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Computational Analysis of miRNA and Target mRNA Interactions: Combined Effects of The Quantity and Quality of Their Binding Sites*

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Abstract MicroRNAs (miRNAs) act by binding to complementary sites on target messenger RNA (mRNA) to induce mRNA degradation and/or translational repression. To investigate the influence of miRNAs at transcript levels, two human miRNAs (miR-1 and miR-124) were transfected into HeLa cells and microarrays used to examine changes in the mRNA profile showed that many genes were downregulated and that the fold decreases in levels of these target mRNAs differed remarkably. Features depicting interactions between miRNAs and their respective target mRNAs, such as the number of putative binding sites, the strength of complementary matches and the degree of stabilization of the binding duplex, were extracted and analyzed. It was found that, for a given target mRNA, both the quality and quantity of miRNA binding sites significantly affected its degree of destabilization. To delineate these types of interactions, a simple statistical model was proposed, which considers the combined effects of both the quality and quantity of miRNA binding sites on the degradation levels of target mRNAs. The analysis provides insights into how any animal miRNA might interact with its target mRNA. It will help us in designing more accurate methods for predicting miRNA targets and should improve understanding of the origins of miRNAs.

Key words microRNA, binding sites, target mRNA, downregulation
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1 Introduction

MicroRNAs (miRNAs)——small single-stranded noncoding RNAs——regulate gene expression posttranscriptionally in animals, plants, even virus[1~4]. Although miRNAs were discovered only 14 years ago, they are now known to be involved in many biological processes, such as controlling leaf and flower development in plants, and in the regulation of signaling pathways, apoptosis, metabolism, cardiogenesis, and brain development in animals[5, 6]. In addition, misregulation of miRNA expression has been proved to be related to many kinds of cancer[7~9].

Given the important biological regulatory roles that miRNAs play, hundreds of miRNAs have now been identified from plants, animals and viruses[10], and each miRNA is thought to have hundreds of target messenger RNAs (mRNAs), at least in mammalian genomes. There are two mechanisms by which miRNAs direct the RNA-induced silencing complex to downregulate gene expression by base-pairing to complementary sequences within the message: mRNA degradation and/or translational repression. In plants, miRNAs usually have sufficient complementarities to mRNAs and regulate gene expression through mRNA degradation. In contrast, miRNAs in animals do not have sufficient complementarities to their targets, and were originally believed to act mainly on repressing translation rather than destabilizing mRNA[11, 12]. Nonetheless, this view has been proved only partially correct, as studies have shown that miRNAs in animals can also promote mRNA degradation[13~17]. Recently, three different miRNA targets were examined by Behm-Ansmant *et al.* [17] and these showed similar repression levels. However, the mechanisms by which repression achieved were all different: the first target

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mRNA was repressed translationally and stable; the second one was destabilized; and the third one was repressed by both destabilization and translational repression. Thus, besides the two mechanisms mentioned before, this study identified a new combination mechanism of miRNA-mediated regulation.

In a previous study, Lim *et al*.[13] transfected two human miRNAs (miR-1 and miR-124) into HeLa cells, and used microarrays to examine changes in mRNA profiles. The analyses showed reductions at transcript levels. By analyzing the expression profiles produced by both miRNAs (miR-1 and miR-124), we found that the fold decreases in the expression levels of their target mRNAs are remarkably different. For both miRNAs, all reactions took place under the same conditions and the only differences lay in the sequences of the target mRNAs. Therefore, if there are any factors affecting the degradation levels, most if not all should be within and/or correlated with the sequences of the target mRNAs. To test this hypothesis, in the present study, we tried to identify miRNA binding features buried in the sequences of the target mRNAs and examined how these features——individually or/and cooperatively——might influence the degree of the interaction between miRNAs and their respective targets at transcript levels. We found that the degradation level of a target mRNA was significantly correlated with both the quantity and the quality of binding sites along the full-length mRNA sequence. Therefore, we have proposed a simple model approximate the effects of these features on the expression level changes of any target mRNA downregulated by a miRNA.

2 Materials and methods

2.1 miRNA targets selection

Lim *et al*.[13] transfected two human miRNA (miR-1 and miR-124) duplexes into HeLa cells separately, and then purified and profiled mRNAs on microarray. By filtering the expression profiles for the genes characterized, 96 (miR-1) and 174 (miR-124) gene sets that were significantly downregulated ($P < 0.001$) at both 12 h and 24 h were selected. The rules used for filtration were extremely strict, so these genes could be considered as true targets with high confidence. By removing those target mRNAs with unclear annotation, 91 (miR-1) and 169 (miR-124) target mRNA sequences were extracted from the RefSeq database (NCBI, human release 23)[18] for further analysis.

2.2 miRNA binding sites selection and sequence features collection

At first, we considered obtaining information on miRNA binding sites from some existing predictions. However, we found some discrepancies between the experimental and the existing prediction results[19]. That means that some experimental targets were not involved in the existing predictions, so information on their binding sites was not available. Considering the limitation of the existing predictive results, we decided to use the miRanda package (version 1.9 for Linux OS) to detect all potential miRNA binding sites along these 260 target mRNA sequences (91 for miR-1; 169 for miR-124) by ourselves[20~23]. First, to avoid missing weak binding sites, we set the thresholds of match score and the optimal free energy to 100 and –10, respectively. Both of these thresholds are calculated by the miRanda package. The match score reflects the complementarity match strength between a mature miRNA and one of its candidate sites; whereas the free energy represents the degree of stabilization of the binding duplex (more detailed definitions could be found in the documentation of the miRanda package). For a given miRNA and one of its target mRNAs, each match predicted by miRanda was then denoted as a candidate that satisfied both thresholds criteria we chose. Then, each candidate site was categorized as a 5′-dominant or 3′-compensatory one by inspecting whether it had perfect base pairing to the seed region of the miRNA, Figure 1 shows two experimentally supported examples of two kinds of miRNA binding sites. Here, the seed region of a miRNA was defined as the consecutive stretch of six nucleotides starting from the second nucleotide at the 5′ end of a miRNA[24]. Many such 5′-dominant sites have been usually discarded in previous analyses because of their low match scores. Therefore, for each target mRNA, we chose all of the 5′-dominant candidate sites as true binding sites. However, the number of detectable 5′-dominant binding sites seems to be an order of magnitude greater than that of 3′-compensatory ones[25], so only the 3′-compensatory candidate sites on the top N_5/10 (sorted ascending by free energy) were selected as true binding sites. For this, N_5 was set as the number of 5′-dominant binding sites on the corresponding target mRNA. Finally, the positional information of each true binding site was simply

tagged as 3′-UTR or non-3′-UTR according to its position along the mRNA sequence. Figure 2 shows the pipeline of binding sites selection and the collection of their features.

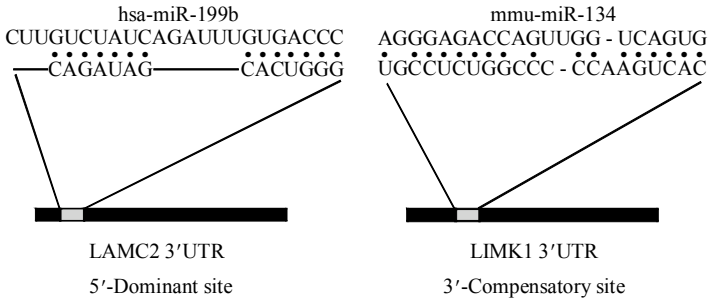

Fig. 1 The categories of miRNA binding sites[26]

Experimentally supported examples of 5′-dominant (left) and 3′-compensatory (right) miRNA binding sites.

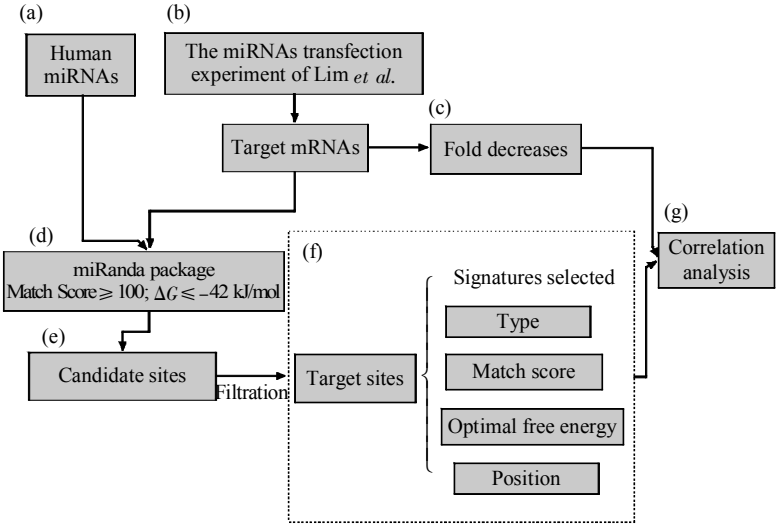

Fig. 2 The pipeline for binding sites selection and features collection

Source data consisted of (a) two human miRNAs (miR-1 and miR-124) and (b) target mRNAs gained from the miRNAs transfection experiments. These were (d) input to the miRanda package and (e) many candidate sites were predicted. Through filtration, (f) high quality binding sites and their respective features were delineated. Finally, potential correlations between these features and (c) the fold decreases extracted from the miRNAs transfection experiment were analyzed statistically (g).

So far, for each of the two human miRNAs and one of its experimental targets, there were several predictive binding sites. For each binding site, four features were distilled: the match score, the optimal free energy, the type (3′-compensatory or 5′-dominant) and the position (3′-UTR or non-3′-UTR). Thereafter, we explored whether there were any correlations between the fold decreases at 12 h and 24 h for these two human miRNAs that could reflect the degree of mRNA downregulation, and the patterns of these features extracted from the target mRNA sequences.

2.3 Correlation analysis

To detect the relationship between the sequence features and the fold decreases of target mRNAs degradation, correlation analysis was used (SPSS 13.0 for Windows). We chose the values of the fold decreases at 24 h and 12 h. To guarantee the reliability of statistical analysis without losing of generality, the targets of the two miRNAs were merged into one dataset for further analysis. As all the data we used for correlation analysis were not normally distributed, Spearman's rank correlation analysis was used. Table 1 lists the results of correlation analyses. The results in Table 1 show that data at 24 h were more significant than those at 12 h. That's easy to explain: miRNAs might need more time to react adequately.

Table 1 The results of correlation analyses at 12 h and 24 h

Spearman′s rank correlation	Fold decreases of target mRNAs				Number of samples
	Correlation coefficient		*P*		
	12 h	24 h	12 h	24 h	
(a) Numbers of binding sites					
5′-dominant binding sites on the 3′-UTR	–0.086	–0.193**	0.165	0.002	260
5′-dominant binding sites on the full-length mRNA	–0.089	–0.204**	0.152	0.001	260
All binding sites on the full-length mRNA (Figure 3a)	–0.093	–0.207**	0.134	0.001	260
(b) Numbers of seed sites					
Seed sites on the 3′-UTR	–0.073	–0.180**	0.243	0.004	260
Seed binding sites on the full-length mRNA	–0.080	–0.176**	0.198	0.004	260
(c) Scores					
Scores calculated based on Equation 1 (Figure 3b)	–0.102	–0.200**	0.101	0.001	260
Scores calculated based on Equation 2 (Figure 3c)	–0.111	–0.226**	0.075	1×10^{-6}	260
(d) Specific sites					
The maximum match score	–0.052	–0.086	0.408	0.165	260
The minimum optimal free energy	0.005	0.135*	0.941	0.029	260

** $P < 0.01$ (2-tailed); * $P < 0.05$ (2-tailed).

3 Results and discussion

3.1 Effects of the number of binding sites on the level of target mRNA degradation

For any given miRNA, many of its known and predicted target mRNAs contain more than one binding site, and the numbers of these binding sites vary remarkably[19, 23, 27]. Some experiments have shown that the number of binding sites in a target mRNA is likely to determine the effectiveness of translational repression [28]. Thus, when we investigated what may lead to the differences of mRNA degradation levels, the first candidate feature we inspected was the number of binding sites.

To test whether the types and the positions of binding sites matter, we constructed three groups of binding sites: (1) 5′-dominant binding sites on 3′-untranslated region (UTR), (2) 5′-dominant binding sites on the full-length mRNA, and (3) all binding sites on the full-length mRNA (including both 5′-dominant and 3′-compensatory). We counted the numbers of the binding sites and tested for any correlations between the binding site numbers and the degradation levels (Table 1a). For each of the three groups mentioned above, there was a significant correlation between the binding site number and the fold decreases in target mRNA expression levels. Further, by scrutinizing the results of correlation analyses, we extended the range of binding sites selected from only 3′-UTR to the full-length mRNA; these correlation tended to be more significant. We speculate that both the 5′-dominant binding sites on 3′-UTR and those on the non-3′-UTR are functional. Subsequently, when we included the 3′-compensatory binding sites for analysis, the correlation became even more significant (Table 1a, Figure 3a), suggesting that the effect of 3′-compensatory binding sites of a miRNA on the downregulation of its target mRNAs should not be neglected.

Therefore, as we expected, for any miRNA target, the number of binding sites on it is critical for transcriptional downregulation of the mRNA. Interestingly, both 5′-dominant and 3′-compensatory binding sites on the full-length mRNA seem to function simultaneously. Table 2 lists the distribution pattern of the binding sites.

Table 2 The distribution pattern of binding sites

Type of binding site	Numbers of binding sites	
	non-3′-UTR	3′-UTR
5′-dominant	161	451
3′-compensatory	15	6

3.2 Effects of the binding sites affinity on the level of target mRNA degradation

In contrast with plants, the miRNA::binding-site duplexes in animals are more variable in structure because of differences in binding site sequences. Recent experiments have shown some correlation between the level of translational repression and the free energy of binding of the seed region of a given

miRNA[29]. It remains unclear whether a similar relationship exists at the transcript level. We used two features to depict the quality of binding sites: (1) the match score, which reflects the level of complementarity matching between a miRNA and one of its binding sites, and (2) the optimal free energy, which reflects the stabilization of the miRNA:: binding-site duplexes. To determine the effect of the quality of binding sites on the downregulation of target mRNA expression level, we constructed two summed statistics to represent the overall effects of all binding sites of a miRNA on one of its target mRNAs, measured by the complementary match (Equation 1) and the optimal free energy (Equation 2).

$$S_{\text{target}} = \sum_{i=1}^{n} S_i \quad (1)$$

$$E_{\text{target}} = \sum_{i=1}^{n} E_i \quad (2)$$

Here S_i and E_i are the complementary match score and the optimal free energy of binding site i, respectively, and n is the number of binding sites for a given target mRNA. As shown in Table 1c, Figure 3b and Figure 3c, there are significant correlations between both of these two summed scores and the fold decreases in target mRNA expression level. This indicates that the affinity of miRNA binding sites on full-length mRNA also contributes to the effects on the target mRNA at the level of degradation.

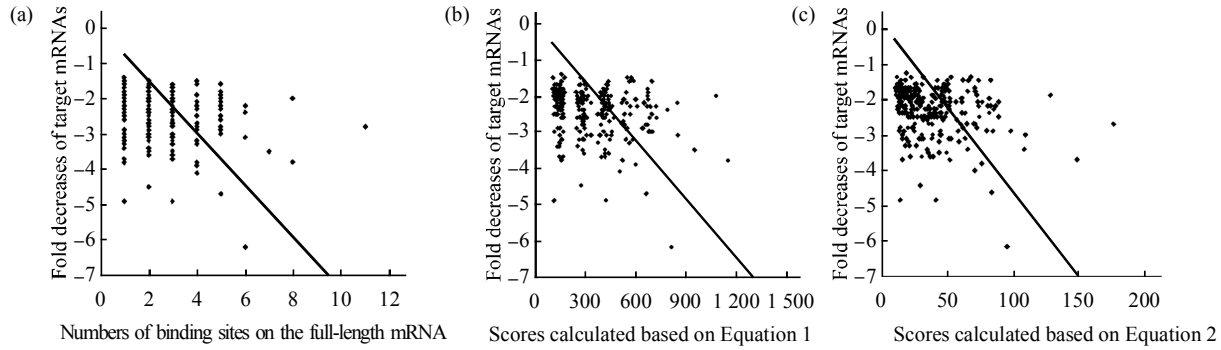

Fig. 3 Both the quality and quantity of miRNA binding sites significantly affected the degradation level of target mRNAs

(a)Scatter plot of fold decreases of target mRNAs versus numbers of all binding sites on the full-length mRNA. (b) Scatter plot of fold decreases of target mRNAs versus Scores calculated based on Equation 1. (c) Scatter plot of fold decreases of target mRNAs versus Scores calculated based on Equation 2.

We know that the 5′-dominant binding sites have a perfect miRNA 5′ match, but insignificant complementarity to the remainder of the miRNA sequence, and that the 5′ seed region is thought to be very important in target recognition for animal miRNA[30~34]. By searching the sequences of the target mRNAs and counting the number of “seed site” motifs that are exactly complementary to positions 2～7 from the 5′ end of the corresponding miRNA, we found that for some targets there are more seed sites than 5′-dominant binding sites. This indicated that some seed sites were filtered by miRanda (a software for miRNA target prediction)[23] because of their low quality in complementarity to the non-seed region. Should these seed sites be excluded? We did another analysis of possible correlations between the numbers of seed sites and the fold decreases (Table 1b). We found that for sequences only from the 3′-UTR or from the full-length mRNA, the correlation between the numbers of 5′-dominant binding sites and the fold decreases in mRNA expression levels (Table 1a) were more significant than those correlations between the numbers of seed sites and the fold decreases. This result suggests that a site only perfectly complementary to the miRNA seed region may not be sufficiently functional to cause downregulation of gene expression levels.

3.3 Cooperative mechanism of miRNA on the level of target mRNA degradation

To investigate how a specific binding site correlates with the target mRNA degradation level, for each target mRNA, the binding site with the maximum complementary match score was chosen for a similar correlation analysis. Interestingly, this maximum score may not represent most effects on the downregulation of mRNA expression level and a similar trend could be observed when the binding site with the minimum optimal free energy was tested (Table 1d).

Correspondingly, the degradation level of a target mRNA is significantly correlated with both the quantity (the number of target sites) and the quality (the complementary match score and the optimal free energy) of binding sites on the full-length mRNA (Table 1a, c), suggesting that multiple binding sites may be associated with a greater mRNA destabilization. This observation has been supported by some new transfection experiments[35].

Based on these findings, we proposed the model outlined above in Equations 1 and 2. We found that the multicollinearity between two variables represented the quality of the binding site. Thus, for match score and optimal free energy, Spearman's rank correlation coefficient r_s was 0.953 (P close to 0). We therefore used stepwise regression analysis to filter variables. The statistical software filtered out the match score, so we proposed Equation 2, which considers the abovementioned factors, to measure the magnitude of downregulation level of mRNA transcription targeted by a given miRNA. The model considers the effects of both 5′-dominant and 3′-compensatory binding sites along the full-length mRNA sequences. The quantity of binding sites likely dominates the degradation level of target mRNA, but the quality of binding sites also matters.

Of course, the correlations shown in Figure 3 are still not very strong based on our models. Because the interaction between miRNA and target is really complex, and many factors affect the degradation level of target mRNAs are still unknown. Now just part of the information in target mRNA sequences was considered, we believe with more and more factors within and without target sequences discovered, our model will be improved someday.

3.4 The complicated recognition mechanism between a miRNA and its targets

There are many ready-made computational target prediction programs, for example PicTar[19]. However, the predictions of PicTar show a low degree of overlap with the experimental results. The percentage of experimentally supported targets predicted by PicTar based on the conservation of five species (NCBI build 35) is only 23% (60/260). There are several plausible explanations. First, because the mechanism of the action of animal miRNAs has been recognized as translation repression and because the 3′-UTR is a safe place to make sure the miRNAs are not pushed away by moving ribosomes, most if not all prediction programs search for binding sites only within the 3′-UTRs[19, 33, 36]. However, when an animal miRNA acts as mRNA destroyer, it is independent of the location of interaction and the interaction sites could be elsewhere on the full-length mRNA just like miRNAs in plants[37]. Several studies have provided evidence that seed matches in non-3′-UTR region can also confer regulation by a miRNA in animal genomes[35, 38, 39]. That means that the predictions present before, such as PicTar, might neglect non-3′-UTR binding sites of some targets and some targets with non-3′-UTR binding sites only. Second, the existing prediction programs may overemphasize the 5′-dominant sites and inevitably lose functional 3′-compensatory sites. Third, almost all the ready prediction programs are based on cross-species conservation requirements. Nonetheless, many species-specific miRNAs have been found and investigated experimentally[40, 41]. For these complicated reasons, we suggest that the biogenesis of miRNA targets for a given miRNA is an ongoing process in which new binding sites for a given miRNA can emerge.

We suggest that the next generation of approaches for predicting miRNA targets might improve accuracy and specificity if the following four points are considered. First, the range of binding sites to be searched should be extended from 3′-UTRs to full-length mRNA sequences. Second, both 5′-dominant and 3′-compensatory sites should be included, but with a cautious selection of the 3′-compensatory sites. Third, species-specific targets should also be searched besides conserved targets across genomes. Last, the putative targets should be sorted according to both the quantity and the quality of their predicted binding sites. In addition, considering the existence of several distinct but perhaps overlapping mechanisms (inducing mRNA degradation and/or translational repression), some targets might not be detected by miRNA transfection experiments, as we reported[13]. That is another reason for the discrepancies between the experimental and the existing predicted results. It will be very important and challenging to define which subsets of mRNAs are subject to different mechanisms[42].

4 Conclusions

In this study, we have explored how animal miRNAs might interact with their target mRNAs in terms of the sequence features of target mRNAs.

Because of the limited experimental data, only two sets of miRNA interaction profiles were analyzed. With the emergence of more high-quality experiments, it is expected that the complicated interactions between a miRNA and its target mRNAs will be better understood. One of the latest research uncovered five general features of binding site context related to the mRNA degradation level[35]. Another study mentioned that mRNAs whose association with Ago2 increased upon miRNA expression were much more likely to have overall mRNA levels decrease in response to the miRNA transfection[38]. These are all good inspirations for us. Maybe we can merge some features buried in context and some facts outside together into our model to make it better in further work.

In addition, the mechanism is complicated. Currently, we are still uncertain about whether each miRNA::binding-site interaction functions independently. If this interaction is independent of the other potential sites on a target mRNA [35], the inclusion of more binding sites increases the probability of a single degradation event and elevates the degradation level of the target mRNA. Nevertheless, if the function of binding multiple copies of any miRNA to the different binding sites of a given target mRNA is cooperative, more binding sites would guarantee the effectiveness of target mRNA degradation. The issue between these two scenarios still needs further investigation.

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miRNA 与其靶 mRNA 的相互作用：绑定位点的质量与数量特征的整合计算分析 *

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摘要 miRNAs 通过完全或不完全的碱基互补绑定到信使 RNA(mRNA)上，通过抑制翻译或者直接导致 mRNA 降解的方式来调节靶基因的表达．为了研究 miRNAs 在转录水平上面的调控作用，两种人类基因组中组织特异的 miRNAs(miR-1 和 miR-124)被转染到 HeLa 细胞中，微阵列(microarray)分析转染前后细胞中各基因 mRNA 表达水平变化情况的结果表明：动物基因组中靶基因与 miRNAs 不完全的碱基互补也会导致 mRNA 的直接降解．通过分析实验得到的 mRNA 表达水平变化数据，发现这相同 miRNA 的不同靶基因 mRNA 表达水平的下调倍数有着明显的差别，推测这些靶基因 mRNA 序列本身存在某些影响其受调节程度的因素．为此，提取和分析这些靶基因 mRNA 的序列特征，通过对这些序列特征与 mRNA 表达水平下调数据进行统计相关分析，最终发现，miRNA 靶基因受调节的程度与以下几个因素相关联：mRNA 序列中 miRNA 靶位点的个数，靶位点与 miRNA 序列碱基互补的程度，以及绑定后形成二级结构的稳定程度(即最低自由能的大小)．在此基础上，初步建立起一个多因子作用下的 miRNA 靶基因 mRNA 表达水平下调程度模型，分析表明：该模型在一定程度上可以反映了部分序列特征对于 miRNA 靶基因 mRNA 表达水平下调程度的影响．

关键词 microRNA，靶位点，靶 mRNA，下调

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