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Large-scale analysis of pseudogenes in the human genome

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Pseudogenes are considered as genomic fossils: disabled copies of functional genes that were once active in the ancient genome. Recently, whole-genome computational approaches have revealed thousands of pseudogenes in the genomes of the human and other eukaryotes. Identification of these pseudogenes can improve the accuracy of gene annotation. It also offers new insight on the evolutionary history and the stability of the genome as a whole.

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Abbreviations

CDS	coding sequence
cyc	cytochrome *c*
Ka	non-synonymous rate of substitution
Ks	synonymous rate of substitution
LINEs	long interspersed nuclear elements
nNOS	*nitric oxide synthase*
SINEs	short interspersed nuclear elements
UTR	untranslated region

Introduction

Mammalian genomes, such as human and mouse, contain a large number of gene-like sequences called pseudogenes. These pseudogenes are inheritable, non-functional, gene homologies that are generally disabled at the transcriptional level [1,2••]. In most cases, pseudogenes cannot produce transcripts as a result of functional promoter scarcity. Very rarely, some pseudogenes have either retained or acquired a functional promoter so they can be transcribed, but these transcripts are not translated because of a lack of translational or splicing signal sequences. As the result of their non-functionality, pseudogenes are generally released from selective pressure and often accumulate mutations such as frameshifts, in-frame stop codons, or interspersed repeats in the original protein-coding sequence (CDS) (see Figure 1). Consequently, we can identify pseudogenes operationally through finding regions of homology that have these non-gene-like features (Table 1).

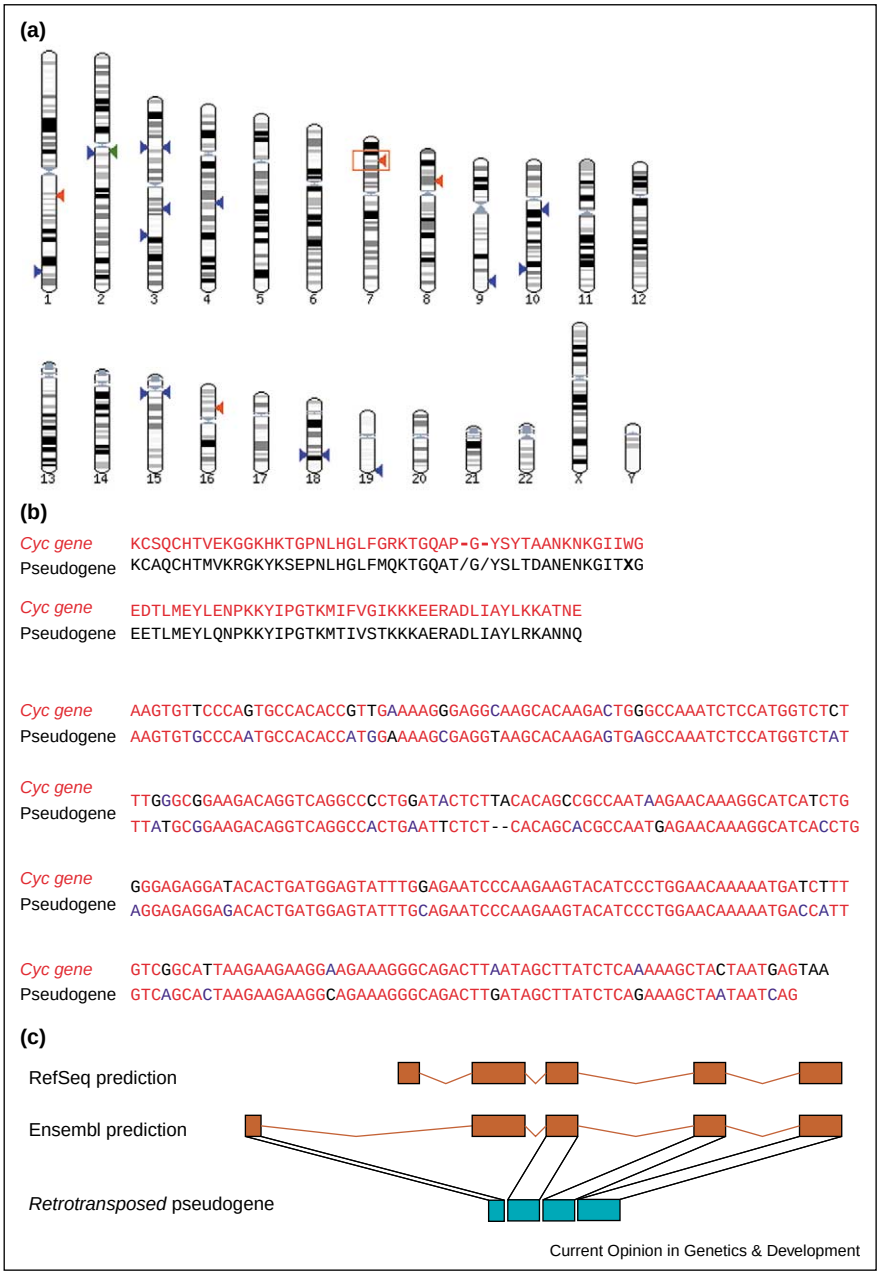
Depending on the mechanism by which they were generated, the majority of the mammalian pseudogenes can be divided into duplicated pseudogenes and retrotransposed pseudogenes (also called processed pseudogenes). Duplicated pseudogenes arose from tandem duplication or unequal crossing-over, thus they often have retained the original exon–intron structures of the parental genes, although sometimes incompletely. By contrast, retrotransposed pseudogenes were created from retrotransposition: the reverse transcription of the mRNA transcript followed by integration into the genome [3,4]. Therefore, retrotransposed pseudogenes are often considered as a special type of retrotransposon, just like long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) in the mammalian genomes [5•]. Retrotransposed pseudogenes also share some of the common characteristics of the LINEs and SINEs, which include a complete lack of introns, the presence of small flanking direct repeats, and a polyadenine tail near the 3′-end. Because of their close homology to functional genes, pseudogenes often introduce errors or contaminations in the sequence databases (Figure 1). In addition to retrotransposed and duplicated pseudogenes, other types of pseudogenes also exist in the human genome (see below).

Over the years, pseudogenes have been comprehensively surveyed in several completely sequenced genomes (Table 2). In 2002, a preliminary survey reported ~400 pseudogenes on the two smallest human chromosomes, 21 and 22 [6•]. Several other studies have focused on the pseudogene population of selected gene families [7•,8–10]. 2003 proved to be an exciting year, as three groups independently published comprehensive surveys of pseudogenes in the entire human genome [11••–13••]. It was also discovered in the same year that a mouse pseudogene actually has a regulatory role [14].

Whole-genome identification of pseudogenes

Traditionally, pseudogenes were often discovered as by-products of gene sequencing or PCR experiments. It is only after the whole-genome sequencing projects that large numbers of pseudogenes were identified and annotated. Using a homology-based approach, Zhang *et al.* [12••] identified ~8000 retrotransposed pseudogenes and ~3000 duplicated pseudogenes in the human genome draft (Build 28, April, 2002 release). Ohshima *et al.* [11••] used basically the same approach in their survey except

Figure 1



Example and prevalence of human pseudogenes. **(a)** A screen shot from the Ensembl website showing the contamination of pseudogenes in the genomic databases. The human cytochrome *c* functional gene (*cyc*) is located in the chromosome 7. Many retrotransposed *cyc* pseudogenes exist in the human genome. The red arrows point to those pseudogenes that are mistakenly annotated as genes by Ensembl. The functional *cyc* gene contains 1 intron in the coding region while the pseudogenes have no introns. **(b)** The amino acid and nucleotide sequence alignments between the functional cyc gene and a pseudogene. The pseudogene contains frame shifts and stop codons. **(c)** Retrotransposed pseudogene can be used to verify the exon structure predictions. The exon structures predicted by RefSeq and Ensembl are compared with a retrotransposed pseudogene. The inconsistency between the predictions and the pseudogene sequence could represent alternative splicing or erroneous predictions.

that they used an older release of the human genome (April, 2001).

In addition to just relying on the existence of truncation or frame disruptions to ascertain the non-functionality of the pseudogenes, Torrents *et al.* [13••] developed a neutrality test by computing the ratio of synonymous to non-synonymous substitution rates (K_A/K_S) for each pseudogene. The K_A/K_S ratio measures how often nucleotide substitutions in a DNA sequence change the amino acid and this

Table 1

Features of pseudogenes (or potential pseudogenes).

	Tokyo [11••]	Yale [12••,26••]	EMBL [13••]	Others*
Level of sequence homology to parent gene	■	■	■	
Sequence completeness relative to parent gene	■	■	○	
Absence of introns	■	■	◆	
Ratio of the non-synonymous to synonymous substitution rates (Ka/Ks)	○	▲	■	[25,50••]
Chromosomal location (in relation to parent gene)	○	▲	◆	
Existence of frame disruptions (frameshifts and stops)	○	■	○	
G+C content of pseudogenes and background	▲	▲	○	
Expression level of the parent gene	○	▲	○	
Occurrence of regulatory regions such as CpG islands	○	○	○	
Codon composition and nucleotide substitutions in relation to parental gene	○	▲	○	[34,51]
Occurrence of polyadenine tail	○	■	○	
Conservation with mouse genome	○	▲	■	
Association with evidence of transcription such as EST matches or micro-array data	○	○	○	[52,53]
Occurrence of SNPs	○	○	○	[54]
Number of pseudogenes per gene family	▲	▲	▲	[7•,8–10,55]

■, Main feature used for the assignment of pseudogenes; ◆, minor feature used for the assignment of pseudogenes; ▲, surveyed after assignment of pseudogenes (in comparison to genes); ○, not currently used or surveyed but potentially could be; *analysis performed by others; EST, expressed sequence tag; SNP, single-nucleotide polymorphism.

ratio is often used to test whether a sequence is under selective constraints [15]. These researchers reported ~20,000 potential human pseudogenes. By correlating with sequence conservation in the mouse syntenic regions, they estimated 70% of these were retrotransposed pseudogenes. The pseudogene annotations can also be validated by the absence 'CpG islands' in their 5′ upstream regions; this is because these 'CpG islands' are often associated with the 5′ end of the functional genes [16]. Operationally, as we have pointed out earlier, pseudogenes can be defined by a variety of different sequence features. The three research groups (Tokyo, Yale, and EMBL) have taken somewhat different approaches towards the definition, resulting in different numbers. The differences are summarized in Table 1. Some features listed in Table 1 were not used in the identifying pseudogenes, but rather in the later stages of analysis and inferences.

Exact number of the pseudogenes in the human genome

It is a little surprising that the total numbers of human pseudogenes reported by the three research groups are quite different. Much of the discrepancy can be attributed to the different criteria used by individual groups. Ohshima *et al.* [11••] applied the most stringent criteria in

Table 2

Annotated pseudogenes in the completely sequenced genomes.

Organism	Genome size (Mb)	No. of genes	No. of pseudogenes	No. of retrotransposed pseudogenes	Ref.
R. prowazekii	1.1	834	241	0	[21]
M. leprae	3.3	1604	1116	0	[22]
Y. pestis	4.6	4061	160	0	[56]
E. coli, K-12	4.6	4400	95	0	[23]
E. coli, O157	5.5	6000	101	0	[23]
S. cerevisiae	12.1	6340	241	0	[18]
C. elegans	102.9	20,009	2168	208	[17]
D. melanogaster	128.3	14,332	110	34	[20]
A. thaliana	115.4	25,464	>700	?	[57]
H. sapiens	3040	~35,000			
			~14,000	~7800	[12••]
			~3600	~3600	[11••]
			~19,000	~13,300	[13••]
M. musculus	2493	~22,000			
			~10,000	~4500	[26••]
			~13,000	N/A	[25]

their procedures as they only presented those pseudogenes that are 90% complete in comparison with their parental genes. Zhang *et al.* [12••] counted those candidates that are 70% complete in coding region as pseudogenes and designate those shorter than 70% as 'pseudogenic fragments'. By contrast, Torrents *et al.* [13••] did not apply any sequence completeness threshold in their procedures. If the 70% completeness cut-off is applied to the pseudogene set derived by Torrents *et al.*, ~7800 of them are indeed longer than this threshold. This is actually remarkably close to the number reported by Zhang *et al.* [12••]. Thus, even though the reported numbers differ, the results from the three groups are actually consistent with each other.

Pseudogenes in other organisms

In addition to human, large numbers of pseudogenes were also identified in the genomes of other eukaryotes including the nematode worm [17], budding yeast [18], puffer fish [19], and fruitfly [20]. Some prokaryotic genomes also reportedly have many pseudogenes [21–23]. Generally, pseudogenes are less common in prokaryotes because their genomes are more compact and have higher DNA deletion rates [24].

The initial annotation of the mouse genome reported ~14,000 putative pseudogenes [25]. A more recent study that revealed ~5000 retrotransposed pseudogenes in mouse [26••] was based on the same criteria as used for the human pseudogenes [12••]. This is significantly lower than the number of retrotransposed pseudogenes in human, even though the mouse genome is only slightly smaller than the human genome. However, this does not mean that retrotransposition is less active in mouse. The mouse genome has higher nucleotide substitution, insertion and deletion rates than human [25,27], thus the pseudogenes in mouse decay faster and are not recognized as easily as those in the human genome.

It is interesting to estimate what fraction of the pseudogenes in the human and mouse genomes are lineage-specific, that is, those pseudogenes that were created after the primate and rodent lineages split at ~75–80 million years ago [25]. Torrents *et al.* [13••] reported that ~76% of their 'pseudocoding regions' in the human genome can also be found within the corresponding mouse synetnic regions. Using an alternative approach, Zhang *et al.* [26••] estimated sequence divergence between the pseudogenes and the parent gene and converted the divergence data to evolutionary time. These researchers concluded that ~60% of the retrotransposed pseudogenes in the human and mouse genomes are lineage-specific.

Retrotransposed pseudogenes are special types of retrotransposons

The human genome contains several millions copies of LINE and SINE elements that comprise >30% of the entire human genomic DNA [5•]. Whereas LINEs are autonomous (i.e. they can retrotranspose their own transcripts), SINEs have to rely on active LINEs to propagate. It is believed that LINE retrotransposons are also responsible for mobilizing mRNA transcripts and generating retrotransposed pseudogenes [4]. Macroscopically, the distribution of the retrotransposed pseudogenes in the human genome is random and dispersed, with the pseudogene abundance on each chromosome proportional to its length. Despite the common mechanism in their biogenesis, LINEs, SINEs and retrotransposed pseudogenes have distinct distributions in the genomic regions of different G+C composition [12••]. This discrepancy has been explained by the different stability of the retrotransposons and pseudogenes in different regions [28].

By calculating the sequence divergence between the sequence of pseudogene and the parental functional gene, one can estimate the age of a pseudogene, that is, the time that has elapsed since it became nonfunctional [11••,12••,29]. The retrotransposed pseudogenes in the human genome have an overall age profile that is similar to that of the *Alu* elements, the predominant SINE element in primates. The rate of new retrotransposed pseudogenes generated in human peaked ~40 million years ago, which coincided with the onset of higher primate radiation [11••,12••].

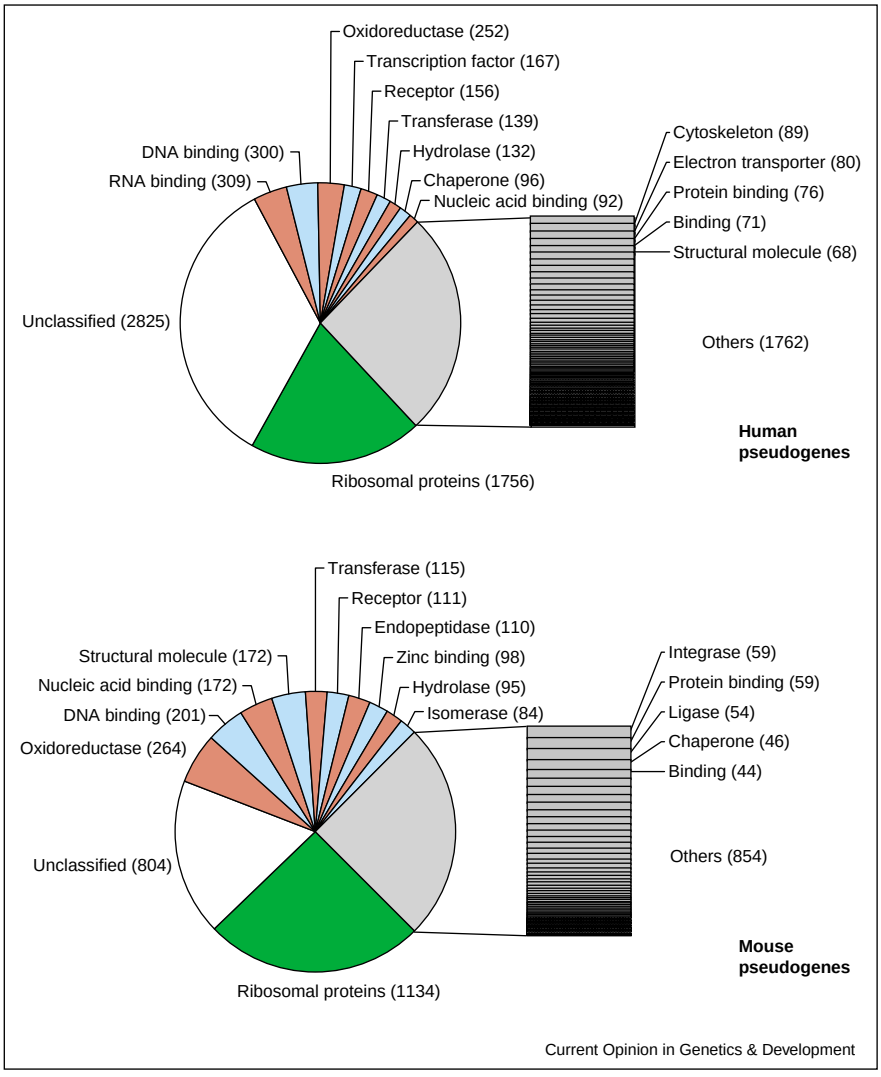
Highly expressed genes tend to have multiple retrotransposed pseudogenes

The number of retrotransposed pseudogenes per gene is highly uneven in the human genome. In fact, only 10% of human genes have at least one retrotransposed pseudogene identified [11••,12••]. Ribosomal proteins, which have 79 genes in the human genome, account for nearly 20% of the entire retrotransposed pseudogenes population [7•]. Other genes that have multiple retrotransposed pseudogenes include housekeeping genes, genes that code for structure protein and metabolic enzymes. In general, the genes that have multiple retrotransposed pseudogenes tend to be highly expressed, have short transcripts, and have lower G+C composition [12••,30]. Figure 2 shows the functional categories of the human and mouse genes that gave rise to multiple retrotransposed pseudogenes. These also include some genes that are involved in cancer or have other medical implications such as cyclophilin, nucleophosmin and prohibitin [12••]. For those human genes that have multiple retrotransposed pseudogenes, their mouse homologues also tend to have many pseudogenes in the mouse genome.

Pseudogenes as tools to study gene and genome evolution

Pseudogenes are often considered as 'genomic fossils' because they provide glimpses of genes that were active

Figure 2



Functional classification of the retrotransposed pseudogenes in **(a)** the human genome and **(b)** mouse genome, according to Gene Ontology functional categories. 'Unclassified' are those pseudogenes that arose from genes that were not yet assigned to a Gene Ontology category. Less-populated categories are lumped together into 'Others'.

millions of years ago. They can be analyzed to infer the evolutionary history of particular genes or gene families. By comparing the sequences of human cytochrome *c* (*cyc*) pseudogenes with the functional *cyc* gene from human and mouse, it became obvious that accelerated evolution in *cyc* had occurred in the primate lineage leading to human [31•]. In another case, it is found that the orthologs of a human keratin pseudogene in the chimpanzee and gorilla are still functional [32].

Because pseudogenes are free to accumulate mutations, they are also very valuable in studying nucleotide substitution, insertion and deletions [33,34]. On a related note, retrotransposition of mRNA transcripts has been suggested as an important mechanism of generating new genes [35–37]. Brosius has argued that mammalian genomes were forged and shaped by 'massive bombardments' of retrotransposed sequences [38,39].

Some pseudogenes are transcribed

Because pseudogenes have high sequence similarity with their parental genes, they can potentially introduce contaminations in hybridization or amplification experiments. Special cautions need to be taken to prevent such interferences [40]. It has been reported that a cytokeratin-19 pseudogene may have interfered with diagnostic assays used to detect micrometastatic tumor cells [41]. In another instance, a novel pseudogene of *phox*, a com-

ponent of phagocyte NADPH oxidase complex, complicates the detection of chronic granulomatous disease [42,43].

The original definition of pseudogenes implies that they are transcriptionally silent but over the years there have been many reported cases where a pseudogene can indeed be transcribed (for a complete list, see [2••]). In one instance, it was found that a tumor suppressor gene, PTEN, has a transcribed retrotransposed pseudogene that has more transcripts than the parental functional gene [44]. In another case, a pseudogene even has developed a tissue-specific expression pattern [45].

Potential functional roles of pseudogenes

Because of their close similarities to the functional genes and high level of sequence conservation, pseudogenes, especially those that are transcribed, have been hypothesized as having regulatory roles [46]. Korneev, Park and O'Shea [47] have reported that, in the neurons of mollusk *Lymnaea stagnalis*, a transcribed pseudogene of neural *nitric oxide synthase* (nNOS) suppresses the synthesis of nNOS protein in an RNAi-like mechanism [47]. The transcript of the pseudogene contains a region with significant antisense homology to the nNOS mRNA transcript and binds to it to form a stable RNA/RNA duplex. In another example, the pseudogene of the mouse gene *Makorin1* modulates the expression of the homologous functional gene in either an RNA-mediated or a DNA-mediated mechanism [14]. At the RNA level, the pseudogene RNA transcript could compete with the functional mRNA for an RNA-digesting enzyme. At the DNA level, the pseudogene locus could potentially compete with the functional *Makorin1* gene for transcription repressors [48].

Pseudogenes have also been proposed to serve as a sequence pool for generating genetic diversity [2••]. Genes and pseudogenes can recombine and produce new genes; such processes have been reported in the human immune system [49].

Conclusions

Pseudogenes are ubiquitous and abundant in mammalian genomes. Their importance and implications have captured the interests of researchers from very diverse disciplines. The fact that pseudogenes have regulatory roles further demonstrates that these sequences should not be treated as 'junk DNA'. With more mammalian genomes, such as that of chimpanzee, being sequenced, a more complete picture of pseudogenes and their functions is starting to emerge.

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