miRNAs are short, nonprotein coding RNAs that regulate target gene expression principally by causing translational repression and/or mRNA degradation. miRNAs are involved in most mammalian biological processes and have pivotal roles in controlling the expression of factors involved in basal and stimulus-induced signaling pathways. Considering their central role in the regulation of gene expression, miRNAs represent therapeutic drug targets. Here we describe how miRNAs are involved in the regulation of aspects of innate immunity and inflammation, what happens when this goes awry, such as in the chronic inflammatory lung diseases cystic fibrosis and asthma, and discuss the current state-of-the-art miRNA-targeted therapeutics.

**miRNA biogenesis & function**

miRNAs are a class of noncoding endogenous nucleotide RNAs that have emerged in recent years as regulators of gene expression. These highly conserved 20–25-mer RNAs regulate mRNA at the post-transcriptional level by binding principally to 3´ untranslated regions (3´ UTR). Formerly thought to repress the translation of target mRNA, it has been recently shown that their main function in mammalian systems is to decrease target mRNA levels [1].

Human miRNAs are present in intragenic regions and introns of coding genes. Primary miRNAs (pri-miRNAs) are mainly transcribed from long primary transcripts by RNA polymerase II [2]. They are then cleaved by Drosha-DGCR8 into precursor miRNAs (pre-miRNAs) after going through a series of endonucleocytic steps [3]. Pre-miRNAs are approximately 70 nucleotides in length and typically have a stem-loop hairpin structure; these are transported into the cytoplasm by the RanGTP-dependent dsRNA-binding protein Exportin 5 [4]. Pre-miRNAs are further processed by the cytoplasmic RNAase III enzyme (or Dicer) into double-stranded miRNA duplexes of approximately 22 nucleotides in length consisting of a mature miRNA and a miRNA* strand [5]. The former strand is incorporated into an miRNA-induced silencing complex (miRISC), which is facilitated by Argonaute protein and transported by Importin 8 to its cognate mRNA, leading to either target degradation or repression [6]. Within each miRNA there exists a 2–8 nucleotide ‘seed region’ which is critical for target selection, binding selectively to miRNA recognition elements within the 3´ UTR of target mRNAs. However, recent studies have suggested several exceptions [7]. For example, miRNA-369-3 has been shown to increase rather than decrease the expression of TNF-α by binding within the 3´ UTR of its mRNA [8].

miRNAs themselves are subjected to regulatory processes. These include induction of miRNA expression by transcription factors in response to inflammatory stimuli or cellular stresses, impaired processing due to Dicer inhibition, post-transcriptional modifications and miRNA localization to stress granules and p-bodies [9,10]. Like other gene regulators, miRNAs display spatial and temporal effects which are crucial in the regulation of many genes involved in a variety of biological processes.

**miRNAs, innate immunity & Toll-like receptor signaling**

The innate immune system is a complex, highly organized system that provides the first-line
defense upon exposure to invading pathogens or allergens. Among its major functions are the recruitment of immune cells to the site of damage, induction of antimicrobial defenses and priming of the adaptive immune system. Innate immunity is predominantly mediated via myeloid cells [11], however, within specific organs other cell types can also play a large part. These cells commonly express pattern recognition receptors which can recognize and discriminate structures present in microbial and/or damaged-self molecules. For example, epithelial cells in the lung express Toll-like receptors (TLRs) [12] which can become activated in response to microbial products such as lipopolysaccharide (LPS) or lipopeptides, or host-derived factors such as HMGB1, leading to activation of transcription factors such as NF-κB and the interferon-regulatory factors [13]. miRNA modulation of innate immune mechanisms is an important fundamental process. At the most basic level miRNAs have an important role in hematopoiesis and in the establishment and maintenance of the cellular fate of immune cells [14–16]. Additionally, multiple miRNAs are involved in regulating components of innate immune signaling pathways and, furthermore, their expression can be induced in response to various proinflammatory stimuli.

MiR-155 is an excellent example of a miRNA that participates in multiple aspects of innate immunity. It is an ubiquitously expressed miRNA [17]. Known targets of miR-155 include the transcription factor PU.1, a key mediator of monocyte and macrophage differentiation [18]; SOCS1 [19]; the inositol phosphatase SHIP1 [20]; FOXP3 [21] and MyD88 [22,23]. Other targets include FADD, IKK-ε and the TNF receptor superfamily-interacting serine threonine kinase Ripk1 [24]. The expression of miR-155 is strongly induced by inflammatory cytokines such as IFN-β and γ and also by TLR ligands through MyD88- or TRIF-dependent pathways [22,24,25].

miR-146a is another miRNA identified to have an important role in the innate immune system. miR-146a is mainly regulated by bacterial products and has been shown to be suppressed by LPS-treatment in a murine macrophage cell line. By directly targeting the 3´ UTR of IRAK1 and TRAF6, this miRNA may play a role in dampening the LPS pathway in the absence of microbial infection, while its downregulation may be needed for the LPS-induced inflammatory response [26]. In human alveolar cells increased expression of miR-146a and b is induced by IL-1β, an event corresponding to a decrease in the expression of IL-8 and RANTES [27].

In the context of TLR and IL-1R signaling the roles of a selection of miRNAs have been elucidated (Figure 1). For example, members of the let-7 family and miR-223 are key miRNAs that directly target TLR4 [28,29]. Transfection of antisense miRNA to let-7e in macrophages increases LPS-induced cytokine responses [30]. Meanwhile, downregulation of let-7i increases TLR4 in human cholangiocytes following LPS treatment [28]. The aforementioned miR-155 targets MyD88 while miR-146a negatively regulates the expression of IRAK1 and TRAF6 [31]. IKK-α and β were recently shown to be targeted by miR-223 and -199, respectively [32,33], and miR-9 could also directly target the NF-κB1 gene [34].

miRNAs that target cytokines induced by TLRs have also been identified. For example, let-7 and miR-106 target IL-6 and IL-10 mRNA, respectively [35,36]. Interestingly, miRNA-induced stabilization of TNF and IL-10 mRNA has also been demonstrated for miR-369-3 and miR-446l [3,37].

**Figure 1. miRNA regulation of TLR2, TLR4 and IL-1R signaling.** miRs are expressed in the nucleus and are exported into the cytoplasm (black arrow, lower right hand corner). Multiple TLRs such as TLR2 and 4 and IL-1 receptor signal via the MyD88/IRAK/TRAF6 pathway to propagate NF-κB-dependent gene transcription, which leads to proinflammatory products. Multiple miRNAs directly target components of these pathways including TLR2, TLR4, IL-1R, MyD88, MAL, IRAK1, TRAF6, TAB 2, IKK-α and IKK-β, and p50 protein. The TOM1/Tollip complex and SOCS1 are examples of negative regulators of this pathway, which have also been identified as targets of miRNA.

TLR: Toll-like receptor.

Data from [22,25,26,31,32,41,73,74].
Cystic fibrosis & asthma: the role of miRNAs in the innate immune response of inflammatory lung diseases

By translating these generic observations regarding the roles of miRNAs in innate immunity to clinical scenarios, the concept of miRNAs as therapeutic drug targets emerges clearly. Cystic fibrosis (CF) is a recessive genetic disease caused by mutations in the CFTR gene. CF exhibits a multitude of clinical manifestations, however, the lung disease is responsible for the major morbidity and mortality in CF sufferers; this is characterized by defective epithelial chloride ion conductance, a decreased airway–surface liquid volume, increased expression and activity of host proteases, impaired antimicrobial defenses, mucus hypersecretion and a propensity to become colonized with microbes that can form biofilms within the lung [38–40]. Colonization with Pseudomonas aeruginosa is associated with intrapulmonary LPS and elevated expression of IL-1β impacting on TLR4 and IL-1R1, respectively. Proteases such as neutrophil elastase induce expression of proinflammatory cytokines such as IL-8 via TLRs, MyD88, IRAK and TRAF6. These signaling intermediates therefore represent potential therapeutic targets for miRNA-based CF therapy.

Indeed evidence that miRNAs represent modulators of the innate immune system in CF is emerging [41]. TOM1, a negative regulator of IL-1β- and TNF-α-induced signaling pathways, is a target for miR-126. This miRNA is highly expressed in the non-CF lung, but suppressed in vivo in the CF lung [42]. Following stimulation with LPS or IL-1β, overexpression of TOM1 was found to downregulate NF-kB activity while TOM1 knockdown resulted in a significant increase in NF-κB-regulated IL-8 production. Overexpression of miR-155 in CF has also been implicated in promoting inflammation by directly targeting and reducing SHP1 expression and promoting PI3K/Akt activation. This caused downstream activation of MAPKs and concomitant stabilization of IL-8 mRNA [43].

Asthma is another common chronic airway disease affecting approximately 300 million people worldwide. Its pathogenesis is attributed to both genetic and environmental factors leading to reversible bronchoconstriction as a result from chronic airway inflammation, mucus hypersecretion and airway hyperresponsiveness to inhaled stimuli. An abnormal Th2 response is usually associated with this condition and Th2-related cytokines such as IL-4, -5, -6 and -13 have all be implicated in asthma [44]. Studies on animal and human models have implicated miRNAs in asthma pathogenesis. miR-21, for example, is highly expressed in IL-13 transgenic mice [45] and has also been implicated as a major regulator of Th1 versus Th2 responses and IFN-γ signaling [46]. Meanwhile, inhibition of miR-126 by administration of an antagonomir has also been shown to suppress eosinophil recruitment into the airways but had no effect on chronic inflammation in the airway wall, or on changes of remodeling in the mouse model of chronic asthma [47]. Another study showed that let-7 inhibits IL-13 expression, identifying it as a major potential regulator of Th2 inflammation [48]. New data on miRNAs including, once again, miR-146a modulating human bronchial epithelial cell survival in response to the cytokine-induced apoptosis [49], strongly support the concept that miRNAs represent valid drug targets for the treatment of asthma (Figure 2).

Figure 2. The current understanding of miRNAs in the pathogenesis of asthma.
Changes in the expression of several miRNAs are associated with the development of asthma. Class 1 HLA-G, an asthma susceptibility gene is a direct target of miR-148a, b and -152 [75]. miR-146a have multiple roles including the regulation of inflammation in both human alveolar and bronchial smooth muscles cells via Bcl-XL and IL-1β-induced cytokine production, respectively [47,27]. miR-21 targets IL-12p35 and proinflammatory tumor suppressor PDCD4 while miR-126 regulates PU.1, regulating cytokine production such as IL-3, -5 and -10 and therefore effectively suppressing the Th2-driven airway inflammation [44,76,77]. Meanwhile, let-7 miRNAs inhibit IL-13 expression and thereby Th2 inflammation [44]. In ovalbumin-induced asthma mice model, upregulation of MMP-12 resulted in decreased miR-672 and -143, while miR-29 regulation of NF-κB-YY1 is implicated in tissue fibrosis and remodeling [78,79]. In another ovalbumin-induced asthma model, the expression of Rhoa is associated with downregulation of miR-133a. miR-25 and -26a directly target KLF-4 and GSK3β, respectively [80–82]. These targets are associated with airway smooth muscle hypertrophy and bronchial smooth muscle cells. Blue arrows reflect 3´ UTR mRNA targeting, while green arrows reflect indirect or unproven 3´ UTR targeting.
Pharmaceutical strategies to modulate miRNAs
miRNA therapeutics are the most recent of a range of RNA therapies that have emerged over the last 10–15 years including siRNA and shRNA based on manipulation of the RNAi machinery of diseased cells (Table 1).

miRNA inhibitors
miRNAs that are overexpressed or have a gain of function in diseased tissue may be therapeutically targeted in a number of ways (Figure S3A). Each of the approaches aims to target endogenous miRNAs of interest through sequence complementarity and subsequently block its processing by RISC or alternatively lead to its degradation. Anti-miRNA oligonucleotides (anti-miRs) have been developed to anneal to miRNAs and subsequently inhibit their function. Many modifications to anti-miRs exist which confer increased serum stability and higher affinity and specificity towards small RNAs [50,51]. More advanced modifications include locked nucleic acids [52] and cholesterol-conjugated anti-miRs termed ‘antago-miRs’ [53].

miRNA mimics
Conversely, miRNAs that are downregulated in disease may be replaced transiently by using miRNA mimics, or more stably using a transgene approach to deliver DNA encoding primary, pre- or mature miRNA generally via plasmid DNA (Figure 3B). Although most of the work with miRNA mimetics to date has been done in the field of cancer treatment, efforts to use these strategies in other settings are underway.

The translation of miRNA modulation into therapeutics in vivo will be dependent on delivery to the desired target cells. As miRNA

Table 1. Therapeutic strategies for miRNA modulation†.

| Vector         | Delivery route          | Species        | Disease/area      | miRNA | Inhibitor/mimic | Ref. |
|----------------|-------------------------|----------------|-------------------|-------|-----------------|------|
| **Viral**      |                         |                |                   |       |                 |      |
| Lentivirus     | Intranasally            | Mouse          | NSCLC             | let-7 | Inhibitor       | [83] |
| Lentivirus     | Intravenous injection   | Mouse          | Gastric cancer    | let-7f| Mimic           | [84] |
| Lentivirus     | Local injection         | Mouse          | Cancer            | mir-101| Mimic           | [85] |
| Adenovirus     | Intranasally            | Mouse          | NSCLC             | let-7 | Inhibitor       | [86] |
| Adenovirus     | Cellular innoculation   | Mouse          | Breast cancer     | mir-145| Mimic           | [87] |
| Recombinant AAV| Intravenous injection   | Mouse          | Hepatocellular carcinoma | shRNA | Mimic | [88] |
| **Nonviral**   |                         |                |                   |       |                 |      |
| Naked          | Intravenous and subcutaneous injection | Rat      | Cardiac disease   | miR-208 | Inhibitor | [89] |
| Naked          | Intravenous injection   | Mouse          | Breast/lung cancer| mir-10b| Inhibitor       | [90] |
| Naked          | Intravenous injection   | Chimpanzee     | HCV               | mir-122| Inhibitor       | [63] |
| Naked          | Intraperitoneal injection | Mouse      | Hypercholesterolemia | mir-122| Inhibitor       | [52] |
| Naked          | Intravenous injection   | African green monkey | Hypercholesterolemia | mir-122| Inhibitor       | [52] |
| Plasmid        | Intratumoral injection | Mouse          | Colorectal cancer | USP22 miRNA | Mimic | [91] |
| Plasmid        | Intravenous injection   | Mouse          | Familial hypercholesterolemia | Synthetic (no endogenous miR) | Mimic | [92] |
| Plasmid DNA-coated gold particles | Biolistic epidermal transfection | Mouse | Infection and inflammation | mir-155 | Mimic | [93] |
| Atelocollagen-mediated | Intravenous injection | Mouse | Bone-metastatic prostate cancer | miR-16 | Mimic | [94] |
| PEI            | Intravenous injection   | Mouse          | Colon carcinoma   | mir-145| Mimic           | [62] |
| iNOP-7         | Intravenous injection   | Mouse          | Hypercholesterolemia | mir-122| Inhibitor       | [95] |
| CPP-targeted PEI | Intravenous injection | Mouse | Neuronal development | mir-124a | Mimic | [96] |

†Methods for therapeutic miRNA modulation in vivo can be broadly categorized into viral and nonviral strategies. The most popular viral delivery methods include lentiviral, adenoviral and adeno-associated virus systems. Various nonviral means of miRNA modulation exist including delivery of ‘naked’ nucleic acids or by using polymeric, liposomal or peptide-targeting systems.

AAV: Adeno-associated virus; CPP: Cell-penetrating peptide; NSCLC: Non-small-cell lung cancer; PEI: Polyethylenimine.
modulation is mediated by large, anionic DNA- and RNA-based constructs, many anatomical and cellular barriers to their delivery exist in vivo including degradation by serum nucleases, clearance by glomerular filtration, limited extravasation, poor cell membrane permeability and limited endolysosomal escape. While localized delivery to the target tissue overcomes some of these barriers, for example, aerosolization for delivery of miRNA therapeutics targeting respiratory conditions, the barriers to delivery are still not trivial [55]. At a cellular level, small RNAs must be delivered to the cytoplasm where the RISC is active; whereas plasmid DNA-based strategies require access to the nucleus for efficacy. Many delivery systems are currently under examination for use in vivo, and categorized into viral and nonviral approaches.

Viral delivery
Stable expression of anti-miRs or mature miRNA may be achieved by using a viral vector such as adenovirus, lentivirus and adeno-associated virus. For the therapeutic targeting of disease associated with overexpressed miRNAs, ‘miRNA sponges’ are an exciting option. With the ability to stably express these are transcripts containing numerous tandem-binding sites targeting an miRNA of interest, allowing for a greater number of target miRNAs to be inhibited than by using synthetic anti-miRs [56]. Conversely, miRNAs that are downregulated in disease (e.g., miR-126 in CF bronchial epithelium) may be replaced transiently by using miRNA mimics, or more stably using the transgene approach to deliver DNA encoding primary, pre- or mature miRNA. Driven by RNA polymerase (Pol) III promoters, miRNAs can be expressed as pre-miRNAs or as artificial shRNA, the latter with the ability to circumvent Dicer processing. Although high stable expression of these transcripts can be achieved using Pol III, a major drawback is risk of severe toxicity due to the saturation of the exportin-5 pathway used by endogenous miRNA [57]. This saturation can have fatal consequences [58]. Perhaps a better method, with the possibility of induced ectopic or tissue-specific expression of the miRNA of interest is the use of an entire primary miRNA driven by the Pol II promoter. Successfully used to overexpress various miRNAs, a pri-miR-Pol II transgene system has also appeared to be of use in the expression of multiple miRNAs from a single transcript [59–61].

Nonviral delivery
Viral delivery of RNA and DNA in vivo has been associated with induction of toxic immune responses, random integration into the host genome and potential saturation of RISC machinery [62]. Therefore, nonviral delivery approaches have been explored. Possibly the most advanced miRNA therapeutic is currently a ‘naked’ locked nucleic acid-anti-miR against liver-expressed miR-122 [63]. Constituting over 70% of the total miRNAs in the liver, this miRNA appears to be required for the replication of HCV [64]. Santaris Pharma has recently entered the locked nucleic acid miravirsen (SPC-3649) into Phase II(a) clinical trials.

Nonviral carriers can offer improved stability and targeting over naked nucleic acids, especially for systemic delivery of miRNA therapeutics. The majority of nonviral RNA and DNA carriers are lipid or polymer based. Cationic lipids or liposomes

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**Figure 3. Approaches to therapeutic miRNA inhibition or restoration in disease.** Many therapeutic options for inhibition of miRNAs upregulated in disease are illustrated in (A). Anti-miRNA antisense oligonucleotides (1) can be synthesized to be complimentary to a mature miRNA of interest and inhibit its function. Modifications of these nucleic acids have been developed such as 2’-O-Methyl, 2’-O-Methoxyethyl and Locked nucleic acids (LNAs), which have additional characteristics such as increased stability in serum [50–51]. miRNA sponges (2) are transcripts containing several tandem-binding sites towards a miRNA of interest, and can be stably expressed through delivery to the nucleus [51]. A different class of oligonucleotides may be delivered to the cytoplasm to bind to miRNA responsive elements on target genes [97]. As these oligonucleotides mask miRNA binding to gene target sites [51], these are aptly termed ‘miRNA masks’ (3). Therapeutic options for restoration of miRNAs that are downregulated in disease are also under development (B). Nuclear delivery (1) of DNA encoding pri-miRNA, pre-miRNA and shRNA may produce sustained restoration of miRNA expression. Transient restoration may be attained through the use of pre-miRNA and mature miRNA mimics with cytoplasmic delivery (2) [54,57].
Using patented encapsulation technologies that rarely, if ever, target only a single mRNA transcript. Thus, inhibit pros, there are downsides to take into consideration also. miRNAs knockdown or replace a particular natural miRNA that is fewer than several splice variants; for miRNA therapies the goal is to either nucleotides in length and part of populations that encompass therapies may be simpler than siRNA therapeutics. For siRNA the moderate effects can be developed there would now appear to be a near equal interchangeability, where siRNA versus miRNA-based therapies targeted strategies. Although these two approaches are not always drug development from siRNA-based approaches to miRNA-research practice has come a measurable shift in the focus of early biology and the race to develop effective miRNA-based therapies may have made significant advances in our basic understanding of the complete miRNA-ome of individual mRNAs and using a translational research approach to determine whether these miRNA signatures are altered in vivo.

With respect to the treatment of respiratory disease, RNA-based therapies are particularly attractive as organ-specific targeting of RNA therapeutics can be achieved via inhalation. Respiratory drug delivery decreases systemic exposure of the patient to the therapy, thereby reducing off-target effects. Indeed, inhaled siRNA has been one of the first RNA-based therapies to reach the clinic with Alnylam’s anti-RSV siRNA treatment now reaching Phase III trials. It seems inevitable that miRNA-targeted strategies to treat chronic inflammatory lung diseases will follow a similar route in the coming years.

Five-year view

Within the next 5 years we are likely to see continued investment of time and effort by researchers and pharmaceutical companies in the development of miRNA-targeted therapies to treat chronic inflammatory disorders. Although organ-specific delivery systems for modulation of miRNAs in vivo are still in development, major advances in this area are inevitable. In our group we have been examining the feasibility of delivering pre-miRs into CF bronchial epithelial cells and our efforts have yielded very promising results to date [McKiernan PJ et al. Delivery of pre-miR-126 to cystic fibrosis bronchial epithelial cells using nanoparticles (2012), Manuscript in Preparation]. Using patented encapsulation technologies that can penetrate mucus, facilitate rapid and efficient cell uptake and minimize toxicity we are developing bioreponsive, inhalable hydrogels that will release miR-targeted nucleic acids directly to sites of inflammation in the lung. These customized drugs will selectively inhibit only abnormal inflammatory processes and mucus hypersecretion leaving intact the normal processes required for normal lung physiology.

In order to develop these and similar drugs effectively, clearly we also need a better understanding of the in vivo pharmacokinetics of viral and nonviral miRNA mimics and inhibitors. For this, preclinical animal models will prove useful. Finally, while we have made significant advances in our basic understanding of the role of miRNAs in cellular physiology, cancer and more recently inflammatory diseases, there will be further intensive research in these areas over the coming years. A particularly exciting aspect of this will be the prospect of developing personalized medicines to treat idiosyncratic disease manifestations.

Expert commentary

The enthusiasm with which researchers have embraced miRNA biology and the race to develop effective miRNA-based therapies over recent years has been astounding. With this change in research practice has come a measurable shift in the focus of early drug development from siRNA-based approaches to miRNA-targeted strategies. Although these two approaches are not always interchangeable, where siRNA versus miRNA-based therapies can be developed there would now appear to be a near equal investment of time and effort in both.

With each comes challenges: however the idea of using miRNAs rather than siRNAs to fight disease may have broader and more moderate effects [72]. In addition, some aspects of miRNA-based therapies may be simpler than siRNA therapeutics. For siRNA the targets are mRNA transcripts that can be up to several thousand nucleotides in length and part of populations that encompass several splice variants; for miRNA therapies the goal is to either knockdown or replace a particular natural miRNA that is fewer than two dozen nucleotides long. However, notwithstanding these pros, there are downsides to take into consideration also. miRNAs rarely, if ever, target only a single mRNA transcript. Thus, inhibiting or enhancing the expression of a miRNA can have off-target effects. One potential solution to this problem could be to target combinations of miRNAs that represent a ‘miRNA signature’ for a given mRNA. Work in our group focuses on identifying the complete miRNA-ome of individual mRNAs and using a translational research approach to determine whether these miRNA signatures are altered in vivo.

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Therapeutic modulation of miRNA for the treatment of proinflammatory lung diseases

Key issues

- miRNAs regulate 30–60% of human mRNA transcripts and play a key role in almost all biological processes.
- In the context of innate immunity miRNAs have roles in regulating immune cell lineage development and maintenance, and in the control of individual genes involved in innate immune signaling pathways. miRNAs can be rapidly induced in response to proinflammatory stimuli to enhance the immune response.
- miRNA expression can be altered in a tissue-specific manner in individuals suffering from chronic inflammatory diseases such as cystic fibrosis or asthma. Key miRNAs that are dysregulated in this way represent new therapeutic targets for drug development.
- Strategies to overexpress or inhibit miRNAs exist but are still in their infancy. Currently, the most significant barriers to miRNA-based medicines are the development of effective pharmaceutical strategies for targeted delivery to specific sites and with acceptably low toxicity.
- One of the most advanced miRNA-based therapies comes from Santaris Pharma and has recently reached Phase II(a) clinical trials. This therapy is a locked nucleic acid (SPC-3649) targeting miR-122 in the liver for the treatment of HCV.

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