Review

RNA Devices for Therapeutic Applications: Progress, Challenges and Future Perspective

Vibha Dwivedi * and Yun-Xing Wang

Protein-Nucleic Acid Interaction Section, Center for Structural Biology, National Cancer Institute, National Institutes of Health, Frederick, MD 21702, USA
* Correspondence: vibha.dwivedi@nih.gov
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Abstract: Advances in RNA structural biology and engineering have stimulated thinking of using engineered RNA devices for precision cell and gene therapy. The RNA devices provide distinct advantages over existing approaches. Riboswitches, the key element in RNA devices, are regulatory structural elements found within mRNA in all three kingdoms of life but mostly in bacteria to sense the concentrations of small molecule metabolites, pathway products, uncharged tRNA and elemental ions for regulation of gene expression. Because of non-immunogenicity and several other advantages, riboswitches are considered to be a promising tool that may have significant implications in medicine and health. This review will provide an overview on the structural studies of key motifs critical for RNA device functions and some of the pioneering works aimed at developing RNA based therapeutics for medicine.

Keywords: RNA aptamer; RNA devices; aptazyme; precision medicine; cell and gene therapy; noncoding RNA

1. Introduction

The ability of an RNA to interact with small molecules, proteins and other nucleic acids and its relative easiness to be incorporated into biological systems makes it an ideal molecule for a wide variety of applications. This review is focused on the RNA device-based cell and gene therapy applications in detail. For broader survey of literature in other areas of RNA devices readers are referred to more extensive excellent review articles [1–5]

One of the RNA molecules, riboswitch is a structural element found naturally in the 5′ untranslated region (5′ UTR) of mRNA. It consists of at least one ligand-sensing domain (aptamer) that changes its conformation and alters gene expression patterns in response to ligand binding without help from protein (Box 1). In the simplified depiction of how a riboswitch works, ligand-induced conformational change results in the formation of a hairpin that halts the progression of RNA polymerase, or sequesters the Shine–Dalgarno sequence, preventing the ribosome from either binding or initiating, or both, consequently rendering gene-expression "OFF" (Figure 1A). On the other hand, the "ON"-switches result in the formation of an anti-terminator stem or the opening of the ribosome binding site from sequestering resulting in the initiation of the transcription or translation of the downstream gene [6,7] (Figure 1B). The first three examples of riboswitches that selectively bind coenzyme B(12) [8], vitamin B(1) [9] thiamine and flavin mononucleotide (FMN) [10], were discovered and reported more than twenty years ago.

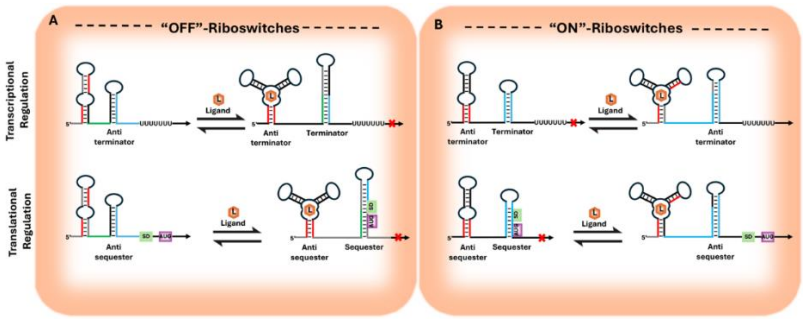

Figure 1. Schematic Illustration of transcriptional and translational control mediated by small ligand molecule and riboswitch: (**A**) An example of 'OFF' riboswitch device: Ligand (Brown Hexagon) binding to the anti-terminator

(Transcriptional regulation) or anti-sequester (Translational regulation) results in the shut-down of gene expression. (**B**) An example of 'ON' riboswitch device: Ligand binding results in the formation of anti-terminator (Transcriptional regulation) or anti-sequester (Translational regulation) allowing the gene expression. Shine Dalgarno sequence location and initiation codon are represented by green and magenta boxes.

Concept Definition Box 1

RNA regulator: It is a broad term and refers to any RNA involved in the regulation of biological processes and functions. Examples are microRNA, RNA involved in RNA interference (RNAi), anti-senses RNA oligos, long non-coding RNA (lncRNA) and any structural elements that recognize and bind small protein or ligands to regulate gene expression such as riboswitch RNA. Riboswitch is one of most studied regulatory RNA structural elements. In the following discussion, terms RNA device and riboswitch are almost interchangeable.
Riboswitch: Riboswitch is a part of mRNA normally found in the 5′ untranslated region of mRNA (5′ UTR) and consists of an aptamer domain and expression platform. The aptamer domain is ligand binding and sensing structural element. The structures or confirmations of aptamer and expression domains are mutually exclusive depending on the presence or absence of ligand.
Aptazyme: aptazyme implied by its name consists of an aptamer and ribozyme. Aptazyme is a synthetic RNA element that is ligand-responsive ribozyme and is designed to be incorporated into genes for regulation.
RNA devices: Because of the capability of switching gene expression ON or OFF in response to ligand binding or other types of external stimuli, RNA regulators could be made in various arrangements analogous to electronic logic devices. In this sense, RNA devices mostly refer to synthetic RNA structural elements. RNA genetic devices are those that are inserted into host genes in cells to control gene expression.

The gene expression processes regulated by riboswitches include transcription [6], translation [6,11], splicing controls [12,13], transcriptional interference [14] and mRNA stability [15–18]. Controlling splicing and mRNA stability using riboswitch or RNA devices are most frequently used in the regulation of gene expression actuated through small molecular ligands.

Riboswitches are also classified based on secondary structure layouts and ligand types [19,20]. In particular, classification based on ligand types with dissociation constants and discovery chronology is illustrated in an excellent review [1]. There are more than fifty classes of natural riboswitches that have been experimentally verified [2] with thiamine pyrophosphate and adenosylcobalamin riboswitches as the two largest classes [19]. However, the experimentally verified riboswitches only represent a very small fraction of the predicted riboswitches, ~28,000, based on bioinformatics analyses [2]. Most riboswitches act alone but riboswitches naturally arranged in tandem do exist [21–26]. This arrangement is also imitated and used to control gene expression in cells and animals [27]. Nevertheless, a natural riboswitch is a prototype of a gene regulator, which is involved in maximizing the natural fitness and survival, not overexpression or down-regulation of gene expression in foreign hosts [28]. In addition to natural riboswitches, there are increasing numbers of synthetic riboswitches identified through various high-throughput screenings [27,29–31].

Synthetic biology is a relatively new field of study that uses multi- and interdisciplinary approaches to modify and create biological systems such as devices, cells, organelles, or even whole organisms. As such, synthetic biology represents a new type of tool to better understand, manipulate, design and simulate intricate counterparts of realistic biological systems, metabolic pathways, structures and functions for applications in biotechnology and medicine. It holds great promise in applications such as bioreactors for manufacturing compounds and pharmaceuticals that could not be produced economically or possibly otherwise [32,33]. Among these systems, the development of novel RNA genetic devices is one of the most active areas of research in frontier RNA biology and RNA biotechnology. There are several distinct advantages of using synthetic RNA as a genetic control element as opposed to other approaches and they control expression of a targeted gene without perturbing innate host regulatory networks and without the involvement of protein cofactors [34–39]. Many of these properties have been exploited for various applications in biotechnologies and gene therapy, with the tetracycline aptamer as the most extensively characterized synthetic riboswitch aptamer reported in literature [40,41]. In this context aptazymes are the most frequently used design of RNA devices for applications of regulating gene expression by randomizing the crucial sequences followed by screening and selection [35,36].

RNA devices are highly modular and portable with a small genetic footprint and thus are relatively easy to implement for a wide range of applications in biosensing, synthetic biology and engineering for biomolecule and pharmaceutical productions. Concerns over immunogenicity preclude approaches involving introducing foreign protein regulators [42–44]. Immune response against foreign epitopes is one of the key hurdles to gene therapy [44–47].

2. Structural Basis of Switching by Ligand in Riboswitch

While it is clear that RNA-based regulators, riboswitch in particular, have enormous potential in applications in biotechnology and medicine, the design and development of novel RNA regulators have largely been relying on high throughput techniques to screen randomized libraries or small pools of RNA regulators [29,36,48]. Those randomized sequences are more based on the modular concept of RNA structures [49], but less on rational design based on the context-dependent structure-function relationship principle. This is at least partially due to the lack of understanding of the structural basis for conformational switching, the core mechanism of RNA-based regulators. The need for structural information underlines the importance of the structural biology studies of RNA regulators [50].

The structural basis of ligand-induced switching in riboswitches has been studied extensively using biophysical/biochemical/computational techniques available currently, including in-line probing, single-molecule Förster resonance energy transfer (sm-FRET), small-angle X-ray scattering (SAXS), X-ray crystallography, NMR, cryo-EM, and MD simulations and there are numerous excellent reviews on the subject [51–59]. However, because of the lack of apo structures, the mechanistic bases for switching were inferred largely from the holo structures, thus based on hypothesis and proposition. These include riboswitches sensing lysine, glycine, cyclic di-GMP, S-adenosylhomocysteine (SAH), and S-adenosylmethionine (SAM), which consists of SAM-I, SAM-III, SAM-IV, SAM-I/IV, SAM-VI sub-classes [60–69]. Of note, the crystal structures of the lysine and glycine riboswitches in the absence of ligand were determined many years ago [60,61]. However, these structures highly resemble their respective ligand-bound conformations, which raises the question if the resemblances are due to buffer conditions and the effects of crystallization, or both. Although structural information provides important ligand-induced local changes even when both apo and holo-structures resemble one another overall, and very likely exist in the solution ensemble, it does not reveal insight into the structural basis for switching. The relatively less ambiguous pictures about the switching mechanisms emerge only when the structures in both apo and holo states become available.

The underlying structural mechanism that regulates gene expression through riboswitch involves a short stretch of switching sequence that can form two mutually exclusive secondary structures, whose formation and stability depend on the presence/absence or concentration of ligand. In most riboswitches, this switching sequence is part of the P1 helix in the aptamer. Ligand binding/unbinding to the aptamers regulates the formation or stability of the P1 duplex [70]. However, it is challenging to determine the structures of aptamers in an apo state due to their often-inherent structural instability in the absence of ligand. Nevertheless, a number of riboswitch structures have been determined in both apo and bound states [60–69,71–90]. Surveying these structures reveals the diverse structural and mechanistic basis of conformational switching via P1 helices, which is one of the focuses of this review.

The purine riboswitches have a highly conserved sequence and three-way junction architecture, where nucleobase specificity, adenine or guanine/hypoxanthine/xanthine, is achieved primarily through the ligand-sensing nucleotide: U74 for the former, C74 for guanine/hypoxanthine/xanthine [71,72,75]. The binding pocket is right at the three-junction, where the ligand is sandwiched between two adjacent base triples that thus facilitate the coaxial stacking between helices P1 and P3 (Figure 2A). This holo conformation provides initial information about the end state upon ligand binding. The full dynamic picture of ligand-induced conformation emerged about 13 years later when the two ligand-free structures of an adenine riboswitch were determined using X-ray free electron laser (XFEL) technology and mix-and-inject technique [73,74]. The comparison of the apo and holo adenine riboswitch structures reveals the structural basis for regulating P1 stability [73,74]. The P1 helices in both the apo states, which are significantly different from the holo-structure [75,76]. The presence of the two apo conformers and the interconversion between the two might serve an additional step of thermodynamic control.

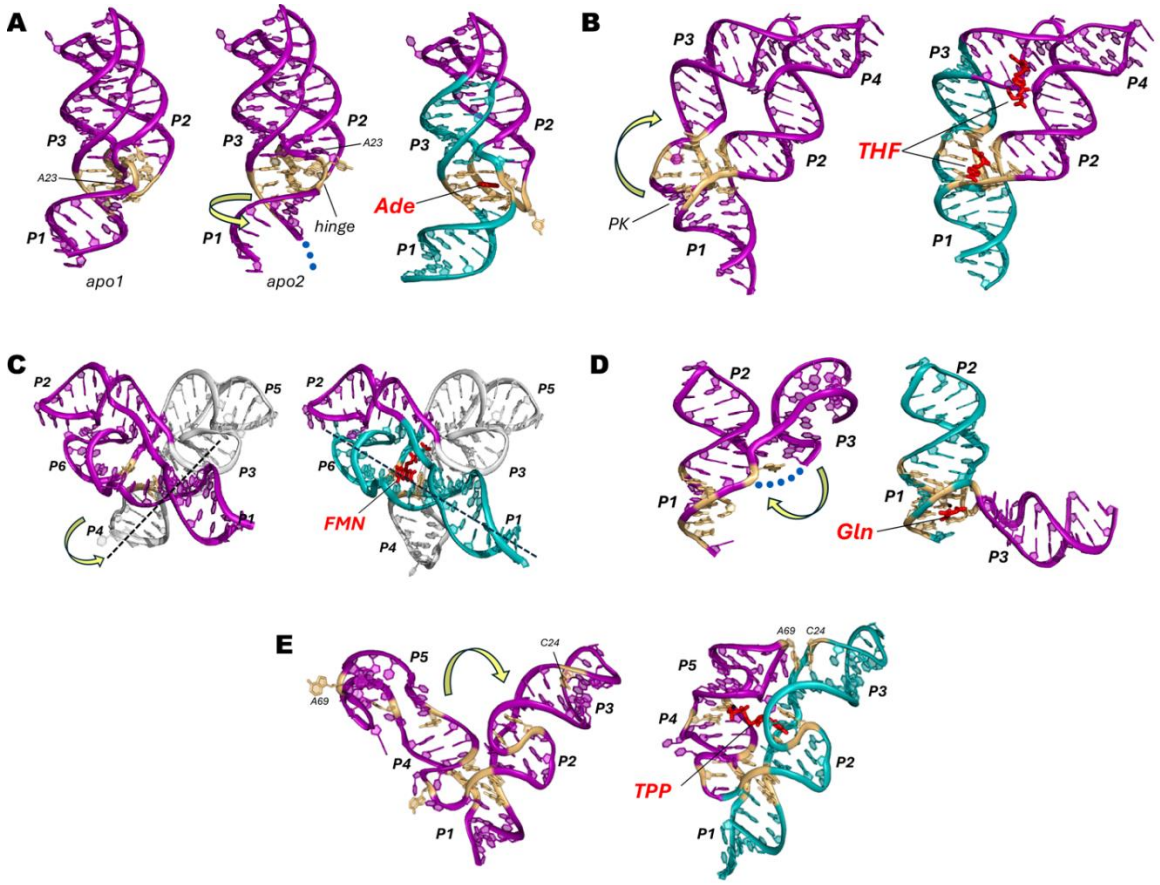

Figure 2. Comparison of apo and holo form of five riboswitch aptamers: (**A**) Adenine riboswitch- apo (PDB ID: 5E54) and holo (PDB ID: 4TZX) structures. (**B**) THF riboswitch- apo (PDB ID: 7KD1) and holo (PDB ID: 4LVV) structures. (**C**) FMN riboswitch- apo (PDB ID: 6WJR) and holo (PDB ID: 3F2Q) structures. (**D**) Gln riboswitch-apo (PDB ID: 5DDO) and holo (PDB ID: 5DDP) structures. (**E**) TPP riboswitch-apo (PDB ID: 84FO) and holo (PDB ID: 2GDI) structures. Ligands in each case is shown in red. Key residues are colored in yellow. Regions of coaxial stacking that stabilize P1 upon ligand binding are colored in cyan. Disordered missing residues in the crystal structure are represented by blue dots. Motion of conformational change from apo to holo states is indicated by the yellow arrow. Modified from a review on Riboswitch Mechanisms for Regulation of P1 Helix Stability [50].

The Tetrahydrofolate (THF) riboswitch uses very different mechanisms for regulating P1-helix stability thus conformational switching even though it resembles the purine riboswitches in both size and shape [81–84]. The most distinct feature is that it contains two cooperative ligand binding sites. The first binding site sits at a three-way junction conjoining P1, P2, and P3, and is formed via a pseudoknot between L3 and J1/2 as a result of THF binding, which directly impacts the Pp1 helix stability (Figure 2B). Specifically, the pterin ring of THF stabilizes the pseudoknot through a network of hydrogen bonds and π-stacking interactions, which are crucial for maintaining coaxial alignment of P1/L3 with P3. Without ligand, these stabilizing interactions are not present, which ultimately results in P1 instability in the apo state. Another binding site consists of a short helix (P4) that forms a three-way junction with P2 and P3 and creates a binding site that is quite far from P1. Since the structures in apo and holo states show very little difference in this region, THF binding to this site co-transcriptionally may simply stabilize the tertiary interaction between P2 and P3 [81,84].

The largest classes of riboswitches that have been extensively studied are those that regulate genes involved in the metabolism, biosynthesis and transport of cofactors critical for cellular functions. Riboswitches in these families regulate the P1 stability in responding to various types of metabolites in a number of metabolic pathways. One of these riboswitches is the Flavin mononucleotide (FMN) riboswitch. It has one of the most unique structures consisting of six helical domains conjoined by a six-way junction that forms the FMN binding pocket [77–79]. It has a pseudo two-fold symmetry where the two three-way junction domains are fused via a six-way junction with opposite orientations (Figure 2C). Domain 1 and 2 consist of P1, P2 and P6, and P3, P4 and P5, respectively. This architectural arrangement results in a relatively overall rigid scaffold, leaving only the protruding helices, P1 and P4, with certain degrees of motion. As a result, both P4 and P1 serve as pseudo-regulatory P1 helices, which form

the structural basis for ligand-induced conformational switching between mutually exclusive coaxial stacking within each domain [79]. Specifically, the switching action is centered around the FMN-binding site involving triple adenine residues: A48, A49, and A85. In the holo state, the nucleobases of A48 and A85 sandwich the FMN isoalloxazine ring, which also interacts with A49 via π-π interactions. Consequently, FMN binding facilitates the continuity and coaxial alignment of P1/P6 in Domain 1, and misalignment of P3/P4 in Domain 2 at the same time. In absence of ligand, the coaxial stacking is favored toward P3/P4 resulting in the misalignment of P1/P6 and the destabilization of P1.

Riboswitches also regulate biosynthesis by recognizing amino acids. One such example is the L-glutamine (Gln) riboswitch that senses Gln. This riboswitch adopts a rather conventional architecture with the common three-helical Y-shape. But it is unique in the following respects. First of all, ligand binding stabilizes the aptamer in an open conformation, rather than closed, which is more common in other riboswitches (Figure 2D). In other Y-shaped aptamers, ligand binding induces or stabilizes tertiary interactions between the P2 and P3 helical arms. In this case, however, the P2 and P3 contacts through A-minor interactions are disrupted upon ligand binding, as the two helices separate from one another resulting in an open conformation. Second, ligand binding at the bottom of the P1 helix brings in the P3 helix and results in the formation of the binding pocket through an induced fit. In this case, Gln binds directly to P1 in the major groove near the P2 through a network of hydrogen bonds with Mg^{2+}-coordinated water molecules [80]. Ligand specificity is achieved through hydrophobic interactions between the ligand and residues in the junction. The stability of the P1 helix is achieved through ligand-mediated interactions with ligand. Specifically, these include the formation of two base triples and the insertion of G23 into the P1 helix to form a Watson-Crick base pair with C60. In apo state, both G22 and G23 are disordered.

The (thiamine pyrophosphate) TPP riboswitch, also known as the *thi*-box riboswitch, is among the first riboswitches ever to be reported [9]. It is widespread and is only riboswitch found in all three life domains, including bacteria, archaea, fungi, and plants [85,86]. In addition to transcription and translation, eukaryotic TPP riboswitches regulate gene expression through alternative splicing [13,85,86]. The structures of the ligand-bound TPP riboswitches from bacteria and plants are remarkably similar [87,88], despite using regulatory mechanisms of transcription termination and alternative splicing, respectively. The crystal structure of the *E. coli* TPP riboswitch in such an apo conformation was determined recently, providing insight into the structural basis for regulating P1-helix stability [89]. In comparison with the apo structure, the ligand-bound architecture of this riboswitch consists of three helical domains intersecting at a three-way junction that is stabilized by a distal tertiary interaction, which is only present in the presence of ligand (Figure 2E) [87–89].

Like in the Gln riboswitch, TPP binds near the tertiary interface, distant from P1, and serves as a linchpin between the two sensing domains comprising P2/P3 and P4/P5, respectively. Interestingly, the TPP riboswitch currently is the only P1 helix-regulating riboswitch whose ligand makes no direct contact with either the three-way junction or P1. The stability of the tertiary structure is dependent on the ligand binding, specifically, P4/P5 interactions with the two TPP-coordinating Mg^{2+} ions and the TPP 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) ring intercalation between G42 and A43 of J3/2. Without the ligand, the riboswitch exists in open and closed conformations regarding P2 and P3. Thus, the conformational stability of the tertiary structure afforded by the TPP ligand is propagated to P1 via the 3WJ, which comprises two stacked A-minor tetrads that facilitate coaxial stacking between P1 and P2. In the absence of ligand, the extensive mobility of sensory domains and large structural changes in P4/P5 results in the disorder of J2/4 and disrupt the P1-stabilizing effect of the base tetrads.

In summary, comparative structural studies of five riboswitches in apo and holo states lay the foundation for understanding the basis for switching and for designing improved switching structural elements. Nevertheless, the insights to the structures and dynamics of the ligand-triggered conformational transitions are obtained from the isolated aptamer domains, not in the context of the fully functional RNA devices consisting of all essential elements. In fact, there has been no reported systematic structural and functional study done with a complete RNA device up to date, which underlines the great urgence and importance of the studies in this area.

3. Development of RNA Devices

The lack of three-dimensional structural information of fully functional RNA devices consisting of all functional elements remains one of the main hurdles for utilizing the structure-function relationship-based design principle for developing riboswitch-based RNA devices. Thus, at the present time, development of RNA devices heavily relies on rational design or high throughput approach, for selection of either components of or whole RNA device [29,31,49]. Most reported applications of RNA regulators for control of gene expression are synthetic aptamers, not the natural counterpart that evolved for natural fitness and evolutionary survival and presumably not for overexpression [91]. Synthetic aptamers were initially selected through SELEX libraries either starting from

natural aptamers or systematic discovery of genomic aptamers (genomic SELEX libraries) [27,29,31,92]. An analysis of the new synthetic paromomycin (PARO) riboswitch and identification of its binding pockets has been done by generating a library between one- and three-point mutations with 3% randomization. These 1520 variants were then screened for Gain of Function (GOF) and Loss of function (LOF) by inserting them in a GFP start codon in pCBB06 plasmid [91]. For proper gene regulation, a screening approach for efficient ribozyme selection is developed in mammalian cells by generating more effective variants of pistol ribozymes. In this approach, each variant had a unique 9-nt barcode sequence, and the dsDNA library with U6 promoter was transfected into mammalian cells. Analysis of total RNA separated by polyacrylamide gel electrophoresis (PAGE) revealed the cleaved and un-cleaved fragment sequences for the selection of better performers [93]. Subsequently, the selected aptazymes were demonstrated to regulate transgene expression in mammalian cells and mice. In order to avoid using barcoding, a novel approach of barcode free amplicon sequencing is also developed in human cells to screen tetracycline and Guanine responsive ON and OFF switches. Each construct of the library was self- barcoding and hence, no pre-selection and cDNA manipulation was required. They transfected RNA-based library into HEK cells and incubated the cells in the absence/presence of tetracycline followed by RNA extraction. The riboswitch flanking primers were used to PCR amplify the fragment which was subsequently subjected to cDNA synthesis followed by cDNA-amplicon seq [29]. This approach has relatively vast applicability as demonstrated by the U1-snRNP library screen in addition to the ribozyme platform.

Above reviewed approaches are based on the rational designing, which is time and labor intensive and performed with limited number of variants. However, the development of computational analysis and high throughput approaches selection procedures are expanded to create desired functional aptamers [31,94]. For example, a computational program developed for ligand -sensitive terminator sequences, was utilized to search sequences connecting theophylline aptamer to a transcription termination sequences through randomizing linker sequence [95]. These programs can consider other parameters such as free energy changes for the optimization of desired sequence and hence used in this context to filter those sequences having lower free energy change than the binding energy of theophylline to its aptamer. For further improvement, biophysical attributes of RNA are also incorporated in the computational programs for example, one of the programs developed to design devices to regulate translational initiation by determining the binding strength of ribosomes to ribosome binding sites in the presence or absence of ligand [94]. Despite the advantages to speed up the process, these computational designing approaches utilizes the trivial secondary structure prediction programs and free energy changes rules in base pairing that are applied to any rational designing and hence, the application of these devices remained limited due to the lack of better understanding of complex dynamic RNA structure to functional role in terms of regulating gene expression. In this context it is important to understand that the systematic study of apo structures of devices and the holo counterparts can reveal the principles of dynamic switching mechanism, which could be utilized to improve designs aimed at obtaining more effective devices. A recent study shows that the conformation dynamic mediated switching rather than structural switching plays a crucial role in determining the functionality of tetracycline inducible active RNA device-D43 tested in mammalian cells (Stagno et al., under revision). Deep understanding of these principles with the proper consideration of other parameters are expected to develop more effective variants.

It is evident that screening procedures performed in vitro may not be replicated in vivo with the same efficiency as the two environments are very different. Hence, with a view to screen the RNA devices in vivo with several variants, high throughput approaches are developed. The selection potential of RNA devices by a high throughput can be one of the practical approaches in searching for and developing more efficient RNA devices that function efficiently in mammalian cells and animals. In this context, connecting the ribozyme to a tetracycline responsive aptamer by a variable linker region and the selection of those variants which prevent self-cleavage in the absence of ligand was done [96]. In this approach, the RNA library was indeed transcribed in vitro and the uncleaved transcripts were separated by PAGE following multiple rounds of enrichment and their subsequent transformation in yeast [96]. The final sequences were selected based on their ability to retain GFP signal which is an indicator of transcript retention in the absence of tetracycline in the yeast model. Unfortunately, when these selected sequences were tested in mammalian cells, none of them showed the desired activity [97]. Therefore, it is also beneficial to consider the mammalian cells for in vivo assays directly rather than in vitro assays, as the device evaluation should be done in a similar environment in which they are finally indented to be used.

For in vivo screening, the RNA device sequences are needed to be transformed to DNA sequences and the switch library is then cloned into expression construct containing reporter gene or gene of interest to express in mammalian cells in the absence or presence of ligand. Each cell having a single RNA device variant can be clonally expanded further and the deep sequencing can tell the device sequences identity. Further integration of the reporter gene assay techniques like fluorescent-activated cell sorting (FACS) have also opened the possibilities for better

screening methods compared to the earlier assay techniques like B-galactosidase where the selection procedure was based on blue and white colony selection resulting in lower throughput and limiting the number of variants tested [98]. Several new RNA switches have been developed by integrating the in vivo screening with FACS based approaches for both transcriptional and translational activation [99,100]. In addition, high throughput RNA-Seq assays in mammalian cells have identified new ribozyme-based RNA devices responsive to theophylline, hypoxanthine, cyclidic-GMP, and folinic acid [30]. Based on modular design, they utilized a hammerhead ribozyme with aptamers located in one of the two stem loops in ribozyme. The randomization of sequence in the non-aptamer loop generated the HTP library having potential switches displaying the crucial properties for selection like, fast cleavage rates in the absence of ligand (probably, due to desired tertiary structure formation) and capability to disrupt these tertiary structures and inhibit cleavage on ligand binding. Several of these approaches also used HHRz with pre-existing theophylline aptamers with cell sort (FACS-seq assay) and NGS, followed by statistical analysis to select the best switch devices. Their reporter construct constitutes 3′UTR of GFP where the variants were inserted and mCherry being the second reporter served as a control for the normalization of GFP expression [49,101].

In summary, integration of high-throughput methods, data-rich assays and in silico design, with novel screening strategies, is expected to provide a platform for a better understanding of the structure-functional aspect of RNA devices in cellular systems to regulate gene expression.

4. RNA Device-Mediated Gene Expression Regulation

One of the most frequently used RNA regulators is aptazyme that can effectively inhibit gene expression due to self-cleavage [102,103], which is the main focus of this review. One pioneering study of utilizing riboswitches as gene control elements is presented by Yen et al. [102]. They showed for the first time that integration of self-cleaving RNA sequences inhibited gene expression, which was reversible in the presence of complementary oligonucleotides or small molecules in mammalian cells and live animals. Recent research suggests the possibility of gene expression control by engineering either aptamer-only and/or engineering aptamer-ribozyme modules. Both of them can be engineered by in vitro or in vivo selection to obtain desired cellular functions and this synthetic approach is seen to be far more applicable than natural metabolite-responsive riboswitches [102]. The first confirmation of ribozyme activity in human HEK293 cells was demonstrated by inserting it in the 3′ UTR of EGFP mRNA with guanine-activated ON-switch [102]. Since then, similar approaches have been utilized multiple times with diverse ligand and aptazyme combinations with the most popularly used tetracycline-mediated aptamer switching [102–104]. Tetracycline aptamer is very well established and characterized, with the binding affinity to its ligand in nanomole scale [105]. In one of the studies, tetracycline-dependent aptazyme displayed 8.7-fold induction of gene expression by using tetracycline dependent hammerhead ribozyme in HeLa cells [39]. Tetracycline aptamer was the first aptamer to be used to inhibit pre-mRNA splicing by inserting it in the 5′ splice site of an actin intron in the GFP reporter gene in yeast [12]. Several other studies have also shown the potential of tetracycline aptamer as a gene control element [39,106–108]. As evident, tetracycline-dependent aptazyme insertion in 3′UTR converts a gene into inducible segment. This approach is utilized for the spatial and temporal expression of poly-glutamine stretch which forms inclusion bodies to augment neurological disorder in Huntington's disease models [102–104]. This type of stable integration is independent of any regulatory protein and hence may be improved further for potential therapeutic applications. In this way, a Huntington's disease model generated in C. elegans could be useful by utilizing aptazyme as a tool to understand disease pathogenesis.

The development of tetracycline aptamers has garnered significant interests among researchers because the drug is already FDA approved which may alleviate potential regulatory hurdles. Various hammerhead, hepatitis-delta-virus, and twister ribozymes as well as U1-snRNP polyadenylation-dependent RNA devices have been developed by engineering tetracycline- and guanine-responsive ON- and OFF-switches by self-barcoding of each construct by cDNA amplicon-sequencing of library in transiently transfected and stimulated human cells, without additional pre-selection or cDNA-manipulation steps [29]. Further modifications involve a different approach, where 62 synthetic riboswitches were designed based on a statistical thermodynamics model to sense various small molecules like theophylline, tetramethylrosamine, fluoride, dopamine, thyroxine, 2,4-dinitrotoluene) with the 383-fold upregulation of gene of interest [94]. In addition to hammerhead ribozyme for function in cis, Hepatitis delta virus (HDV) ribozymes have also shown theophylline and guanine induced 29.5 fold change in mammalian cells [109]. In the same study, two engineered ribozymes were placed in tandem to develop a functionally more efficient device. Additionally, allosteric HDV ribozyme switches (with theophylline aptamer) are utilized to activate RNAi for the regulation of endogenous mammalian genes [110]. In this approach, upon ligand binding, co-transcription

cleavage of the ribozyme module takes place to promote RNAi complex formation without the involvement of immunogenic proteins.

In addition to controlling mRNA cleavage, riboswitches are also used for controlling ribosomal frameshifting, microRNA maturation pathway and other gene regulatory pathways [35]. Those applications can be further extended to control alternative splicing. In this context, tetracycline-inducible riboswitches are developed that allow the exon skipping in the presence of ligand by controlling the accessibility of the 3′ splice site. One example is the cellular signaling cascade that is controlled by regulating CD20 expression to induce cell death in cancer research [111]. In addition, adding aptamer in conjunction with polyA signal (PAS) cleavage in 5′UTR of luciferase GFP transgene is seen to be highly efficient in enhancing the reporter gene expression in live mice and the endogenous CD133 gene in human cells [103]. The dual mechanism used in this study includes the ligand-induced aptamer clamping to avoid PAS cleavage and alternative splicing that removes the PAS altogether, hence allowing gene expression (Figure 3C). Remarkably, this approach leverages the switch-mediated induction up to 900-fold with relatively lower effective dosages of tetracycline [103]. This induction dynamic range is among the highest ever reported in the literature. Moreover, the induction of gene expression is also seen to be reversible on the removal of tetracycline. Another example of the functional versatility of RNA devices is displayed by a rationally designed hammerhead ribozyme capable of binding to naphthyridine carbamate tetramer with Z-stilbene linker (Z-NCTS) which acts as a molecular glue to bring two isolated loops into contact with each other to perform the function in mammalian cells [112]. These approaches rely on the mechanism of ligand-dependent self-cleavage in the ribozyme module. Due to the cleavage and removal of poly A tail, the transcript become susceptible to 5′ and 3′ exonucleases and subsequently degraded in case of 'OFF' switch device. This mechanism can be applied with various permutative combinations to make 'ON' or 'OFF' switch devices more robust. Figure 3 shows the schematics of device mediated control of gene expression with an example of device insertion in of 3′ or 5′ UTR of a gene (Figure 3A,B) to create an 'OFF' switch device. Also, some additional modifications like introducing a PAS cleavage site, which is known to increase the device efficiency manyfold [103], is shown as an example to prove that 'ON' switch devices can be further modified accordingly (Figure 3C). In short, recent developments of RNA devices by either rational designing or high throughput screening approach shows a remarkable modulation of the engineered gene that can be utilized for the remediation of several disease conditions.

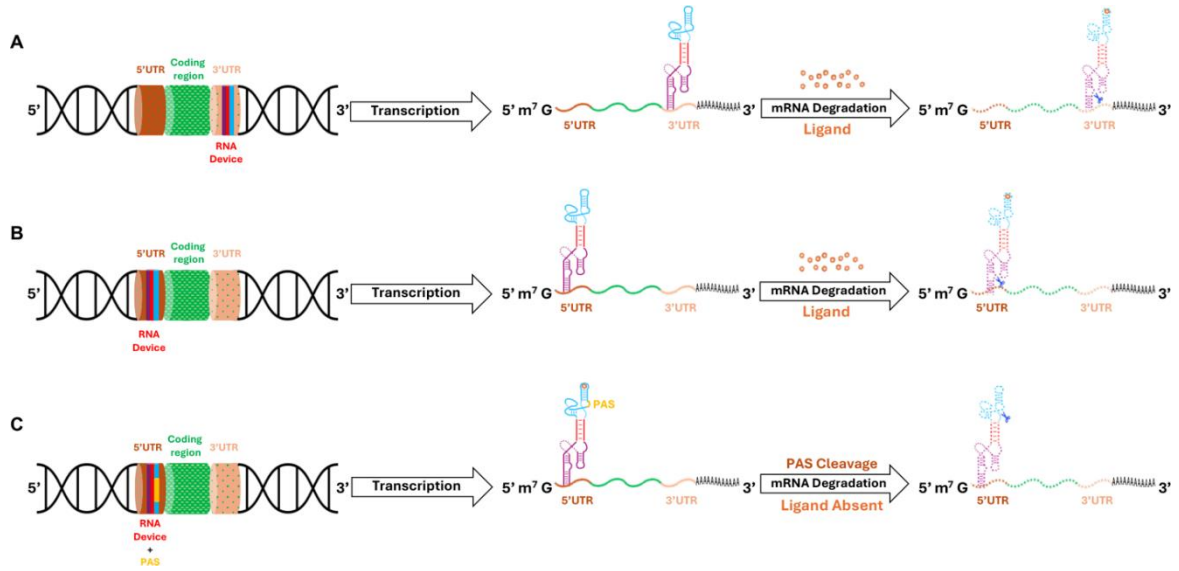

Figure 3. Schematics of device mediated control of gene expression: (**A**) Device integration in the 3′UTR of gene (Green), in the presence of ligand that bind to aptamer domain (Blue), undergo self-cleaving activity in the ribozyme module (Magenta) that subsequently results in the complete degradation of RNA message. (**B**) Similarly, device can be introduced into 5′ UTR to cleave the transcript. (**C**) Additionally, for an example, introducing PAS (Yellow) in the aptamer domain of the RNA device inserted in 5′ UTR is reported to stabilize the transcript in the presence of ligand and allow gene expression which can be reversed on ligand removal.

5. RNA Devices for the Next Generation Cell and Gene Therapy

One of the prominent features of living organisms is to have proper gene regulation to perform cellular functions and adapt to different environmental conditions. Among several regulatory mechanisms available to regulate gene expression, RNA-based molecules are an emerging class of regulators that can be controlled using

external ligand without disturbing the incongruous regulation network. Recent developments in designing synthetic RNA devices have allowed dynamic modulation of gene expression through diverse mechanisms. The existing mammalian RNA devices can be further subjected to modification for improved dynamic range and ligand sensitivity for cellular and therapeutic applications.

The functional versatility of riboswitch due to its diverse ligand sensitivity is applicable for a variety of gene therapy applications. One of them is endogenous gene expression controlled by miRNA-based riboswitches as they operate in trans [113]. On the other hand, UTR-embedded aptazymes that function in cis are popularly used for gene regulation through mRNA degradation [35]. Small regulatory ON switches in the ligand bound state, capable of forming a stem between a microRNA target sequence (miR-T) and a complementary competing strand can be used to regulate the long-term expression of a gene of therapeutic interest [113].

In a pharmacologic gene regulation approach, the creation of artificial transcription factors (activate the transgene expression upon binding of the pharmacologic agent like antibiotics, hormones, etc.) by fusing a DNA-binding domain, a drug-binding domain and a transcription activation domain, displays several limitations in usages for human gene and cell therapies due to the toxicity of inducer molecule and the immunogenicity of the chimeric transcription factor [42–44]. Immune response against foreign epitopes is one of the key hurdles to gene therapy [44–47. Among all the inducible systems available for gene expression, the most popular one is the tetracycline and the rapamycin-dependent systems. However, they also resulted in an immune response against protein in the long term, particularly in the case of Tet-On tetracycline regulatable system in nonhuman primates (NHP) [44]. These responses subsequently lead to the complete loss of transgene regulation and expression. Thus, we expect that this type of approach to involve non-indigenous proteins would find limited applications in medicine.

For efficient transportation of rationally or high throughput selected riboswitches, several animal model-based and clinical trial studies rely on the adeno-associated virus (AAV) for in vivo transgene delivery (Box 2). However, there are several limitations of this AAV-mediated gene therapy, for example, the packaging capacity of the vector, robust delivery method and safe expression. In this context, riboswitches turned out to be promising candidates for AAV-mediated gene therapies [114]. For example, for angiogenesis gene therapy, RNA device integration and gene transfer mediated by AAV have shown a 6 to 10-fold reversible induction for gene VEGF-B which is a prominent gene for cardiovascular diseases [115]. Several previously reported riboswitches are compared in this study for their performance in regulating gene expression. They also established the importance of the position of riboswitch in the gene cassette and the compatibility of riboswitch with WPRE, for the first time. Along with the development of the therapeutic approaches involving AAV-mediated therapies, the issue of immune response came into the picture and hence, further modifications were performed in a vector to create a balance between AAV-mediated tolerance and the activation of immunity [45]. Apparently, it was also observed that the AAV-associated immune response was more prominent in some primates compared to the mice model [46]. First proof of the clinical applicability of AAV viral vector mediated transgenic expression in mice was shown with the tetracycline-dependent ribozyme K19 with 15-fold induction along with the reversibility and re-inducibilty [116]. Another vector, the vesicular stomatitis virus (VSV) vector with Guanine-responsive aptazyme is shown to repress transgene expression as much as 26.8-fold in the presence of guanine [117]. This reversible repression is crucial for the further development of robust VSV vectors for application in vaccine and gene therapy. In addition, an application of the reversible ON-switch approach for gene therapy for anemia has been demonstrated by regulating the erythropoietin level by inducer oligonucleotide. This riboswitch enhances the activity up to 223-fold [118]. Applications of RNA-based regulatory systems further extended to primary human T cell proliferation in response to drug input in vivo [34]. Two drug candidates ASP2905 and ASP7967, developed by Astellas Pharma, that bind to RNA sequences, AC17-4 have shown a change from 10–300 fold induction of a reporter gene [119]. It is notable that ASP2905 is developed for the treatment of neurological diseases and its role in modulating a switch element will be further applicable to several other disease conditions.

Over the past decade, immunotherapeutic approaches to treat cancer have immensely developed and many of them have been implemented into the practice as well following successful clinical trials. Various treatment modalities that come under the same umbrella named cancer immunotherapeutics have indeed achieved substantial attention and success, but the complete understanding of impeccable treatment procedures is still lacking in the field. With a view that even successful laboratory experiments on model organisms are not seamlessly capable of performing well in patients due to several immune-related adverse events (IRAEs). Therefore, these are the high times to try considering integrating riboswitch devices in the genes key to the cancer-immunotherapy pathway. One of the approaches will be to regulate the expression of genes key to the checkpoint inhibitor or activator to avoid the under/overexpression of crucial genes and subsequently avoid T cell exhaustion.

Concept Definition Box 2

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system can recognize and cleave complementary DNA sequences. This system is naturally present in bacteria to memorize and destroy viruses, however, it is widely used in genome editing by incorporating an approach of using gRNA and Cas enzymes for precise incision and insertion/deletion of required sequences.
IRAE: Immune-related adverse events are the phenomenon when immunotherapy is linked to the occurrence of side effects. They occur due to immune-related toxicity and elicit autoimmune responses that can exaggerate the health issues of the patient under clinical trial.
AAV/Lentiviral mediated gene transfer: Adeno associate virus or lentiviral vectors are used to deliver the copies of engineered therapeutic genes to the desired cell/tissues/animals. These vectors can be easily manipulated and packaged into viruses (which are non-virulent in spreading a disease) to transduce other cells.
Checkpoint Inhibitors/Activators: Immune checkpoints are positive and negative receptors expressed on the surface of immune cells to activate or inhibit the immune response, respectively. These are crucial to maintain immune tolerance and avoid overactivation on healthy cells.
CART-cell: Chimeric antigen receptor T cells are the modified/engineered cells that have the potential to bind and destroy cancer cells. Mostly, these therapeutic procedures are customized for each patient and are based on the principle of combining antibody type specificity with effector T cell function.
Leukapheresis: This is the first step in CART-cell therapy and a method to separate white blood cells. These cells can be subsequently engineered and clonally expanded to re-infuse in the patient's body.

There are several advantages of using RNA devices as gene control element rather than alternative gene therapy approaches as given in Table 1. In this respect, riboswitch incorporation in genes have minimal immunogenicity. Gene expression controlled by riboswitches is reversible and externally tunable using small-molecule drugs without perturbing innate host regulatory networks and without the involvement of protein cofactors [34–39].

Table 1. Comparison of various gene therapy approaches.

Alternative Gene Therapy Approach Perspective	Limitations	Remediation by using RNA devices
Viral vectors mediated therapy [45]	Non-efficient packaging efficiency and immunotoxicity [46]	Modular and portable, Minimal Immunogenicity
Non-Viral vectors [120]	Less efficient for targeting CNS and protein corona formation due to LNPs can impact cellular uptake [120]	Fast regulatory Response
Gene gun [121]	Applicable to superficial tissues only and penetration is challenging [121]	Can be engineered in circulating cells as well
Mesenchymal stem cells [122]	Cannot be expanded outside the body	Engineered cells can be expanded in laboratory
RNA and small molecule-based therapy [3,123,124]	Robust delivery methods are required, immunogenic, and unstable, results in perpetual down/up regulation of the gene [125]	Reversible and reinducible, Small genetic footprint
CRISPR gene therapy [126]	Off target effects, PAM requirement, DNA damage toxicity and immunotoxicity [127]	Controlled expression of a targeted gene without perturbing innate host regulatory networks

As discussed in this review, a substantial amount of research has been carried out in the field of applying RNA devices to regulate gene expression aimed at eventual applications for precision cell and gene therapy. However, a foolproof system is still under development. The pre-requisite of validation, using RNA devices for disease management is still awaiting systematic in vivo studies in model organism and human clinical trials and therefore the research in this area is at exploring stage, the subject under this review is still in its very early infancy.

6. Limitations and Challenges of Using RNA Devices for Gene Expression/Gene Therapy

Despite an enormous potential in regulating gene expression and a broad-spectrum applicability in almost all the fields of Biotherapy, a lack of better understanding of certain aspects of conformational switching in the context of the fully functional devices in vivo environments hamper the development of clinically applicable RNA devices

for precision cell and gene therapy. These include a lack of a way to fine-tune expression or activity of target genes, requiring a non-nature promoter and enhancer sequences, and use of foreign regulatory proteins that could trigger an immune response against foreign protein epitopes [42–44]. Common to all these conventional approaches is the insertion of large genomic footprints that could alter the native genomic structure, neighboring coding and non-coding sequences [128]. In addition, switches involving tertiary interactions are difficult to design by rational approaches due to the lack of accuracy in existing software to predict tertiary interaction and ligand binding. Most of the riboswitches designed to date either have a low dynamic range or high background activity or respond only to high ligand concentrations. In vitro assays for riboswitch selection are indeed needed to be complimented with in vivo assays to simulate the cellular environment, particularly magnesium ion concentration. Moreover, the effect of device insertion on viability and motility should also be tested simultaneously if they are screened *in vivo*. The recent report of control of mammalian gene expression using a combination of polyA signal cleavage and tetracycline riboswitch is highly promising but its relatively large genetic footprint and complex scheme may limit its clinical application [103]. As mentioned above Yokobayashi group has also developed a method to sequence in-vitro transcribed switches using several ribozyme variants but their method requires the separation of isolated RNA into cleaved and un-cleaved fragments on gel which could be error-prone and difficult to implement.

Several rational designing approaches are limited by the number of variants that can be tested and validated in vitro or *in vivo*. This approach is also time-consuming and labor-intensive. To overcome these challenges several groups have adopted a high throughput approach for the selection of ribozyme/aptamer-ribozyme in mammalian cells [29,48]. However, they often have various drawbacks particularly, due to the lack of a better screening platform using mammalian cell models. Integration of FACS-seq with the high throughput approaches mediated by RNA-seq method rendered the better screening of variants in the first place as shown in various publications but the greater noise was noticed in FACS-seq compared to RNA-seq probably due to high rates of lentiviral recombination, random integration in the genome and epigenetic silencing. From the same group, another approach including FACS-seq is generated that requires expensive sequence barcoding of variants and yet is not shown to be transferable to humans. Therefore, the real application of the selected variants has never been tested in human subjects to combat any disease condition.

The current approaches for cell and gene therapy face several formidable challenges, as in vivo screening through high throughput approach indeed requires additional steps of packaging the library into carrier vectors like AAV, Lenti etc. which creates an additional challenge. The most popularly tested AAV is reported to elicit immune response in primates [43,46] and lentiviral approach though capable of carrying larger inserts, still has an issue with high rates of recombination. Despite these challenges an approach of using trans-splicing ribozyme to inhibit hepatocellular carcinoma by 86% is reported [129]. In this approach human telomerase reverse transcriptase (hTERT) is targeted with the help of AAV mediated transfer of ribozyme under the control of miR-122 which was efficiently cleaved with the selected ribozyme. In another study aptamer mediated targeting of non-small cell lung cancer is tested by induction of apoptosis in NH-H460 human cells [130]. These RNA aptamers were shown to inhibit the viability of these cells in a dose dependent manner with IC_{50} in Nanomole range (100nM).

Given the advantages outlined in the introduction, ligand responsive RNA devices hold great potential over all existing cell and gene therapy approaches using adeno-associated virus (AAV), tet-on system or CRISPR–Cas9 [42–46,103]. However, the efficiency of knock-in (KI) rate is extremely low, almost impractical to obtain large number, at least several ten million of cells with double-allele KI for in cell and animal tests. Thus, double allele KI and generating sufficient cells with the insert is one of the major bottlenecks if RNA device-mediated precision gene therapy can be practical. Another formidable challenge is engineering RNA devices into a target gene in cells followed by amplification and screening the cells with inserts.

Additionally, all currently reported illustrations of RNA device mediated gene regulations show some severe limitations such as narrow dynamic range, use of high toxic or suboptimal dosage of ligands and high background (leakage) [115,117], all of which could attributed to under-developed screening methods using eukaryotic or more specifically human cells. Thus, screening new RNA devices based on riboswitches should focus on developing more efficient screening methods using human cell systems.

7. Future Perspectives of Using RNA Devices for Precision Cell and Gene Therapy

All current RNA devices were developed based on the modular concept of RNA structures aided by mutagenesis or high throughput screening of libraries in bacteria [17,23], yeasts [49] or mammalian cells [29]. For an efficient synthetic riboswitch design by rational methods, some of the important parameters of the communication module, like hydrogen bonding, base stacking, and distance to the enzymatic core are very crucial and all these parameters were taken into consideration to generate 32 variants in study reported by Zhong et al.

[36] which is one of the first attempts using a rational design approach based on the thermodynamic principle. These tetracycline induced aptazymes were also proved to regulate the gene expression in mammalian cells and mice model [36]. It was observed that the base pairing within the communication module mostly affects the ribozyme activity, and they devised a parameter that correlates with the ribozyme performance, 'Weighted Hydrogen Bond Score (WHBS) to further narrow down the selection procedure. However, as more structural information becomes available, it is anticipated that structure-based rational design in combination with consideration of thermodynamics principle aided high throughput cell assays for the development of more efficient RNA devices will become feasible in the near future.

One key aspect of an efficient RNA device is transmitting the signals of conformational changes in the aptamer domain to the expression platform (ribozyme in the case of aptazyme) through a communication module [30]. The principal requirement for an effective communication module is low signal attenuation. To achieve this, the structural basis for an effective communication module is required to be understood. Thus, it is highly desirable to determine three-dimensional structures of complete RNA devices, consisting of an aptamer, an effective communication module and an expression platform (ribozyme in the case of an aptazyme) that is fully functional in mammalian cells and animals. So far, there is no such a structure ever determined. Furthermore, high throughput approaches can be adapted by using libraries containing randomized sequences of the communication module and the functional devices can be selected by appropriate screening procedures, particularly, in mammalian cells.

Besides the applications that are reviewed in the previous section, one perspective new application is to integrate the RNA devices in primary human T cells to turn genes important for T cell functions ON/OFF using ligands. This strategy may lead to the development of various therapeutic T cells for immunotherapy and infection control. For example, a functional switch device that meets all the above-mentioned parameters, can be integrated in the 3′ or 5′ UTR of a checkpoint inhibitor gene and in the presence of ligand the gene can be shut down and regulated so on, in a reversible and re-inducible manner, if required (Figure 4). Recent development in the CART-cells [131,132] further supports that this system can be feasibly applied for gene therapy by clonal expansion of the engineered T cell population and re-infused to the patients to improve the immune response to kill the target cells expressing ligands that bind to the checkpoint inhibitors as shown in schematics in Figure 4. Having met these demands, the RNA device applications would be dramatically directed towards the development of various treatment procedures. Therefore, the future of RNA devices as molecular tools for gene therapy is very promising with the development of interdisciplinary approaches for designing functional devices and their in vivo screening for health welfare.

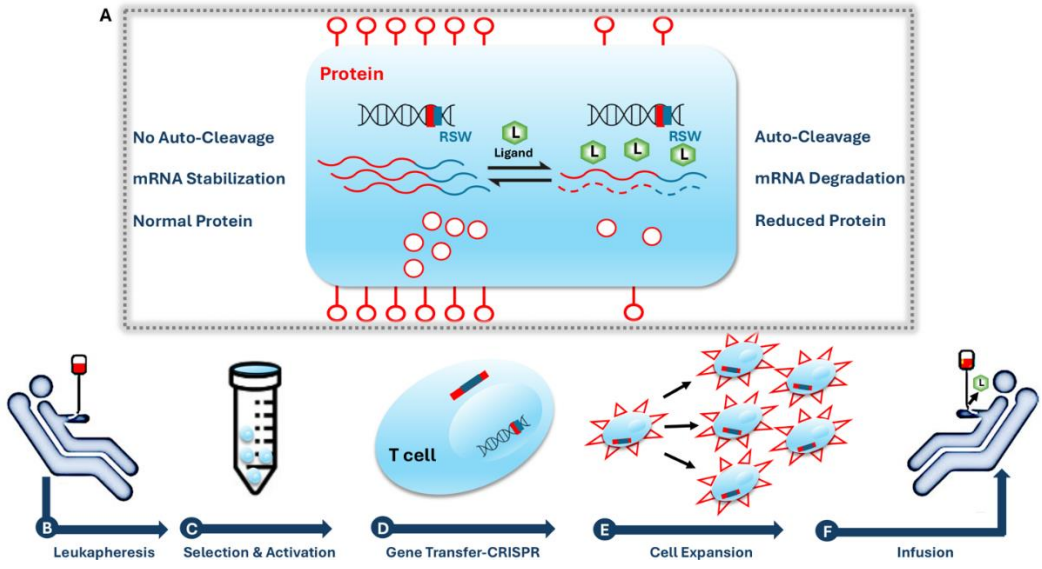

Figure 4. Clinical application of RNA devices for gene therapy: (A) Strategy for switch-mediated reduction in the level of receptor protein. CRISPR-mediated integration of riboswitch device (blue) in the 3′UTR of a gene of interest (red) would result in the transcripts with riboswitch sequence that should allow the normal protein expression. In the presence of ligand (green), during transcription as soon as the riboswitch sequence is transcribed, due to self-cleavage activity the transcript will be degraded resulting in diminished protein level. (**B)** A patient's blood will be taken out to harvest primary T cells, (**C**) the cells will be activated in laboratory and (**D**) engineered by CRISPR-mediated integration of devices. (**E**) The positive cell population expressing engineered devices will be clonally expanded and then F) re-infused to the patient.

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