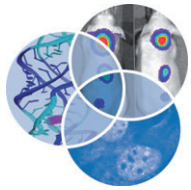
Focus Article



Can circulating miRNAs live up to the promise of being minimal invasive biomarkers in clinical settings?

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MicroRNAs have been discussed as non- or minimal invasive biomarkers with a remarkable extracellular stability. Despite a multitude of studies in basic research, there are only few independent validation studies on blood-born miRNAs as disease markers. Toward clinical applications numerous obstacles still need to be overcome. They are of technical origin but also fundamentally associated with the source and the nature of miRNAs. Here, we emphasize on potential confounding factors, the nature and the source of miRNAs. We recently showed that age and gender could influence the pattern of circulating miRNAs. On the cellular level, the miRNA pattern differs between plasma and serum preparations. On the molecular level, one has to differentiate between extracellular miRNAs that are encapsulated in microvesicles or bound to proteins or high-density lipoproteins. Using whole blood as source for miRNAs helps to minimize miRNA expression changes due to environmental influences and allows attributing miRNA changes to their cells of origin like B-cells and T-cells. Moreover, unambiguous annotation and differentiation from other noncoding RNAs can be challenging. Even not all miRNAs deposited in miRBase do necessarily represent true miRNAs, just a fraction of miRNAs in the reference database have been experimentally validated by Northern blotting. Functional evidence for a true miRNA should be obtained by cloning the precursor miRNA and by subsequent detection of the processed mature form in host cells. Surprisingly, attempts to finally confirm a true miRNA are frequently postponed until evidence has been established for a likely value as biomarker.

How to cite this article:
WIREs RNA 2016, 7:148–156. doi: 10.1002/wrna.1320

miRNAs AS FUNDAMENTAL REGULATORS OF PROTEIN EXPRESSION

MicroRNAs (miRNAs) are endogenous small noncoding (nc)RNAs that are crucial for gene regulation in a broad range of eukaryotic species from plants to animals.[1] An analysis of the transcriptional landscape of the mammalian genome indicated that miRNAs represent one of the most abundant classes of gene-regulatory molecules in mammals.[2] In humans more than two thousand five hundred miRNAs have been identified and stored in the reference database miRBase. This database has been established as online repository for all miRNA sequences and annotations.[3] MiRNAs exert their function through interacting with their mRNA targets. The base paring of the miRNA, which is canonically at the 3′- untranslated region (3′-UTR) of their targets, triggers mRNA decay or protein translational inhibition resulting in

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Conflict of interest: The authors have declared no conflicts of interest for this article.

widespread changes of protein synthesis.[4,5] Early statistical analysis suggested that more than 60% of human mRNAs are subject to post-transcriptional regulation mediated by miRNAs.[6] As addressed below such predictions do, however, require experimental confirmation. Independent of the number of target gene, it is largely agreed on, that the interaction between mRNAs and miRNAs is regulated within a complex network with single genes targeted by several miRNAs and with single miRNAs simultaneously targeting extended number of genes.[7,8]

miRNA BIOMARKERS BETWEEN BASIC RESEARCH, INDEPENDENT VALIDATION, AND CLINICAL TRIALS

As early as a decade ago, there was first evidence that miRNAs are mediators of regulation of cell growth and are crucial for regulatory pathways like the apoptosis pathways.[9] The expression of miRNAs has been found to be specific for tissues, developmental stages, and various diseases.[10–12] As of now (accessed August 2015), there are around 44 entries in PubMed related to miRNAs, the annual number of publications has increased from around 200 a decade ago to 9000 in 2014. Of the cumulative 44,000 manuscripts listed in PubMed, the majority has a direct association to human diseases. A yet increasing number of publications report miRNAs that are supposed to be useful in clinical settings. The majority of these reports concern the use of miRNAs as biomarkers for cancer.[13,14] The raising number of studies that focus on miRNA as biomarkers reflect a commonly held believe that miRNAs are ideally suited for a wide range of applications including not only diagnosis, but also prognosis and treatment monitoring for various diseases.[15,16] In contrast to the numerous manuscripts that describe miRNAs associated with diseases, is the paucity of clinical trials that have been launched to investigate the value of miRNAs as biomarkers. A total of 275 clinical trials with the keyword 'miRNA' or 'microRNA' are listed in the U.S. National Institutes of Health database ClinicalTrials.gov (accessed August 2015). Of those, 157 clinical trials analyze miRNAs in blood, serum, or plasma. Over all studies, the median enrollment as stored in clinicaltrials.gov is 62 individuals. The discrepancy between basic research studies on miRNAs with a potential clinical impact and the number of clinical trials that investigate miRNAs, provokes the question to what extend miRNAs live up to the promise of being applicable as markers in clinical settings. The slow progress toward a clinical implementation is, among many other factors, largely attributed to the tremendous heterogeneity among the studies in miRNA biomarker research. This diversity concerns the sources of miRNAs, but also other aspects including miRNAs processing, data evaluation and last but not least the question for which the miRNA biomarkers should yield an answer. In this review we try to dissect through factors that we feel are crucial for the translation of basic miRNA research into clinical applications of miRNA biomarkers. This review will not provide summaries of miRNAs that have been associated with specific diseases. There are excellent reviews that survey the role of ncRNAs including miRNAs in diseases and the potential of these RNAs in body fluids.[12,17]

THE IMPACT OF BLOOD DONORS ON miRNA ABUNDANCE

The origin of miRNAs should be clearly defined to judge their value as biomarkers. The first questions concerning the origin of miRNAs are about the donor and his influence on the miRNAs as biomarkers. Others and we recently reported an influence of age on the miRNA pattern in human blood.[18,19] Our array-based analysis detected miRNAs that were significantly correlated to the age after adjustment for multiple testing. Regarding the gender, we found a significant correlation for some miRNAs prior but not after adjustment for multiple testing. To determine the influence of the confounding factors age and gender on miRNA pattern, we implemented a web-based tool, which is freely available for noncommercial use at http://www.ccb.uni-saarland.de/mirnacon/. Knowing the influence of confounding factors like age and gender on specific miRNAs helps to better gauge the diagnostic value of these miRNAs in a given context for examples in cases with patients that belong to a certain age group like childhood cancer patients. The influence of the daytime on blood-born miRNAs seems to be limited as shown by others and us.[20] Other factors that may influence the miRNA patterns include, for example, the food uptake. Although blood drawing for clinical purposes is usually done in fasting conditions, it may nevertheless be important to clarify the influence of the fasting status on the miRNA abundance in blood. The influence of a diet on the miRNome of individuals is currently controversially discussed. There is circumstantial evidence that exogenous miRNAs can be transferred into blood and subsequently into tissues to regulate endogenous transcripts. However, the

uptake of exogenous miRNAs and regulatory effects in cells, remains to be confirmed by independent verification.[21]

THE IMPACT OF PLASMA OR SERUM ISOLATION ON miRNA ABUNDANCE

Besides the influence of the donor on the miRNA measurements, the choice of the analyzed tissue impacts the usefulness of miRNA biomarkers. Many studies report miRNAs as non- or minimally invasive markers identified not only in solid tissues but also in various body fluids including blood, urine, or saliva.[17,22] Blood borne miRNAs are frequently referred to as circulating miRNAs. The remarkable extracellular stability of miRNAs makes them an intriguing class of biomarkers and has prompted a yet increasing number of studies that evaluate the use of circulating miRNAs. The stability has been confirmed under various conditions including freeze–thaw cycles, extreme pH values, and storage at room temperature.[23]

While the definition of circulating miRNAs may at first sight appear sufficient to define a miRNA source, it is generally too vague to allow for a comparison of different blood-born miRNA biomarker studies. Circulating miRNAs can stem either from plasma or from serum, both of which are readily available and therefore frequently used as source for miRNAs in clinical settings. While plasma is collected in presence of anticoagulants including EDTA (K2 ethylene ediamine tetra acetic acid), Na citrate, and heparin, serum is gained by clot separation after coagulation. As result of coagulation, miRNA expression is activated in and released from platelets. In addition, miRNA is also released from red and white blood cells contributing to an overall increased miRNA concentrations in sera.[16] Other studies show higher miRNA concentrations in plasma than in sera[23] indicating that most differences between the miRNA abundance in plasma or serum remain to be elucidated. The combined use of both sources within one miRNA expression study should be discouraged unless a differential influence of plasma or serum on the expression of a particular miRNA has been excluded.

In many clinical institutions extended collection of serum samples are available. Although it might be tempting for retro-perspective studies to make use of serum samples that have been collected over longer periods of time, caution is mandatory to avoid data that may be flawed by various factors. First, in many instances the specifics of the blood drawing are not documented. Even the size of the needles can have an impact on miRNA levels. Small gauge needles can cause activation of platelets and subsequently release of miRNAs. Immediately after blood drawing, the storage time and temperature of the sample should be standardized to avoid the impact of miRNAs that are released from blood cells. Likewise lysis of erythrocytes can influence the overall miRNA abundance in human plasma or serum. A lack of information on these factors renders a collection of serum or plasma samples of minor value for the search of miRNA biomarkers. The conditions of sampling do not only directly affect the miRNA abundance in serum, but have also meaning for the measurement of the miRNA expression pattern. Both heparin and citrate inhibit enzymes used in qRT-PCR[24,25] and in next-generation sequencing (NGS). Preferentially, miRNA expression analysis should be done on serum that was prepared from tube with EDTA as anticoagulant to minimize downstream effects on the measurement of the miRNAs.

DIFFERENTIATION BETWEEN EXTRACELLULAR miRNA THAT ARE BOUND TO MEMBRANE VESICLES, SINGLE PROTEINS, OR TO HIGH-DENSITY LIPOPROTEIN

In serum and plasma miRNAs are found in different components.[26] Extracellular miRNA are frequently encapsulated in membrane vesicles (microvesicles) but are also found in apoptotic bodies.[27,28] The majority of circulating miRNAs are, however, bound to different proteins including nucleoplasmin and Argonaute2, and high-density lipoprotein (HDL).[29,30] Each of these components can be specifically targeted to identify miRNA biomarkers.[31] While there are known antibodies for immune precipitation of Argonaute2 and HDL allowing for a rather straight forward isolation of bound miRNAs, the isolation of miRNAs from extracellular vesicles appears far more challenging. Potential miRNA biomarkers have been reported to be isolated from exosomes leaving the question of how exosomes are defined.[31] Generally they are considered as vesicles in the size range between 50 and 100 nanometers, but the criteria used in different studies are fare from being consistent. Besides the size, surface markers identified by antibodies are used to define and to distinguish exosomes from other microvesicles. The specificity of the antibodies that are directed against exosomal surface proteins, awaits further

confirmation. Without having a clear nomenclature, and without having protocols that are sufficient to isolate pure or at least highly enriched exosomes, it is difficult to compare studies that report miRNA biomarkers isolated from exosomes.[32,33]

Independent of the localization, that is, protein or vesicle bound, miRNAs in serum or plasma can hardly be traced back to their cell of origin. A larger number of studies report elevated levels of miRNAs both in serum or plasma of patients and in the cells of the diseased organ. Especially for cancer these correlations may readily suggest a causative link that explains the elevated miRNA serum/plasma levels by leakage or active transport of the according miRNA out of the cancer cell. Even if some steps of such a mechanism are verified by *in vitro* experiments, for example, a miRNA that is overexpressed in cancer cells and subsequently encapsulated in tumor cell derived microvesicles, the final and conclusive *in vivo* evidence remains a demanding task.[34]

THE IMPACT OF WHOLE BLOOD ISOLATION ON miRNA ABUNDANCE

Besides serum or plasma, whole blood can be used as source for miRNAs. Mainly two systems are in use including PAXgene™ blood RNA tubes (Franklin Lakes, New Jersey, USA) and Tempus blood RNA tubes (Invitrogen).[35–37] Both systems contain stabilizing reagents that directly lyse the blood cells, inhibit *ex vivo* transcription, and inactivate cellular RNases. Using whole blood collected in these tubes helps to avoid changes in RNA expression that occur immediately after phlebotomy due to environmental influences and omits anticoagulant, which are needed for the abovementioned serum-based miRNA studies and which entail *ex vivo* post-phlebotomic expression changes. However, the use of these special RNA-stabilizing blood collection tubes is not always possible for example in retrospective studies or in studies, which require intact cells. While it is known that the transcriptome is *ex vivo* altered due to exposure to EDTA, in our hands the blood miRNome is also affected by even a very short contact with EDTA.

For PAXGene blood tubes a high stability of miRNA patterns over time and dependent on freeze/thaw cycles has been demonstrated, making them generally suited for clinical biomarker studies.[38] MiRNA expression patterns that stem from whole blood, which was stabilized in PAXgene™ or Tempus blood RNA tubes are mostly determined by the blood cells. As result, these miRNA patterns largely mirror changes of the immune system in response to a disease development. Others and we have previously reported disease-specific miRNA expression profiles in whole peripheral blood for lung cancer,[39] multiple sclerosis,[40] melanoma,[41] COPD,[42] myocardial infarction, and heart failure,[43,44] Alzheimer Disease,[45] and other diseases.[46] Since these disease-specific miRNAs mainly stem from the cellular components of the blood, there is the possibility to dissect the cellular origin of disease-specific miRNA expression patterns.[47] We recently analyzed the miRNome of eosinophilic and neutrophilic granulocytes, monocytes, B-cells, T-cells, and natural killer cells, each in lung cancer patients and healthy individuals.[48] We found specific miRNA expression patterns for each immune cell type. The miRNA expression was also depending on the line of defense and the cell functions. This clearly shows that standardized procedures for sample collection are mandatory to avoid bias and misinterpretation of results.

THE CHALLENGE TO DIFFERENTIATE SMALL NONCODING RNAs BY SIZE, SEQUENCE, AND OTHER FEATURES

One of the ambiguities in studies on miRNA biomarkers frequently originates from unclear definitions of miRNAs. In general, miRNAs are defined as small ncRNA molecules that are approximately 22 nucleotides in length. MiRNAs belong to the group of ncRNAs, which also include small interfering RNAs (siRNA), small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), large intergenic ncRNAs (lincRNAs), and long ncRNAs (lncRNAs) among others.[12,49] lncRNAs that are longer than 200 nucleotides can readily be separated from other types of ncRNAs, which are smaller and mostly more similar in size. PiRNAs, which bind the PIWI subfamily of Argonaute proteins, are of 24–30 nt in length and small interfering RNAs (siRNA), which are involved in the RNA interference (RNAi) pathway, are 20–25 nt in length. SnoRNAs, which are components of small nucleolar ribonucleoproteins, show a size range from 60 to 300 base pairs.

Since it is not always possible to simply define ncRNAs by size, the different ways the ncRNAs are processed offer further means to define miRNAs and other classes of ncRNAs. NcRNAs can either be derived from single-stranded precursors with a hairpin structure or from dsRNA precursors, referred to as hairpin RNAs and small interfering RNAs, respectively. MiRNAs that belong to the

hairpin RNAs are expressed as primary miRNAs (pri-miRNA), subsequently processed by the enzyme Drosha to precursor miRNAs (pre-miRNA), exported from the nucleus by exportin 5, and finally processed by Dicer and to mature miRNAs. By combining the information on precursor and on mature miRNAs several features can be delineated to characterized miRNAs including the minimum free energy, the base composition or the number of neighboring precursors within a given genomic region. The latter criterion acknowledges the reported clustering of miRNA coding sequences in the genome. Likewise, other small similar sized ncRNAs have also complex sequence and structural features, thereby complicating the classification and separation of the different classes of ncRNAs. This can be exemplified for snoRNAs.[50,51] Based on sequence features, there are two classes of snoRNAs. C/D box snoRNAs have consensus C (RUGAUGA, R = A or G) and D (CUGA) box motifs. H/ACA box snoRNAs have a double-hairpin structure with two single-stranded H (ANANNA, N = A, C, G or U) and ACA box domains.[52] Some snoRNAs have very short sequences that extend little beyond the C and D boxes. Since many of these features are likely awaiting identification, the grouping of ncRNAs is a yet ongoing process. While in most studies on miRNA biomarkers there is no need to test the above criteria in detail, there should be a general awareness that the definition and classification of a miRNA by sequence is not necessarily a simple task.

The miRBase is the primary online repository for all miRNA sequences and annotations. Presently, miRBase Release 21, http://www.mirbase.org/ contains more than 28,500 miRNA entries, including 2578 human mature miRNAs. However, not all miRNAs deposited in miRBase do necessarily represent true miRNAs. Since miRBase was established in 2002 the methods by which miRNAs are discovered and verified have fundamentally changed. While in the beginning many miRNAs have been confirmed by Northern blotting, the overwhelming majority of the recent miRNA Database entries stems from deep-sequencing technologies, which on one hand have caused a rise in the rate of novel miRNA discovery, but on the other hand have also introduced a great deal of uncertainty about the nature of the deposited ncRNAs. For many miRNAs the number of reads is not sufficient to verify or refute miRNA annotations. To address this problem miRBase has established a catalogue of criteria to define a subset of miRNAs that are called high confidence miRNAs.[3] While in miRBase version 20 just around 20% fall in this category, the refined miRBase version 21 lists already 77% of the human miRNAs in the high confidence set. This in turn means that at least still over 500 miRNA biomarker candidates deserve further confirmation.

Already 2003, researchers lead by Tuschl described specific criteria for the experimental verification of miRNAs. In addition to expression criteria also the biogenesis of miRNAs was described to be of importance.[53] In this article, the typical length of miRNA precursors in animals was described to be 60–80 nucleotides. Of the 1881 human miRNA precursors annotated in the current release version 21, 219 miRNAs exceed 100 bases with the longest precursors having as much as 180 bases. The knowledge of the biogenesis of miRNAs helps to predict and analyze the specific regulatory effects of different miRNAs on their targets.

THE CHALLENGE TO IDENTIFY AND CONFIRM miRNAs BY EXPERIMENTAL ASSAYS

Many studies that are aiming to identify informative miRNAs start with a screening or biomarker discovery assay. In this step usually many more miRNAs as compared to samples are measured ('$p \gg n$ problem'). In most cases this first step involves either microarray platforms or NGS approaches. In the early stages of miRNA profiling, microarrays have been widely used to generate miRNA patterns. Array-based technologies allow the screening of an extended number of miRNAs, require a relative low amount of input RNA and show a reasonable reproducibility. Among the most frequent platforms are the Affymetrix Gene Chip arrays that require a minimum of 100 ng total RNA and use a biotin/streptavidin detection system. Also frequently used are the Agilent oligonucleotide arrays that require 100 ng of total RNA and use Cyanine 3-pCp for detection of hybridized miRNAs. The Exiqon miRCURY LNA™ miRNA arrays, which are less frequently used, only require 30 ng of input RNA.[20,54,55] The miRNA expression microarrays are based on miRNA sequences that are deposited in one of the most recent miRBase versions. In contrast to the hybridization based approaches, NGS does not require a priori knowledge of target miRNAs and therefore allows discovery of novel miRNAs.[56] Among the commonly used high-throughput NGS platforms are the Illumina HiSeq2000, the ABI SOLiD and the Roche GS FLX+ (454). The Illumina MiSeq, the Invitrogen Ion Torrent and the Roche GS Junior 454, each have a comparable smaller capacity.

For both, NGS and microarrays, technological bias is well known. Hafner et al., for example, described a RNA-ligase dependent biases in miRNA patterns that have been generated by deep-sequenced small RNA cDNA libraries.[57] Likewise, biased detection toward miRNAs in array profiles depending on the guanine content has been described.[58] Both arrays and NGS are primarily used for biomarker discovery/screening assays and are less suited for clinical care. As for the NGS approach, the Roche platforms (GS FLX+ 454 and GS Junior 454) yield longer reads but show higher error rates, while the Illumina HiSeq2000 yield shorter reads but allow higher throughput. The ABI SOLiD also yields shorter reads but has the advantage of a low Error rate. The Ion Torrent system has the advantage of short run times; major disadvantages of the later system will come to light as soon as there will be a more widespread use. Since this review is on the translation of miRNAs as biomarkers into the clinical practice, we will not further discuss the pros and cons of the different hybridization and NGS based methods for miRNA detection. An excellent review on reproducibility of miRNA platforms is presented by Mestdagh et al.[59]

TRANSLATION TO CLINICS AND VALIDATION OF miRNAs

Complex technologies such as NGS are established in a fraction of clinics. This adds to the slow translational process. Many gold standard biomarkers that are in clinical routine use such as cardiac troponin are measured using immunoassays. For miRNAs, a respective assay has also been developed,[60] which has however still limitations such as a restricted degree of multiplexing and limited technical sensitivity. For translation in to a clinical application, qRT-PCR seems thus to be currently the method of choice. Beyond an accurate quantification it can also be employed in the context of a routine testing without requiring equipment that is usually not found in clinical settings. There are two commonly applied methodologies both of which include Reverse Transcription (RT) for cDNA synthesis.[61] The SYBR Green approach uses universal polyA tailing and an oligo(dT) primer, which converts mature miRNA species, but also precursor miRNA, other ncRNAs, and mRNAs into cDNA. Subsequently, the PCR is done by using miRNA-specific forward primer and a universal reverse primer in combination with SYBR Green. The Life Technologies miRNA TaqMan assay uses a unique stem-loop RT primer, which significantly enhances the specificity of the reaction. Since the RT primer hybridizes to the miRNA to generate sequence extension next to the 3′ of the miRNA, this approach discriminates isomiRs. In summary, while the SYBR Green method is cheaper than TaqMan and does not require probe design, the TaqMan method is less prone to false positive results. However, using appropriate primer selection software, both methods show comparable performances.

Once there is sufficient evidence by either next generation sequencing, array analysis or PCR to consider a miRNA as potential biomarker, the nature of this miRNA still requires final confirmation:

(i) One option is electrophoresis separation of qRT-PCR products followed by blotting and hybridization with a probe directed against the qRT-PCR product. However, signals that are found after gel electrophoresis can represent artificial amplifications including primer dimers. Sequencing of the products is mandatory to exclude artificial amplification products.

(ii) As stated above, for further confirmation of a true miRNA it is helpful to determine the length of the mature miRNA, of the precursor, of 3p and 5p mature forms, and of the loop.

(iii) Beyond these criteria, the gold standard still remains the identification of an endogenous miRNA by Northern blotting.[62] In opposite to a PCR analysis this approach does not include an enzymatic amplification step. Northern blotting provides a high degree of certainty but also requires larger amounts of RNA, which in many cases cannot be obtained from the cells under investigation. For a radioactive detection by Northern blotting, the necessary amount of RNA lies in the order of five microgram.

(iv) Functional evidence for a true miRNA can be obtained by cloning of the premature miRNA and by its subsequent expression in host cells. Confirmed processing of the premature into the mature miRNA provides evidence for a true miRNA. However, a caveat of the latter system lies in the heterologous expression of the premature RNA in a likely not physiological amount.

(v) Further confirmation for a true miRNA can be obtained by comparative expression analyses in Dicer knockout cells versus wild type cells. While in the knockout cells miRNA precursor should not be processed, in the wild type the mature miRNA should be detected.

CONCLUDING REMARKS

In this review, we have focused on specific problems toward the identification of miRNA biomarkers and implications for testing in clinical care. Known technological bias including the comparability of different miRNA discovery platforms have already been extensively addressed. Beyond these challenges we emphasize on questions that concern the source and the definition of miRNAs. The source can have a fundamental impact on the miRNA abundance and without acknowledging this fact, specific miRNA abundances can erroneously be attributed to a disease status. We specifically addressed the influence of the patient (the blood donor), of the cellular level and of the molecular level. One of the foremost reasons that many miRNA biomarker—and other biomarkers as well—do not life up to their expectations lies in the insufficient acknowledgement of these confounding factors.

Equally important for the search of miRNA biomarkers is the confirmation that the RNA under investigation is indeed a true miRNA. While discussing several features that can help to characterize a miRNA, we also highlight that the final confirmation of a miRNA can be quite demanding and that just few miRNAs have been experimentally validated to a comprehensive extent. This may explain why confirmation of a true miRNA is frequently not provided even if there is evidence for a value as biomarker. Classifying a short RNA as miRNA by sequencing data only is certainly not sufficient. A misclassification can readily lead to wrong assumptions on the regulatory functions of a short miRNA. The concept of an impaired protein synthesis by either RNA degradation or translation repression is specifically known for miRNAs but not necessarily for other classes of short RNAs.

Nonetheless, own and others studies provide evidence that miRNAs from body fluids can add to diagnosis and prognosis of human pathologies, although considerable additional work is required. This includes the correct classifying of a short RNA as miRNA and an independent validation of its value as biomarker under specific consideration of confounding factors.

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