The p53/microRNA Network in Cancer: Experimental and Bioinformatics Approaches

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Abstract/Summary

In the recent years, microRNAs (miRNAs) were identified as important components of the signaling cascades that mediate and regulate tumor suppression exerted by p53. This review illustrates some of the main principles that underlie the mechanisms by which miRNAs participate in p53's function and how they were identified. Furthermore, the current status of the research on the connection between p53 and miRNAs, as well as alterations in the p53/miRNA pathways found in cancer will be summarized and discussed. In addition, experimental and bioinformatics approaches, which can be applied to study the connection between p53 and miRNAs are described. Although, some of the central miRNA-encoding genes that mediate the effects of p53, such as the *miR-34* and *miR-200* families, have been identified, many additional analyses remain to be performed to fully elucidate the connections between p53 and miRNAs.

Keywords

p53 • microRNA • miRNA • Tumor suppression • SILAC • Next generation sequencing • Genome-wide analysis • miR-34 • miR-34a • miR-34b/c

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5.1 Introduction to p53 Biology

The p53 transcription factor is encoded by a tumor suppressor gene, which is presumably the most commonly mutated gene in human cancer [1]. In addition, many of the cancers without *p53* mutation may harbor alterations up- or down-stream of

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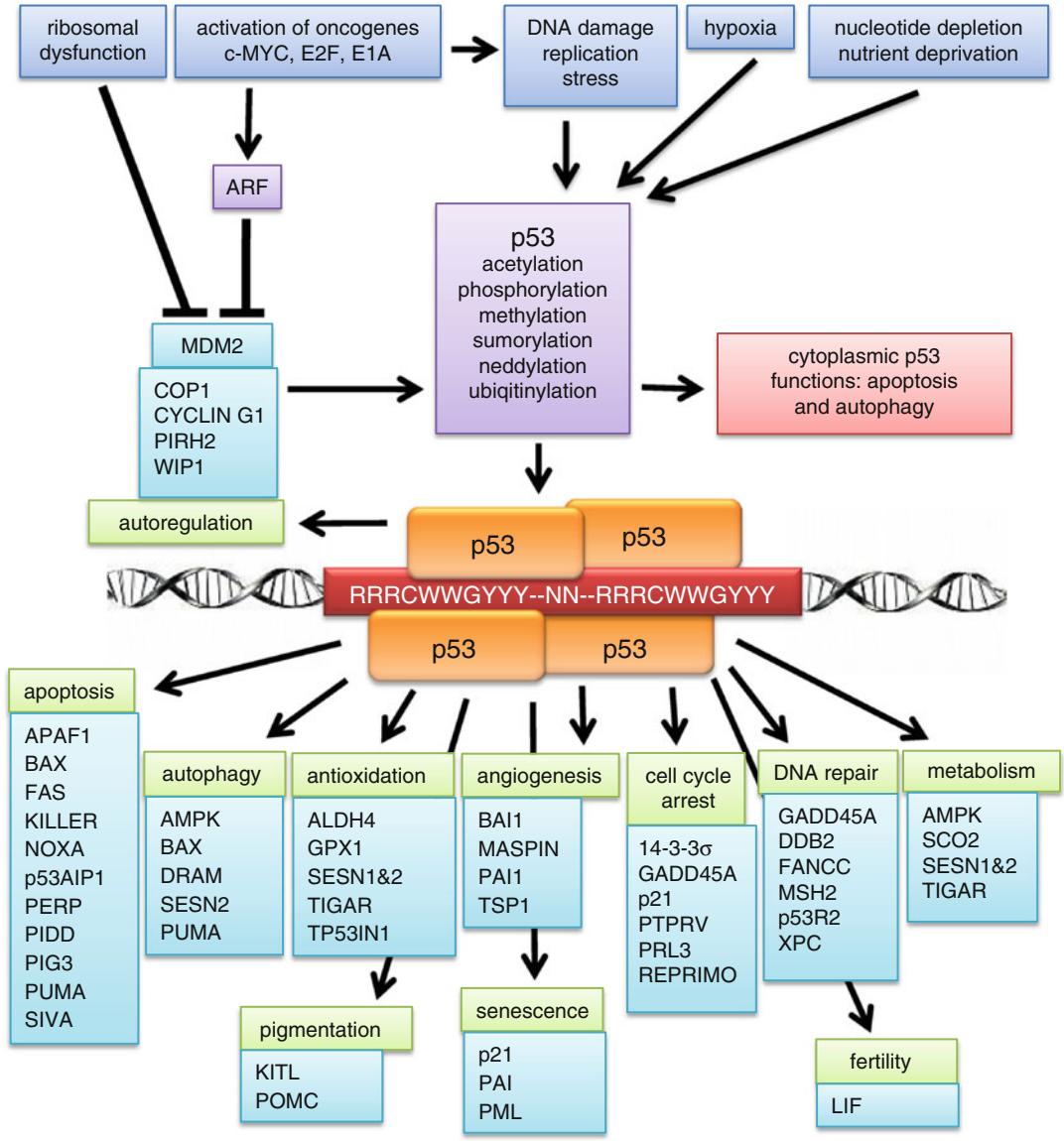

Fig. 5.1 p53 as a central mediator of stress responses. In this model the types of stress and cellular events (*dark blue*) leading to activation of p53 and the protein encoding genes activated by p53 are depicted. p53 is shown as a symbolic tetramer occupying a p53 binding motif (in *red*) containing two palindromic DNA sequences (*white letters* with R = purines (A or G), Y = pyrimidines (C or T), W = A or T and N = bases representing spacers between the two palindromic half-sites). Processes (*green*) regulated by p53 and the respective p53 target genes (*light blue*) implicated are indicated

p53, which also impede the ability of p53 to suppress tumor cell growth. p53 and its loss may represent attractive targets for tumor therapy [2]. Most *p53* mutations target the DNA binding domains of p53, suggesting that the regulation of specific target genes is central for the tumor suppression mediated by p53. However, alternative functions of p53 in the cytoplasm and in mitochondria have also been described [3]. p53's transcriptional activity is induced by various forms of cellular stress that cause diverse post-translational modifications of p53, which are thought to allow a fine-tuning of the cellular response to the type and extent of stress

experienced by the respective cell ([4] summarized in Fig. 5.1). For example repairable DNA damage may cause a transient cell cycle arrest, whereas extensive damage may induce apoptosis via generating different levels of p53 activity. DNA damage in the form of double-strand DNA breaks was one of the first inducers of p53 to be discovered. Subsequently, ribosomal, replication, metabolic, oxidative and transcriptional stress, as well as hypoxia were found to cause an increase in p53's transcriptional activity. These alterations stimulate distinct signaling cascades, which activate enzymes that modify p53 or regulate co-factors binding to p53. For example DNA double strand breaks lead to activation of the ATM kinase, which phosphorylates p53 at multiple N-terminal residues [5] and thereby increases its transactivation activity. Furthermore, p53 may be activated by inhibition of the MDM2 protein, which represents an E3-ubiquitin ligase that marks p53 for proteasomal degradation. p53 forms tetramers, that bind to palindromic recognition sites often organized in tandem repeats with spacers of varying length between them (Fig. 5.1). Promoters display gradual responsiveness to p53 either due to different numbers of p53 binding motifs or due to the presence of high affinity versus low affinity sites [6]. For example, the *p21* gene has a high affinity p53-binding site and mediates cell cycle arrest, whereas genes that mediate cell death harbor low affinity p53 binding sites. Therefore, apoptosis is presumably only induced when p53 is strongly activated. p53 directly activates a large set of genes, which mediate numerous cellular functions that contribute to tumor suppression. Many, but not all of these protein coding target genes are depicted in Fig. 5.1. The activation of p53 target genes is either caused by an increase in p53 abundance after p53 stabilization, anti-repression of specific genes after removal of repressive MDM2/MDMX from p53 by acetylation and/or phosphorylation, as well as by formation of promoter-specific transcriptional complexes [4]. Furthermore, p53 may mediate the specific repression of genes. However, the mechanisms of gene repression by p53 are less well understood and may be indirect to some extent [6]. In the recent years, miRNAs were shown to represent important mediators of gene repression caused by p53.

5.2 p53 and the miRNA World: Current State of the Art

miRNAs have presumably evolved to allow organisms to effectively deal with stress [7, 8]. In line with this notion the p53 stress-response pathway is heavily interconnected with miRNAs not only by regulating their expression and processing, but also since p53 itself represents a down-stream target of miRNAs (see Figs. 5.2, 5.3 and 5.4). The protein-coding genes regulated by p53 elicit several cellular phenotypes/processes, which contribute to tumor suppression, as for example induction of cell cycle arrest, senescence and apoptosis, as well as inhibition of metastasis, angiogenesis and glycolysis [9–15]. Interestingly, these processes are also regulated and in some cases induced by p53-regulated miRNAs [10, 12]. In the last 5 years the characterization of a number of miRNAs directly regulated by p53 and the cellular effects of these connections have been reported. For an overview see Fig. 5.3.

5.2.1 The *miR-34* Genes

In 2007 the miR-34 genes, *miR-34a* and *miR-34b/c*, were reported to be directly regulated by p53 by a number of laboratories using diverse approaches [16–21]. For example, we determined the abundance of miRNAs in libraries representing small RNAs generated after p53 activation using a next generation sequencing approach [16]: we found that miR-34a showed the most pronounced increase among all detected miRNAs after p53 activation, which is mediated by p53 binding sites in the promoter region of its host gene. When ectopically expressed, miR-34a and miR-34b/c displayed tumor suppressive activities, i.e. they caused induction of apoptosis and senescence, inhibition of cell cycle progression, and a decrease of angiogenesis (reviewed in [10, 12, 22]). These effects were mediated by direct down-regulation of the expression of numerous key regulators and effectors of these processes as BCL-2, Cyclin E, CDK4 and CDK6. Meanwhile, a large number of additional miR-34 targets have

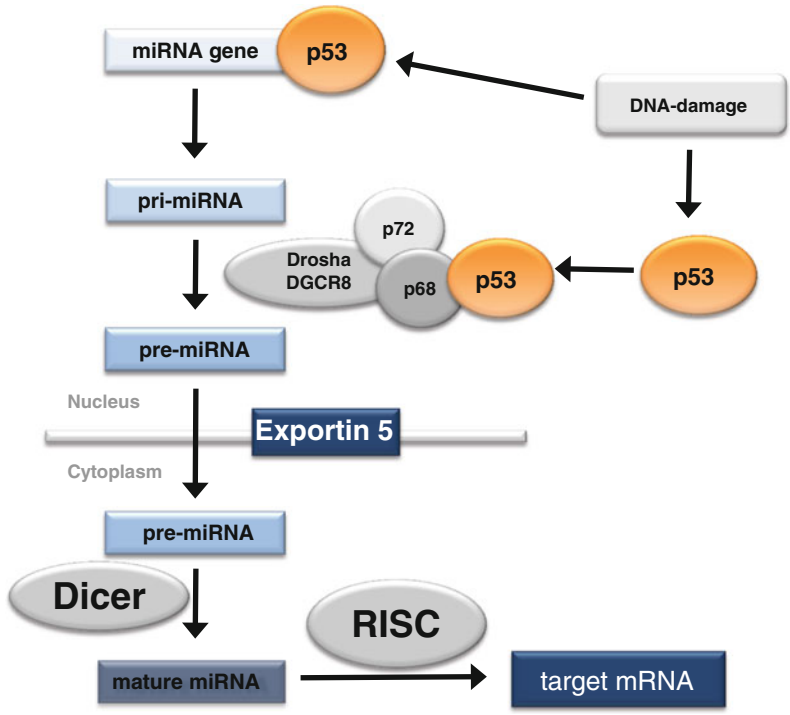

Fig. 5.2 Effects of p53 on the miRNA processing pathway. The synthesis of miRNAs in mammalian cells and the known modes of regulation by p53 are depicted

been identified using a variety of approaches (reviewed in [12]; see also [23, 24] and references therein). Among the miR-34 targets SIRT1, c-MET, Axl, c-/N-MYC, LDH-A and SNAIL seem to be especially relevant for the suppression of cancer. In fact their common up-regulation in tumors could be due to the frequent inactivation of the p53/miR-34 axis during tumor development ([25, 26]; see also next sub-chapter). These targets contribute to the suppression of migration and invasion (SNAIL, c-MET, Axl) and metabolism (LDH-A). In the case of c-MET it was recently shown that p53 down-regulates c-*MET* expression via SP1-mediated occupancy and repression of the c-*MET* promoter and by inducing miR-34a/b/c, which directly target the 3′-UTR of the c-*MET* mRNA [27]. p53 may suppress metastasis by antagonizing epithelial-mesenchymal transitions, which have been implicated in the early, invasive stages of metastasis. Instead, p53 activation promotes mesenchymal-epithelial transition (MET) and favors the epithelial state of cells [28]. We recently found that p53-induced MET is mediated by induction of *miR-34a* and *miR-34b/c* in colorectal cancer cell lines [29]. miR-34a and miR-34b/c achieve this effect by negatively regulating a master-regulator of EMT, the SNAIL transcription factor [29, 30]. In addition, we found that the *miR-34a* and the *miR-34b/c* genes are directly repressed by SNAIL [29]. Therefore, miR-34a/b/c and SNAIL form a double-negative feed-back loop (summarized in [31]). Stemness represents another important oncogenic trait of cancer cells which is suppressed by miR-34. It was shown that miR-34 directly suppresses CD44, which blocks the expansion of cancer-initiating tumor stem cells in a mouse model of prostate cancer [32]. When miR-34a is ectopically expressed, stemness markers as CD133, CD44 and BMI-1 are down-regulated in colorectal cancer cells [29]. Furthermore, it was recently reported that, similar to p53, the miR-34 miRNAs provide a barrier for somatic cell reprogramming and the generation of IPS

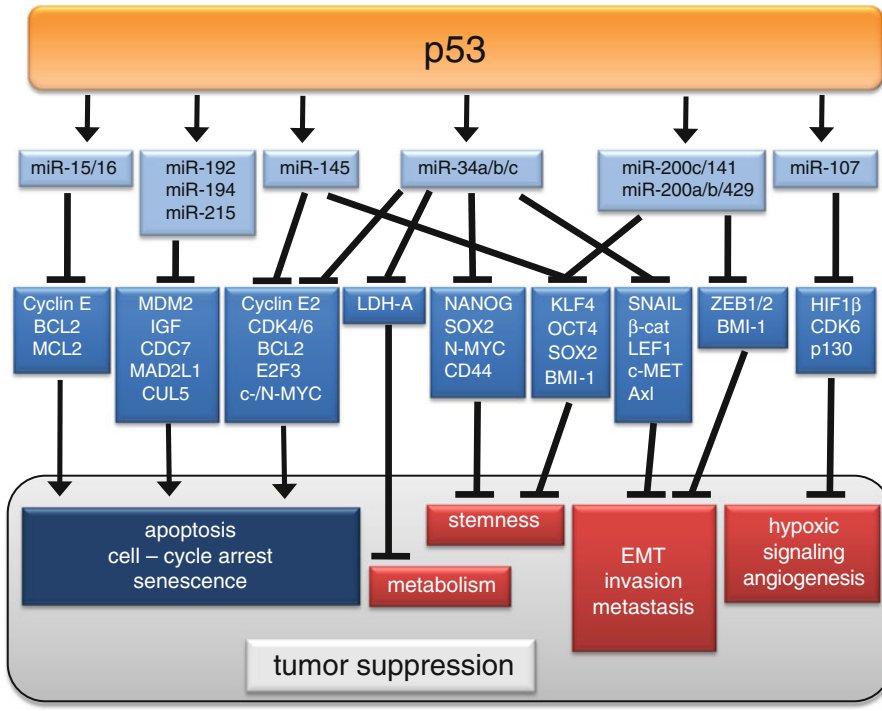

Fig. 5.3 Regulation of miRNA expression by p53. Model summarizing direct transcriptional activation of miRNA-encoding genes, the affected miRNA targets and the reported cellular effects, which collectively contribute to tumor suppression by p53. The *arrows* or inhibition symbols pointing to the cellular processes represent the summation of the regulations resulting from the activity of the indicated p53-induced miRNAs. For details see the main text

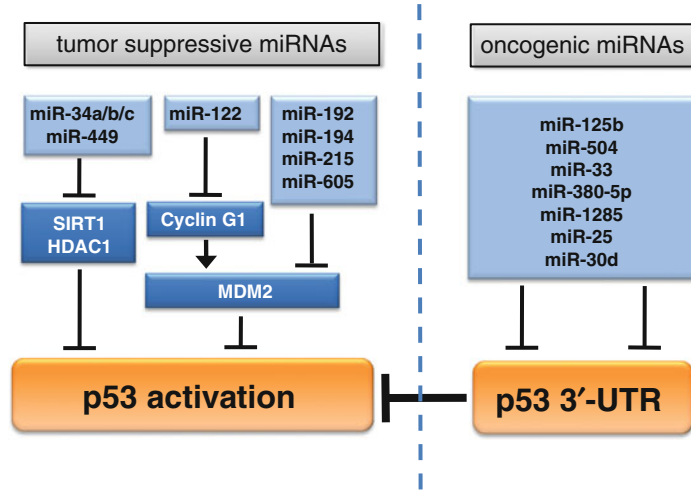

Fig. 5.4 Regulation of p53 by miRNAs. Model summarizing the regulation of the 3′-UTR of *p53*, as well as the down-regulation of p53-modifying enzymes by miRNAs. Oncogenic miRNAs with seed-matching sequences in the 3′-UTR of *p53* or tumor suppressive miRNAs directly down-regulating the indicated proteins are depicted. For details see the main text

(induced pluripotent stem) cells from mouse embryo fibroblast [33]. miR-34 mediated this effect by direct down-regulation of NANOG, SOX2 and N-MYC. Therefore, cancer cells with loss of miR-34 expression may also be more prone to become tumor initiation cells, which exhibit features of stem cells. Furthermore, miR-34 inhibits components of the wnt/β-catenin/TCF

pathway, as β-catenin, LEF1 and WNT1 [34, 35]. Thereby, miR-34 may contribute to the suppression of stemness- and EMT-related features of cancer cells.

The miR-34 family also includes miR-449. Although the seed sequences of miR-34a/b/c and miR-449a/b/c are highly conserved, the regulation of the genes encoding these miRNAs is divergent as miR-449 expression is induced by E2F1, but not by p53 and/or DNA damage [36]. Therefore, the regulation of similar targets by miR-34 and miR-449 miRNAs may occur under rather distinct circumstances. Furthermore, miR-449 presumably has a restricted expression pattern, since it was found to be highly expressed in differentiating lung epithelia and at comparatively low levels in other tissues [36].

5.2.2 The miR-200 Family

More recently the two genes encoding the miR-200 family, which give rise to the miR-200c/141 and the 200a/200b/429 miRNAs, were identified as direct p53 target genes that enforce mesenchymal-epithelial transitions (MET) [37, 38] by targeting the EMT-regulators ZEB1 and ZEB2 [39]. In addition, miR-200c down-regulates KLF4 and the polycomb repressor BMI-1, both stemness factors, and thereby contributes to the loss of metastatic capacity of tumor initiating cancer stem cells [37]. Therefore, induction of the miR-200 family represents a new mechanism by which p53 suppresses metastasis (reviewed in [28, 40]).

5.2.3 The miR-192 Family

The three members of the miR-192 family were found to be encoded by p53 target genes using a microarray analysis to monitor miRNA expression after treatment with the MDM2 inhibitor Nutlin-3a [41]. These authors also found that ectopic miR-192 expression induces p21 in a p53-dependent manner. Later it was shown that the miR-192 family targets the IGF pathway and also MDMD2, which results in the activation of p53 [61]. Furthermore, this tumor suppressive loop is impaired in multiple myeloma, which shows down-regulation of the miR-192 family. In addition, ectopic miR-192 leads to a G_1 and G_2/M cell cycle arrest by targeting CDC7, MAD2L1 and CUL5 [42].

5.2.4 Additional p53-Regulated miRNAs

miR-107 is encoded by an intron of the p53-induced *PANK1* gene [43]. Ectopic expression of miR-107 decreases HIF1β expression, which diminishes the response to hypoxia and blocks tumor angiogenesis and growth. In addition, miR-107 targets the cell cycle regulators CDK6 and p130/pRBL2 [44].

miR-145 represents a p53-inducible miRNA, which was shown to contribute to repression of c-MYC by p53 via directly targeting the c-*MYC* 3′-UTR [45]. Interestingly, miR-145 also negatively regulates OCT4, SOX2 and KLF4, and thereby represses pluripotency in human embryonic stem cells [46]. Therefore, miR-145 may at least in part explain why deletion of p53 strongly enhances the generation of IPS cells and potentially promotes the expansion of cancer stem cells [47].

miR-15a and miR-16-1 are encoded by an intron of the *DLEU2* mRNA. Initially, miR-15a/16-1 were shown to be processed at an increased rate after p53 activation [16, 48]. Later, the *DLEU2* gene was shown to be a transcriptional target of p53 [49]. Since miR-15/16 target BCL2 and Cyclin E, they affect both, apoptosis and the cell cycle.

5.2.5 Direct Regulation of p53 Expression by miRNAs

Seversal recent publications demonstrated that miRNAs contribute to the tight control under which p53 is placed in the cell by directly interacting with the 3′-UTR of *p53* mRNA (summarized in Fig. 5.4). By computational analysis of putative miRNA binding sites using TargetScan

and mirBase prediction software a binding site of miR-125b was identified in the 3′-UTR of *p53* [50]. MiR-125b is expressed at high levels in the brain and conserved between human, zebrafish and other vertebrates. Ectopic expression of miR-125b decreased p53 protein levels and apoptosis in human cells, whereas inhibition of miR-125b had the opposite effect in lung fibroblasts and zebrafish brain. When zebrafish were treated with DNA damaging agents miR-125b expression was down-regulated, presumably allowing the observed increase in p53 protein. Analysis of 89 colorectal cancer samples revealed that elevated expression of miR-125b is associated with increased tumor size and invasion, and also correlates with poor prognosis and decreased survival [51]. These results are in accordance with a negative regulation of p53 by miR-125b.

By an *in silico* search two miR-504 seed-matching sequences were identified in the 3′-UTR of p53 [52]. Accordingly, ectopic expression of miR-504 down-regulated p53 protein levels, reduced p53-dependent apoptosis and cell cycle arrest, and resulted in increased tumor formation *in vivo*.

miR-33 also targets p53 by binding to two seed-matching motifs in the 3′-UTR of p53 [53]. Interestingly, miR-33 is down-regulated in hematopoietic stem cells (HSC) and up-regulated in more differentiated progenitor cells in super-p53 mice, which are endowed with an extra copy of p53. Ectopic expression of miR-33 in HSC results in increased stemness and decreased recipient survival. In mouse embryonic fibroblasts miR-33 promotes neoplastic transformation presumably via down-regulation of p53.

miR-380-5p was found to down-regulate p53 in neuroblastomas, which commonly express wild-type p53 [54]. Neuroblastomas with elevated expression of miR-380-5p showed a decreased patient survival. Furthermore, miR-380-5p was highly expressed in mouse embryonic stem cells and its ectopic expression cooperated with HRAS in transformation, abrogation of oncogene-induced senescence and promoted tumor formation in mice. Finally, *in vivo* delivery of a miR-380-5p antagonist decreased tumor size in an orthotopic mouse model of neuroblastoma.

In a systematic, bioinformatics screen 107 potential p53-targeting miRNAs were identified using TargetScan [55]. When these candidates were experimentally tested in a dual-reporter assay, miR-1285 turned out to be the most effective repressor of p53's 3′-UTR reporter activity. In line with these results, miR-1285 decreased p53 mRNA- and protein-levels by directly binding to the 3′-UTR of p53 via two seed-matching sequences.

In a similar bioinformatics screen using less stringent criteria and four different miRNA target prediction methods (Miranda, TargetScan, PicTar and RNA22) 67 candidate miRNAs with the potential to directly inhibit p53 expression were identified [56]. In a subsequent, experimental screen only eight of these had an inhibitory effect on p53-mediated transactivation. Of these only three were effective in a dual reporter assay employing the *p53* 3′-UTR: miR-200a, -30d and -25. By mutation of the respective corresponding seed-matching sequences in reporter constructs only miR-30d and miR-25 were validated as direct regulators of the *p53* 3′-UTR. In contrast, miR-200a presumably affects the *p53* 3′-UTR by indirect regulation, e.g. via modulation of transcription factors that regulate miRNAs, which directly target p53. In a cellular assay ectopic miR-30d and miR-25 decreased p53 levels, p53 target expression and downstream effects of p53 as apoptosis, cell cycle arrest and senescence. The opposite was observed, when both miRNAs were inhibited by antagomirs. In line with these observations, miR-25 and miR-30d were found to be up-regulated in multiple myeloma cells, which showed a concomitant down-regulation of p53 mRNA expression. Furthermore, inhibition of miR-25 and miR-30d induced p53 and apoptosis in a multiple myeloma cell line. Therefore, miR-25 and miR-30d presumably represent oncogenic miRNAs. These examples show that the bioinformatics identification of miRNA/target mRNA interactions has to be validated experimentally as it currently generates mainly false predictions.

5.2.6 Indirect Regulation of p53 by miRNAs

Several examples of p53 being subject to indirect regulation by miRNAs via down-regulation of upstream regulators of p53 have been documented. One of the first cases was the regulation of SIRT1 by miR-34a [57]. An *in silico* search for miR-34a targets, which might affect p53 resulted in the analysis and experimental confirmation of SIRT1 as a miR-34a target. As a consequence of SIRT1 down-regulation by miR-34a an increase in p53 activity and enhanced expression of its targets p21 and PUMA, as well as increased apoptosis was observed. Since miR-34a itself is induced by p53 the regulations connecting miR-34a, SIRT1 and p53 constitute a positive feed-back loop. In tumors this self activating loop may be disrupted by the silencing of *miR-34* genes by CpG methylation [12, 25, 26] and mutation/inactivation of p53.

As mentioned above, miR-449 is similar to miR-34, but regulated by other factors, as for example E2F1. Interestingly, when miR-449 was expressed ectopically it also indirectly activated p53 via directly suppressing the expression of SIRT1 [58]. This may allow additional pathways to increase p53 activity.

Also miR-122 leads to an up-regulation of p53 [59]. However, this is accomplished even more indirectly, since the miR-122-mediated down-regulation of Cyclin G1 presumably inhibits recruitment of PP2A phosphatase to MDM2 resulting in decreased MDM2 activity and increased p53 levels/activity. In line with this scenario ectopic miR-122 expression increased the sensitivity of hepatocellular carcinoma derived cell-lines to doxorubicin.

More recently, miR-885-5p was shown to activate p53 and the expression of p53 target genes [60]. However, although miR-885-5p was shown to target CDK2 and MCM5, the mechanism of the miR-885-5p effect on p53 remained unclear.

miR-192/194/215 are transcriptionally induced by p53 and negatively modulate MDM2 activity [61]. Interestingly, their ectopic expression enhanced the therapeutic effectiveness of MDM2 inhibitors against multiple myeloma (MM), an incurable B cell neoplasm, in experimental settings. A similar feedback loop was recently described for miR-605, which is also induced by p53 and negatively regulates MDM2 expression [62].

5.2.7 Direct Involvement of p53 in miRNA Processing and Maturation

Since the levels of certain processed miRNAs were increased after p53 activation even in the absence of an induction of the corresponding primary miRNAs (pri-miRNA), the possibility that p53 may directly affect the processing of miRNAs was analyzed [48]. Indeed, these authors found that p53 interacts with the miRNA processing complex DROSHA through association with the DEAD-box RNA helicase p68 (indicated in Fig. 5.2). Thereby, p53 enhances processing of specific pri-miRNAs with growth suppressive function (e.g. miR-16-1, miR-143, miR-145) to precursor miRNAs (pre-miRNAs) resulting in a significant increase in the corresponding miRNAs. Therefore, direct transcriptional regulation of any miRNA-encoding gene by p53 should not be deduced from the detection of an increase in mature miRNA levels by techniques like miR-Seq. Such analysis should be complemented by quantifications of the pri-miRNA levels and detection of p53 occupancy at the promoter of the respective pri-miRNA encoding gene.

Another link between p53 and miRNA-processing has been observed in conditional DICER knock-out mice [63]. DICER deficiency and therefore incomplete miRNA maturation induces p53 and p19/ARF, which leads to reduced proliferation and premature senescence. Interestingly, deletion of *Ink4/Arf* or *p53* prevents premature senescence induced by deletion of DICER. Therefore, a p53-dependent checkpoint seems to monitor proper miRNA processing.

5.2.8 The p53-Relatives p63 and p73 in the Regulation of miRNAs

The p53 family members p63 and p73 have also been implicated in the regulation of miRNA

expression and processing. TAp63 was shown to coordinately regulate DICER and miR-130b to suppress metastasis [64]. In contrast to *p53,* the *p63* and *p73* genes are not affected by mutations in tumors. p73 promotes genome stability and mediates chemosensitivity, whereas p63 largely lacks these p53-like functions and instead promotes proliferation and cell survival. p63 and p73 were shown to be connected via miRNA regulations: p63 represses the expression of miR-193-5p, which targets p73, thereby causing an increase in p73 expression, whereas p73 induces miR-193-5p [65]. Interestingly, therapeutic inhibition of miR-193-5p effectively blocked tumor progression when combined with an otherwise ineffective chemotherapy in an orthotopic tumor model.

5.3 Alterations of the p53/miRNA Network in Human Cancer

Similar to protein coding genes miRNA-encoding genes may harbor oncogenic or tumor suppressive functions. As discussed above, p53-induced miRNAs promote tumor suppressive processes, as cell cycle arrest, senescence, inhibition of EMT and metastasis. During cancer initiation or progression cells with inactivation of miRNA-encoding genes may have a selective advantage, since they presumably display a weakened or missing induction of these tumor suppressive mechanisms. In tumors miRNA-encoding genes may be inactivated by a number of different mechanisms. The p53-inducible miRNAs discussed above are likely to be down-regulated in at least half of all tumors due to the mutational inactivation of p53. However, in tumors retaining wild-type p53 the p53-regulated miRNA-encoding genes represent good candidates for being subject to inactivating events. These include loss by deletion or other structural changes as translocations. In addition, down-regulation of miRNA expression by epigenetic silencing via CpG methylation and/or deacetylation of promoter regions has been described. Furthermore, indirect down-regulation due to mutations of other up-stream regulatory transcription factors and alterations in the miRNA processing machinery has been observed. Another mode of inactivation may be due to the aberrant expression of a seed-match containing RNA, a so-called competing endogenous RNA (ceRNA), which sequesters the respective miRNA [66]. This mechanism was originally observed in plant cells [67]. The existence of cancer-relevant ceRNAs in human cells was documented by the identification of RNAs, which regulate expression of the PTEN tumor suppressor via this route [68]. A further possibility of miRNA inactivation was suggested to occur by mutation of seed sequences or altered processing of miRNAs. However, such alterations were only rarely observed until now [69, 70]. Furthermore, an escape from miRNA action by deletion or mutation of seed matching sequences in the respective target mRNA is conceivable. Indeed, such alterations have been observed in mRNAs encoding oncogenic factors [71, 72]. For an overview of reported alterations in the p53/miRNA network detected in cancer see Table 5.1.

5.3.1 Cancer-Specific Alteration of the miR-15/16 Encoding *dLEU2* Gene

The first report of a genetic inactivation of a miRNA was the observation that the *dLEU2* gene, which is located on chromosome 13q14 and encodes the miR-15a and miR-16-1 miRNAs, is commonly deleted in chronic lymphocytic leukemia (CLL)[75]. More recently, it was shown that experimental deletion of *miR-15a/16-1* or of the entire *dLEU2* gene predisposes mice to CLL [123]. Therefore, *dLEU2* is presumably the tumor suppressor gene located in the 13q14 region. Importantly, this study provided the first proof for a *bona fide* tumor suppressor gene function of an miRNA.

5.3.2 Cancer-Specific Alterations of the *miR-34* Family

The *miR-34a* and *miR-34b/c* genes are frequently silenced by CpG methylation in a variety of tumor types [12, 25, 26, 94]. *MiR-34a* methylation was initially shown to occur in numerous cell lines

Table 5.1 Alterations of p53-regulated miRNAs in human tumors

miRNA-gene	Tumor type	Mechanism	Frequency [%]	n=	References
miR-15a/16-1	Prostate cancer	Deletion	80	20+15	[73]
	Chronic Lymphocytic Leukemia/CLL	Germline Mutation in the primary precursor	15	75 cancer (+ control: 160 normal)	[74]
	Chronic Lymphocytic Leukemia/CLL	Deletion	68, 51	60, 322	[75, 76]
	Mantle Cell Lymphoma/MCL	Deletion	55	53	[77]
	Mantle Cell Lymphoma/MCL	Downregulation	71	30	[76]
	Non Small Cell Lung Cancer/NSCLC	Deletion or down-regulated	74	23	[78, 79]
	Pituitary tumors [Cushing's Disease / CD]	n.d.	–	14 (+7controls)	[80]
	Ovarian	Downregulation		38	[81]
	Non-Hodgkin's Lymphoma/NHL	Deletion	43	43	[82]
	Hodgkin's disease/HD	Deletion	29	7	[82]
	Multiple Myeloma/ MM	Downregulation	54	37	[83]
	Pituitary adenoma	Deletion	n.d.	20	[84]
	Pancreatic cancer	Downregulation	70	10	[85]
	Prostate cancer	Downregulation	100	23	[86]
	Prostate cancer cell lines	Downregulation	–	50	[87]
miR-16-2	Progression of prostate carcinogenesis	Downregulation	–	63	[88]
miR-34a	Non Small Cell Lung Cancer/NSCLC	Downregulation	91	23	[79]
	Acute Myeloid Leukemia/AML	Hypermethylation	0	20	[89]
	Non-Hodgkin's Lymphoma/NHL	Hypermethylation	18.8	32	[89]
	Acute Lymphoblastic Leukemia/ALL	Hypermethylation	0	20	[89]
	Chronic Lymphocytic Leukemia/CLL	Hypermethylation	4	50	[89]
	Chronic Myeloid Leukemia/CML	Hypermethylation	0	11	[89]
	Multiple Myeloma/ MM	Hypermethylation	5, 5	55	[89]
	Colorectal cancer cell lines	Hypermethylation, p53 mutation	23	13	[25]
	Prostate cancer	Hypermethylation	79	24	[25]
	Breast cancer cell lines	Hypermethylation	25	24	[25]
	Kidney cancer cell lines	Hypermethylation	21	14	[25]

(continued)

Table 5.1 (continued)

miRNA-gene	Tumor type	Mechanism	Frequency [%]	n=	References
	Bladder cancer cell lines	Hypermethylation	33	6	[25]
	Lung cancer cell lines	Hypermethylation	29	24	[25]
	Melanoma	Hypermethylation	63	32	[25]
miR-34b/c	Gastric cancer	Hypermethylation	70	118	[90]
	Non Small Cell Lung Cancer/NSCLC	Hypermethylation		161	[91]
	Primary melanoma cell lines	Downregulation		2	[92]
	Head and Neck Cancer/H&N	Downregulation	n.d.	10	[93]
	Colon cancer	Hypermethylation	90	111	[94]
	MYC translocation-negative Burkitt Lymphoma	Downregulation	100	5	[95]
miR-34a/b/c	Malignant Pleural Mesothelioma/MPM	Hypermethylation	28(a)/85(b/c)	47	[96]
	Ovarian cancer	p53 Mutation, Hypermethylation	100(a)/72(b/c), 62(a)/69(b/c)	89, 13	[26, 97]
	Colorectal Cancer	Hypermethylation	74(a)/99(b/c)	114	[26]
	Pancreatic cancer	Hypermethylation	64(a)/100(b/c)	11	[26]
	Mammary cancer	Hypermethylation	60(a)/90(b/c)	10	[26]
	Urothelial Cancer/UC	Hypermethylation	71(a)/57(b/c)	7	[26]
	Renal cell cancer	Hypermethylation	58(a)/100(b/c)	12	[26]
	Soft tissue Sarcomas	Hypermethylation	64(a)/45(b/c)	11	[26]
	Esophageal Squamous Cell Carcinoma/ESCC	Hypermethylation	67(a)/41(b/c)	54	[98]
miR-107	Head and Neck/Oral cancer/HNOC	Downregulation		4	[99]
	Acute Promyelocytic Leukemia/APL	Downregulation		26	[50]
	Pancreatic carcinoma cell lines	Hypermethylation		2	[100]
	Tongue squamous cell carcinoma/TSCC	Downregulation	n.d.	4	[99]
	Chronic Lymphocytic Leukemia/CLL	Hypermethylation	n.d.	50	[101]
	Pancreatic cancer	Upregulation		44 (+12 Controls)	[102]
miR-141	Colorectal cancer	Downregulation		10	[103]
	Mesenchymal breast cancer cell lines	Hypermethylation	100	4	[104]
	Epithelial breast cancer cell lines	Hypermethylation	0	4	[104]
	Lung cancer	Downregulation		10	[103]
	Bladder cancer	Hypermethylation		10 (+5 Controls)	[105]
miR-145	Prostate cancer	Hypermethylation, p53 Mutation	81	27	[106]

(continued)

Table 5.1 (continued)

miRNA-gene	Tumor type	Mechanism	Frequency [%]	n=	References
	Prostate cancer	Downregulation		63	[88]
miR-192	Colorectal cancer	p53 Mutation		34	[107]
	Colorectal cancer (MSI)	Downregulation		54 (+20 Controls)	[108]
	Multiple Myeloma/ MM	Hypermethylation		47 (+5 Controls)	[61]
miR-194	Colorectal Cancer with liver metastasis	p53 Mutation, SNP		30	[109]
	Multiple Myeloma/ MM	Hypermethylation		47 (+5 Controls)	[61]
miR-200a	Ovarian cancer	Downregulation		55	[110]
miR-200b	Colorectal cancer	Loss		30	[109]
	Ovarian cancer	Downregulation		55	[110]
	Lung cancer	Hypermethylation	25	24 (+ Controls)	[111]
	Bladder cancer	Hypermethylation		10 (+5 Controls)	[105]
miR-200c	Lung cancer	Hypermethylation	29	24 (+ Controls)	[111]
	Colorectal cancer	Hypermethylation, p53 mutation			[112]
	Mesenchymal breast cancer cell lines	Hypermethylation	100	4	[104]
	Epithelial breast cancer cell lines	Hypermethylation	0	4	[104]
	Lung cancer	Hypermethylation	25, -	24, 69	[111, 113]
	Bladder cancer	Hypermethylation		10 (+5 Controls)	[105]
miR-215	Colorectal cancer	Downregulation		34	[107]
miR-429	Colorectal cancer cell lines	Hypermethylation	50	2	[114]
	Breast cancer cell lines	Hypermethylation	50	2	[114]
	Lung cancer cell lines	Hypermethylation	33	3	[114]
	Ovarian cancer	Downregulation		55	[110]
Exportin 5	Breast cancer	Mutation		441 (+479 Controls)	[115]
	Hereditary nonpolyposis colon cancer	Downregulation	26	38	[116]
	Sporadic colon cancer (MSI+)	Downregulation	22	211	[116]
	Sporadic gastric cancer (MSI+)	Downregulation	28	58	[116]
	Sporadic endometrial tumors (MSI+)	Downregulation	13	30	[116]
Dicer/Drosha	Ovarian cancer	Downregulation	60/51	111	[117]
Dicer	Cystic nephroma, Wilm's tumor	Germline mutation	0	50	[118]
	Pulmonary pediatric cancer	Mutation	91	11 (+360 Controls)	[119]

(continued)

Table 5.1 (continued)

miRNA-gene	Tumor type	Mechanism	Frequency [%]	n=	References
Dicer	Lung cancer	Downregulation	67		[120]
	Colorectal cancer	Upregulation		237	[121]
	Acute Myeloid Leukemia/AML	Upregulation	86	71	[122]

Summary of the reported alterations in p53-regulated miRNAs in cancer. *Frequency* relates to the alteration indicated in the third column, *n* number of tumor samples/patients analyzed, *MSI* micro-satellite instable.
n.d. = not determind.

derived from different tumor types, as well as in primary prostate cancer and melanoma [25]. Also the expression of the miR-34 family members miR-34b and miR-34c, which are encoded by a common transcript, is down-regulated in many types of cancer [26]. A high frequency of silencing of the *miR-34b/c* promoter by CpG methylation has been found in colorectal cancer cell lines and colorectal tumor samples [94]. We also found CpG methylation of *miR-34b/c* in all 114 cases of primary colorectal cancers analyzed [26]. Interestingly, *miR-34b/c* methylation correlated with metastasis and poor survival for several types of cancer [124]. The reintroduction of *miR-34b/c* into cancer cell lines exhibiting *miR-34b/c* silencing inhibited their motility, reduced tumor growth, suppressed metastasis formation in a xenograft model and was associated with down-regulation of the respective target genes (e.g. c-MYC, E2F3, CDK6).

The *miR-34a* gene is located on chromosome 1p36, a region which is commonly deleted in human cancers, as for example in neuroblastoma [125], which often display loss of *miR-34a* expression [126].

5.3.3 Cancer-Specific Alterations of the *miR-200* Family

The miR-200 family encodes a highly conserved group of miRNAs, which control EMT by down-regulating the EMT-inducing transcription factors ZEB1 and ZEB2 [39]. The miR-200 family can be sub-divided into two clusters: miR-200c and miR-141 (located at chromosome 12p13), and miR-200a, miR-200b and miR-429 (located at chromosome 1p36). Expression of the miR-200c/141 cluster is frequently silenced by CpG methylation in breast cancer [104]. Interestingly, a correlation between methylation of the miR-200c promoter and invasiveness was determined in breast cancer cell lines. Down-regulation of the miR-200c/141 cluster was also described for breast cancer initiating cells [127] and EBV-associated gastric carcinomas [128]. As mentioned above, loss of 1p36 is a recurrent aberration especially in neuroblastoma, indicating that there may be two distinct mechanisms that down-regulate the expression of the miR-200 family.

5.3.4 Cancer-Specific Alterations of the *miR-192* Family

The p53-regulated miR-192 family is comprised of miR-192, miR-194-2, and miR-215, which induce p21 expression and cell cycle arrest in a p53-dependent manner [41]. The miR-192 family is down-regulated by an unknown mechanism in multiple myeloma (MM), which rarely shows mutation or deletion of p53 [61]. Reactivation of p53 in MM resulted in re-expression of miR-192, miR-194-2, and miR-215 and down-regulation of MDM2, which represents a target of these miRNAs [61]. Moreover, ectopic expression of miR-192 family members inhibited cell growth, migration and invasion of MM. Furthermore, the miR-192 family members are down-regulated in colon cancer, and induce apoptosis and senescence, although to a lesser extent than miR-34a [41]. The mechanism by which down-regulation of the miR-192 family occurs remained unclear in

this study, but p53 inactivation [129] and a single nucleotide polymorphism (SNP) located within the miR-194-2 precursor [130] may contribute to this phenomenon.

5.3.5 Other p53-Induced miRNAs Inactivated in Cancer

Recently, the p53-inducible miR-145 was shown to be down-regulated by CpG methylation and p53 mutation in prostate cancer samples and cell lines [106].

5.3.6 Alterated Regulation of the miRNA Processing Machinery in Cancer

miR-107 was shown to directly target DICER1 mRNA, which encodes a central component of the miRNA processing machinery [131]. Ectopic expression of miR-107 enhances migration *in vitro* and allows metastatic dissemination of otherwise non-aggressive cells *in vivo*, whereas the loss of miR-107 opposes migration and metastasis of malignant cells. Moreover, it was shown that high levels of miR-107 are associated with metastasis and poor outcome in breast cancer. However, these observations are not compatible with mediation of p53-induced tumor suppression by miR-107. Nonetheless, these findings suggest that the deregulation of the miRNA processing machinery in cancer leads to metastasis and poor outcome, and predicts an anti-cancer activity of the majority of the miRNAs. In support of this conclusion, *DICER1* was characterized as an haplo-insufficient tumor suppressor gene in a tumor mouse model [132]. Furthermore, decreased expression of DICER1 correlates with poor prognosis in human lung cancer [120]. Interestingly, the p53 family member p63 transcriptionally controls DICER1 expression. Mutant p53 presumably interferes with this regulation, which leads to a reduction in DICER1 levels and reduces the levels of certain cancer-relevant miRNAs [64]. Mutant p53 may also interfere with the post-translational regulation of DROSHA by wild-type p53 and thereby affect the processing of selected, tumor suppressive miRNAs [48].

5.3.7 Mutations in the miRNA Processing Machinery in Cancer

Another possibility how the abundance of p53-regulated miRNAs could be altered in cancer is to constitutively change the processing of pri-miRNAs to miRNAs by genetic alterations in components of this pathway. For example, mutations of the nuclear export protein Exportin-5 resulted in the trapping of pre-miRNAs in the nucleus and reduced miRNA-processing [116]. As a result, numerous miRNAs were not fully processed and a diminished inhibition of the respective miRNA targets was detected. Notably, restoration of Exportin-5 function reversed the impaired export of pre-miRNA and had tumor-suppressive effects. Recently, several studies supported the notion that variations in the expression and mutations of miRNA processing components as Exportin-5 and DICER1 affect the outcome of breast [115], ovarian [117], cystic nephroma [118] and pediatric pulmonary cancer [119].

5.4 Approaches to Study p53-Regulated miRNAs and Their Targets

Although, numerous connections between p53 and miRNAs have been identified, the examples described above also illustrate that we have only begun to understand the role of miRNAs in tumor suppression mediated by p53. Therefore, additional efforts are necessary to obtain more details of the p53/miRNA network. A feasible strategy for a comprehensive, genome-wide identification of p53-regulated miRNAs and their associated target genes is the combination of the approaches depicted in Fig. 5.5. This strategy may in principle also apply to other transcription factors of interest besides p53. These analyses generate a large amount of bioinformatics data, which can be processed with the help of the algorithms indicated

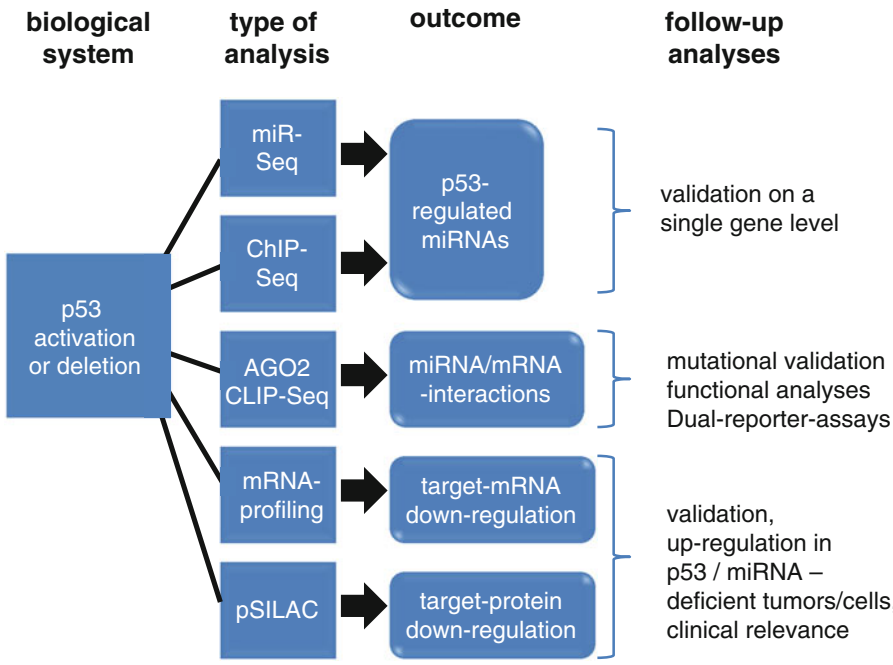

Fig. 5.5 Analysis of p53-regulated miRNAs and their targets. Summary of experimental approaches for the comprehensive identification and characterization of p53-regulated miRNAs. The approaches are described in detail in the text

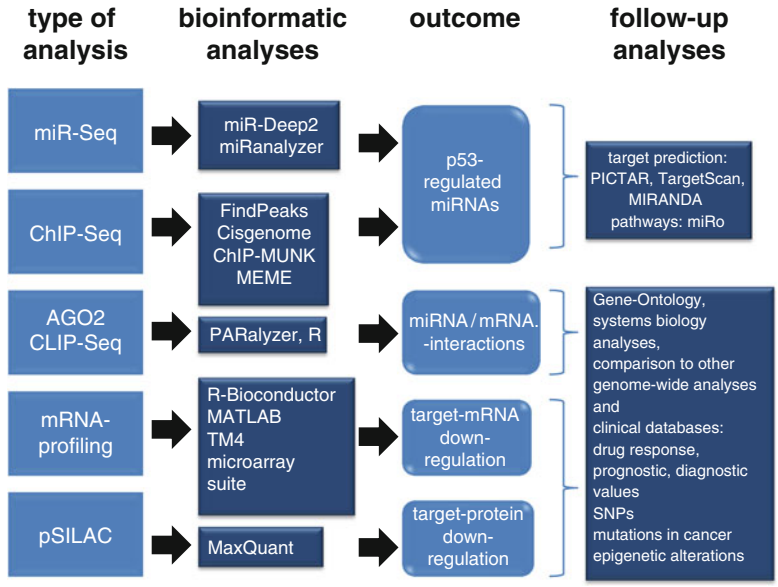

Fig. 5.6 Bioinformatics characterization of p53-regulated miRNAs and their targets. Summary of bioinformatics approaches for the comprehensive characterization of p53-regulated miRNAs. As indicated, the programs and websites Bioconductor [133], Cisgenome [134], ChIP-Munk [135], FindPeaks [136], MaxQuant [137], Meme [138], MirDeep2 [139], miRanalyzer [140], miRo [141], PARalyzer [142] and TM4 microarray suite [143] facilitate the analyses of data obtained by the experimental analyses described in the main text and in Fig. 5.5

in Fig. 5.6. The experimental strategy can be subdivided into two main parts: (1) the identification of p53-regulated miRNAs and (2) the identification of target mRNAs of the p53-regulated miRNAs. So far the studies in this area have rather focused on the identification and characterization of single miRNAs regulated by p53 or they have carried out one type of genome-wide approach, with subsequent confirmation of a limited number of candidates. In the following section we will describe which approaches have been applied to identify and characterize p53-regulated miRNAs and their associated targets in the past and which lessons have been learned from these analyses.

5.4.1 Identification of p53-Regulated miRNAs

In order to experimentally identify p53-regulated miRNAs cellular systems in which p53 activity can be turned on using conditional systems or pharmacological p53 activators should be employed. Endogenous p53 can either be activated by addition of DNA damaging substances or by p53-activators as the MDM2 inhibitor Nutlin-3a. Isogenic cells with and without wild-type p53 should be analyzed in parallel in order to identify p53-dependent regulations. For example, the colon cancer cell lines HCT-116 with either wild-type p53 expression or p53-knockout are useful for this purpose [144]. Alternatively, the miRNA expression in tissues of p53 knock-out mice or derived cells, e.g. mouse embryonic fibroblasts (MEFs), represent useful systems in order to identify p53-mediated miRNA regulations, as documented previously [18].

A highly specific activation of p53 can be achieved using ectopic expression of p53. However, certain post-translational modifications of p53 induced by treatment with DNA-damaging agents may not occur hereby. Therefore, differences in the pattern of miRNAs regulated by p53 may occur when compared to activation of p53 by stressors as oncogene activation and DNA damaging agents. In the past, we have used an episomal, doxycyclin-inducible expression system to re-express p53 in p53-deficient H1299 lung cancer cells [16].

Differential expression of miRNAs upon p53 activation can be monitored using specifically designed miRNA microarrays. A number of commercially available microarray platforms can be used for this purpose: for example the Human miRNA Microarray 1.0 (Agilent), the miRCURY LNA miRNA Array v9.2 (Exiqon), the Array Matrix 96-well MiRNA Expression Profiling Assay v1 (Illumina Sentrix), the mirVana miRNA Bioarrays v2 (Ambion), the miRNA 4X2K Microarray (Combimatrix) and the NCode Multi-Species miRNA Microarray v2 (Invitrogen).

Several previous studies have used microarrays to identify *miR-34* and *miR-215/miR-192* as direct p53 targets. A custom-made array was used to identify *miR-34*a as a p53 target gene [20], a 4X2K Microarray (CombiMatrix) that contained probes against mouse miRNAs identified *miR-34b/c* as a p53 target gene [21] and customized miRNA arrays were used to detect *miR-34a* [17] and *miR-192/miR-215* [41] as p53 target genes. More recently, two studies employed miRNA microarrays to identify members of the *miR-200* family as p53 targets [37, 38].

In addition, induction of mature miRNAs after p53 activation can be measured by stem-loop RT-qPCR assays. Hannon and colleagues used a panel of 145 TaqMan assays to monitor changes in mature miRNA levels after p53-activation [18]. This approach may also be used to verify the microarray expression data at the level of individual, processed miRNAs. In order to determine, whether p53 regulates miRNA expression at the transcriptional level, induction of the pri-miRNA transcript can be measured using total mRNA preparations after reverse transcription into cDNAs and standard real-time quantitative PCR (qPCR).

A subset of miRNAs lie within intronic sequences of host genes, and therefore differential expression of the host mRNAs can in principle be monitored by standard gene expression arrays used for mRNAs. However, induction of the primary host transcript does not necessarily lead to a significant induction of the mature miRNA. Therefore, the induction of the mature miRNA should be validated by stem-loop RT-qPCR assays. The above mentioned methods have in common that they only detect previously known miRNAs.

For the unbiased detection of all miRNA expressed in a certain state several Next Generation Sequencing (NGS) based approaches are currently

being used. Small RNAs are isolated, ligated to adapters, reverse transcribed and amplified to generate libraries, which may be analysed using different NGS platforms, e.g. Solexa-sequencing (Illumina), 454-sequencing (Roche) or the SOLID system (Applied Biosystems). The adapters often contain distinct bar-codes, which allow multiplexing of several samples in one sequencing run generating up to several hundred million reads. The coverage which can be achieved by these analyses is presumably close to complete. In 2007 we reported a 454-sequencing approach to identify *miR-34a* as direct p53 target [16]. Although only ~200,000 sequencing reads per run were reached at that time, these were sufficient to identify many of the miRNAs displaying the most pronounced regulation by p53.

Since p53 may enhance the synthesis of miRNAs via directly influencing pre-miRNA processing the detection of differential expression of the mature miRNA is not sufficient to deduce a direct transcriptional regulation of the corresponding pri-miRNA by p53 [48]. Therefore, it is advantageous to obtain both miRNA and pri-miRNA profiles simultaneously in order to distinguish transcriptional from other modes of miRNA abundance regulation by p53.

5.4.2 Confirmation of Direct Regulation by p53 Using ChIP Approaches

The detection of p53 occupancy at the respective promoters of the genes encoding p53-regulated pri-miRNAs or other pre-cursor mRNAs can be achieved by chromatin-immunoprecipitation (ChIP) based techniques. These can either be performed on a gene-by-gene basis using qPCR-ChIP or on a genome-wide level by coupling ChIP with techniques as NGS, SAGE or hybridization to a promoter array. The disadvantage of the latter method is the limitation to previously characterized promoters.

The consensus sequence necessary for p53 binding consists of two copies of the RRRCWWGYYY motif separated by a small spacer of 0–21 nucleotides (R = pyrimidine; Y = purine; W = A/T; see also Fig. 5.1). However, among the validated p53 response elements identified in p53 target gene promoters, the majority displays slight deviations from the consensus sequence, indicating a certain flexibility in p53's binding requirements. Based on the consensus motif, potential p53 binding sites can be predicted using a variety of search algorithms. For example, the p53MH algorithm [145] and the MatInspector software (Genomatix) have been applied to identify p53-binding sites in the promoters of miRNA-encoding genes. The P53MH algorithm was used to identify a p53-binding site in the *miR-34b/c* promoter [21] and in the *miR-194-1/miR-215* cluster [41], whereas the two p53-binding sites in the *miR-145* promoter were identified using the MatInspector software [45].

Initially, binding of p53 to the predicted binding site was experimentally tested *in vitro* by gel shift assays. Furthermore, in order to test the requirement of the p53 response element, the promoter region encompassing the p53 binding site or its mutant version can be placed upstream of a *luciferase* ORF or an equivalent reporter gene. The responsiveness of these constructs to p53 can then be interrogated by co-transfection with p53-encoding plasmids into mammalian cells and a subsequent reporter assay. In order to test whether p53 binds to the predicted binding site in a native chromatin environment *in vivo*, chromatin immunoprecipitation (ChIP) assays have to be performed. This can either be done on a single gene basis by ChIP followed by semi-quantitative PCR or qPCR. Alternatively, p53 binding sites can also be identified on a genome-wide scale. In the initial genome-wide binding studies, immunoprecipitated DNA from the ChIP experiment was hybridized to high-density oligonucleotide tiling arrays (ChIP-on-Chip). For example, a ChIP-on-Chip approach was used to map p53 binding sites on human chromosomes 21 and 22 and identified 48 high confidence sites [146]. These results suggested the existence of ~1,600 putative p53 sites in the human genome. Indeed, when the same approach was applied to the complete genome 1,546 p53-binding sites were identified in actinomycin D treated U2OS cells [147].

The ChIP-PET method is an extension of the ChIP-on-Chip approach and is related to SAGE [148]. Short tags derived from immunoprecipitated

DNA fragments are converted into a DNA library. After further ligations the paired end ditags form concatemeres, which are subjected to capillary sequencing. The obtained tag-sequences are subsequently mapped to the genome and quantified. The ChIP-PET method was used to monitor p53 binding across the whole genome and identified more than 500 high-confidence p53 binding sites [149]. This resource was used by other laboratories to identify p53 binding sites in the *miR-34a* and *miR-34b/c* promoters [19, 20].

The methods mentioned above are currently replaced by a combination of ChIP and NGS (ChIP-Seq). Since the new sequencing devices achieve several hundred millions reads in one run it is possible to multiplex several time-points and experimental replicas in one single sequencing run. The identification of occupied p53-binding sites in the genome may be combined with detection of histone modifications indicating active transcription units and enhancers. This allows the assignment of orphan miRNAs derived from pri-miRNA transcribed from active promoters present in the vicinity, which have not been characterized before. Furthermore, the results obtained using the expression studies described above have to be compared to the DNA binding patterns of p53 in a genome-wide manner using bioinformatics approaches (see also Fig. 5.6).

5.4.3 Identification of miRNA Targets

After obtaining a set of p53-regulated miRNAs, the next step is to identify the physiologically relevant target mRNAs of these miRNAs. We suggest the systematic identification of miRNA-regulated target genes following p53 induction by an integrated approach that involves

(A) Identification and mapping of miRNA binding sites using biochemical techniques involving RISC isolation.
(B) Testing the functionality of these binding sites in the regulation of their respective target mRNAs using either microarrays or NGS as well as dual reporter assays.
(C) Proteomic approaches to measure changes in target abundance on the protein level indicating translational regulation in cases without decrease in the corresponding mRNA.

Similarly to the identification of p53-induced miRNAs described above, these approaches ideally should be performed in parallel as they complement each other. The identification and mapping of miRNA binding sites on mRNAs provides information as to whether a miRNA directly binds to its cognate target mRNA, but does not provide information about the regulation of the bound mRNA. Conversely, microarray and proteomic approaches provide information on the regulation of a given mRNA or protein, but do not *per se* distinguish between direct and indirect targets. Therefore, a combined approach that maps binding sites of p53-regulated miRNAs on mRNAs and validates the functionality of these binding sites regarding target regulation may comprehensively uncover the network of protein expression that is regulated by p53-induced miRNAs.

MiRNAs typically regulate their targets via association of a ~7 nucleotide stretch, the so-called seed-sequence, located in their 5′-portion with a complementary sequence in the 3′-UTR of the target mRNA. Additional base pairing may occur via nucleotides in the middle and 3′-portion of the miRNA. Since miRNAs only pair imperfectly with their respective target mRNAs, the number of theoretically possible targets is typically large and presumably most of the predicted targets are not significantly regulated by the respective miRNA. Several bioinformatics algorithms have been developed to predict miRNA targets with the intention to reduce the rate of false positive predictions by incorporating features as conservation between species. However, even these algorithms often predict hundreds of target mRNAs for a particular miRNA, most of which are presumably false positive hits.

Due to differences in the parameters used to weigh individual features involved in miRNA/mRNA interaction, different target prediction algorithms often result in only partially overlapping sets of predicted target genes. The algorithms TargetScan and Pictar [150, 151] place more

weight on perfect, evolutionarily conserved seed matches, whereas PITA and RNA22 [152, 153] prioritize the ΔG of the miRNA/mRNA duplex and the accessibility of the site within the mRNA. Although algorithms like TargetScan and Pictar have been shown to have high predictive power when tested on experimentally obtained proteomic data [154–156], they may be less useful in the prediction of miRNA target sites that lack a perfect seed-sequence, are not evolutionarily conserved, or lie outside the 3′-UTR of the target gene. Therefore, the combined use of several different algorithms may be helpful to identify target mRNAs of a given miRNA.

The sets of predicted target mRNAs generated by different algorithms are typically being used to filter sets of differentially regulated genes that were identified by experimental perturbation of miRNA function, followed by unbiased genome- or proteome-wide measurements of changes in mRNA or protein abundance. As outlined in Fig. 5.5, miRNA binding sites can be mapped by isolation of miRNA target mRNAs via the association of RISC/miRNA/mRNA-complexes. This is typically accomplished by immunoprecipitation of RISC components such as Ago2, which can either be done via endogenous proteins or ectopically expressed epitope-tagged versions of the respective proteins [157–159]. The RISC/mRNA/miRNA complexes are precipitated and the associated mRNAs are identified either by hybridization to microarrays or by NGS technologies. However, this method does not directly lead to the identification of the actual miRNA binding site, since all RISC-bound mRNAs containing different miRNAs and their targets are immunoprecipitated and sequenced.

An improved version of these initial approaches is high-throughput sequencing of RNAs isolated by crosslinking and immunoprecipitation (HITS-CLIP) [160]: miRNA-bound RNAs are cross-linked to RISC by UV irradiation. The RISC/miRNA/mRNA complex is then immunoprecipitated with antibodies against RISC components such as Ago2. A RNAse-digest eliminates all mRNA fragments not protected by the RISC/miRNA complex. All miRNA seed-matching regions occupied by miRNA/RISC complexes are determined by NGS. Thereby, information is obtained not only regarding the bound mRNA target but also concerning the miRNA matching sequence, which allows to deduce the putative identity of the miRNAs. In the case of p53-induced miRNAs these miRNAs should be among those which are detected at increased levels after p53 activation.

In another version of an AGO2-IP based approach, named photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP), cells are cultured with photo-reactive 4-thiouridine before UV-cross-linking [161]. 4-thiouridine is incorporated into the cellular RNA during transcription and leads to improved protein/mRNA cross-linking efficiencies. Since 4-thiouridine results in C-to-T transitions in the regions previously protected by AGO2/RISC complexes during reverse transcription, it allows to map the position of miRNA/RISC binding on the mRNA.

However, none of these approaches have been specifically applied to identify mRNA targets of p53-induced miRNAs yet. Furthermore, as all these approaches essentially rely on the isolation of the RISC complex, all miRNAs and their bound mRNA targets associated with RISC will be identified. Therefore, identification of mRNA targets of a particular miRNA from the obtained NGS data largely depends on the subsequent extraction of sequence features associated with that particular miRNA, i.e. either the presence of a hexameric seed sequence or the presence of other sequence features predicted to be targeted by miRNAs by algorithms, such as PITA or RNA22. A more direct, alternative approach involves the use of biotinylated miRNAs, which can be purified together with RISC in a tandem affinity purification approach [162, 163]. However, this approach may have limitations as the high concentrations of biotinylated miRNAs reached after transfection may result in false positive results.

As explained above, information on the miRNA binding site does not automatically mean that this particular binding site is physiologically relevant for target regulation. Therefore, miRNA-induced changes in either mRNA or protein abundance have to be confirmed by perturbation of

miRNA expression. Experimental studies to identify target mRNAs of p53-regulated miRNAs should involve ectopic expression of miRNAs either by transfection of synthetic pre-miRNA molecules or inducible expression of pri-miRNA transcripts [16, 19, 23]. Furthermore, synthetic miRNA inhibitors (antagomirs) can be used to block miRNA function. Alternatively, and more elegantly, knock-out cell lines for individual miRNAs can be used to address this question. In addition, HCT116 DICER^{ex5}, a human colorectal cancer cell line harboring a hypomorphic *DICER* allele [164], has been used to validate the regulation of targets of p53-regulated miRNAs [18, 42].

A number of studies applied microarrays to identify targets of p53-induced miRNAs. For example, in the case of miR-34 [17–19, 165] and miR-215/miR-192 [42] mRNA expression profiles were generated after ectopic expression of the respective miRNA. However, mRNA-profiling based approaches are limited as they cannot detect miRNA targets that are solely regulated at the level of translational repression. On the other hand, assuming that miRNAs in most cases only cause modest decreases in protein translation, the miRNA-mediated regulation of proteins with long half-lives may not be detected by measuring steady-state protein levels using standard proteomic quantification as SILAC (***s***table ***i***sotope ***l***abeling by ***a***mino acids in ***c***ell culture) [166]. This problem was solved by the introduction of pSILAC (***p***ulsed SILAC), which facilitated the quantification of differences in protein translation rates caused by miRNAs [156]. With this approach, induction of miRNA expression is followed by a pulse of isotope-labeled amino acids which are incorporated into newly synthesized proteins. Subsequent mass spectrometric analysis of the proteome therefore allows to detect changes in protein translation rates caused by miRNA expression. In a recent study we applied this approach to identify target genes of the miR-34a miRNA [23]. Notably, numerous of the identified miR-34a targets were confirmed in an miRNA capture approach using biotinylated miR-34a as a bait [24]. Other quantitative proteomic methods like isotope-coded affinity tag (ICAT)-labeling following transfection with miR-34a have been used to identify miRNA targets [167]. One major drawback of all proteomic methods is their still limited ability to cover the entire proteome of the cell, as well as their strong bias for highly expressed proteins.

All transcriptome- or proteome-wide approaches to identify miRNA targets require subsequent validations such as qPCR or Western blot analyses to verify that a given mRNA or protein is indeed regulated following miRNA induction.

Direct regulation by a miRNA is often determined in dual-reporter assays. For this the 3′-UTR of the putative target mRNA is placed downstream of a *firefly* luciferase reporter gene. This reporter-construct is co-transfected either with miRNA mimics or miRNA inhibitors, and a *Renilla* luciferase vector for standardization. In case of specific, direct regulation the 3′-UTR reporter is repressed by ~20–80 %. In order to map and validate the seed-matching sequences these should be mutated in the context of the 3′-UTR sequence. The resulting constructs should ideally show resistance towards the respective miRNAs.

5.4.4 Follow-Up Analysis

Once p53-mediated regulation of miRNAs and their targets have been confirmed numerous additional analyses are possible to interrogate the physiological and pathophysiological relevance of the identified regulations. Co-expression of the p53-induced miRNA and a miRNA-resistant target mRNA can be used in rescue-experiments to determine the relevance of the respective down-regulation for cell biological phenotypes, as cell cycle arrest and/or apoptosis. Furthermore, the relevance of the respective miRNAs for p53-mediated effects can be tested using antagomirs specific for the respective miRNA. Finally, the importance of miRNA/target regulations for p53-mediated tumor suppression can be tested in miRNA knock-out mice in combination with tumor mouse models. However, these studies may

take years. A recently published collection of ES cell lines with deletion of 392 miRNAs was generated to facilitate the rapid generation of knock-out mice and may therefore accelerate this type of analysis [168]. Furthermore, the inactivation of the respective miRNA encoding genes by CpG methylation or mutations in different tumor types may be analyzed. The miRNA inactivation can be correlated with the putative up-regulation of miRNA targets in the affected tumor samples and pathological features of the affected tumors. Detection of CpG-methylation and miRNA/target expression may also have prognostic and diagnostic value for cancer patients in the future.

5.4.5 Outlook

In the future technological developments may result in an increased sensitivity of mass-spectral analyses which could facilitate similar coverage rates of proteomic quantifications as are now reached by DNA-sequencing/hybridization based approaches. Furthermore, the integration of different bioinformatics platforms into a common program for mRNA/miRNA/DNA binding and protein quantification would make integrated analyses less complicated and laborious. Another useful tool would be a comprehensive ontology-like database for miRNA functions and targets. The miRo website is an example of such a tool [141]. In the future, more publicly available datasets of miRNA expression in cancer patient cohorts which allow to determine correlations with mutations, epigenetic changes and clinical data will become available. Taken together, these possibilities will hopefully lead to the rapid translation of knowledge derived from analysis of the p53/miRNA network into diagnostic and therapeutic applications.

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