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Perspective

Unmet Expectations: miR-34 Plays No Role in p53-Mediated Tumor Suppression In Vivo

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In vivo modeling of tumor suppressor p53 functions and regulation has a history of unexpected and even enigmatic outcomes [1], despite the status of p53 as the most frequently mutated gene or dysfunctional pathway in human cancers [2,3]. Beginning with the surprising viability of the first mice deleted for *Trp53* [4,5], various hypotheses of compensation, cell type–specificity, stimulus-dependent response, or modifier influences were posed to explain how an exquisitely regulated transcription factor, implicated in a vast array of pathways [6], appeared to have no impact on development. Limited background-specific developmental and fertility problems do occur, especially in female p53-null mice [7,8], and deletion of potentially compensatory p53 family members, p63 and p73 isoforms, leads to profound developmental and tissue-specific phenotypes [9,10]. But overall, the most striking result of p53 loss in vivo is early tumor predisposition in p53$^{-/-}$ mice, which lack genomic surveillance provided by p53-mediated regulation of cell cycle arrest, apoptosis, and senescence.

As reported by Concepcion et al. in this issue of *PLoS Genetics* [11], expectations built on cell-based studies of p53 response are again unrealized in mouse models. Previously, multiple in vitro analyses suggested that microRNA (miR)-34 family members are important players in a p53-regulated network of genomic surveillance [12–17] (Table 1). Together, these studies strongly supported the view that p53 response to multiple stimuli depended on miR-34, and that ectopic expression of miR-34 was sufficient to elicit p53 response, consistent with miR-34 functioning as a bonafide tumor suppressor. However, Concepcion et al. report that complete inactivation of the entire family of miR-34 genes (miR-34a/b/c) or knockout of each individual miR-34 gene in mice leads to little or no change in p53-mediated functions in tumor suppression [11].

Interest in a miR-34 axis as mediator of p53-response begins with the niche that miRNAs fill in regulation of RNA expression. miRNAs are small, regulatory non-

coding RNAs that generally mediate post-transcriptional silencing of a number of specific target mRNAs [18]. More than 50% of human miRNA genes are found within cancer-associated or fragile sites of the genome, which suggests that miRNAs play essential roles in tumorigenesis [19]. The identification of miRNAs as regulatory targets of p53 [20] suggested their potential involvement in tumor suppression, and expanded the repertoire of p53 downstream targets to both coding and non-coding genes. Further, the view that p53 both positively and negatively regulates gene expression could now rely on increased expression of miRNAs as a mechanism for p53-mediated, indirect repression of gene expression [13,20], in addition to the few documented cases of direct repression by p53 binding to chromatin [21–25].

The members of the evolutionarily conserved miR-34 family, which arise from three different transcripts at two different gene loci in vertebrates, were the first of several non-coding RNAs identified as directly activated by p53 in response to genotoxic stress [13,26]. miR-34a is at 1p36, a region commonly deleted in tumors, and miR-34b and miR-34c share a common primary transcript arising from 11q23 [27,28]. miR-34a, b, and c are expressed at very low levels in several types of cancers [28]. Previous reports show that p53 directly activates miR-34a/b/c expression and, dependent on cellular context, they act downstream of p53 in mediating cell cycle arrest or apoptosis [29]. The current list of validated miR-34 downstream targets includes several genes that are repressed

during cell cycle arrest or apoptosis when p53 is activated [28].

Given the rationale provided by these studies in cultured cells (Table 1), multiple laboratories created genetic knockout models of either miR-34a or miR-34b/c, or a compound mutant animal harboring homozygous deletion of all three miR-34 family members (miR-34^{TKO}) [11,30]. Surprisingly, mice bearing the miR-34 deletion(s) developed normally, are born at the expected Mendelian ratio, and are fertile [11]. The authors subjected the mice and derived mouse embryonic fibroblasts (MEFs) to a battery of tests to assess any impact on p53-dependent tumor suppression. MEFs obtained from mir-34^{TKO} mice have a slightly higher proliferation rate, but reach senescence with kinetics similar to wild-type MEFs. In response to genotoxic threats, miR-34–deficient MEFs are indistinguishable from wild type: they undergo p53-dependent cell cycle arrest and apoptosis. With ectopic expression of oncogenic K-Ras, p53-deficient MEFs are readily transformed, which is not true of K-Ras–expressing miR-34$^{-/-}$ MEFs.

In the intact mouse, the story is similar: aging cohorts of mir-34^{TKO} mice remain healthy with no spontaneous tumors, in contrast to p53-null mice [4]. In fact, miR-34–deficient mice remain remarkably healthy and tumor-free for at least 60 weeks after irradiation. Assays of apoptosis in response to irradiation proved positive in tissues of these mice, which additionally exhibited no acceleration of tumor progression in Eμ-models of B-cell lymphomagenesis. All of these assessments of p53 functions in vivo undermine the view that

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Table 1. A list of different in vitro and in vivo model systems used to study miR-34 functions.

Model System	Description	miR-34	Functional Outcome	Ref.
mESCs	Mouse embryonic stem cells	Genetrap-mediated deletion	Decreased spontaneous apoptosis during differentiation	[12]
NSCLCs	Non-small cell lung cancer cells	Overexpression	Inhibits growth	[12]
SW480	p53 mutant colon cancer cells	Overexpression	G1-arrest	[12]
Wi38	Human diploid fibroblasts	Depletion	Protection from Staurosporine-induced apoptosis	[12]
IMR90	Primary lung fibroblasts	Overexpression	Growth inhibition (G1 and G2 arrest), and senescence	[13]
A549	Human alveolar adenocarcinoma cells	Overexpression	G1-arrest	[13]
HCT116	Human colon cancer cells	Overexpression	G1-arrest	[13]
TOV21G	Human ovarian cancer cells	Overexpression	G1-arrest	[13]
MEFs	Mouse embryonic fibroblasts	Overexpression	Apoptosis	[13]
H1299	Human lung cancer cells	Overexpression	Reduced colony formation	[14]
U2OS	Human osteosarcoma cells	Depletion	Reduction in Etoposide-induced apoptosis	[14]
HCT116 ($p53^{+/+}$ or $p53^{-/-}$)	Human colon cancer cells	Long-term overexpression	Apoptosis	[15]
HCT116, RKO	Human colon cancer cells	Overexpression	Suppression of proliferation and induction of senescence	[16]
Mouse xenograft model	HCT116 or RKO cells were inoculated into nude mice	Subcutaneous administration of miR-34a/atelocollagen complexes	Suppression of cell proliferation and reduction in tumor volume	[16]
H1299	Human lung cancer cells	Overexpression	Apoptosis	[17]
U2OS	Human osteosarcoma cells	Overexpression	G1-arrest and reduction in colony formation	[17]
MiaPaCa2, BxPC3	p53 mutant human pancreatic cancer cell lines	Overexpression	Inhibited clonogenic cell growth and invasion, induced apoptosis and G1 and G2 arrest; sensitized the cells to chemotherapy and radiation	[34]
OSN1, OSN2	Neoplastic epithelial ovarian cells	Overexpression	Suppression of proliferation and reduced colony formation	[35]
Kelly, NGP	Neuroblastoma cells with MYCN amplification (+MNA)	Overexpression	Reduction in proliferation and increased apoptosis	[36]
SK-N-AS	Neuroblastoma cells without MYCN amplification (-MNA)	Overexpression	Reduction in proliferation and increased apoptosis	[36]
Mouse $mir34a^{-/-}$; $mir34b/c^{-/-}$	miR-34 knockout mouse in C57BL/6 background	Germline deletion of miR-34	Efficient reprogramming, no effect on proliferation	[30]
Mouse mir34^TKO	miR-34 knockout mouse in129SvJae and C57BL/6 mixed background	Germline deletion of miR-34	Normal p53 activity	[11]

miR-34 functions as a tumor suppressor or is an essential component of the p53-tumor suppression network.

Although miR-34 proved nonessential in the most highly studied examples of p53 function (senescence, cell cycle arrest, apoptosis, and tumor suppression), it remains possible that miR-34 is involved in other p53-influenced processes, such as metabolism, autophagy, stem cell quiescence, differentiation, and embryogenesis [6]. For example, specific links between miR-34– and p53-regulated functions have been forged in stem cells [26]. miR-34–deficient MEFs are more efficiently reprogrammed to induced pluripotent stem cells (iPSCs), by expression of pluripotency factors and c-myc [30], compared to wild-type counterparts. While this study of miR-34 as a barrier to reprogramming does not establish a direct tie to p53, it complements multiple reports that depletion of p53 or dysfunctional p53 pathways enhance the efficiency of reprogramming differentiated, somatic cells to iPSCs [31]. Recently, we showed that p53 promotes human embryonic stem cell differentiation by direct activation of p21 and miRNAs, including miR-34a, which repress pluripotency factors and SIRT1 [32]. Taken together, these results indicate that miR-34 has pro-differentiation effects in maintenance of nontransformed, somatic cells, some of which are p53-dependent.

In the future, miR-34–deficient mouse models will be valuable in addressing whether miR-34 functions downstream of p53 in a tissue- and/or context-specific manner. miR-34a, miR-34b, and miR-34c share the same seed sequence and target the same RNAs, although differences in target accessibility or binding affinities may dictate their effectiveness. Genome-wide expression analysis may be needed to determine family member–specific effects, such as the reported regulation of c-MYC by miR-34b/c and not miR-34a [33]. Questions of specificity in gene targets for each member of a miRNA family and potential compensation by other miRNAs may be addressed by studies in these and other miRNA mouse models, perhaps still under development. Non-coding RNAs are thought to act in networks that impact diverse cellular pathways, suggesting considerable challenges ahead in asking the right questions and understanding the functional significance of these RNAs.

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