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The effects of the Wnt/β-catenin signaling pathway on the in vitro differentiation of rat BMSCs into leydig cells

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Late-onset hypogonadism (LOH) refers to sexual and non-sexual symptoms in men caused by age-related decreases in circulating testosterone. Leydig cells (LCs) transplantation is considered to be one of a viable approach for LOH therapy, but the limited source of LCs limits the application of this approach. The aim of this study was to induce the directed differentiation of rat bone marrow mesenchymal stem cells (BMSCs) into LCs in vitro, and explore the potential involvement of Wnt/β-catenin signaling pathway in the differentiation process. BMSCs were extracted from rats and characterized by flow cytometry for positive rates of mesenchymal stem cell markers CD29, CD44, CD90, and the hematopoietic marker CD45. BMSCs were divided into three groups: Control, Wnt agonist (CHIR-99021), and Wnt inhibitor (LGK-974), each incubated for 14 days. ELISA and RT-qPCR were used to verify the protein and mRNA expression of β-catenin, LRP5 and TCF, the key factors in Wnt/β-catenin signaling pathway. The average fluorescence intensity of 3β-hydroxysteroid dehydrogenase (3β-HSD) on the surface of LCs was detected by immunofluorescence (IF) assay. The content of testosterone secreted in cell culture medium was detected by ELISA. The results of flow cytometry indicated that we successfully extracted and cultured BMSCs. Moreover, post 14 days of incubation, the changes of β-catenin, LRP5 and TCF, at the protein and mRNA level demonstrate successful intervention in the activation and inhibition of the intracellular Wnt/β-catenin signaling pathway. Compared with the control group, the LCs surface marker 3β-HSD expression intensity in the CHIR-99,021 group was significantly increased by 69% ($p < 0.01$), while significantly decreased by 59% in LGK-974 group ($p < 0.01$). The ELISA results indicated a higher testosterone concentration in the CHIR-99,021 group (359.58 ± 17.46 pg/mL) than in the control (225.31 ± 15.42 pg/mL) and LGK-974 groups (183.67 ± 4.47 pg/mL), and the difference was statistically significant ($p < 0.05$). This study successfully demonstrates the directed differentiation of BMSCs into LCs under the action of inducers. We verified that the Wnt/β-catenin signaling pathway is involved in this differentiation process. The idea proposed in our study for efficiently inducing differentiation of BMSCs into LC in vitro, may provide a safe and sustainable LC source for developing clinically feasible cell transplantation-based LOH therapies.

Keywords Late-onset hypogonadism, Bone mesenchymal stem cell, Leydig cell, Wnt/β-catenin, Testosterone

With age, male organs gradually degenerate, leading to a decline in Leydig cells (LCs) numbers and a 1–2% annual decrease in testosterone levels starting around age 40[1,2]. Mechanically, the Sirt1 and Nsf2 responsible for maintaining oxidant/antioxidant environment tend to reduce in aging LCs, which then translates into an increased ROS level. The accumulation of ROS can affect cholesterol translocation within mitochondria, which is the rate-determining step in steroid production, thereby reducing testosterone production[3]. Furthermore, the upstream regulator that influences the production of testosterone is luteinizing hormone (LH). In most men,

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serum LH levels typically do not change with age, but there is reduced responsiveness of the aging LCs to LH that also results in reduced testosterone production[4]. Age-related declines in testosterone lead to a condition known as late-onset hypogonadism (LOH). LOH patients often experience symptoms such as erectile dysfunction, anemia, osteoporosis, reduced endurance, and mental health issues due to androgen deficiency[5]. Therefore, therapies aimed at restoring serum testosterone levels to normal may alleviate some of these symptoms.

Currently, LOH is primarily treated with exogenous supplemental testosterone therapy also known as testosterone replacement therapy (TRT)[6,7]. However, this exogenous androgen supplementation does not conform to the biological rules of androgen secretion in the hypothalamic-pituitary-gonadal axis. Nevertheless, TRT has been found to improve sexual activity, libido, and erectile dysfunction, but its efficacy for other LOH symptoms, like obesity, osteoporosis, poor concentration, and memory, remains controversial[8]. In addition, TRT also has defects in adverse reactions, and may even induce the risk of prostate cancer and cardiovascular disease[9]. LCs are the main source of testosterone in men, so LCs transplantation is currently considered by most researchers to be one of the viable approaches for replenishing testosterone levels in the body, which, unlike exogenous TRT, could provide a more natural regulation of testosterone levels, thereby reducing side effects observed with TRT[10,11]. However, LCs only exists in testicular tissue and its source is very limited, which is the biggest obstacle restricting LCs transplantation therapy. Therefore, there is an urgent need to develop a more reliable and sustainable LCs source.

Bone mesenchymal stem cells (BMSCs) are pluripotent stem cells. Studies have shown that they have long-term self-renewal properties in vitro and can be directed to differentiate into a variety of cells with the help of inducible factors[12,13]. Xu et al. reported that BMSCs can differentiate into osteoblasts to promote bone healing during bone injury repair[14]. Prior to this, our research isolated BMSCs from rats and used human chorionic gonadotropin (hCG), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) to stimulate the differentiation of BMSCs into LCs in vitro. The amount of released testosterone, however, is only 0.34 nmol/L, which is hardly sufficient for the normal human body, according to our earlier research. As a result, we tried to find a breakthrough in the transformation mechanism. The Wnt pathway has been shown to play an important role in stem cell maintenance and cell fate determination. Shi et al. reported that Wnt/β-catenin signaling pathway can promote osteogenic differentiation of BMSCs through positive feedback loops[15]. Here, we are planning to focus on the effects of Wnt/β-Catenin signaling on the differentiation of BMSCs into LCs.

Wnt/β-catenin signaling is a highly conserved intercellular signaling cascade that has become an important factor in stem cell biology. Numerous studies have confirmed that the Wnt/β-catenin signaling pathway plays an important role in the multidirectional differentiation of BMSCs, including the formation of cardiomyocytes and chondrocytes and the increment of bone mass[16–19]. The Wnt pathway primarily affects cell behavior by activating target gene transcription through nuclear heterotopic of β-catenin. Upon binding of the extracellular Wnt signal to the cell surface coreceptors Frizzled and LRP5/6, the phosphorylation of LRP5/6 and the subsequent recruitment of signal transduction DVL and AXIN to the Wnt binding receptor aid in inhibiting the activity of GSK3β, thereby preventing the phosphorylation and degradation of intracellular β-catenin. Subsequently, β-catenin enters the nucleus and binds to TCF/LEF for transcriptional activation[20]. In this process, GSK/3β mainly phosphorylates β-catenin and degrades it. Currently, the GSK/3β inhibitor CHIR-99,021 is one of the compounds that activate Wnt signaling[21]. LGK-974 is a compound that inhibits Wnt signal cascade, mainly inhibits the binding of Wnt secreted protein with receptor Frizzled (Fig. 1)[22]. The classical Wnt signaling pathway dependent on β-catenin is believed to be closely related to cell behaviors such as proliferation, differentiation, and maturation[23].

To sum up, Wnt/β-catenin signals play an important role in the development and differentiation of mesenchymal stem cells, and previous studies of our group also suggested that Wnt/β-catenin signals may be activated during the transformation of BMSCs into LCs. In this study, we used the Wnt agonist CHIR-99,021 and the inhibitor LGK-974 to illustrate the hypothesis that the Wnt/β-catenin signaling pathway may more precisely promote the LC-directed differentiation of BMSCs. This study findings are envisaged to pave the way for cell-based LOH therapies enabling naturally restoring testosterone levels in aging men.

Materials and methods

Reagents

Isoflurane was purchased from RWD company; DMEM complete medium from Gibco; Trizol reagent from Invitrogen; Protease inhibitor PMSF and anti-fluorescence quenching sealing tablets were provided by Sevin Innovation Biotechnology company. PDGF, hCG, bFGF, CHIR-99,021 and LGK-974 were purchased from MCE. APC anti-mouse/rat CD29 antibody (102216), FITC anti-rat CD44H (Pgp-1, H-CAM) antibody (203906), PE anti-rat CD45 antibody (202207), Brilliant Violet 421™ anti-rat CD90/mouse CD90.1 (Thy-1.1) antibody (202529) was provided by BioLegend. RIPA protein lysate, testosterone (Testo) detection kit (CEA458Ge), β-catenin detection kit (SEB021Ra) and LRP5/6 detection kit (SED102Ra) were provided by Wuhan Yunclon Technology company. Rabbit Anti-HSD3B1 Polyclonal Antibody (bs-3906R) and Goat Anti-Rabbit IgG H&L/FITC (bs-0295G-FITC) were provided by Beijing Bioss Biotechnology company. The TCF ELISA kit (MM-61435R1) was provided by Wuhan Enzyme Free Biotechnology company. 4% paraformaldehyde, 0.25% trypsin digestion solution and DAPI dye solution are provided by Beyotime Biotechnology Company; qPCR primer was provided by BGI Genomics. Both RNA reverse transcription Kit (RR037Q) and the Real Time PCR reaction kit (RR820A/B) was provided by Takara.

Animal studies

The 4-week-old male SD rat ($n=18$) was purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). All the animals were kept at a suitable temperature (25 ± 1 °C) free of pathogens, and the light-dark cycle was 12/12 hours. Water and a standard diet are provided at will, and cages are changed twice a week to

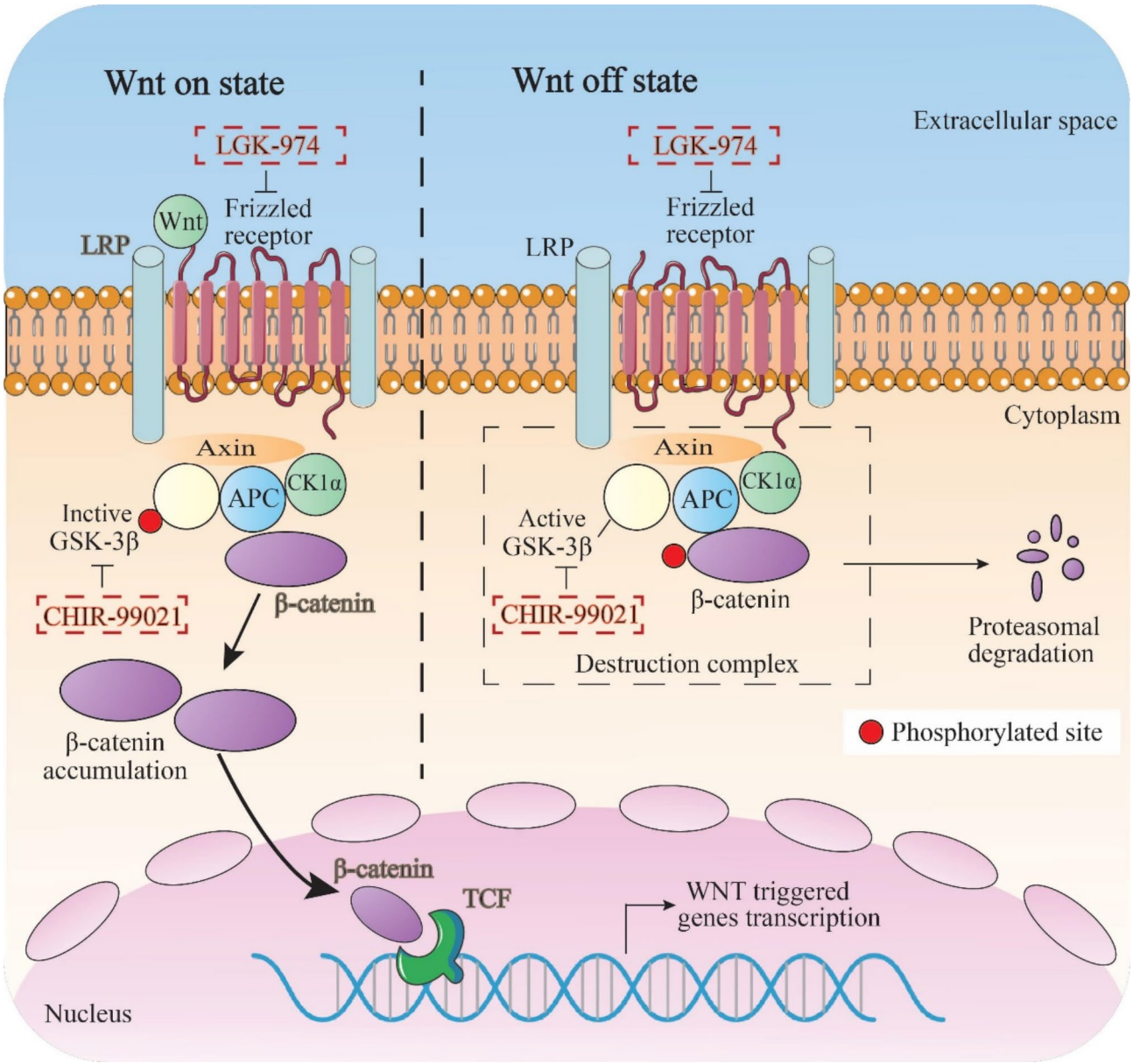

Fig. 1. Schematic diagram of the mechanism of Wnt/β-catenin signaling pathway and the targets of CHIR-99,021 and LGK-974.

maintain sanitary conditions. All procedures involving animals are approved by the Animal Experiment Ethics Committee of the host institution (permission no. DW2022023). All the methods are reported in accordance with ARRIVE guidelines. All stages of the experiments were conducted under the same ethical standards without any exception.

BMSCs isolation, identification and culture

Animals were euthanized using the following method to ensure minimal distress, and monitored continuously for any signs of pain or distress. Healthy SD rats were randomly selected and placed in an airtight container along with isoflurane (100% concentration) impregnated cotton ball. After a few minutes, the rats reached euthanasia due to excessive central nervous system depression caused by inhalation of excessive anaesthetics. Soak the rats in 75% alcohol on a super-clean work table for 15 min. The rat femur and tibia were separated with scissors and washed 3 times with PBS. The epiphyses were cut at both ends with scissors, and the complete medium of DMEM was absorbed with a syringe. The cells in the bone marrow cavity were slowly blown off, and the cell suspension was collected and centrifuged. The cells were resuspended by adding DMEM complete medium and transferred to Petri dishes and placed in a constant temperature incubator at 37℃ with 5% CO_2, and the medium was changed on the next day. Change the medium every 2–3 days and observe under the microscope after 7 days. When the cells were 70–80% fused, the cells were digested with 0.25% trypsin and passaged 1:2.

The third-generation BMSCs of the logarithmic growth stage were obtained and digested with 0.25% trypsin routine. Then, they were incubated with the antibodies APC anti-mouse/rat CD29 (1:200), FITC anti-rat CD44H (Pgp-1, H-CAM) (1:200), PE anti-rat CD45 (1:200), Brilliant Violet 421™ anti-rat CD90/mouse CD90.1 (Thy-1.1) (1:200) at 4℃ for 30 min, respectively. Flow cytometry was performed on the FACSCanto II flow cytometry system (BD Biosciences, USA) and the data were analyzed using FlowJo software.

Grouping induces BMSCs

BMSCs were divided into three groups: Control group, Wnt agonist group and Wnt inhibitor group. Each group was given 0.1ng/mL hCG + 10.0ng/mL PDGF + 10.0ng/mL bFGF (the induction solution was changed every 3d, and cell changes were observed by microscope during induction). CHIR-99,021 (10μM) and LGK-974 (1μM) were added to the Wnt agonist group and Wnt inhibitor group, respectively. The control group was not given any treatment, and each group was incubated for 14 days.

ELISA assay for β-catenin, LRP-5, TCF, Testo

Following 14 days of cell induction, the amount of testosterone in each group's culture medium was measured, and cell proteins were extracted for detection the expression level of β-catenin and LRP5. The specific procedures were carried out in strict accordance with the instructions. Rat T-cell factor TCF was detected in cellular proteins using an enzyme-free kit. The prepared samples were reacted with standards at 37 °C for 30 min, with enzyme reagents at 37 °C for 30 min, with colorant solutions A and B at 37 °C for 10 min, and finally with stop solution for 15 min. The OD values were measured at 450 nm using an microplate reader (Tecan, Switzerland).

RT-qPCR

Total RNA was extracted from three groups of cells using Trizol Kit (Invitrogen). The RNA content was determined by Thermo Fisher Scientific spectrophotometer. The RNA reverse transcription and real-time quantitative PCR reaction was carried out in strict accordance with the instructions of Takara. Finally, the LightCycler 480II real-time PCR instrument (Roche, Switzerland) was used for real-time PCR detection. Using β-actin as the internal reference, the relative expression levels of the above genes were calculated by $2^{-\Delta\Delta Ct}$ value, and the experiment was repeated for 3 times. The primer sequence is as follows:

Gene	Primer sequence(5'-3')
β-actin	F: CACGA TGGAG GGGCC GGACT CATC
	R: TAAAG ACCTC TATGC CAACA CAGT
β-catenin	F: TTCCT GAGCT GACCA AACTG
	R: GCACT ATGGC AGACA CCATC
TCF	F: CTGCC ATCTG ACTTC TCCAA
	R: CCTAC CCTCT ACCTC ACCCA
LRP-5	F: GATTG AGCGT GTGGA GAAGA
	R: GAGAA CTCCT CCAGG CTGAC

Immunofluorescence assay

After 14 days of induction, all groups of cells were fixed in 4% paraformaldehyde for 30 min, washed three times with PBS, and sealed with goat serum for 30 min. Rabbit Anti-HSD3B1 Polyclonal Antibody (1:100) was added and incubated overnight at 4℃. Goat Anti-Rabbit IgG H&L/FITC (1:100) of the corresponding species of the primary antibody was added and incubated at room temperature in a dark space for 1 h, DAPI dye solution was added and incubated at room temperature in a dark space for 30 min, the tablets were sealed and stored in the refrigerator at 4℃. The sections were observed under laser confocal fluorescence microscope (Leica TCS SP8 X, Germany) and three fields of view were randomly selected to collect images (1000×). Image J was used to calculate the average fluorescence intensity of 3β-HSD.

Statistical analysis

All the experimental data were analyzed and processed by GraphPad Prism 9.0 statistical software. All experiments in this paper were repeated three times, and the experimental data were expressed as mean ± SD. One-way ANOVA analysis of variance was used for comparison among multiple groups, and $p < 0.05$ was statistically significant.

Results

Characterization of isolated BMSCs

After inoculation, most of the rat BMSCs were small round and suspended in culture medium. After 48 h of complete culture medium incubation, some of the cells were observed to grow in a long spindle shape under optical microscope. In the first week, the formation of cell colonies was observed, and most of the spindle cells were arranged radially. When the cell fusion reaches 70–80%, the cell cytoplasm is abundant, the nucleolus is clear, and the growth is spindle shape or star shape. The expression of mesenchymal stem cell markers CD29, CD44 and CD90 was detected by flow cytometry, and the positive rates were 99.96%, 94.16% and 99.93%, respectively. And the hematopoietic marker CD45 expression was negative (1.72%) (Fig. 2), which was consistent

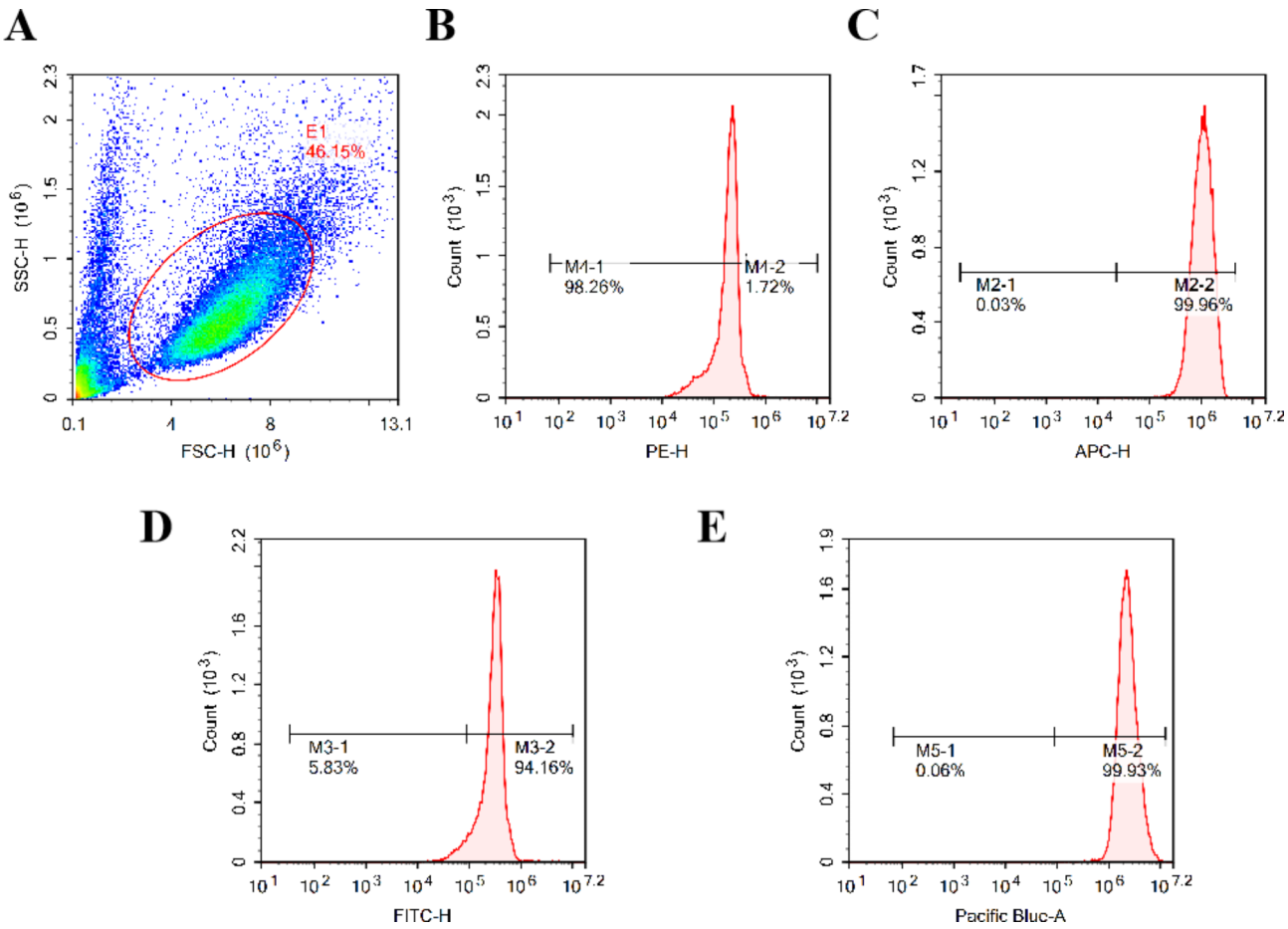

Fig. 2. The expression of BMSCs surface markers CD29, CD45, CD90 and CD44. (**A**) The set gate and collected cell scatter diagrams. (**B**) PE-H-CD45 antibody. (**C**) APC-H-CD29 antibody. (**D**) FITC-H-CD44 antibody. (**E**) Pacific Blue-A-CD90 antibody.

with the characteristics of BMSCs. Therefore, the above results indicated that we successfully extracted and cultured BMSCs.

Cytomorphological changes

CHIR-99,021 and LGK-974 were co-incubated with inducers in BMSCs complete culture medium for 14 days. Firstly, we observed the morphological changes of the cells in each group. We found that compared with normal BMSCs, after adding hCG, PDGF and bFGF inducible factors, the cell shape began to appear round, cell fusion began and the nucleus becomes round (Fig. 3A, a-b). The majority of the cells in CHIR-99,021 group were significantly altered, with nearly round or oval cells, larger and rounded nuclei, and almost sheet-like distribution between cells, which was consistent with the morphological characteristics of LCs (Fig. 3A, c). On the contrary, in the LGK-974-treated group, the cells showed irregular growth, the cell morphology was almost unchanged, and some cells were fragmented (Fig. 3A, d). This indicates that the addition of Wnt agonists and Wnt inhibitors alters the differentiation and development process of BMSCs into LCs.

Expression of wnt pathway-related proteins and genes

To clarify the working status of the Wnt agonist CHIR-99,021 and the inhibitor LGK-974 in BMSCs, we examined the expression of key molecules in the intracellular Wnt/β-catenin signaling pathway, including β-catenin, LRP5 and TCF, at the protein and mRNA level. We normalized the expression quantity so that the comparison would be more intuitive. ELISA results showed that the levels of β-catenin and TCF proteins were increased by 38% and 28%, and the levels of cell membrane receptor LRP5 protein were also increased by 23% in CHIR-99,021 group ($p < 0.01$). After the use of LGK-974, the average levels of all three proteins were significantly reduced by 29%(β-catenin), 26%(TCF), 65%(LRP5), respectively ($p < 0.05$) (Fig. 3B). RT-qPCR also detected the mRNA expression of the above three genes, and the results were consistent with those of protein levels (Fig. 3C). Therefore, ELISA and RT-qPCR results demonstrate that we successfully intervened in the activation and inhibition of the intracellular Wnt/β-catenin signaling pathway.

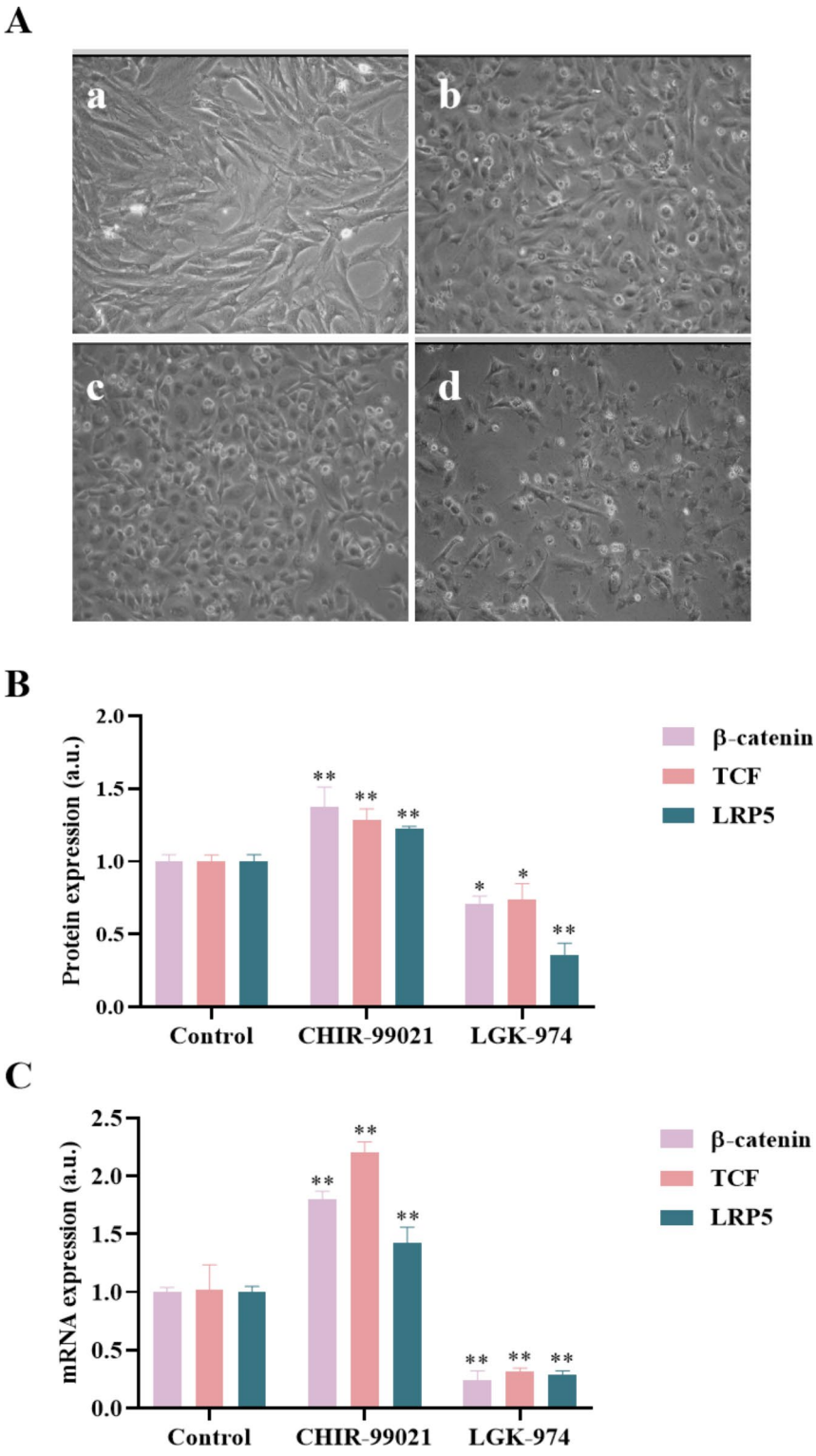

Fig. 3. Cell morphology of BMSCs in different groups and relative expression levels of LRP5, β-catenin, TCF in each group. (**A**) a: Rat BMSCs (200×); b: Control group (200×); c: CHIR-99,021 group (200×); d: LGK-974 group (200×). (**B**) The protein expression levels of LRP5, β-catenin, and TCF in control group, CHIR-99,021 group and LGK-974 group. (**C**) The mRNA expression levels of LRP5, β-catenin, and TCF in three different groups of cells. (*, $p < 0.05$ vs. control group; **, $p < 0.01$ vs. control group)

The wnt pathway promotes the differentiation and development of BMSCs into LCs

In order to elucidate the directional development of BMSCs to LCs via Wnt/β-catenin signaling pathway under inducers, we examined the expression of LCs specific surface marker 3β-HSD and the content of testosterone in culture medium. The expression level of 3β-HSD on cell surface of each group after induction was detected by IF method. The results showed that compared with the control group, the expression intensity of 3β-HSD after treatment with CHIR-99,021 was increased by 69%, while the expression intensity of 3β-HSD after treatment with LGK-974 was decreased by 59%, with statistical significance ($p < 0.01$) (Fig. 4A-B). In addition, we also detected the content of testosterone in the culture medium by ELISA assay, and the results were in line with our expectation. The testosterone concentration was found to be 225.31 ± 15.42pg/mL in the control group, 359.58 ± 17.46pg/mL in the CHIR-99,021 group, and 183.67 ± 4.47pg/mL in the LGK-974 group, respectively ($p < 0.05$) (Fig. 4C). These findings indicated that Wnt/β-catenin signaling pathway activation can improve BMSC differentiation and development efficiency into LCs, hence raising testosterone levels.

Discussion

LOH is a condition in which men experience a decline in secondary physical characteristics and maintenance due to aging, which manifests itself in a variety of symptoms such as decreased libido, osteoporosis, erectile dysfunction, anemia, depression, sweating and hot flushes[24]. Past cross-sectional studies have suggested that the etiology of LOH may be related to obesity, disease, smoking, and alcohol consumption. But recent studies have shown that the aging process is directly related to LOH. A large proportion of men over the age of 60 who generally have low serum testosterone concentrations are considered hypogonadal. At ages 60 and 70, 20% and 30% of men, respectively, meet the LOH criteria[25]. The above data suggest that LOH significantly affects quality of life and organ function in older men.

Under normal circumstances, the hypothalamus releases gonadotropin-releasing hormone (GnRH) rhythmically, which interacts with the pituitary gland to stimulate the secretion of luteinizing hormone (LH), and LH binds to the receptors on the LCs, where the 17-hydroxy steroid dehydrogenase in the LCs catalyzes the synthesis of testosterone[26,27]. Thus, testosterone secretion from LCs is regulated by the hypothalamic-pituitary-gonadal axis, and exogenous supplementation of testosterone for LOH is inconsistent with the biology of androgen secretion. Furthermore, there is still no universal diagnostic standard for LOH worldwide, despite the fact that a decrease in serum testosterone levels is the basis for the diagnosis of LOH in the majority of research[28]. Therefore, there is still a need to further explore new therapies to replace traditional TRT treatment.

In regenerative medicine, BMSCs are not only capable of multispectral differentiation and self-renewal, but also resistant to hypoxia-induced apoptosis and oxidative stress-induced senescence. Therefore, BMSCs are widely used in gene therapy and cell therapy to help patients treat diseases and improve their quality of life. Kale et al. transplanted BMSCs into rat testis, and the transplanted cells successfully survived and differentiated into steroid hormone-secreting cells[29]. Feng et al. demonstrated that transplantation of BMSCs into rat testes could promote their differentiation into LCs, thereby increasing the level of testosterone secretion level[30]. Studies have shown that BMSCs can differentiate into LCs that produce testosterone under the induction of gonadotropin/luteinizing hormone, human chorionic gonadotropin, platelet-derived growth factor and interleukin-1α at different concentrations[31]. These studies confirm that BMSCs have far-reaching applications in the male reproductive system. In this study, we induced the differentiation of BMSCs into LCs using hCG, PDGF and bFGF. It is well known that testicular LCs are round or oval in shape, with a rounded nucleus and a slightly larger volume. The morphological characteristics of the induced cells observed under the optical microscope in this study were similar to those of the LCs, among which the morphological characteristics of the LCs in the CHIR-99,021 group were particularly obvious, while most of the cells in the LGK-974 group were fragmented and grew irregularly.

Mesenchymal stem cells express a variety of Wnt ligands, receptors, and regulators, and both β-catenin-dependent and independent Wnt pathways are thought to play a critical role in their ability to self-renew, proliferation, and differentiation. Chen et al. reported that Wnt/β-catenin signaling pathway plays an important role in promoting the proliferation and osteogenic differentiation of BMSCs[32]. Guo et al. found that induced pluripotent stem cell-derived conditioned media promoted LCs proliferation by upregulating the Wnt/β-catenin pathway, and moreover, promoted testosterone production by increasing the expression of steroid-producing enzymes, which may be applied in regenerative medicine in the future[33]. As a member of the Wnt family, Wnt5a was found for the first time to regulate the proliferation, apoptosis and stem-cell character of human BMSCs by activating the β-catenin signaling pathway, providing a new understanding of the niche regulation of human testis[34,35].

Combined with the results of this study, LCs surface marker 3β-HSD was detected after treatment with Wnt agonist and inhibitor. The results were consistent with our expectations, with a significant increase in 3β-HSD in CHIR-99,021 group, with a 69% increase compared to the control group. As we all know, 3β-HSD in LCs is one of the key enzymes in testosterone biosynthesis, which converts pregnenolone to testosterone in the smooth endoplasmic reticulum. Since testosterone production comes primarily from the secretion of LCs, the amount of 3β-HSD indirectly reflects the quantity of LCs[36]. To further verify the experimental findings, we also assessed the amount of testosterone secreted in the induced differentiated cells. We collected media with the same number of cell samples and detected a testosterone concentration of 359.58 ± 17.46pg/ml in the CHIR-99,021 group, which was significantly higher than that in the control group (225.31 ± 15.42pg/ml). In contrast, the inhibited group had low testosterone levels (183.67 ± 4.47pg/ml). These findings fully indicated that LC-directed differentiation of BMSCs could be more efficiently facilitated by activating the Wnt/β-catenin signaling pathway in conjunction with the induced microenvironment.

However, like most previous studies, our results were still derived from in vitro experiments of rat BMSCs[32–35]. Therefore, due to the lack of trustworthy techniques for activating the Wnt/β-catenin pathway in specific cells of

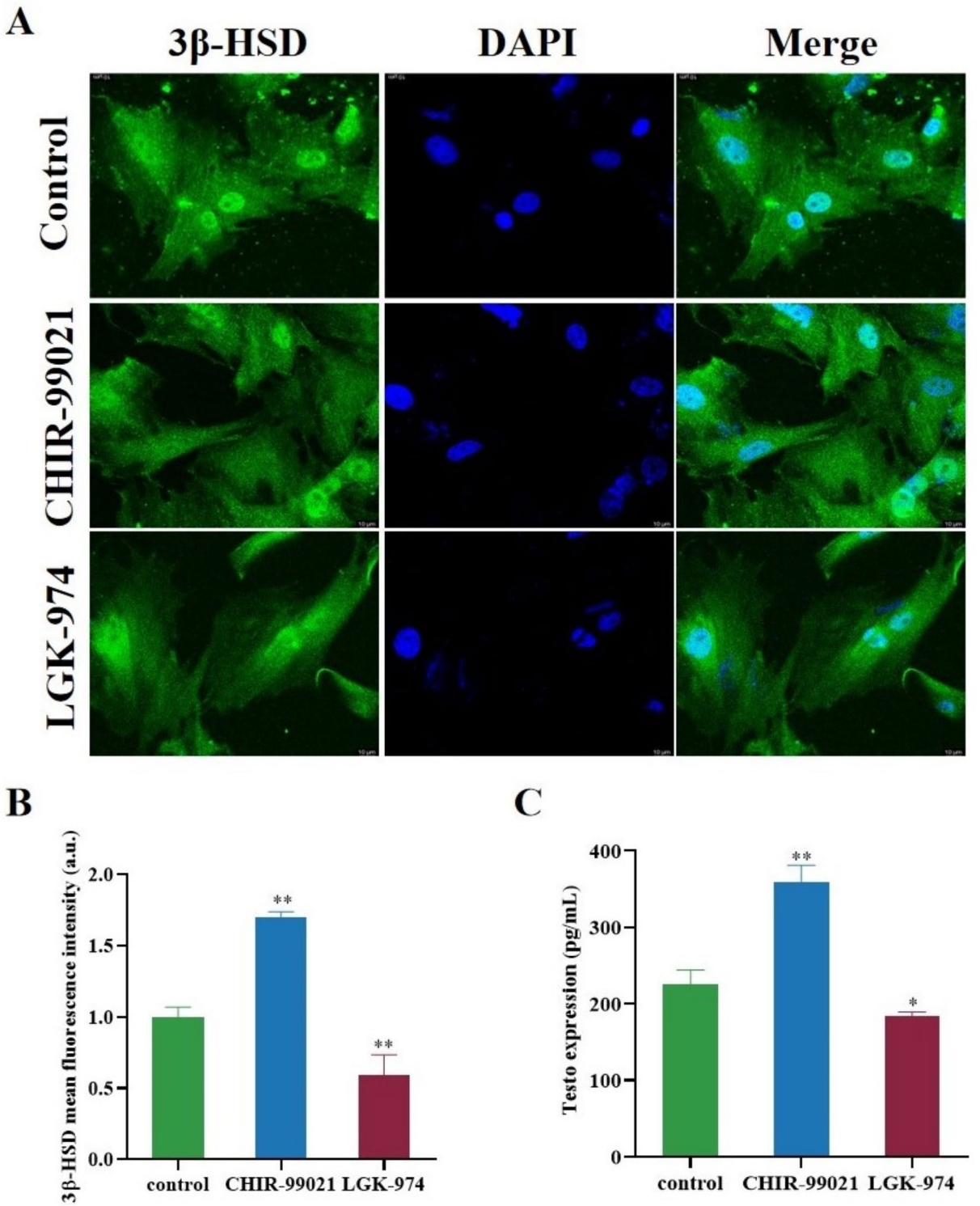

Fig. 4. 3β-HSD immunofluorescence staining of testicular LCs and the content of testosterone in culture medium. (**A**) The expression of 3β-HSD in control group, CHIR-99,021 group and LGK-974 group. The green fluorescence was positive expression of 3β-HSD, and the DAPI staining was blue fluorescence with nuclear staining (1000×). (**B**) The relative mean fluorescence intensity of 3β-HSD in each group. (**C**) The testosterone secretion levels in three groups of cells. (*, $p < 0.05$ vs. control group; **, $p < 0.01$ vs. control group)

the human body, the practical application of activating this pathway in vivo to improve BMSCs differentiation still needs to be further investigated[37]. Nevertheless, clinical research on the autologous transplantation of chemically induced pluripotent stem-cell-derived islets (CiPSC islets) beneath the abdominal anterior rectus sheath for type 1 diabetes treatment has recently been reported, demonstrating patient's complete withdrawal from insulin injection therapy post 75 days of transplantation and showed no indication of transplant-related abnormalities within 1 year[38]. For the clinical use of cell-based LOH therapies, health practitioners might be able to obtain autologous BMSCs from patients, expand and induce differentiation into LCs in vitro, and transplant them into the patient's body to achieve long-lasting and natural testosterone secretion. However, before this, the preclinical trial evaluation is still required using human-derived BMSCs and in vivo environments.

Conclusion

In conclusion, this study successfully demonstrates the directed differentiation of BMSCs into LCs under the action of inducers. Currently, there are few studies have investigated the mechanism of BMSCs differentiation into LCs. The innovation of this study is that we first explored the effects of Wnt/β-catenin signaling pathway in BMSCs differentiation into LCs. We explained this result in terms of LCs surface marker 3β-HSD expression and secreted testosterone content, which fully indicated that Wnt/β-catenin signaling pathway was involved in the differentiation of BMSCs into LCs. Although we used rat bone marrow mesenchymal stem cells, our study proposes a potential mechanism for differentiating BMSCs into LCs, which may contribute to developing safe and sustainable LC sources to enable clinical cell transplantation-based LOH therapies.

Data availability

Data underlying the results presented in this paper are not publicly available at this time but may be obtained from the corresponding authors (Liu Chun: sxtyliuchun@126.com and Liu Yuxiang: liuyuxiang@tmu.edu.cn) upon reasonable request.

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Author contributions

Conceptualization: P.Y., Y.G.; Methodology: J.Z., P.Y., Y.G.; Formal analysis and investigation: P.Y., Y.G.; Writing - original draft preparation: P.Y., S.M., Y.G.; Writing - review and editing: C.L., Y.L.; Funding acquisition: C.L., Y.L.; Supervision: C.L., Y.L.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

The procedures of this study were approved by the Ethics Review Committee of the Second Hospital of Shanxi Medical University (permission no: DW2022023), and conducted according to the ARRIVE guidelines.

Additional information

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