarian, Myometrial and Cervical Cancers

Ovarian cancer is a frequent cancer type in females. Due to its aggressive nature, it causes approximately 125.000 deaths yearly worldwide. Poor prognosis is mainly due to late-stage diagnosis, tumor resistance to chemotherapy and unclear pathology at early stages [51]. There are various histological subtypes of ovarian cancer, such as serous, mucinous and endometrioid cases which differ in their morphology and molecular genetic characteristics [52, 53]. New cancer diagnostic tools including expression profiling technologies, discovery of new biomarkers and application of new therapies and drugs have improved diagnosis and therapy outcome.

MicroRNA array analysis was applied to evaluate miR expression profiles in heterogeneous ovarian cancer types compared to the normal ovarian samples. The results showed differential miR expression when compared to healthy tissue but also among the various different histological subtypes. For instance, miR-200a and miR-200c were upregulated in serous, endometrioid and clear cell carcinomas, whereas miR-200b and miR-141 were overexpressed in the endometrioid histotype compared to normal tissue. The latter histotype showed also upregulation of additional miRs, including miR-21, miR-203 and miR-205 [40]. Other potentially confounding factors, such as patient age, grade or stage of the disease did not significantly influence miR expression profile in the serous histotype. Another independent study revealed that miR-21 was the most frequently upregulated microRNA in the serous ovarian carcinoma biopsies compared to the normal control ovarian tissue. This data further suggest miR-21 relevance to ovarian cancer [54]. MiR-21 was also overexpressed in leiomyomas compared to normal myometrium as assessed using microRNA arrays and confirmed by real-time PCR [55].

Human cervical cancer is frequently caused by human papilloma virus (HPV) [56]. MiR-21 expression was increased in cervix cancer cell lines compared to the normal cervical tissue. Indeed, Northern blot analysis of miR-21 in cervical cancer biopsies and matched control samples

demonstrated significantly increased expression in 21 out of 29 tumor samples [41].

### miR-21 in Colon Cancer

There are increasing numbers of colon cancer cases in the Western countries associated with nutrition factors such as fat-rich and fiber-poor diet as well as sedentary life style [57]. The absence of clear symptoms during the initial cancer stages and high mortality rates at the late stages are the characteristic feature of colon carcinoma. There is still a need to identify reliable and easy detectable disease markers as well as to develop new therapeutic strategies and suitable therapeutic targets. Cellular and tissue localization pattern using *in-situ* hybridization technique (IHC) and microRNA-21-specific locked-nucleic acid (LNA) probe showed that miR-21 was highly expressed in colorectal epithelial cells and fibroblasts in colorectal adenocarcinoma [58]. Notably, miR-21 expression levels inversely correlated with protein expression levels of PDCD4 in cancer tissue and cell lines suggesting that miR-21 is negative regulator of PDCD4 *in vivo* and *in vitro* [58, 59].

MiR expression profiling in colon cancer has been performed in two independent colon adenocarcinoma patient cohorts. Five microRNAs (miR-20a, miR-21, miR-106a, miR-181b and miR-203) were overexpressed in colon adenocarcinoma samples [42]. Further analysis showed higher miR-21 expression levels in advanced cancer stages and high expression levels were associated with worse outcome, poor response to therapy and more rapid disease recurrence. Increased miR-21 expression positively correlated with the cancer stage, lymph node positivity and development of distant metastases [60]. Indeed, end-stage colon adenocarcinoma patients, with high tumor-specific miR-21 expression levels were associated with poor response to anticancer drug 5-fluorouracil (5-FU) therapy and more rapid disease recurrence [42]. Surprisingly, a remarkable number of microRNAs that are already overexpressed in neoplastic tissues including miR-21 were upregulated in the colon cancer cell lines C22.20 and HC.21 treated with 5-FU [61]. This unexpected effect may be due to cell-specific defense mechanisms to survive 5-FU treatment. These cell defense mechanisms involved in neoplastic tissue resistance to 5-FU needs to be better understood.

### miR-21 in Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC), is a common cause of mortality from cancer worldwide [62]. Major risk factors for the development for HCC include liver cirrhosis as well as chronic liver diseases due to chronic hepatitis B and C infections, metabolic syndromes, chronic alcoholism, hereditary hemochromatosis and many others. Meng *et al.* analysed microRNA expression profile in HCC tumor tissue *versus* normal liver and showed a greater than 5-fold increased miR-21 expression in the tumor compared to normal tissue samples [63]. MicroRNA profiling studies with human HCC and healthy liver biopsies showed numerous other miRNAs to be upregulated especially in patients with cirrhosis and concomitant hepatitis infections whereas neither cirrhosis nor hepatitis alone were sufficient to induce significant changes in miR expression. MiR-21 was significantly upregulated in HCC specimens from patients infected with

hepatitis B but not hepatitis C compared to adjacent benign tissue [43]. These findings were confirmed by Connolly *et al.* who showed that miR-21 was among the most highly overexpressed miRs in hepatitis B positive cirrhotic liver and HCC biopsies compared to the healthy liver tissue [64]. Genetic manipulation to repress miR-21 in HCC cell line HepH2 resulted in a reduction of cell proliferation, retardation of cell cycle progression, increased cellular apoptosis and reduction in cell colony size. MicroRNA expression profiles were also assessed in the hepatocellular carcinoma model in rats. Male rats fed with folate and methyl-deficient (FMD)-diets developed preneoplastic nodules and hepatocellular carcinomas after 36 and 54 weeks, respectively. Microarray data and subsequent validation by Northern blot analysis demonstrated low basal miR-21 expression levels in the healthy liver, its upregulation in preneoplastic nodules and the highest expression levels in carcinomas [65].

### miR-21 Expression in Brain Cancer

Significant up-regulation of miR-21 was observed in glioblastoma multiforme, the most prevalent and aggressive type of primary brain tumors, compared with its expression in non-neoplastic tissue samples. Sequence-specific knock-down of endogenous miR-21 in human glioblastoma cell lines U87, A172, LN229 and LN308 resulted in marked increase in apoptosis and enzymatic activity of caspase-3 and caspase-7 [24]. For *in vivo* experiments, miR-21 was selectively inhibited in U87 cells using miR-21-specific locked-nucleic acid (LNA)-antimiR-21 or control LNA and implanted intracranially into athymic nude mice. Significant reduction in glioma tumor size was observed in animals which received (LNA)-anti-miR-21 transfected cancer cells compared to the cells transfected with control oligonucleotides. Intracranial implantation of U87 cells that express cytotoxic agent tumor necrosis factor-related apoptosis inducing ligand S-TRAIL in combination with miR-21 knockdown resulted in even superior tumor size reduction compared to S-TRAIL alone [66]. This data demonstrate that combination of miR-21 knockdown with S-TRAIL overexpression seems to be an interesting approach in the treatment of certain brain cancers.

### miR-21 IN THE CARDIOVASCULAR SYSTEM

MiR-21 is expressed not only in cancerous tissues but also in the cardiovascular system. In contrast to data from the cancer field where miR-21 was described to play a main role in cancer progression, its function in the cardiovascular system appears to be more complex. Initial reports about the role of miR-21 in the cardiovascular system, showed miR-21 to be upregulated in murine hearts subjected to cardiac stress, such as thoracic aortic banding leading to left ventricular pressure overload or to phenylephrine (PE)-treatment [26, 27]. Different data about the role of miR-21 in cardiomyocyte size regulation and/or hypertrophy exist; Cheng *et al.* showed that inhibition of miR-21 in PE- and angiotensin II (Ang-II) stimulated cardiomyocytes resulted in significant decrease in cardiomyocyte cell size, whereas Tatsuguchi *et al.* demonstrated that inhibition of miR-21 using antisense probe resulted in increased hypertrophy in neonatal cardiomyocytes and miR-21 overexpression slightly

decreased cardiomyocyte size [26]. Thum *et al.* did not find direct effects of miR-21 on the regulation of cell size in cardiomyocytes *in vitro* or *in vivo* using a cardiomyocyte-specific miR-21 transgene [67]. Cardiac stress *in vitro* by H<sub>2</sub>O<sub>2</sub> treatment resulted in upregulation of endogenous miR-21 in time and concentration dependent manner in neonatal cardiomyocytes [68]. A further role for miR-21 as regulator of cardiomyocyte branching and outgrowths was described by Sayed *et al.* who showed that overexpression of miR-21 in neonatal cardiomyocytes did not influence hypertrophic growth in the presence of growth factors but rather increased cellular branching indicating that miR-21 upregulation in hypertrophied hearts could participate in the remodelling of gap junctions by regulation of cell-cell connectivity [69]. Further independent studies showed miR-21 upregulation in failing murine and human myocardium where miR-21 expression was upregulated especially in cardiac fibroblasts and a further increase in miR-21 expression was observed after cardiac stress, such as myocardial infarction and/or heart failure [67, 70]. Concomitantly, miR-21 expression levels positively correlated with development of cardiac fibrosis which was efficiently reversed upon miR-21 knockdown *in vivo* using miR-21 inhibitors [67]. Increased miR-21 expression was also demonstrated in the infarct region of 7-days ischemia-reperfused mouse hearts. In this study, an elegant approach using laser-capture microdissection technique was applied to obtain tissue samples from the area of interest. This study demonstrated strongest miR-21 signal in cardiac fibroblasts of the infarcted area of the heart and low expression in cardiomyocytes [70]. Differential spatiotemporal distribution of miR-21 was observed in the hearts subjected to acute ischemia/reperfusion: miR-21 was highly upregulated in the border zone and downregulated in infarcted area at six hours after ischemia/reperfusion. Further *in vivo* experiments demonstrated that myocardial infarct size was significantly reduced in rat hearts overexpressing miR-21. *In vitro* and *in vivo* experiments showed that miR-21 had anti-apoptotic effects in cardiomyocytes subjected to experimental hypoxia/reoxygenation and acute myocardial infarction whereas miR-21 inhibition increased apoptosis rates. Time-, cell- and disease-specific differences should be taken into account in the future development of miR-21-based therapeutic approaches in cardiovascular disease (see also further reviews in this issue) [71-73].

Data about miR-21 effects and functions in other cardiac cell types, such as endothelial cells and smooth muscle cells are rather sparse. Selective knock-down of miR-21 reduced cell proliferation and increased apoptosis in cultured vascular smooth muscle cells (VSMCs). For further data about miR-21 and other microRNAs in endothelial and smooth muscle cells see this issue [74, 75]. *In vivo*, the miR-21 inhibitor 2'-OMe-miR-21 also decreased cell proliferation and increased apoptosis in injured cell walls following carotid artery injury in rats. These findings strongly suggest miR-21 as a putative target for treatment of proliferative vascular diseases such as atherosclerosis and stroke. Increase of miR-21 expression was observed in hydrogen peroxide-treated vascular smooth muscle cells. MiR-21 depletion and overexpression *in vitro* experiments indicated that miR-21 mediated protective effects on cellular injury, apoptosis and cell death

[30]. Taken together, current data demonstrate that regulation of miR-21 expression and function in the cardiovascular system is very complex and requires further investigations.

## MiR-21 TARGETS

MicroRNAs bind to 3'-UTR region of the target messenger RNA (mRNA) and downregulate protein expression by translational repression or by mRNA degradation mechanisms [76]. Bioinformatic tools in combination with molecular biology approaches helped to identify a plethora of the proteins whose expression is regulated by miR-21. In the following section we summarized some important miR-21 targets and the respective regulatory mechanisms. As discussed above, multiple studies showed that miR-21 promotes cell proliferation, migration and invasion, prevents apoptosis in diverse cancers and cancer cell lines and in the cardiovascular system [49, 61, 77]. The role of miR-21 target in cell cycle regulation and their aberrant expression in various cancers suggest their role as a novel class of oncogenes or tumor suppressor genes depending on the targets they regulate. The information about miR-21 targets which are described in the literature are listed in Table 3. Selected miR-21 targets are described in more detail below.

### Phosphatase and Tensin Homolog (PTEN)

Phosphatase and tensin homolog (PTEN) is a phosphatidylinositol-3,4,5-trisphosphate 3 (PIP3)-phosphatase (PTEN) that inhibits phosphoinositide-3-kinase (PI3K) pathway while dephosphorylating (PIP3) and thus prevents Akt activation [78, 79]. The PI3K pathway is constitutively active in many cancers types and regulates cell survival. MiR-21-mediated regulation of PTEN expression has been shown in several cancers and cell types including cholangiocytes [80], vascular smooth muscle cells [81], hepatocellular carcinoma [63], and breast cancer [82]. For instance, demonstration of PTEN as a *bona fide* miR-21 target was shown in cholangiocarcinoma cell line Mz-ChA-1. Functional analysis showed that miR-21 overexpression in cholangiocarcinoma cells resulted in increased cell survival and resistance to chemotherapy agent gemcitabine-induced apoptosis [80]. Similar observations were reported in hepatocellular carcinoma where miR-21 was highly expressed and, concomitantly, expression of PTEN was decreased compared to normal hepatocytes [63]. MiR-21-mediated downregulation of PTEN led to increased phosphorylation of the focal adhesion kinase (FAK), and Akt that are further key mediators of tumor cell survival and invasion. PTEN suppressed expression of several matrix metalloproteinases (MMPs), such as MMP2 and MMP9, *via* FAK dephosphorylation [83, 84]. In line with these observations, MMP2 and MMP9 expression was significantly higher in the malignant cells compared to normal hepatocytes and miR-21 transfection of normal hepatocytes with miR-21 precursors increased MMP9 mRNA expression. It remains to be determined whether this mechanism is also active in other cell types. This data suggest that miR-21 acts as a negative PTEN regulator leading to increased cell migration and metastasis formation. MiR-21-dependent regulation of PTEN was also observed in dedifferentiated VSMC compared to differentiated VSMCs [81].

Table 3. MiR-21 Targets

miR-21 target	Validated by	Function	References
PTEN	Luciferase reporter assay, real-time PCR, western blot, immunocytochemistry; cell proliferation, migration and invasion assays	Tumor suppressor, decreases tumor cell proliferation, migration, and invasion.	[63, 78, 112]
PDCD4	Computational analysis, luciferase reporter assay, western blot; cell invasion, intravasation and distal metastasis formation	Tumor suppressor, reduces cancer cell invasion, reduce intra-vasation and lung metastasis in chicken embryo metastasis assay.	[113] [59, 86, 108, 114-116]
Tropomyosin	Two- dimensional protein electrophoresis of breast tumor samples, luciferase reporter assay	Actin- binding protein, suppresses anchorage-independent cell growth	[117]
Sprouty 1	Western blot following miR-21 modulation, luciferase reporter gene assay, protein modulation in the heart following miR-21 silencing <i>in vivo</i>	Negative regulator of ERK-MAP kinase activity	[67]
Sprouty 2	luciferase reporter assay, immunocytochemistry following miR-21 expression modulation, migration assay	Inhibitor of branching morphogenesis and neurite outgrowths	[69]
RECK	Computational analysis, western blot, cell migration and invasion assays, clinical studies	Tumor suppressor, negative regulator of MMP9	[90, 93]
Bcl2	Luciferase reporter assay, western blot	Apoptosis inhibitor	[30]
MARCKS	Reported RISC-coimmunoprecipitation, western blot, cell motility, apoptosis and proliferation assays	PKC substrate, an actin filament crosslinking protein	[109]
HNRPK	Computational analysis, real- time PCR, luciferase reporter gene assay	One of the major pre-mRNA-binding proteins, regulation of cell cycle	[118]
IL-12p35	Computational analysis, luciferase reporter gene expression	Is involved in allergic airway inflammation	[110]
JAG1	Computational analysis, luciferase reporter gene assay, cell differentiation assay	Ligand for the receptor Notch 1, mediates Notch signalling, is involved in cardiovascular development, enhances FGF- induced angiogenesis	[119]
BTG2	Computational analysis, RT-PCR in laryngeal carcinoma samples, EGFP reporter gene assay, cell proliferation assay	Antiproliferative protein	[111]
LRRFIP1	EGFP reporter gene assay, RT-PCR, western blot	Inhibitor of NF- $\kappa$ B signalling	[120]
BMPRII	Computational analysis, luciferase reporter gene assay	Receptor for BMP2 and BMP7 and less to BMP4, mutation in BMPRII causes primary pulmonary hypertension	[121]
TGFBR2	Real-time PCR, western blot, luciferase reporter gene assay	Ser/Thr protein kinase, a member of TGF $\beta$ receptor subfamily	[122]
Cdc25A	MicroRNA analysis, computational analysis, RT-PCR, western blot, cell proliferation assay, colony formation assay	Tyrosine protein phosphatase, oncogene	[106, 123]
TAp63	Luciferase reporter assay, real-time PCR, apoptosis and cell growth assays, cycle analysis	A homolog of p53, mediates cell arrest and apoptosis	[118]

### Programmed Cell Death 4 (PDCD4) Protein and Tropomyosin1(TPM1)

Programmed cell death 4 protein (PDCD4) is probably one of the most analyzed miR-21 targets so far. PDCD4 is a tumor and metastasis suppressor which is downregulated in multiple cancer forms and upregulated during apoptosis [59, 77, 85]. PDCD4 was identified as direct miR-21 target in multiple cancer forms. Studies showed significant increase of endogenous PDCD4 protein expression following miR-21 inhibition *in vitro* as well as direct miR-21 binding to 3' region of PDCD4 mRNA [86]. MiR-21 suppression in

metastatic breast cancer cell line MDA-MB-231 resulted in downregulation of PDCD4 and other tumor suppressor, tropomyosin 1 (TPM1). In metastasis assay, miR-21 inhibition reduced cell invasiveness and metastatic growth in PDCD4- and TPM1- dependent manner [87]. Downregulation of tropomyosin 1 (TPM1) expression was detected in breast cancer cell lines as well as in human breast carcinoma tissue samples [88, 89]. TPM1 was shown to induce anoikis (detachment induced apoptosis) whereas downregulation of TPM1 in tumors may destabilize microfilament architecture, promote resistance to anoikis, cancer cell survival outside the normal microenvironment and malignant growth.

### Tissue Inhibitor of Matrix Metalloproteinases (TIMP)

The negative regulators of matrix metalloproteinases (MMPs), RECK and TIMP3, are additional targets for miR-21 [90-93]. Functional analysis demonstrated that RECK regulates metalloproteinase-9, a key enzyme involved in tumor invasion and metastasis whereas TIMP3 regulated multiple MMPs such as MMP-1, MMP-2, MMP-3, MMP-9 and others [90, 94]. TIMP3 and RECK downregulation by miR-21 was demonstrated in several cancer forms, such as gastric cancer [93], gliomas [90] and others. MiR-21 inhibition led to elevated levels of TIMP3 and RECK protein and, concomitantly, reduced migratory capability and invasiveness of glioma cells [90]. MiR-21 regulated MMP and TIMP expression in cardiovascular disease remains to be investigated.

### Sprouty Proteins

Sprouty-1 (Spry1) and sprouty-2 (Spry2) are members of a highly conserved protein family which negatively regulate receptor tyrosine kinase (RTK) signalling by suppression of growth factor-mediated mitogen-activated protein kinase (MAPK) activation. There are four mammalian homologues (Sprouty 1-4) whose expression pattern and RTK signalling modulation depends on growth factor environment and cellular context. Sprouty-1 expression is enriched in cardiac fibroblasts but not in cardiomyocytes [67] whereas Spry2 is more abundantly expressed in cardiomyocyte, endothelial and smooth muscle cells [69, 95]. Transplantation of Spry1 deficient fibroblasts into left ventricular myocardium increased cell survival and Spry1 was described to have a functional role in the development of cardiac fibrosis whereas Spry2 regulates morphological changes in cultured neonatal cardiomyocytes [67, 69].

### miR-21 AS TARGET FOR THERAPEUTIC APPROACHES

There is ample experimental evidence that miR-21 is an interesting therapeutic target in cancer and cardiovascular disease. Modulation of miR-21 expression as potential cancer therapy was investigated in various cancer types, such as pancreatic cancer, lung cancer, glioblastoma and others [96-99]. Inhibition of miR-21 using specific antisense oligonucleotide alone resulted in increased apoptosis rates, reduced cell proliferation and upregulation of PTEN and RECK proteins in pancreatic adenocarcinoma cell line HS766T. Cell treatment with antisense- miR-21 in combination with an anti- cancer drug gemcitabine sensitized HS766T cells to gemcitabine and resulted in superior effects compared to gemcitabine alone [97]. Similarly, Moriyama *et al.* demonstrated that miR-21 overexpression in multiple pancreatic cancer cell lines increased cell invasiveness, proliferation and chemoresistance to gemcitabine whereas miR-21 downregulation in the same cells resulted in decreased cell proliferation, Matrigel invasiveness and increased sensitivity to gemcitabine [96]. The question whether gemcitabine alone can modulate miR-21 expression needs to be answered. Another anticarcinogen, indole-3-carbinol (I3C), efficiently downregulated endogenous miR-21 expression in vinyl carbamate (VC)- induced lung tumors in mice compared to the animals treated with VC alone.

Concomitantly, miR-21 target proteins PTEN, PDCD4 and RECK were upregulated upon I3C treatment in VC- induced lung tumors indicating a direct link between I3C- dependent miR-21 suppression and increased protein levels of miR-21 target proteins [98]. Further studies of the mechanisms involved in miR-21- dependent cell sensitization to chemotherapy revealed that miR-21 inhibitor efficiently sensitized human glioblastoma cells carrying PTEN-mutant as well as PTEN-wild type gene to taxol, a potent anticancer drug. In glioblastoma cells carrying mutant PTEN, miR-21 inhibitor in combination with taxol showed additive effects whereas miR-21 inhibitor and taxol acted synergistically in the cells carrying wild-type PTEN, indicating that other, PTEN-independent mechanisms, are affected by miR-21 downregulation [99].

A novel cancer therapy employing co-delivery of anti-cancer drug 5-fluorouracil (5-FU) with antisense-miR-21 oligonucleotide to human glioma cells U251 was described by Ren *et al.* [100]. In this study, authors applied the poly (amidoamine) PAMAM dendrimer to enhance the delivery of antisense-miR-21 and 5-FU to the cells. Delivery of miR-21 inhibitor significantly improved glioblastoma cell sensitivity to 5-FU, increased apoptosis rates and reduced migration of the tumor cells. This co-delivery approach might be of clinical importance in treatment of solid tumors over-expressing miR-21. Antisense-miR-21 was also successfully co-delivered with traditional Chinese medicine arsenic trioxide (ATO) into chronic myelogenous leukemia cells (cell line K562) using the poly (amidoamine) PAMAM dendrimer as carrier moiety [101]. Though both substances were able to induce cellular death and apoptosis, a combination of antisense-miR21 and arsenic trioxide was superior in growth inhibition and apoptosis and these effects were at least partially mediated by miR-21 target protein PDCD4.

A new strategy for silencing of microRNAs *in vivo* using chemically modified and cholesterol-conjugated RNAs termed 'antagomirs' was demonstrated by Krutzfeldt *et al.* [102, 103]. Recently, "antagomir"- based therapeutic approaches using antagomir against miR-21 (antagomir- 21), was applied for treatment of cardiac fibrosis in pressure- overload cardiac disease model in mice. In this model, antagomir application efficiently inhibited interstitial fibrosis and improved cardiac function by activating sprouty-1, a negative regulator of ERK-MAP kinase pathway [67].

Thus, miR-21 inhibition alone or in combination with conventional therapies results in positive therapeutic outcome in diverse pathologies such as cancer and cardiovascular disease suggesting miR-21 as promising therapeutic target in pathologies associated with abnormal miR-21 expression levels.

### CONCLUSIONS

Here we summarized current knowledge about miR-21, which is probably one of the most studied miRs to date. MiR-21 initially was described as an "oncomir" which is indeed overexpressed in multiple cancer forms and tightly associated with cancerogenesis. MiR-21 affects cell survival mechanisms by regulating multiple apoptotic proteins, negative regulators of metalloproteinases, cell cycle proteins

and others. Since miR-21 overexpression is strongly associated with tissue malignancy, abnormal miR-21 levels might serve as a marker of cancer pathologies and also as target for future anti-cancer therapies. In the cardiovascular system, miR-21 is upregulated under many pathophysiological conditions and contributes to the onset of cardiac fibrosis and dysfunction of circulating angiogenic cells [67,124]. Recent data demonstrated that inhibition of miR-21 is promising therapeutic onset for treatment of cardiac fibrosis. It strongly suggests that this strategy might be useful for treatment of other organs with abnormal fibrosis, such as the kidney or the liver.

MiR-21 is a small RNA molecule with great diagnostic and therapeutic potential. Further studies are required to understand miR-21 expression regulation, to detect gene networks which are regulated by this microRNA and to develop potent miR-21-based therapeutics.

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