

Discovery of Siglec-14, a novel sialic acid receptor undergoing concerted evolution with Siglec-5 in primates

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ABSTRACT Immune receptors that show high mutual sequence similarity and have antagonizing signaling properties are called paired receptors, and are believed to fine-tune immune responses. Siglecs are sialic acid-recognizing receptors of the immunoglobulin (Ig) superfamily expressed on immune cells. Human Siglec-5, encoded by *SIGLEC5* gene, has four extracellular Ig-like domains and a cytosolic inhibitory motif. We discovered human Siglec-14 with three Ig-like domains, encoded by the *SIGLEC14* gene, adjacent to *SIGLEC5*. Human Siglec-14 has almost complete sequence identity with human Siglec-5 at the first two Ig-like domains, shows a glycan binding preference similar to that of human Siglec-5, and associates with the activating adapter protein DAP12. Thus, Siglec-14 and Siglec-5 appear to be the first glycan binding paired receptors. Near-complete sequence identity of the amino-terminal part of human Siglec-14 and Siglec-5 indicates partial gene conversion between *SIGLEC14* and *SIGLEC5*. Remarkably, *SIGLEC14* and *SIGLEC5* in other primates also show evidence of gene conversions within each lineage. Evidently, balancing the interactions between Siglec-14, Siglec-5 and their common ligand(s) had selective advantage during the course of evolution. The “essential arginine” critical for sialic acid recognition in both Siglec-14 and Siglec-5 is present in humans but mutated in almost all great ape alleles.—Angata, T., Hayakawa, T., Yamanaka, M., Varki, A., Nakamura, M. Discovery of Siglec-14, a novel sialic acid receptor undergoing concerted evolution with Siglec-5 in primates. *FASEB J.* 20, 1964–1973 (2006)

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IMMUNE CELL SURFACE receptors sharing similar sequences and having counteracting signaling properties are called “paired receptors.” Known paired receptors belong to two classes of molecular families, namely, the Ig superfamily and the C-type lectin family (1). These receptor pairs (or receptor families) include the killer

cell Ig-like receptors (KIR) of primates (2), the leukocyte Ig-like receptors (LILR) of primates (3), the paired Ig-like receptors (PIR) of rodents (4), the myeloid-associated Ig-like receptors (MAIR) of rodents (5), the Ly49 family of rodents (6), and the CD94/NKG2 family of both primates and rodents (7). Although Ly49 and CD94/NKG2 families belong to C-type lectin family, to our knowledge none of them has been unequivocally shown to recognize glycan ligands. Rather, their ligands have been shown to be major histocompatibility complex class I proteins. The functional significance of the presence of paired inhibitory and activating receptors remains elusive, but it is proposed that these paired receptors are involved in fine-tuning of immune responses (8).

Siglecs are a family of vertebrate lectins belonging to the Ig superfamily that recognize glycan chains containing sialic acids. Most of them are expressed on cells involved in immunity (9–11). Thirteen functional Siglecs have been identified so far in great apes, of which humans lack Siglec-13 (12). CD33rSiglecs (CD33rSiglecs) are a major subset of the Siglec family, showing a higher degree of mutual sequence similarity, and many have been shown to associate with protein tyrosine phosphatase SHP-1 via immunoreceptor tyrosine-based inhibitory motif (ITIM). In fact, antibody (Ab)-mediated cross-linking of CD33rSiglecs leads to negative regulation of cellular activities, such as cell death or reduced cell proliferation (13–17). Most CD33rSiglec genes are clustered on a defined chromosomal region, *i.e.*, a Siglec gene cluster (located on cytological band 19q13.3–13.4 in human, near the gene

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clusters containing KIR and LILR genes), indicating that this subset has expanded via repeated gene duplications (12, 18). Sequence comparison of Siglec gene clusters in five mammalian species revealed that CD33rSiglecs have undergone rapid evolution via multiple mechanisms, including gene duplications, deletions and chimerism, as well as possible positive selection in the domain involved in glycan recognition (12). We also recently demonstrated human-specific gene conversion between *SIGLEC11* and the adjacent pseudogene *SIGLECP16*, resulting in human-specific expression of Siglec-11 in brain microglia (19).

Siglec-5, also known as CD170, is a CD33rSiglec reported to be expressed on granulocytes and monocytes (20), as well as on plasmacytoid dendritic cells, monocyte-derived dendritic cells, and macrophages (21). Siglec-5 is potentially involved in the negative regulation of innate immune responses (22), and also suggested to be a useful diagnostic and therapeutic marker for acute myelogenous leukemia (23). In a previous study we identified *SIGLEC5**, a genomic segment adjacent to *SIGLEC5* gene in the Siglec gene cluster that showed extreme sequence identity with a part of *SIGLEC5* (12, 18). However, it was unclear if this genomic segment is a part of an active genetic element. It was evident that the genomic region downstream of *SIGLEC5** lacked sequences similar to the exon containing ITIM typically found in CD33rSiglecs, suggesting a possibility that the Siglec protein encoded by *SIGLEC5**, if any, may have a different signaling potential from that of Siglec-5, or any other human CD33rSiglecs.

Here we show that this segment is in fact a part of a novel Siglec gene, *SIGLEC14*. We propose that Siglec-5 and Siglec-14 are paired inhibitory and activating receptors, by comparative analyses of the expression profiles and glycan binding preferences of Siglec-14 and Siglec-5, and demonstration of Siglec-14 association with the DAP12 activating adaptor protein. We also demonstrate that *SIGLEC14* and *SIGLEC5* have undergone concerted evolution via gene conversion in multiple primate species. We discuss the evolutionary dynamics behind the birth of Siglec-14 and its concerted evolution with Siglec-5, based on these results.

MATERIALS AND METHODS

Cloning of human Siglec-14

We previously identified a segment adjacent to *SIGLEC5* gene in human genomic DNA sequence that showed near-complete (>99%) sequence identity with *SIGLEC5*, and named it *SIGLEC5** (18). By basic local alignment search tool (BLAST) search using the putative exon 4 sequence (5'-atgctccagaaacctgcatcagcatctctcagaaatggcacagccacag-3') unique to *SIGLEC5** as query, an expressed sequence tag (EST) clone (IMAGE ID: 5756894; GenBank accession number: BM922958) that could contain a cDNA for Siglec-5* was identified. The corresponding physical clone was obtained from OpenBiosystems (Birmingham, AL, USA), and its plasmid insert sequenced. This plasmid contained a full-length

cDNA of a novel putative Siglec, which we named Siglec-14 (GenBank accession number: AY854038).⁴

Full-length protein expression construct

The open reading frame of Siglec-14 was amplified by polymerase chain reaction (PCR) using the plasmid containing full-length Siglec-14 cDNA described above as a template, Phusion DNA polymerase (Finnzymes, Espoo, Finland, USA) and the primer pair HsSig5/14 Expr F (5'-ccctctagaccaccATGCTGCCCCCTGCTGCTGCTGCC-3'; *Xba*I site underlined) and HsSig14 Full R (5'-cccgaagctTCAGCCAGGCCTCTCAGCCC-3'; *Hind*III site underlined). The PCR product was digested with *Xba*I and *Hind*III, cloned into *Xba*I-*Hind*III sites of pcDNA3.1(-) (Invitrogen, Carlsbad, CA, USA), and the insert sequence was confirmed. The resulting construct was named Siglec-14/pcDNA. An expression construct for Siglec-14 R362A point mutant was prepared using QuickChange II Site-directed Mutagenesis Kit (Stratagene, San Diego, CA, USA), following the manufacturer's instructions. Siglec-14/pcDNA was used as template, and primers Sig14 R362A S (5'-CTCGTCCCTCACCTGATCgcccGGGGCTCTCATGGGGGCT-3'; Ala codon indicated with lowercase letters) and Sig14 R362A AS (complementary to Sig14 R362A S) were used in PCR. The entire coding sequence of Siglec-14 was sequence-verified to ascertain the introduction of the desired mutation and absence of any additional mutations. The resulting construct was named Siglec-14 (R362A)/pcDNA. We also prepared Siglec-14/pIRES2-EGFP by subcloning *Pme*I fragment (containing full-length cDNA) of Siglec-14/pcDNA into *Sma*I site of pIRES2-enhanced GFP (Clontech, Palo Alto, CA, USA), and the direction of the insert was verified. A similar construct named Siglec-5/pIRES2-EGFP, with full-length Siglec-5 cDNA instead of Siglec-14, was also prepared.

Soluble Siglec-14-Fc fusion protein expression construct

The extracellular domain-coding segment (containing 3 Ig-like domains) of Siglec-14 cDNA was amplified by PCR using the plasmid containing full-length Siglec-14 cDNA as a template, Pfu DNA polymerase (Stratagene) and the primer pair HsSig5/14 Expr F (see above) and HsSig14 Fc R (5'-atcGGAAGAGGAGCTTCTCTGCACA-3'; *Eco*R V half-site underlined). The PCR product was digested with *Xba*I, cloned into *Xba*I-*Eco*R V sites of EK-Fc/pcDNA3.1(-) (24), and the insert sequence was confirmed. The resulting construct was named Siglec-14-Fc/pcDNA. A similar construct for expression of Siglec-5-Fc fusion protein (containing the first 3 Ig-like domains) was prepared by PCR using the primer pair HsSig5/14 Expr F and HsSig5 Fc R (5'-atcGAGGGAGTAACTGAGAGATTCAGA-3'; *Eco*R V half-site underlined) in the same manner. The resulting construct was named Siglec-5-Fc/pcDNA.

Preparation of Siglec-Fc fusion proteins

Recombinant Siglec-14-Fc and Siglec-5-Fc proteins were prepared in a similar manner as described previously (24). Briefly, 293T cells were transfected with Siglec-14-Fc/pcDNA or Siglec-5-Fc/pcDNA using LipofectAMINE 2000. Culture medium was changed next day to 2% low IgG-FBS (HyClone, Logan, UT, USA) in Opti-MEM (Invitrogen), and the cells were cultured for 6 days with a medium exchange at day 3. Collected culture medium was clarified of debris by centrifugation, and Fc fusion proteins were purified by absorption to protein A-Sepharose (Amersham Biosciences, Arlington Heights, IL, USA), elution with 0.1 M glycine-HCl buffer, pH 3.0, followed by immediate neutralization with 1/10 volume of 1 M Tris-HCl buffer, pH 8.0.

Glycan binding assay using human Siglec-5 and Siglec-14 recombinant proteins

Binding analysis using recombinant Siglec-Fc proteins and biotinylated polyacrylamide probes multiply substituted with sialylated oligosaccharides (PAA-Bio; Glycotech, Rockville, MD, USA) was performed as described previously (25, 26). In brief, each well of 96-well plate (Nunc #269620) was coated with 100 μ l of 5 μ g/ml protein A (Sigma, St. Louis, MO, USA) in 50 mM sodium bicarbonate buffer, pH 9.5 at 4°C overnight, washed twice with ELISA buffer (20 mM HEPES-NaOH buffer, pH 7.45, 125 mM NaCl, 0.02% NaN₃, 1% BSA), and blocked with ELISA buffer at room temperature for 1 h. Thereafter, wells were sequentially incubated at room temperature, followed by three washes with ELISA buffer (150 μ l/well) after each step, with (100 μ l/well in each case): recombinant Siglec-14-Fc, Siglec-5-Fc or human IgG (as negative control) in ELISA buffer (2 μ g/ml) for 2 h; PAA-Bio probes in ELISA buffer (5 μ g/ml) for 2 h; streptavidin-alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA, USA) in ELISA buffer (1 μ g/ml) for 1 h. Finally, 100 μ l/well of alkaline phosphatase substrate (10 mM *p*-nitrophenyl phosphate in 100 mM Na₂CO₃, 1 mM MgCl₂) was added to each well and color development was monitored at 405 nm with VersaMax tunable microplate reader (Molecular Devices, Palo Alto, CA, USA). Binding between each receptor-ligand pair was tested in triplicate wells.

Analysis of Siglec-5 and Siglec-14 mRNA expression

Expression analysis of Siglec-5 and Siglec-14 mRNA was done by RT-polymerase chain reaction (RT-PCR) using human tissue total RNA panel (Clontech) as templates. Oligo dT (dT₁₂₋₁₈)-primed first-strand cDNA was synthesized from total RNA (0.2 μ g) by reverse-transcription using Superscript II (Invitrogen) following the manufacturer's instructions, and used in the PCR reactions. Each reaction tube contained the following (in 25 μ l): first-strand cDNA (equivalent of 10 ng RNA); primers, 0.2 μ M each; dNTP, 0.2 mM each; ExTaq DNA polymerase, 0.5 U (Takara, Otsu, Shiga, Japan); and 1 \times ExTaq PCR buffer (Takara). Thermal cycling parameters were as follows: 94°C, 2 min; (95°C, 15 s; 60°C, 1 min) \times 35 cycles. The following primers were used in the PCR: Sig5 RealT F (5'-TCTGTATCTGTCTGTGCCTCATCTT-3') and Sig5 RealT R (5'-GGGTCTTCATCATCCATTTTCTCT-3') for Siglec-5 transcript amplification; Sig14 RealT F (5'-TTCCTCGCCTGCACAGTTG-3') and Sig14 RealT R (5'-TTTCCTCCCGGAACCA-3') for Siglec-14 transcript amplification. PCR products were separated by agarose gel electrophoresis, the gel was stained with ethidium bromide and photographed under an UV illuminator.

Analysis of cross-reactivity of anti-Siglec-5 monoclonal antibodies with Siglec-14

293T cells were transfected with Siglec-5/pIRES2-EGFP, Siglec-14/pIRES2-EGFP or pIRES2-EGFP using LipofectAMINE 2000. Cells were washed, lifted and dispersed with 10 mM EDTA in PBS 24 h after transfection. Dispersed cells were incubated with one of the commercial mouse monoclonal antibodies against Siglec-5 (R&D Systems, Minneapolis, MN, USA; clones 194111, 194117, and 194128; 0.2 μ g/10⁶ cells) or with one of the privately prepared antibodies (clones IA5 and 8H2, kindly provided by Paul Crocker, University of Dundee, UK; 1 μ l hybridoma culture sup/10⁶ cells) in 1% BSA/PBS on ice for 30 min, then with Alexa Fluor 750-allophycocyanin goat antimouse IgG (Molecular Probes; Eugene, OR, USA; 0.1 μ g/10⁶ cells) in 1% BSA/PBS on ice for 30 min. Cells

were washed twice with 1% BSA/PBS, suspended in 1 μ g/ml propidium iodide in 1% BSA/PBS, and analyzed with FACSAria (BD Cytometry Systems, San Jose, CA, USA). Cells positive for green fluorescent protein, representing the cells expressing Siglec-IRES-EGFP bicistronic mRNA, were gated for the analysis of Ab staining.

Preparation of C-terminal FLAG-tagged human DAP12 expression construct

DAP12 cDNA was amplified by PCR from first strand cDNA prepared from THP-1 human monocytic cell line with primers DAP12 F1 (5'-CAGCATCCGGCTTCATGG-3') and DAP12 R1 (5'-TGCTGACTGTCATGATTCGGG-3'), and product was purified by agarose gel electrophoresis. The purified product was subjected to nested PCR with primers DAP12 ExprF (5'-cccctcgagccaccATGGGGGACTTGAACCCTG-3'; *Xho*I site underlined) and DAP12 R FLAG (5'-ggggaattcTTACTTGTGTCATCGTCGTCCTTGTAGTCTTTGTAATACGGCCTCTGTGTG-3'; *Eco*R I site underlined, complementary FLAG tag-coding sequence *Italicized*), digested with *Xho*I and *Eco*R I and cloned into pBluescript (Stratagene). The *Xho*I-*Eco*R I fragment of a sequence-verified clone was subcloned into pcDNA3.1(-), and the resulting plasmid was named DAP12-FLAG/pcDNA.

Coimmunoprecipitation of DAP12-FLAG and Siglec-14

The plasmid DAP12-FLAG/pcDNA was transfected to 293T cells singly or in combination with Siglec-14/pcDNA or Siglec-14 (R362A)/pcDNA using LipofectAMINE 2000 reagent (Invitrogen). The cells were lysed in lysis buffer (10 mM Tris-HCl buffer, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA with protease inhibitor cocktail) 24 h after transfection, precleared with centrifugation, and the supernatant was used for immunoprecipitation with monoclonal anti-FLAG Ab conjugated to agarose beads (ANTI-FLAG M2 Affinity Gel, Sigma) overnight at 4°C. The proteins precipitated with the beads were resolved by SDS-PAGE and blotted to a PVDF membrane. The membrane was blocked and incubated with either 1) biotinylated goat anti-human Siglec-5 polyclonal antibody (pAb), followed by peroxidase-labeled streptavidin (Amersham), or 2) rabbit anti-FLAG pAb, followed by peroxidase-labeled goat anti-rabbit IgG (Sigma). The Ab binding signals were detected by using enhanced chemiluminescence (ECL) Western blot Detection Reagents (Amersham).

Sequencing of great ape *SIGLEC5* and *SIGLEC14* loci

Using the genomic DNA prepared from EBV-transformed great ape B cells (kindly provided by Peter Perham, Stanford University) as templates, ~1.5 kb genomic DNA fragments containing first 4 exons of *SIGLEC5* and *SIGLEC14* were amplified by PCR using the following primer pairs: Sig5-F (5'-CTCAGCTGGACAAGCCTCAC-3') and Sig5-R (5'-CCTGAAGATGGTGATGGTCTG-3') for *SIGLEC5*; Sig5*-F (5'-CAAGTCTGGACAAGCCCTC-3') and Sig5*-R (5'-GATGCTGATGGCGAGGTTCTG-3') for *SIGLEC14*. The PCR products were directly sequenced [Data deposition: AY854038 (Human Siglec-14 cDNA), DQ238604 and DQ238605 (Gorilla *SIGLEC5* and *SIGLEC14*), DQ238606 and DQ238607 (Orangutan *SIGLEC5* and *SIGLEC14*)] (see Supplemental Table 1 for the primer sequences).

Molecular phylogenetic analysis

Genetic distances were calculated with multiple-hit corrections (27). Phylogenetic trees were constructed by using the

neighbor-joining method (28) and reliability assessed by bootstrap values with 1,000 replications.

Timing estimation of the most recent gene conversion in each lineage

Intronic regions were used for the timing calculations. The genetic distance of the intronic parts downstream of converted region was assumed to be the human-chimpanzee standard genetic distance around *SIGLEC5* and *SIGLEC14* loci. The human-chimpanzee divergence time is considered as ~6 million years (myr) based on the fossil findings (29, 30). By assuming that the human-chimpanzee standard distance corresponds to 6 myr, the timing of gene conversion was calculated in each lineage.

RESULTS

Identification and cloning of human Siglec-14

We previously reported the presence of a genomic DNA segment located upstream of human *SIGLEC5* that shows >99% identity over ~1.3 kb with the 5'-end of *SIGLEC5* containing exons 1–3, and named it *SIGLEC5** (18). This region is followed by a short (TG)_n tract and another short segment (~0.2 kb) showing a lesser sequence identity (78%, excluding indels) with a part of *SIGLEC5* containing exon 4 (Fig. 1). The entire region showing high sequence identity with *SIGLEC5* is ~1.5 kb, and the high-identity region abruptly ceases at both 5' and 3' ends. A homology searching of nucleotide sequence database using the putative exon 4 sequence of *SIGLEC5** as a query revealed the presence of a cDNA clone (IMAGE ID: 5756894) representing this locus. Sequencing of the insert of this clone revealed an open reading frame encoding a novel Siglec, which we named Siglec-14. Thus, *SIGLEC5** is now renamed *SIGLEC14* (Fig. 1).

Human Siglec-14 is a type-I transmembrane protein with 396 amino acid residues, consisting of three Ig-like domains, a transmembrane domain and a very short cytoplasmic tail (Fig. 2). As expected from the unusual sequence identity of the genomic segments containing exons 1–3 of Siglec-14 and Siglec-5 mentioned above, the predicted amino acid sequence of the signal peptide and the first 2 Ig-like domains of Siglec-14 is nearly identical with that of Siglec-5 (Fig. 2). The third Ig-like domain and beyond, however, was unique to Siglec-14.

An existing entry in GenBank was found to contain a partial cDNA of Siglec-14 (GenBank accession number: AY358369), but lacked the first 4 nucleotides of the open reading frame. However, the 3'-end sequence of this entry did not match that of standard human genome sequence. Whether this discrepancy is due to polymorphism in human population or sequencing/assembly error remains an open question.

Expression pattern of Siglec-14 mRNA is similar to that of Siglec-5

RT-PCR analysis of human tissue total RNA revealed that Siglec-14 mRNA was expressed mainly in hemato-

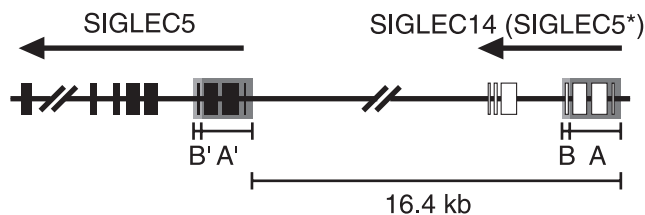


Figure 1. Schematic representation of the human genomic region containing *SIGLEC14* and *SIGLEC5*. Open and closed boxes represent exons of *SIGLEC14* and *SIGLEC5*, respectively. The regions A and B in *SIGLEC14* and A' and B' in *SIGLEC5* are indicated with dark shadows (A/A') and light shadows (B/B'). A and A' show >99% sequence identity, while B and B' show 78% identity (excluding indels).

poietic tissues, such as bone marrow, spleen and fetal liver, while some expression was also detected in lung and testis (Fig. 3). This expression pattern generally paralleled that of Siglec-5 mRNA (Fig. 3). This result suggests that Siglec-5 and Siglec-14 are expressed simultaneously in the same tissues, possibly on the same cells.

Siglec-14 shows glycan binding preference similar to Siglec-5

Because of the near-identity between Siglec-5 and Siglec-14 in the first 2 Ig-like domains involved in glycan recognition (Fig. 2), glycan binding preferences of these Siglecs are predicted to be similar. This was the case indeed, as demonstrated using recombinant soluble Siglec proteins (Fig. 4). However, Siglec-14-Fc showed stronger binding signals compared with Siglec-5-Fc, suggesting that Siglec-14 has more robust glycan binding property. This conclusion was further supported by the fact that COS-7 cells expressing full-length Siglec-14 showed more robust erythrocyte binding compared with Siglec-5-expressing cells (data not shown). Taken together, Siglec-14 and Siglec-5 likely recognize the same set of ligands containing sialic acids, with different avidity. The difference in binding avidity between Siglec-5 and Siglec-14 may be explained by the difference in the length of linker peptide connecting Ig-like domains 2 and 3 (Siglec-5 has 13-amino acid linker, while Siglec-14 has 18-amino acid linker), which may affect the orientation of the glycan binding domains. However, we cannot rule out the possibility that it is due to the difference in protein stability or folding efficiency.

Siglec-14 associates with DAP12 via an arginine residue in transmembrane domain

The cytoplasmic tail of Siglec-14 lacks ITIM typically found in CD33rSiglecs, but its transmembrane domain contains a positively charged residue (Arg³⁶²). This configuration appears to be similar to that of some activating members of paired receptors that associate with adapter molecules such as DAP12 (31). To test if Siglec-14 associates with DAP12, we expressed Siglec-14 and FLAG-tagged DAP12 in 293T cells, and analyzed

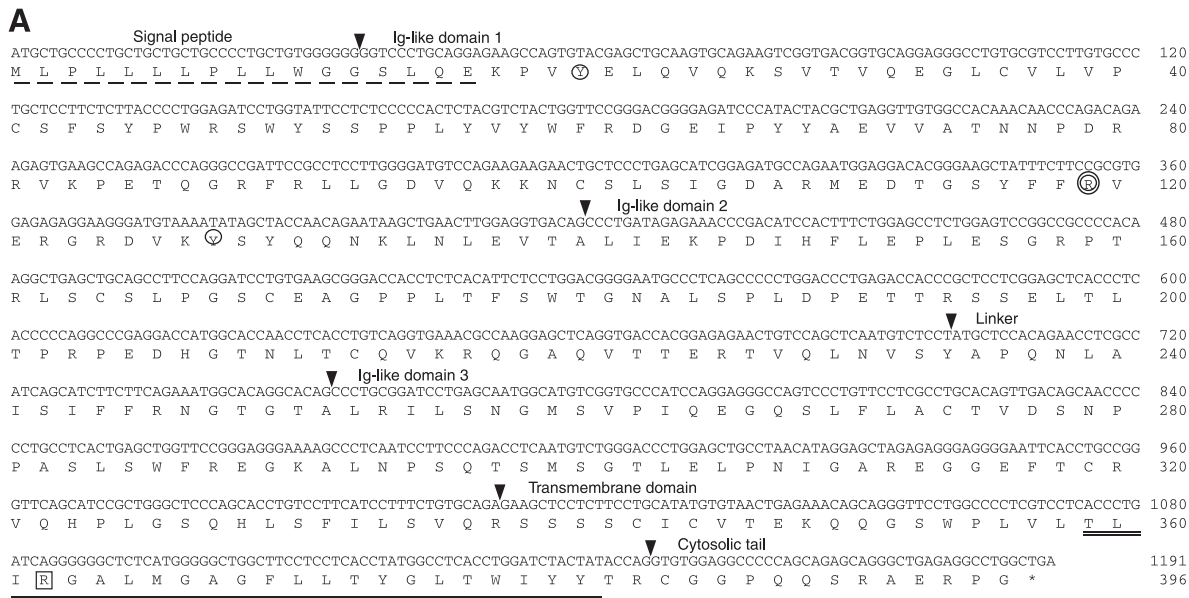


Figure 2. Primary sequence of Siglec-14. *A*) Nucleotide and amino acid sequences of human Siglec-14. Double circle: ‘‘essential’’ arginine residue involved in sialic acid recognition; circle: conserved aromatic amino acid involved in sialic acid recognition; square: the arginine residue in the transmembrane domain; dotted underline: signal peptide; double underline: transmembrane domain; arrowhead: exon junction. *B*) Sequence comparison of Siglec-14 and Siglec-5. The arrowheads represent domain borders defined by exon junctions. The sequences of the first and second Ig-like domains of Siglec-14 and Siglec-5 are nearly identical, while those in and beyond the third Ig-like domain are quite different.

their association by immunoprecipitation. Immunoprecipitates by an anti-FLAG Ab contained Siglec-14, and Siglec-14 R362A mutant showed negligible association with DAP12 (Fig. 5). Therefore, Siglec-14 can associate with DAP12 via its arginine residue in transmembrane domain. This result suggests that Siglec-14 functions as an activating receptor.



Figure 3. Tissue distribution of Siglec-14 and Siglec-5 transcripts. RNA samples from various human tissues were analyzed by RT-PCR as described in Materials and Methods. Siglec-14 mRNA was strongly expressed in hematopoietic tissues (bone marrow, spleen and fetal liver). The distributions of Siglec-14 and Siglec-5 transcripts were similar.

Most anti-Siglec-5 antibodies cross-react with Siglec-14

Because of the extreme sequence identity of Siglec-5 and Siglec-14 at the amino-terminal region, some of the anti-Siglec-5 antibodies may cross-react with Siglec-14. By expressing Siglec-5 or Siglec-14 full-length protein on 293T cells and probing with anti-Siglec-5 antibodies, we found that 4 out of the 5 monoclonal antibodies (clones 194128, 194111, 1A5, and 8H2) against Siglec-5 cross-react with Siglec-14 (Fig. 6). Although the result was ambiguous with regard to one of the antibodies (clone 194117), Western blot analysis of Siglec-5- and Siglec-14-Fc fusion proteins with this Ab revealed that it is actually specific to Siglec-5 (data not shown), suggesting that it recognizes a cryptic epitope that is better exposed by denaturation. We also performed a standard ELISA of immobilized Siglec-5- and -14-Fc fusion proteins to assess Ab cross-reactivity, which essentially confirmed the results stated above (data not shown). Therefore, some prior reports of the expression pat-

tern of Siglec-5 in humans might represent Siglec-5 expression, Siglec-14 expression, or both.

SIGLEC5 and SIGLEC14 have undergone gene conversions in primates

The extreme sequence identity between the ~1.3 kb segment of human *SIGLEC14* and *SIGLEC5* (designated A in *SIGLEC14* and A' in *SIGLEC5*; Fig. 1) is most likely explained by a recent gene conversion between these loci in the human lineage. A part of *SIGLEC14* in chimpanzee and baboon genomes also shows extreme sequence identity with a part of *SIGLEC5* in the respective species (12). The boundaries for the *SIGLEC14* and *SIGLEC5* genomic regions showing high sequence identity (corresponding to the region A/A' in human *SIGLEC14*/*SIGLEC5* pair) were approximately the same in all three species including humans. We therefore hypothesized that similar gene conversions may have also happened in other primates. To test this hypothesis we amplified and sequenced corresponding genomic DNA fragments from gorilla and orangutan, aligned them with equivalent sequences in human, chimpanzee, and baboon (Supplemental Fig. 1), and used in the reconstruction of molecular phylogenetic tree. Remarkably, our analysis revealed that region A of *SIGLEC14* in every species shows closer sequence identity with region A' of *SIGLEC5* in respective species, rather than with region A of *SIGLEC14* in other species (Fig. 7). This fact strongly suggests that at least one independent gene conversion event involving *SIGLEC5* and *SIGLEC14* has taken place in each of the five primate lineages examined in this study.

The intraspecies genetic distances of intronic part of the converted region between *SIGLEC14* and *SIGLEC5* are 0.009 ± 0.005 in human, 0.000 ± 0.000 in chim-

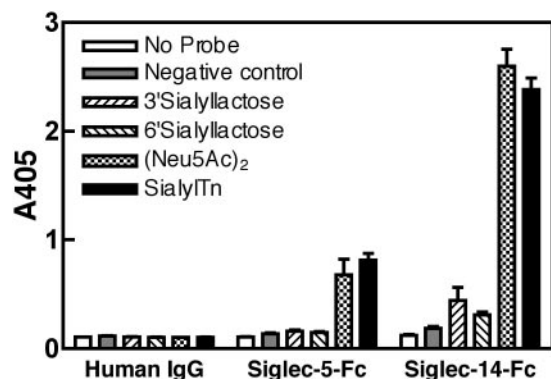


Figure 4. Glycan binding preference of Siglec-14 and Siglec-5. Binding of polymer probes substituted with multiple copies of oligosaccharides (indicated in the inset) to recombinant Siglec-Fc fusion proteins immobilized onto 96-well plate was analyzed as described in Materials and Methods. Both Siglec-14 and Siglec-5 showed preference toward (Neu5Ac)₂ and Sialyl-Tn structures. The binding signal for Siglec-14 was stronger than that for Siglec-5. Error bars represent sd. 3'Sialyllactose, Neu5Ac α 2-3Gal β 1-4Glc; 6'Sialyllactose, Neu5Ac α 2-6Gal β 1-4Glc; (Neu5Ac)₂, Neu5Ac α 2-8Neu5Ac; Sialyl-Tn, Neu5Ac α 2-6GalNAc.

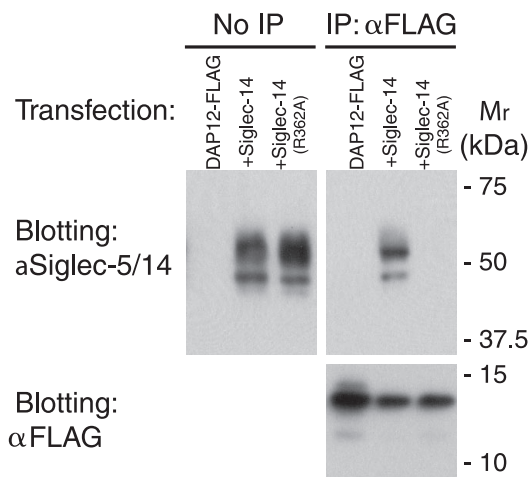


Figure 5. Association of Siglec-14 with DAP12. 293T cells were transfected with FLAG-tagged DAP12 protein expression construct alone or with Siglec-14 (wild-type) or with Siglec-14 (R362A) expression construct. Cell lysates were subjected to SDS-PAGE and Western blot either before immunoprecipitation (No IP) or after immunoprecipitation with immobilized anti-FLAG Ab (IP: α FLAG). Membranes were probed either with anti-Siglec-5/14 pAb (blotting: α Siglec-5/14) or with anti-FLAG pAb. (blotting: α FLAG). While approximately equal amounts of Siglec-14 (wild-type) and Siglec-14 (R362A) are expressed, only the wild-type (WT) Siglec-14 was coprecipitated with DAP12. Data shown is representative of three experiments.

panzee, 0.016 ± 0.007 in gorilla, 0.022 ± 0.008 in orangutan, and 0.012 ± 0.006 in baboon (Table 1). The genetic distance of the intronic part of nonconverted region between human and chimpanzee *SIGLEC5* is 0.019 and can be considered as the human-chimpanzee standard distance (HCSD) of the genomic region around *SIGLEC14* and *SIGLEC5*. Assuming this standard genetic distance (0.019) corresponds to 6 myr, the timing of gene conversion in each lineage was calculated as follows: 2.92 ± 1.70 myr in the humans, 4.87 ± 2.20 myr in the gorillas, 6.88 ± 2.61 myr in the orangutans, and 3.90 ± 1.95 myr in the baboons. The timing of the gene conversion in chimpanzee lineage was calculated as 0 myr, because the short (~300 bp) intronic part used in the calculation was identical between *SIGLEC14* and *SIGLEC5*, suggesting a very recent gene conversion event.

Most SIGLEC14 and SIGLEC5 alleles in great apes have “the essential arginine” mutated

We found that in all great apes other than humans, the “essential arginine” residue, required for optimal sialic acid recognition (9–11) is mutated in both Siglec-14 and Siglec-5. In fact, this “essential arginine” codon is conserved only in one allele of gorilla *SIGLEC14*. As we have reported in our previous paper (12), arginine-mutated (wild-type) chimpanzee Siglec-5 showed much reduced (by ~80%) binding to the sialylated glycans compared with an artificial arginine-restored counterpart.

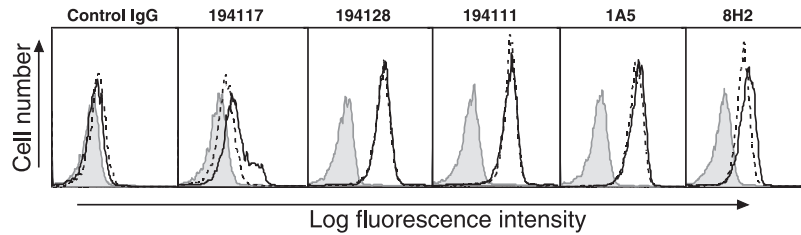


Figure 6. Cross-reactivity of anti-Siglec-5 monoclonal antibodies with Siglec-14. Cells transfected with Siglec-14/pIRES2-EGFP (dotted line), with Siglec-5/pIRES2-EGFP (solid line), or with pIRES2-EGFP (shaded) were probed with available monoclonal antibodies and analyzed by flow cytometry. The populations positive for green fluorescent protein were gated and plotted. Most monoclonal antibodies, except for the clone 194117 (R&D Systems), recognized both Siglec-5 and Siglec-14.

Because of the difficulty in reconstructing the evolutionary path of a region undergoing frequent gene conversions, it is not feasible to determine if the presence of the “essential arginine” residue in human Siglec-14 and Siglec-5 is actually a result of conservation or that of “restoration” by a reverse-mutation. Nevertheless, this seems to be yet another example where humans differ from great apes in an aspect of sialic acid biology.

Direction of gene conversion between *SIGLEC14* and *SIGLEC5*

It was proposed that an activating receptor (Ly49H) was generated from an inhibitory receptor (Ly49I-like ancestor) to counter a viral pathogen that exploit the latter (32), and that this mechanism may apply to many other activating receptors that have inhibitory counter-

parts (33). Assuming that an inhibitory receptor recognize endogenous ligand(s) while its activating counterpart functions as pathogen recognition molecule (33), and that the threat by the pathogen that initially led to the birth of the latter persists, then selective pressures will force the latter to stay similar to the former (not the other way around), so that the former remains functional while the latter provides effective protection against the pathogen that may co-evolve with the former. If this model is widely applicable, the direction of gene conversions between *SIGLEC5* and *SIGLEC14* should be constant, *i.e.*, the former should convert the latter. To determine the direction of gene conversion between regions A in *SIGLEC14* and A' in *SIGLEC5* in the human and chimpanzee lineages, we analyzed the relationships among the HCSD and interspecies distances of intronic parts of A and A'. If the direction of gene conversion was the same in both human and chimpanzee lineages, the *interspecies* distances between two homologs (*i.e.*, the distances between hA-cA, hA'-cA', hA-cA', and hA'-cA) should be the same as HCSD (Supplemental Fig. 2). On the other hand, if the directions were the opposite, *interspecies* distances between two homologs become larger than the HCSD (Supplemental Fig. 2). Actual data suggest that the latter is the case (*i.e.*, the gene conversion in the human and chimpanzee lineages occurred in the opposite directions).

We next compared the position of 5' conversion boundaries of A/A' in human and chimpanzee. The distribution of nucleotide differences suggests that the 5' gene conversion boundary is around site 40 and 150 for human and chimpanzee lineages, respectively (Supplemental Fig. 1). Thus, the region between the sites 40 and 150 (40–150 region) underwent the gene conversion only in the human lineage. The 40–150 region has 10 different sites and one 4-bp insertion/deletion difference between the two chimpanzee loci. Of these, 5 contiguous sites (sites 103, 115, 117, 119 and 137) in the 3' half (sites 101–150) of 40–150 region of chimpanzee *SIGLEC14* are the same as those of human loci, suggesting that *SIGLEC14* converted *SIGLEC5* in the human lineage. On the other hand, the 5' half (sites 40–100) of 40–150 region of chimpanzee *SIGLEC5* shows concentrated distribution of sites that are the same as those of human loci, suggesting that *SIGLEC5*

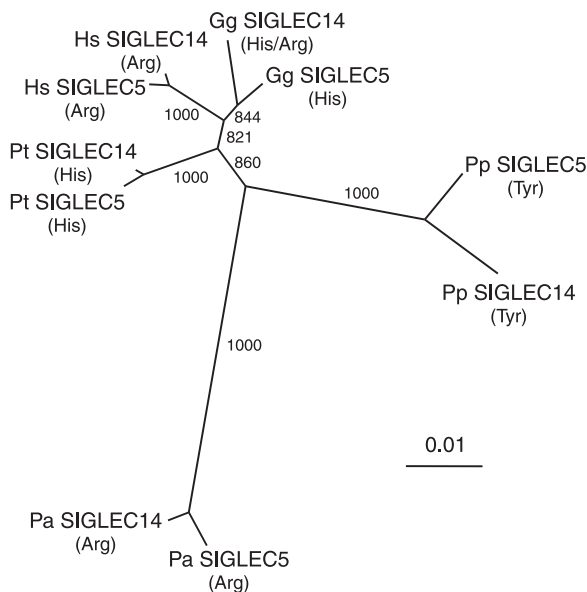


Figure 7. Molecular phylogenetic tree of regions A (*SIGLEC14*) and A' (*SIGLEC5*) of Old World primates. The numbers besides internodes represent bootstrap support for 1,000 replications. Below each gene is indicated the amino acid residue occupying the site of the “essential arginine” residue. Hs, *Homo sapiens* (human); Pt, *Pan troglodytes* (chimpanzee); Gg, *Gorilla gorilla* (gorilla); Pp, *Pongo pygmaeus* (orangutan); Pa, *Papio anubis* (baboon).

TABLE 1. *Pairwise genetic distances of the converted intronic parts between SIGLEC5 and SIGLEC14*

| | Hs 14 | Pt 5 | Pt 14 | Gg 5 |
|-------|---------------|---------------|---------------|---------------|
| Hs 5 | 0.009 ± 0.005 | 0.031 ± 0.010 | 0.031 ± 0.010 | 0.022 ± 0.008 |
| Hs 14 | | 0.028 ± 0.010 | 0.028 ± 0.010 | 0.025 ± 0.009 |
| Pt 5 | | | 0.000 ± 0.000 | 0.028 ± 0.010 |
| Pt 14 | | | | 0.028 ± 0.010 |
| Gg 5 | | | | |
| Gg 14 | | | | |
| Pp 5 | | | | |
| Pp 14 | | | | |
| Pa 5 | | | | |

converted *SIGLEC14* in the human lineage. Taken together, it is likely that the conversion of *SIGLEC5* by *SIGLEC14* in the region downstream of site 100 in the human lineage occurred first, then the conversion in the opposite direction in the region downstream of site 40. Taken together, the data suggest that directions of gene conversions between *SIGLEC14* and *SIGLEC5* have not always been the same, even in a single lineage.

DISCUSSION

Here we described the discovery of a novel Siglec, Siglec-14, that has undergone concerted evolution with Siglec-5 in multiple primate lineages. The extreme sequence identity between human Siglec-14 and Siglec-5, similarity in their tissue distributions and ligand recognition properties, and their counteracting signaling properties justifies calling them “paired receptors.” Although some paired receptors belong to C-type lectin family (e.g., Ly49 and CD94/NKG2), none of them has been definitively proven to recognize glycans. To our knowledge Siglec-14 and Siglec-5 are the first examples among paired receptors that have clear glycan recognition property.

Siglec-14 is also the first human CD33rSiglec shown to have an activating signaling potential. Most CD33rSiglecs in primates and rodents have ITIMs, and many of them associate with SHP-1 and are involved in inhibitory signaling. The exceptions are rodent CD33 and Siglec-H, both of which lack canonical ITIMs but have a lysine residue in the transmembrane domain (11). It is therefore possible that these rodent Siglecs may also associate with activating adapter protein and have functions similar to those of Siglec-14. In fact, it was recently reported that mouse Siglec-H also associates with DAP12 (34), although it appears to be unable to recognize glycans that were tested (35). We previously reported the minimal phenotype of CD33 null mice and discussed the lack of significant phenotype in the context of the lack of ITIM in mouse CD33 (36). In retrospect, it is possible that the studies done on these mice were not optimal to observe immune dysregulation as consequences of CD33 deletion, and the mouse

model warrants further investigation in the light of CD33’s possible function as a stimulatory rather than an inhibitory receptor.

In our previous studies we proposed that the loss of Neu5Gc in humans (37–39) caused profound changes in Siglec biology, such as altered distribution of sialoadhesin/Siglec-1 (which preferentially bind Neu5Ac) (40), elimination of sialic acid binding ability of Siglec-XII (which preferentially bind Neu5Gc) (41), and adaptive evolution of some CD33rSiglecs (change from strong Neu5Gc preference to accommodation of Neu5Ac) (42). Given that Siglec-14 and Siglec-5 in great apes lack the essential arginine residue whereas human counterparts have it, it is tempting to speculate that human Siglec-14 and Siglec-5 prefer ligand(s) containing Neu5Ac exclusively, which increased in quantity in humans as a consequence of loss of Neu5Gc. One such candidate is α 2–8-linked oligo Neu5Ac (43), which is a preferred ligand for both human Siglec-14 and Siglec-5 (Fig. 4). Quantitative studies of the presence of α 2–8-linked oligo Neu5Ac in great ape and human tissues will be necessary to test this hypothesis. Our future investigation will focus on the search for the endogenous ligand(s) of Siglec-14 and Siglec-5, and a mechanism by which the recognition of ligands by Siglec-14 and Siglec-5 coordinately regulate innate immunity.

There is still no definitive functional explanation for why paired receptors exist or why they should be present in pairs. As mentioned, it has been proposed that an activating receptor may have initially evolved from an inhibitory counterpart, because of a selective advantage in controlling the virus that exploit the inhibitory receptor (32). By an analogy, bacterial pathogens that carry sialic acid (e.g., some strains of *Neisseria meningitidis* shown to interact with Siglec-5 via sialylated lipopolysaccharides; ref. 44) might have imposed such a selective pressure, and led to the emergence of Siglec-14. Under the assumptions that 1) an inhibitory receptor recognizes endogenous ligand(s) while its activating counterpart functions mainly as a “pathogen receptor,” and that 2) the threat of the pathogen that led to the birth of the latter persists, we would expect that the inhibitory receptor undergoes

TABLE 1. (continued)

| Gg 14 | Pp 5 | Pp 14 | Pa 5 | Pa 14 |
|---------------|---------------|---------------|---------------|---------------|
| 0.025 ± 0.009 | 0.048 ± 0.012 | 0.064 ± 0.015 | 0.064 ± 0.015 | 0.064 ± 0.015 |
| 0.028 ± 0.010 | 0.051 ± 0.013 | 0.061 ± 0.014 | 0.061 ± 0.014 | 0.061 ± 0.014 |
| 0.031 ± 0.010 | 0.035 ± 0.011 | 0.044 ± 0.012 | 0.058 ± 0.014 | 0.058 ± 0.014 |
| 0.031 ± 0.010 | 0.035 ± 0.011 | 0.044 ± 0.012 | 0.058 ± 0.014 | 0.058 ± 0.014 |
| 0.016 ± 0.007 | 0.031 ± 0.010 | 0.048 ± 0.012 | 0.061 ± 0.014 | 0.061 ± 0.014 |
| | 0.041 ± 0.012 | 0.058 ± 0.014 | 0.071 ± 0.015 | 0.071 ± 0.015 |
| | | 0.022 ± 0.008 | 0.068 ± 0.015 | 0.068 ± 0.015 |
| | | | 0.078 ± 0.016 | 0.078 ± 0.016 |
| | | | | 0.012 ± 0.006 |

The genetic distance of the nonconverted intronic region between human *SIGLEC5* and chimpanzee *SIGLEC5* was 0.019 ± 0.001 . Hs, *Homo sapiens*; Pt, *Pan troglodytes*; Gg, *Gorilla gorilla*; Pp, *Pongo pygmaeus*; Pa, *Papio anubis*. Numbers “5” and “14” represent *SIGLEC5* and *SIGLEC14*, respectively.

sequence changes to avoid exploitation by the pathogen (while maintaining its endogenous functions); the pathogen evolves to catch up with the change of the inhibitory receptor; and the activating receptor is kept similar to the inhibitory receptor so that it can be engaged by the pathogen. If gene conversion is the mechanism to keep the two receptors stay similar, then the inhibitory receptor gene should always convert the activating receptor gene. However, the directions of gene conversions between *SIGLEC14* and *SIGLEC5* have not been constant. Therefore, while the birth of Siglec-14 might have been driven by a bacterial pathogen that carries sialic acid, the concerted evolution between Siglec-5 and Siglec-14 has likely been driven by some other force.

Given that the inhibitory receptor should have endogenous ligand(s), the activating counterpart of the pair should also be able to recognize the same ligand, at least initially. A marked imbalance in the ligand binding, especially excessive binding by the activating receptor, may cause immune over-reaction leading to autoimmunity that would be detrimental to the host. Keeping the balance in ligand binding by activating and inhibitory pair within a certain range may be required, and that might have been the driving force behind concerted evolution of Siglec-14 and Siglec-5. Almost all *SIGLEC14* and *SIGLEC5* in great ape species had the “essential arginine” residue mutated, while apparently maintaining an open reading frame. This frequent occurrence of “quasi-functional” paired receptors, both of which lost optimal binding to their ligands, may be explained by the above-mentioned model. Loss of the ligand recognition caused by a point mutation of the “essential arginine” codon in *SIGLEC5*, selected for by some advantage under certain environmental conditions, may have been “repaired” by gene conversion by *SIGLEC14*, or counterbalanced by gene conversion of *SIGLEC14* by *SIGLEC5*, so that the mutation will not lead to autoimmunity. Likewise, keeping the binding specificities of these receptors might have been necessary. If this model is applicable to other paired receptors, we should find other cases of frequent gene conversions among members of paired receptors. Because paired receptor genes form gene clusters and

show close sequence similarity, conditions are favorable for such gene conversions to occur. Recent completion of the draft sequencing of chimpanzee genome (45) could provide a platform to reveal additional paired receptors that have undergone independent gene conversions in multiple primate lineages. **FJ**

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