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MicroRNA-449 in cell fate determination

Muriel Lizé,[1,*] Alexander Klimke[2] and Matthias Dobbelstein[1,*]

[1]Department of Molecular Oncology; Göttingen Center of Molecular Biosciences (GZMB); Ernst Caspari Haus; University of Göttingen;
[2]Research Group Developmental Biology; Max Planck Institute for Biophysical Chemistry; Göttingen, Germany

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The microRNAs 449a, b and c (miR-449) are potent inducers of cell death, cell cycle arrest, and/or cell differentiation. They belong to the same family as the p53-responsive microRNAs miR-34. Instead of p53, however, the cell cycle regulatory transcription factor E2F1 induces miR-449. All members of this microRNA family are capable of mediating cell cycle arrest and apoptosis and might thereby contribute to tumor suppression. Underlying mechanisms include the downregulation of histone acetyl transferases and consecutive activation of p53, but also the targeting of cyclin dependent kinases and their association partners. Thus, miR-34 and miR-449 provide an asymmetric feedback loop to balance E2F and p53 activities. More recently, it was discovered that miR-449 displays strong tissue specificity, with high levels in lung and testes. Two model systems (Xenopus embryos and cultured human cells) revealed that miR-449 is essential for the development of ciliated epithelia, and this appears to depend on miR-449-mediated modulation of the Notch signaling pathway. Here we summarize our current knowledge on cell fate determination by miR-449, and we propose future directions to explore the function of miR-449 in cell regulation and organismal development. MiR-449 helps to ensure proper cell function but also to avoid cancer, marking a close link between cell differentiation and tumor suppression.

MicroRNAs

MicroRNAs (miRNAs) are small, non-coding RNAs representing a novel class of regulators for gene expression. Many miRNAs are encoded within regular protein-coding genes.[1-5] Some microRNAs are ubiquitously distributed, others are expressed only in specific tissues.[6,7] They act as regulators of mRNA stability and/or protein synthesis through specific hybridization of their "seed sequences" (region from base 2 to base 8 of a mature microRNA) with mRNA target sequences, allowing each microRNA species to regulate a characteristic set of transcripts.[8-10] The synthesis of their precursors resembles that of mRNAs, starting with the polymerization of a microRNA precursor called pri-miRNA through the RNA polymerase II. The specific nucleases Drosha and Pasha ("Partner of Drosha" or DGCR8, DiGeorge syndrome critical region 8) then recognize and cleave the stem-loop portion of the pri-miRNA to produce the pre-miRNA which is then transported to the cytoplasm for further processing through the ribonuclease Dicer. Mature microRNAs are around 20 bases long and can target mRNAs for degradation (perfect complementarity) or translational repression (imperfect match).[10-13] Unfortunately, the ability of miRNAs to regulate translation impedes the accurate prediction of targets by mRNA quantitation alone. Computational predictions of targets are available but do not always reflect the in vivo situation. microRNAs have been shown to be essential for cell fate determination, e.g., in the differentiation of the hemapoietic lineages.[14] An important question of cell biology is how specific microRNAs affect cell fate, e.g., differentiation and apoptosis.

*Correspondence to: Muriel Lizé and Matthias Dobbelstein;
Email: mlize@uni-goettingen.de and mdobbel@uni-goettingen.de
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The miR-34/449 Family of MicroRNAs

The characterization of the miR-34 family of microRNAs started with the discovery of miR-34a as a p53-responsive gene capable of inducing apoptosis and cell cycle arrest in tumor cell lines.[15-18] MiR-34a is encoded separately; its homologs miR-34b and c share a common primary transcript. MiR-34 a, b and c are abbreviated as miR-34 from here on. MiR-34 targets the histone deacetylase SIRT1,[19-21] leading to the accumulation of acetylated and therefore highly active p53. Additionally, miR-34 downregulates several cyclin-dependent kinases (CDK), cyclins and E2Fs,[22,23] leading to the inhibition of the E2F pathway, and to cell cycle arrest (**Table 1**).

Later on, the miR-449 cluster, encoding the highly conserved miR-449a and miR-449b (and the much later described miR-449c), was found to contain similar sequences and secondary structures as the miR-34 family, and they were therefore classified as one family of microRNAs. These microRNAs will be named miR-449 from here on. In particular, they share the same seed sequence, suggesting similar mRNA targets.[8,24-27] In line with the tumor-suppressive role of miR-34,[18,28] miR-449 was shown to be downregulated in cancers of prostate and stomach in comparison to the corresponding normal tissue.[29,30] The expression of miR-449 was first described in embryonic mouse brains.[31]

In the human genome, the miR-449 cluster is located on chromosome 5 in a highly conserved region within the second intron of the CDC20B gene, a homolog of CDC20. CDC20 is an activator of the anaphase promoting complex (APC) during mitosis[32] but it is currently unclear whether the same is true for the miR-449 host CDC20B. The CDC20B gene is largely conserved

Table 1. Previously described target mRNAs of the miR-34/449 family and their effect on cell fate

Targets	Reported miRNA regulators	Affected signaling pathways	Cell fate when downregulated
BCL-2 (B-cell CLL/Lymphoma 2)	miR-34[134]	Intrinsic apoptosis[135]	Apoptosis[135]
CCND1 (Cyclin D1)	miR-34[22]	Rb/E2F[136]	Cell cycle arrest[136]
CCNE2 (Cyclin E2)	miR-449[30] and miR-34[137-139]	Rb/E2F[136]	Cell cycle arrest[136]
CDC25A (Cell division cycle 25A)	miR-449 target[35] and miR-34[139]	Rb/E2F[136]	Cell cycle arrest[136]
CDK4 (Cyclin-dependent kinase 4)	miR-34[139]	Rb/E2F[136]	Cell cycle arrest[136]
CDK6 (Cyclin-dependent kinase 6)	miR-34 and miR-449[22,35,139]	Rb/E2F[136]	Cell cycle arrest[136]
c-Myc	miR-34[140]	E2F and p53[141]	Cell cycle arrest, senescence[140]
DLL1 (Delta-like 1)	miR-34 and miR-449 predicted,[93] miR-449 confirmed[38]	Notch[142]	Differentiation[142]
E2F3 (E2F transcription factor 3)	miR-34[139,143]	E2F[144]	Cell cycle arrest[144]
E2F5 (E2F transcription factor 5)	miR-449[55] and miR-34[143]	E2F[144]	Differentiation[55]
GMNN (Geminin)	miR-449[30]	Cell cycle,[145] replication[146]	Apoptosis,[147] differentiation[148]
HDAC1 (Histone deacetylase 1)	miR-449[95]	p53, chromatin structure and general transcription[149]	Differentiation, apoptosis[97,149]
HDMX (Mouse double minute 4 homolog; MDM4)	miR-34[150]	p53, Ras[151]	Senescence[151]
HMGA2 (High mobility group AT-Hook 2)	miR-34[134]	Chromatin structure[74]	Differentiation[74]
MET (Met protooncogene; hepatocyte growth factor receptor; HGFR)	miR-34[28,138] and miR-449[30]	Notch, STAT and Ras/MAPK; invasion[152]	Apoptosis, cell cycle arrest[152]
N-Myc (V-MYC avian myelocytomatosis viral-related oncogene, neuroblastoma-derived; MYCN)	miR-449[153]	E2F and p53[141]	Differentiation, apoptosis[141]
Notch1 (Drosophila notch homolog 1)	miR-34[28,134] and miR-449[38]	Notch[142]	Differentiation[142]
SIRT1 (Sirtuin 1)	miR-34[19] and miR-449[37]	p53, chromatin structure and general transcription[154]	Apoptosis,[154] senescence[155]

in vertebrates, which may reflect its importance. Interestingly, CDC20B was detected in lung tissue and airway epithelia, suggesting a role for miR-449 and/or its host CDC20B in such tissues. For instance, CDC20B was found upregulated after virus infection in the trachea of chicken[33] and, most notably, its expression is induced more than 180-fold during human mucociliary differentiation.[34]

We recently reported, in parallel to other groups, that miR-449 expression is under the control of the S-phase promoting transcription factor E2F1,[35,36] and that miR-449 is able to induce apoptosis in tumor cell lines regardless of their p53 status,[36] a tumor suppressor often deregulated in cancer. We additionally found miR-449 to be expressed at high levels and specifically in testicular and pulmonary tissues. Its expression levels correlated with the differentiation of bronchial epithelia. Moreover, the treatment of aeroepithelial cells with DNA-damaging tobacco smoke led to a further induction of miR-449.[37] This suggests that miR-449 is a tumor suppressive microRNA playing a role in the proper differentiation of airway epithelium, and in the first line defense of the respiratory tract against toxic agents. A recent publication by Marcet et al. provided evidence that miR-449 is required for the differentiation of ciliated cells in human airway epithelia, and in epidermal cells of *Xenopus laevis*. This report further suggested that miR-449 controls ciliary differentiation through the regulation of the notch pathway. Additionally, they found the miR-449 host gene product, CDC20B, to be localized in multiciliated cells, near the basal bodies where the cilia arise from. The fact that miR-449 and its host gene CDC20B are coregulated argues in favor of a function of CDC20B in the same tissues. However, the function of this gene product in differentiation, as well as its potential tumor suppressive role, remains to be determined.

In this review, we will now discuss the role of the miR-34/449 family of microRNAs in cell fate determination, especially in ciliary differentiation, cell cycle and apoptosis.

MiR-449—A Prerequisite for the Differentiation of Ciliated Cell Progenitors

Due to their "open" nature and their proximity to the environment, airways are exposed to many risks. The air we breathe transports viruses, bacteria, small particles, smoke, solvents, toxins etc. These are potential threats to the respiratory tract and the integrity of the cells it is made of, especially to its surface, the bronchial epithelium. To allow proper respiration and host defense, epithelial cells within the respiratory system must differentiate in a highly ordered fashion.[39-41] Particularly, the airway epithelium covering the trachea and bronchia must ensure proper air flow, prevent the loss of fluid, and contribute to mucociliary clearance and host defense by avoiding the accumulation of toxic substances from the environment. Thus, it acts as a protective

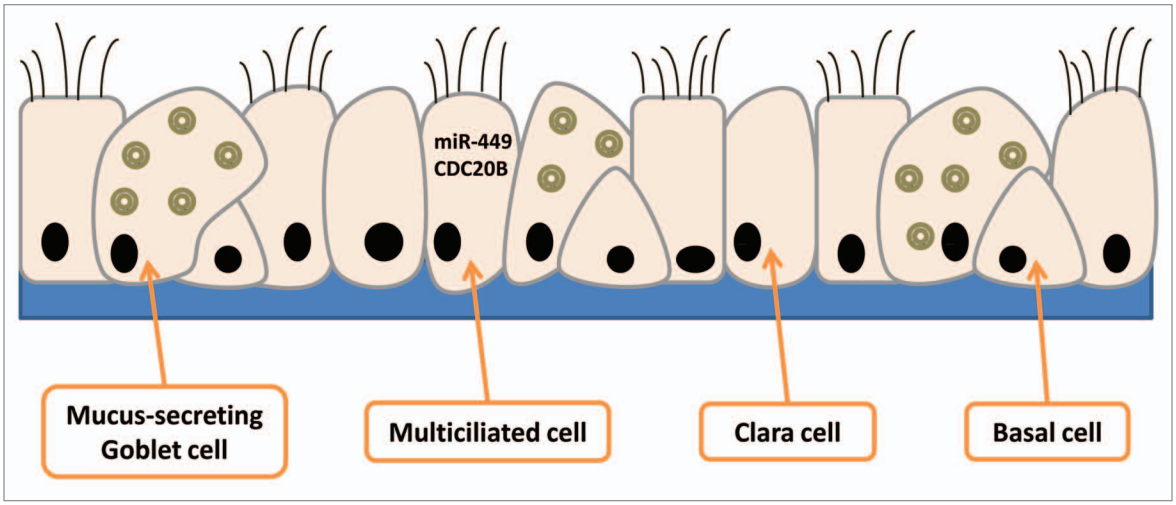

Figure 1. Airway epithelium. The bronchial epithelium is a pseudostratified epithelium consisting of Goblet, basal, Clara and ciliated cells (inspired by the review by Maeda et al. 2007). MiR-449, CDC20B and FoxJ1 are expressed specifically in the ciliated cells.

barrier. This epithelium forms a pseudo-stratified layer consisting of basal cells; Goblet cells for mucus secretion; Clara cells with helper functions in secretion, detoxification and cell renewal; and ciliated cells (**Fig. 1**). The latters are responsible for the mucociliary transport, the most important mechanism of defense in this tissue. Defects in mucociliary clearance are associated with severe respiratory disorders like cystic fibrosis or chronic obstructive pulmonary diseases (COPD).[42]

The process of mucociliary differentiation starts during the pseudo-glandular phases of lung development (around E12 in the mouse) and continues until lung maturity (P5-P20 in the mouse).[43] The differentiation of the airway epithelia occurring shortly before and after birth is essential for survival and is accompanied by spectacular changes in cell function. However, the posttranscriptional mechanisms regulating the proper progression through this process are only partially understood at this stage. The differentiation of bronchial epithelia can be recapitulated in vitro. Human primary bronchial epithelial cells (aero-epithelial cells or AECs) can be maintained in a monolayer culture. The subsequent lift of AECs from a liquid environment to the air-liquid interface (ALI) initiates a 21 d long mucociliary differentiation program, reflecting the physiological processes occurring in the lung.[44] By analyzing the changes in gene expression happening during this period, a number of differentially regulated genes were previously identified, including the transcription factor FoxJ1,[44-46] the miR-449 host gene CDC20B,[34] and miR-449 itself[37] (**Fig. 2B**). The spatiotemporal expression of the transcription factor FoxJ1 strikingly correlates with ciliogenesis.[47] Foxj1 mutant mice display a complete absence of motile cilia, a random determination of left-right asymmetry, and mislocated basal bodies. Thus, Foxj1 is required early for ciliogenesis, already for the proper cellular localization of the basal bodies.[45,46,48-52] A newly published study shows that miR-449 expression not only correlates with mucociliary differentiation, but also regulates this process by directly targeting the notch pathway.[38] In this study, the mucociliary epidermis of Xenopus embryos served as model for mucociliary epithelia, because mammalian bronchi and frog skin display a similar mix of mucus-secreting goblet cells and ciliated cells.[53]

Consistent with the idea of miR-449 being expressed mostly in ciliated cells, miR-449 and its host gene CDC20B were found almost exclusively in tissues containing high amounts of cilia, e.g., the brain (choroid plexus, ependymal cilia), the reproductive systems (female reproductive tract: fallopian tube cilia; male reproductive tract: sperm flagella, epididymis stereocilia), the respiratory tract, and the mucociliary epidermis found in xenopus embryos.[37,38]

Cilia are filamentous organelles attached to the cell surface. They are assembled when cells exit the cell cycle to differentiate. In humans, cilia are classified into four main types depending on their features, motile vs. non-motile and mono- vs. multiciliated (reviewed by Fliegauf et al.). The bronchial epithelium harbors multiciliated cells, these cilia being motile. Therefore, it was hypothesized that miR-449 is being expressed in multiciliated cells only.[38] However, we showed that miR-449 is expressed at high levels in human and murine testis,[36] using RNA preparations devoid of the multiciliated cells-rich epididymis. Thus, miR-449 might be strongly expressed also in non-multiciliated cells, and their precise identity remains to be determined.

Additional studies found miR-449 expression in the mouse brain,[31] more precisely in the choroid plexus.[55] The choroid plexus produces the cerebrospinal fluid, and it is covered by a layer of ciliated epithelial cells responsible for its proper transport. Dysfunctional cilia on the choroids plexus result in hydrocephalic mice.[56] Moreover, normal ciliogenesis was shown to be a requirement for correct neural tube closure, presumably because cilia are required for the establishment of a correct planar cell polarity, linking ciliated cells to brain development.[57-61]

It is tempting to speculate that miR-449 governs the differentiation of several types of ciliated cells; therefore it would remain of interest whether other tissues containing ciliated cells also contain functional miR-449. Candidates include the photoreceptor

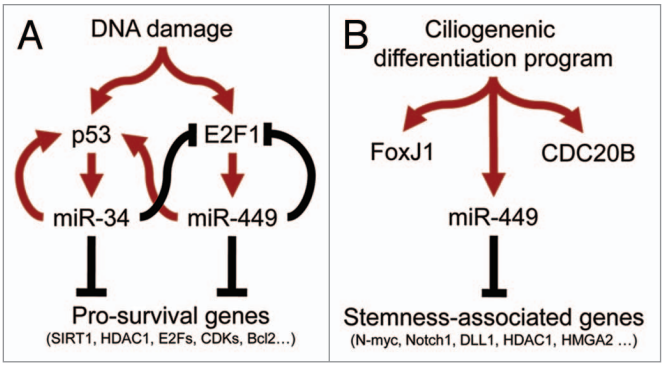

Figure 2. MiR-449 in the regulation of cell fate in the context of (A) DNA damage (original model shown in Lizé, Cell Death and Differentiation 2010) and (B) Ciliogenesis.

connecting cilia of the eye, the sensory stereocilia of the inner ear, or the renal or pancreatic cilia. A role for miR-449 in early development is also conceivable, as its expression might correlate with the formation of the nodal cilia of the embryo, which is crucial for the determination of the left-right asymmetry. Sensitive techniques like in situ hybridization of LNA probes,[62] or in situ hybridization with probes detecting the host gene CDC20B, on tissue sections would provide valuable information regarding this question.

MiR-449—Additional Functions Affecting Cell Fate

MiR-449 was shown to provoke cell cycle arrest in a variety of cell types, with some preference for cells expressing wild-type p53. This correlates with the downregulation of several cell cycle regulators, like the cyclin-dependent kinases (CDKs) CDK6 and CDK4, cyclins (cyclin D1, Cyclin E2), the CDK-regulating cdc25 family of phosphatases (activating CDKs by dephosphorylation of the active site), geminin (GMNN), and the E2F transcription factors E2F1, E2F3 and E2F5.[30,35,36,55,63] Under normal circumstances, the S-phase promoting transcription factors E2F1-E2F3 are activated by the hyperphosphorylation of pRb by CDKs, thereby allowing the cell to transcribe genes necessary to proceed in the cell cycle. MiR-449 prevents the cell from entering the S-phase through at least two mechanims: by downregulating E2Fs directly and by inhibiting the activity of E2F transcription factors through the reduction of CDKs. The additional induction of the p53 pathway including its target, the CDK inhibitor p21, potentiates these effects toward cell cycle arrest. Cell fate determination appears to be determined in the G_1-phase of the cell cycle, where a choice has to be made between proliferation, quiescence, differentiation, apoptosis or senescence.[64] Thus, miR-449 represents a potential regulator of cell fate.

Besides cell cycle arrest, with or without quiescence or differentiation, miR-449 can also induce apoptosis.[30,36] This happens in part through the activation of the p53 pathway by downregulating the histone deacetylases HDAC1 and SIRT1. However, miR-449 also promotes apoptosis in cells depleted of p53, even though the underlying mechanisms are not fully understood.

It appears that miR-449 induces cell differentiation or apoptosis in a mutually exclusive fashion. Differentiated bronchial epithelia harbor high levels of miR-449 without showing any sign of cell death. In contrast, the reintroduction of miR-449 kills tumor cells efficiently or drives them into a permanent arrest called senescence.[30,63] This selective proapoptotic activity would render miR-449 an attractive means to eliminate tumor cells, if an efficient delivery system was available. Indeed, a new lung cancer therapeutic approach based on the targets of miR-34 was shown to kill tumor cells efficiently.[65] Since miR-449 and miR-34 have several common targets, this argues for a potential future use of miR-449 or its targets in lung cancer therapy.

Of interest, other members of the miR-34 family have also been involved in differentiation and development. For instance, a target of miR-34, HMGA2 (high mobility group AT-hook 2), is known to be reduced in differentiated cells, and has been involved in the maintenance of an undifferentiated state in cancer.[66-68] HMGA2 is aberrantly overexpressed in 90% of lung cancer.[68,69] Notch signaling is regulated by miR-449 and has a role in development and differentiation (further discussed in the coming chapter). Also, the activation of p53 by miR-449 can contribute to the suppression of cellular dedifferentiation[70] through the consequent downregulation of the pluripotency factor Nanog.[71]

Signaling Pathways Modulated by miR-449 and Their Impact on Cell Fate

miR-449 or its homolog miR-34 regulate several targets, governing partially overlapping signaling pathways (**Table 1**). We will exemplify the best-described pathways in the context of cell fate determination.

Notch Pathway

Notch signaling (reviewed previously in refs. 72 and 73) functions between two cells via receptor-ligand interactions. There are four notch homologs in mammals, Notch1–Notch4, and five well-described notch ligands, Jagged1 and Jagged2 (JAG1 and JAG2), and delta-like 1, 3 and 4 (DLL1, DLL3–4). The binding of a ligand to its receptor leads to the cleavage of the receptor, which can then enter the nucleus and induce the expression of transcriptional repressors from the Hairy Enhancer of Split family.[74] An active Notch signaling is typically found in immature and proliferating cells.[75,76]

Notch signaling has previously been linked to ciliated cell fate. For instance, it regulates the specification of ciliated cells in the epidermis of *Xenopus laevis* embryos.[77,78] Notch signaling also controls the differentiation of multiciliated cells in the kidney through jagged2/notch3 interaction.[79,80] Finally, notch signaling is required for the differentiation of epithelial cells from progenitors into diverse cells in the bronchial epithelium.[81-83] This partially happens through direct cell-cell interaction, by a mechanism referred to as "lateral inhibition," meaning that a cell adopting a particular fate can keep its neighboring cells from doing so. The transmembrane proteins Notch and Delta are well-defined mediators of this

kind of interaction.[84] Notch-mediated lateral inhibition leads to a typical "salt and pepper" pattern of cell types.[79,85-88]

Interestingly, notch signaling was found to regulate the specification of ciliated cells in amphibian epidermis[77,78] as well as in human mucociliary airway epithelia.[83] Notch signaling appears to be relevant in lung development and lung cancer.[89] Notch proteins and their ligands are increasingly expressed during the development of the lung until adulthood;[90,91] this negatively correlates with miR-449 expression being the highest in lung before birth and gradually decreasing until adulthood.[37] Moreover, notch signaling can contribute to oncogenic ras-mediated tumorigenesis in lung cancer,[92] and miR-449 appears to be downregulated in cancer.

Not only the notch1 receptor but also one of its ligands, DLL1, represents a target of miR-449 [38] and potentially of miR-34.[93] Thus, miR-449 targets a receptor (Notch1) and a ligand (DLL1), allowing the reduction of Notch signaling and lateral inhibition from both interacting cells. Notch inactivation by miR-449 is expected to release transcriptional repression from genes necessary for differentiation; the exact targets and their precise mechanistic role remain obscure.

The E2F and p53 Pathways

MiR-449 provides a negative feedback on E2F and a positive feedback on the p53 pathway, reinforcing the E2F1-p53 interdependence. This was summarized in our recently published study.[36]

In response to DNA damage, the transcription factors p53 and E2F1, both deregulated in cancer, are activated and can induce proapoptotic genes. However, while E2F1 stabilizes p53 through the induction of $p14^{arf}$ and additionally enhances the transactivation of p53-responsive genes via the induction of the p53-homolog TAp73, p53 activation results in the inactivation of E2F1 through increased transcription of the gene encoding the CDK inhibitor p21.

MiR-449 recapitulates and enforces this asymmetric, mutual regulation. In response to DNA damage, E2F1 transactivates miR-449 which in turn activates the p53 pathway, thereby inducing the expression of miR-34 (**Fig. 2A**). Both miR-449 and miR-34 then inhibit the E2F pathway in a negative feedback loop. The attenuation of E2F activity by miR-449 occurs through several different mechanisms, pointing to the importance of this effect. First, miR-449 targets CDK2 and CDK6, two cyclin-dependent kinases responsible for the phosphorylation of pocket proteins (e.g., Rb). As a result, hypophosphorylated Rb can bind to and inactivate E2F. Furthermore, another miR-449 target, SIRT1, has been linked to cell cycle regulation through E2F inhibition, since SIRT1 deacetylates Rb.[94] A reduction of SIRT1 leads to the accumulation of acetylated, active Rb (through the obstruction of inhibitory phosphorylation sites), thereby inactivating E2F and promoting growth arrest. In p53 wild type cells, the negative regulation of E2F1 by miR-449 is further supported by the upregulation of the p53-reponsive CDK inhibitor p21. This is at least partially due to the downregulation of SIRT1, which allows the accumulation of a highly active, acetylated form of p53.[19] This p53 activation may result in the induction of apoptosis, as described earlier as a consequence of SIRT1 downregulation.[22]

However, since both miR-34 and miR-449 induce cell death in p53 deficient cells, they must also trigger proapoptotic mechanisms independently of p53. MicroRNAs commonly regulate large numbers of target genes, as demonstrated by the enormous amount of targets found by computational predictions. We expect that miR-449 also has a broad range of targeted mRNAs, and that the outcome of miR-449 expression depends on the combination of several effects with regard to the cellular context. As the majority of tumor cells have lost proper p53 activity, the mechanisms behind miR-449-mediated p53-independent apoptosis could obtain therapeutic relevance.

MiR-449 as a Regulator of General Gene Expression and Chromatin Structure

The miR-449-mediated downregulation of the histone deacetylases HDAC1 [95] and SIRT1 [36,95] can be expected to affect the expression of a broad range of genes, through chromatin modification (i.e., acetylation of histones) and regulation of various transcription factors (e.g., acetylation of p53, Sp1). Histone deacetylases play an important role in cell fate determination.[96-98]

HDACs, in contrast to microRNAs from the miR-34 family, are overexpressed in various cancers.[99-106] This mostly correlates with poor prognosis.[101,105-108] HDAC inhibitors efficiently kill a variety of tumor cells, and accordingly, HDAC inhibitors are currently subject to clinical trials.[109-112] Another type of histone deacetylases, SIRT1, is also targeted by miR-449.[36] SIRT1 is highly expressed in many cancers, and its knockdown induced apoptosis even in cells lacking wild-type p53.[113,114] The specific inhibition of SIRT1 also sensitizes tumor cells to chemotherapeutics.[115]

The mechanism leading to HDAC overexpression in cancer is not yet clear but might involve the loss of miR-449 and/or miR-34. MiR-449 is generally reduced in tumor cells, mainly through epigenetic silencing.[35,36,95] Tumor cells might be selected for miR-449 silencing since they would otherwise probably undergo cell cycle arrest or apoptosis. The fact that the transcription factor E2F1 is frequently overactive in cancer and can induce miR-449[35,36,116] may further increase the need for miR-449 silencing. The loss of miR-449 observed in cancer may contribute to enhanced expression of HDAC1 and SIRT1, thereby eventually contributing to carcinogenesis through "cell reprogramming."[117] Intriguingly, miR-449 itself is induced by HDAC inhibition.[36] Thus, miR-449 induction may contribute to cell death upon HDAC inhibitor treatment.

Hence, HDACs keep miR-449 low in tumor cells while miR-449 keeps HDACs low in non-cancerous cells. This mutual regulation might provide a "switch" for general gene expression, such as often observed in tumorigenesis vs. differentiation.

Regulation of miR-449 Expression

MiR-449 is strongly induced during the mucociliary differentiation of airway epithelium, and its expression in the murine lung is highest around birth, when this process is needed the most.[37,38]

MiR-449 expression was found to be coregulated with its host gene CDC20B in several studies,[35-37] in part through transactivation by the transcription factor E2F1. However, the strong increase of miR-449 expression observed in mucociliary differentiation can hardly be attributed to E2F1, since this factor remains unchanged in its levels during this process.[37] Therefore, the regulatory mechanisms responsible for tissue specificity and for the huge induction of miR-449 in differentiation remain largely unknown. However, it seems conceivable that transcription factors responsible for mucociliary epithelia might contribute to the miR-449/CDC20B induction seen in mucociliary differentiation.[34,37] These include FoxJ1, a forkhead transcription factor which is specifically expressed in ciliated cells and governs the formation of cilia;[45-48,50,51,118-123] it is also expressed in the choroid plexus[124] as well as in the reproductive epithelium.[123] Also, Sox factors,[40,125-128] especially sox7,[129] and sox17,[127] and other Fox-Factors,[40,130] represent interesting candidates for the regulation of miR-449 and CDC20B expression.

Open Questions for Ongoing Research

It remains to be determined what additional cell types could be responsible for the high overall miR-449 levels in tissues other than lung, e.g., in testis. It is very possible that a regulator of multiciliated differentiation, like miR-449, could play a role in the formation of the flagella in spermatids. The fact that miR-449 is highly expressed in testicular tissue also could suggest that miR-449 is involved in the differentiation of male germ cells. There has been a recent report of a role for miR-34c in late spermatogenesis, mainly through the regulation of notch2 and TGIF2, an inhibitor of the TGFβ pathway.[131] As miR-449 possesses a very similar seed sequence as miR-34 members, it might also contribute to spermiogenesis.

Moreover, it is still unclear how precisely miR-449 and its host gene CDC20B contribute to ciliogenesis. Ciliogenesis is highly cell specific, whereas notch signaling is found in many cells, including non-ciliary cells. Additionally, it is still not clear how the inhibition of the notch patway through miR-449 leads to mucociliary differentiation downstream of notch cleavage. Furthermore, since miRNAs possess many targets, we might still miss information on additional signaling pathways influenced by the miR-34/449 family. New approaches like the AGO-RIP method,[132,133] where a compound of the RISC complex, the protein Argonaute 2 (Ago2) is coprecipitated with its bound RNA, provide a promising procedure to uncover additional targets.

The mechanisms regulating miR-449 expression in specific tissues will be important to understand the corresponding differentiation program. It has been shown that miR-449 and its host gene CDC20B are coregulated by E2F1 in the context of DNA-damage. However, nothing is known about the regulatory mechanisms in the context of differentiation. The Fox and Sox families of transcription factors, known to regulate different cell fates in different tissues, might represent promising candidates.

The decision between simple cell cycle arrest, permanent exit for differentiation or senescence, or even apoptosis, in response to miR-449, has to be tightly regulated. Future research may reveal what additional signals influence this decision, and why miR-449 preferentially induces the death of non-differentiated cells.

Loss of miR-449 may represent a prerequisite of tumorigenesis. A lack of notch inhibition arising from miR-449 silencing could contribute to cancer progression by enhancing the self-renewal capacity of the tumor cells. Until now, there is no study directly indicating a causal role of miR-449 in tumorigenesis, even though it was reported that miR-449 levels are decreased in tumor tissues. Especially tissues harboring high levels of miR-449 under normal conditions (especially testicular and pulmonary tissues) should be considered in such studies. Could the re-introduction of miR-449 (or the mimicking of its effects through siRNAs against its main targets) even becomes a potential therapeutic approach in cancers arising from such miR-449-null tissues? In such a scenario, the ability of miR-449 to induce apoptosis in a p53-independent manner could be of high relevance, as functional p53 activity is lost in cancers.

To answer all the questions about the role of miR-449 in differentiation, development and tumorigenesis, animal models with targeted gene disruptions or tissue-specific miR-449 overexpression will be required.

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