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Mini-Review

Are morpholino technology dilemmas an affidavit of the non-translational structural role of mRNA?

Malgorzata Kloc[1,2,*], Jacek Z. Kubiak[3,4] and Rafik M. Ghobrial[1,2]

[1]Department of Surgery, The Houston Methodist Hospital; [2]The Houston Methodist Research Institute, Houston, TX, USA. [3]CNRS UMR 6290, Institute of Genetics and Development of Rennes, Cell Cycle Group; [4]University of Rennes 1, IFR 140 GFAS, Faculty of Medicine, 35 043, Rennes, France.

ABSTRACT

Morpholino technology has been widely used by developmental biologists for over a decade. However, in recent years numerous authors have expressed concerns about the usefulness of morpholinos and their worrisome side effects and discrepancies between phenotypes produced by morpholinos and those resulting from mutation(s) in the gene of interest. Such excellent and extremely informative studies looked at various potential explanations and remedies but they have not discussed the possibility that some of the off-target effects and discrepancies present in morpholino knockouts may result from the unintended interference with mRNA structure and function. Here, to supplement the ongoing worldwide discussion on the subject, we present another potential explanation for such unexpected effects of morpholino technology.

KEYWORDS: morpholino, mRNA, structural RNA

INTRODUCTION

Morpholino antisense oligonucleotide (MO) technology has been widely used by developmental biologists to produce the loss-of-function phenotypes and thus assess the function of the gene of interest in various model organisms [1-3]. Morpholinos block production of the protein of interest either by inhibiting translation (ATG or UTR MOs) or by interfering with mRNA maturation when designed to target the pre-mRNA splice sites (SPL MOs, PhotoMOs) [4-7]. Since its inception over a decade ago, the initial enthusiasm toward morpholino technology has been fading due to the mounting evidence of the so-called non-specific toxicity or off-target effects of morpholinos [1- 3, 8-11]. Some of the artifacts of the MOs and off-target effects are the result of the non-specific binding to inadvertent targets because of huge molar excess of injected MOs. Schulte-Merker and Stainier [9] calculated that a typical injection of 1 ng of 25–mer MOs introduces a 2×10^4-fold molar excess of MO over the intended target mRNA. Although some of these undesirable effects can be dealt with by applying proper controls such as designing different MOs or using rescuing RNA [9, 10], even more worrisome are the reports of discrepancies between phenotypes produced by morpholinos and those resulting from mutation(s) in the gene of interest [9, 11]. The principle of morpholino technology is based on the assumption that the phenotype depends exclusively on the function of protein, an assumption, which may not be universally correct. Over the years several laboratories showed that in *Xenopus*, HeLa cells and *Drosophila* certain cellular structures and functions are independent of proteins and depend on the non-translational structural role of their cognate mRNAs instead. These include organization of cytokeratin and actin cytoskeleton and anchorage of various localized RNAs and germ cell determinant (germinal granules) at the cortex of *Xenopus* oocytes [12-15], the dynamics of microtubules of the mitotic spindle

*Corresponding author: mkloc@houstonmethodist.org

and asters in HeLa cells and *Xenopus* oocyte extracts [16], and formation of germ cells and scaffolding of cytoplasmic complexes essential for oocyte development in *Drosophila* [17]. In addition, studies on *Xenopus* showed that different fragments of the mRNA exert different phenotypic effects on cytokeratin cytoskeleton of the oocyte [18]. These data gave rise to the hypothesis of binary phenotype, which proposes that at least in some cases the normal phenotype depends not only on the function of protein but also on the non-translational structural role of its cognate mRNA (Fig. 1) [19-22].

DISCUSSION

What is the non-translational structural role of mRNA and how different types of morpholinos can interfere with this function?

The non-translational structural role of mRNAs, which we are referring to, is the ability of mRNA molecule to direct or influence protein polymerization/multimerization (such as formation of cytokeratin and actin filaments or microtubules), to organize and keep proteins in proper spatial (three dimensional) arrangements and/or to conglomerate different proteins into the higher order complexes (Fig. 2). All these functions are completely independent of the translational function of the given mRNA and may reside in any part (non-coding or coding regions or both) of the mRNA molecule.

There are numerous examples of the non-translational structural role of mRNA in various organisms (Fig. 2). For example in icosahedral viruses (such as human hepatitis A virus, poliovirus or tobacco mosaic virus), which are built from a single-stranded sense RNA surrounded by a proteinaceous shell [22, 23] the viral RNA not only carries the genetic information, but also directs the geometry of capsid protein assembly and the ultimate size of the virus [23-26]. The engineered

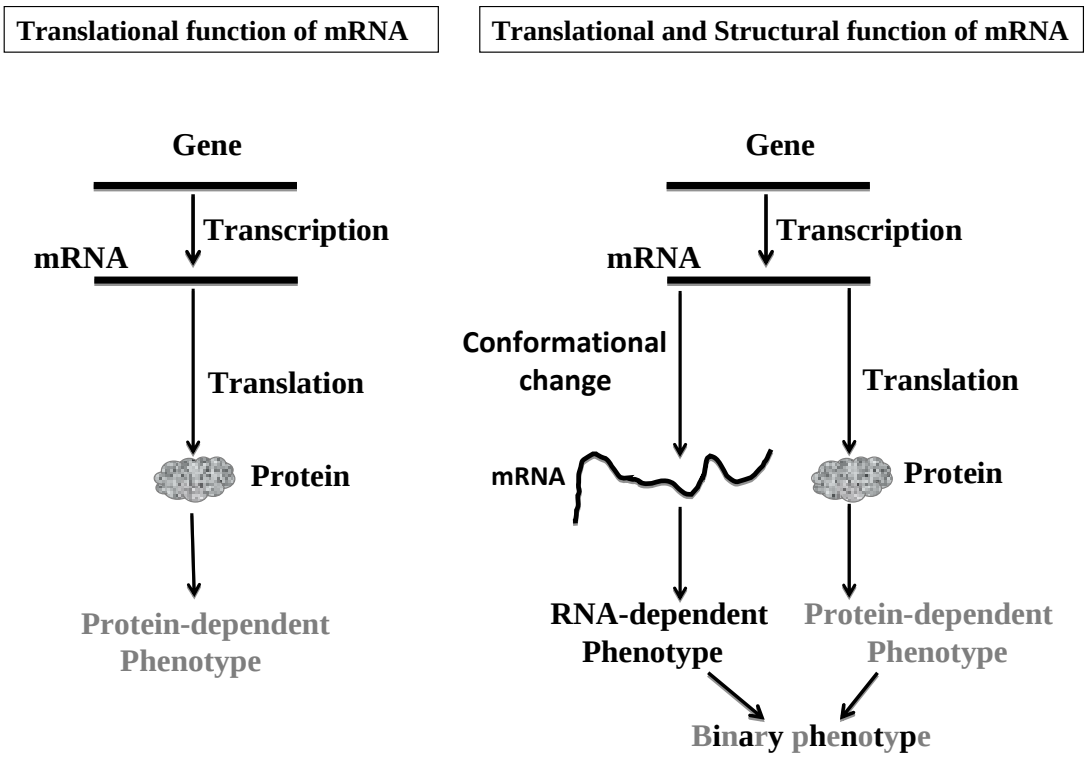

Fig. 1. The diagram shows two different scenarios of mRNA function and resulting phenotypes. Left panel shows commonly accepted (canonical) translational function of mRNA, which results in the production of protein and the phenotype depending on the presence/function of the protein. Right panel shows the scenario in which the same mRNA can, depending on conformation, have two different functions: canonical (translational) and non-canonical (non-translational = structural) function, which results in a combinatorial (binary) phenotype.

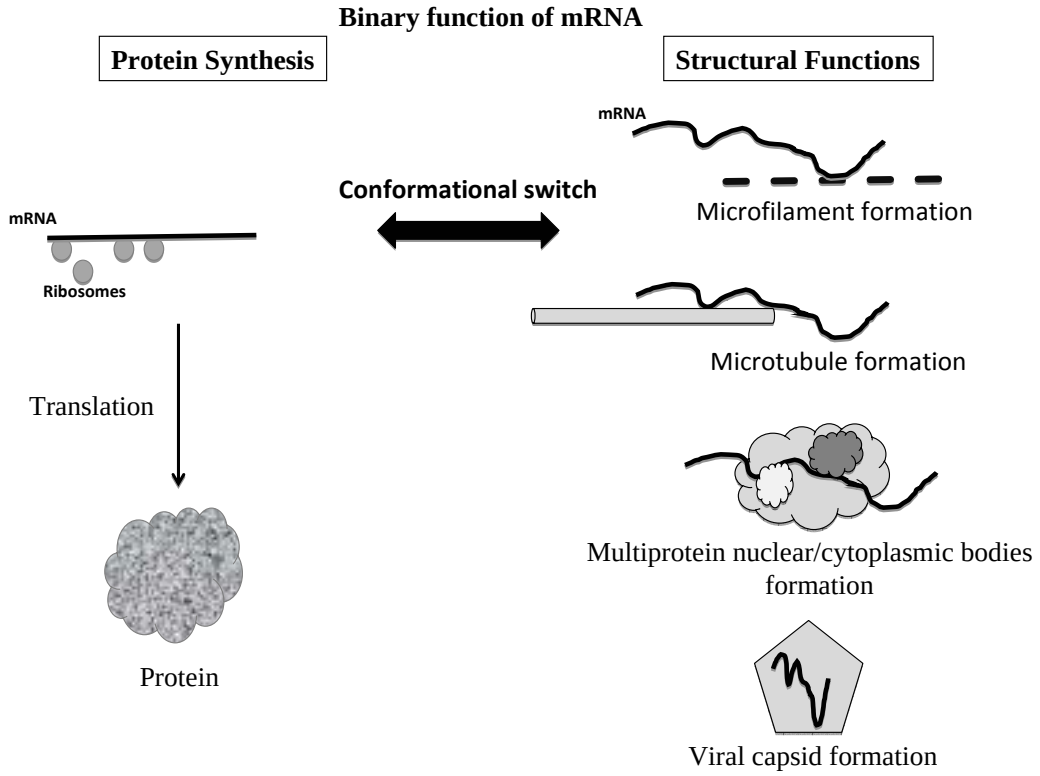

Fig. 2. The diagram shows binary (canonical and non-canonical) function of mRNA. Conformational switch enables the same mRNA to be translated and make protein or to play a non-translational structural role in the formation of various structures such as microfilaments, microtubules, nuclear/cytoplasmic bodies or viral capsids.

viral RNAs (containing various combinations of fragments of two different viruses) form abnormal capsids. This indicates that RNA is able to orchestrate protein assembly according to alternate structural pathways [24-26]. Another example of the non-translational structural role of mRNAs, this time in Eukaryotes, is their ability to form various nuclear bodies such as histone locus bodies (HLBs), Cajal bodies and nuclear speckles (Fig. 2) [27, 28]. Shevtsov and Dundr [29] showed that the histone H2b mRNA is able to orchestrate the formation of HLBs and Cajal bodies, and that the RNA polymerase II mRNA directs the formation of nuclear speckles in HeLa cells by serving as a scaffold for the recruitment of pre-mRNA splicing factors. Similarly, in *Drosophila*, the oskar mRNA serves as a scaffold for the assembly of cytoplasmic complexes (Fig. 2) [17, 30], and the loss-of-function mutants of oskar can be rescued by the expression of the 3' UTR of oskar mRNA [17]. Another example of structural (non-translational) function of mRNAs is their involvement in the assembly and maintenance of the cytoskeleton, namely, microtubules and microfilaments (Fig. 2). Blower *et al.* [16] showed that the Rae1 mRNA, which is located on the microtubules of mitotic apparatus in HeLa cells and *Xenopus* is required for the assembly and maintenance of microtubules in spindle and asters. Studies from our laboratory have shown that VegT and Fatvg mRNAs play a role in the organization of cytokeratin and actin filaments in *Xenopus* oocytes [13-15, 20, 21]. These mRNAs are localized on the cytoskeletal filaments and their removal causes either collapse of cytokeratin network or hyper polymerization of cytokeratin and actin filaments [14, 15, 20, 21]. We also showed that the VegT mRNA binds to the unpolymerized cytokeratin and facilitates/accelerates its polymerization into filaments both *in vivo* and *in vitro* [22] and that the multiple cytokeratin polymerization/ deplymerization signals reside in the coding region of VegT mRNA molecule [18].

The non-translational structural role of various mRNAs exemplified above implies that any direct

or indirect (through genetic mutations) interference with sequence, structure, maturation or expression level of certain mRNAs may have devastating and unforeseen effects on cellular structures, functions and eventually on the cellular/organismal phenotype.

Morpholinos work by a steric-blocking mechanism, either by targeting sequence in the post-spliced mRNA in the region adjacent to the AUG translational start site or by blocking nuclear processing events such as pre-mRNA splicing. Morpholinos directed against any splice junction generate a complete or partial single exon deletion or a complete or partial single intron insertion. Morpholinos designed to block an internal exon can produce a range of outcomes including exon skipping, intron insertion, partial exon deletion and partial intron insertion (in the case of unexpected activation of cryptic splice site) [4]. All these modifications resulting from the interference with mRNA splicing events will produce a range of modified RNA molecules, which are not only untranslatable (which is the predicted and intentional effect of MOs) but may be defective (in different ways) in its potential structural function (which is the unpredicted and unintentional effect of MOs). What follows is that morphant phenotypes produced by MOs designed to interfere with mRNA splicing/ maturation may result from the unintentional interference with two independent components (protein- versus mRNA-based) of combinatorial (binary) phenotype (Fig. 3). In addition, one can imagine that the unexpected phenotype may also represent a summation of unforeseen interactions between these two independent sub phenotypes. For example, the morpholino-based screen of novel genes involved in craniofacial morphogenesis in zebra fish clearly shows that, in many cases, the ATG/UTR MOs produce phenotypes that are different from those produced by SPL MOs, and in some instances they produce phenotype only when they are injected together [7]. These studies showed that out of 40 screened zebra fish genes only 11 genes produced morphant phenotype when ATG MOs were used and only 8 out of these 11 genes produced the same morphant phenotype when ATG and SPL MOs were used. This in our opinion

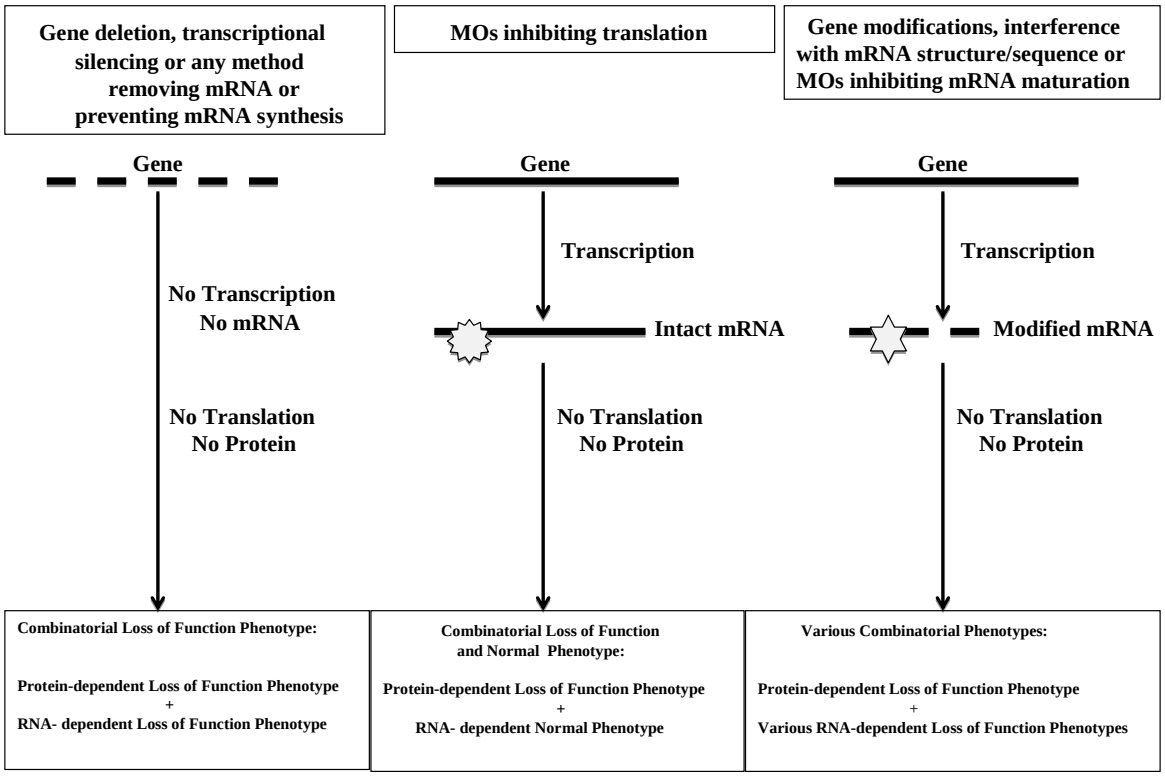

Fig. 3. The diagram summarizes the different phenotypic effects of various silencing methods.

may suggest a possibility that some of these unaccounted for genes (mRNAs) have a non-translational structural role.

Some of the valid questions are: what is the prevalence of mRNAs with non-translational structural role, and what are the features of these mRNAs?

Although there is no information on what features mRNAs should have to perform non-translational structural function, one of the possibilities is the formation of specific and unusual stem-loop structures such as the A'-form RNA helices, which facilitate specific interactions with target proteins [31, 32]. Another possibility is the conformational change (known to occur in regulatory RNAs and riboswitches), which allows the same RNA to adopt two mutually exclusive (ligand-bound and ligand-free) conformations [33, 34]. By analogy, one can hypothesize that the structural mRNA can adopt mutually exclusive conformations, one translation-competent and another specialized for binding and orchestrating the structure of specific target proteins [22]. There is also increasing number of evidences that the coding region of mRNA may contain concealed information pertaining to epigenetic regulatory function, splicing, co-translation folding and RNA secondary structure [35-37], which implies that defining an mRNA as unequivocally 'protein-coding' is probably inaccurate [38]. This, in turn, reinforces the notion that any interference with mRNA structure may have profound and unpredicted effects.

So far all known structural mRNAs belong to the category of 'localized RNAs', either affiliated with cytoskeleton or specific subcellular structures (such as germinal granules or nuclear bodies) in somatic cells, germ cells and embryos [20-22, 39]. One of the possible explanations is that during evolution the structural functions of non-localized mRNAs got replaced by appropriate proteins, while localized mRNAs, confined to the specific cellular structures retained their functional binarity [22]. This poses the question as to how prevalent are localized RNAs? Recent genome-wide screenings have shown that localized mRNAs are far more common than previously thought. For example, the actin filament-rich protrusions of mammalian migrating fibroblasts contain about 50 different localized mRNAs [40]. Genome-wide screening in *Xenopus* and HeLa cells showed that ~10% of the all mRNAs present in the cells were enriched 1.5-fold or more on the microtubules of the mitotic apparatus [39]. In *Drosophila,* there are 33 different localized mRNAs associated with the microtubules of cell division apparatus, and in the fly embryos 71% of the 3,370 expressed genes encode localized mRNAs [41]. Although further studies are needed to prove this, there is a possibility that many of these localized mRNAs may have non-translational structural functions vulnerable to splicing/maturation-interfering morpholinos.

CONCLUSION

In summary we argue that the MO phenotype is reliable only when one can show that it results exclusively from the absence of a given protein and that there is certainty that the potential structural function of mRNA remains intact.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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