

Pseudogenes: Pseudo-functional or key regulators in health and disease?

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ABSTRACT

Pseudogenes have long been labeled as “junk” DNA, failed copies of genes that arise during the evolution of genomes. However, recent results are challenging this moniker; indeed, some pseudogenes appear to harbor the potential to regulate their protein-coding cousins. Far from being silent relics, many pseudogenes are transcribed into RNA, some exhibiting a tissue-specific pattern of activation. Pseudogene transcripts can be processed into short interfering RNAs that regulate coding genes through the RNAi pathway. In another remarkable discovery, it has been shown that pseudogenes are capable of regulating tumor suppressors and oncogenes by acting as microRNA decoys. The finding that pseudogenes are often deregulated during cancer progression warrants further investigation into the true extent of pseudogene function. In this review, we describe the ways in which pseudogenes exert their effect on coding genes and explore the role of pseudogenes in the increasingly complex web of noncoding RNA that contributes to normal cellular regulation.

Keywords: pseudogenes; functional; noncoding RNA; transcription; RNA

INTRODUCTION

The human genome, like that of other mammals, is littered with a variety of repetitive elements and noncoding genes. One such element is the pseudogene, a poor facsimile of an original protein-coding gene that has lost the ability to produce a functional protein (Mighell et al. 2000). Because they do not code for proteins, pseudogenes are often assumed to be nonfunctional and labeled as “junk DNA.” While some pseudogenes are transcriptionally silent, others are active, raising the question of whether their noncoding transcripts are a spurious use of cellular energy or instead harnessed by the cell to regulate coding genes (Balakirev and Ayala 2003). This question is particularly pertinent given the recent flurry of evidence suggesting that long noncoding RNAs play a critical role in regulating genomic function (Mattick and Makunin 2006; Guttman et al. 2009; Caley et al. 2010).

Pseudogenes can arise through a variety of mechanisms. Spontaneous mutations in a coding gene that prevent either

transcription or translation of the gene (Fig. 1A) result in the formation of “unitary” pseudogene (Zhang et al. 2010). Duplicated pseudogenes are created via tandem duplication or uneven crossing-over (Mighell et al. 2000). These duplicated genes lose their protein-coding potential due to the loss of promoters or enhancers or crippling mutations such as frameshifts or premature stop codons (Fig. 1B). However, they do tend to retain their characteristic intron-exon structure. In contrast, retrotransposed or “processed” pseudogenes (PPs) are produced when an mRNA transcript is reverse-transcribed and integrated into the genome at a new location (Fig. 1C), and therefore, they do not normally contain introns (Maestre et al. 1995; D’Errico et al. 2004). Other common features of PPs are their polyA tracts and direct repeats at either end of the pseudogene (Maestre et al. 1995; D’Errico et al. 2004). The retrotransposition of mRNAs into the genome appears to be mediated by long interspersed nuclear element 1 (L1) (Esnault et al. 2000; Ding et al. 2006), and the transcriptional activity of the resulting PP depends on whether the integration event occurs close to another promoter (Zheng et al. 2007). The collection of processed pseudogenes in the human genome has been generated from just 10% of the coding genes (Ohshima et al. 2003; Zhang et al. 2003). Highly expressed housekeeping genes are more likely to produce PPs, as are other highly transcribed shorter RNAs (Gonçalves et al.

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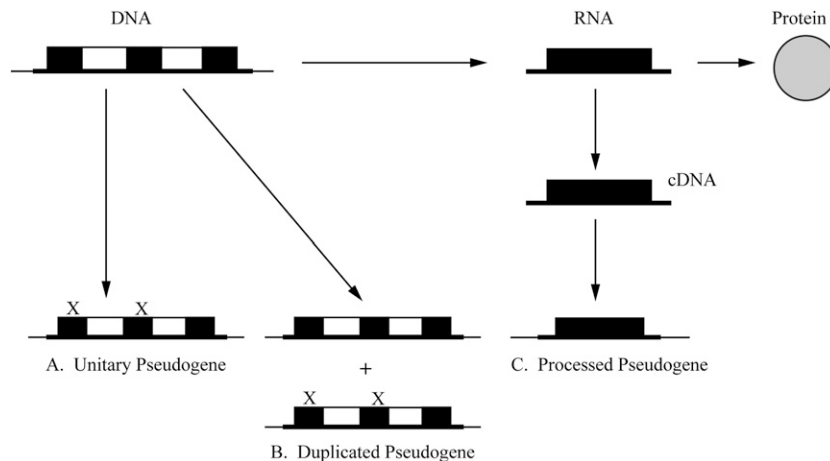


FIGURE 1. Types of pseudogene. (A) Mutation of existing genes gives rise to unitary pseudogenes. (B) Duplicated pseudogenes are produced following mutation of copied genes. (C) Reverse transcription of mRNA into cDNA followed by retrotransposition into genomic DNA leads to the generation of processed pseudogenes. Filled boxes represent exons; open boxes represent introns; X represents a crippling mutation that ablates protein-coding potential.

2000). This is exemplified by the small number of genes encoding ribosomal proteins that account for $\sim 20\%$ of human PPs (Zhang et al. 2002).

The term pseudogene was coined in 1977, when Jacq and co-workers discovered a version of the gene coding for 5S rRNA that was truncated but retained homology with the active gene in *Xenopus laevis* (Jacq et al. 1977). During the following two decades, pseudogenes were discovered in a sporadic fashion (Mighell et al. 2000). The acceleration in next-generation sequencing technologies coupled with the monumental human genome project has yielded the full genome sequences of a range of organisms, permitting much more thorough analyses of pseudogene prevalence (Ohshima et al. 2003; Torrents et al. 2003; Zhang et al. 2003). Remarkably, pseudogenes are almost as numerous as coding genes, with predictions ranging from 10,000 to 20,000 human pseudogenes (Zhang and Gerstein 2004). The majority of human pseudogenes are PPs (Ohshima et al. 2003; Torrents et al. 2003; Zhang et al. 2003), while the number of unitary pseudogenes in the human genome is <100 (Zhang et al. 2010). Pseudogenes are present in a wide range of species, including plants (Loguercio and Wilkins 1998; Benovoy and Drouin 2006), bacteria (Ochman and Davalos 2006) [though they are not as numerous in unicellular organisms (Lawrence et al. 2001)], insects (Ramos-Onsins and Aguadé 1998; Harrison et al. 2003), and nematode worms (Harrison et al. 2001), but they are particularly numerous in mammals (Zhang and Gerstein 2004).

EVOLUTION AND CONSERVATION OF PSEUDOGENES

Pseudogenes are sometimes considered to represent “neutral sequence,” in which mutations that accumulate are

neither selected for or against (Li et al. 1981). However, this premise relies on the assumption that pseudogenes are functionally inert. There is recent evidence that some pseudogenes are functionally active, and therefore, studying their evolution and conservation could support a functional role and give insight into their potential mechanism of action.

The synonymous to nonsynonymous substitution rate (K_A/K_S) is a measure of the proportion of mutations in DNA sequence that also alter amino acid sequence; it is often used to assess whether a sequence is under evolutionary constraint (Hurst 2002; Torrents et al. 2003). It has been reported recently (for review, see Balakirev and Ayala 2003) that synonymous point mutations occur far more frequently than do nonsynonymous base changes in the *Drosophila Est-6* pseudogene (Balakirev et al. 2003). In chickens, it has been observed that, within the multiple *IgIV* and *IgHV* pseudogenes, the number of stop codons contained in the “coding” sequence is far lower than would be expected were nucleotide substitutions occurring at random; in addition, the majority of stop codons introduced by point mutations are then “corrected” and eliminated by further point mutations within the same codon (Rothenfluh et al. 1995). This feature, which is also observed in *V_H* pseudogenes in mice (Schiff et al. 1985), may indicate either that some presumed pseudogenes are, in fact, protein-coding, or that the conservation of open reading frames plays a role in any putative function of pseudogenes involved in somatic gene rearrangements (Balakirev and Ayala 2003).

A recent report described a region of the 3' UTR of the *PTENP1* pseudogene that shared over 95% sequence homology with the *PTEN* coding gene (Poliseno et al. 2010). The functional implications of this pattern and high degree of conservation are discussed in further detail below.

Pseudogenes gradually accumulate mutations, and the number of mutations can give us an estimate of their age. Fascinatingly, the appearance of Alu elements in Old World primates coincided with the peak of processed pseudogene generation and subsequent radiation of primates ~ 40 million years ago (Ohshima et al. 2003; Zhang et al. 2003). Conservation of pseudogenes across different species has also been observed. Analysis of the rhesus macaque major histocompatibility complex (MHC) extended class II region revealed two pseudogenes that were found to be homologous to the human HIV TAT-specific factor-1-like and zinc finger-like pseudogenes, which was suggestive of evolutionary conservation (Sudbrak et al. 2003). Investigations by Podlaha and colleagues (Podlaha and Zhang 2004) demonstrated that

the *Makorin1-p1* pseudogene is conserved across *Mus musculus* and *Mus pahari* strains. This prompted a genome-wide survey for pseudogenes conserved between humans and mice in which human pseudogenes, along with their parent genes, were compared with the corresponding mouse orthologues and their pseudogenes (Svensson et al. 2006). Interestingly, many of the pseudogenes examined were found to have very few mutations within the regulatory regions they shared with their parent genes, which might suggest that these regulatory regions are of importance to the pseudogene and that the pseudogene may be functional. Of the groups of genes and pseudogenes examined, sequence analysis suggested that 30 of them represented pseudogenes that were present in both mice and humans and had arisen before the two species diverged. Comparison of transcribed human pseudogenes shows that ~50% are conserved with rhesus monkey, but only 3% are conserved in mouse (Khachane and Harrison 2009). Analysis of these pseudogenes showed that, in spite of a K_A/K_S substitution rate indicative of noncoding RNAs, the levels of GC and the rate of mutation in these pseudogenes is constrained relative to the intergenic regions that surround them (Khachane and Harrison 2009). The collective evidence that some pseudogenes exhibit sequence conservation hints at a potential functional role in the organisms that harbor them.

TRANSCRIPTION OF PSEUDOGENES

Most pseudogenes lose the ability to be transcribed, either due to mutations in their promoter, or (in the case of PPs) integration into silent regions of the genome. Making accurate measurements of pseudogene transcription is complicated by the similarity they share with their parent genes (Ruud et al. 1999; Harper et al. 2003). However, there are numerous examples of pseudogenes that are transcribed, including pseudogenes for the tumor suppressor *PTEN* (whose transcripts are more numerous than the parent gene) (Fujii et al. 1999), the adrenal steroid hydroxylase *P450c21A* (Bristow et al. 1993), human leukocyte interferon (Goeddel et al. 1981), *GAPDH* (Tso et al. 1985), glucocerebrosidase (Sorge et al. 1990), and *Oct4* (Redshaw and Strain 2010). Microarray technology permits analysis of pseudogene transcription on a much larger scale. Estimates of the proportion of transcribed human pseudogenes vary from 2% to 20% (Yano et al. 2004; Harrison et al. 2005; Zheng et al. 2005, 2007).

Investigating the pattern of transcription across tissues and cell lines can give insight into potential functionality. Other noncoding RNAs exhibit tissue specific expression patterns and have also been shown to have functional roles, including antisense RNAs (Dahary et al. 2005; Katayama et al. 2005), intergenic transcripts and long noncoding RNAs (Bertone et al. 2004; Cheng et al. 2005; Guttman et al. 2009) and miRNAs (Zhang 2008). In a survey of transcription in the ENCODE regions of the genome, 14

pseudogenes were found to be transcribed (Zheng et al. 2007). Five of these were transcribed exclusively in the testes, and a further four were also active in testes and other tissues (Zheng et al. 2007). This pattern of transcription is consistent with previous results, suggesting a possible biological significance for testes-specific pseudogene transcription (Kleene et al. 1998; Reymond et al. 2002). There are also several examples of pseudogenes whose spatio-temporal expression pattern is different from that of their parent gene (Olsen and Schechter 1999; Elliman et al. 2006).

Specific changes in pseudogene expression can also occur under different physiological conditions, including diseases such as diabetes (Chiefari et al. 2010) and cancer (Suo et al. 2005; Zou et al. 2009; Poliseno et al. 2010). Examples of dynamic pseudogene transcription have been observed in other organisms. Robust pseudogene transcription has been measured in *Mycobacterium leprae*, the organism that causes leprosy, with levels of specific transcripts varying during the process of infection (Suzuki et al. 2006). Induction of stress in *Arabidopsis thaliana* leads to changes in expression of numerous genes and pseudogenes (Zeller et al. 2009).

The transcriptional activity of a pseudogene will, in part, depend on the promoter it is utilizing. Some have their own promoters, while others use the promoters of nearby genes (Harrison et al. 2005; Vinckenbosch et al. 2006). A processed pseudogene is largely at the mercy of its integration site when it comes to promoter activity. Therefore, the difference in transcription pattern between the pseudogene and its parent gene does not necessarily reflect a functional role but may be instead merely the result of being driven by a new promoter. If the latter were true, then one could predict that the act of transcribing a pseudogene would be evolutionarily neutral (or even selected against to conserve cellular energy). Analysis of conserved transcribed pseudogenes shows that ~50% are indeed conserved across millions of years of primate evolution (though far fewer are conserved between species more distant to human, such as rodents) (Khachane and Harrison 2009). That transcription of some pseudogenes is tissue-specific, dynamic, and has been maintained over millennia suggests that their transcripts may play some functional role in cells. There is no substitute, however, for functional experiments to test whether pseudogenes and their transcripts have a direct active role.

EVIDENCE FOR PSEUDOGENE FUNCTION

Many multicellular organisms appear to conserve the presence of pseudogenes and impose constraint on the sequences themselves. Various unicellular organisms, on the other hand, actively expel genes that have become pseudogenized (Kuo and Ochman 2010). The question remains, then, of what is the potential benefit to mammals and other complex organisms in retaining and arguably embracing genes that

have lost protein-coding potential? One proposed function of pseudogenes is to act as a source of genetic diversity, for example, in the generation of antibodies or antigen variation (Balakirev and Ayala 2003). However, it is in the context of noncoding RNA that pseudogenes offer potentially much more dynamic mechanisms to regulate ongoing nuclear processes. In the last decade, a new layer of complexity has been revealed in the regulation of gene expression and nuclear function. Many noncoding RNAs, both long and short, have been shown to regulate a variety of processes in cells. The noncoding RNAs produced from some pseudogenes also appear to use a variety of fascinating mechanisms to control gene function.

There is evidence that pseudogenes and their parent genes form regulatory pairs that can influence each other. Specific knockdown of the ATP-binding cassette (ABC) transporter pseudogene *ABCC6P1* causes a decrease in the expression of *ABCC6* mRNA (Piehler et al. 2008). *Oct4*, a pluripotency-associated transcription factor, has several known pseudogenes. Overexpression of a putative pseudogene transcript (*Oct4P1*) leads to inhibition of mesenchymal stem cell differentiation while stimulating proliferation (Lin et al. 2007). An inverse correlation was observed between *BRAF* pseudogene transcription and the quantity of *BRAF* mutations during the progression of papillary thyroid carcinoma (Zou et al. 2009). Forced transcription of the *BRAF* pseudogene stimulated MAP kinase signaling, transformed NIH3T3 cells in culture, and induced tumors in nude mice (Zou et al. 2009). Fascinatingly, the Xist noncoding RNA, which mediates dosage compensation by coating the inactive X chromosome in mammals and mediating epigenetic repression, may have evolved by pseudogenization of a protein-coding ancestor called *LnX3* (Duret et al. 2006).

It has long been hypothesized that antisense pseudogene transcripts could combine with sense genic transcripts to regulate levels of expression (Fig. 2A) (McCarrey and Riggs 1986). Examples of this mechanism have now been reported. Knockdown of an RNA antisense to an *Oct4* pseudogene leads to an apparent increase in levels of both *Oct4* and two of its pseudogenes (Hawkins and Morris 2010). Simultaneous transcription of both neural nitric oxide synthase (*nNOS*) and a related pseudogene in the same neurons of *Lymnaea stagnalis* (a species of snail) leads to the formation of a duplex between the two strands and a reduction in *nNOS* translation (Korneev et al. 1999).

In two landmark reports, it was shown that in mouse oocytes, portions of many pseudogene transcripts are

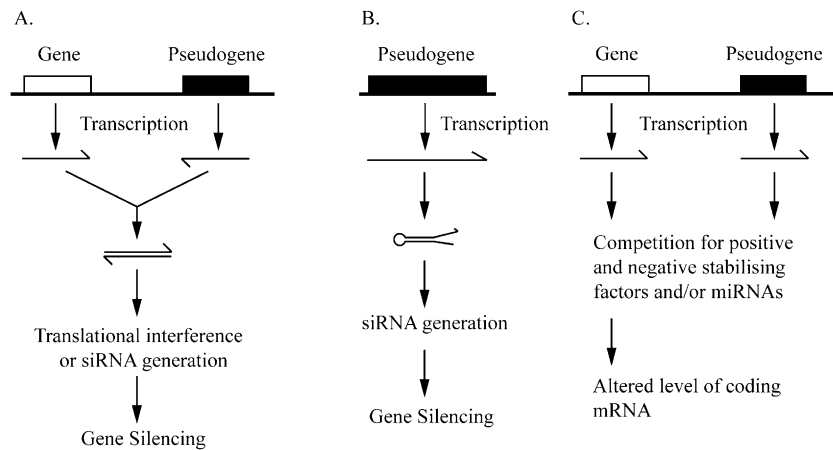


FIGURE 2. Potential mechanisms of pseudogene function. (A) Antisense RNA generated from pseudogenes can combine with sense-stranded mRNA from a homologous coding gene and either inhibit translation or lead to the formation of siRNAs that can inhibit expression of the coding gene. (B) Similarly, siRNAs can be generated from hairpin structures within folded pseudogene transcripts. (C) RNA from coding genes and their homologous pseudogenes can compete for *trans*-acting stability factors and degradation by miRNAs. When the levels of pseudogene transcripts are altered, this, in turn, affects the stability of the coding gene mRNA and therefore, expression levels.

processed into small interfering RNAs (siRNAs) (Tam et al. 2008; Watanabe et al. 2008). These siRNAs were derived either from pseudogenes with internal secondary structure (Fig. 2B), or from dsRNAs composed of sense and antisense transcripts (pseudogene-pseudogene and pseudogene-coding mRNA combinations were observed). The loss of Dicer (a protein necessary for the production of siRNAs) led to a decrease in the levels of pseudogene-derived siRNAs and an increase in the level of coding gene mRNAs with homology with the siRNA sequences (Tam et al. 2008; Watanabe et al. 2008). This suggests that the siRNAs generated from the dsRNA are capable of repressing gene expression. For example, siRNAs generated from a hairpin structure in the *Au76* pseudogene RNA were able to inhibit expression from the parent coding gene *Rangap1* (Watanabe et al. 2008). Several siRNAs were detected bearing sequence similarity to the histone deacetylase complex, *HDAC1* (Tam et al. 2008). All of the siRNA sequences were derived from a series of *HDAC1* pseudogenes (none from the *HDAC1* gene itself), yet upon Dicer knockout, the levels of *HDAC1* mRNA increased, suggesting that the coding gene is regulated by a RNA-induced silencing complex (RISC)-based process. In another case, the siRNAs were generated from regions of dsRNA formed between *Ppp4r1* mRNA and an antisense RNA generated from a pseudogene with 90% homology (Watanabe et al. 2008). The siRNAs produced from this pairing appear to repress the *Ppp4r1* gene. A similar survey in a species of rice shows that a small proportion of pseudogenes are transcribed and processed into siRNAs after pairing with the coding gene or a paralogous pseudogene transcript (Guo et al. 2009). These findings suggest a potential mechanism by which antisense transcripts

may operate. However, it remains to be seen whether similar processes occur in mammalian somatic cells.

Several groups have proposed that pseudogenes may interfere with factors that regulate mRNA stability (Hirotsune et al. 2003; Piehler et al. 2008; Chiefari et al. 2010). It is known that the stability of a given mRNA depends on *cis*-acting sequences and their interaction with *trans*-acting molecules (Ross 1996). If a pseudogene and parent coding gene harbor similar *cis*-acting sequences, then it is feasible that they may compete for the same *trans*-acting elements (Fig. 2C). Thus, regulated changes in pseudogene transcription could lead to altered coding-gene mRNA stability and, therefore, expression. Such a role was suggested for the imprinted *Makorin1-p1* pseudogene in modulating the expression of its imprinted parent gene *Makorin-1* (Hirotsune et al. 2003). Reduction of *Makorin-p1* RNA led to a drop in mRNA from the protein-coding gene and a significant disease phenotype in mice (Hirotsune et al. 2003). This finding remains controversial, however, as a subsequent study showed that the pseudogene is, in fact, transcriptionally silent, and neither gene is imprinted (Gray et al. 2006). Decreased production of high mobility group A1 (HMGA1) protein can lead to the deregulation of the insulin receptor (*INSR*) gene and subsequent development of type 2 diabetes mellitus (Brunetti et al. 2001). In two recently tested type 2 diabetes patients, the low level of HMGA1 was also associated with a high level of HMGA1 pseudogene (*HMGA1-p*) mRNA (Chiefari et al. 2010). Knockdown of *HMGA1-p* RNA led to partially restored *HMGA1* mRNA levels, suggesting that the two transcripts may compete for a *trans*-acting stability factor.

miRNAs are a class of noncoding RNA that affect mRNA stability. Their specificity and function is mediated by base pairing with the target (primarily at the 3' UTR); their primary effect is to cause degradation of the mRNA and therefore reduce levels of expression (Bartel 2009; Guo et al. 2010). In a recent report, a gene-pseudogene pair was shown to be co-regulated by the same miRNAs (Poliseno et al. 2010). *PTEN* is a tumor suppressor that is often mutated at one allele (while wild type at the second allele) at cancer presentation (Salmena et al. 2008). The severity and susceptibility to cancer is influenced by the dosage of *PTEN* (Alimonti et al. 2010). Therefore, maintaining precise levels of *PTEN* protein is critical for preventing oncogenesis. *PTENP1*, a pseudogene of the *PTEN* tumor suppressor, is transcribed at a high level (Fujii et al. 1999). As described above, the 5'-most region of the *PTENP1* 3' UTR is ~95% similar in sequence to the 3' UTR of *PTEN* (Poliseno et al. 2010). A number of miRNAs are capable of targeting this region on both the gene and pseudogene. Knockdown of the *PTENP1* transcript leads to reduced levels of *PTEN* mRNA and protein and inhibition of growth; conversely, transfecting cells with the 3' UTR of *PTENP1* caused *PTEN* expression to increase (Poliseno et al. 2010). This suggests that the *PTENP1* pseudogene acts

as a “miRNA decoy,” binding to and thereby reducing the effective cellular concentration of miRNAs, therefore allowing *PTEN* to escape miRNA-mediated repression. A functional link between the *PTEN/PTENP1* pair is consistent with the findings that their levels are often correlated in prostate cancer samples and that focal deletions containing *PTENP1* occur frequently in sporadic colon cancer cases (Poliseno et al. 2010). A similar relationship was also demonstrated between the oncogene *KRAS* and its pseudogene *KRASP1* (Poliseno et al. 2010). Interestingly, the ability of the *HMGA1-p* pseudogene to destabilize *HMGA1* mRNA depends on the 3' UTR region (Chiefari et al. 2010), suggesting that both positive and negative stabilization factors are able to interact competitively with the untranslated regions of genes and pseudogenes to regulate expression output.

CONCLUSIONS

Caution must be exercised when interpreting the results of functional experiments on pseudogenes. In some cases, what appears to be a nontranslated pseudogene can, in fact, code for truncated proteins (Kandouz et al. 2004; Zhang et al. 2006). Nevertheless, the evidence that some pseudogenes can exert regulatory effects on their protein-coding cousins is mounting. Such functions appear to be mediated by noncoding RNAs produced from active pseudogenes. While not all pseudogenes (or even all transcribed pseudogenes) will have biological functions, it is likely that, where an unexpected regulatory benefit results from the formation of a pseudogene, the effect will be conserved. For the large part, pseudogenes have been overlooked in the quest to understand the biology of health and disease, to the extent that pseudogene probes are often absent from commercially available microarrays. As evidence emerges that pseudogenes are deregulated in disease, and indeed that their deregulation can contribute to diseases such as diabetes and cancer, the prevalent attitude that they are nonfunctional relics is slowly changing. With the advent of affordable next-generation sequencing, the study of transcriptomics, and in particular, pseudogenes (and other transcribed noncoding elements), should experience a quantum leap forward. In the coming decade, the extent and mechanisms of pseudogene function should become clearer.

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