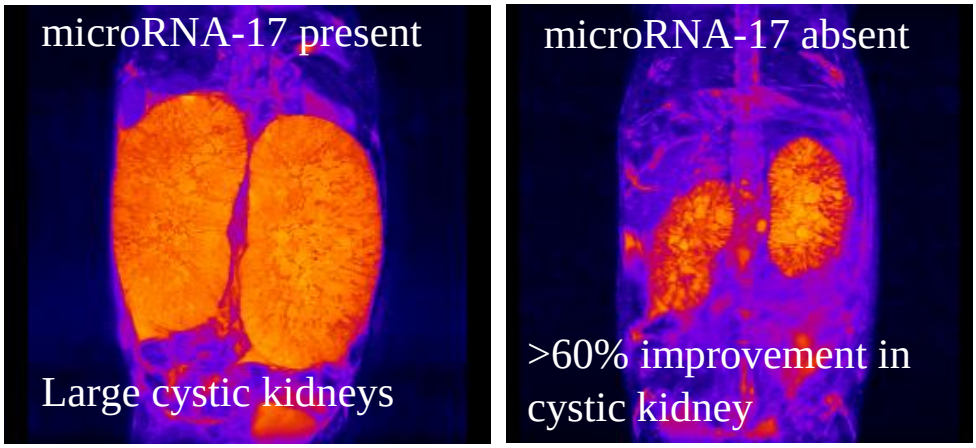
MicroRNAs: New Drug Targets for Kidney Diseases



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This is to acknowledge that Vishal Patel, M.D. has disclosed that he does have financial interests or other relationships with commercial concerns related directly or indirectly to this program. Dr. Patel will not be discussing off-label uses in his presentation.

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Biography: Dr. Patel's primary research interest is to study the role of non-coding RNAs and RNA metabolism in the context of the healthy and diseased kidney. His group has uncovered new mechanisms by which microRNAs regulate kidney tubule development and homeostasis. They have also identified microRNAs as novel drug targets for polycystic kidney disease (PKD), a fatal but common genetic disorder, and co-developed a microRNA drug that is currently being tested in early-stage human clinical trials. This is the first instance where drug development starting from proof-of-principle mouse studies to lead compound optimization has explicitly been geared towards PKD. The research in Dr. Patel's lab is funded by a R01 grant from the NIH and a SRA grant with Regulus therapeutics. He has previously been supported by NIH K08, R03, and challenge grants and a PKD foundation grant.

In 2017, Dr. Patel launched and now is the director of a new comprehensive polycystic kidney disease clinic and translational research center at UT Southwestern, first such clinic in the state of Texas. Dr. Patel has mentored several students and post-docs, including two physician-scientists who have both attained independent academic positions. He is a member of the scientific advisory board for the PKD foundation and executive advisory committee for the NIH-funded (P30) PKD center at University of Alabama Birmingham. He also serves as a scientific consultant for Regulus Therapeutics. Dr. Patel volunteers as an advocate for patients with PKD by organizing and presenting educational seminars for families affected by PKD and speaking at national and local events to increase awareness and raise funds for PKD research.

Purpose and Overview: The purpose of this presentation is to review following topics briefly: (i) Basic miRNA biology, (ii) Role of miRNAs in healthy and diseased kidney (Polycystic Kidney Disease), and (iii) Potential of RNA-based therapeutic approach.

Educational objectives: At the end of this lecture, the listener should be able to: (i) Understand the basic mechanism of action of microRNAs. (ii) Appreciate that microRNAs play a critical role in normal renal physiology and in the pathogenesis of diseases such as polycystic kidney disease. (iii) Have a general idea of the state of the RNA and microRNA drug development field.

Introduction:

miRNA discovery was first reported in 1993 with the publication of two landmark papers by Victor Ambros and Gary Ruvkun in the same issue of the journal Cell [1, 2]. *Lin-4*, the first discovered miRNA, was shown to be essential for *C. elegans* larval development. For 7 years after this discovery, it was believed that miRNAs were peculiar to nematodes because *lin-4* is not conserved in other organisms. However, this perception quickly changed after the discovery of another miRNA, *let-7*, which is also required for *C. elegans* larval development. Unlike *lin-4*, *let-7* is conserved – in size, nucleotide sequence, and expression profile – among all animals with bilateral symmetry. These observations led to the examination of roles of miRNAs in other taxa. We now know that 1000s of conserved miRNAs are encoded by the human genome, and miRNAs are implicated in diverse biological processes[7] ranging from implantation of the embryo, stem cell biology, and development of vital organs to metabolism and innate immunity. Furthermore, aberrant miRNA expression is observed in numerous human diseases, and correction of miRNA expression is emerging as a novel therapeutic strategy. By 2008, miRNA biotech companies began forming, and many clinical trials are on-ongoing. Because of the realization that miRNAs are integral to all forms of life and perhaps can be even used as drug targets to treat human diseases, Victor Ambros and Gary Ruvkun were awarded the Lasker prize in 2008.

MicroRNA: Biogenesis and function:

The human genome contains 1000s of miRNA genes, the majority of which are conserved in other species. Nearly half of the miRNAs are actually embedded within introns or sometimes even in the exons of known protein-coding genes. Usually, these miRNAs are transcribed along with their host gene and then spliced out of host gene mRNA. miRNA genes can also be found in intergenic regions that produce one or multiple microRNAs. These miRNA genes do not have the makeup of a traditional protein-coding gene but are regulated in exactly the same way. They have independent promoters, and the same machinery (RNA Pol2) that is required for the expression of traditional genes also regulates miRNA gene transcription.

miRNA biogenesis begins in the nucleus with the transcription of relatively long, polyadenylated transcript called primary miRNA[8]. This transcript is sequentially processed by two RNA enzyme complexes. First, the DROSHA/DGCR8 complex in the nucleus processes pri-miRNA to produce

a smaller RNA stem loop called precursor miRNA. This is in turn processed by the second RNA enzyme complex comprising of Dicer, which eventually produces the mature miRNA. The mature miRNA associated with a multiprotein complex called the RISC complex. Watson-Crick base pairing between seed sequence and complementary sequences located in the 3'-UTR of target mRNAs results in destabilization of that mRNA target. In this manner, miRNAs function as sequence-specific inhibitors of post-transcriptional gene expression[9].

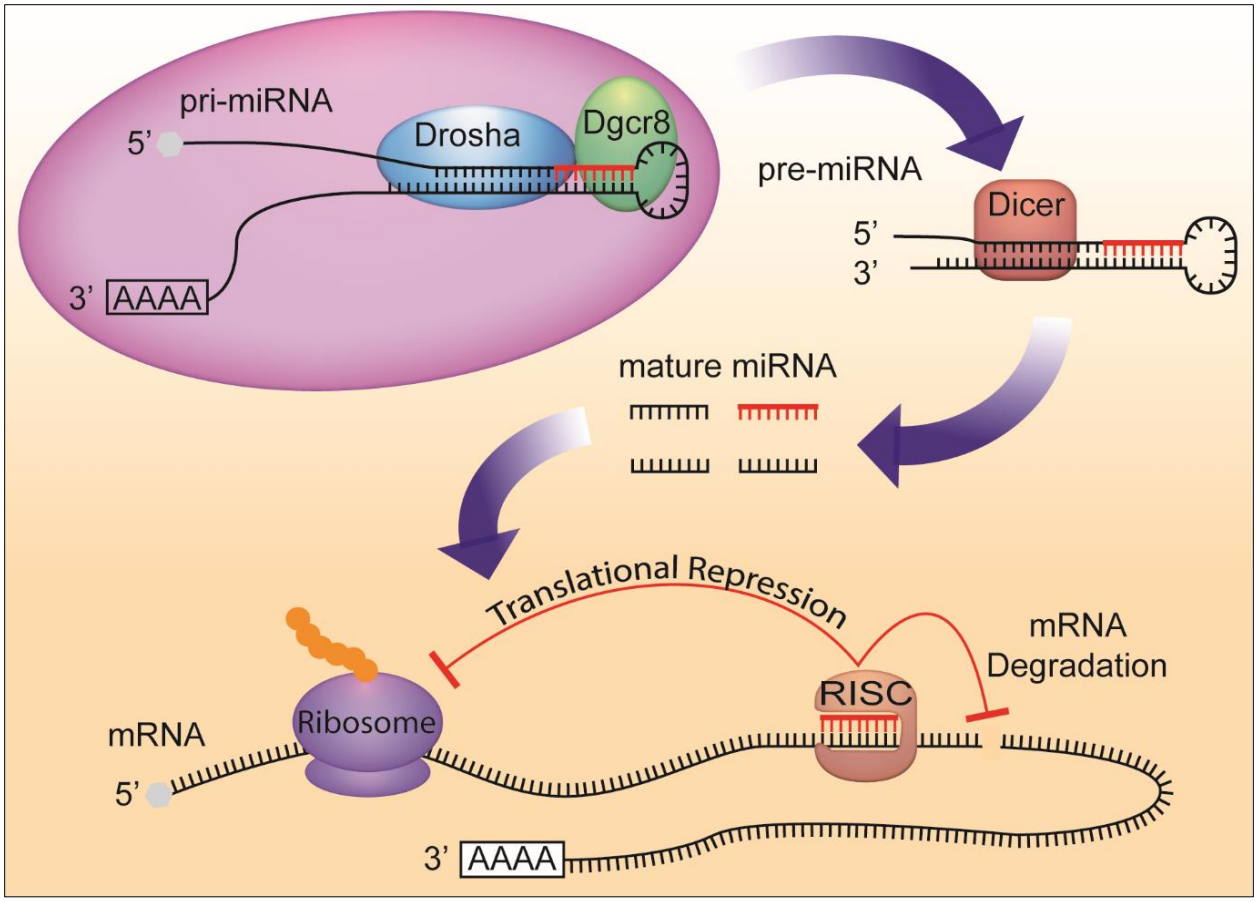

Figure 1: MicroRNA biogenesis and function. From: Yheskel & Patel, Current Opinion in Nephrology and Hypertension (2017)[3]

Gene expression can be regulated by transcription, such that, the same gene could be off, highly expressed or expressed at intermediate levels giving cells their unique identities. However, for any given transcriptional state, the presence of miRNAs can generate a much more complicated pattern of gene expression. The emergence of this much needed complexity may have allowed for the

evolution of higher organisms and sophisticated organs with diverse cell types. There are nearly 5000 miRNAs. Each miRNA is predicted to regulate 1000s of target mRNAs. On the other hand, on an average, a mRNA has 3-4 miRNA binding sites. Therefore, there are potentially millions of unique mRNA-miRNA cross talk events in any given cell.

The physiological function of microRNAs in the kidney:

miRNAs have been implicated in virtually all aspects of its development and homeostasis. Kidney development proceeds is a series of well-defined stages. The ureteric bud, a precursor of collecting ducts, sends molecular signals to nephron progenitor cells to produce the precursors of glomeruli and nephron (renal vesicle followed by comma-shaped bodies and S-shaped bodies). Conversely, the nephron progenitors send molecular signals to the ureteric bud to branch. This process is repeated numerous times to eventually endow the kidney with nearly 1 million nephrons.

Two examples of how miRNAs affect kidney development are presented. First, if all miRNAs are removed from the UB, it fails to send the signal to progenitor cells to start forming nephron precursors[10]. As a result, the kidney fails to develop. The second example has to do with the last stage of kidney development. We found that if all miRNAs are removed after nephrons have already formed, the kidney forms as expected. But the tubules rather than elongating into straight narrow tubes, grow haphazardly and eventually form kidney cysts[5] (Figure 2).

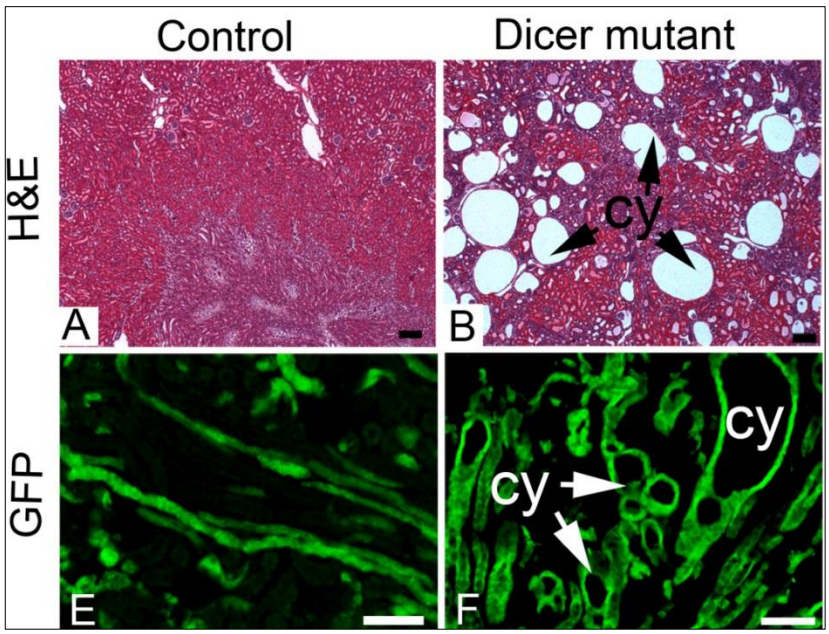

Figure 2: Inhibiting microRNAs in elongating renal tubules results in tubular dilation and cyst formation. Taken from Patel et al, Journal of Ameri. Soc. Nephrology (2012)[5]

The full scope of miRNA function in the adult kidney is still not known. That's understandable considering the complex structure and function of the kidney. However, there are tantalizing clues that miRNAs may be just as critical in the adult kidney as the developing kidney. We recently collaborated with the Eurenomics, a consortium involving investigators from several European institutions, to provide the first comprehensive global miRNA expression patterns in the mature nephron[6].

Impressively, solely on the basis of miRNA expression profiles, various nephron fractions can be faithfully divided into four distinct groups (glomerulus, proximal convoluted tubules/ proximal straight tubules, loop of Henle/ distal convoluted tubule, and collecting duct) (Figure 3). We typically think that what makes the various parts of the nephron unique is the presence of podocyte-specific proteins in the glomerulus or renal tubule specific channels and transporters. Our observations suggest that the segmental kidney miRNAs expression patterns also contribute to deciding how a glomerulus is different from the renal tubules or how a proximal tubule is different from collecting duct.

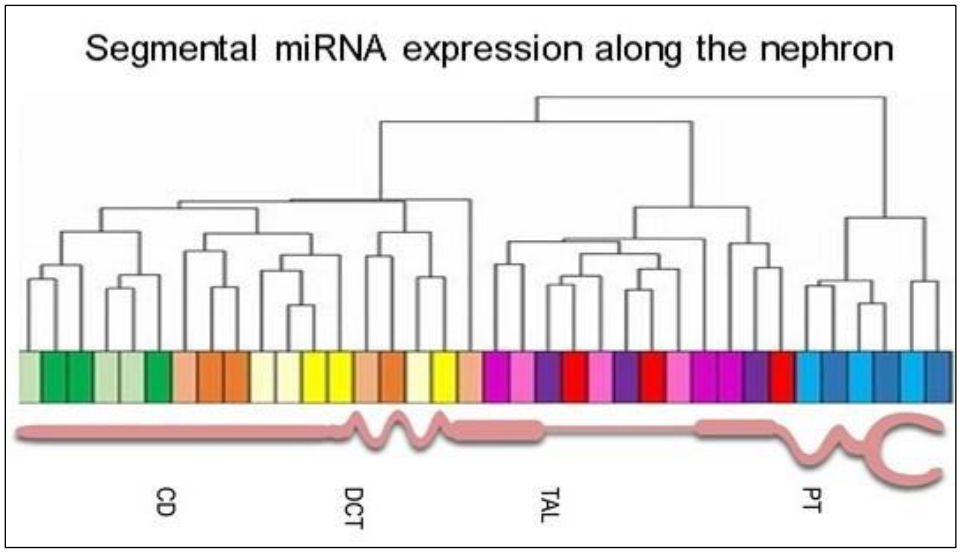

Figure 3: microRNAs exhibit segment expression pattern along the nephron. Taken from Hajarnis et al, Journal of Ameri. Soc. Nephrology (2017)[6]

So, what are these segment-specific functions of miRNAs? We recently found that ablating miRNAs from collecting duct invokes a tubule injury-like response that culminates into fibrosis resembling CKD. Surprisingly, loss of miRNAs from PT has no effect. This suggests that the anti-fibrotic miRNAs reside in the collecting duct, and shines a new light on the underappreciated role

of CDs in promoting CKD. Segment-specific functions of miRNAs in other parts of renal tubules are still not known.

Pathological roles of microRNAs in autosomal dominant polycystic kidney disease: miRNAs have emerged as key regulators of numerous diseases, especially various types of cancers, heart diseases, kidney diseases etc[11, 12, 13, 14]. I will discuss one kidney disease called ADPKD as a case study of how miRNAs can promote disease progression[3, 15].

Autosomal dominant polycystic kidney disease (ADPKD) is among the most common monogenetic human disease[16]. ADPKD is characterized by the presence of numerous fluid-filled cysts in the renal parenchyma. The cysts arise from renal tubules and are lined by abnormally functioning epithelial cells. The cyst epithelial cells secrete excessive fluid and display high rates of proliferation, which results in the expansion of cysts. The expanding cysts compress the surrounding normal nephrons causing renal failure. ADPKD is caused by mutations of *PKD1* or *PKD2*. Approximately half of the individuals affected with ADPKD develop end-stage renal failure. ADPKD is the 4th most common cause of end-stage renal disease in the United States and accounts for ~5% of people on dialysis or requiring kidney transplantation. Significant advances have been made towards identifying a cure for ADPKD [17]. Recently, tolvaptan, a vasopressin receptor antagonist, was shown to retard cyst growth in patients with ADPKD. Despite recent progress, pathogenesis of ADPKD is not fully understood, and no FDA-approved drugs are available.

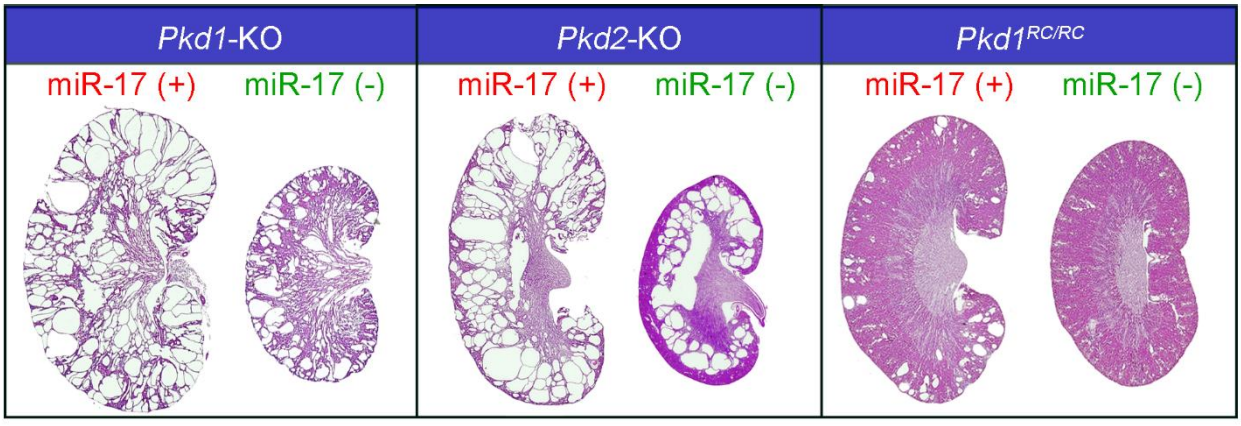

Figure 4: Deleting miR-17 slows cyst growth in multiple models. Taken from Hajarnis et al, Nature Communications 2017 [4]

In a pair of publications (PNAS

2013 and Nature Communications 2017)[18, 19], we have provided several converging lines of evidence conclusively linking the miR-17~92 miRNA cluster to PKD pathogenesis. We have shown that: (i) miR-17 family is upregulated in multiple orthologous mouse models of ADPKD as well as other ciliopathy models, (ii) miR-17 levels are also increased in human ADPKD samples, (iii) kidney-specific overexpression of miR-17 cluster is sufficient to produce PKD-like, tubular cysts, (iv) Conversely, genetic deletion of miR-17 attenuates cyst proliferation and cyst growth, improves renal function, and markedly prolongs survival of four orthologous ADPKD mouse models, including two long-lived models that we followed for close 18 months. These studies provide a strong scientific rationale for targeting miR-17 as a novel therapeutic approach for PKD. Other miRNAs such as miR-21 have also been implicated in ADPKD pathogenesis.[20]

What are underlying molecular mechanisms by which miR-17 promotes ADPKD progression? Mutations of *Pkd1* or *Pkd2* is associated with increased expression of c-Myc. c-Myc binds to the miR-17~92 promoter and enhances its transcription in cystic kidneys. The miR-17~92 primary transcript is processed to yield the individual mature miRNAs. In the cytoplasm, the mature miRNAs (miR-17 and miR-19) bind to *Pparα* 3′-UTR. PPARα is known to regulate the expression of key metabolic genes involved in the mitochondrial OXPHOS pathway. miR-17 and miR-19 binding to *Pparα* 3′-UTR lead to reduced *Pparα* expression, which in turn affects mitochondrial metabolism in kidney epithelial cells.

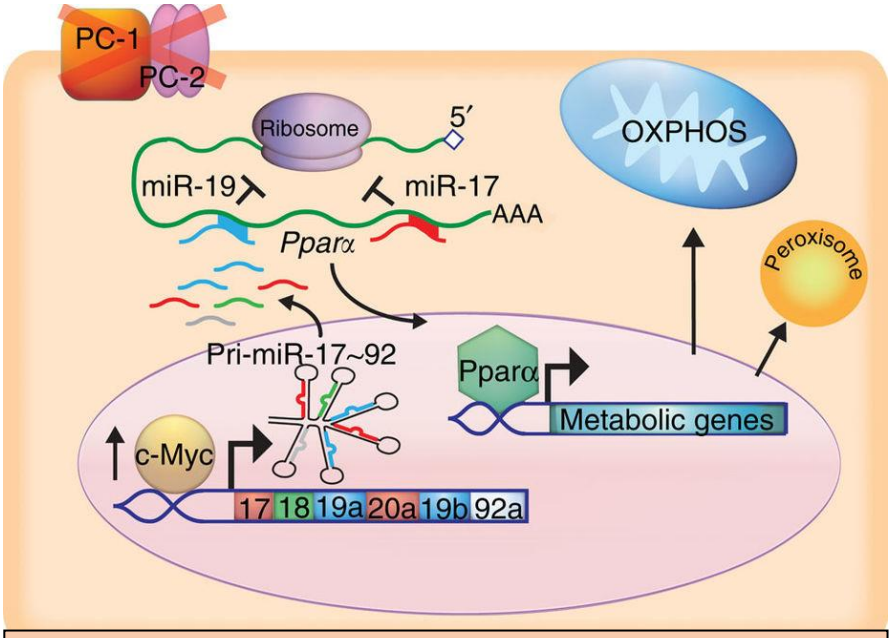

Figure 5: Mechanism by which miR-17 promotes cyst growth and disease progression in polycystic kidney disease.

RNA-based therapeutic approaches:

The idea behind RNA-based drugs predates the discovery of miRNAs by many years, in fact, a

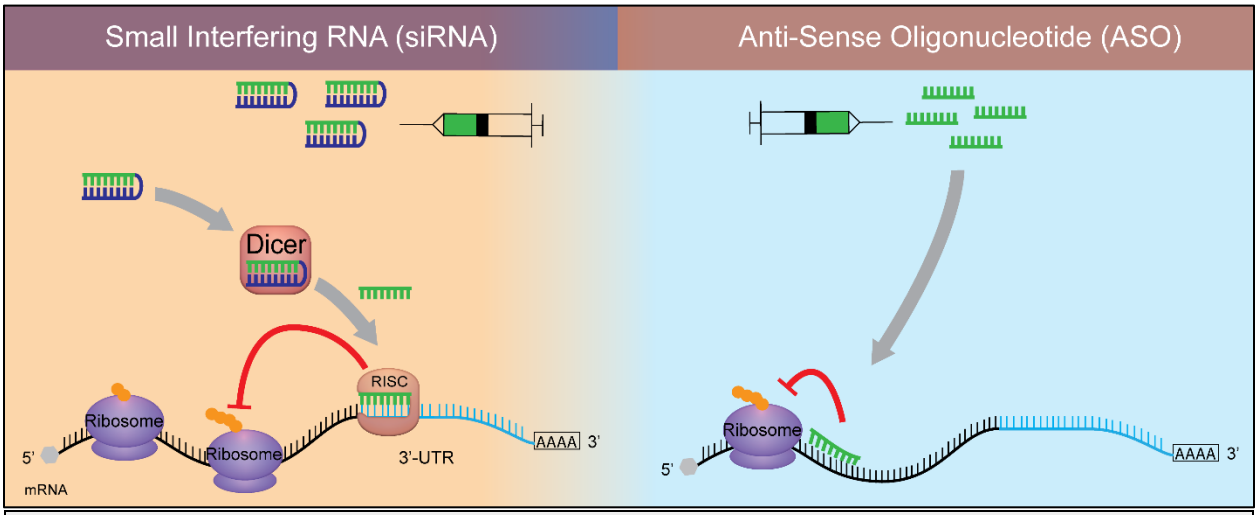

Figure 6: Mechanism of action of RNA-based drugs

couple of decades. The premise is pretty simple. Produce synthetic oligonucleotides with sequences that are complementary to pathogenic mRNAs (Figure 6). These oligonucleotides hybridize with cognate mRNAs and inhibit their translation into proteins. The RNA-based approach can be applied in two ways: first, is the anti-sense oligonucleotide (ASO)-based approach. Once inside the cell, ASOs bind to target mRNAs and physically prevent ribosomes from translating the mRNA. Thus, the ASOs act as steric inhibitors of mRNA translation. The ASOs can also be designed such that upon binding to target mRNAs, they recruit RNAase H, which degrades the mRNA. The second approach is the siRNA-based approach. This method takes advantage of the fact that each cell in our body has endogenous siRNA machinery. These are double stranded RNA drugs. Once inside the cell, Dicer processes the double stranded RNA to produce active, single-stranded siRNA. This is in turn loaded on the RISC (RNA-induced silencing complex), which catalyzes the reaction to inhibit mRNAs.

ASOs can be used to inhibit miRNAs as well. In healthy cells, there is an optimal balance between mRNAs that are being translated versus the ones that are undergoing miRNA-mediated translational repression. In a diseased state, when a particular miRNA is activated, this balance is tipped in favor of excessive miRNA-mediated translational repression. ASOs can be designed to harbor sequences that are complementary to any miRNAs. Once inside the cell, ASOs bind to

cognate miRNA and displace them from target mRNAs. Thus, restoring the balance between mRNA translation and repression (Figure 7).

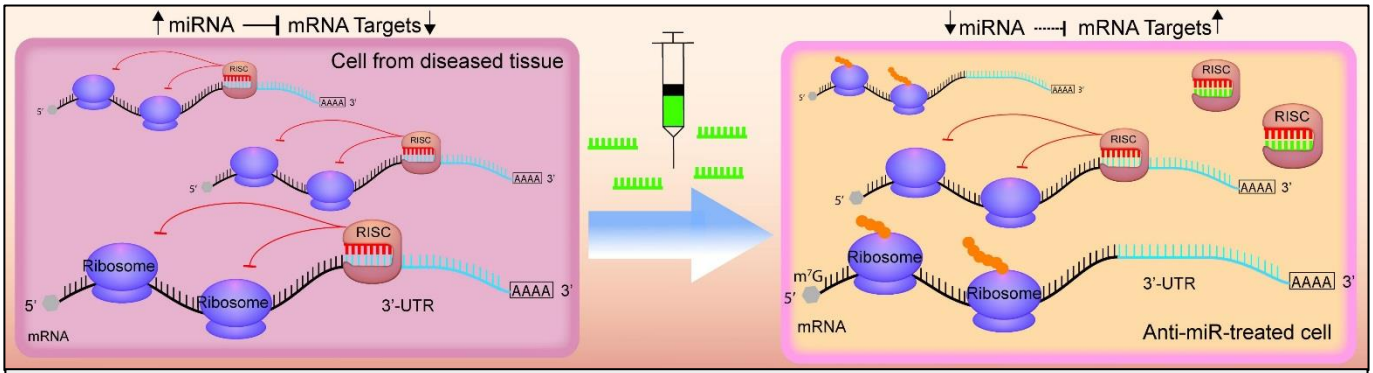

Figure 7: Mechanism of action of anti-miRs: Anti-miRs (green) binds to cognate miRNAs (red) and displace them from target mRNAs thereby relieving the miRNA-mediated mRNA inhibition.

Properties of RNA drugs:

Unmodified RNA is quickly degraded by nucleases and evokes an intense immune reaction. Thus, they are not suitable drugs. RNAs are composed three chemical units: the phosphate backbone, ribose sugar, and nucleotide, each of which can be modified to impart 'drug-like' properties. Amongst the more common modifications are:

(A) Phosphorothioate (PS) RNA backbone modification: These are RNA analogs where non-bridging oxygen in the internucleotide linkage is replaced by sulfur. Because they are non-natural analogs of RNA, PS-RNA oligonucleotides are not readily hydrolyzed by ribonucleases. This stabilization can lead to enhanced biological activity.

(B) 2′ O-Methyl Ribose modification: This chemical modification also offers stability against nucleases, as well as increased stability of binding to target mRNAs or miRNAs.

(C) Locked nuclei acid (LNA) modification: This modification locks the ribose ring in a conformation that is ideal for Watson-Crick binding. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the oxygen at 2nd position with carbon on the fourth position. This modification enhances the stability of ASO binding to target mRNAs or miRNAs.

RNA drugs are hydrophilic, have a relatively large molecular weight, and appropriate chemical modifications render them stable for long periods if adequately refrigerated. These drugs can be

dissolved in normal saline and administered systemically (intravenous and subcutaneous) or locally (skin, eyes, brain, lungs). They are quickly cleared from the plasma (3-5hrs) and accumulate primarily in the liver, kidney, spleen, and macrophages. In tissues, they can last for up to 2-3 weeks. Some ASOs are loosely protein bound, which prevents them from being freely filtered via the kidney. They are taken up by the cell via endocytosis pathways. Eventually, these drugs are degraded by nucleases and excreted via urine and bile.

State of the RNA drug field

1978, it was first reported that ASO could be used to inhibit viral replication in vitro. This has initiated decades-long work to use ASO as therapeutic agents. The field has had its fair share of ups and downs (Figure 8). Through the 80's and 90's, the field was enjoying remarkable successes. Beginning with the invention of automated Oligo synthesis followed by identification of crucial chemical modifications (PS, LNA, etc.). Remarkably, these advances lead to the development, testing, and ultimately approval of the first ASO drug called Fomivirisen. True to the original observation that ASOs can inhibit viral replication, this drug was developed to treat CMV retinitis

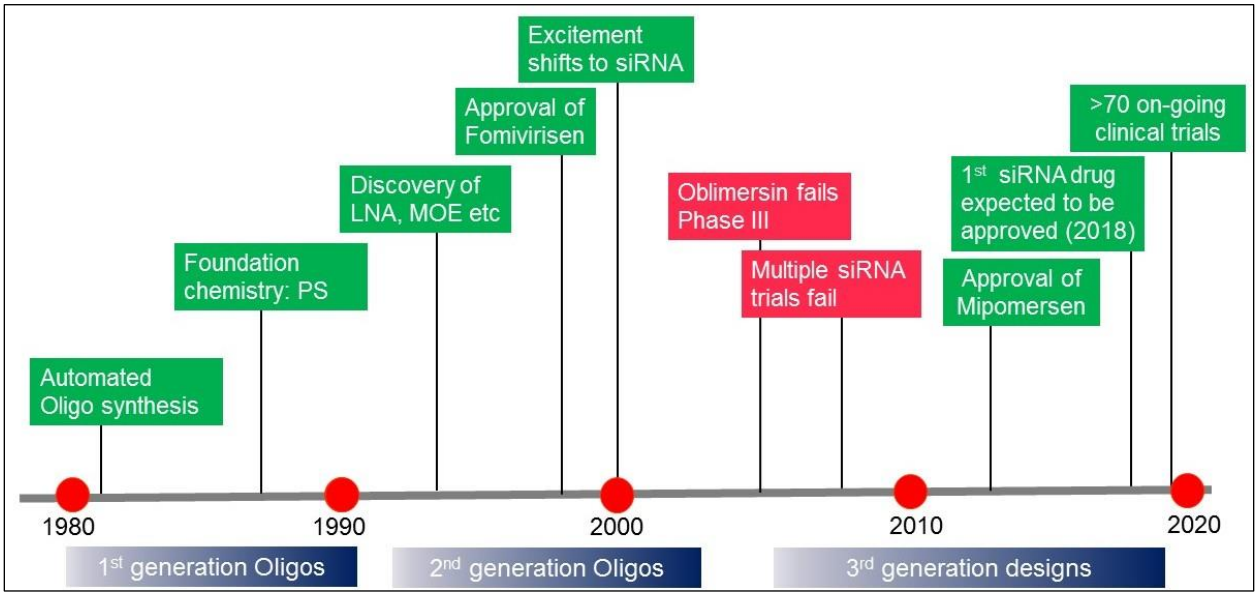

Figure 8: Timeline for RNA drug development.

in immunocompromised patients, mainly AIDS. The drug is safe and effective. However, if you dig deeper, the shortcomings of this medicine and the ASO field were obvious. First, strategically, the drug was developed for an indication that was rapidly declining as newer HIV medications

were being introduced. Second, this drug represented a considerable limitation; it needed to be directly injected in the eye. In the 2000s, due to several setbacks, the momentum started to shift away from the field. First, an ASO drug Oblimersin failed in Phase 3[21]. This drug actually was an improvement over Fomivirisen in that it was systemically delivered. It also was not overtly toxic, but it was unable to demonstrate clinical efficacy; perhaps owing to poor target choice. Second, multiple siRNA drug trials failed as well. The culprit here was poor delivery and chemical designs. This decade has seen a resurgence in the interest in developing RNA-based drugs. The catalyst may have been the development of the third generation ASO with chemical modifications that improve stability, delivery and reduce toxicity and better identification of putative drug targets. A second ASO drug, Mipomersen was approved by the FDA for the treatment of familial forms of hypercholestolemia[22]. This drug, however, causes liver toxicity and fatty liver and therefore has not been approved in Europe. In 2017, a siRNA-based drug called Patisiran was shown to demonstrate remarkable safety and efficacy in the treatment of a rare but lethal genetic disorder called familial amyloid polyneuropathy (FAP), also called transthyretin-related hereditary amyloidosis. This drug has received breakthrough therapy designation by the FDA and is expected to be approved for marketing in 2018. The will be the first siRNA-based drug to come to the market, a significant milestone for the siRNA field. There are nearly 70 on-going clinical trials testing various RNA drugs.

MicroRNA drugs in clinical trials:

Several drugs designed to either inhibit or mimic miRNA function are currently now in various stages of clinical testing[23]. The first miRNA drug ever to be tested in humans was Mirvirasen, an anti-miR-122 LNA-modified ASO, for the treatment of Hepatitis C. This drug showed remarkable efficacy in suppressing HCV in phase 2A clinical trial[24]. However, patients also experienced some some side effects including injection site reactions and flu-like symptoms. A follow-up trial has been not performed perhaps owing to the competitive Hepatitis C treatment landscape. Other miRNAs drugs have been developed for the treatment of various cancers and tissue fibrosis[25]. With regards to the kidney, two miRNA drugs are now being tested in clinical trials. The first drug is an anti-miR-21 compound for the treatment of Alport syndrome, and the second miRNA drug is an

anti-miR-17 compound being developed for the treatment of ADPKD. The full list current on-going miRNA clinical trials are shown in figure 9.

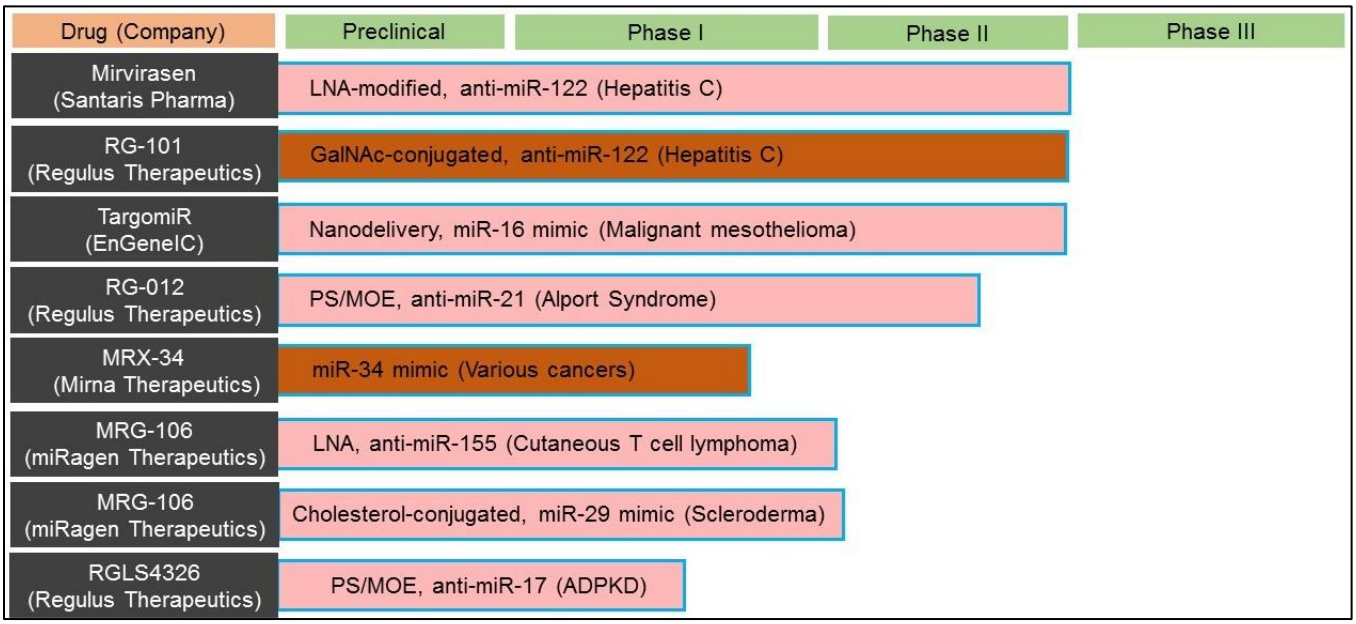

Figure 9: miRNA drugs currently in clinical trials. Clinical trial that have been stopped are shown in orange bars.

There are significant challenges that lie ahead for the miRNA drug development field. So far, two trials have been halted because of toxicity due to these drugs. Therefore, modifying drug chemistry to minimize toxicity will continue to be a significant future focus. Another challenge is improving delivery in organs such as brain and heart. Newer delivery approaches will be needed to target heart and brain diseases.

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References:

1. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**, 843-854 (1993).

2. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell* **75**, 855-862 (1993).

3. Yheskel M, Patel V. Therapeutic microRNAs in polycystic kidney disease. *Curr Opin Nephrol Hypertens* **26**, 282-289 (2017).

4. Patel V. microRNA-17 family promotes polycystic kidney disease progression through modulation of mitochondrial metabolism. *Curr Opin Nephrol Hypertens* **8**, 14395 (2017).

5. Patel V. MicroRNAs regulate renal tubule maturation through modulation of Pkd1. *Drug discovery today Disease models* **23**, 1941-1948 (2012).

6. Hajarnis S*, et al.* Suppression of microRNA Activity in Kidney Collecting Ducts Induces Partial Loss of Epithelial Phenotype and Renal Fibrosis. *J Am Soc Nephrol*, (2017).

7. Stefani G, Slack FJ. Small non-coding RNAs in animal development. *Nat Rev Mol Cell Biol* **9**, 219-230 (2008).

8. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281-297. (2004).

9. Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. *Nature* **455**, 64-71 (2008).

10. Nagalakshmi VK, Ren Q, Pugh MM, Valerius MT, McMahon AP, Yu J. Dicer regulates the development of nephrogenic and ureteric compartments in the mammalian kidney. *Kidney Int* **79**, 317-330 (2011).

11. van Rooij E, Olson EN. microRNAs put their signatures on the heart. *Physiol Genomics* **31**, 365-366 (2007).

12. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* **435**, 839-843 (2005).

13. Patel V, Noureddine L. MicroRNAs and fibrosis. *Curr Opin Nephrol Hy* **21**, 410-416 (2012).

14. Medina PP, Nolde M, Slack FJ. OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. *Nature* **467**, 86-90 (2010).

15. Noureddine L, Hajarnis S, Patel V. MicroRNAs and Polycystic Kidney Disease. *Drug discovery today Disease models* **10**, e137-e1743 (2013).

16. Patel V, Chowdhury R, Igarashi P. Advances in the pathogenesis and treatment of polycystic kidney disease. *Curr Opin Nephrol Hypertens* **18**, 99-106 (2009).

17. Torres VE, Harris PC. Polycystic kidney disease in 2011: Connecting the dots toward a polycystic kidney disease therapy. *Nat Rev Nephrol* **8**, 66-68 (2012).

18. Hajarnis S, *et al.* microRNA-17 family promotes polycystic kidney disease progression through modulation of mitochondrial metabolism. *Nat Commun* **8**, 14395 (2017).

19. Patel V, *et al.* miR-17~92 miRNA cluster promotes kidney cyst growth in polycystic kidney disease. *Proc Natl Acad Sci U S A* **110**, 10765-10770 (2013).

20. Lakhia R, *et al.* MicroRNA-21 Aggravates Cyst Growth in a Model of Polycystic Kidney Disease. *J Am Soc Nephrol*, (2015).

21. Oblimersen: Augmerosen, BCL-2 antisense oligonucleotide - Genta, G 3139, GC 3139, oblimersen sodium. *Drugs R D* **8**, 321-334 (2007).

22. Wong E, Goldberg T. Mipomersen (kynamro): a novel antisense oligonucleotide inhibitor for the management of homozygous familial hypercholesterolemia. *P T* **39**, 119-122 (2014).

23. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov* **16**, 203-222 (2017).

24. Janssen HL, *et al.* Treatment of HCV infection by targeting microRNA. *N Engl J Med* **368**, 1685-1694 (2013).

25. van Zandwijk N, *et al.* Safety and activity of microRNA-loaded minicells in patients with recurrent malignant pleural mesothelioma: a first-in-man, phase 1, open-label, dose-escalation study. *Lancet Oncol* **18**, 1386-1396 (2017).