Nestin-dependent mitochondria-ER contacts define stem Leydig cell differentiation to attenuate male reproductive ageing

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Male reproductive system ageing is closely associated with deficiency in testosterone production due to loss of functional Leydig cells, which are differentiated from stem Leydig cells (SLCs). However, the relationship between SLC differentiation and ageing remains unknown. In addition, active lipid metabolism during SLC differentiation in the reproductive system requires transportation and processing of substrates among multiple organelles, e.g., mitochondria and endoplasmic reticulum (ER), highlighting the importance of interorganelle contact. Here, we show that SLC differentiation potential declines with disordered intracellular homeostasis during SLC senescence. Mechanistically, loss of the intermediate filament Nestin results in lower differentiation capacity by separating mitochondria-ER contacts (MERCs) during SLC senescence. Furthermore, pharmacological intervention by melatonin restores Nestin-dependent MERCs, reverses SLC differentiation capacity and alleviates male reproductive system ageing. These findings not only explain SLC senescence from a cytoskeleton-dependent MERCs regulation mechanism, but also suggest a promising therapy targeting SLC differentiation for age-related reproductive system diseases.
The reproductive system is of crucial importance to many processes related to the development, maturation and aging of males. It has been reported that reproductive system aging implicates other organs, such as the heart and bones, which may lead to overall senescence of the whole organism. Testosterone is the major anabolic hormone in men and a key player in maintaining normal reproductive and sexual function. However, the population mean of serum testosterone level declines with age, leading to aging of the reproductive system. Therefore, it has been suggested that testosterone deficiency may be the driving force that propels the overall senescence of males.

Leydig cells (LCs) account for most testosterone production in men. During aging, the testosterone-synthesizing capacity in LCs gradually decreases, which is considered the major reason for the age-related drop in testosterone levels. Although numerous studies have explored approaches to restore testosterone production in aged LCs, few of them have yielded results with clinical significance, indicating the complexity of regulating the cellular state of LCs. However, it is still feasible to restore testosterone production by reinforcement of newly differentiated LCs. Stem Leydig cells (SLCs), which are mesenchymal stem cells (MSCs), reside in the testicular interstitium. When cultured in vitro, SLCs can be induced to differentiate into LCs with normal testosterone-producing capacity. In addition, in living organisms, SLCs are capable of proliferating and differentiating into functional LCs, but SLCs are likely to be damaged under pathological conditions or senescence. These findings have led us to hypothesize that during aging, the loss of differentiation capacity in SLCs may account for the reduction in the healthy LC population. By uncovering the mechanism of SLC senescence, we may be able to restore the differentiation capacity of aged SLCs, thus re-establishing a functional LC population and ultimately, alleviating the aging process.

The mechanism of stem cell aging is heterogeneous. Segel et al. identified the mecharonesponsive ion channel Piezo1 as a key mediator of oligodendrocyte progenitor cell aging, which corresponds to the fact that the prefrontal cortex progressively stiffens during senescence. For bone marrow-derived mesenchymal stem cells, their aging is regulated by autophagy levels, and inhibiting autophagy could turn young cells into an aged state with degenerative properties. However, the aging mechanism of various kinds of stem cells, such as SLCs in the reproductive system, remains unknown. Multiple organs in the reproductive system display particularly high steroidogenesis activity due to the production of sex hormones. In the testis, the production of testosterone by LCs requires the delicate cooperation of enzymes that are located on different organelles, especially mitochondria and the endoplasmic reticulum. The multistep process that converts cholesterol into the final steroid hormone product involves the transportation of testosterone precursors through close contact sites between these two organelles, namely, mitochondria-ER contacts (MERCs). Considering its central role in controlling steroidogenesis, MERCs should have particular significance in the reproductive system. Moreover, Ziegler et al. demonstrated that MERCs also participate in the senescence of human cells, suggesting that in stem cells, the age-related decline in differentiation capacity may be associated with dysfunctional MERCs. Together, these findings suggest that maintaining normal MERCs could be an ideal approach to preserve the differentiation capacity of SLCs, thus mitigating reproductive system senescence.

Nestin is a type VI intermediate filament (IF) protein originally identified as a marker for neural stem cells in early development. It was later found to be expressed in many other stem/progenitor cell populations, promoting its application as a marker to isolate various adult stem cells, including stem Leydig cells. As an intermediate filament, Nestin is highly dynamic in expression level, which ensures its ability to mediate intracellular activity in response to various stimulations. In recent years, emerging studies have further revealed Nestin’s function as an important regulator, altering various cellular activities, especially in proliferative cells, which seems distinctive among IFs. In our previous work, we demonstrated that Nestin colocalizes with mitochondria, and that knockdown of Nestin influences the mitochondrial function, including oxygen consumption rates, ATP generation and mitochondrial membrane potential. Moreover, Nestin has also been found to participate in the maintenance of stemness in neural stem/progenitor cells. Therefore, uncovering the function of Nestin in regulating intracellular homeostasis and the differentiation capacity of stem Leydig cells will provide new insights into therapies for male age-related reproductive diseases.

Here, we report that SLCs from aged mice exhibit lower differentiation capacity. It is postulated that this reduction in stemness is due to impaired MERCs, which in turn results in imbalanced intracellular homeostasis, including disorderred redox homeostasis and increased ER stress. We further identifies Nestin as an important regulator of MERCs, which reacts upon elevated ROS stress by competing with Nrf2 in binding Keap1. Intriguingly, melatonin treatment significantly rescues MERCs deficiency in aged SLCs, thus restoring their differentiation capacity. Moreover, in vivo study shows that improving MERCs are effective in improving overall male function in aged mice, providing a promising path to stem cell therapy in reproductive system aging.

**Results**

**SLCs exhibit lower capacity for differentiation during aging.** To determine the exact reason for the age-related decline in testosterone levels, we investigated changes that occurred in the testosterone-producing cell population during aging. H&E staining of mouse testicular mesenchyme revealed a significant decrease in interstitial cell number during aging, mostly resulting from the reduction of LCs (Fig. 1a). Along with the reduction in serum testosterone levels (Fig. 1b), lowered mRNA expression of LC markers, such as HSD3b, CYP11A1, and HSD17b3, was also noticed (Fig. 1c–e). In addition, the density of the cells expressing these markers also decreased in aged testes (Fig. 1f–i). The aforementioned result confirmed the reduction in the LC population as the major cause of the age-dependent decline in testosterone production.

SLCs are capable of proliferating and differentiating into functional LCs, playing an important role in maintaining the number of LCs and intratesticular environmental homeostasis. Thus, to determine whether the shrinkage in LC colonies is caused by dysfunction in generating new LCs, we tracked the cell fate of Nestin-expressing SLCs by labeling them with RFP (Fig. 1j). Interestingly, we found that there was a prominent reduction in newly differentiated LCs, shown as RFP+ cells (Fig. 1j). Consistently, we found that there was a prominent reduction in newly differentiated LCs, shown as RFP+ and RFP+LHR+ cells, relative to the total number of RFP+ cells in old mice, suggesting that the impaired differentiation of SLCs into LCs chiefly contributed to the decreasing number of LCs in senescent testes (Fig. 1k–l).

To further confirm the effect of aging on SLC differentiation, we harvested Nestin+ SLCs from mice of different ages and induced them to differentiate in vitro (Supplementary Fig. 1a). Consistent with previous experiments, we identified that SLCs from old mice produced fewer mature LCs (Supplementary Fig. 1b–j). Together, these results indicate that the age-dependent decrease in the differentiation capacity of SLCs leads to a reduced LC population, thus resulting in insufficient testosterone production during aging.
SLCs establish fewer MERCs during ageing. The mitochondria-ER contacts (MERCs) are crucial for carrying out various cellular functions, including mitochondrial dynamics, Ca\(^{2+}\) signaling and lipogenesis. Considering the active lipid metabolism in the reproductive system, we speculated that MERCs are particularly important in regulating the differentiation of SLCs into LCs. Therefore, we further investigated whether the loss of differentiation capacity of aged SLCs results from the decoupling between mitochondria and the ER. Colocalization analysis revealed that mitochondria and ER displayed less contact during ageing (Fig. 2a–d).
mitochondria and the ER was less than 100 nm\textsuperscript{22} and we found that fewer effective apposition sites could be observed in SLCs from older mice, and a smaller proportion of mitochondria was in close contact with the ER (Fig. 2e–g and Supplementary Fig. 2a, b). This phenomenon was robust, as we repeated our quantification using a variety of interorganellar tethering lengths—mito-ER distances of 100, 50, and 25 nm (Supplementary Fig. 2c–f), which all pointed to the same conclusion. Moreover, we applied surface-surface contact site area assay and proximal ligation assay (PLA) to further illustrate the age-related decline in MERCs, which were also consistent with our previous results, indicating fewer MERCs (Fig. 2h–j and Supplementary Fig. 2g–j). Together, these results suggest that senescence may exert its influence on SLCs by scissoring mitochondria and ER apart.

Fewer MERCs result in mitochondria and ER dysfunction. Intracellular homeostasis is largely dependent on the normal function of mitochondria and the ER\textsuperscript{23,24}. The disruption of MERCs usually leads to impaired intracellular homeostasis, mainly characterized by an imbalanced oxidative state and increased ER stress\textsuperscript{25,26}. First, we observed that there was an increase in intracellular ROS levels, especially mitochondrial ROS, and a reduced antioxidation ability in SLCs from aged mice (Fig. 3a–e and Supplementary Fig. 3a–m). In addition, mitochondria became smaller during SLC senescence (Fig. 3f–i and Supplementary Fig. 4a, b). Moreover, higher apoptosis, lower mitochondrial membrane potential and enhanced mitophagy but total RFP + cells of different age Nexitin-CreER\textsuperscript{ERT2}, Rosa26\textsuperscript{RFP} mice. I Quantification of percentage of RFP + LHR + cells in total RFP + cells of different age Nexitin-CreER\textsuperscript{ERT2}, Rosa26\textsuperscript{RFP} mice in (k) (n = 5 biological repeats for each group; One-way ANOVA). Two-sided comparison; All data are mean ± SD; Error bars represent SDs. *p < 0.05, **p < 0.01, ***p < 0.001; Source data are provided as a Source Data file. See also Supplementary Fig. 1.

Targeting Nestin degradation restores aged SLCs homeostasis. Melatonin, an endogenous hormone synthesized by the pineal gland, has been proved to be downregulated in aged organism\textsuperscript{35,38}, and could have antioxidant capacity in a variety of ways to reduce elevated ROS levels during ageing\textsuperscript{37–39}. In addition, melatonin is capable of protecting mitochondria\textsuperscript{40}, relieving ER stress\textsuperscript{41}, and even enhancing Nrf2 expression while inhibiting Keap1\textsuperscript{12}, besides it has been safely used clinically\textsuperscript{43,44}. Given the above reasons, we finally chose melatonin for treatment and further explored its potential for restoring SLC functions. We found that melatonin successfully restored Nestin protein levels
in aged SLCs in vivo (Fig. 5a), which resulted from inhibited ubiquitin-proteasomal degradation (Fig. 5b). As we expected, melatonin treatment in vivo could also restore MERCs in aged SLCs, providing stronger proof of the relationship between Nestin and MERCs (Fig. 5c). Furthermore, in addition to decreased ROS stress, restored redox homeostasis and alleviated ER stress were observed in SLCs after melatonin treatment, and mitochondrial dysfunction was also prevented (Fig. 5d–f, Supplementary Fig. 8a–o and Supplementary Fig. 9a–h). Moreover, the expression of numerous LC markers and testosterone production were both rescued after melatonin treatment (Fig. 5g and Supplementary Fig. 10a–h). In addition, melatonin’s efficacy was
investigated ex vivo (Fig. 5h). Nestin expression was also rescued in the aged group by melatonin treatment (Supplementary Fig. 10i, j), followed by increased HSD3β expression and testosterone production (Fig. 5i–k). In addition, the same phenomena such as increased MERCs and enhanced differentiation capacity in aged SLCs were found in other classic antioxidants, such as NAC and VitE45,46 (Supplementary Fig. 11a–h). Furthermore, we confirmed the efficacy of melatonin in a senescence-inducing model by applying H2O2 to young SLCs (Supplementary Fig. 12a–o). Finally, the functional link between melatonin and MERCs was further verified by loss-of-function experiments (Supplementary Fig. 13a–g). Altogether, these multimodal data repeatedly draw a clear association between melatonin availability and MERCs formation through Nestin.

Melatonin promotes male reproductive functions in old mice.

We next applied melatonin treatment to mice to combat the age-related decline in numerous male functions, such as testosterone production and spermatogenesis. The results revealed that Nestin expression was successfully restored after melatonin treatment (Fig. 6a, b). In addition, melatonin treatment restored MERCs in aged SLCs in situ (Fig. 6c–e). Moreover, more mature LCs could be seen in the testicular interstitium from melatonin-treated mice, together with elevated LC markers expression and enhanced testosterone production (Fig. 6f, g and Supplementary Fig. 14a–d). Given that the action of testosterone is known to be critical for the completion of meiosis and spermatogenesis in aged SLCs were found in other classic antioxidants, such as increased MERCs and enhanced differentiation capacity in aged SLCs in situ (Fig. 6c–e). Moreover, more mature LCs could be seen in the testicular interstitium from melatonin-treated mice, together with elevated LC markers expression and enhanced testosterone production (Fig. 6f, g and Supplementary Fig. 14a–d). Given that the action of testosterone is known to be critical for the completion of meiosis and spermatogenesis in old mice and found that the expression of the meiotic marker SYCP3 was increased in the seminiferous tubules (Fig. 6h–j), which was also evidenced by H&E histological analysis of the testes (Supplementary Fig. 14a, f). Notably, the epididymal sperm number and sperm motility appeared to be significantly higher in the melatonin-treated aged mice (Fig. 6k and Supplementary Fig. 14g–h). In addition, we further detected the weight of organs associated with the reproductive system and found an increased testes volume and weight, accompanied by a decrease in the weight of the epididymis, seminal vesicle, and prostate present in melatonin-treated old mice (Supplementary Fig. 14i–m). Altogether, these results indicate that melatonin treatment significantly restored reproductive function in old male mice, thus showing great potential for combating various age-related male diseases.

Furthermore, we sought to confirm whether Nestin was necessary to rescue LC numbers by melatonin treatment. We specifically knocked out Nestin in SLCs by constructing PDGFRα-CreERT2, NestinloxP/loxP, Rosa26RFP mice, which received melatonin treatment with PDGFRα-CreERT2, Rosa26RFP control group was in parallel (Fig. 6l). The efficacy of Nestin knockout was confirmed by measuring Nestin with the SLC marker PDGFRα in vivo (Fig. 6m, n). Consistent with our hypothesis, Nestin-knockout mice presented with fewer LCs in the testicular mesenchyme and lower serum testosterone levels, which could not be rescued by melatonin treatment (Fig. 6o–q). These findings confirmed that melatonin rescues the differentiation capacity of aged SLCs via a Nestin-dependent pathway.

Nestin knockdown diminishes Melatonin’s anti-senescence effect. To further examine the role of Nestin in the pathogenesis of SLC differentiation in vivo, we constructed PDGFRα-CreERT2, Rosa26CAG-LSL-Cas9-tdTomato mice, delivered AAV8-Control or AAV8-Nestin sgRNA, and then treated them with melatonin 8 weeks later (Fig. 7a). By conditional knockout of Nestin expression in PDGFRα+ SLCs in the testes (Supplementary Fig. 15a–f), ER and mitochondria exhibited significant detachment compared to the control group, which failed to be rescued by melatonin treatment (Fig. 7b, c). Functionally, melatonin treatment also failed to improve the differentiation capacity of SLCs into the LCs, testosterone production, and spermatogenesis in conditional Nestin knockout mice (Fig. 7d–h and Supplementary Fig. 15g, h). Moreover, disordered redox homeostasis and ER stress failed to be restored in the SLCs after melatonin treatment in vivo (Fig. 7i–j and Supplementary Fig. 15i, j).

Furthermore, we verified Nestin’s pivotal role in regulating SLC differentiation towards LCs in a chemically damaged model45,49. Before melatonin treatment, the existing LCs in the testicular mesenchyme were eliminated by intraperitoneal injection of ethylene dimethanesulfonate (EDS) (Fig. 7k and Supplementary Fig. 15k). However, considering that this treatment resulted in the partial elimination of LCs in the adult testes in mice50,51, we further detected the number of LCs and testosterone levels recovered after melatonin treatment, yet the effect was much less significant in conditional Nestin knockout mice (Fig. 7l–n).

Altogether, the results indicated that melatonin treatment can effectively attenuate reproductive ageing in old mice. Importantly, Nestin plays an irreplaceable role in the process, probably by regulating MERCs (Fig. 8).

Discussion

In this study, we identified mitochondria-ER contacts (MERCs) deficiency as the main reason for the reduced differentiation...
capacity in aged SLCs. This finding added to the understanding of the testosterone production drop in the aged population, apart from LC degeneration. Furthermore, we revealed a novel function of the intermediate filament Nestin in regulating stem cell differentiation, contributing to our understanding of cytoskeletal function.

Reproductive system ageing is often accompanied by decreased circulating testosterone levels. Typically, the functional decline of LCs, rather than the loss of their quantity, is considered to be the main culprit for reduced testosterone production. Influenced by this theory, numerous studies have focused on the rejuvenation of aged LCs, yet none has revealed results that are promising.
Fig. 3 Fewer MERCs result in mitochondrial dysfunction and ER stress in aged SLCs. a Flow cytometry of intracellular ROS level stained with DHE of Nestin-GFP + SLCs from different age groups in vivo. b Quantification of mean fluorescence intensity of DHE in (a). (n = 5 biological repeats for each group; One-way ANOVA). c qPCR analysis of relative mRNA expression of anti-oxidative genes of Nestin-GFP + SLCs from different age groups. (n = 5 biological repeats for each group; Multiple t tests). d, e Western Blot analysis and quantification of cellular anti-oxidative protein expression of Nestin-GFP + SLCs from different age groups. (n = 5 biological repeats for each group; Multiple t tests). f Representative immunostaining pictures of mitochondrial morphology of primary Nestin-GFP + SLCs from different age groups in vitro. Mitochondria are marked with Tom20. Scale bar, 20 μm. g A plot of Aspect Ratio (AR) against Form Factor (FF) showed that particles in primary Nestin-GFP + SLCs from different age groups in (f). Lower values of FF and AR, indicating small, circular mitochondrial. h Representative TEM images of mitochondrial morphology of primary Nestin-GFP + SLCs from different age groups in vitro. Scale bar, 1 μm for original pictures and 500 nm for enlarged pictures. i Quantitative analysis of the average value of mitochondrial length (micrometers) in (h). (n = 5 biological repeats for each group; n = 126-184 mitochondria; One-way ANOVA). j qPCR analysis of relative mRNA expression of ER stress-related genes of primary Nestin-GFP + SLCs from different age groups in vitro. (n = 3 biological repeats for each group; Multiple t tests). k-o Western Blot analysis and quantification of ER stress-related proteins of primary Nestin-GFP + SLCs from different age groups in vitro. (n = 3 biological repeats for each group; Multiple t tests). Two-sided comparison; All data are mean ± SD; Error bars represent SDs. *p < 0.05, **p < 0.01, ***p < 0.001; Uncropped western blots and source data are provided as a Source Data file. See also Supplementary Figs. 3–5.

enough for clinical application. Consequently, we sought to restore testosterone production by increasing the supply of healthy, fully-functional LCs, regardless of the degenerated intrinsic LCs. By targeting MERCs in aged SLCs, enhanced differentiation towards LCs was achieved, contributing to a fortified testosterone-producing LC colony. Elevated circulating testosterone levels were observed after treatment, which indicates that the healthy LC supplied from enhanced SLC differentiation could, at least partly, offset the impact of intrinsic LC degeneration. Considering the potential risk of exogenous testosterone replacement therapy54,55, our stem cell-targeting approach may be a promising path to recover endogenous testosterone in treating reproductive system diseases and ameliorating male ageing.

The relationship between stem cell ageing and differentiation is complicated. On the one hand, the loss of differentiation capacity is considered one of the most important signs of stem cell ageing56; on the other hand, excessive differentiation has been shown to deplete the stem cell pool, leading to the overall ageing of organs and systems57. In addition, for some types of stem cells, their ageing usually accompanies skewed differentiation into certain downstream lineages58. However, it remains unclear whether the senescence of stem cells damages their intrinsic differentiation modulators, or whether the extended period of differentiation causes the degeneration of stem cells. In exploring the reason for reduced testosterone production in aged mice, we solely focused on the loss of differentiation capacity of SLCs. Moreover, we observed an age-related decrease in Nestic expression as a potential regulator of MERCs, further influencing the differentiation of SLCs. These findings strongly suggest that the senescence of SLCs may be the root cause of reproductive system ageing, which will be elucidated deeply in future studies.

The MERCs have been proven to have great significance in maintaining lipid and calcium (Ca^{2+}) homeostasis, initiating autophagy and mitochondrial division, and sensing metabolic shifts59. More recently, Latorre-Muro et al. showed that MERCs are also required for the regulation of mitochondrial cristae formation60. These findings imply the central role of MERCs in regulating intracellular homeostasis and maintaining the functional state of the two organelles. Considering mitochondria, an adequate level of MERCs is essential to mitochondrial health. MERCs are closely associated with mitochondrial dynamics and mitophagy, which are necessary to optimize mitochondrial quality control and maintain normal intracellular ROS level61. Given the importance of ROS in regulating stem cell activity, the age-dependent deterioration of stem cell function should be closely linked to mito-ER crosstalk. Consistent with this, our findings proved that impaired MERCs in aged SLCs lead to smaller mitochondrial morphology, reduced mitochondrial mass, ROS accumulation and decreased ATP production, which also corresponds to the reduced differentiation ability in aged SLCs. Regarding the regulation of ER, it is commonly believed that increased ER stress usually accompanies the tightening of MERCs28, which seems contradictory to our result that impaired MERCs appear with increased ER stress simultaneously. However, impaired MERCs may serve as a perfect example of a dysfunctional regulatory system in aged stem cells. When ER stress occurs in healthy SLCs, MERCs tighten to increase ATP production, thus facilitating the synthesis of ER chaperones that contribute to the normal folding of proteins28. Moreover, previous studies have confirmed that MERCs are the initiation point of autophagy62,63, which eliminates misfolded proteins and alleviates the unfolded protein response in the ER. As a result, these studies suggest that MERCs is a cellular response that will be activated transiently and alleviates ER stress when it occurs. Together, this finding further echoes the fact that MERCs play a central role in regulating the homeostasis of the reproductive system, thus controlling the differentiation of SLCs.

Various kinds of cytoskeletons have been proven to be involved in the regulation of MERCs. Friedman et al. proposed that acetylated microtubules provide tracts for mitochondria and ER transportation within the cell, thus increasing the chance for establishing contact between the two organelles59. Korobova et al. also suggested that actin fibers with related regulatory factors are a classical regulatory factor of MERC54,65. In this study, however, no change in the expression of morphology of these two kinds of cytoskeletons was observed, suggesting that neither microtubules nor actin fibers accounted for the mito-ER detachment in aged SLCs, probably due to different cell types or different conditions. Intermediate filaments (IFs) are an enormous family of cytoskeletal proteins, comprising Lamin, Desmin, Keratin, Vimentin and Nestin, which are highly variable among different tissues and different developmental stages of a single cell. Unlike microtubules and actin fibers, the structure and function of the IF skeleton is controlled by the dynamic expression of IF proteins66. In addition, various studies have demonstrated that IF expression is altered as the cellular state changes67–69, indicating that IF has regulatory functions in mediating cellular function that far exceed the basis supporting the function of the cytoskeleton. Nevertheless, due to the heterogeneity between different IF subfamilies, their functions have not been fully elucidated. Here, we reported that Nestin expression actively mediates the differentiation of SLCs through MERCs, adding to the evidence that IF proteins can regulate cellular function via expression changes, thus more widely expanding our knowledge about IF’s function. Moreover, we also
found the reduction of MERCs due to the degradation of Nestin might trigger a different compensatory change of MAM protein levels, consistent with the conclusion that the change in MAM protein levels does not always correlate with the MERCs number, indicating other molecular mechanisms regulating the number of MERCs or the other cellular distribution and biological function, which enlightens us to seek for deeper understanding of the relationship between Nestin and MAM proteins in the future studies. Therefore, considering Nestin’s proven effect in regulating stem cell function, more Nestin-targeting therapies in rejuvenating aged stem cells are worth exploring.
In various organs and systems, the senescence of stem cells often accompanies elevated intracellular ROS levels. Interestingly, melatonin, the indoleamine produced by the pineal gland, has been well stated for its antioxidant properties. It has been reported that melatonin can protect stem cells by scavenging radicals, upregulating antioxidant enzyme expression, reducing the activity of pro-oxidant enzymes, and maintaining mitochondrial homeostasis. More encouragingly, data from animal and human studies have proven that short-term use of melatonin elicits only mild adverse effects, such as dizziness, headache, nausea, and sleepiness, even in extreme doses. Here, we explored melatonin’s effect in restoring Nestin expression and MERCS, yielding satisfactory results. To date, approaches to regulate intracellular Nestin levels are limited to gene ablation and RNA interference, which greatly hinders Nestin-targeted therapy from clinical application. Intriguingly, this finding provided evidence that Nestin expression can be altered by small molecular drugs, which is far more practical than gene therapy. Considering the important function of Nestin in multiple organs and systems, there should be wider application of Nestin-targeted therapy in other degenerative diseases, which can be explored in future studies.

In conclusion, our identification of the pivotal role of Nestin-dependent MERCS in SLC senescence opens the door for further understanding of male reproductive system ageing, providing insights into therapies for age-related diseases.

Methods

Animals. Animal use and all experiments involving animals were approved by the Ethical Committee of Sun Yat-sen University. Only male mice were used throughout the experiment.

C57BL/6 mice were purchased from Beijing Vital River Laboratory, aged 2 months, and 24 months were used for establishing comparisons between different age groups. Nestin-GFP mice (homozygous transgenic mice expressing enhanced GFP controlled by a Nestin promoter on the C57BL/6 genetic background) were kindly provided by Dr. Masahiro Yamaguchi.

PDGFRα-CreERT2 mice81,82 were gifts from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. NestinCreLoxP/l mice were purchased from VIEWSOLID BIOTECH (Beijing, China).

Rosa26CreERT2 mice were purchased from VIEWSOLID BIOTECH (Beijing, China).

NestinCreLoxP/l mice were purchased from VIEWSOLID BIOTECH (Beijing, China).

NestinCreLoxP/l mice were purchased from VIEWSOLID BIOTECH (Beijing, China).

Microinjection. Microinjection was performed using a Picospritzer (General Valve, USA) and a stereomicroscope (Carl Zeiss, Germany).

RNA transfection. For loss of Nestin function, retrovirus vectors (pSM2) encoding Nestin shRNA were used according to previous articles. Scramble shRNA was used as a control. All shRNAs were constructed in our laboratory. Details on the plasmids are provided in Supplemental Table 2.

Retrovirus transduction. Retrovirus vectors (pSM2) encoding Nestin shRNA were used according to previous articles. Scramble shRNA was used as a control. All shRNAs were constructed in our laboratory. Details on the plasmids are provided in Supplemental Table 2. ShRNA transfections were performed using the MegaTran 1.0 Transfection Reagent (OriGene) according to the manufacturer’s instructions. Briefly, the lentiviruses were used to infect SLCs with Polybrene (8 μg/ml) for 4 h. The original medium was replaced with a fresh medium 12 h later. Mice-targeted Nestin was constructed using Invitrogen’s Gateway System. The efficiency of knockdown was assessed by Western blot. The shRNAs were purchased from Ribobio, and their encoding vectors were transfected into cells using the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen).
LC differentiation of Nes-GFP+ cells in vitro. Nes-GFP+ cells were cultured in fresh differentiation-inducing medium containing phenol red-free DMEM/F12 and M199 medium (1:1; Gibco), 2% FBS, 10 ng/ml PDGFAA (PeproTech), 1 ng/ml LH (R&D Systems), 250 nM Smoothened Agonist HCl (SAG; Millipore), 1 μM Forskolin (Fsk; Sigma-Aldrich), 50 ng/ml insulin-like growth factor 1 (IGF1, PeproTech), and 5 μg/L insulin-transferrin-sodium selenite (Sigma), and they were incubated for 14 days.

Culture of seminiferous tubules. Seminiferous tubules were isolated and cut into equal lengths (5 cm) and then they were cultured in 24-well plates in phenol red-free DMEM/F12 and M199 medium (1:1; Gibco) supplemented with 0.1% BSA, LH (10 ng/mL), penicillin/streptomycin (100 U/mL and 100 μg/mL), and 5 μg/L insulin-transferrin-sodium selenite (Sigma) for up to 2 weeks at 37 °C in a humidified 5% CO₂ water-jacketed incubator, according to the established protocol.
RNA isolation and Real-time quantitative PCR. Total RNA was extracted from testis tissue or cells using the TRizol reagent (Molecular Research Center, Inc.) according to the manufacturer’s protocol. Quantification was performed with a NanoDrop 2000 spectrophotometer and 1 µg of total RNA was used to reverse transcribe with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1622). cDNAs were used as the template for real-time quantitative PCR (qPCR) reactions with the FastStart Essential DNA Green Master Mix (Roche, 480129204001). All samples were run in triplicate and the results were normalized to 18S rRNA as relative mRNA levels. The primers designed and used for qPCR are described in Supplementary Table 2.

Western blot. For Western blot analysis, cell lysates were collected and washed twice with cold phosphate-buffered saline (PBS). Then, the cells were directly lysed twice with cold phosphate-buffered saline (PBS). Then, the cells were directly lysed with a dounce homogenizer in 1 ml LP2 lysis buffer (20 mM Tris-HCl (pH 7.7), 100 mM NaCl, 10 mM NaF, 20 mM β-glycerophosphate, 5 mM MgCl2, 0.1% Triton X-100, 5% glycerol), supplemented lysate buffer (20 mM Tris-HCl (pH 7.7), 100 mM NaCl, 10 mM NaF, 20 mM β-glycerophosphate, 5 mM MgCl2, 0.1% Triton X-100, 5% glycerol), supplemented with benzonase (30 U/l; Santa Cruz) and complete protease inhibitor cocktail (Roche), and incubated at 4 °C for 1 h. Subsequently, lysates were terminated by centrifugation at 16,000 × g for 30 min, resulting in lyses. 1 ml of lystate with 10 µl of protein-oxidation carrying beads rotated for 4–12 h at 4°C. Precipitated products were then washed with lysis buffer and eluted with 2.5× SDS protein gel loading solution containing 10% β-mercaptoethanol for Western blotting. Related antibodies used can be found in the Supplementary Table 2. Chemiluminescent substrate (Millipore) was used for quantification of Nestin expression in primary SLCs from 2 months old, 24 months old, and melanoten-treated 24 months old mice. (n = 3 biological repeats for each group; Unpaired t-test).

**Flow cytometric assay.** Testicular cells derived from Nestin-CreERT2; Rosa26RFP and Nestin-CreERT2; Cas9-tdTomato mice were washed with FACS buffer (1% bovine serum albumin in PBS containing 0.5 mM EDTA) twice. Cells were stained with primary and secondary antibodies in the dark on ice at 4°C. Cells were then washed twice with FACS buffer and run on CytoFLEX (Beckman Coulter, USA). Data were analyzed using the FlowJo software (Tree Star Inc., Ashland, Oregon).

**Immunohistochemistry (IHC) and Immunofluorescent staining (IF).** For IHC staining, mouse testis tissues were fixed with 4% neutral-buffered paraformaldehyde. Paraffin-embedded tissues were sectioned by 5 mm on a Leica RM2255 rotary microtome, which was subjected to immunostaining using an UltraSensitiveTM SP (Mouse/Rabbit) IHC Kit (MXB, KIT-9710). Following deparaffinization and antigen retrieval, the testis tissue specimens were incubated overnight at 4 °C with corresponding antibodies listed in Supplementary Table 2.  Signal amplification and detection were performed using a DAB system according to the manufacturer’s instructions (MXB, MAX-001).

For immunofluorescent staining, the tissue sections and cells were incubated with the primary antibodies listed in Supplementary Table 2, stained using a Zeiss 880 Laser Scanning Confocal Microscope, a Zeiss 880 Laser Scanning Confocal Microscope with Airyscan, Dragonfly CR-DFLY-202 2540 and a Nikon A1R N-SIM N-STORM Microscope.

**Histology analysis.** After sacrifice, mouse testis and cauda epididymis tissues were perfused with 4% paraformaldehyde and then subjected to paraffin embedding. Sections (2 mm) were cut and stained with hematoxylin and eosin (H&E). The sections were analyzed, and images were captured using a Zeiss Axiocan.Z1 microscope.

**Counting Leydig cells.** To count CYP11A1 + or HSD3β + or HSD1β3 + Leydig cells, a fractionator technique was used for the above tissue-array sections according to established protocol88,89. In brief, under the live image of a digital camera with a ×10 objective and fixed point of the “upper” sections, we counted cells of the total microscopic field. We calculated the total number of Leydig cell number counted in a known fraction of the testis by the inverse of the sampling probability.

**Mitochondria and endoplasmic reticulum staining and analysis.** The mitochondrial structural network was detected by 200 nM Mitotracker Red and ER tracker Green (Cell Signaling Technology) in a culture medium at 37 °C for 30 min. After washing, the cells were stained with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100 and then stained with other antibodies. Cells were imaged using a Zeiss 880 Laser Scanning Confocal Microscope with Airyscan and Nikon A1R N-SIM N-STORM Microscope. Acquired images for mitochondria morphology were analyzed10. The colocalization indexes Pearson’s and Manders’ coefficients were calculated with the ImageJ colocalization analysis plugin according to previous articles36,37. MERCs were also reconstructed and analyzed via surface-surface contact site area tool by Imaris (Bitplane) followed the manufacturer instructions.

**In situ proximity ligation assay.** Proximity ligation was performed in Duolink® Blocking Solution–fixed tissue or cells. According to the established protocol15, samples were incubated with specific primary antibodies to the putative interacting partners, followed by Duolink® PLA Probe were added to the reaction and incubated. Two oligonucleotides and ligase were the ligation solution. In this assay, the oligonucleotides hybridize to the two proximity ligation probes and

**SA-β-gal staining.** According to the manufacturer’s protocol of senescence-associated β-galactosidase kit (Boyehite, C6002), SLCs seeded in 12-well plates were fixed with PBS and fixed in 4% paraformaldehyde for 15 min. After washing, SLCs were incubated overnight with the working solution of β-galactosidase plus X-Gal at 37°C. The senescent cells were observed under an optical microscope (Leika, DMi8) and counted from three random fields of vision.
join to a closed-loop if they are in close proximity. Amplification solution, consisting of nucleotides and fluorescently labeled oligonucleotides, was added together with polymerase. The oligonucleotide arm of one of the proximity ligation probes acts as a primer for "rolling-circle amplification" using the ligated circle as a template, and this generates a concatemeric product. Fluorescently labeled oligonucleotides hybridize to the rolling circle amplification product (Duolink; SigmaAldrich). The proximity ligation signal was shown as a distinct fluorescent spot and was analyzed by confocal microscopy. Control experiments included routine immunofluorescence staining of the proteins of interest under identical experimental conditions. PLA spots were counted in cell lines using software Blobfinder version 3.290. PLA scores were calculated by normalizing the number of PLA spots counted in each sample to the average number of PLA spots counted in the control sample, which was set to 100.
Fig. 6 Treatment with Melatonin increases testosterone level and promotes spermatogenesis in old mice. a, b Western blot analysis and quantification of Nestin expression in the testes from different age group. (n = 3 biological repeats for each group; Unpaired t test). c, d Analysis of TEM from (e). Percentage of mitochondria coverage covered by ER (n = 115 cross-sections from 5 mice for each group) and mito-ER proximity were quantified (n = 110 cross-sections from 6 mice for each group); Unpaired t test. e Representative TEM images of testicular mesenchyme. (mitochondria, yellow; ER, red; contact sites, blue). Scale bar, 500 nm for original pictures and 250 nm for enlarged pictures. f Measurement of serum testosterone concentration. (n = 6 biological repeats for each group; Unpaired t test). g qPCR analysis of relative mRNA expression of testosterone production-related genes in the testes. (n = 6 biological repeats for each group; Unpaired t test). h Representative immunostaining pictures of SYCP3-positive cells and Sox9-positive Sertoli cells in seminiferous tubules of testes. Scale bar, 40 μm. i Quantification of the number of SYCP3-positive cells and Sox9-positive Sertoli cells per seminiferous tubule. (n = 6 biological repeats for each group; Multiple t tests). j Quantification of the ratio of SYCP3-positive cells vs Sox9-positive Sertoli cells per seminiferous tubule. (n = 6 biological repeats for each group; Unpaired t test). k Representative H&E staining pictures of cauda epididymis. Scale bar, 500 μm for original pictures and 50 μm for enlarged pictures. l Schematic of the experimental animal model for conditionally knocking out Nestin in PDGFRα+ cells. Nestin KO: Nestin knockout. m Representative immunostaining pictures of Nestin in testicular mesenchyme of Control and Nestin KO mice. Scale bar, 40 μm for original pictures and 20 μm for enlarged pictures. n Representative immunostaining pictures of Nestin in primary SLCs from Control and Nestin KO mice. Scale bar, 20 μm. o Representative immunostaining pictures of testicular mesenchyme from Control and Nestin KO mice, treated by ethanol or melatonin. LCs are identified as H5D3J+ i cells. Scale bar, 40 μm for original pictures and 40 μm for enlarged pictures. p Quantification of LCs cell numbers in (l). (n = 6 biological repeats for each group; Multiple t tests). q Measurement of serum testosterone level of Control and Nestin KO mice, treated by ethanol or melatonin. (n = 6 biological repeats for each group; Multiple t tests). Two-sided comparison; All data are mean ± SD. Error bars represent SDs. *p < 0.05, **p < 0.01, ns, p > 0.05; Uncropped western blots and source data are provided as a Source Data file. See also Supplementary Fig. 14.

Transmission electron microscopy. Cells grown in chamber slides were fixed in 2.5% glutaraldehyde in PBS for 2 h at room temperature, then stored at 4 °C overnight before processing. Thin sections on grids were observed in a Tecnai 12 BioTwin transmission electron microscope (FEI) at 120 kV. Images were acquired with a charge coupled device camera (AMT) according to previous article22.

For tissue microscopy, the testis was isolated from mice, cut in 1 mm thick sagittal sections and small portions of upper layers of the cortex were dissected for further processing (for injured cortices, the examined area was dissected according to the location of the lesion track). For EPON embedding, the fixed tissue was washed with 0.1 M sodium cacodylate buffer, incubated with 2% OsO4 in 0.1 M cacodylate buffer (Osmium, Science Services; Caco Applichem) for 2 h at 4 °C and washed again three times with 0.1 M cacodylate buffer. Subsequently, tissue was dehydrated using an ascending ethanol series with 15 min incubation at 4 °C each in EtOH solution. Tissues were transferred to propylene oxide and incubated in EPON (Sigma-Aldrich) overnight at 4 °C. Tissues were placed in fresh EPON at RT for 2 h, followed by embedding for 72 h at 62 °C. Ultrathin sections of 70 nm were cut using an ultramicrotome (Leica Microsystems, UC6) with a diamond knife (Diatome, Biel, Switzerland) and stained with 1.5% uranyl acetate at 37 °C for 15 min and lead citrate solution for 4 min. Electron micrographs were taken with a JEM-2100 Plus Transmission Electron Microscope (JEOL), equipped with Camera OneView 4 K 16 bit (Gatan) and software DigitalMicrograph (Gatan).

For analysis, the length of each mitochondrion was determined in ImageJ following a manual drawing of single organelles. The extent of mitochondria-ER contact sites (defined as sites of contact within a reciprocal distance of 100, 50, and 25 nm) is calculated as the ratio of ER-OMM apposition length/total OMM length. Minimal mitochondria-ER proximity is determined by measuring the minimal distance between mitochondrial and ER within a mitochondria-ER contact site. All parameters obtained from one field of view (usually containing several mitochondria and multiple contact sites) were averaged together.

ROS Generation. Intracellular ROS levels were determined by DHE staining according to the manufacturer’s instructions. Briefly, treated cells were incubated in DMEM containing 10 μM DHE (Sigma-Aldrich) at 37 °C for 30 min at dark. After washing, labeled cells were evaluated using flow cytometry. Data were analyzed by FlowJo Software (Tree Star Inc., Ashland, Oregon). 150 μM antimycin treatment was used as the positive control.

CellROX® Deep Red Reagent (Thermo Fisher Scientific) was also used to stain Intracellular ROS levels according to manufacturer’s instructions. Briefly, CellROX Reagent was added at a final concentration of 5 μM/mL and incubated for 10 min at 37 °C. After fixing with 3.7% formaldehyde, cells were analyzed by confocal microscopy. 200 μM TBHP treatment was used as the positive control.

The levels of mitochondrial ROS were incubated in SLC medium using the fluorescent probes MitoSOX® Red (Invitrogen) for 30 min. After washing, labeled cells were evaluated using flow cytometry. Data were analyzed by FlowJo Software. (Tree Star Inc., Ashland, Oregon).

Measurement of total GSH and GSSG. Total glutathione (GSH + GSSG, IGSH) and GSSG levels were determined by the method of Rahman et al29. Briefly, cells are harvested and washed twice with PBS. Next, we centrifuged and discarded the supernatant, resuspended the cells in 1 ml of ice-cold extraction buffer, containing 0.1% Triton-X and 0.6% sulfoalicylic acid in KPE (0.1 M potassium phosphate buffer with 5 mM EDTA disodium salt, pH 7.5). After that, sonicate the suspension in icy water for 2–3 min (Tissue Homogenizer) and then mixed with working buffer, 100 mM DTNB. Either 1× assay buffer or thioredoxin reductase inhibitor was added to the wells. Enzymatic activity was determined by subtracting the time-dependent increase from total activity in absorbance at 412 nm every 10 s for 2 min in a spectrometer in the presence of the thioredoxin reductase inhibitor. All samples were run in duplicate. All reagents used in this assay were purchased from Sigma-Aldrich.

Thioredoxin reductase activity. The activity of thioredoxin reductase was measured using the Thioredoxin Reductase Assay Kit (Sigma-Aldrich) according to the manufacturer’s instructions. In brief, cytosolic fractions were added to 96 well plate and then mixed with working buffer, 100 mM DTNB. Either 1× assay buffer or thioredoxin reductase inhibitor was added to the wells. Enzymatic activity was determined by subtracting the time-dependent increase from total activity in absorbance at 412 nm every 10 s for 2 min in a spectrometer in the presence of the thioredoxin reductase inhibitor. All samples were run in duplicate.

Mitochondrial membrane potential assessment. For measurement of mitochondrial membrane potential, cells were stained with 200 nM Mitotracker Green (Thermo Fisher Scientific), 200 nM of tetramethylrhodamine, ethyl ester (TMRE, Thermo Fisher Scientific) at 37 °C for 30 min. Mitochondrial depolarization was calculated as % of the area of green mitochondria compared to that of all the mitochondria visualized on Mitotracker Green and TMRE merged images. Data are presented as depolarized mitochondria (green) / total mitochondria (green + yellow) × 100%. 20 μM FCCP was used as the positive control.

Metabolism assays. Intracellular ATP production was measured using a CellTiter-Glo Luminescent Cell Viability Assay kit. (Promega, Madison, WI, USA), per the manufacturers’ instructions. Extracellular lactate production and intracellular glucose uptake were measured using lactate and glucose assay kits (Promega, Madison, WI, USA, respectively).

Testosterone concentration assay. The cell culture supernatants and sera were collected at each experimental time point for the quantitative determination of testosterone. Testosterone levels were measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions.

Semen collection and analysis. The epididymides were collected and used for semen analysis according to a previous article30. In brief, each fresh epididymis was cut into small pieces and dispersed in 0.5 ml of F12 medium (GIBCO) containing 0.1% bovine serum albumin (Invitrogen) prewarmed to 37 °C and incubated for 15 min to facilitate the transmigration of sperm from the epididymis. Each epididymal sperm suspension was subjected to sperm counting and sperm motility analyses by computer-aided semen analysis (CASA) system. In brief, cauda epididymides were removed and incised with micro scissors, and incubated in 0.5 ml buffer containing 0.5% BSA (Sigma) for 15 min at 37 °C to allow for sperm release.
The tissue was removed, and sperm samples were diluted and analyzed using a Hamilton Thorne’s Ceros II system. At least six fields were assessed for each sample, and the sperm concentration and percentages of motile and progressively motile spermatozoa were determined.

Statistics and reproducibility. All experiments were carried out with at least three biological replicates and showed successful reproducibility. All data are reported as the mean ± SD of at least three independent experiments. Sample sizes are all presented in the figure legends. Statistical analysis between two groups was performed using unpaired t-test. Statistical analysis between multiple groups was performed by one-way ANOVA, with Tukey’s multiple comparison test. All data were analyzed using Prism software (GraphPad Software). A two-sided p-value < 0.05 was considered to be statistically significant. The level of significance defined as p < 0.05 (*), p < 0.01 (**), p < 0.001 (***)
Fig. 7 AAV-mediated downregulation of Nestin separates MERCs and diminishes Melatonin’s effect in attenuating male reproductive ageing. a Schematic of the experimental animal model for conditionally knocking out Nestin in testis. b Representative TEM images of testicular mesenchyme. (mitochondria, yellow; ER, red; contact sites, blue). Scale bar, 500 nm for original pictures and 250 nm for enlarged pictures. c Analysis of TEM from (b). Percentage of mitochondria coverage covered by ER (n = 115 cross-sections from 4 mice for each group) and mito-ER proximity were quantified (n = 130 cross-sections from 5 mice for each group; Unpaired t test). d Flow cytometry of the percentage of tdTomato+LHR+ cells in vivo. e Quantification of percentage of tdTomato+LHR+ cells in total tdTomato+ cells in (d) (n = 3 biological repeats for each group; Unpaired t test). f Serum testosterone level of wild type, AAV8-sgCon, AAV8-sgNes, and AAV8-sgNes+Mel groups. (n = 6 biological repeats for each group; Unpaired t test). g Representative immunostaining pictures of SYCP3-positive cells in seminiferous tubules of testis. Scale bar, 100 μm. h Quantification of SYCP3-positive cells per tubule cross-section. (n = 6 biological repeats for each group; Unpaired t test). i Flow cytometry of intracellular ROS level stained with DHE of tdTomato+ cells in vivo. j Quantification of mean fluorescence intensity of ER stress markers in Supplementary Fig. 15j. (n = 3 biological repeats for each group; Multiple t tests). k Schematic of the chemical-damaged animal model treated with EDS and conditionally knocking out Nestin in testis. l Representative immunostaining pictures of testicular mesenchyme from EDS(+)EDS(+)EDS(+)AAV8-sgCon(+)AAV8-sgNes(+) groups 4 weeks after EDS treatment. LCs are identified as HSD3β+/CYP11A1+/HSD17β3+ cells. Scale bar, 20 μm. m Quantification of LCs cell numbers in (l) with different markers. (n = 3 biological repeats for each group; Multiple t tests). n Measurement of serum testosterone level. (n = 5 biological repeats for each group; Multiple t tests). Two-sided comparison; All data are mean ± SD; Error bars represent SDs. *p < 0.05, **p < 0.01, ***p < 0.001, ns, p > 0.05; Source data are provided as a Source Data file. See also Supplementary Fig. 15.

Fig. 8 Schematic illustration of Nestin-mediated MERCs loss during reproductive ageing and melatonin’s anti-senescence effect. Loss of intermediate filament Nestin compromises the differentiation capacity of SLCs via separating MERCs during senescence. Pharmacological intervention by melatonin restores Nestin-dependent MERCs, improving SLC differentiation capacity and alleviating male reproductive system ageing.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The authors declare that data supporting the findings of this study are available within the article and its Supplementary information files or from the corresponding author (S.A.S.) upon request. All uncropped western blots and data values for all figures are provided in the source data file. Source data are provided with this paper.

Code availability
All software and bioinformatic tools used in the present study are publicly available.
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