Ablation of the p16INK4a tumour suppressor reverses ageing phenotypes of klotho mice

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The p16INK4a tumour suppressor has an established role in the implementation of cellular senescence in stem/progenitor cells, which is thought to contribute to organismal ageing. However, since p16INK4a knockout mice die prematurely from cancer, whether p16INK4a reduces longevity remains unclear. Here we show that, in mutant mice homozygous for a hypomorphic allele of the α-klotho ageing-suppressor gene (kklklkl), accelerated ageing phenotypes are rescued by p16INK4a ablation. Surprisingly, this is due to the restoration of α-klotho expression in kklklkl mice and does not occur when p16INK4a is ablated in α-klotho knockout mice (klklklkl), suggesting that p16INK4a is an upstream regulator of α-klotho expression. Indeed, p16INK4a represses α-klotho promoter activity by blocking the functions of E2Fs. These results, together with the observation that the expression levels of p16INK4a are inversely correlated with those of α-klotho throughout ageing, indicate that p16INK4a plays a previously unrecognized role in downregulating α-klotho expression during ageing.
issue repair and regeneration are essential for longevity in complex animals, and often depend on the proliferative activity of stem or progenitor cells. In many tissues, the proliferative activity of such cells declines with age, contributing to many aging-associated pathologies5–9. In mammals, the p16INK4a tumour-suppressor gene elicits irreversible cell-cycle arrest known as cellular senescence5–10, and its expression increases with age in many tissues11–13, along with the accumulation of dysfunctional senescent stem/progenitor cells14–16. However, recent studies using middle-aged mice lacking p16INK4a (p16/−/− mice) revealed that the aging-associated induction of p16INK4a expression reduces the proliferative and regenerative capacities of certain stem/progenitor cells during the aging process14–16. These findings have led to speculation that the induction of p16INK4a expression and the consequent cellular senescence are causally implicated in aging-associated declines in stem/progenitor cell functions, thereby reducing longevity. However, since p16−/− mice die of cancer long before they reach the age at which most normal mice start to die17, it remains unclear whether p16INK4a truly limits longevity in mammals. One approach to circumvent this problem would be the use of short-lived mouse strains with accelerated-aging phenotypes. However, attempts towards extending the maximum lifespan of accelerated-aging mouse strains by p16INK4a ablation have so far been unsuccessful18,19, raising the question of whether p16INK4a truly limits longevity in mammals.

Mutant mice homozygous for a severely downregulated hypomorphic allele of the z-klotho gene (referred to as kk/kl or klotho) manifest multiple age-related disorders that are also observed in humans, including infertility, growth retardation, osteoporosis, pulmonary emphysema, skin atrophy, ectopic calcification and shortened lifespan20. Furthermore, the levels of z-klotho expression decline with age in both humans and mice21–1, and overexpression of z-klotho extends the maximum lifespan in mice22, suggesting that z-klotho acts as an ageing-suppressor gene in mammals23. The z-klotho gene encodes a single-pass transmembrane protein that is predominantly expressed in the kidney20, and to a lesser extent in the brain24. Two forms of the z-klotho protein exist: a membrane-bound form and a secreted form25,24,26,27. As increases in senescent progenitor cells and decreases in stem cell numbers were observed in several tissues in kk/kl mice28,29, we wondered whether p16INK4a contributes to the accelerated-aging phenotypes in kk/kl mice, by eliciting cellular senescence in certain stem/progenitor cells.

In the present study, we explore the roles of p16INK4a in accelerated-aging phenotypes of klotho mice. We show that ablation of the p16INK4a gene reverses various ageing phenotypes, including maximum lifespan, of kk/kl mice. Surprisingly, however, this is due to the restoration of z-klotho expression in kk/kl mice and does not occur when p16INK4a is ablated in knockout mice lacking z-klotho (kl−/−), indicating that p16INK4a is an upstream regulator of z-klotho expression. Thus, although p16INK4a has an established role in the implementation of cellular senescence in stem/progenitor cells5–10, which are likely to reduce longevity13, our results reveal that p16INK4a has an additional function in promoting ageing phenotypes by downregulating z-klotho expression in mice. Our findings advance our understanding of the molecular mechanisms underlying the development and progression of aging in mammals.

**Results**

**Ablation of p16INK4a reverses ageing phenotypes of klotho mice.** To investigate whether p16INK4a contributes to the accelerated-aging phenotypes in kk/kl mice, we first generated kk/kl mice lacking p16INK4a (kk/kl p16−/− mice) by cross-breeding heterozygous klotho (kl+/-) mice with heterozygous p16INK4a knockout (p16+/-) mice, and tested whether the accelerated-aging phenotypes of kk/kl mice can be reversed by p16INK4a ablation. Various accelerated-aging phenotypes of kk/kl mice, such as growth retardation, osteoporosis, pulmonary emphysema and severe atrophy of the intestinal wall and skin, were remarkably mitigated in the kk/kl p16−/− mice as compared with those in the kk/kl p16+/- (referred to as kk/kl or kk/kl p16+/- littermates with the same genetic background (98.375% C57BL/6, 1.625% C3H/Blj) (Figs 1 and 2, and data not shown). Moreover, the maximum lifespan of the kk/kl p16−/− mice was significantly extended as compared with those of the kk/kl or kk/kl p16+/- littermates, irrespective of gender (Fig. 1b, and data not shown). These results indicated that the accelerated-aging phenotypes of the kk/kl mice depend strongly on the p16INK4a status. The simplest explanation for these results would be that the z-klotho deficiency caused the ageing phenotypes by elevating p16INK4a expression, and thus p16INK4a ablation would mitigate the ageing phenotypes in kk/kl mice.

To test this hypothesis, we took advantage of using the p16-luc mouse12, in which the levels of p16-luc expression can be monitored throughout the body using a bioluminescence imaging (BLI) technique. The p16-luc mice were crossed into the klotho genetic background and were subjected to BLI. Unexpectedly, however, we were unable to detect any substantial increase of p16INK4a expression throughout the body in kk/kl mice as judged by BLI (Fig. 3). Because z-klotho is predominantly expressed in the kidney20,24 (see also Supplementary Fig. 1), we further examined the p16INK4a expression in the kidney using quantitative reverse transcription–PCR (qRT–PCR) analysis. Again, however, there was a slight but insubstantial increase of p16INK4a expression in kk/kl mice, as compared with p16INK4a expression in those of wild-type (wt) mice.

![Figure 1](https://example.com/figure1.png) **Figure 1 | Extension of maximum lifespan of kk/wt mice by p16INK4a ablation.** (a) Representative photographs of 11-week-old mice of each genotype (n = 3). (b) Kaplan–Meier plot showing survival of WT (male, n = 20; female, n = 20), p16−/− (male, n = 21; female, n = 22), kk/wt (male, n = 25; female, n = 24) and p16−/− kk/wt (male, n = 25; female, n = 28).
Figure 2 | Reversing the ageing phenotypes of klotho mice by p16INK4a ablation. (a) Histological analysis of 11-week-old female WT, p16−/−, klotho and p16−/− klotho mice. Representative images of bone radiographs of femurs (X-P), HE of tissues indicated top and von Kossa staining (von Kossa) of the kidney for detecting ectopic calcification were shown. (b) The histograms indicate the quantitative analysis of X-ray transparency of femur (WT (n = 3), p16−/− (n = 3), klotho (n = 6) and p16−/− klotho (n = 3)), the mean linear intercept (Lm) in lung tissue (WT (n = 9), p16−/− (n = 9), klotho (n = 7) and p16−/− klotho (n = 9)), intestinal villi length (WT (n = 14), p16−/− (n = 7), klotho (n = 11) and p16−/− klotho (n = 3)), epidermal and subcutaneous fat layer thickness (WT (n = 6), p16−/− (n = 6), klotho (n = 8) and p16−/− klotho (n = 3)) and the percentages of calcified areas in kidneys (WT (n = 3), p16−/− (n = 3), klotho (n = 3) and p16−/− klotho (n = 3)). For graphs of X-ray transparency of femur and Lm in lung tissues, data were analysed by Mann-Whitney U-test and are displayed as mean ± s.e.m. For graphs of intestinal villi length, epidermal and subcutaneous fat layer thickness and the percentages of calcified areas in kidneys, data were analysed by Student’s t-test and are displayed as mean ± s.e.m. For all graphs: *P < 0.05, **P < 0.01.

(Supplementary Fig. 2). These results raise a question as to how the p16INK4a ablation reversed the accelerated-ageing phenotypes of klotho mice.

p16INK4a ablation restores z-klotho expression in klotho mice.

To explain the effects of the p16INK4a ablation on the accelerated-ageing phenotypes of klotho mice, we next took a closer look at the biochemical characteristics of klotho mice. In klotho mice, the level of 1α-hydroxylase gene expression is reportedly increased in the kidneys, resulting in an elevated serum level of 1,25-dihydroxyvitamin D, the active metabolite of vitamin D that regulates calcium and phosphate homeostasis. Since these changes are known to be associated with the accelerated-ageing phenotypes in klotho mice, we examined the levels of these biochemical hallmarks in klotho mice. Notably, the levels of these hallmarks were substantially reduced in klotho p16−/− mice, as compared with those in klotho mice (Fig. 4a,b). Moreover, the aberrant activation of calpain-1 and the ectopic calcification in kidneys, signs of the abnormal calcium homeostasis observed in klotho mice, were absent in klotho p16−/− mice (Figs 2 and 4c), implying that the z-klotho function might be somewhat restored in the klotho p16−/− mice. Since the klotho mice are not a complete null, but have a severe hypomorphic mutation for z-klotho expression, the entire z-klotho-coding sequence is intact in klotho mice. Thus, we next wondered whether p16INK4a ablation could restore the levels of z-klotho expression in klotho mice. Indeed, the levels of both z-klotho mRNA and protein were substantially increased in the kidneys of p16−/− mice, as compared with those in klotho mice, albeit to lesser extents as compared with those in wt mice (Fig. 4a,c). Notably, z-klotho expression was observed only in the renal distal convoluted tubules in p16−/− mice (Fig. 5a), which are the major sources of z-klotho expression in wt mice. Thus, it appears that p16INK4a ablation restores the normal z-klotho expression pattern in mice,Importantly, in stark contrast to the klotho mouse, the p16INK4a ablation failed to reverse the accelerated-ageing phenotype in mice lacking the z-klotho gene (z-klotho knockout mice (kl−/−); ref. 30; Supplementary Fig. 3). These results indicate that p16INK4a ablation mitigates the accelerated-ageing phenotypes of klotho mice, by restoring z-klotho expression.
p16\textsuperscript{INK4a} downregulates \textit{x}-klotho expression in wt mice. The obvious next question is whether p16\textsuperscript{INK4a} downregulates \textit{x}-klotho expression in wt mice. Note that there is an inverse correlation between the levels of p16\textsuperscript{INK4a} expression and \textit{x}-klotho expression during the ageing process in kidneys (Fig. 5b). However, because p16\textsuperscript{-/-} mice die prematurely from cancer\textsuperscript{17} (see also Fig. 1b), we cannot examine whether p16\textsuperscript{INK4a} ablation ameliorates the ageing-associated decline of \textit{x}-klotho expression in mice harbouring wt \textit{x}-klotho. To circumvent this problem, we employed the mouse model of chemically induced kidney injury. It was previously reported that patients with chronic renal failure develop multiple age-related disorders resembling those of \textit{klotho} mice, with a marked reduction of \textit{x}-klotho expression in kidneys\textsuperscript{33,34}. Moreover, treatment with cisplatin, a chemotherapeutic agent that causes severe adverse actions with nephrotoxicity, is known to provoke a significant reduction of \textit{x}-klotho expression in kidneys, accompanied by the accumulation of DNA damage\textsuperscript{35}. Since persistent DNA damage induces p16\textsuperscript{INK4a} expression in many different cell types\textsuperscript{12}, we analysed the effect of p16\textsuperscript{INK4a} expression on the levels of \textit{x}-klotho expression in the cisplatin-induced kidney injury model. Indeed, the cisplatin treatment resulted in a marked reduction of \textit{x}-klotho expression in the renal distal convoluted tubules of wt mice, coinciding with the accumulation of γH2AX foci, a sign of the DNA damage response, and the induction of p16\textsuperscript{INK4a} expression (Supplementary Fig. 4). Notably, however, the cisplatin-induced \textit{x}-klotho expression was substantially attenuated in the p16\textsuperscript{-/-} mice (Supplementary Fig. 4), although this level of \textit{x}-klotho restoration was insufficient to block the cisplatin-induced nephrotoxicity in this experimental condition (Supplementary
Fig. 5), indicating that p16INK4a has the potential to downregulate \( \alpha \)-klotho expression in wt mice as well.

**p16INK4a downregulates the \( \alpha \)-klotho promoter in murine cells.**

To further verify this notion, we next sought evidence that p16INK4a downregulates \( \alpha \)-klotho promoter activity. As \( \alpha \)-klotho expression is rather limited in the renal distal convoluted tubules, we were unable to find any established murine cell lines expressing substantial levels of \( \alpha \)-klotho (Supplementary Fig. 6). Therefore, primary mouse renal tubular epithelial cells (mRTECs) were prepared from the kidneys of wt mice, and were used for a promoter–reporter analysis. The luciferase activity of a reporter plasmid containing the region 1,035 nucleotides upstream of the mouse \( \alpha \)-klotho translation start site was reduced by the ectopic expression of p16INK4a in a dosedependent manner in early-passage primary mRTECs (Fig. 6a), indicating that p16INK4a indeed downregulates \( \alpha \)-klotho expression at the promoter level. Although the \( \alpha \)-klotho promoter sequences are not well conserved between human and mouse, both include potential binding sites for E2F transcription factors, which are critical downstream mediators of the p16INK4a–retinoblastoma tumour suppressor pathway, at the same position from the \( \alpha \)-klotho translation start sites (Figs 6a and 7b). Notably, newborn mice lacking both E2F1 and E2F3a, a subset of the activator E2Fs (referred to as E2F1\(^{-/-}\) E2F3a\(^{-/-}\) mice), reportedly exhibited normal weight and appearance; however, by their third week of life the proliferative index of most tissues was significantly reduced, and 90% of the mice became severely runted and died within 2 months. Furthermore, white adipose tissues were absent and lung alveolar branching was severely reduced in E2F1\(^{-/-}\) E2F3a\(^{-/-}\) mice. Since these phenotypes are reminiscent of the \( kl/kl \) phenotypes, we analysed whether E2F1 and/or E2F3 activate the \( \alpha \)-klotho promoter activity. Indeed, the ectopic expression of either E2F1 or E2F3 increased the activity of the \( \alpha \)-klotho promoter in cultured primary mRTECs (Fig. 6b). However, this was not the case when the E2F-binding element was disrupted by a nucleotide substitution in the reporter plasmid (Fig. 6b, -674 E2F-Mut). Moreover, increasing amounts of E2F3 blocked the trans-repressing activity of co-transfected p16INK4a in early-passage primary mRTECs (Supplementary Fig. 7). Note that endogenous E2F1 and E2F3 were found to bind to the \( \alpha \)-klotho promoter, as judged by a chromatin immunoprecipitation (ChIP) analysis using cultured primary mRTECs or kidney tissues prepared from wt mice (Fig. 6c). These results, in conjunction with the observation that the levels of endogenous \( \alpha \)-klotho mRNA and protein expression were substantially reduced in the kidneys of E2F1\(^{-/-}\) E2F3a\(^{-/-}\) mice (Fig. 6d), strongly suggest that p16INK4a downregulates \( \alpha \)-klotho expression, at least partly by blocking the function of activator E2Fs in wt mice.

**p16INK4a downregulates \( \alpha \)-klotho expression in human cells.**

Finally, to further support our murine data and to extend the analysis to human physiology, we tested whether p16INK4a downregulates \( \alpha \)-klotho mRNA expression in human cells. Similar to murine cells, primary human RTECs (hRTECs), but not other human cell lines, express substantial levels of \( \alpha \)-klotho (Supplementary Fig. 8). We thus used primary hRTECs in the following experiments. We found that the levels of \( \alpha \)-klotho mRNA expression declined when cultured primary hRTECs were rendered senescent by serial passage, accompanied by the induction of p16INK4a mRNA expression (Fig. 7a, left). However, the short interfering RNA (siRNA)-mediated depletion of p16INK4a substantially increased the levels of \( \alpha \)-klotho mRNA expression in late-passage hRTECs, coinciding with the increased expression of cdc6, an established E2F target gene (Fig. 7a, middle). Conversely, the ectopic expression of p16INK4a reduced the levels of \( \alpha \)-klotho mRNA expression in early-passage hRTECs (Fig. 7a, right). Together, these results suggest that p16INK4a downregulates \( \alpha \)-klotho expression, by blocking the function of the activator E2Fs in human cells, as well as in mouse cells. Indeed, the ectopic expression of either E2F1 or E2F3 substantially increased the transcriptional activity of the human \( \alpha \)-klotho promoter in cultured...
hRTECs (Fig. 7b, -1,028 wt and -473 wt). These effects were blunted when the putative E2F-binding site within the human \(\alpha\)-klotho promoter was mutated or deleted in the reporter plasmid (Fig. 7b, -473 E2F-Mut and -360 wt). Furthermore, the ChIP analysis revealed that endogenous E2F1 and E2F3 bind to the human \(\alpha\)-klotho promoter in cultured hRTECs (Fig. 7c). Interestingly, the G to A single-nucleotide polymorphism (SNP), in the putative E2F-binding site of the human \(\alpha\)-klotho promoter, reportedly impaired the DNA–protein interaction and is associated with the reduction of bone mineral density (BMD) in aged postmenopausal women. Indeed, the G to A substitution in the E2F-binding site of the reporter plasmid greatly reduced the response to E2F overexpression (Fig. 7b, -473 E2F-SNP). These results, in conjunction with previous observations that there is the potential inverse correlation between the levels of renal \(p16^{INK4a}\) expression and \(\alpha\)-klotho expression in elderly people, suggest that \(p16^{INK4a}\) is likely to have the potential to downregulate \(\alpha\)-klotho expression by blocking the transcriptional activity of E2Fs in human kidneys.

**Discussion**

The ageing process is multifactorial, with genetic background and environmental stress as two critical components. The mutation of the \(\alpha\)-klotho gene causes multiple premature ageing phenotypes, including a shortened lifespan in mice, and some SNPs in the human \(\alpha\)-klotho gene are associated with reduced lifespans. Moreover, the levels of plasma \(\alpha\)-klotho decrease with increasing age and are associated with longevity in humans, indicating that the \(\alpha\)-klotho gene is an important factor in the ageing process.
antiageing gene in both mouse and human. However, it remained unclear how the α-klotho gene could be linked to environmental stress. Here we show that the p16\(^{INK4a}\) tumour-suppressor gene, a stress sensor known to induce cellular senescence\(^5\)–\(^12\), downregulates α-klotho gene expression in both mouse and human renal tubular epithelial cells. Ablation of the p16\(^{INK4a}\) gene mitigates various accelerated-ageing phenotypes of klkl/kl mice, including shortened maximum lifespan, by partially restoring α-klotho expression (Figs 1,2 and 4). Furthermore, cell culture studies reveal that p16\(^{INK4a}\) represses α-klotho gene expression at the promoter level by blocking the function of activator E2F, most likely through activation of retinoblastoma expression at the promoter level by blocking the function of

Methods

Mice. All efforts were made to minimize animal suffering and to reduce the number of animals used. Wt mice (C57BL/6), klotho (klkl/kl) mice\(^39\) (mixed C57BL/6) and C3H/J genetic background; 50% C57BL/6, 50% C3H/J) and α-klotho knockout (kl\(^{-/-}\)) mice (C57BL/6) were purchased from CLEA Inc., Japan. Klkl/kl mice were backcrossed with C57BL/6 mice for five generations and used in this study. Klkl/kl p16-luc mice were generated by crossing klkl/kl mice with p16-luc mice\(^12\), E2F1\(^{-/-}\) and E2F3\(^{-/-}\) mice\(^36\) were provided by Dr Gastabo Leone. p16\(^{-/-}\) mice\(^43\) were provided by Dr Norman E. Sharpless. These mice were...
Figure 8 | Dual roles for the p16INK4a/ RB pathway in organismal ageing. The p16INK4a has an established role in provoking cellular senescence, which is likely to cause stem cell ageing and thereby contributing to organismal ageing. Here we show that, in addition, p16INK4a also contributes to organismal ageing through blocking the expression of ageing suppressor, z-klotho.

Bone radiography. BMD was analysed using X-ray radiography. Femur and tibia were resected from 11-week female mice, and placed on wrapped films (FUJIFILM INDUSTRIAL X-RAY FILM ENVLEOPAX IX FR; FUJIFILM Corporation, Japan), and exposed to X-irradiation at 20 kVp, 9 mA for 15 s using a SOFTEX CMB-2 (SOFTEX CO., LTD., Japan). Films were developed using a Fuji Medical Films Processor SEPROS SV (FUJIFILM Corporation) and inspected for BMD.

Bioluminescence imaging. For the detection of luciferase expression, mice were anaesthetised, injected intraperitoneally with D-luciferin sodium salt (75 μg kg−1) 5 min before beginning photon recording. Mice were placed in the light-tight chamber and a grey-scale image of the mice was first recorded with dimmed light followed by acquisition of luminescence image using a cooled CCD (charged-coupled device) camera (PIXIS 1024B, Princeton Instruments)2,24. The signal-to-noise ratio was increased by 2 × 2 binning and 5-min exposure. For colocalization of the luminescent photon emission on the animal body, grey scale and pseudo-colour images were merged by using IMAGE-PRO PLUS (Media Cybernetics).

Histology and immunofluorescence analysis. Samples were fixed in 10% formalin for a 24 h or longer, progressively dehydrated through gradients of alcohol and embedded in paraffin. Samples were then sectioned on a microtome (5-μm thick) and were deparaffinized and rehydrated. Fixed sections were incubated with 5% silver nitrate to aid in nuclei release52. After this procedure, ChIP was performed using the EZ-Chip kit (Millipore) according to the manufacturer's instruction. The immunoprecipitation of cross-linked chromatin was conducted with anti-mouse E2F1 (1:1,000, Santa Cruz, sc-193X), anti-mouse E2F3 (1:1,000, Santa Cruz, sc-878X), anti-human E2F3 (1:1,000, Santa Cruz, sc-193X), anti-human E2F3 (1:1,000, Santa Cruz, sc-878X) and rabbit IgG (1:1,000, Cell Signaling Technology, 2729) as a negative control. After immunoprecipitation, DNA was extracted using the QiAquick PCR purification kit (Qiagen) and an aliquot was amplified using qPCR using following primers flanking the putative mouse E2F-binding site position at −388 to −396 bp of mouse z-klotho gene promoter: 5'-TGTTCTCTGAAAGATTCCCC-3' and 5'-TCCCCCTGGCTTCCGGGAC-3', or primers flanking the putative human E2F-binding site position at −391 to −397 bp of human z-klotho gene promoter: 5'-TGGGAGAAAGTACGACGAC-3' and 5'-TGGGAGAAAGTACGACGAC-3'.
RNA interference. RNA interference (RNAi) was performed using the RNAiMAX transfection reagent (Life Technologies) and siRNA oligos against target genes. The sequences of target siRNAs are as follows:

Human p53 5'-GAGGAGGGUGGCGGCGACTT-3' (sense) and 5'-GAGGCCCGACCACUCCUGTT-3' (antisense) Control 5'-AUGAAGCGUAAAAGGUCAATT-3' (sense) and 5'-UUGAAGCAUUGAGCUCUAU-3' (antisense).

Luciferase-reporter assays. The human and mouse z-klotho gene promoter sequence was amplified with PCR using genomic DNA extracted from the mouse tail or BAC clone (RP11-720E2) containing the entire human z-klotho gene as a template. Deletion mutants were prepared with standard PCR procedures. Promoter sequences containing point mutations were generated using the Quick Change Site-directed Mutagenesis kit (Agilent Technologies). The promoter fragments were inserted into pGL3 basic firefly luciferase reporter plasmid (Promega). All inserted DNAs were sequenced and verified. Transfection of reporter plasmids was performed using the X-treamGENE9 DNA transfection reagent (Roche) according to the manufacturer’s instructions. The luciferase assays were performed using the Luciferase assay systems kit (Promega). Cytomegalovirus promoter-renilla luciferase plasmid or SV40 promoter-galactosidase plasmid was used as an internal control.

Cisplatin (cis-diaminedichloro-platinum II) treatment. Cisplatin was purchased from Wako pure chemical, Japan and dissolved in saline. Mice were intraperitoneally injected with Cisplatin solution (12 mg kg⁻¹) three times (every other day) for a week and killed. The kidneys were immediately taken from mice and used for analysis.

Statistical analysis. The significance of differences was analysed by Student's t-test, Welch’s t-test or Mann–Whitney U-test. P-values of less than 0.05 were considered to be significant. Statistical analyses were performed using the GraphPad Prism programme Ver. 5.01 (GraphPad Software Inc.).

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Author contributions
N.O. and E.H. designed the experiments. S.S., Y.K., A.T., Y.I., A.H., M.T., K.Y. and N.O. performed experiments. H.S. helped in calcium analysis. H.K. and Y.I. helped in histopathology. S.S., Y.N., N.O. and E.H. analysed the data. E.H. wrote the manuscript.

Additional information
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