Novel recombinant feline interferon carrying N-glycans with reduced allergy risk produced by a transgenic silkworm system

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Abstract

Background: The generation of recombinant proteins for commercialisation must be cost-effective. Despite the cost-effective production of recombinant feline interferon (rFeIFN) by a baculovirus expression system, this rFeIFN carries insect-type N-glycans, with core α1,3 fucosyl residues that act as potential allergens. An alternative method of production may yield recombinant glycoproteins with reduced antigenicity.

Results: A cDNA clone encoding the fifteenth subtype of FeIFN-α (FeIFN-α15) was isolated from a Japanese domestic cat. This clone encoded a protein of 189 amino acids with a molecular mass of 21.1 kDa. The rFeIFN-α15 was expressed using a transgenic silkworm system, which was expected to yield an N-glycan structure with reduced antigenicity compared with the protein produced by the baculovirus system. The resulting rFeIFN-α15 accumulated in the sericin layer of silk fibres and was easily extracted and purified by column chromatography. The N-terminal amino acid sequence of purified rFeIFN-α15 was identical to the mature form of natural sequence. Moreover, its N-glycans did not include detectable core α1,3 fucosyl residues. Its anti-vesicular stomatitis virus activity (2.6 × 10⁸ units/mg protein) was comparable to that of the baculovirus-expressed rFeIFN.

Conclusions: The lower allergy risk of rFeIFN produced by the transgenic silkworm system than by the baculovirus expression system is due to the former lacking core α1,3 fucosyl residues in its N-glycans. The rFeIFN-α15 produced by the transgenic silkworm system may be a prospective candidate for the next generation of rFeIFN in veterinary medicine.

Keywords: Feline interferon, Transgenic, Silkworm, N-glycan, Core α1,3 fucosyl residues, Fucose, Bombyx mori

Background

Recombinant proteins generated for medical use should retain as much of their original structures as possible, including amino acid sequences and attached glycans. Any structural changes in these proteins may affect their bioactivity and/or bioavailability and may also increase the side effects associated with their use [1–4]. To avoid these shortcomings, despite their high production costs, most recombinant proteins used in humans are produced using mammalian cell lines.

A cost-effective method of producing recombinant proteins for veterinary medicine involves the use of the silkworm moth (Bombyx mori). Larvae or insect cell lines are infected with recombinant baculovirus [5–8], with the recombinant proteins accumulating in larval body fluid or the cultured cells. The post-translational modification pathways in insects such as silkworms are similar to those in mammalian cells, and various recombinant glycoproteins have been successfully produced. For example, commercially available recombinant FeIFN-ω (NCBI Number: E02521) was produced in silkworms using the baculovirus expression system [9–12].

Despite the cost advantages of the baculovirus expression system, there is concern with the structures of the N-glycans attached to the recombinant protein products. These are insect-type N-glycans, with a high percentage of core α1,3 fucosyl residues. These core α1,3 fucosyl residues have been found to induce allergic reactions
when administered to mammals [13]. In fact, the rFeIFN-ω produced by this system is thought to contain core α 1,3 fucosylated N-glycans [14, 15]. Although the rFeIFN-ω has over 20 years of clinical experience, eliminating the insect type N-glycan is thought to be beneficial to improve the quality of veterinary medications in terms of potential allergenicity of core α 1,3 fucosyl residues [13, 16].

For this purpose in addition to the low cost feature of silkworm systems, we decided to use a transgenic system using silkworms [17–20]. In this system, foreign DNA carried on a vector plasmid is injected into silkworm eggs and is incorporated into the silkworm genome. Recombinant proteins synthesised in the middle silk gland (MSG) are secreted into the sericin layers of silk fibres of cocoons, allowing these proteins to be easily solubilised in aqueous buffer.

The N-glycan profile of recombinant glycoproteins produced by this transgenic silkworm system was assessed using recombinant mouse IgG [20]. These experiments showed that the N-glycans attached to the recombinant IgG did not include typical insect-type oligosaccharides, paucimannosidic glycans with core α 1,3 fucosyl residues [21–23]. Because the sericultural industry requires the production of large numbers of silkworm cocoons annually, the transgenic silkworm system may become a basic technology for the cost-effective production of recombinant proteins carrying non-allergic N-glycans.

Using a cDNA clone encoding a newly discovered FelIFN α subtype, FelIFN-α15, isolated from the blood of a Japanese domestic cat, this study evaluated the properties of rFeIFN produced by the transgenic silkworm system.

Results
Isolation of a clone of FelIFN
Total mRNA was isolated from leucocytes of a domestic cat, and a FelIFN clone was isolated by PCR using primers designed from the sequences of FelIFN 7 and FelIFN 8. The open reading frame of the FelIFN clone consisted of 567 base pairs, predicted to encode a 189 amino acid protein with a molecular mass of 21.1 kDa (Fig. 1). An alignment with reported sequences (Fig. 1) showed that the amino acid sequence of this FelIFN clone was very similar to those of other FelIFNs, including type α subtypes 1–14 and type ω. Although subtypes α5, α7, and ω contained a five amino acid insertion, this clone did not (Fig. 2). The clone had a predicted signal peptide sequence at amino acids 1–23 (underlined) and a consensus site for asparagine-linked glycosylation (double underlined). The FelIFN is almost identical to α3, 9, 13, and 14 with the exception of four amino acid residues. Based on its amino acid sequence, the gene encoding this clone was defined as FelIFN-α15.

Purification of rFeIFN-α15 from cocoons
Using transformation vectors (Fig. 3), rFeIFN-α15 was expressed in the cocoons of transgenic silkworms. The resulting rFeIFN-α15 protein in the cocoons was dissolved in phosphate-buffered saline (PBS) and purified by two steps of column chromatography (Table 1). Starting with approximately 100 mg cocoons, 411 μg of crude proteins were extracted, including 267 μg rFeIFN-α15, making the latter 65% pure, as determined by silver staining of SDS-PAGE gels. The crude extracts were chromatographed on a Blue Sepharose column, yielding 93% pure rFeIFN-α15; this was followed by chromatography on a ceramic hydroxyapatite column, yielding 67 μg of 99% pure rFeIFN-α15 (Table 1 and Fig. 4). Electrophoresis of the purified rFeIFN-α15 on SDS-PAGE gels followed by silver staining showed two main bands, a lower band at less than 20 kDa and an upper band at around 20 kDa, both close to the expected molecular weight of mature rFeIFN-α15 (18.9 kDa). The upper and lower protein bands are likely the glycosylated and non-glycosylated proteins, respectively. The net recovery of rFeIFN-α15 from the crude extract was calculated to be 25%.

N-terminal amino acid sequence of rFeIFN-α15
The five N-terminal amino acids of purified rFeIFN were analysed by Edman degradation (Table 2). Because the
cDNA sequence indicated that the N-terminal residue of mature FeIFN-α15 was cysteine, the first degradation cycle did not show any peak as expected. The second to fifth amino acids were found to be D, L, P, and Q, consistent with the deduced amino acid sequence from the cDNA. The signal sequence of this protein was likely processed correctly for secretion into the cocoons.

Glycosidase digestion of rFeIFN-α15
Glycosylation of purified rFeIFN-α15 was assessed by blotting with an anti-IFN antibody and concanavalin A (Con A), which recognises mannose residues (Fig. 5). Treatment of the sample with glycosidase F in the presence of both SDS and NP-40 (Fig. 5, lane 1) resulted in an almost complete absence of the upper, 20-kDa protein band compared with the untreated control (Fig. 5, lane 3), probably because treatment with this enzyme removed all N-glycan residues and shifted its molecular weight to that of the lower, <20-kDa protein band. This lower band was stained by both CBB (lane 1) and anti-FeIFN antibody (lane 4), but not by ConA (lane 7). In contrast, treatment with the enzyme in the absence of SDS and NP-40 or non-treatment with the enzyme resulted in both protein bands being stained by CBB (lanes 2 and 3) and

**Fig. 2** Homology of amino acid sequences of feline IFN alpha subtypes 1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 and IFN omega and IFN alpha subtype 15 (this study). The GenBank accession numbers of these proteins are AY117395, AY117394, AY117393, AY117392, AY117391, AB094996, AB094997, AB094998, AB094999, AB095000, AB095001, AB095002, AB095003, and E02521, respectively [9, 23, 24].
anti-FelIFN antibody (lanes 5 and 6), although only the higher molecular weight band reacted with ConA (lanes 8 and 9). These results indicated that 20-kDa and < 20-kDa protein bands represented the N-glycosylated and non-glycosylated forms of rFelIFN-α15, respectively.

**Structure of N-glycans of rFelIFN-α15**

Treatment of purified rFelIFN-α15 with pepsin followed by digestion with glycosidase A resulted in the release of N-glycans. The reducing ends of the N-glycans were labelled by digestion with glycosidase A. The N-glycans of rFelIFN-α15 were obtained by column chromatography and were summarized in Table 3. Five PA-oligosaccharide fractions were obtained by column chromatography and were shown to be Man2Man3GlcNAc2-PA (M5), GlcNAcMan3GlcNAc2-PA (GNa), GlcNAcMan3GlcNAc2-PA (GNb), GlcNAcMan3GlcNAc2-PA (GN2), and Man3GlcNAc2-PA (M3). The most abundant N-glycan, constituting 58.2% of the total, was M5 mannose-type, with about 30% being complex and hybrid types. About 10% of the total N-glycan was paucimannose-type (M3). There was no evidence of the existence of high-mannose-type (M6 or higher) or core-fucosylated glycans.

**In vitro anti-vesicular stomatitis virus activity**

The titre of rFelIFN-α15 against vesicular stomatitis virus (VSV), measured using Fcwf-4 cells, was found to be 6.2 × 10^7 units/mg in the crude extract and 2.6 × 10^8 units/mg in the protein obtained after Blue Sepharose column chromatography (Table 4). It was reported that the anti-VSV titre of the baculovirus-produced rFelIFN-ω measured by using Fc9 cells was 2.4 × 10^8 units/mg [14]. Although the feline cell lines used in the measurements differed, the rFelIFN-α15 and rFelIFN-ω are likely to possess VSV activities at similar levels.

**Discussion**

To date, 14 subtypes of FelIFN-α have been reported [24, 25]. In the present study, we cloned the fifteenth subtype of feline interferon α gene, named FelIFN-α15. We compared the protein product of this gene with rFelIFN-ω (NCBI Number: E02521), classified as a FelIFN-α [24–26], produced in silkworm larvae using the baculovirus expression system. Expression of rFelIFN-ω from its original signal sequence in silkworm larvae resulted in processing of the N-terminal peptide at a site different from the natural version [27]. Because the peptide sequence of human calreticulin has been reported to be processed at the correct site in MSG [28], we used the signal sequence of human calreticulin for the expression of FelIFN-α15 in this study. We found that this signal sequence was processed at the correct site and that the mature form of rFelIFN-α15, carrying its natural N-terminal sequence, was secreted into cocoons.

The N-glycan structure of rFelIFN-α15 was assessed using glycosidase F, an enzyme that can cleave N-glycan linkages in the presence of both SDS and NP-40 and when the α 1,3 fucose is not attached to the core N-acetylglucosamine (GlcNAc). The results obtained indicated that N-glycans attached to the rFelIFN-α15 did not contain core α 1,3 fucosyl residues. Furthermore, comparison of the N-glycan structures of the rFelIFN-α15 produced by the transgenic silkworm system during expression in MSG and of the ω type produced by the baculovirus expression system in entire silkworm larvae [10] showed noticeable differences between them. The N-glycan profile of rFelIFN-α15 by the transgenic silkworm system in MSG consisted of about 30% hybrid-
and complex-types, 58% M5, and 10% paucimannose-type. No high-mannose-type (M6 or higher) nor core α 1,3 fucosylated glycans were detected. This profile differs markedly from the typical insect profile. Although the precise N-glycan structures of rFeIFN-ω have not been determined, the sugar chains were reported to consist of mannose, GlcNAc, and fucose, in a 2:2:1 ratio [14]. These data suggest that the N-glycans of rFeIFN-ω had a typical insect-type profile, with FM2 (Man2GlcNAc2-Fuc) thought to be a main N-glycan [14]. The N-glycan structures of recombinant mouse IFN-β produced by the baculovirus expression system were reported to consist of 20% high-mannose-type (more than M6), 51% paucimannose-type, and 12% core α 1,3-fucosylated glycans [29]. Thus, the N-glycan pattern of rFeIFN-α15 synthesised in silk glands in this study differed from those of previously reported rIFNs synthesised in the baculovirus expression system, in which recombinant proteins were expressed in baculovirus-infected organs, such as fat bodies or haemolymph except silk glands.

These findings and those regarding IgG production in MSG [20] suggest that the mechanisms of N-glycosylation, especially core α 1,3 fucosylation, in MSG differ from those in other organs. The silk gland is specialised to produce proteins required for weaving cocoons, and MSG is involved in the production of sericin, which covers the fibroin core. This specific functional feature of MSG may be related to the specificity of N-glycan structure. Although detailed analysis of the glycosylation mechanism of MSG is necessary, it is thought that the specificity of the N-glycan structure in MSG provides a significant benefit with regard to the production of safe recombinant proteins with insects.

The anti-VSV titres of rFeIFN-α7 and ω, both of which had a five amino acid insertion, were approximately 10 times higher than the titres of rFeIFN-α8 and -α10 without the insertion [30]. As these rIFNs were produced in E. coli without any N-glycans on them, the high anti-VSV titres of rFeIFN-α7 and -ω have been attributed to the five amino acid insertion. However, our study showed that the anti-VSV titre of rFeIFN-α15 (without the insertion) was comparable to that of rFeIFN-ω (with the insertion), suggesting that anti-VSV activity may be affected by both the N-glycan profile and amino acid insertions.

In this study, the anti-viral activity of the recombinant rFeIFN-α15 was evaluated by in vitro assay with a single kind of virus, VSV. To demonstrate the clinical usability of the rFeIFN-α15, in vivo studies for anti-viral activities to several kinds of feline infective viruses should be evaluated.

**Conclusion**

This study showed that the rFeIFN-α15 produced by the transgenic silkworm system had the N-terminal amino acid sequence identical to the natural one and had no core α 1,3 fucosyl residues in its N-glycans. Additionally, the anti-VSV activity of the rFeIFN-α15 assessed in vitro was comparable to that of rFeIFN-ω produced by the baculovirus expression system in silkworms. These results indicate that rFeIFN-α15 may be useful in veterinary medication, but further studies including an assessment of in vivo anti-viral activity would be required to verify its availability.

**Methods**

**Experimental animals**

*B. mori* strain w1-pnd was obtained from the National Institute of Agrobiological Science (Tsukuba, Japan). The larvae were reared at 25 °C on an artificial diet (Silk MatePM; Nosan Corp, Kanagawa, Japan). Feline blood was obtained from a Japanese domestic cat.

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**Table 2** N-terminal amino acid sequence of purified rFeIFN-α15

| Cycle no. | Amino acid | pmol |
|-----------|------------|------|
| 1         | ND         | –    |
| 2         | D          | 26.1 |
| 3         | L          | 18.9 |
| 4         | P          | 10.5 |
| 5         | Q          | 15.3 |

Purified rFeIFN-α15 was separated on a SDS-PAGE gel, and its N-terminal amino acid sequence was determined by five cycles of automated Edman degradation. The N-terminal sequences of the two main bands on SDS-PAGE were determined. Results obtained from analysis of the larger, 20-kDa band are shown; results obtained with the < 20-kDa band were identical. ND: No amino acid was detected during the first cycle of degradation, suggesting that it was cysteine.
acting as a donor for blood transfusion. Fcwf-4 cells, the cell line derived from feline embryo fibroblasts, were purchased from the ATCC (ATCC no. CRL-2787).

**Cloning of FeIFN-α and vector construction**

Leukocytes were isolated from feline blood, and total RNA was extracted from the cells using TRIzol® LS Reagent (Thermo Fisher Scientific K.K., Yokohama, Japan). IFN-α cDNA was synthesised using a One-Step RT-PCR Kit (Invitrogen, Carlsbad, USA) with the two amplification primers, 5'-ATGGCGCTGCCCTCTTCCTTGG-3' (forward) and 5'-TTTCTCGCTCCTTAATTTTCTGC-3' (reversed) derived from the common sequences of IFNA7 and IFNA8 (GenBank Accession Nos. NM_001009197 and NM_001009193, respectively). The cDNA fragments obtained by reverse transcription polymerase chain reaction (RT-PCR) were sequenced. The cDNAs encoding the open reading frames of full-length FeIFN-α (IFNα family) were cloned. FeIFN-α15, a novel type of FeIFN-α, was also isolated (GenBank Accession No. AB608218). The predicted signal sequence region of FeIFN-α15 was replaced with that of human calreticulin, as this signal sequence has been reported to be processed at the correct site in MSG [28]. The PCR product amplified using the following primer sets was inserted into a cloning vector. The 5’-untranslated region (UTR) sequence of *Bombyx mori* nuclear polyhedrosis virus (NPV) gene was used for the construction of the vector. The 5’-untranslated region (UTR) sequence of *B. mori* nuclear polyhedrosis virus (NPV) gene was used for the construction of the vector.

Table 3. Structure of N-glycans attached to the rFeIFN-α15 produced by the transgenic silkworm system. The ratio (%) of each glycan was calculated from the corresponding peak area on the chromatogram obtained by HPLC.

| Glycan structure | Abbreviation | Peak area |
|------------------|--------------|-----------|
| Mano6            | M5           | 58.2 %    |
| Mano3            |              |           |
| Mano6            |              |           |
| Mano3            |              |           |
| Mano3            |              |           |
| Mano3            |              |           |
| GlcNAcβ2Mano6    | GNa          | 6.7%      |
| Mano3            |              |           |
| GlcNAcβ2Mano3    | GNb          | 7.9%      |
| GlcNAcβ2Mano6    | GN2          | 14.9%     |
| GlcNAcβ2Mano3    |              |           |
| Mano3            | M3           | 10.4%     |
| Mano3            |              |           |

![Fig. 5 Glycosidase F digestion of rFeIFN-α15.](image)
various assays except for N-glycan analysis. For N-glycan analysis, purified rFeIFN-α15 was used for 50% reduction in CPE. Commercially available rFeIFN-ω was used as a standard.

Table 4 Anti-VSV activity of rFeIFN-α15 as measured using CPE assay

| Sample                        | Unit/mg |
|-------------------------------|---------|
| Crude extract                 | 6.2 × 10^8 |
| Blue Sepharose™ column elute  | 2.6 × 10^8 |

The inhibitory activity of rFeIFN-α against VSV infection of FcwF-4 cells was determined by a 50% reduction in CPE. Commercially available rFeIFN-ω was used as a standard.

Generation of a TG silkworm line expressing rFeIFN-α15

pFeIFN-α15/MSG1.1MG was injected, along with the helper vector pHA3PIG [33], into pre-blastoderm silkworm embryos as described [33]. The hatched G0 larvae were reared to moths at 25 °C. G1 embryos were generated by mating siblings or w1-pnd and screened for ocular expression of monster green fluorescent protein (MGFP), an efficient marker for detection of transgenic silkworms [28, 32].

N-terminal amino acid sequence of rFeIFN-α15

The N-terminal sequence of purified rFeIFN-α15 was determined with a protein sequencer, HP G1005A (Agilent Technologies International Japan, Tokyo, Japan), using a standard Edman degradation protocol.

Glycosidase digestion and blotting assays

Purified rFeIFN-α15 was digested with glycosidase F (Takara Bio, Shiga, Japan) at 37 °C for 17 h in the presence or absence of 0.5% SDS and 1.0% NP-40. The samples were separated on SDS-PAGE, followed by blotting to Immobilon-P membranes (Merck Millipore, Tokyo, Japan). For immunoblotting, the membranes were incubated with primary anti-FeIFN-α polyclonal rabbit antibody, generated by us, and secondary horseradish peroxidase (HRP)-conjugated anti-rabbit goat IgG (Sigma-Aldrich, Tokyo, Japan), with HRP detected with chemiluminescent reagents (ECL Western Blotting Detection System) (GE Healthcare). For lectin-blotting, the membranes were treated with biotinylated Con A (Seikagaku Corporation, Tokyo, Japan) and the resulting biotin residues reacted with streptavidin HRP conjugate (Sigma-Aldrich). HRP was detected with 3,3-diaminobenzidine (Wako Chemicals, Tokyo, Japan).

N-glycan analysis of rFeIFN-α15

N-glycan analysis of purified rFeIFN-α15, including glycosidase digestion of the protein, pyridylation of the released glycans, and separation of PA-oligosaccharides by column chromatography, was performed as previously described [34].

Mass spectrometric analyses of PA-oligosaccharides

PA-oligosaccharides were subjected to matrix-assisted laser desorption/ionisation time-of-flight mass spectrometric (MALDI-TOF MS) analysis, as previously described [35].

Antiviral activity assay of rFeIFN-α15

The antiviral activity of rFeIFN-α15 was determined in vitro using a standard VSV assay [36]. FcwF-4 cells were inoculated into 96-well plates at a density of 2 × 10^4 cells
per well and cultured in Dulbecco’s Modified Eagle’s medium supplemented with 5% foetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin and streptomycin (Thermo Fisher Scientific K.K.) for 16 h in an atmosphere containing 5% CO₂. The crude extract or proteins eluted from the Blue Sepharose™ column were serially diluted with the media supplemented with 1% FBS, and each 100 μL of the media was added into the Fcw f-4 cell-containing wells. They were cultured for 20 h. Intercat1 (Toray Industries, Tokyo, Japan), a commercially available rFeIFN-ωα, was used as a positive control. After each supernatant was removed, the plates were further incubated for 48 h at 37 °C, followed by infection with 100 μL of VSV (VR-158) (Summit Pharmaceuticals International Corporation, Tokyo, Japan) at a concentration of 1000 TC ID₅₀/ml. When the cytopathic effect (CPE) was greater than 95% of the control cells (without FeIFN), all the plates were stirred gently, and the cells were washed three times with PBS, fixed in 10% (w/v) formalin and stained with 0.1% (w/v) crystal violet. The stained cells were washed with deionised water and allowed to dry. The crystal violet was eluted with 100 μL of methanol, and the absorbance of each well was measured at 595 nm with a micro-titre plate reader (Spectra Max 250; Molecular Devices Japan, Tokyo, Japan). Titres of the samples were corrected by the titres of Intercat1.

**Abbreviations**

BmNPV: *Bombyx mori* nuclear polyhedrosis virus; Con A: Concanavalin A; CPE: Cytopathic effect; FBS: Foetal bovine serum; FeIFN: Feline interferon; fBl: polyA: fibroin L-chain polyA signal sequence; FM2: Man2GlcNAc2Fuc; GlcNAc: N-acetylglucosamine; GN2: GlcNAc2Man3GlcNAc2-PA; GNb: GlcNAcMan3GlcNAc2-PA; HPLC: High-performance liquid chromatography; h3r: h3r enhancer; ie1: IE1 trans activator gene; M3: Man3GlcNAc2-PA; M5: Man2Man3GlcNAc2-PA; MALDI-TOF MS: Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometric; MGFp: Monster green fluorescent protein; MSG: Middle silk gland; P3xP3: 3xP3 promoter; PA: aminopropylidine; PAG: Polyacrylamide gel electrophoresis; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction; Pser1: Ser1 promoter; SDS: Sodium dodecyl sulphate; SV40 polyA: SV40 polyA signal sequence; TG: Transgenic; UTR: Untranslated region; VSV: Vesicular stomatitis virus

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**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**

MS and NY carried out the in vitro experiments. TM and IT designed and generated TG silkworm and checked the expression level of the protein from the TG line. MY prepared the polyclonal antibody against FeIFN-ωα, coordinated the collaboration and advised on the study. MS and SS designed the study, analysed the results, and drafted the paper. SS supervised the study. All authors have read and approved the final manuscript.

**Ethics approval and consent to participate**

The study was carried out using a part of blood which had been collected for transfusion at the Veterinary Hospital. The blood was obtained with the consent of the Doctor of Veterinary Medicine who owned the cat. As stored blood was used, no separate ethical approval was required for the study. All efforts were made to minimize the discomfort of the animals during sampling.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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