Oncology target discovery by RNAi: the past, present, and future perspectives

Despite an investment of billions of US dollars in the search of novel therapies, cancer still remains the leading cause of death in the world. This emphasises the need to identify novel tumour dependencies and molecular targets. Functional genomic tools such as interfering RNAs hold the promise to identify such functional targets in context of key signalling pathways and disease-relevant model systems, expanding the frontiers of the drug discovery world. For this reason, RNA interference technology was embraced with enthusiasm and adapted to high-throughput platforms to address the unmet clinical needs. Despite the potential held by the RNAi screening technology prophesied over the past 15 years, the drug discovery world still remains in dearth of novel targets. The promising candidates from RNAi screens are either refuted or rarely followed up by independent groups, while none of them has contributed towards clinical drug development. The pitfalls and challenges pertaining to RNAi screening have gradually emerged, and still need to be addressed in their entirety. There is a need to manage expectations with regards to the legitimacy of novel targets identified from RNAi screens. Most of the RNAi screens performing measuring cell death or relative hairpin depletions have met with limited success. Perhaps a different approach, such as drug modifier screens, would be more beneficial, especially in terms of developing combination therapy in cancer.

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The International Human Genome Sequencing Consortium announced the completion of the human genome sequence in April 2004[1], laying a strong foundation for the emergence of a new era in functional genomics and proteomics. The simple knowledge of the nucleotide arrangement in a gene would not suffice our understanding of disease development and progression. Therefore, the emphasis now lay on discovery of novel technologies that would allow scientists to better decipher the association among the newly-sequenced genes and a disease state, in context of biological functions and cellular pathways, so as to establish their feasibility as clinical targets. The classical genetic methods used to determine gene expression and function were comprised of

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random mutagenesis studies, which would measure the effect of a mutated gene by means of a change in its phenotypic response[2]. Other approaches have also been tested to study gene function in the past, which included linkage analysis, homologous sequence matching, anti-sense RNAs and microarray expression[2-4]. However, these approaches were limited in terms of the scale at which they could be deployed, were time-consuming and expensive or were simply marred with issues associated with their reliability and quality of probes used for testing[2,5]. Amidst the ongoing struggle to find a robust technology to study genes, RNA interference (RNAi) came as a breath of fresh air. RNAi held the promise to revolutionise the field of functional genomics by addressing the limitations of the existing techniques[6] and simplifying the process to understand the role of possibly every gene in the human genome.

The discovery of RNAi has its roots back in the year 1990, when Napoli and co-workers introduced a chimeric *chalcone synthase* (CS) gene, responsible for petal pigmentation in petunia, in an attempt to overexpress this gene in the plant[7]. Unexpectedly, they observed a complete blockage in petal pigmentation, a phenomenon they hypothesised to be a co-suppression of the CS gene. Following this early observation of RNAi, unknown at the time, a few other scattered groups also reported similar findings; Romano and Macino (1992) in *Neurospora crassa*, Guo and Kemphues (1995) in *Caenorhabditis elegans*, and Bhadra and co-workers (1997) in Drosophila melanogaster[8-10]. However, it was not until 1998, that Fire and Mello, independently, reported on the occurrence of a gene silencing phenomenon triggered by long double stranded RNAs (dsRNAs) in *Caenorhabditis elegans* and termed it as RNAi[11]. This discovery remains the hallmark in the arena of functional genomics and was considered so groundbreaking that Fire and Mello were honoured for their discovery with a noble prize in Physiology and Medicine in 2006. The discovery of RNAi not only revealed a naturally occurring phenomenon of gene regulation, but also opened doors to a novel genomic technology to explore the entire human genome – one gene at a time, to study gene expression and function, carving the path towards identification of novel clinical targets and ultimately to accelerate the process of drug discovery.

1998-2009: an era of initial excitement

RNAi was embraced as the holy grail of scientific research, ushering the drug discovery world into an era of modern medicine. The sheer numbers of publications on the use of RNAi as a technology, which number approximately 600 so far, showcase the excitement surrounding its widespread use within a few years[12]. Past one decade can indeed be considered as the period of initial enthusiasm. A year after Fire and Mello reported their discovery of interference conferred by long dsRNAs[11], Roland Kreutzer and Stefan Limmer demonstrated that even short dsRNAs were capable of silencing genes, which heightened the expectations of their potential use in mammalian cells[13]. This was quickly followed by another milestone in the field of RNAi, which was the feasibility of synthetically designing small interfering RNA (siRNA) duplexes bearing the capability to elicit gene silencing; this early rationale of designing 21-22 base pair long siRNA duplexes was introduced in 2001 as Tuschl's rules, an article which has been cited 2,183 times[14]. Technological advancements made post this early finding, enabled design of siRNA duplexes harbouring chemical modifications for improved efficacy[15].

The period of 2003-04 marked the early beginnings of implementing RNAi as a technology using high-throughput approaches; large-scale dsRNA screens were successfully completed in *Drosophila* cells and *Caenorhabditis elegans*. Aza-Blanc and co-workers (2003) took this a step further by publishing the first siRNA duplex screen targeting a focused set of 510 kinases in HeLa cell line to study TRAIL-induced apoptosis; the screen was performed in singles, which is one hairpin per well, and using cellular viability as the end point readout[16]. They reported an identification of two promising targets obtained from the screen, *DOBI* and *MIRSA*[16]. The silencing rendered by the siRNA duplexes was soon recognised to have a limitation of being transient[17]. This motivated several groups to develop plasmid vectors which would express a short-hairpin RNA (shRNAs), viewing this as a potent solution to enable prolonged inhibition of the target mRNA. Brummelkamp and Berns (2004) became the first group to perform a loss-of-function screen using these shRNA hairpins in immortalised human fibroblasts in order to identify new members of the *p53* pathway[18-19]. For the screen reported by Brummelkamp and co-workers, various shRNA hairpins were cloned into retroviral vector systems, to target a focused set of 638 genes, a collection of shRNA hairpins that they called the NKI library. Their screen was conducted in a 96-well pooled format, which comprised of multiple hairpins targeting one gene per well. The barcode microarray hybridisation was introduced as a

method to deconvolute the data outputs and relative hairpin depletion was measured as the end point readout[19]. Following suit to these two flavours of the siRNA duplex and shRNA hairpin technologies, Kittler and co-workers introduced another form of synthetic RNAi for mammalian cells, which was a collection of endoribonuclease-prepared short interfering RNA (esiRNA) targeting approximately 15,500 genes; esiRNAs were claimed to confer a higher target specificity, and latter screened on high-throughput platforms by the same group[20,21].

In the following years, RNAi technology was rapidly transitioned to high-throughput screening formats to be used by various pharmaceutical companies and academic screening centres[12]. A major contributor to this swift adaptation was the already established chemical screening platform including sophisticated robotics, instrumentation, automated liquid handling systems and even data analysis practices. In 2007, Whitehurst and colleagues performed the first genome-wide RNAi screen, a drug modifier siRNA duplex lethality screen using human non-small-cell lung cancer line to seek novel gene targets that would sensitise the cells to the treatment of taxol[22]. This attempt, for the first time, showed not only the feasibility to perform RNAi screens at a genome-wide level but, in addition, highlighted the plausibility of utilising RNAi to identify drug synergisers, opening new avenues to explore combination therapy in cancer.

Furthermore, microRNAs (miRNAs), although first characterised in 1993, were ignored until the early 2000s when their role as being the key regulators of cellular processes emerged, making them an area of 'hot topic' in cancer research[23,24]. As could be expected, by approximately 2008, miRNA mimic libraries were already being developed and made commercially available[25]; a literature search reveals approximately 20 published screens performed using the miRNA mimics to date. Taken together, RNAi has indeed come a long way from its initial discovery in plants to its use as a technology in nematodes, followed by human cell lines, even those that were hard to transfect, and now even whole mammalian systems, as was shown recently in an *in vivo* RNAi screen in mice[26,27]. RNAi is versatile in nature with regards to its application as a tool, and has been used to elucidate pathways and identify novel targets in the fields of oncology, virology and so on. The shRNA hairpins, predominantly designed using The RNAi Consortium (TRC) guidelines or the miR-30 backbone derived, and the siRNA duplexes, designed to harbour chemical modifications, have emerged as the leading players in the field of RNAi screening[15,28-30]. These technologies have evolved over time and can be used to screen in high content assay systems; although measuring cellular viability or cell death still prevails as end point measurements of choice. In addition, screens performed in pooled formats are found to be more prominent, perhaps due to the ease of their execution and low initial cost associated; it is now possible to screen single pools comprised of thousands of hairpins[12,31]. This led to advancements in the methodologies to deconvolute data outputs from pooled screens, which progressed from barcode hybridisation to half-barcodes and Next Generation Sequencing[32,33]. Nonetheless, the data originating from screens carried out in humongous pools appear to be marked with higher noise, relative to those performed using the concept of one hairpin per well, called arrays or singles[12,31]. A likely explanation to this might be attributed to the possibility of multiple distinct hairpin integrations per cell within a pool, also bearing in mind the additional errors introduced at the stage of data deconvolution[12].

Blindfolded with this initial euphoria, critical observations indicating potential hazards of the RNAi technology were readily overlooked, most important being the discovery of Off-target Effects (OTEs). The first evidence towards the existence of OTEs was provided by Jackson and co-workers in 2003, where they demonstrated that HeLa cells treated with siRNA duplexes targeting *MAPK14*, not only show a reduction in the expression profiles of the target gene but, in addition, exhibit a knockdown of up to nine unrelated genes, which bore only a partial sequence complementarity with the duplex[34]. The following year, Scacheri and co-workers reported on an unexpected reduction in protein levels of two alternate targets, *p53* and *p21* besides the target *MEN1*, post treatment with siRNA duplexes[35]. It is interesting to note that *p53* and *p21* are key cell cycle regulators and oncogenic drivers, therefore, highlighting the risk of misinterpreting RNAi data outcomes especially in context of tumour dependencies in cancer. A number of other studies professing implications of OTEs were reported since then[36-39]. Attempts were made to understand and address the problems pertaining to OTEs, but, unfortunately, were never considered significant enough to develop and incorporate a specific strategy for their removal from RNAi screen data outputs. Perhaps, the advocates of RNAi were more enthusiastic about its potential to discover novel therapeutic targets in detrimental diseases such as cancer.

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Growing transitional gap between laboratory and clinic: how to fill the pipeline?

Drug discovery, in the ancient times, was dominated by testing chemical entities for a therapeutic effect in organisms[40]. The conception and design of high-throughput screening (HTS) platforms in the 1990s, created a new paradigm for drug discovery, where millions of compounds could be tested in a matter of hours and to identify promising compounds for lead development and clinical testing[40,41]. Despite an investment of billions of dollars and a time commitment of a minimum of 10-15 years, the number of drugs transitioning from research laboratories to clinic is not at par with the soaring number of compounds available for HTS[41]. This growing transitional gap is in part due to the limitations in the level of diversity attainable in chemicals designed using the synthetic or combinatorial chemistry. The versatility in the current chemical libraries is negligible in comparison to the total unexplored chemical space[41]. To address this issue the focus shifts to Mother Nature; plant extracts and natural products can be incorporated as a part of the large screening libraries so as to diversify the compound collections being tested. However, purification of the active chemical component from complex natural products still remains a challenging task to accomplish[42].

In the past, we were handicapped by the unavailability of genomic information, which has now grown by leaps and bounds since the landmarked completion of the human genome sequencing. This led to a gain in momentum towards a rational target-based drug discovery, which took the place of the classical black box-based drug discovery[43,44]. The standard workflow of this modern day target-based drug discovery initiates with target nomination and validation; followed by identification of chemical inhibitors against the nominated targets, hit identification, lead optimisation and finally pre-clinical testing[43]. The fundamental requirement for this approach is identification of a tractable molecular target, which would specifically inhibit disease relevant pathways in the cells and reverse the disease state. To this end, the explosion of genomic data has indeed widened the probable drug discovery landscape; a plethora of genes are available to be explored for therapeutic value.

Targeted drug discovery gathered a lot of attention for developing therapies in cancer[45,46]. There have been significant advances in our understanding of cancer genomics and associated pathophysiology, which is marked by genetic aberrations, such as loss-of-function mutations like *BRCA1*, and *RB*, gain-of-function mutations, some of which are identified solely from genomic sequencing like *IDH1*, or over-expression of genes like *HER-2*. Similarly, the target-based drug discovery has shown progress over the years to identify novel therapeutics with targeted selectivities as opposed to the generic cytotoxicities of the conventional chemotherapy. Up to 63 targeted drugs have been approved for cancer treatment in patients till date, as an example imatinib targeting *BCR-Abl* is used for the treatment of chronic myelogenous leukaemia. However, this approach has met with limited success in terms of diversity in the target classes, which are mostly protein kinases such as *EGFR*. Unfortunately, our current knowledge of the druggable-genome is still limited and can be predicted to be only about 20% of the total known genes[47-48]. At this stage, integrating functional genomics and chemical genomics into high-throughput systems would open doors to address this challenge in the target-based drug discovery workflow. This is where the genomic tools such as RNAi bear the potential to search for novel molecular targets allowing for their functional classification and validation in context of intricate biological networks and pathways so as to filter out rational targets for drug discovery. The approach of using RNAi also makes it feasible to identify differentially expressed genes in cancer such as those harbouring an oncogenic addiction or rewired metabolic pathways.

Genome-wide RNAi screens are synonymous to grand fishing expeditions, scanning through the entire genome in search of oncology targets; typically, an unbiased RNAi screen would produce hundreds of gene candidates, which are prioritised based on their functional classification and relevance to the disease-related pathways[17,31]. These selected candidates are then followed up in smaller scale confirmatory screens, preferably using siRNA duplexes and also validated for specific knock downs using qRT-PCR. This refined set of targets are now ready to be progressed for chemical screening so as to identify small molecule inhibitors active against these targets, especially those that are already in pre-clinical trials, and to test if their blockage would exhibit a therapeutic effect. A more pragmatic approach would be to identify and test FDA-approved drug inhibitors against these newly discovered targets. This would present a phenomenal opportunity to drive translational medicine, where the clinic could benefit directly from the laboratory research within a shortest span of time. In addition, cross-referencing outcomes from multiple high-throughput platforms, such as

genome-wide RNAi screens and chemical inhibitor screens, would also be enabling for rapid drug discovery. The possibilities and expectations from RNAi technology are multifold, but the question arises – has the RNAi truly delivered its promise of filling the drug discovery pipeline with novel clinical targets?

Emergence of data reproducibility issues; gene targets discovered by RNAi

The targets discovered in any scientific research are reliable and meaningful only if they exhibit conforming results each time; consistency is the key. Along similar lines, the data quality and reproducibility of RNAi screens can be discerned at two distinct levels – 1) Hit lists identified by independent research groups have a decent level of overlap among them, and 2) Validated targets identified by one group are reproducible when followed up by a different group. Before applying these principles to assess the outcomes from RNAi screens, it is important to bear in mind that the data reproducibility would be impacted to some extent by the inherent heterogeneity and noise associated with RNAi screens, caused by variable transfection efficiencies, random off-target effects (OTEs), and so on. Nonetheless, what we observe in the end with regards to data discrepancies among RNAi screens is completely unanticipated and shocking, as detailed below.

In its early years, the RNAi screening centres seemed to be operating in isolation and the journals were swarmed with the articles publishing promising hit candidates from RNAi screens. The first ever effort in terms of consolidating screening results came about as late as 2009 when Bushman and co-workers compared hits from HIV host-virus interaction studies. Among those were three genome-wide siRNA duplex screens. Their comparative analysis of the three siRNA duplex screens revealed only three genes in common: *MED6*, *MED7* and *RELA*, all of which are components of the host transcriptional machinery[49]. Two years later Pache and co-workers expanded this comparison by including an additional genome-wide shRNA hairpin screen for targets in HIV infection[50].

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Surprisingly, no genes overlapped among the four screens being compared[50]; the receptor-co-receptor complex of *CXCR4* and *CCR5*, which is critical for the entry of the HIV virus into the host cells, was also missing[12]. These four screens were performed in different cell lines measuring either the ß-galactosidase activity or cellular viability in different laboratories, and analysed using different statistical methods; Pache and co-workers held these factors responsible for the observed data discrepancy[50]. These analyses were the first red flags on the reproducibility of RNAi screens but, unfortunately, remained widely ignored.

In 2013, an extensive endeavour was undertaken to compare 30 published RNAi lethality screens comprised of more than a hundred different hits lists, with the motivation to compile a portfolio of genes essential for cellular survival[31]. Surprisingly, not only was the overlap marginal across the board, but also key essential genes such as *PLK1* and *KIF11* routinely used controls in RNAi screens and a benchmark for accessing the performance of lethality screens, failed to qualify among the top scoring hits in the overlap[31]. As mentioned earlier, instead of addressing the issue of data reproducibility head-on, explanations with regards to experimental variations had been provided prior to this vast comparative study of lethality screens[50]. These explanations were soon to be dismissed based on a comparative report of two genome-wide RNAi screens conducted to identify novel modulators of miRNA biogenesis. The two screens were performed using a similar experimental set-up, assay system, data analysis methodology and personnel, and the only point of difference was in the choice of technology used; one screen was siRNA duplex while the other was shRNA hairpin[51]. Even under such controlled conditions, the data reproducibility was questionable. Although marginal commonality was observed at the levels of cellular pathways and biological functions, the actual number of overlapping genes was only 29, which included only one known modulator of miRNA biogenesis, *DROSHA*[51]. This accounted for a very dismal overlap, provided approximately 15,000 genes were common to both the libraries screened[51]. Interestingly, both of these screens were gain-of-function, and therefore expected to be cleaner relative to loss-of-function screens. Taken together, these studies strongly suggest that the issue of data reproducibility at the level of screens conducted by independent groups is far more widespread and serious than expected. Importantly, if the search is indeed for clinical targets, what significance would identification of common pathways hold on the path to drug discovery?

Furthermore, the issue of data reproducibility translates into a high risk of unsuccessful outcomes from studies undertaken by independent groups to validated targets from non-reproducible hit lists. The most popular example in this context is that of *STK33*, a promising target identified for its essentiality in survival of *KRAS*-dependent cancer cell lines by a reputed academic RNAi screening centre; 5,025 hairpins were screened in pools targeting 1,011 genes and *STK33* was identified as the strongest hit[52]. Pharma companies, in practice, refer to the findings from basic research laboratories in order to scout promising targets for follow-ups. Similarly, a group in Amgen led by Babij picked up *STK33* for independent testing so as to pursue it further as a clinical target. Unfortunately, they failed to reproduce any association between the *KRAS*-status of cancer cells and a selective killing after *STK33* knockdown[53]. These findings from Babij and co-workers were initially met with a dismissive criticism from the original group[54]. Shortly after, another group from the same academic institution described that a selective *STK33* inhibitor, BRD8899, did fail to block proliferation of *KRAS*-dependent cells[55]. These findings on *STK33* brought into light the nuisances associated with RNAi technology and, for the first time, raised a question on its merits. Since then, a handful of cancer-relevant targets identified from RNAi screens have already been refuted, such as *TBK1*[12], while the drug discovery pipeline still awaits legitimate clinical targets.

Revisiting RNAi a decade later

RNAi was adapted enthusiastically to high-throughput platforms for target hunting, prior to a complete realisation of its potentials *vis-a-vis* its pitfall. It is only now that we are growing to become more aware and accepting of the associated challenges. Importantly, the sensitivity and specificity of RNAi as a technology platform is on fire. The basic principle behind using RNAi is to silence specific genes in the genome, but the troubling question is whether we in fact know the actual number of protein-coding genes in the human genome. The initial prediction of up to 100,000 genes has now been contained to fall within the range of 20-25,000 genes, but the list is constantly being revised[56,57]. Furthermore, these protein-coding genes constitute only about 2% of the total human genome[12]. The remaining 98% of the DNA is non-coding, previously determined to be 'junk DNA', and now known to be comprised

of regulatory non-coding RNAs (ncRNA) that tightly control gene expression at various levels[58]. Clearly, the existence of this endogenous regulatory machinery is bound to undermine the specificity and sensitivity of exogenous RNAi, as we seek to disrupt the complex intracellular network in search of a specific phenotypic response.

It is an underlying assumption that RNAi being a ubiquitous mechanism would produce similar degrees of phenotypic perturbations in a battery of cell lines. In 2010, Boettcher and co-workers reported on differential knockdown efficiencies exhibited by miR-30-based shRNA hairpins after their transfection into an ovarian cell line versus a breast cancer cell line[32]; this was the first indication towards existence of cell-line specific behaviour of exogenous RNAi. To validate each and every RNAi duplex in a plethora of routinely used cell lines would be a daunting task to accomplish given the genome-scale coverage of most of the commercially available siRNA and shRNA libraries. Nonetheless, efforts are ongoing to validate these commercially available RNAi duplexes to assert their target specific knockdowns. Such attempts to validate the duplexes have been made by Ambion (now a part of Thermo Fisher Scientific, Waltham, MA) for their collection of Silencer® Select pre-designed siRNAs, and Sigma-Aldrich (St Louis, MO) in collaboration with the Broad Institute (Cambridge, MA) for their TRC-based shRNA hairpins libraries[28,59]. Despite this vertical progress in the number of duplexes being validated, the horizontal progress in terms of the versatility of cell lines utilised for validation is limited. As an example, the TRC-based shRNA hairpins have been validated mostly in the A549 cell line, a human lung carcinoma cell line that is more susceptible to viral infections than the others. Similarly, the Silencer® Select pre-designed siRNAs have been validated in HeLa and U-2 OS human osteosarcoma cell lines. To highlight this further by means of an example, the TRC library carries 23 hairpins targeting *PLK1*, 20 of which have been experimentally validated in MCF7 cell line. However, when tested as part of a genome-wide simple lethality screen, these validated hairpins failed to render a similar phenotypic effect in HeLa cell line, and only four out of these 20 validated hairpins were scored as active[60]. This is a rather surprising result given the generic essentiality of *PLK1* in cellular survival and that the end point measurement in this screen was cell death, as quantified by reduction in total nuclei count.

Even after years of exploiting RNAi as a technology at a high-throughput scale, it is perplexing that we are still not well-versed with the underlying mechanisms of the RNAi pathway. The naturally occurring RNAi pathway was conventionally believed to be fairly conserved; the role of *DICER1* enzyme was determined to be key in processing miRNAs inside the cell, and was also assumed to play a similar role in intracellular processing of the synthetic shRNA hairpins[61]. Recent discoveries, on the contrary, demonstrate that the engagement of *DICER1* in hairpin cleavage is not indispensable, thereby revealing the existence of a dicer independent pathway[62-64]. It has been predicted that the stem and loop length of a hairpin oligonucleotide might perhaps be a determinant of the fate of its intracellular hairpin cleavage[62-64]. Additionally, no association has been found between *DICER1* status and target knockdown in a report comparing mRNA reduction profiles obtained from three pairs of *DICER1* mutated or wild-type cell lines transduced with a standard TRC shRNA hairpin (TRCN#40273)[65]. While we were sill questioning the very basics of RNAi pathway, a recent report published by Chu and co-workers made an unexpected observation in terms of variability's in RNAi performance; their work with western corn rootworm revealed that efficacy of RNAi duplexes is also subject to variations depending on the levels of intracellular gene expression and physiological conditions[66]. These findings taken together highlight the gaps in our comprehensive knowledge with regards to the RNAi technology, which still remains an ongoing learning process.

Besides all these newly-identified challenges in terms of the biology of RNAi, there is another issue that arose purely due to the negligence on the part of the global RNAi community- a complete lack of standardisation in RNAi screening practices. Although RNAi was accepted as a technology of choice worldwide, individual RNAi screening centres seemed to operate in isolation[12], a cross talk among groups using this technology did not seem to exist. As an example, up to 33 different methods of nominating a hit have been found from a mere 80 RNAi screening publications[67]. Ironically, almost none of the data analysis methodologies were tailored specifically for analysing RNAi screening data, which should have inherently included a metrics to incorporate its combinatorial nature such as an H score, and a technique to filter out potential seed-based off-targeting duplexes[67]. Besides, no standardised repository exists to date that would mandate the submission of the entire raw data obtained from RNAi screens, so as to allow for their unbiased comparison across the

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board. Efforts ongoing to set up such database resources have met limited success.

After a decade of RNAi screening, we are still not well-positioned to ask ourselves the question as to whether the technology has delivered. Perhaps since most of the concepts of RNAi screening were borrowed from the standard chemical screening practices, RNAi was never really given the time to come of age as an independent technology. We might even speculate that RNAi is on its way to extinction and joining the ranks of the dinosaurs, which once were the most dominant terrestrial vertebrates.

Hairpins in a petridish: pools of ambiguity and lacking scientific merits

RNAi screens when conducted in pooled formats are traditionally believed to be robust and cost-effective in identifying novel targets, which, however, seems to be questionable especially in the context of shRNA hairpin screens. Commercially available genome scale shRNA libraries are either designed using the TRC guidelines or the miR-30 backbone, and comprise up to a million targeting hairpins[28-29]. These libraries can be utilised to perform an RNAi screen using either of the two available formats; 1) arrayed, where one RNAi duplex targeting one gene is introduced in each well of a 96-well, or 384-well microtiter plate, or 2) pooled, where thousands of different hairpins targeting multiple genes simultaneously are introduced in the same petridish[31]. Here, it is important to note that there is no methodology in existence that would ensure the efficiency of hairpin transduction; specifically, it is difficult to determine whether a single or multiple plasmid vectors have integrated into a cell. Logically driven, it can be argued that an arrayed format provides a relatively controlled environment for the intracellular entry of a single type of hairpin designed to target a specific gene *in lieu* of a pooled format, where thousands of distinct hairpins are available for cellular integration within the same space. The possibility of multiple different integrations into the same cell within a constituent pool introduces a high level of complexity in the observed phenotypic readout. It would be hard to comprehend whether the results relate to targeted silencing or a synergistic and/or antagonistic silencing of multiple genes.

The ambiguity associated with the phenotypic outcomes from shRNA hairpin screens gets further amplified due to the newly-described phenomenon of Alternate Targeting Sequence Generator (ATSG)[65]. Prior to this discovery, Gu and co-workers had shown that a hairpin cleavage inside the cell might lead to production of multiple different sequence variants, besides the intended targeting sequence[68]. They had used miR-30-based shRNA hairpins in HEK293 cells to provide the first experimental evidence in the form of northern blots highlighting the inefficiencies in hairpin processing, but did not explain whether these variants of inefficient processing were functional. The discovery of ATSG has established that not only are these variants functional inside the cell, but also exert an unanticipated impact on the overall data outputs. ATSG explains that the alternate targeting sequences (ATS) resulting from inefficient hairpin cleavage lead to down regulation of unintended mRNAs and therefore non-specific gene silencing, as confirmed by qRT-PCR experiments[65]. This phenomenon has also been shown to exhibit a cell-type specific behaviour for the 27 different cell lines used in the experiment. ATSG was initially described in context of the arrayed screening platforms where one hairpin was shown to have approximately six alternate targets. Earlier in 2013, Ramji and co-workers had also reported on a similar observation while studying the NFκB signalling pathway in a melanoma cell line. For the same target, a plasmid-based shRNA hairpin led to non-specific gene silencing, while its siRNA counterpart rendered a target specific silencing[69]. This perplexing finding might as well be explained by the existence of ATSG.

At this stage, the question arises as to how deeply would ATSG impact the outcomes from the shRNA hairpin screens conducted in pooled formats and at a genome-wide scale. Taking into account the possibility of multiple different hairpins integrating into a single cell, the magnitude of ATS and non-specific shadow targets would grow exponentially. Each cell could be viewed as a black box, making it impossible to determine the reliability of the observed phenotype, and therefore a heightened risk of misinterpreting the data outputs from pooled screens (**Figure 1**). These pools of ambiguity are detrimental to the entire research community and question the legitimacy of hits identified through this process. This issue is more crucial given the high stakes in RNAi screening ventures, especially at a time when the world of drug discovery has hit stagnation and is in a dire need of novel therapeutic targets.

Interfering RNA in oncology: managing expectations

Genetic aberrations are the hallmarks of cancer. Multiple mutations are accumulated in the tumour cells in the form of deletions, insertions,

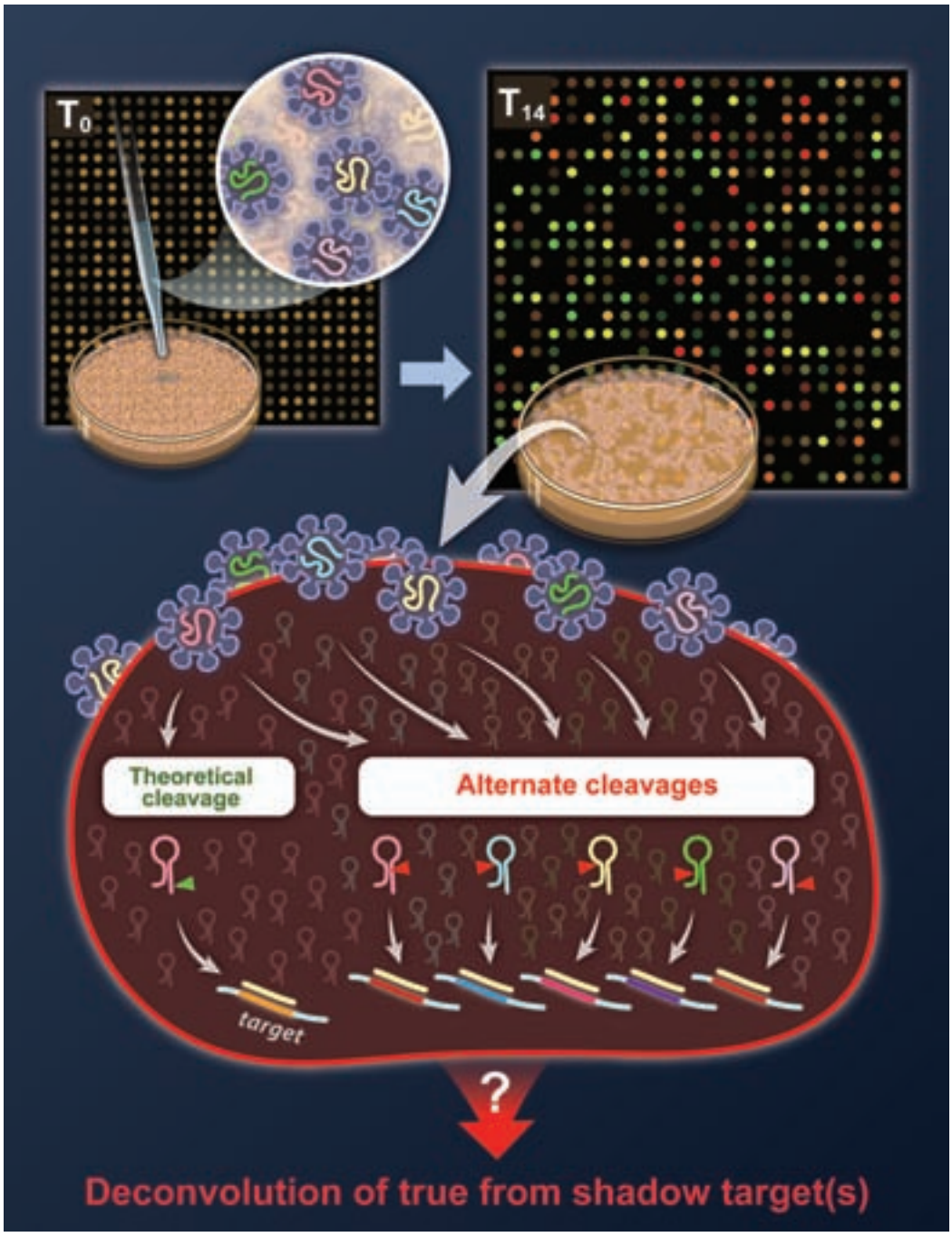

Figure 1
Pooled shRNA screening yields a plethora of non-specific targeting RNAi sequences in the cell. Pools comprising multiple different hairpins targeting multiple different genes collectively with end point readout of relative hairpin depletion or abundance. The validity of the outcome is illusive due to the recently discovered alternative targeting sequence generator, in addition to the likelihood of multiple integrations per cell and producing undesired targeting sequences

rearrangements, copy number variations, or epigenetic modifications triggering modulations in expression of genes, or proteins, or ncRNAs such as miRNAs[46, 70]. However, it is important to note that despite this hyper-genomic variability, all of these mutations might not be functionally relevant. Rachael Kobos and co-workers, in an effort to identify targets for sarcomas harbouring chimeric *ASPSCR1-TEF3* fusion proteins, devised a multiplexed strategy to screen a selected set of genes which were either up-regulated or down-regulated only in response to this fusion protein[71]. To this end, the group used genomic data corresponding to gene expression profiles and genome-wide transcription factor binding sites so as to compile a set of 130 genes, and tested them for activity in a siRNA duplex screen using a caspase-3 dependent apoptotic assay. Surprising, only 12 genes out of the 130 were identified as hits[71], a relatively low rate of success highlighting that only

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a selective number of variabilities hold a biological relevance in cancer progression.

The complexity of developing therapy for cancer grows even more due to the fact that the oncogenic driver mutations initiating tumorigenesis might not even play a role in its maintenance, while these accumulating mutations, including chemotherapy-resistant mutations, might confer a sustain uninhibited proliferation of cancer cells. More so, cancer cells have also been found to exhibit genomic variations among sub populations derived from the same cell of origin[46]. Altogether, the cancer genome is dynamic and these genetics alterations are diverse causing high genomic instability and also a reason behind non-respondent patient populations. RNAi screens have been conducted in the past to identify synthetic lethalities that underscore the tumour dependencies required for sustained proliferation of cancer cells, either within the same pathway of the oncogenic driver or in parallel pathways. This provided an opportunity to target novel avenues for therapeutic intervention and to reverse resistance selectively in cells harbouring oncogenic mutations such as *KRAS*-dependence or *p53*-dependence. However, RNAi screens, measuring total cell death or relative hairpin depletions, have not proven to be immensely successful as was anticipated; targets identified in RNAi screens are already being refuted and none of the targets identified from RNAi screens has reached the clinic[12]. A most useful strategy at this point would be to identify those hits from the RNAi screening outputs that have known chemical inhibitors and re-test for a cancer-specific response, the fastest way to test target legitimacy and the fastest route to enter clinical trials.

Meanwhile, a promising application of RNAi screening in cancer therapeutics would be to identify novel targets that sensitise the cells to the effect of the administered drugs. Several RNAi screens have been performed in the past to search for genes whose down-regulation would synergise with drugs, such as Bortezomib in myelomas, Toxol in breast cancer, Erlotinib in lung cancers and so on[72-74]. To this end, one cancer that could reap tremendous benefits from this approach is the Triple Negative Breast Cancer (TNBC). TNBC is characterised by the absence of estrogen receptors, progesterone receptors and *HER2*[75-76], making it extremely difficult to treat patient population with conventional chemotherapy. mTOR and/or PI3K signalling pathways are known to be modulated in TNBC, but mTOR and PI3K Inhibitors alone do not seem to produce significant effect on reducing cell growth and proliferation, and additional targets need to be identified to enhance the efficacy of the existing inhibitors. Another example would be that of cancers with altered Notch Signaling pathway, where γ-secretase has been viewed as a putative target for therapeutic intervention[77]. However, almost all of the small molecule inhibitors targeting γ-secretase have failed in clinic[78]; highlighting this unmet need to identify adjuncts to develop a more efficient therapy. Clearly, RNAi could play a role within this niche to identify drug sensitisers for cancers that are problematic to manage and this could go long way in developing combination therapies to improve patient prognosis.

Future perspectives

RNAi technology, at the time of its inception, was believed to hold a grand promise for functional genomics studies. However, 15 years into its trials and tribulations with no clinical target in near sight, the pitfalls of RNAi screening have become more evident than ever. It seems to have reached a cross junction of its pursuit as a technology in the future. Big Pharma took its time in adopting RNAi until about 2006, but seems to have acted faster in abandoning this technology – Pfizer, Roche and Merck had made an exit from their RNAi screening operations by 2010, and now Novartis is following in their footsteps by completely bailing out of its RNAi initiatives[79]. It is incumbent upon the academic screening centres to take necessary steps to mend the fence. Perhaps the preliminary steps required are towards standardisation of the RNAi practices and establishing a close-knit RNAi community; united we stand, divided we fall. Some of the RNAi screening centres have participated in an RNAi Global Initiative, with a motivation to establish standards for reporting RNAi screening data, called the Minimum Information About an RNAi Experiment (MIARE)[80]. The National Center for advancing translational Science (NCATS, Bethesda, MD) has taken steps to release the sequences of the siRNA duplexes of its screening libraries, so as to allow researchers to not only obtain the screen data but also give them an opportunity to assess sequence-based OTEs[81]. Since approximately 600 RNAi screens have already been published so far, and thousands of targets have been reported in association with various disease states, it might be worthwhile to apply a stringent analytical method to re-score hits from these published screens and to build a centralised repository comprising gene networks and frequent hitter targets that correlate with various types of cancer.

It is also important to note that the full-blown use of RNAi was initiated without the mechanistic

knowledge of the intracellular RNAi pathway, which is hijacked by the exogenous RNAi duplexes and hairpins. *Ago2*, a major splicer enzyme of the RNAi pathway was not discovered until 2004. This was followed by the discovery of a few constituents of the RISC complex in 2005, while the involvement of *DICER1* in the pathway still remains controversial[62-65,82]. It is rather surprising that without a full realisation of the fate of exogenous RNAi post its introduction into the cell; we had already adapted it to undertake large-scale endeavours, incurring huge investment of time and taxpayers' dollars. This, perhaps, is also the very reason as to why the phenomena like ATSG triggering random gene silencing remained unrecognised for so long[65]. The recent discovery of ATSG has now put us in a tough spot so as to question the merits of the hit lists identified from shRNA screens so far, especially those performed in pools of hundreds to thousands of hairpins.

While further research in the area of shRNA hairpin technology would most likely revolve around deciphering better methods to design more efficient shRNA hairpins than the ones currently in use, siRNA duplexes, with predefined targeting sequences, would become the preferred technology for years to come. Challenges pertaining to OTEs and ATSG dictate that executing RNAi screens using arrayed formats is perhaps a safer approach compared to using complex pools of ambiguity. In addition, based on our past experience, it might be best to restrain from simple lethality screens measuring cell death or relative hairpin depletions, but to move forward in the direction of gain-of-function phenotypic screens, with much cleaner outputs, and drug-modifier screens which would enable the development of combination therapy as well as predictive biomarker discovery for novel cancer therapies.

While the RNAi technology is in troubled waters amid its unaddressed challenges and commercial abandonment, the current trend reflects a turnaround. Genome editing technologies such as the zinc finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR) have been around since the turn of this century[83-84]. But as the issues pertaining to the use of RNAi technology are becoming more evident, genome editing technologies are rapidly picking up the pace as alternate investigational tools, predominantly the CRISPR/Cas system. However, even CRISPR/Cas system, at this time, has its own limitations, which include non-specific effects, requirement of a PAM motif and delivery of the system into cells, cell type specificities and perhaps also the propensity of long RNAs to fold into complex tertiary structures. As we undertake this daunting task to explore alternate technologies, it is important to remember the lessons learnt from our experience with adopting RNAi whole-heartedly in its neonatal stage. Perhaps the wisdom lies in primarily understanding the implications of the usage of the blossoming technologies, prior to rushing into their transition to high-throughput platforms and application towards target discovery.

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