The evolution of RNAi technologies in the drug discovery business

In the past decade, the pharmaceutical industry has exploited the naturally occurring cellular RNAi pathway to enhance drug discovery research. The RNAi pathway, triggered by dsRNA, selectively, although not always specifically, degrades mRNA leading to substantial decreases in post-transcriptional gene expression. Researchers have capitalised on this intrinsic pathway by synthesising RNAi reagents to modify the expression of any desired gene. RNAi libraries consisting of synthetic siRNAs or plasmid based shRNAs are amendable to large-scale genome-wide screening campaigns to search for new therapeutic targets. Such loss of function screens can reveal novel targets and synthetic lethal interactions for cancer therapy. These screens have also been used to identify novel host factors for diseases such as Hepatitis C and HIV. Selective gene silencing can deconvolute molecular pathways implicated in disease onset and progression.

The use of RNAi screening is an efficient and cost effective method for identifying possible targets for low molecular weight compounds or antibody therapies. RNAi has been demonstrated as a powerful tool for both the identification and validation of genes in cell based assays and in vivo applications. Recent developments in RNAi formulation and delivery have advanced the use of RNAi as a therapeutic for targets that would otherwise not be druggable using conventional methods. This review discusses how RNAi has contributed to the drug discovery process and the future possibilities of using RNAi in drug discovery and as a therapeutic modality itself.

RNAi is the mechanism by which dsRNA inhibits gene expression. A dsRNA molecule is processed by the enzyme Dicer into a small 19-23 base pair duplex known as a short-interfering RNA (siRNA). The siRNA then enters a multi-protein complex known as the RNA induced silencing complex (RISC). Once incorporated into RISC, a helicase unwinds the sense and antisense strands of the siRNA. The antisense strand then binds the complementary mRNA. The target mRNA is cleaved by an RNase inside the RISC complex, which results in silenced gene expression through increased mRNA degradation and reduced protein production. Researchers can utilise chemically synthesised siRNAs that are designed to target a specific gene of interest. The creation of genome-scale siRNA libraries allows for the potential identification of new drug targets that regulate disease relevant pathways.

Traditionally, siRNAs are transfected into target cells lines via a reagent that carries the siRNA across the lipid bilayer of the cell. The transfection conditions need to be optimised in order to ensure the phenotype being screened is appropriately measured and that the signal-to-background ratio is above a certain quantifiable threshold. There are numerous limitations preventing the accurate measurement of mRNA levels used to assess small interfering RNA (siRNA) efficacy after transfection. One approach to overcome these difficulties is to use a phenotypically neutral assay to quantify the knockdown of an exogenous luciferase gene delivered by a lentiviral vector. In this assay, the efficacy of a luciferase siRNA is compared to a negative control siRNA across many distinct assay parameters including cell type, cell number, lipid type, lipid volume, time of the assay and concentration of siRNA. Once the siRNA transfection is optimised as a 384-well luciferase knockdown, the biologically relevant phenotypic analysis can proceed using the optimal siRNA transfection conditions. While this and other similar approaches play a crucial role in RNAi screening assay development, certain cell lines such as primary cells are not easily transfectable using lipid complex mediated uptake. One way researchers overcome this hurdle is to use RNAi libraries comprised of vector based plasmids encoding short-hairpin RNA (shRNA). Either constitutive or inducible shRNA hairpin vectors can be integrated stably into the genome for long term or regulatable knock down studies.

Both formats of RNAi libraries, either siRNA or shRNA, have their advantages and disadvantages. SRNAs are straightforward to design and synthesise at scale for screens. The siRNA reagents are most useful in transfectable cell lines but gene silencing is transient. SRNAs can also give rise to off target effects which can lead to undesired phenotypes making it difficult...
to interpret the results. In contrast, shRNAs in viral vectors have the advantage of being transduced into difficult to transfect cell lines. The hairpin can be stably integrated into the host genome resulting in long term gene silencing. This makes them more suitable for assays that require longer incubation times such as soft agar, colony formation or in vivo xenograft studies. They can be cloned into inducible vectors, allowing for regulated expression of the respective target gene. A major disadvantage of viral vectors is the requirement of a viral packaging system and special bio-safety facilities required for amplification. ShRNA libraries are amendable to pooled screening strategies where multiple shRNAs are pooled and assayed in parallel in contrast to running each shRNA in an individual well. A well-by-well based shRNA screen is greatly limited by the upfront efforts to design and validate multiple shRNAs targeting each gene separately.

Another application of RNAi screening is performing screens in a format more compatible with in vivo growth conditions. Demonstration of anchorage independent growth of transformed cancer cells grown in a semi-solid, 3-dimensional (3D) medium, such as soft agar, is an ideal assay for determining tumorigenicity of cells in vitro. The soft agar assay (SAA) can yield results that closely match those of xenograft assays where tumorigenic cells are injected into nude mice. This is done to discover genes that regulate anchorage-independent growth in cancer cells that traditional 2D growth assays might miss. Performing SAAs in a high throughput manner has been shown to be both technically feasible and robust. These protocols are currently being used to screen cell lines that are sensitive to anti-cancer compounds when grown in a 3D format. Running soft agar assays in high throughput RNAi screens is a more physiologically relevant way to identify pathways and proteins whose decreased expression leads to slowed or inhibited tumour growth than traditional in vitro based assays run on plastic.

The miniaturisation of cell based assays to 1536 well format has greatly increased the throughput capacity cell based RNAi assay. The smaller scale format uses fewer reagents thereby significantly reducing screening time and costs. To date, successful high density chemical compound screens have been successfully run in 1536 well format where greater than one million small molecular compounds were analysed in one screen.

The use of high density, ultra high-throughput RNAi screens in this format opens the possibilities for expanded coverage of siRNAs used and conditions tested. The increase in the number of wells that can be screened at once leads to the novel screening method of matrixed based siRNA screening where combinations of genes whose loss of function reveal desired phenotypes. Continued advancements in automated microscopy platforms and the use of fluorescence tags and antibodies have allowed researchers to perform higher throughput high content RNAi screens to identify pathways and proteins that modify cellular function. Advanced time-resolved live cell imaging assays
can advance the understanding of complex cellular processes that cannot be studied using traditional endpoint assays. Researchers can now perform genome-wide RNAi screening assays that are informative on cell growth rates, migration, subcellular localisation, protein-protein interactions and when monitoring dynamic changes in intracellular structures.

An example of where RNAi screening has been successful at identifying drug targets is in the area of host factors that regulate viral replication. Novel host factors that regulate viral replication is a new paradigm to treat multiple genotypes of a pathogenic virus and a strategy to overcome viral resistance induced by therapeutics directed to viral proteins. Several RNAi based screens have revealed an important role of phosphatidylinositol-4-kinase III alpha (PI4KCA) in HCV replication. Although the RNAi libraries used by each research group varied in design and format, PI4KCA was consistently identified as a host factor that potently regulated HCV replication. Pharmacological inhibition of this enzyme may represent a new class of antiviral therapy for multiple genotypes of HCV. Other siRNA and shRNA screens have also identified other cellular host factors critical for all stages of the HIV life cycle.

These studies have yielded a significant number of known and novel host factors important for HIV replication that could be further developed as therapeutic targets. Thus, RNAi based screening has led to new insights into the complexity of HCV and HIV life cycles and the host factors that regulate them. The new pathway insights identified through these screens increases the potential for novel antiviral strategies to be explored.

RNAi screening has been an essential tool for discovering genes and/or pathways that drive cancer cell survival. Selective silencing of survival pathways can mimic the effect of a targeted cancer therapy leading to selective killing of cancer cells that harbour certain mutations. Loss of function screens combined with a cell viability endpoint assay plays a significant role in the identification of novel tumour growth factor pathways. RNAi screens have also been used in combination with compound treatment to identify the mechanism of action of compounds in late stage drug development pipelines. Successful target gene silencing should confer resistance or be ‘rescued’ from compound induced cell death. For example, RNAi based screens have uncovered the mechanism of action of SMAC mimetic compounds. These studies have demonstrated that TNFa and the Nkx8 pathway are critical to the activity target for SMAC mimetic compounds. Conversely, compound sensitisation RNAi screens are used to identify target genes that, when knocked down, can increase the efficacy of the compound in resistant cell lines. This is particularly important for targeted therapeutics where understanding the MOA is critical.

RNAi screening can also identify genes that, when inhibited, result in greater sensitivity to currently standard of care chemotherapy. A partial response or resistance to chemotherapy is a major hurdle in the successful treatment of cancers. Improvements to existing therapies through new drug combinations are of high clinical interest. Paclitaxel has been used extensively for treating multiple forms of cancers; however, many patients develop resistance to treatment. Recent RNAi screens have identified genes whose loss of function enhances sensitivity to Paclitaxel in breast cancer cells. Specific chemical inhibitors against the top proteins identified in these screens showed synergistic drug activity when used in combination with Paclitaxel. Lower doses of chemotherapies can be used in combination with specific RNAi silencing of genes known to be highly over-expressed in tumour cells. George et al showed that knockdown of the BCL-2 gene sensitises human glioblastoma cells to Paclitaxel treatment and could present a novel approach to treatment of malignant brain tumours. Irons et al showed the utility in performing parallel compound and RNAi screens in combination with Tamoxifen treatment, an inhibitor of the oestrogen receptor used to treat breast cancer. These high throughput screening efforts led to the discovery that targeting of the PDK-1 signalling pathway via siRNA or with small molecular inhibitors increased sensitivity to Tamoxifen.

A synthetic lethal interaction is when the combined inhibition or mutation of two or more genes results in cell death whereas cells survive perturbation of either gene alone. RNAi screening is well suited to identify these interactions. For example, several groups are using pooled shRNA strategies to identify proteins that are synthetic lethal in both cell and in vivo based experiments. Large pools of shRNAs are introduced into target cells lines over time and the cells that survive are deep sequenced to ‘retrieve’ shRNAs present in the surviving cells. Another technique is to measure shRNA enrichment using a barcode microarray where specific DNA sequences are barcodes in the shRNAs. Several research groups have used this approach to identify synthetic lethal interactions with the KRAS oncogene. Additionally, shRNA pooling strategies have also been utilised for in vivo to explore tumour growth in mouse models of cancer. In this study, researchers were able to screen nearly 1,000 genetics interrogations in one tumour bearing mouse.

SiRNAs have also proven to be efficacious in mouse models. Huang and colleagues were able to develop an intraperitoneal lipid delivery system to target Claudin-3 (CLDN3) siRNAs to ovarian xenografts tumour cells in mice. CLDN3 has been shown to suppress tumour growth in ovarian cancer cells in vitro. The successful silencing of CLDN3 in vivo resulted in reduced cell proliferation and tumour growth, an increase in apoptotic cells, and reduced metastasis. This suggests that lipidoid delivery of siRNAs in vivo has the potential to become a therapeutic in ovarian as well as other forms of cancer that specifically over express proteins that can be targeted by in vivo siRNAs. Localised or topical delivery of siRNAs, that is delivery of siRNAs directly to damaged tissues, has shown to be well suited for treating diseases associated with the eyes, skin or mucosal membranes as the delivery formulations continue to improve. Systemic delivery of siRNAs pose some significant challenges as the siRNA therapeutics need to travel through the patient’s body and to the target organ(s), evading uptake by non-targeted tissues. Systemic delivery of siRNAs needs to be carried out in vehicles that remain stable in the blood and endothelial system. siRNA therapies are moving into clinical trials to treat diseases such as age related macular degeneration, diabetic macular edema, respiratory syncytial virus (RSV), hepatitis B (HBV), HIV and therapeutic siRNAs targeting solid tumours.

The complex regulatory network of microRNAs (miRNAs) presents a challenge to develop drugs to target these molecules, including the identification of novel tumour suppressors and the pathways they regulate. MicroRNAs (miRNAs) are small non-coding, single stranded RNAs that regulate gene expression post transcriptionally by binding near complementary protein mRNAs sequences.
thereby inducing RISC mediated mRNA degradation. miRNAs can also regulate gene expression by binding to imperfect complementary sequences in the 3’ UTR of the target mRNA which also triggers a decrease in gene expression through a similar RISC complex used in the RNAi pathway. Researchers are setting up platforms and using miRNAs as a way to explore biomarkers of disease and response to drug treatments. miRNAs profiling of cancer cells and primary tumour samples have found miRNAs that regulate tumour progression and metastasis in breast cancer. miRNAs provide a new class of molecules to study in the hopes of identifying agents that play a role in development of human diseases and a new area for drug companies to explore for novel biomarkers and potential therapeutic opportunities.

In summary, the use of siRNA reagents has evolved from a research tool into a promising therapeutic. RNAi has the ability to be used as an effective screening tool to interrogate all the genes of the human genome to answer questions about the mechanism of action of compounds and disease processes. Identification of novel drug targets, the pathways that regulate compound resistance, synthetic lethal interactions and genes that are essential for disease progression are essential to the drug discovery pipelines and the betterment of human health. RNAi can be used to unravel the mechanism of action of compounds being developed in the drug discovery pipeline, helping pharmaceutical companies deliver more novel and higher quality therapies into the clinic. Focused research in the design and delivery of siRNA in vivo provides a promising new field of therapeutics that may be able to drug the undruggable targets.

References


