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Review

Widespread genome transcription: New possibilities for RNA therapies

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ABSTRACT

Comprehensive analysis of mammalian transcriptomes has surprisingly revealed that a major fraction of the RNAs produced by mammalian cells and tissues is comprised of long non-coding RNAs (lncRNAs). Such RNAs were previously disregarded as useless, but recent functional studies have revealed that they have multiple regulatory functions. A large subset of these lncRNAs are antisense to protein-coding genes; such RNAs are particularly attractive to researchers because their functions are better understood than other lncRNAs and their action can be easily modulated and engineered by modifying the antisense region. We discuss various aspects of regulation by antisense RNAs and other small nucleic acids and the challenges to bring these technologies to gene therapy. Despite several remaining issues related to delivery, RNA stability, side effects, and toxicity, the field is moving quickly towards future biotechnological and health applications. Therapies based on lncRNAs may be the key to increased cell-specificity of future gene therapies.

Contents

1. A multitude of regulatory RNAs

One of the surprises of genomics has been the discovery that mammalian genomes produce a large amount of non-coding RNA. Apart from the production of small RNAs, such as microRNAs (miRNAs), which often function as negative regulators of RNA stability or translation, there are many long non-coding RNAs (lncRNAs) that have been well categorized both in mouse [1] and human [2–4]. Results of large-scale analyses suggest a striking but inconvenient truth: there are more non-coding RNAs than the ~20,000 protein-coding genes. The FANTOM3 project, by using physical cDNA clone analysis alone, identified more than 23,000 lncRNAs [1]. The GENCODE project (release 20), as part of the ENCODE project [5], identified almost 24,000 loci producing lncRNAs, but the total census of non-coding RNAs may be above 37,000 if miRNAs, RNA from processed pseudogene transcripts,

Abbreviations: Aβ, amyloid beta; AGO, argonaute; AON, antisense oligonucleotide; AS RNA, antisense RNA; ATL, adult T-cell leukemia; EBOV, Ebola virus; eRNA, enhancer RNA; ES, embryonic stem; HBZ, HTLV-1 basic leucine zipper factor; HTLV-1, human T-cell leukemia virus type 1; IL, interleukin; iPSC, induced pluripotent stem cell; lncRNA, long non-coding RNA; LTR, long terminal repeat; MARV, Marburg virus; miRNA, microRNA; NAT, natural antisense transcript; X-SCID, X-linked severe combined immunodeficiency; UTR, untranslated region.

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and other types of lncRNAs were to be included. The number of lncRNAs may still rise if next-generation sequencing studies focus on cell types that are not yet completely characterized. For example, in human embryonic stem (ES) cells and induced pluripotent stem cells (iPSCs), deep sequencing of cytoplasmic and nuclear transcripts has uncovered more than 3000 lncRNAs that were not previously identified in the GENCODE catalogue, including a large fraction of RNAs derived from long terminal repeats (LTRs) and other retrotransposon elements [6]. The function of the majority of lncRNAs is still unknown; however, whenever the function has been determined, lncRNAs show a remarkable diversity of function and mechanisms of action in various cell compartments.

After synthesis, a large number of lncRNAs never leave the nucleus, which makes this organelle a large source of uncharacterized lncRNAs [6,7]. A fraction of these lncRNAs closely interact with the chromatin and help to direct the epigenome. For instance, lncRNAs can mediate the interaction between polycomb repressive complex and target genes in ES cells [8], or mediate X-chromosome inactivation [9] by physically causing chromatin condensation around interacting regions in *cis* on X-chromosome [10,11]. Alternatively, lncRNAs can act in *trans*: e.g., *HOTAIR*, whose mis-expression is associated with cancer [12]. To further complicate the interpretation and analysis of lncRNAs, active enhancers also produce lncRNAs, often called eRNAs [6,13], whose function is mostly unknown. At least in some cases, eRNAs could be essential structural molecules that promote nuclear interactions through the multi-protein complex called Mediator, thus enhancing the set of potential regulatory lncRNAs [14]. LncRNAs can also have other structural functions in the nucleus: for instance, *NEAT1* is an essential RNA component of structures known as paraspeckles [15]. There are numerous cases where lncRNAs are processed to produce smaller RNAs, which have various regulatory functions, including not only miRNAs [16] and small nucleolar RNAs (commonly known as snoRNAs) [17] but also other classes of small RNAs [18,19]. Due to space limitations we cannot extensively review all aspects of RNA processing and we apologize for not citing some important works of colleagues: please see other reviews [20–23] for extensive discussions of lncRNAs and their processing.

Antisense transcripts [24] are a broad and very important component of the non-coding transcriptome. The mechanisms of action and regulation of antisense lncRNAs are somehow better understood than those of other lncRNAs. Because antisense sequences can easily be designed, lncRNA action based on sense-antisense transcript pairing is highly flexible in principle. Accordingly, we will focus on the biology and current applications of antisense lncRNAs, and how these transcripts could be used for future therapies.

2. Role of some lncRNA in diseases

Some antisense lncRNAs can positively regulate transcription [25,26]. A prime example is the regulation of the gene encoding β-secretase 1 (BASE1) (also known as β-site amyloid precursor protein-cleaving enzyme), which is implicated in the pathogenesis of Alzheimer's disease. Transcription of *BASE1* is positively regulated by its noncoding antisense transcript (*BASE1*-AS). Knockdown experiments with small interfering RNA (siRNA) against *BASE1*-AS RNA caused reduction of amyloid β (Aβ) 1–40 and Aβ 1–42 protein production in human SH-SY5Y cells. These results suggest that *BASE1*-AS transcript is positive regulator of transcription of the coding gene. *In vivo* experiments showed that 14 days continuous treatment with siRNA targeting *BASE1*-AS transcript reduces the *BACE1* mRNA expression level in mouse brain regions (cortex, striatum, dorsal hippocampus, and ventral hippocampus). Cell stress with high temperature, serum starvation, Aβ 1–42 accumulation, exposure to H_2O_2, or treatment with a high glucose concentration caused a 30–130% increase in *BACE1*-AS levels associated with a 20–60% increase in *BACE1* mRNA levels. When *BACE1*-AS transcript levels in the cerebellum, hippocampus, entorhinal cortex, and superior frontal gyrus were compared between Alzheimer's disease patients and control subjects, the *BACE1*-AS levels were elevated in Alzheimer's disease subjects by up to six fold, with an average of about two fold across all brain regions [25]. This study is particularly remarkable because the lncRNA expression level can positively control the mRNA expression level.

Some of the mechanisms of gene regulation based on sense-antisense pairing in the nucleus appear to involve transcriptional interference [27] or the siRNA pathway. For instance, in the nucleus, argonaute (AGO) proteins, a part of the RNA-induced silencing complex (RISC), are involved both in specific transcriptional activation and repression [28], suggesting broad involvement of the siRNA machinery on the chromatin. Importantly, although AS RNAs are commonly believed to be mostly negative regulators of sense mRNA counterparts [25], there are many more mechanisms of action, many of which are not yet fully understood.

Apart from regulating transcript stability, antisense lncRNAs can regulate transcription initiation [24,27]. This phenomenon could be used in future AS RNA therapies: in fact, inhibition and degradation of natural antisense transcripts (NATs) could upregulate some specific mRNAs for therapeutic purposes. The group led by Claes Wahlestedt found that inhibition of the NAT of the gene for brain-derived neurotrophic factor (BDNF) increases the level of the sense mRNA transcript and BDNF protein expression, leading to neuronal outgrowth and differentiation *in vitro* and *in vivo*. In a follow-up study, the same group investigated two additional RNAs, which are antisense to the mRNAs encoding glial-derived neurotrophic factor (GDNF) and ephrin receptor B2 (EPHB2). Inhibition of these NATs resulted in increased sense mRNA transcript levels [29], showing that this is a general phenomenon. The application of these approaches for increasing protein production is becoming recognized as important for human therapy. Accordingly, the company OPKO-CURNA in USA is exploiting this approach, which was named "Inhibition of Antisense Transcripts to Upregulate its Sense mRNA". These new RNAs, named AntagoNATs, are designed to target the NAT region overlapping the mRNA and thus inhibit the sense-antisense RNA pairing (Fig. 1A).

Since many antisense and other lncRNAs are regulated by cellular stress, it is very important to understand their dynamic expression and regulation when cells or tissues are under stress, including during heat shock [30] or immune challenge [31].

Genetic mutations can affect antisense regulation. For example, α-thalassemia is a disorder caused by reduced production of functional globins due to gene mutation. In particular cases, the mutation increases expression of an antisense transcript, called *LUC7L*, which induces transcriptional silencing of globin genes by methylation of the CpG island [32].

3. SINEUPs: surprising AS RNAs that enhance translation

Reduction in the expression of the *UCHL1* gene (also called *PARK5*), is positively correlated with familial Parkinson's disease [33]. A surprising novel mechanism of sense-antisense action came from the study of AS *Uchl1* RNA (AS-*Uchl1*) in mouse [34]. Large-scale analysis of full-length cDNA sequences by the FANTOM3 consortium reported widespread sense-antisense transcription (>72% of identified genes in mouse) [24]. Using the FANTOM3 cDNA clones, Carrieri et al. investigated the functional role of AS RNAs, including AS-*Uchl1*, in the mouse dopaminergic neuronal cell-line, MN9D. AS-*Uchl1* overlaps the 5′ untranslated region (UTR) and

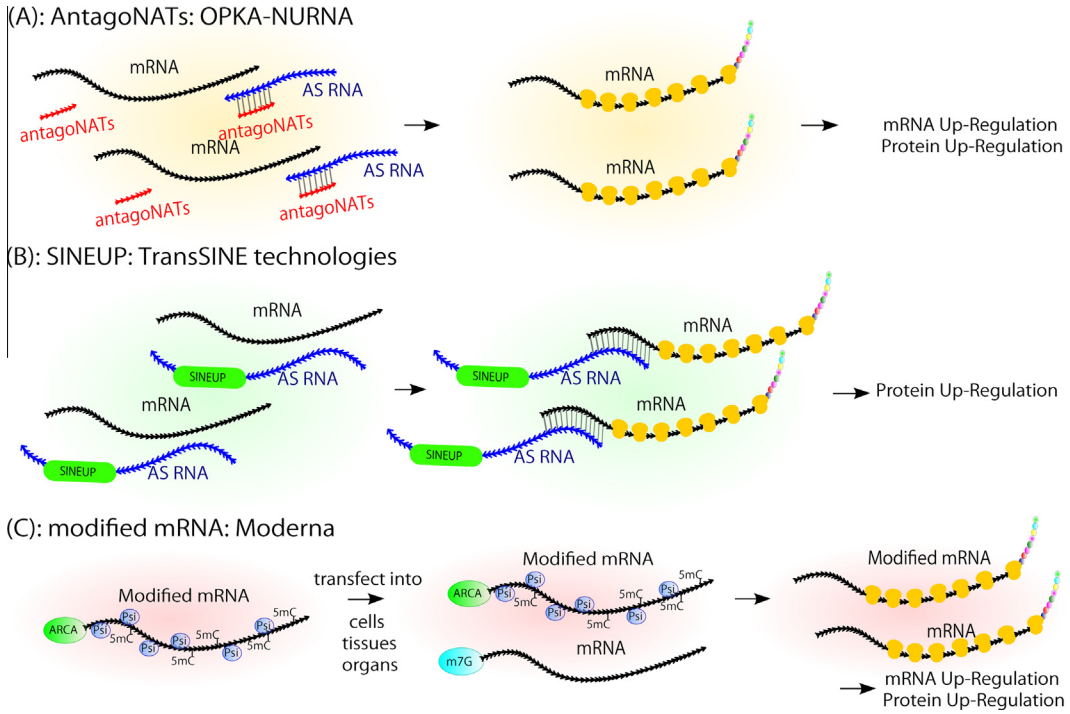

Fig. 1. Novel RNA therapeutics. (A) AntagoNATs: inhibitors of natural antisense transcripts (antagoNATs) are designed against non-coding AS RNAs in the region that overlaps with mRNA, causing the mRNA to be overexpressed with resultant up-regulation at the protein level. (B) SINEUP: a SINE element is present in the antisense RNA (AS RNA). When the mRNA and AS RNA overlap, the AS RNA can work as a guide for mRNA translation, thereby enhancing protein production. (C) Modified mRNAs are synthesized with anti reverse cap analog (ARCA) and pseudouridine to increase stability. After transfection into cells or tissues, or injection into organs, protein is produced mostly from the modified mRNA, but also from the endogenous mRNA.

translational start site of the *Uchl1* mRNA [34]. Surprisingly, while *Uchl1*-AS does not have any effect on the stability of the mRNA, it up-regulates the translation of the UCHL1 protein. Distinct from the region of overlap, AS-*Uchl1* contains an inverted short interspersed element (SINE) B2 repeat, which is transcribed in the antisense orientation relative to the canonical sequence reported in the FANTOM databases. Deletion mutant analysis revealed that both the overlapping region and the SINEB2 region are essential to up-regulate translation. This RNA, together with similar RNAs identified in the study, are called SINEUPs, because they contain SINE elements that UP-regulate protein translation (Fig. 1B). AS-*Uchl1* works in signaling stress response. In MN9D cells treated with rapamycin, which inhibits the CAP-dependent translational pathway through mammalian target of rapamycin (mTOR) and induces stress response pathways, AS-*Uchl1* is exported from the nucleus to the cytosol, where it pairs with sense *Uchl1* mRNA. This pairing stimulates the interaction between the mRNA and actively translating polysomes, thereby increasing UCHL1 translation [34]. In summary, in the cytosol SINEUPs can stimulate protein translation from the mRNA with which they pair [34]. SINEUPs that are artificially introduced by using overexpression vectors can simply stimulate translation by specifically hybridizing to the 5′ UTRs of target mRNAs, as demonstrated for the reporter molecule, green fluorescent protein [34]. Because SINEUPs can be designed to increase translation of various mRNAs, it is theoretically possible to construct a genome-wide resource by simply designing short overlapping regions complementary to the 5′ UTRs of all mammalian mRNAs.

How many natural SINEUPs exist? In the first bioinformatics screening for AS RNAs carrying SINE elements in their non-overlapping regions [34], about 100 potential SINEUPs were identified in the mouse transcriptome. While the AS RNA that pairs with *Uxt*-1 mRNA has been proven to be another true SINEUP, many more candidates await experimental validation. We believe that their cell or tissue specificity and strength may differ. Remarkably, the SINEUPs that were discovered in the mouse are also effective in human cells [34]. Because SINEB2 regions are not present in the human genome, some conserved structure (rather than sequence) is likely to be functional. This finding raises the fascinating question of how specific non-conserved domains of mouse lncRNAs can be functional in human and other mammalian cells (unpublished observations). Perhaps there are structures that we have not yet identified, which are conserved in apparently different lncRNAs across different species. Human natural SINEUPs have not yet been discovered; further studies are needed to fully clarify the broadness and applicability of SINEUP technology in vertebrates and beyond.

4. AS RNA in viral pathogenesis

The active domains of SINEUPs originate from SINEB2 retrotransposons, which include ancient retroviral elements and other transposable, parasitic elements that have been integrated in the genome, often to become symbiotic regulatory elements [34]. Non-coding RNA activity by viruses, and in particular retroviruses, can still be observed in common parasites of living primate species. Several reports suggest up-regulation of antisense RNAs after viral infection [35–39]; some of these lncRNAs map to the opposite strand of the 5′ LTR, 3′ LTR, or both of a virus genome and they function to silence viral genome amplification. One striking example is the report that an HIV-1-encoded antisense lncRNA, which localizes to the 5′ LTR promoter region of the HIV-1 genome, works as an epigenetic viral transcription regulator to suppress viral

replication [35,37,40,41]. Saayman et al. conducted chromatin immunoprecipitation (ChIP) to investigate whether HIV-1-encoded antisense lncRNA can epigenetically regulate viral transcription in an HIV-1 latent T cell clone (ACH2). They discovered that HIV-1-encoded antisense lncRNAs associate with DNA methyltransferase 3a (DNMT3a), causing destabilization of viral transcripts. Mechanistic studies further revealed a role for the HIV-1-encoded antisense lncRNA, together with small inhibitory RNAs, such as *as154* or *aspro5*, which target the *rev* and *ref* regions, respectively, of the antisense lncRNA. Inhibition of antisense transcripts resulted in reduction of both the Enhancer of Zeste Homolog 2 (EZH2), which is a polycomb-group protein that acts as a histone methyltransferase and histone deacetylase 1 (HDAC-1). These results imply that binding of viral transcripts to the antisense lncRNA may guide epigenetic silencing by directing the chromatin-remodeling protein complex to specific targets [36].

Another interesting example of a viral lncRNA is an AS RNA expressed by human T-cell leukemia virus type 1 (HTLV-1). HTLV-1 infection causes adult T-cell leukemia (ATL), a chronic disease of the spinal cord named HTLV-1-associated myelopathy, and HTLV-1 uveitis [42,43]. The HTLV-1 genome expresses an antisense HTLV-1 basic leucine zipper factor (*HBZ*) RNA, which can down-regulate viral transcription, reduce leukemogenesis, and promote T-lymphocyte proliferation [38,44–46]. *HBZ* RNA has two isoforms: a spliced one (s*HBZ*) with two exons and an unspliced one (us*HBZ*) with one exon, which produce polypeptides of different length (206 and 209 amino acids, respectively). 5′ RACE (Rapid Amplification of cDNA ends) experiments confirmed that transcription start sites are located in the first exon of s*HBZ*, which resides in the U3-R region of the 3′ LTR of the HTLV-1 proviral genome [39,47]. Interestingly, s*HBZ* expression is 4 times that of us*HBZ* in HTLV-1 carriers and in ATL cases. This suggests that inhibition of the first exon of s*HBZ* might stop ATL cell proliferation, with important therapeutic implications.

A third viral example is from the *Sendai virus*: when infecting Namalwa cells, *Sendai virus* expresses an antisense lncRNA that can stabilize human interferon-α1 mRNA by binding and sequestering a miRNA, via single-strand loop regions [31], creating a sort of viral-derived miRNA–lncRNA sponge.

In summary, several viral-derived lncRNAs can specifically modify the transcription, RNA stability, the epigenome and the translation rate through various mechanisms. AS lncRNAs have become attractive target for therapies because of their ability to up- or down-tune regulatory networks already existing in the cells.

5. Use of exogenous RNAs to interfere with cell programs

Utilization of AS RNAs as a tool to modify the activity of mRNAs or even DNA has been very appealing for long time. Antisense approaches present the theoretical advantage that target specificity can simply be achieved by altering the sense-antisense sequences. Unfortunately, many of the initial trials were not successful due to a variety of issues, including difficulties in hitting the target mRNAs due to secondary structures, and ineffective sense-antisense pairing due to mRNA association with proteins *in vivo*. Additionally, long sense-antisense RNAs can trigger an interferon response [48], and efficient delivery of nucleic acids in the cells of a tissue or organ has been problematic [49]. Nonetheless, recent studies suggest that it is good time to revisit the field and address old challenges. In particular, there has been considerable activity in the biotech industry focusing on developing novel RNA therapeutics: for example, AntagoNATs (OPKO-CURNA, http://www.opko.com/therapeutics/opko-curna/; Fig. 1A), SINEUPs (TransSINE technologies, http://www.transsine.com/; Fig. 1B), and modified mRNA (Moderna, http://www.modernatx.com/, Fig. 1C). We will discuss some of the key events in the development of these technologies and the current status of RNA-based therapy. Issues of delivery are shared antisense lncRNAs, miRNAs, siRNAs, and other artificial short AS RNAs. We will discuss some of these small RNAs, to gain better understanding of the potential and challenges of antisense therapies.

The classic genetic disorders, β-thalassemia, Duchenne muscular dystrophy, and spinal muscular atrophy have been successfully targeted with antisense oligonucleotides (AONs) to either prevent or induce exon skipping [50,51]. Most exon-skipping therapies in use today originated as AON therapies with pre-mRNA as the target. AONs are designed to target splicing sites to modify the structure of the mature mRNA. They act by selecting specific exons, artificially designed pseudo exons, or intra-exonic cryptic splice sites, generally to eliminate exons containing nonsense mutations [52] For instance, by modifying exon junctions, AONs can be used to produce antisense-induced splicing variants that no longer contain premature stop codons (i.e., stop codons upstream of the final exon). This prevents nonsense-mediated mRNA decay, a mechanism that detects and degrades mRNAs containing premature stop codons [53], and results in up-regulation of the mRNA transcript level, and production of a protein that is at least partially functional (see the AON review [54] for more details).

AONs should (1) be able to bind to specifically targeted mRNA sequences; (2) be nuclease (e.g., RNaseH) resistant, especially for pre-mRNA splicing strategies; (3) be deliverable into specific cells and tissues; and (4) be safely metabolized. There have been many efforts to chemically modify oligonucleotides to increase the stability of RNA binding and, most commonly, to improve nuclease resistance. One important breakthrough has been the use of phosphorothioate-modified AONs [55]. A therapeutic phosphorothioate AON, called Vitravene [56], was developed by ISIS Pharmaceuticals in US as an antiviral agent against cytomegalovirus retinitis in AIDS patients and gained US food and drug administration approval in 1998 [57]. Other phosphorothioate modifications have been introduced into AONs: e.g., 2′-O-methyl and 2′-O-methoxyethyl groups; these modifications show nuclease resistance and increased binding capacity to the target sequences [50,58]. Other chemically-modified AONs, named phosphorodiamidate morpholino oligomers (PMO), are a charge-neutral class of agents that have been studied for their resistance to nucleases and proteinases, and their ability to very efficiently inhibit translation of targeted mRNA by steric blockage [59–61]. Comprehensive pharmacokinetic analysis of PMOs has been reported in several species [59,62]. PMO have been used successfully to inhibit viral mRNA translation, for example in infection by the lethal filoviruses, Ebola virus (EBOV) and Marburg virus (MARV). Filovirus genomes (~19 kb in length) contain, in order, a 3′-UTR, genes encoding nucleoproteins, VP35 and VP40, genes encoding glycoproteins, VP30 and VP24, a gene encoding RNA-dependent RNA polymerase (L protein), and a 5′-UTR [63,64]. EBOV and MARV are divergent in their genome sequence and host tropism. EBOV-infected rhesus macaques (or rhesus monkeys; *Macaca mulatta*) were tested with PMOs whose antisense sequences were specifically designed to bind to the *VP35*, *VP24*, and *L* transcripts of EBOV. The combination of PMOs efficiently blocked viral replication and protected 75% of the macaques from lethal EBOV infection [65]. Additionally, PMO chemical modifications that included conjugation with peptides of various lengths and compositions were tested against VP24 in EBOV-infected cells and mice [66]. Positively-charged PMOs (PMO*plus*) that are a combination of EBOV-specific sequences (AVI-6002) or of MARV-specific sequences (AVI-6003) against *VP24* and *VP35* transcripts, respectively, protected >60% of EBOV-infected rhesus macaques and 100% of MARV-infected cynomolgus macaques (*Macaca fascicularis*) by acting as specific virus replication inhibitors [67].

Although chemically-modified AONs are complex to produce, they have the advantages of high specificity and effectiveness [68]. Unfortunately, they are problematic because of their toxicity [49]. Although some nucleic acids are too toxic for use in long-term therapies, they may still play an extremely important role in short-term approaches against infectious diseases; for instance, AONs could be used to neutralize viruses during the peak of infection. This could become particularly important in association with next-generation sequencing, which will help to promptly identify viral causative agents responsible for future pandemic infections. Newly-discovered infectious agents could be coupled promptly to antisense therapy because (a) the design of virus-neutralizing AONs does not require an in depth understanding of the biological mechanisms of viral infections, and (b) varying the antisense sequences to compensate for virus mutations could cope well with the problem of target variability. Changing the sequence of AONs is likely to be much faster than developing novel conventional drugs specific for mutated viral proteins.

6. In search of stable expression

Due to the instability of RNAs, gene therapy using vectors (and in particular viral vectors) to express RNAs has been attractive because it is possible to provide stable, continuous expression of therapeutic, native RNAs. However, other problems have arisen, due mainly to the lack of control of insertion of expression vectors into the genome. X-linked severe combined immunodeficiency (X-SCID) is caused by mutation of the gene encoding the common gamma chain (γc, also known as IL-2Rγ), which is component of receptors for interleukins (IL)-2, -4, -9, -11, -15, and -21. In X-SCID patients, γc mutations block T-cell, B-cell, and NK-cell differentiation. Mutations in the γc gene on chromosome X (X13q band) were first identified in an analysis of three X-SCID patients in 1993; all three patients had different point mutations that caused premature stop codons in the γc gene [69]. In 1999, Cavazzana-Calvo et al. [70] started the first clinical trial for X-SCID gene therapy with a Moloney murine leukemia virus-based retroviral vector (MFG) containing γc cDNA under the control of the retroviral LTR [71]. Following *in vivo* infusion of *ex vivo*-infected bone marrow $CD34^+$ cells, two patients, aged 11 months and 8 months, respectively, showed increased T lymphocyte, including CD4+ and CD8+ subsets [72]. The γc protein was present on the membranes of T cells in the second patient. Promising results prompted the extension of the clinical trial to additional patients; ultimately, treatment helped 4 out of the total of 5 patients to live in normal environmental conditions at least 4 years after gene therapy. Unfortunately, one patient died from bacillus Calmette–Guerin infection in early treatment even though T-cell immunity was partially restored. Worryingly, another patient developed T-cell leukemia resulting from unexpected insertion of therapeutic vector sequence near the *LMO2* gene [73]. In a subsequent second trial in France, 9 out of the total of 10 patients were successfully treated by gene therapy; however, 4 of these 9 patients developed T-cell leukemia 31–68 months after gene therapy [74]. FDA put a hold on the clinical trials in 2002 [75]. These patients also showed unexpected insertion of vector sequence [76], suggesting that alternative solutions that are not based on viral vectors are needed.

7. Are RNA-based therapies feasible?

As discussed above, additional efforts are needed to develop vectors that are incapable of inserting their genome into the host genome, to (a) minimize the risk of mutation of important genes, and (b) deliver RNA molecules directly into the target cells. Important advances in this regard have come from the technologies used to develop iPSCs; we believe that the production of iPSCs from individual patients will become a fundamental tool for the next generation of personalized medicine. Yamanaka and colleagues first created iPSCs by overexpressing KLF4, c-MYC, OCT4, and SOX2 (KMOS), (known as the Yamanaka factors) with the use of retroviral vectors [77,78]. As shown for X-SCID clinical trials, gene therapies with viral vectors are not safe; in particular, the continuous overexpression of these factors could be oncogenic. To safely create iPSCs, Rossi and colleagues used modified mRNAs encoding the four Yamanaka factors from human ESC-derived dH1f fibroblasts, which display relatively efficient viral mediated iPSC conversion [79–81]. They synthesized mRNA from PCR templates by using anti-reverse cap analog (3′-O-Me-m^7G[5′]ppp[5′]G), ATP, GTP, 5-methyl cytidine triphosphate, and pseudouridine (Fig. 1C). Phosphatase treatment slightly reduced the cytotoxicity upon transfection. The combination of 5-methyl cytidine and pseudouridine modifications was very important for reducing interferon responses and toxicity to cells. Transfection and cell growth conditions were optimized, while daily mRNA transfection was required for high level expression of four Yamanaka factor mRNAs in dH1f fibroblast cells to properly initiate the reprogramming process to produce iPSCs. Daily transfection was not further required during the reprogramming process. Clearly, using modified mRNAs for transfection to generate iPSCs (named RNA-induced pluripotent stem cells [RiPSCs]) is highly effective and is safe compared with the use of viral vectors. However, this process is relatively complex.

Kormann and colleagues successfully used modified mRNA encoding mouse erythropoietin (EPO) *in vitro* and *in vivo* to stimulate red blood cell production [82]. Both *in vitro* and *in vivo* experiments showed reduction of immune response and satisfactory induction of expression of EPO when compared with un-modified mRNA. Furthermore, therapeutic studies of regeneration of cardiac tissues in a mouse myocardial infarction model have been performed by using modified mRNA encoding human vascular endothelial growth factor-A. [83].

Although long-term preservation of RNA expression is still a potential issue, these studies suggest a broad feasibility of RNA-based therapies. Developing such therapeutics is particularly important when considering that there are 6000 rare diseases [84] for which there is no existing therapy and no critical mass of patients to pay for the cost of traditional drug development. The biotechnology industry is quickly developing around these areas. For instance, Moderna has been pioneering RNA therapies based on modified mRNA technology. New regulations to facilitate trials of treatments for rare diseases with RNA will be needed. To expand the tools available, we believe that not only mRNAs, but also lncRNAs and small RNAs should be considered. In this regard, non-coding RNAs (miRNAs, SINEUPs) are particularly attractive because they usually modulate the activity of mRNAs that are already expressed in a given cell or tissue, and they have potentially much higher specificity than mRNA overexpression technologies, which can cause ectopic protein expression in unintended cells or tissues, with unpredictable consequences.

8. We need to better understand RNA structures

Therapeutic AS RNAs including AONs should specifically bind to target RNAs to produce double-stranded RNA. AS RNAs should be designed to target a single-stranded, preferably looping region, because strong stem-regions tend to react poorly with AS RNAs. However, one major difficulty is accurately predicting the structure of the target mRNAs. Additionally, even if the structure can be predicted or detected *in vitro*, the information may not be sufficient to predict the structure *in vivo* because the presence of RNA-binding proteins can change the folding pattern. One

approach to RNA structure detection is to conduct nuclear magnetic resonance (NMR) analysis, but this technique can only be used for RNAs that are no longer than ~100 nt. Consequently, structure determination of lncRNAs, which are in the order of thousands of nucleotides in length, would require potentially misleading analyses of separate segments of the RNA, followed by the combination of separate structures and resolution of possible discrepancies. Although NMR is quite accurate for small length of RNA, it is very laborious. A simpler approach to mapping secondary structures of RNAs is to use a predictive RNA secondary structure program, such as RNAfold [41] or CentroidFold [85]. Such programs have been improved by the use of free energy minimization algorithms. Mathews and colleagues reported that RNA secondary structure prediction using such an algorithm was successful in 73% of regions targeted by AS RNAs [86]. Such programs are particularly useful when no other options are available; however, they are limited by calculation time and the very large number of predicted potential structures, which may not necessarily reflect actual folding *in vivo*. Alternatively, a recent and very promising approach is to digest folded RNA with RNase V1 or S1 nucleases and to conduct sequencing to detect single-stranded and double-stranded regions [87]. By using this approach, a comprehensive map of the RNA secondary structures of human lymphoblastoid cell RNA, taken from a family trio (mother, father and their child) was produced; AGO-bound target sites on the mRNA showed strong structural accessibility from −1 to 3 nucleotides relative to the 5′ end of the miRNA-target site on the mRNA compared to predicted targets not bound by AGO. An alternative to the double RNAse-digestion approach consists of comprehensive chemical modification by dimethyl sulfate followed by sequencing analysis [88]; dimethyl sulfate is highly reactive towards single-stranded loop regions of RNAs but unreactive towards highly structured stem regions. This approach was validated by analyzing mRNAs of known structure, *HAC1*, *RPS28B* and *ASH1*, from *Saccharomyces cerevisiae* [89]. Like the double RNAse-digestion approach, the dimethyl sulfate-sequencing approach has been used to distinguish single-stranded and double-stranded regions and provide a draft genome-wide RNA structure map [90]. Although structure may not be fully predicted by these approaches, we believe that they provide a fundamental step towards RNA therapeutics, and in particular antisense-based therapeutics, by providing comprehensive catalogs of single-stranded RNA targets [91].

9. Perspectives

RNA is fast becoming a major target for cellular manipulations and gene therapy. A particular important role will be played by non-coding RNAs, because their large number and broad regulatory functions mean that they have the potential to provide a large collection of regulatory tools. For instance, pre-mRNA and mRNA can be targeted by using chemically-modified AONs, siRNA, splice-switching oligonucleotides, translation-suppressing oligonucleotides, or external guide sequences, as described in more depth elsewhere [92]. Experience delivering these small RNAs has helped in the development of delivery techniques that will be useful for antisense lncRNAs, such as SINEUPs, antagoNATs, or combinations of multiple types of lncRNAs. AS RNA approaches are particularly promising because these RNAs often work as regulators of specific existing mRNAs or genomic loci, which means that they have the potential to increase the specificity of future therapies and diminish side effects. In fact, where sense mRNAs are not expressed, antisense lncRNAs are less likely to have an effect because their natural mRNA target is absent. We can envisage the engineering of lncRNAs with multiple types of domains: one domain might carry sequences needed to pair specifically with other nucleic acids (e.g., for triple-helix formation with genomic DNA), and other domains could interact with specific proteins, targeting them to specific loci; however, such non-coding RNA domains are at the moment mostly unknown. Future studies to determine RNA structures and identify functional domains are needed to elucidate the world of regulatory RNAs. We anticipate that there will be a research gold rush similar to that experienced for protein research a decade ago.

We should remember that there is a lot of work ahead: there is no finalized gene therapy tool as yet. Many technologies have their drawbacks, with delivery and stability issues being the most difficult to overcome. RNA therapy should be broadly considered in combination with different technologies or conventional drugs to maximize synergistic effects and find better therapeutic solutions.

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