Up-regulation of bone morphogenetic protein and its signaling molecules following castration of bulls and their association with intramuscular fat content in Korean cattle

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We evaluated whether castration affects bone morphogenetic protein 2 (BMP2) level and the expression of its signaling molecules in Korean cattle bulls. We also checked whether castration affects the expression of muscle fiber type and oxidative and glycolytic enzyme genes. Enzyme-linked immunosorbent assays revealed that steers had higher plasma BMP2 and leptin concentrations than bulls. Quantitative real-time PCR showed that steers had higher mRNA levels of the lysyl oxidase gene, a downstream target of the BMP signaling pathway, in the longissimus thoracis (LT) muscle. Steers had higher adipogenic peroxisome proliferator-activated receptor gamma and lipogenic fatty acid binding protein 4 mRNA levels in the LT than bulls. Steers had lower mRNA levels for several muscle fiber type 1 genes and fiber type 2A myosin heavy chain 2 gene than bulls. Steers had higher mRNA levels of the glycolytic enzyme phosphoglycerate kinase 1 gene than bulls. Transcript levels of oxidative enzyme genes did not differ between bulls and steers. Regression analysis revealed a positive association between plasma BMP2 levels and intramuscular fat (IMF) content in the steer group. These findings suggest that upregulation of the BMP signaling pathway in response to castration induces increased adipogenic gene expression, contributing to the increased IMF deposition observed in castrated animals.

Marbling refers to the dispersion or scattering of fat inside lean meat, and the degree of marbling is the major determinant of the quality grade of Korean cattle beef1. Castration significantly increases the marbling score (MS) and intramuscular fat (IMF) accumulation, improving beef quality in Korean cattle12. Previously, we showed that the activation of adipogenesis and lipogenesis is involved in increased IMF deposition post-castration2–4. Both hyperplasia (adipocyte number) and hypertrophy (adipocyte size) contribute to IMF deposition5,6. Adipocyte hyperplasia may result from new preadipocyte recruitment and commitment by mesenchymal stem cells (MSCs) from the vascular stroma of adipose tissue7, adipocyte proliferation via mitotic clonal expansion of the committed preadipocytes during differentiation8, and new adipocyte differentiation from preadipocytes9.

The bone morphogenetic protein (BMP) signaling pathway is thought to be required for the differentiation of MSCs into the adipocyte lineage based on cell culture studies10–12. Lysyl oxidase (LOX) is a downstream target gene of the BMP signaling pathway, and is implicated as an early marker of adipogenic commitment in cell culture10. Little is known about the involvement of BMP signaling molecules in marbling formation in bovine species. It is known that leptin can induce adipocyte differentiation in preadipocytes13,14. Limited information is available regarding whether castration affects plasma leptin levels.

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Castration reduces circulating testosterone\(^2\) and affects muscle fiber types in cattle.\(^{15}\) Thus, castration may affect the expression of genes involved in myofiber type composition and muscle metabolism through this endocrine change.

In this study, we aimed to understand changes in the expression of BMP2-adipogenesis signaling molecules caused by castration in cattle. We also evaluated whether castration of bulls affects the expression levels of muscle fiber type and metabolism-related genes.

**Results and Discussion**

**Carcass characteristics of Korean cattle bulls and steers.** The carcass weights of bulls and steers averaged 445 ± 9.37 and 434.3 ± 9.47 kg, respectively (Table 1). Steers had greater (\(p < 0.001\)) back fat thickness and greater (\(p < 0.001\)) MS and quality grade than bulls. Steer LT had a 4.0-fold greater (\(p < 0.001\)) IMF content (14.5 ± 5.48%) than bull LT (4.18 ± 1.63%). Bulls and steers were slaughtered at a similar weight, so steers were about 6 months older at slaughter. Age affects marbling development;\(^3\) thus, in addition to castration itself, age differences may affect differences in MS and IMF content between bulls and steers.

**Comparison of plasma BMP2 levels and expression of BMP2-adipogenesis signaling molecules in the LT between bulls and steers.** Hyperplasia requires the proliferation and differentiation of preadipocytes into new adipocytes, a process known as adipogenesis.\(^{8,14}\) BMPs are a group of growth factors in the transforming growth factor-beta superfamily.\(^{17}\) BMP2 induces adipocyte development, including adipogenesis and preadipocyte commitment.\(^{10}\) In this study, steers had higher (\(p < 0.001\)) plasma BMP2 concentrations than bulls (Fig. 1a). Consistent with our results, a study on humans found that circulating serum BMP2 levels were higher in a moderate obesity group than in lean healthy controls.\(^{18}\) After castration of bulls, their circulating serum testosterone levels decreased.\(^1\) A previously published mouse experiment showed that BMP signaling was blocked after testosterone injection.\(^{19}\) Strong negative correlations have been observed between testosterone and body fat.\(^{20}\) In this study, the decreased testosterone levels following castration may have triggered the higher circulating plasma BMP2 levels in steers, contributing to increased IMF deposition in castrated animals. Regression analysis revealed a positive association (\(p < 0.05\)) between plasma BMP2 levels and IMF content in the steer group, but not in the bull group (Table 2). The explanation for the absence of a significant association in the bull group may be that IMF content was relatively homogeneous among animals in the bull group, as the standard deviation of IMF content in the bull group was much lower than that in the steer group (Table 1).

### Table 1. Carcass characteristics of the Korean cattle bulls and steers. Values are mean ± standard deviation.

| Variables                  | Bulls (n = 10) | Steers (n = 10) | P-value |
|----------------------------|---------------|----------------|---------|
| Carcass weight, kg         | 445 ± 9.37    | 434 ± 9.47     | 0.42    |
| Back fat thickness, mm     | 4.7 ± 0.63    | 11.7 ± 0.80    | <0.001  |
| Rib eye area, cm           | 86.4 ± 2.97   | 95.9 ± 6.89    | 0.23    |
| Yield index\(^1\)          | 69.0 ± 0.36   | 66.6 ± 0.97    | 0.04    |
| Yield grade\(^2\)          | 30.0 ± 0.00   | 22.0 ± 2.00    | 0.003   |
| Marbling score\(^3\)       | 1.1 ± 0.10    | 7.1 ± 0.35     | <0.001  |
| Quality grade\(^4\)        | 11.0 ± 1.00   | 44.0 ± 1.63    | <0.001  |
| IMF content, %             | 4.18 ± 1.69   | 14.5 ± 5.48    | <0.001  |

\(1\) Yield index = 68.184–0.625 × back fat thickness + 0.13 × rib eye area – 0.024 × carcass weight + 3.23. \(^2\) Yield grade: 30 = A; 20 = B; 10 = C. \(^3\) Marbling score: 1 = min; 9 = max. \(^4\) Quality grade: 50 = 1++; 40 = 1+; 30 = 1; 20 = 2; 10 = 3.
BMP2 occurs through the activation of PPARG22. PPARG regulates the expression of several adipocyte-secreted molecules in the LT than bulls. In murine mesenchymal progenitor C3H10T1/2 cells, the induction of adipogenesis by BMP2 occurs through the activation of PPARG22. PPARG regulates the expression of several adipocyte-secreted proteins, including adiponectin23. In human mesenchymal cells (hMSCs), a combination of BMP2 and 3-isomethyl-1-methylxanthine induces adiponectin expression24. In our study, the up-regulation of BMP2 and LOX following castration may have led to the activation of preadipocyte commitment and subsequent adipocyte hyperplasia by adipogenesis through activation of PPAR and adiponectin in castrated animals. Steers had higher acetyl CoA carboxylase alpha; FABP4, fatty acid binding protein 4.

| Variables          | Bull (n = 10) |        |        | Steer (n = 10) |        |        |
|--------------------|--------------|--------|--------|---------------|--------|--------|
|                    | Coefficient  | P-value| R²     | Coefficient   | P-value| R²     |
| Plasma leptin      | −0.186       | 0.607  | 0.035  | 0.477         | 0.164  | 0.227  |
| Plasma BMP2        | −0.028       | 0.940  | 0.001  | 0.636         | 0.048  | 0.404  |
| LOX mRNA level     | 0.209        | 0.563  | 0.044  | −0.199        | 0.582  | 0.040  |
| PPARG mRNA level   | 0.444        | 0.199  | 0.197  | 0.465         | 0.176  | 0.216  |
| Adiponectin mRNA level | −0.160  | 0.659  | 0.026  | −0.505        | 0.137  | 0.255  |
| ACC mRNA level     | 0.593        | 0.071  | 0.352  | −0.331        | 0.351  | 0.109  |
| FABP4 mRNA level   | −0.120       | 0.741  | 0.014  | −0.364        | 0.301  | 0.133  |

Table 2. Regression analysis of levels of plasma bone morphogenetic protein 2 (BMP2) and its signaling molecules in the longissimus thoracis with intramuscular fat content (IMF%) in Korean cattle. BMP2, bone morphogenetic protein 2; LOX, lysyl oxidase; PPARG, peroxisome proliferator activated receptor gamma; ACC, acetyl-CoA carboxylase alpha; FABP4, fatty acid binding protein 4.

The LOX gene is a downstream target of the BMP signaling pathway10. Steers had higher (p < 0.05) LOX mRNA levels in the LT than bulls (Fig. 1b). In mesenchymal stem cells derived from human adipose tissue, LOX expression was upregulated by BMP2 induction23. BMP2/4 induces the expression of LOX, which contributes to preadipocyte commitment by murine mesenchymal progenitor C3H10T1/2 cells11. Steers had higher (p < 0.05) PPARG mRNA levels in the LT than bulls. Steers also had higher (p < 0.05) adiponectin mRNA levels in the LT than bulls. In murine mesenchymal progenitor C3H10T1/2 cells, the induction of adipogenesis by BMP2 occurs through the activation of PPARG22. PPARG regulates the expression of several adipocyte-secreted proteins, including adiponectin23. In human mesenchymal cells (hMSCs), a combination of BMP2 and 3-isomethyl-1-methylxanthine induces adiponectin expression24. In our study, the up-regulation of BMP2 and LOX following castration may have led to the activation of preadipocyte commitment and subsequent adipocyte hyperplasia by adipogenesis through activation of PPAR and adiponectin in castrated animals. Steers had higher acetyl CoA carboxylase (p < 0.05) and fatty acid binding protein 4 mRNA levels than bulls. Thus, de novo fatty acid synthesis and fatty acid transport may be subsequently activated through BMP signaling, resulting in increased IMF deposition in castrated animals.

Comparison of expression levels of muscle fiber type and metabolism-related genes in the LT between bulls and steers. In our previous study, castration profoundly reduced circulating testosterone in cattle1. This endocrine change may affect muscle fiber type composition and muscle metabolism. In the present study, we checked whether castration affects the expression levels of genes related to muscle fiber type and muscle metabolism. Steers had lower mRNA levels of muscle fiber type 1 (slow twitch, red muscle, oxidative) genes, including troponin C1 (p < 0.05), troponin T1 (p < 0.001), and myoglobin (p < 0.05), than bulls (Fig. 2a). Previously, a lower proportion of type 1 fiber was observed in the longissimus dorsi muscle of steers compared to that of bulls in French Montèe’ liard cattle at 16 months of age11. In our study, steers had lower (P < 0.01) mRNA levels of the fiber type 2A (fast twitch, oxidative, glycolytic) myosin heavy chain (MYH1) isof orm MYH12 gene than bulls. mRNA levels of the fast type 2B (fast-twitch, glycolytic) MYH4 gene did not differ (p > 0.05) between bulls and steers. Collectively, our results reveal that castration decreases transcription of muscle fiber types 1 and 2A genes in the LT.

These changes in fiber type gene expression may affect muscle metabolism. Thus, we compared the transcript levels of oxidative and glycolytic enzyme genes in the LT between bulls and steers. mRNA levels of oxidative enzyme genes, including citrate synthase and isocitrate dehydrogenase 1, did not differ between bulls and steers (Fig. 2b). Steers had higher (p < 0.05) mRNA levels of the glycolytic enzyme phosphoglycerate kinase 1 gene than bulls (Fig. 2b). Steers also tended to have higher (p = 0.10) mRNA levels of the glycolytic enzyme hexokinase 2 gene than bulls. mRNA levels of muscle type phosphofructokinase 1 and lactate dehydrogenase A genes did not differ between bulls and steers. Consistent with our results, a previous study reported that steers exhibited higher glycolytic enzyme activities than bulls; however, the same study found that steers had lower oxidative enzyme activities than bulls, which contrasted with the results of the present study26. This inconsistency may be due to a difference in cattle breed (Korean cattle vs. French Montèe’ liard cattle). In skeletal muscle forkhead box O1 transgenic mice, expression of muscle fiber type I genes, including slow isoforms of troponins and myoglobin, was down-regulated, but expression of mitochondrial oxidative enzyme genes involved in electron transport system (cytochrome c oxidase II and IV) did not differ, between forkhead box O1 mice and the control25. Collectively, our results indicate that castration moderately affects gene expression levels of glycolytic enzymes but does not affect gene expression of oxidative enzymes.

Comparison of plasma leptin levels between bulls and steers. In this study, steers had higher (p < 0.05) plasma leptin concentrations than bulls (Fig. 1c). White adipose tissue is the main leptin-producing tissue28. Rodent primary culture experiments have shown that leptin is secreted after the differentiation of preadipocytes into adipocytes29. Consistent with our study, higher leptin concentrations were indicative of greater adiposity in beef cattle28. Therefore, higher body fat content in steers may be one explanation for the higher plasma leptin concentrations observed in our study and other studies. Positive correlations between adipocyte size and leptin expression have been found in human29,30 and rodent studies31. Similarly, plasma leptin concentrations
are strongly related to adipose cell size in cattle. Because larger adipocytes accommodate more leptin mRNA, adipocyte size may influence leptin synthesis and secretion. Several studies have reported that hypertrophy is actively involved in marbling and IMF deposition. Our previous study also revealed that castration induces hypertrophy of subcutaneous and abdominal fat in Korean cattle. Therefore, we speculate that increased adipocyte hypertrophy may have contributed to the increased plasma leptin concentrations in castrated animals, although we did not measure the size of intramuscular adipocytes in this study.

**Conclusions**

Our findings suggest that upregulation of the BMP signaling pathway in response to castration induces increased adipogenic gene expression in bulls, contributing to the increased IMF content observed in castrated animals. Our findings also indicate that castration affects the expression of some of muscle fiber type genes and has moderate effects on the expression of glycolytic pathway genes, but not oxidative enzymes. Our results reveal a new adipogenesis pathway for bovine IMF deposition.

**Materials and Methods**

**Animals and tissue samples.** All experimental procedures involving animals were approved by the Seoul National University Institutional Animal Care and Use Committee (SNUICUC: SNU-161117-3) and conducted in accordance with the Animal Experimental Guidelines of the SNUICUC. This study examined 10 Korean cattle bulls and 10 steers. We used bulls and steers, which have clear differences in IMF content, as an experimental model to understand the involvement of BMP2 signaling molecules in IMF deposition and the metabolic differences between bulls and steers. A feeding regime was followed as previously described with modifications. Briefly, 20 bulls were weaned at an average age of 3 mo and fed with 30% concentrates and 70% roughage until 6 mo of age. Ten bulls were castrated at 6 mo, the age at which Korean cattle bulls are routinely castrated. We castrated bulls under the guidance of an expert veterinarian. Bulls and steers were fed with concentrates composed of 15% crude protein (CP) and 71% total digestible nutrients (TDN) from 7 mo to 13 mo of age, 13% CP and 72% TDN until 19 mo of age, and 11% CP and 73% TDN after 20 mo of age. For roughage, timothy (2.5–3.5 kg/d; approximately 1.2% BW/animal) was fed from 7 mo to 13 mo of age, rice straw (2.0–3.0 kg/d; approximately 0.5% BW/animal) until 19 mo of age, and rice straw (1.0–1.5 kg/d; approximately 0.15% BW/animal) after 20 mo of age.

Before slaughter, blood was collected from the jugular vein into EDTA Vacutainer tubes, and plasma was prepared as previously described. Bulls and steers were slaughtered at 25.7 ± 0.52 and 31.8 ± 0.11 months, respectively. Carcass traits, including MS, beef quality grade, ribeye area, fat thickness, yield index, and yield grade, were determined as previously described. Longissimus thoracis (LT) muscles from the left carcass side between the 12th and 13th ribs were collected immediately after slaughter and stored at −70 °C until analysis. The IMF content of the LT muscles was measured following the procedure of Folch et al. Briefly, LT tissues were ground into a fine powder, which was homogenized in a 2:1 chloroform-methanol mixture (vol/vol). The fat content was measured after the fat-containing solvents were evaporated.
| Gene name (Symbol) | Gene bank accession no. | Primer | Sequence (5’-3’) | Tm, °C | Length (bp) |
|-------------------|-------------------------|--------|------------------|--------|------------|
| β-actin (ACTB)*   | NM_179797.3             | Forward | ATGCCAAGGATGTCAGATGTCTGG   | 60.5   | 80         |
|                   |                         | Reverse | ATCCACCAGCTGCTTCTCA        |        |            |
| Lysyl oxidase (LOX)| NM_173932.4             | Forward | ACAAAGCTGTCCTGGTGA          | 60.2   | 102        |
|                   |                         | Reverse | CTTTGGGAGTTTTTGCC          |        |            |
| Peroxisome proliferator activated receptor gamma (PPARG) | NM_181024.2 | Forward | AATCCCTGGTCTTCGTTG         | 59.6   | 88         |
|                   |                         | Reverse | AAAGTTGTAAGGGCAAGG          |        |            |
| Adiponectin       | NM_174742.2             | Forward | CGTCGACTTCTTCTCTCACTTT     | 59.4   | 55         |
|                   |                         | Reverse | TTCTCTGATGCAAAAGCCACC      | 60.3   | 97         |
| Acetyl-CoA carboxylase alpha (ACC) | NM_174224.2 | Forward | AGCAGTGGTAGAAGAGGAAGG      | 59.4   | 55         |
|                   |                         | Reverse | TTCAGCTCAGAGGTTTGCC        | 60.3   | 115        |
| Fatty acid binding protein 4 (FABP4) | NM_174314.2 | Forward | GCTGCCATCTTCTCTCACTTT      | 58.1   | 60         |
|                   |                         | Reverse | TTCTCTGATGCAAAAGCCACC      | 60.3   | 140        |
| Troponin C1, slow skeletal and cardiac type (TNNC1) | NM_001034351.2 | Forward | GTGAGCAGGGCAAGTTGAGT      | 60.9   | 97         |
|                   |                         | Reverse | CGAGAAGATGTCGTCAAG         | 59.6   | 111        |
| Troponin T1, slow skeletal type (TNNT1) | XM_010815469.3 | Forward | GCCCTGATACAGCACAACCAAGCC   | 55.0   | 89         |
|                   |                         | Reverse | GACTTGCTGATCAGGTCACC       |        |            |
| Myoglobin (MB)    | NM_173881.2             | Forward | GATGACATGCAACAGGAGG        | 60.3   | 99         |
|                   |                         | Reverse | GAAGTTGAGAGTGTCAAG         |        |            |
| Myosin heavy chain 2 (MYH2) | NM_001166227.1 | Forward | AAGAGCCCTCTTGGTACTGGG      | 60.0   | 138        |
|                   |                         | Reverse | ATGGGCACCTTGGTCTGCG         |        |            |
| Myosin, heavy chain 4, skeletal muscle (MYH4) | NM_174224.2 | Forward | GTTCCAAGTGCGAAGAGGG       | 60.3   | 50         |
|                   |                         | Reverse | GATGCGCTGCAAAAGTTGG        | 59.6   | 96         |
| Citrate synthase (CS) | NM_001044721.1 | Forward | CATGGCTCTACTCAGTGC        | 59.3   | 97         |
|                   |                         | Reverse | TCCGTTGAGAAGACACTGG         |        |            |
| Isocitrate dehydrogenase (NADP(+)) 1 (IDH1) | NM_181012.3 | Forward | TCCGAAATATCCCTGGTGGC       | 59.5   | 149        |
|                   |                         | Reverse | CCTCTGAGGAAACAGTAAGG        | 60.0   | 149        |
| Hexokinase 2 (HK2) | XM_015473383.2 | Forward | TGCTGCGGCTACTTCTCTTACG    | 60.1   | 126        |
|                   |                         | Reverse | CATGCTCTTTGGGAGAAAAGGC     | 60.2   | 126        |
| Phosphofructokinase, muscle (PFKM) | NM_001075268.1 | Forward | GCAATCTGCGAAAGAGGCCCC      | 52.4   | 126        |
|                   |                         | Reverse | CACGCAAGTGAACACTGGCG       | 60.0   | 126        |
| Phosphoglycerate kinase 1 (PGK1) | NM_001034299.1 | Forward | GTGCGGAGGAGAGGGAAGG       | 59.4   | 89         |
|                   |                         | Reverse | GAAGTTGAGAGTGTCAAG         | 59.2   | 99         |
| Lactate dehydrogenase A (LDHA) | NM_174099.2 | Forward | GCTATACCTGTCGAGCAGGCGCAG   | 60.9   | 110        |
|                   |                         | Reverse | TTTGACTCTTCGAGGATGAGG       | 60.0   | 110        |

Table 3. Sequences of the primers used in real-time PCR analysis. *ACTB = Internal control gene.

Blood plasma analysis. The plasma BMP2 and leptin concentrations were quantified using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer’s instructions. BMP2 was analyzed using a Bovine Bone Morphogenetic Protein 2 ELISA Kit (MyBioSource, San Diego, CA, USA) and leptin was analyzed using a Bovine Leptin ELISA Kit (MyBioSource, San Diego, CA, USA). The intra- and inter-assay coefficients of variation for the Leptin and BMP2 kits were both less than 15%.

RNA extraction and quantitative real-time polymerase chain reaction. Total RNA was extracted from LT tissue using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA), based on the manufacturer’s instructions. The RNA was quantified using a NanoPhotometer (Implen, Munich, Germany), and the quality was assessed using ethidium bromide staining of the 28S and 18S agarose gel electrophoresis bands and a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), as previously described39. cDNA was synthesized from reverse-transcribed total RNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer’s instructions.

Quantitative real-time PCR was performed using QuantiTect SYBR Green RT-PCR Master Mix (QIAGEN, Hilden, Germany), as previously described39. We followed the “Minimum Information for Publication of Quantitative Real-Time PCR Experiments” (MIQE) guidelines for qPCR as closely as possible40. All qPCR analyses were conducted in a 25-μL total reaction volume that contained 20 ng cDNA, 12.5 μL SYBR Green RT-PCR Master Mix, and 1.25 μL of 10 μM primers. The thermal cycling parameters were: 95 °C for 15 min, followed by 40 cycles at 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. Table 3 lists the primers used. We used two different exons for forward and reverse primers to prevent amplification of the DNA template. We indicated the melting temperatures (Tm) of all primers. The Tms of all of the primers were 52.4–60.9 °C. An annealing temperature of 55 °C was used for amplification of all genes, resulting in a single major peak in all cases. The ΔΔCT method was used to determine the relative fold change in gene expression41. We evaluated whether β-actin, a ribosomal protein, lateral stalk subunit P0, and 18S RNA were suitable reference genes. β-actin expression was generally uniform in the LT between bulls and steers and was therefore used as the reference gene. We also used β-actin as a reference gene in the LT in two previous studies24,22.

Statistical analyses. Differences between bulls and steers were examined using the general linear model procedure in SAS 9.1 software (SAS Institute, Cary, NC, USA). The IMF contents were not normally distributed.
due to marked differences between the bull and steer groups. Thus, we performed a linear regression analysis to separately examine the relationship between gene expression and IMF content (%) within the bull and steer groups using SAS 9.4 software. This resulted in the following equation:

\[ \text{IMF}_{pi} = \beta_0 + \beta_1 \text{Expression}_{i} + \epsilon_i \]

where \( \text{IMF}_{pi} \) is the variable of IMF%, Expression_{i} is the variable expression level, \( \beta_0 \) is the intercept, \( \beta_1 \) is the coefficient of expression level, and \( \epsilon_i \) is random error.

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Author contributions
D.J.S.J. performed most of the experiments, performed the statistical analysis, and wrote the draft of the paper. M.B. guided all experiments and revised the paper. All authors approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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