# Hammerhead ribozymes in therapeutic target discovery and validation

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**Gene function assessment is a main task in biological networking investigations and system biology. High throughput technologies provide an impressive body of data that enables the design of hypotheses linking genes to phenotypes. When a putative scenario is depicted, gene knockdown technologies and RNA-dependent gene silencing are the most frequent approaches to assess the role of key effectors. In this paper, we discuss the relevance of hammerhead ribozymes in target discovery and validation, describing their properties and applications and highlighting their selectivity. In particular, similarities with siRNAs are presented and advantages and drawbacks are discussed. A description of the perspectives of ribozyme application in wide range studies is also provided, strengthening the value of these inhibitors for target validation purposes.**

If, on the one hand, human genome sequencing has concluded an important chapter of studies, on the other hand it has opened the door to new challenges and possibilities. Unexpectedly, only 2% of the human genome sequence encodes for proteins whose functions are still to be clarified. Polymorphism genotyping, expression profiling, proteomics, metabolomics and other wide-range post-genomic disciplines are trying to find an answer to the problem and to offer a different vision for the future. Unlike the traditionally 'reductionist' view of biomedical research, these disciplines are characterized by a new 'integrationist' approach, which relates to a global view of processes and organisms and requires increasingly complex interpretative models. In this context, high throughput technologies play a central role in providing an impressive bulk of data capable of supplying information for innovative hypothesis generation on gene networking and system biology [1,2]. In particular, a series of powerful tools (molecular, bioinformatic, instrumental) have been designed to screen complex expression libraries for essential genes and provide functional links from genes to phenotypes. These new technologies have made it possible to identify the role of a number of key factors responsible for the onset of disease, as well as new putative targets in therapeutic treatment. The subsequent step is the validation of the supposed targets, often performed by the pharmaceutical industry during drug development. This phase can be expensive and time-consuming and, therefore, technological alternatives producing fast and early validation are necessary to meet the increasing demand for gene function assignment.

## Knockdown technology overlapping gene function definition and target validation

Two main features are necessary for the reliable function definition and validation of a given gene: (a) the possibility to modify its activity significantly; (b) confidence that such modification is limited to the gene of interest.

To this end, gene knockdown technology is a widely employed tool that relies on selective gene targeting by oligonucleotides, causing a strong inhibition of gene activity and possible reversion of the pathological phenotype. This strategy offers some advantages compared to the knockout technology because it acts on mature differentiated cells, hence avoiding developmental constraints and lethal events. RNA-dependent gene silencing is the most common gene knockdown approach and is based on cell administration of antisense, ribozyme or RNA-interfering short oligonucleotides (siRNAs) [3,4]. Ribozymes and siRNAs, owing to

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their high efficiency, are the most frequently exploited inhibitors compared with the less active antisense oligonucleotides.

All oligonucleotide inhibitors recognize their RNA target through complementary interactions generating double helical structures and, therefore, binding specificity depends on the nucleotide sequence. Some sequences can, however, be shared among different RNAs, raising a key question on how to design target-specific oligonucleotides with no side effects. Such phenomena, also referred to as 'off-target-effects', represent crucial issues in the selection of knockdown tools. In this paper, we describe the main features of hammerhead ribozymes and discuss their properties and possible applications in target discovery and validation in the light of recent research.

## Hammerhead ribozymes realization and applications

The hammerhead ribozyme, the smallest known RNA catalytic motif with endoribonucleolytic activity [5,6], displays a secondary structure where a 17-nucleotide, highly conserved catalytic domain connects three base-paired stems (Figure 1). The cleavage reaction occurs downstream to a NUH site within the RNA target sequence. Stems I and III ensure binding specificity while stem loop II has structural significance and is involved in active conformation establishment. Hammerhead ribozymes have been highly characterized and their cleavage mechanism defined [7,8]. For many years conflicting results made biochemical data and crystal structure of minimal hammerhead sequence irreconcilable [9]. Recently, Martick and Scott resolved the crystal structure of a natural 63 nucleotide hammerhead ribozyme from *Schistosoma Mansoni* satellite RNA [8]. This ribozyme shows a tertiary stabilizing motif-connecting loop II to a bulged loop exposed on the extended distal region of stem I. This structure supports the active geometry, suggesting acid–base cleavage catalysis promoted by 'in-line' geometry without involvement of divalent metal ions. Instead, minimal hammerhead ribozymes are found predominantly in an inactive conformation requiring a structural change in order to be activated. This would explain why they are 500–1000-fold less active than full-length ones [10]. These findings reconcile three-dimensional and biochemical observations explaining some experimental failures. Hammerhead ribozymes react independently of any cellular machinery, so they can also operate *in vitro*. Many studies have been performed on minimal ribozymes directed against RNA sequences with the purpose of designing strategies, for basic applications and therapeutic perspectives. Some ribozymes have come through clinical trial and their subsequent registration as drugs goes some way to validate their therapeutic value [11]. Although the therapeutic applications of hammerhead ribozymes are still sparse, due to unresolved *in vivo* delivery problems, they are widely used as gene-expression modulators for disease phenotyping or therapeutic target assessment [12]. We shall now describe some aspects concerning design and preparation of effective hammerhead ribozymes.

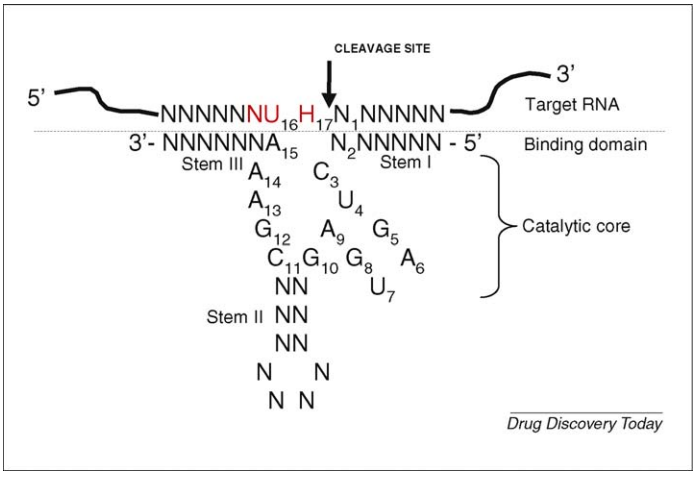


**FIGURE 1**

Features of hammerhead ribozymes. A generic diagram of a hammerhead ribozyme bound to its target substrate: NUH is the cleavage triplet on target sequence, stems I and III are sites of the specific interactions between ribozyme and target, stem II is the structural element connecting separate parts of the catalytic core. Arrows represent the cleavage site, numbering system according to Hertel *et al.* [60].

### *The tricky task of hammerhead ribozyme design and characterization*

Ribozyme design and characterization includes: (a) computational analysis of target sites and definition of related ribozymes, (b) synthesis and purification of selected sequences (c) characterization of ribozyme cleavage properties and (d) chemical stabilization of active sequences or cloning in expression vectors. Since cleavage site accessibility is necessary for ribozyme target binding, some predictive computing methods were implemented to map 'open' regions verifying their uniqueness over all known transcriptomes. These methods identified some potentially effective ribozymes, with no obvious off-target effects [13,14], demonstrating an extremely reduced number of useful sites within a given target. This represents a considerable drawback, especially when short or incomplete RNA sequences have to be targeted. Moreover, the significance of predictive computing analyses is dramatically reduced in the last instance.

Considering recent findings on the cleavage reaction mechanism, search for ribozymes containing stem I distal stabilizing motifs should be introduced, reducing the identification of useful targets and limiting, if not impairing, the design of full-length hammerhead ribozymes. Therefore, minimal hammerhead sequences, although less active, will have a more practical value unless artificial motifs mimicking tertiary stabilization become universally applicable [15]. With regard to synthetic ribozymes, cleavage constraints have shown to expand from simple NUH to NAH and NCH triplets, introducing point mutations at position 15 of the catalytic core [16,17]. The introduction of these 'non canonical' triplets enhances the probability of finding useful ribozymes in whatever RNA sequence.

Several experimental combinatorial approaches mapping accessible triplets for ribozyme cleavage were developed. Antisense oligonucleotide libraries were used to find 'open' regions taking advantage of the finding that oligonucleotides bind target sequences mainly at unpaired, single-stranded regions [18,19]. An alternative *in vitro* approach was exploited by repeated selection cycles of transcription, binding and RT-PCR amplification performed on a library of randomized RNA oligonucleotides, so-called 'guide-RNA' [20]. Using a combinatorial library of ribozymes, as an alternative to antisense, Lieber and Strauss refined

the approach since the identification of ribozymes is not only driven by accessibility but also the ability to catalyse cleavage [21].

Production of working hammerhead ribozymes concerns not only their design and synthesis, but also the laborious assessment of their cleavage activity, which limits their application to high throughput analyses. Experimental ribozyme assessment requires synthesis of both inhibitor and related target. Cleavage properties are usually evaluated on minimal target sequences (multiple turnover) or on full-length RNAs (single turnover), measuring endonucleolytic kinetic reactions [22,23]. Target substrate synthesis represents another time-consuming aspect, since short substrates need different chemical syntheses, one for each ribozyme, while full-length substrate preparation is underpinned by cDNA cloning and transcription.

Chemical ribozyme stabilization, ensuring long intracellular half-life, is necessary but further characterizations is necessary to establish factors affecting catalytic properties [24].

### *Hammerhead ribozyme administration and conventional applicative studies*

Crucial points for successful ribozyme-based gene knockdown are (a) delivery efficiency, (b) ribozyme half-life, (c) turnover rate of target RNA and (d) the half-life of related proteins. An excessively rapid RNA turnover may prevent the biological effect of molecular inhibitors and, the protein gene product should be reduced to induce phenotypic changes. In order to obtain valid functional gene knockdown, proper timing for effect observation should be considered. Ribozyme efficacy can be assessed by establishing the physiological degradation of the protein present in the cell. The time necessary for this operation to take place depends on the turnover rate of the specific protein.

A common approach for gene function inhibition is the transfection of expression vectors that endogenously produce antisense, siRNA and ribozyme oligonucleotides. This strategy ensures a sustained knockdown effect, providing a suitable reply to some of the issues mentioned. Unfortunately, transfection efficiency, frequently low, may involve a limited number of cells. Instead, administration of exogenously synthesized inhibitors is more efficient but requires long-lived oligonucleotides or repeated administration to produce long-term biological effects. Experimental applications can be divided as follows: endogenous ribozymes, representing the most common approach, and exogenous ribozymes, more easily produced on large-scale by chemical synthesis and delivered into the cells by suitable vehicles (liposomes, polyplexes, dendrimers, and so on). [12]. Exogenous ribozymes represent a greater challenge because they must be resistant to cell ribonucleases. To obtain a persistent effect, chemical modifications that ensure longer half-lives have been exploited in various biological conditions [11,24].

Literature on ribozyme applications essentially describes the correlations between expression level of target gene and amount of a measurable biological event demonstrating, for example, the involvement of signal transduction elements in cell growth and differentiation [25–28] (the role of apoptosis regulators cell survival effectors [29–32], and the contribution of DNA repair factors in drug resistance [33,34]). Similarly, it was shown that, in cells with integrated provirus, ribozymes directed to mRNAs encoding retro-viral regulatory proteins produce long-term protection against infection [35,36]. Ribozymes were also used in cardiovascular disease and angiogenesis studies to assess the role of growth factors and cell signaling in coronary lumen thickening and neo-vasculature formation [37–39]. Lists of genes successfully knocked down by ribozyme administrations are reported in a number of published reviews [11] (Table 1). These studies highlighted the correlation between

**TABLE 1**

**Typical examples of successful applications of hammerhead ribozymes. Most of the data are derived from [10] and [11], the others are expressly specified.**

| *Target* | References |
|---|---|
| **Growth factors, receptors, transduction elements** | |
| **PDGF-A** | [11] |
| **VEGF** | [10,11] |
| **TGF-β** | [10,11] |
| **HGF** | [11] |
| **TNF** | [11] |
| **IL-6** | [11] |
| **ErbB-4** | [11] |
| **HGH** | [11] |
| **PDGFR-β** | [10] |
| **Flt-1** | [10] |
| **HGFR** | [11] |
| **KDR** | [10] |
| **RAGE** | [11] |
| **GF receptor** | [11] |
| **Oestrogen receptor** | [11] |
| ***CCTIS*** | [11] |
| **G protein** | [11] |
| **Oncogenes, protoncogenes, fusion genes** | |
| **H-ras** | [10] |
| **K-ras** | [10] |
| **N-ras** | [10] |
| **fos** | [10] |
| **myc** | [10,11] |
| **fms** | [11] |
| **myb** | [10,11] |
| **met** | [10] |
| **RET** | [10,11] |
| **Her2/neu** | [10,11] |
| **bcr/abl** | [10,11] |
| **PML/RAR** | [10,11] |
| **Apoptosis, survival factors, drug resistance** | |
| **Bcl-2** | [10,11] |
| **Survivin** | [10] |
| **hTert** | [10] |
| **Telomerase RNA** | [10] |
| **Caspase** | [11] |
| **MGMT** | [10] |
| **hOGG1** | [30] |
| **MDR-1** | [10,11] |
| **Transcription factors** | |
| **HIF-1** | [10] |
| **E2F1** | [10] |
| **p300** | [11] |
| **NFκB** | [57] |
| **Extracellular matrix, matrix modulating factors** | |
| **Osteopontin** | [11] |
| **Tenascin C** | [10] |
| **MMP-9** | [11] |
| **MMP-2** | [10] |
| **uPAR** | [58] |
| **Type-1 collagen** | [59] |

**TABLE 1 (*Continued*)**

| Target | References |
|---|---|
| **Circulating factors** | |
| **Apolipoprotein-A** | [10] |
| **Apolipoprotein-B** | [11] |
| **α-Lactalbumin** | [11] |
| **Antiboby k-chains** | [11] |
| **Pleiotropin** | [11] |
| **Viral genome, viral genes** | |
| **Hepatitis HCV** | [10] |
| **Hepatitis HBV** | [10] |
| **Cytomegalovirus HCMV** | [10] |
| **Herpes HSV** | [10] |
| **Immunodeficiency HIV** | [10] |
| ***Tat*** | [10] |
| ***Rev*** | [10] |
| ***Env*** | [10] |

gene inhibition and changes in the biological phenomena observed, but could not provide insights into the molecular mechanisms. Recent studies have focused on factors related to targeted-gene activity in an attempt to clarify its functional role. For instance, BMP-7 inhibition in prostate cancer cells reduces their invasiveness by reducing noggin and follistatin expression [40] and Inhibitor of κB kinase (IKK) knockdown in a nude mouse melanoma graft demonstrated the involvement of NFκB activation in reducing tumor growth [41]. Several studies evaluated the effects of single-gene knockdown on general profiles of RNA transcripts or protein expression exploiting wide-range 'omic' technologies [42–44]. These investigations addressed the need for gene function characterization and networking, paving the way for system biology development. Some authors improved a multiple gene knockdown strategy to trace the whole pathway involved in a single biological process and to define the genes involved in a distinct phenomenon. For instance, Musio *et al.* described a panel of genes engaged in genome stability control [45].

## Hammerhead ribozymes *versus* siRNAs in therapeutic target discovery and validation studies

Comparing ribozymes with the more popular siRNAs and their relative properties, demonstrates the advantages and disadvantages of both techniques. At the outset of their discovery, the impressive development of siRNA applications to functional studies seemed to overwhelm the value of the already established ribozymes. The realization of efficient siRNAs is easier and faster than that of hammerhead ribozymes and seems to be independent of molecular target tridimensional constraints. The relevance, however, of off-target effects in RNA interference mechanisms has reinforced the importance and utility of hammerhead ribozymes [46]. The RNA interference system is mediated by specific cell machinery, the RISC complex that enables binding to the RNA target [47]. Both siRNAs and miRNAs share a similar mechanism, but, while siRNAs need a complete match to allow a cleavage reaction, the miRNA modulator activity requires only a 7 nucleotide long-matching stretch (*seed* sequence) as the remaining sequence may match imperfectly. In this case, RISC protein components contribute to complex stability and ensure binding to different targets with the same *seed* sequence. Thus, miRNAs can modulate the activity of several genes concomitantly [48]. Owing to the miRNA-like siRNA modulatory activity on an unknown number of mRNAs, siRNAs may induce uncontrollable off-target effects [49], compromising the exact gene function definition. This makes it difficult to find a truly inactive siRNA for control experiments [46].

Hammerhead ribozymes display a completely autonomous catalytic activity binding and cleave target sequences even in the absence of cell components. Binding depends on complementary match with the homologous target site and is responsible for ribozyme specificity. The target sequence may be considered a twin-lock requiring two independent keys to be cleaved, represented by the two non-contiguous binding arms of the ribozyme sequence (Figure 1). The unique nature of the complete target sequence may ensure almost absolute specificity. Partial homologies due to a single binding arm produce unstable, inactive complexes that cannot fold the catalytic core correctly (Figure 2).

The ability of hammerhead ribozymes to discriminate between targets differing by a single nucleotide when placed inside or in close proximity to the cleavage site has been widely demonstrated [50,51]. Although tolerance for mismatches is very low when the binding sites are shorter than 12 nucleotides [52], studies based on thermodynamics, utilizing transcriptome databases enabled the rejection of target sequences displaying partial homologies with unrelated RNAs [14]. Ribozyme specificity can be experimentally verified by two related controls: an inactive version, able to bind but unable to cleave the target; and a scrambled version, potentially able to cleave but unable to bind to substrate.

Both siRNAs and ribozymes, exogenously delivered, act on RNA at the cytoplasmic level by impairing its translation. While the RISC machinery strictly limits the activity of siRNA in the cytoplasm, different cell compartments may be addressed in the case of ribozymes. For instance, expression vectors encoding U6 ribonucleoparticle-embedded ribozyme will be localized into the nucleus, while those encoding tRNA will direct transcripts to the endoplasmic reticulum. Future developments may be hypothesized by which composite vehicles containing signal peptides should translocate exogenous ribozymes in different compartments including the nucleus. Hammerhead ribozymes, although slightly less effective than siRNAs, can be considered better precision molecular instruments and display higher developmental potentialities in dissecting any single gene from the entire metabolic context.

## Perspectives for hammerhead ribozymes in gene knockdown applications

Over the past decade, high throughput technology has revolutionized basic research producing large amounts of data and suggesting new strategies of development. Instead of a conventional 'hypothesis-driven' investigation, where siRNAs and ribozymes are used to confirm previously postulated mechanisms, the 'hypothesis-generating' approach is starting to emerge. This method combines gene knockdown experiments with large scale expression or protein profiling in order to obtain all data necessary for the complete understanding of a given biological model. It requires the use of a number of different oligonucleotide inhibitors, each specifically selective against a given target. Hence, innovative methods capable of providing effective hammerhead ribozymes quickly would revolution their, as yet, still hesitant application.

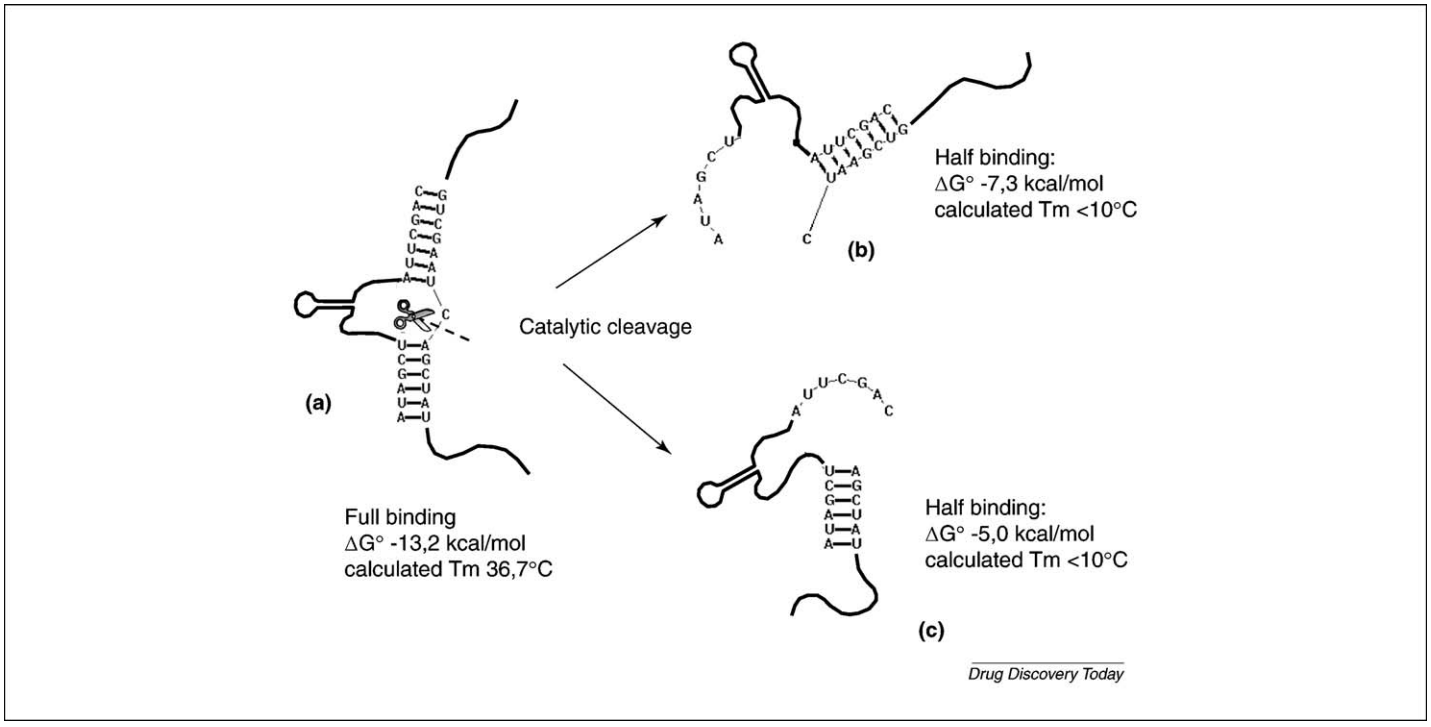


**FIGURE 2**

Target–ribozyme interactions. (a) A scheme of ribozyme binding to full substrate. The calculated energy of this binding ensures the formation of a stable complex. At the denaturating temperature, Tm, will allow this complex to survive to biological conditions. Conversely, after cleavage, binding energies calculated on single, (b) and (c), ribozyme arms are very low and no longer stable. These properties will ensure both the efficient release of cleavage fragments and the prevention of binding to unrelated targets. RNAs complementary to one binding arm only will not be bound or cleaved by the hammerhead catalytic sequence.

At present, siRNAs represent helpful tools, because they are cheap and can be readily supplied by biotech companies that have already tested their specificity. Although there can be issues with selectivity and the associated risk of providing false positive results, siRNAs should be utilized for wide-range screening tests in order to identify candidate therapeutic gene targets. Still, the highly specific hammerhead ribozymes, exactly tailored to the target sequences, would better accomplish the successive target validation step ensuring more confident results. In conclusion, siRNAs and hammerhead ribozymes may be 'brothers in arms' as defined by Bhindi *et al.* [4].

Two main working hypotheses on hammerhead ribozyme applications can be proposed and represent the next phases of their application in functional studies applied to target discovery and validation: (1) the combinatorial approach and (2) the 'chemical-omics' approach.

### *Combinatorial approach to target discovery*

The establishment of a given phenotype depends on the environmental conditions to which cells and tissues are exposed. It concerns the activation of a defined transcription program carried out in a coordinated manner, able to ensure the maintenance of cell homeostasis. Cells may change their properties in response to perturbations from a starting equilibrium undergoing physiological or pathological phenotype switches. According to the combinatorial strategy, libraries of randomized hammerhead ribozymes able to target any transcribed RNA can be used to produce a more or less marked switch. When administered to cells, ribozymes can cause changes related to key functions responsible for a defined phenotype. Cells displaying changed properties can be isolated and ribozymes recovered and sequenced. Database analyses (BLAST search) would enable the identification of targeted RNAs and genes involved in the observed phenomenon. As expected, this 'transcriptome wide' gene knockdown approach needs very well defined models in which phenotypic changes are distinctive of basic processes like apoptosis, cell invasion, viral replication or cell differentiation. By using such a combinatorial strategy, Taira and co-worker described the *Fas*-mediated apoptosis in HeLa cells transfecting a library of hammerhead ribozymes inserted in a retroviral vector [53]. They identified 4 well-known factors such as FADD, caspases 3, 8 and 9 and some other novel genes. The analysis of genes involved in muscle differentiation was also performed by means of a ribozyme library [54]. In addition to the major muscle regulatory factors (MyoD1, Mylk, Myosin, myogenin, Myf5), the authors identified a panel of 7 previously described genes and 6 novel genes.

### *'Chemical-omics' approach to target discovery*

The alternative approach to target discovery combines the high throughput wide spectrum 'omics' technologies (genomics, proteomics, metabolomics) with RNA-based gene inactivation methods including hammerhead ribozymes.

Up until now, there has been a gap between molecular biologists studying protein expression profiling and genomic technologists interested in the application of gene knockdown technology for basic research. The former take advantage of gene knockdown methods merely as useful molecular tools to confirm the actual involvement of a given factor in a presumed pathway, the latter

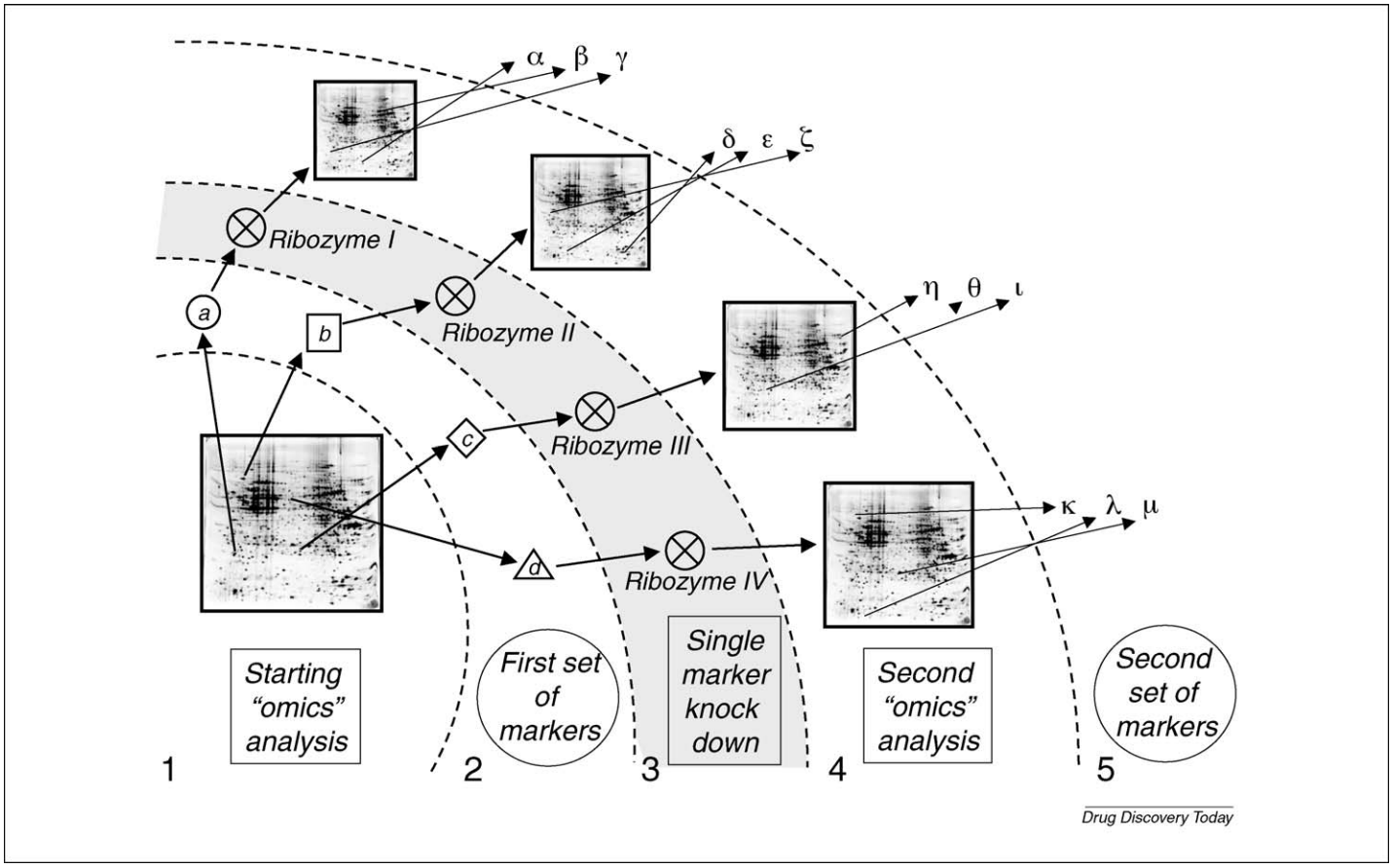


**FIGURE 3**

*'Chemical omics' approach*. According to this target discovery strategy: (1) a first round of 'omic' study (proteomic, genomic, metabolomic, . . .) will enable the discovery of a set of (2) putative markers. A series of hammerhead ribozymes will then be prepared in order to target each marker. (4) A second 'omic' study round will be performed on (3) knocked down samples obtained after ribozymes administration. (5) A new series of markers will then be produced. An expanding analytical process of this type may be further repeated. Finally, a robust bioinformatic algorithm will make it possible to connect the different markers and draw new hypothetical links and pathways.

utilize them to add more insight into gene function. A systematic combination of both approaches would open up new perspectives. According to this model, initially, a round of expression profiling analyses aimed at generating a first set of inherent marker functions would investigate a given phenotypic switch. Each identified marker would then be targeted by first generation ribozymes specifically tailored on related mRNA sequences. A second round of expression profiling analysis would again investigate samples from each single knockdown function generating a second set of markers. Second generation ribozymes would, in turn, be designed and their knockdown effects again investigated by profiling analyses. Such a process may be further reiterated, Figure 3. This systematic approach would allow the monitoring of how cells respond to the selective silencing of identified markers/targets and to draw a map of possible gene interactions. Bioinformatics analyses of data and molecular modeling would help to hypothesize mechanisms underlying phenotypic switches. Further *in vitro* and *in vivo* investigations should be performed to validate the new hypotheses in sight of therapeutic strategies.

## Concluding remarks

The high selectivity of hammerhead ribozymes in gene inhibition makes them distinctive over the other nucleic acid-based inhibitors including siRNAs. This almost ideal characteristic justifies their application in therapeutic target discovery and validation and deserves extensive efforts to overcome the still complicated methods for the design and characterization of catalytically effective molecules. In the near future, improved automated methods for ribozyme realization, including artificial tertiary stabilizing motifs [15], and chemical modifications capable of greatly increasing the intracellular half-life of catalytic oligonucleotides are expected. The combined delivery of 'facilitator oligonucleotides' and ribozymes was shown to overcome the limitations due to target accessibility [55]. It is conceivable that all these advancements, along with innovative techniques for cell delivery, will allow the extensive use of hammerhead ribozymes also in high throughput gene knockdown approaches. Fast and highly productive methods of inhibition are suitable for therapeutic target discovery that intends to assess the functions of a number of different genes in concurrent experiments. For this instance, either ribozymes or siRNAs may be used depending upon their availability. When the aim is target validation, a truly selective inhibition of a single gene is required to demonstrate with high confidence its involvement in the supposed pathological pathway. To this end, the hammerhead ribozyme is the elective choice because its selective action can be experimentally checked. Once

successfully used in target validation, the ribozyme itself can also be exploited as an innovative therapeutic solution. This scheme can be improved by new molecular technologies combining, for instance, hammerhead ribozymes with aptamers. It has been demonstrated that by combining aptamers with hammerhead ribozymes it is possible to obtain allosteric catalytic molecules (allosteric ribozymes) whose inhibitory activity is triggered by their binding to the 'antigenic' factor [56]. Applying allosteric ribozymes to therapeutic treatments, we can idealize an innovative oligonucleotide drug that can be switched on only in the presence of the target protein, thus inhibiting both the protein and its related RNA. In conclusion, hammerhead ribozymes are enjoying a renaissance in both applied and basic research.

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