REVIEW

TALEN-engineered human cell lines with microRNA-21 null mutations

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Dysregulation of microRNA-21 (miR-21) is associated with many types of cancer as well as with kidney and cardiovascular diseases. Aberrant expression of miR-21 leads to multiple phenotypic alterations including cellular proliferation, invasiveness, apoptosis, and fibrosis. We recently used transcription activator-like effector nucleases to engineer human cell lines with miR-21 null mutations. As expected, loss of miR-21 resulted in decrease cell proliferation and reduced transforming activity in culture and in xenografts. Besides an increase of apoptotic gene expression, miR-21 knockout cells also had significantly increased expression of genes involved in extracellular matrix interaction. Results from small RNA sequencing suggest that miR-21 deletion changed the microRNA expression profile. These results raise intriguing possibilities that loss of miR-21 expression may influence cellular interactions and that cells with long term miR-21 deficiency may compensate for the loss of this highly expressed microRNA by changing the abundance of alternate microRNAs or the AGO2 protein in order to maintain the microRNA-AGO2 homeostasis. Further characterization and utilization of miR-21 knockout human cells will shed new light on this pathologically important microRNA.

Keywords: microRNA-21; cancer; Transcription activator-like effector nucleases; null mutations; extracellular matrix; microRNA-AGO balance; fibrosis; oncomiR

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microRNA-21 in cancer and fibrotic disease

MicroRNA-21 (miR-21) was one of the earliest discovered oncogenic microRNAs (oncomiRs) and is overexpressed in the majority of cancers [1]. Circulating miR-21 has the potential to be a diagnostic or prognostic marker for many of those cancers [2, 3], and miR-21 overexpression is associated with drug resistance of cancer cells [4]. Though this miRNA was initially linked to cancer, further studies have revealed miR-21 to be associated with other pathological processes, including cardiovascular disease [5], kidney fibrosis [6], and wound healing [7]. MiR-21 expression increases in cardiac fibroblasts during heart disease and an overexpression of miR-21 impairs the ability of the heart to contract [5, 8]. Up regulation of miR-21 in kidney fibrosis disrupts organ structure and contributes to the disease [6]. Recently, miR-21 was shown to play a different role by acting as an anti-inflammatory factor in macrophages [7]. The multitude of functions miR-21 can have in both disease and healthy states makes this miRNA intriguing to study.

Due to miR-21's importance for cancer and other diseases, there has been much effort to understand the function of this miRNA. Several direct targets of miR-21 have been characterized, including programmed cell death protein 4 (PDCD4), phosphatase and tensin homolog (PTEN), sprout homolog 1 (SPRY1), and peroxisome proliferator-activated

receptor α (PPARα). Down regulation of PDCD4, a tumor suppressor and apoptotic factor, by miR-21 has been linked to cancer development and metastasis [9, 10]. Also identified as a miR-21 target in cancer, PTEN down regulation has been linked to increased cancer cell proliferation and motility [11]. In cardiac fibroblasts, miR-21 mediated down regulation of PTEN has been associated with increased matrix metalloproteinase expression and extracellular matrix defects [8]. SPRY1 down regulation has also been seen in cardiac fibroblasts, leading to decreased apoptosis of these fibrotic cells [5]. In the kidneys, miR-21 targeting of PPARα, not SPRY1, is thought to be the primary mediator of fibrosis [6]. Many other direct and indirect miR-21 targets have been reported [12] and there are likely more to be discovered.

TALEN-mediated gene editing of microRNA-21

The advent of novel sequence-specific gene editing techniques such as zinc finger nucleases, transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9 nucleases have provided a mechanism by which new information about a gene can be discovered through precise editing of that gene. TALENs are recombinant proteins composed of a transcription activator-like (TAL) effector fused to FokI's DNA cleavage domain [13-15]. TAL effectors are derived from DNA binding proteins secreted by the plant bacterium *Xanthomonas* [16] and composed of repeating units containing 34 to 35 amino acids in which only residues 12 and 13, called the repeat-variable diresidue (RVD), are different. The RVD sequence determines the affinity of each repeating unit for a specific DNA base-pair [17, 18]. TAL effectors can be engineered by ligating multiple repeating units together to bind at specific sites in the genome [14]. The other part of a TALEN is composed of the DNA cleavage domain of FokI [19]. FokI is a type II restriction endonuclease which only cleaves when in a dimer [20]. The necessity of dimer formation for cleavage give TALENs additional specificity, especially compared to the off-target prone CRISPR-Cas9 system [21, 22], as a pair of TALENs must both bind DNA with appropriate spacing and orientation. These TALENs create DNA double strand breaks at specific sites and the repair of these sites by non-homologous end joining can result in a small deletion or insertion in the target gene [23]. Target gene knockout can also be achieved using two pairs of TALENs to cleave the DNA on either side of the target region and delete the sequence in between.

Our group and others have designed and used TALENs to target the human microRNA-21 gene [23-26]. We performed TALEN-mediated knockout of miR-21 in HeLa cells using three TALEN pairs to remove or disrupt the stem-loop sequence containing miR-21 [25]. Single clones homozygous for miR-21 knockout were isolated. Phenotypic studies showed the complete knockout of miR-21 resulted in a decreased growth rate, decreased colony forming activity, and less transformed xenografts. The knockout also increased sensitivity of HeLa cells to the chemotheraputic cisplatin, which is consistent with conventional miR-21 knockdown studies [27]. To determine which gene expression changes were associated with these phenotypes, we examined the changes in expression of miR-21 target genes using RNA-seq. A significant enrichment of mRNAs important for cell adhesion, extracellular matrix (ECM), and integrin binding was detected. Surprisingly, although much focus has been placed on miR-21 as an anti-apoptotic factor, these ECM pathways are more highly enriched than apoptosis and cell death pathways. This indicates changes in interaction with the ECM may be more prevalent than increased apoptosis in cells with long term miR-21 deficiency.

Potential microRNA-AGO2 rebalance resulted from the loss of the abundant miR-21

The small RNA profile of our cells was examined to determine what changes in miRNA expression profile occurred after miR-21 knockout. We found 11 miRNAs significantly upregulated and 10 miRNAs significantly downregulated in both of our knockout lines. Interestingly, only one of these differentially expressed miRNAs, miR-487b, is also significantly changed when miR-21 is knocked out in HEK293 cells [24].The small overlap between the different cell lines indicates the change in miRNA profile in response to miR-21 knockout may be the result of missing an abundant miRNA, rather than removal of miR-21's function. We hypothesize that the loss of an extremely highly expressed miRNA (in HeLa cells >50% of the total microRNA reads are of miR-21) could change the miRNA dynamics within the cell. The limiting factor in miRNA abundance is the availability of the AGO2 protein, which is necessary to prevent the degradation of miRNAs [15]. Reciprocally, a dependence of AGO2 on miRNA abundance for stability can be seen in mouse cells [28]. Since human AGO2 is able to bind non-miRNA small RNAs [29], the effect of miRNA abundance on human AGO2 stability is less clear.

There are two possible mechanisms by which removal of a highly abundant microRNA could change global microRNA dynamics: 1) removal of miR-21 results in decreased total miRNA levels and decreased AGO2, 2) removal of miR-21 results in increased stability of alternate miRNAs as more AGO2 is available for binding. To distinguish between these mechanisms, we have examined the AGO2 levels in miR-21 knockout cells and detected a small decrease. However, additional experiments are needed to determine if there was an increase of alternate miRNAs or other small RNAs bound

to AGO2. To further examine the hypothesis, total miRNA abundance of wild type and miR-21 knockout cells will need to be determined. It remains challenging to obtain an accurate measurement of the total miRNA abundance in a cell. The use of Agilent's small RNA tool for the 2100 Bioanalyzer allows for quantification of total RNA from 20-40 nts in length, but this method is highly sensitive to the variation in RNA quality and can overestimate the fraction of miRNAs [30]. A preferred method would be to spike a known quantity of a short RNA not found in human cells into the miRNA-seq samples and, with this internal standard, information about the changes in abundance of each miRNA after knockout may be obtained. Further work to determine how miRNA dynamics change in response to miRNA knockout will not only be important for miR-21, but also for siRNA and shRNA therapeutics as these also compete with microRNAs for AGO2 binding [31].

Due to the strong association of miR-21 with cancer and its function as an anti-apoptotic factor, miR-21 has become a drug development target [32]. For example, a miR-21 targeting antisense oligonucleotide, RG-012, is currently under development by Regulus Therapeutics for use in treating Alport syndrome, a genetic disorder associated with kidney disease [33]. Our study, along with previously published data, show that miR-21 has multiple important roles in healthy and diseased human cells. This makes it necessary to carefully examine the possible side effects of miR-21 targeting drugs, especially as miR-21 is important for limiting inflammatory response [7]. Also, complications of long-term miR-21 treatment should be examined as miR-21 knockout mice did not show the same decrease in cardiac fibrosis seen when miR-21 was knocked down by short term use of antisense lock nucleic acids (LNA) [34, 35]. Though it is possible this discrepancy is due to potential off-target effect associated with antisense nucleic acids, it is equally possible that an unidentified compensatory mechanism for long term loss of miR-21 may exist. This putative mechanism should be investigated before anti-miR-21 therapeutics can be effectively used to treat chronic conditions. It is therefore important to understand the potential inflammatory side effects of targeting this miRNA, as well as the resistance and other possible complications from long term down-regulation of miR-21. The use of new gene editing technologies, such as TALENs, allows for a deeper and comprehensive understanding of the role of miR-21 in human disease and treatment.

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Conflict of Interest

The authors declare no competing financial interests.

Abbreviations

miR-21, microRNA-21; oncomiR, oncogenic microRNA; TALEN, transcription activator-like effector nuclease; RVD, repeat-variable diresidue; ECM, extracellular matrix; LNA, lock nucleic acids.

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