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Endosialin defines human stem Leydig cells with regenerative potential

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STUDY QUESTION: Is endosialin a specific marker of human stem Leydig cells (SLCs) with the ability to differentiate into testosterone-producing Leydig cells (LCs) *in vitro* and *in vivo*?

SUMMARY ANSWER: Endosialin is a specific marker of human SLCs which differentiate into testosterone-producing LCs *in vitro* and *in vivo*.

WHAT IS KNOWN ALREADY: Human SLCs have been identified and isolated using the marker platelet-derived growth factor receptor α (PDGFRα) or nerve growth factor receptor (NGFR). However, the specificity was not high; thus, LCs and germ cells could be mistakenly sorted as SLCs if PDGFRα or NGFR was used as a marker for human SLCs isolation.

STUDY DESIGN, SIZE, DURATION: Firstly, we re-evaluated the specificity of PDGFRα and NGFR for SLCs in adult human testes. Then we analysed the previously published single-cell sequencing data and found that endosialin may identify human SLCs. Subsequently, we sorted endosialin$^+$ cells from four human donors and characterized their self-renewal and multipotent properties. To assess whether endosialin$^+$ cells have the potential to differentiate into functional LCs *in vitro*, these cells were stimulated by differentiation-inducing medium. We next assessed the *in vivo* regenerative potential of human endosialin$^+$ cells after xenotransplantation into the testes of immunodeficient mice.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Single-cell sequencing analysis, immunofluorescence and flow cytometry were used to characterize human testis tissues. *In vitro* colony formation, multipotent differentiation (adipogenic, osteogenic and chondrogenic) and Leydig cell-lineage induction were used to assess stem cell activity. Xenotransplantation into 3-week-old immunodeficient mice was used to determine *in vivo* regenerative potential. Endpoint measures included testosterone measurements, cell proliferation, immunofluorescence, flow cytometry and quantitative RT–PCR.

MAIN RESULTS AND THE ROLE OF CHANCE: The results indicate that endosialin is a specific marker of SLCs compared with PDGFRα and NGFR. Additionally, endosialin$^+$ cells isolated from human testes show extensive proliferation and differentiation potential *in vitro*: their self-renewal ability was inferred by the formation of spherical clones derived from a single cell. Moreover, these cells could differentiate into functional LCs that secreted testosterone in response to LH in a concentration-dependent manner *in vitro*. These self-renewal and differentiation properties reinforce the proposal that human testicular endosialin$^+$ cells are SLCs. Furthermore, transplanted human endosialin$^+$ cells appear to colonize the murine host testes, localize to peritubular and perivascular regions, proliferate measurably and differentiate partially into testosterone-producing LCs *in vivo*.

LARGE SCALE DATA: NA.

LIMITATIONS, REASONS FOR CAUTION: Owing to the difficulty in collecting human testis tissue, the sample size was limited. The functions of endosialin on SLCs need to be elucidated in future studies.

WIDER IMPLICATIONS OF THE FINDINGS: A discriminatory marker, endosialin, for human SLCs purification is a prerequisite to advance research in SLCs and logically promote further clinical translation of SLCs-based therapies for male hypogonadism.

STUDY FUNDING/COMPETING INTEREST(S): A.P.X. was supported by the National Key Research and Development Program of China (2017YFA0103802 and 2018YFA0107200). C.D. was supported by the National Natural Science Foundation of China (81971314) and the Natural Science Foundation of Guangdong Province, China (2018B030311039). The authors declare no conflict of interest.

Key words: stem Leydig cells / male hypogonadism / endosialin / testosterone / testis

Introduction

Leydig cells (LCs) that reside in the testicular interstitium are primarily responsible for testosterone production (Teerds and Huhtaniemi, 2015; Kaufman *et al.*, 2019). Male hypogonadism occurs when LCs fail to restore serum testosterone levels, caused by congenital, acquired or systemic disorders (Basaria, 2014). Administration of exogenous testosterone, known as testosterone replacement therapy, is the most widely used treatment for hypogonadism, and it can help to reverse many of the symptoms caused by low testosterone levels (Bhasin *et al.*, 2018). However, exogenous testosterone replacement can induce many adverse effects, such as polycythaemia (Ponce *et al.*, 2018), sleep apnoea (Fernández-Balsells *et al.*, 2010), infertility (Patel *et al.*, 2019) and increased risks of cardiovascular events (Spitzer *et al.*, 2013). Notably, exogenous testosterone has yet to mimic the physiological patterns of testosterone secretion (Giannoulis *et al.*, 2012). Stem Leydig cells (SLCs), which are capable of regenerating new LCs through proliferation and differentiation, may play a critical role in maintaining LCs homoeostasis in the adult testis (Ge *et al.*, 2006; Jiang *et al.*, 2014; Landreh *et al.*, 2014; Yu *et al.*, 2017). SLCs from rodents have been successfully isolated, expanded and differentiated *in vitro* (Ge *et al.*, 2006; Jiang *et al.*, 2014). Recent studies have demonstrated that SLCs can replace senescent and chemically disrupted LCs to produce testosterone, indicating that SLCs transplantation is a promising therapy for hypogonadism (Ge *et al.*, 2006; Jiang *et al.*, 2014; Zang *et al.*, 2017; Arora *et al.*, 2019). In contrast to rodent SLCs, human SLCs remain largely uncharacterized and have not yet been used in the clinic, mainly because of the lack of an effective isolation system (Landreh *et al.*, 2014; Zhang *et al.*, 2017; Eliveld *et al.*, 2019). Thus, there is an urgent demand for practical methods for the identification and isolation of human SLCs.

Several options for identifying and isolating putative human SLCs have been described in the past few years. Using a testicular biopsy explant culture system, Landreh *et al.* (2014) discovered that human testicular peritubular cells can host putative SLCs that exhibit steroidogenic potential under forskolin stimulation *in vitro*. However, peritubular cells are mixtures of different types of cells in addition to SLCs, such as myoid cells and perhaps LCs (Landreh *et al.*, 2014). Therefore, this method for isolation of human SLCs is suboptimal. Until recently, our group (Zhang *et al.*, 2017) sorted putative human SLCs for the first time through flow cytometric detection of a cell surface protein nerve growth factor receptor (NGFR). The $NGFR^+$ SLCs exhibited the clonogenic self-renewal capacity and showed multi-lineage differentiation potential. More importantly, the putative human SLCs differentiated into functional LCs that expressed LCs lineage markers and produced testosterone *in vitro* and *in vivo*. These findings suggested that NGFR could serve as a surface marker of human SLCs. Unfortunately, it has been demonstrated that NGFR is also expressed on pachytene spermatocytes and round spermatids (Jin *et al.*, 2006; Levanti *et al.*, 2006; Lin *et al.*, 2015), which leads to germ cell infiltration in the NGFR-based sorting system and thus lowers the purity of the SLCs population, reducing the utility of the isolated cells in clinical applications. Eliveld *et al.* (2019) isolated a similar putative SLCs population from human testes through detection of platelet-derived growth factor receptor α (PDGFRα). These cells showed characteristics of mesenchymal stem cells (MSCs) and were capable of differentiating into steroidogenic cells with LCs characteristics. Nevertheless, it is worth noting that PDGFRα is also expressed on LCs at several developmental stages (Brennan, 2003; Ge *et al.*, 2006; Schmahl *et al.*, 2008). Therefore, the purity problem of the putative human SLCs remains to be solved. Furthermore, none of these studies thoroughly examined markers of other testicular cell types in the populations claimed to be SLCs, nor did they provide evidence of whether SLCs retained their crucial adult stem cell characteristics after being transplanted *in vivo* (Li *et al.*, 2018). To solve the problem of SLCs impurity, which may weaken therapeutic effects and introduce safety problems, distinctive cell surface markers for the identification and isolation of human SLCs are needed.

To achieve this goal, single-cell RNA sequencing (scRNA-seq) approaches can be used to effectively delineate cell types and reveal cell markers (Rennert *et al.*, 2016; Griffiths *et al.*, 2018). We analysed the previously published single-cell transcriptome data on human testes (Sohni *et al.*, 2019) and found that the positive rate of endosialin was the highest on putative SLC populations. Endosialin, also referred to as CD248, is a type I transmembrane glycoprotein ubiquitously expressed in normal somatic tissues and during development (Opavsky *et al.*, 2001; Lax *et al.*, 2010; Hong *et al.*, 2019). Endosialin is also expressed on some MSCs (Naylor *et al.*, 2012) and cancer stem cells (Rouleau *et al.*, 2012; Sun *et al.*, 2015), indicating that endosialin might serve as a surface marker of stem cells. However, the expression pattern of endosialin in human testes and whether endosialin can serve as a discriminatory marker of SLCs remain to be determined. Here, we systematically examined endosialin in adult human testes and subsequently evaluated the feasibility of applying endosialin to identify and isolate human SLCs. In addition, we conducted an *in vitro* differentiation culture assay and introduced an *in vivo* xenotransplantation model to assess the regenerative potential of endosialin$^+$ SLCs.

Materials and methods

Human testis samples

Adult human testis samples were obtained from two brain-dead donors (56 and 60 years old) and two prostate cancer patients undergoing bilateral orchidectomy (57 and 67 years old). Informed consent was obtained from all patients receiving surgery and from the family members of the brain-dead donors. Ethical approval was obtained from the ethics committee of the First Affiliated Hospital of Sun Yat-sen University (Assurance # 2019-148).

Animals

Three-week-old male NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Nju (NCG) mice were obtained from the Model Animal Research Center of GemPharmatech Co., Ltd (Nanjing, China). All mice were maintained under controlled temperature (24 ± 1°C) and relative humidity (50–60%) with an alternating 12-h light/12-h dark cycle and were given free access to a standard rodent diet and drinking water. All procedures were approved by the ethics committee of the First Affiliated Hospital of Sun Yat-sen University (Assurance # 2019-013).

Isolation and culture of endosialin$^+$ cells from human testes

Human primary endosialin$^+$ cells were isolated from the testis tissues of clinical donors. In detail, the testes were mechanically cut and enzymatically disassociated with 1 mg/ml type IV collagenase (Gibco, Grand Island, NY, USA) and 200 μg/ml DNase I (Roche, Indianapolis, IN, USA) in Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12; 1:1, Gibco) at 37°C for 20 min with slow shaking (100 cycles/min). The samples were filtered through a 50 μm filter and centrifuged at 256 *g* for 4 min at 4°C. The cell pellets were rinsed two times with Ca^{2+}-/Mg^{2+}-free phosphate-buffered saline and then incubated with an anti-endosialin antibody conjugated with AF647 (BD Biosciences, Franklin Lakes, NJ, USA) and an isotype antibody in the dark for 20 min. The endosialin$^+$ cells were enriched by flow cytometry (Influx Cell Sorter, BD Biosciences) followed by culture in expansion medium composed of DMEM/F12 (Gibco), 1% non-essential amino acids (Gibco), 1% GlutaMAX (Gibco), 1 × insulin-transferrin-selenium (ITS) supplement (Gibco), 1% N2 (Gibco), 2% B27 (Gibco), 20 ng/ml PDGF-BB (PeproTech, NJ, USA), oncostatin M (PeproTech), basic fibroblast growth factor (PeproTech), epidermal growth factor (PeproTech), 1 ng/ml leukaemia inhibitory factor (Millipore, Bedford, MA, USA), 1 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 mM mercaptoethanol (Gibco). The cells were cultured at 37°C with 5% CO_2, and the medium was changed every 3 days.

Cell proliferation assay

Cell proliferation was assayed as previously reported (Mou *et al.*, 2016). Briefly, endosialin$^+$ cells at passage 1 (P1) were seeded into 24-well plates at a density of 1×10^4 cells per well and cultured with expansion medium for 7 days before being digested with 1 mg/ml type IV collagenase (Gibco) in PBS and re-seeded at a density of 1×10^4 cells per well. Cell proliferation was examined using a Cellometer Auto T4 automated cell counter (Nexcelom Bioscience, Lawrence, MA, USA) when the cells were passaged every 6–8 days.

In vitro differentiation of endosialin$^+$ cells

For adipogenic differentiation, we applied a previously reported method (Jiang *et al.*, 2014). Endosialin$^+$ cells were cultured in high-glucose DMEM (H-DMEM, Gibco) supplemented with 10% foetal bovine serum (FBS, HyClone, Logan, UT, USA), 10 μg/ml insulin (Sigma-Aldrich), 100 nM dexamethasone (Sigma-Aldrich), 200 μM indomethacin (Sigma-Aldrich) and 500 μM 3-isobutyl-1-methylxanthine (Sigma-Aldrich). The cells were maintained in differentiation culture for 2 weeks, and the medium was changed every 3 days. The adipogenically differentiated cells were confirmed by Oil Red O staining (Sigma-Aldrich) and quantitative RT–PCR of adipogenic lineage-specific genes (primers are listed in Supplementary Table SI).

For osteogenic differentiation, we applied a previously reported method (Jiang *et al.*, 2014). Endosialin$^+$ cells were seeded in H-DMEM (Gibco) containing 20% FBS (HyClone), 10 mM β-glycerophosphate (Sigma-Aldrich), 100 μg/ml ascorbic acid and 100 nM dexamethasone (Sigma-Aldrich). The cells were cultured for up to 2 weeks, and the medium was changed every 3 days. Osteogenic differentiation was confirmed by Alizarin Red S (Sigma-Aldrich) staining and quantitative RT–PCR for osteogenic lineage-specific genes (primers are listed in Supplementary Table SI).

For chondrogenic differentiation, we applied a previously reported method (Jiang *et al.*, 2014). Endosialin$^+$ cells were induced by using a cell pellet culture system. The cells were suspended in a 15 ml conical tube (NEST, Wuxi, China) containing 3 ml of induction medium consisting of DMEM (Gibco) with 3% FBS (HyClone), 1× ITS (Gibco), 1 mM pyruvate (Sigma-Aldrich) and 10 ng/ml transforming growth factor-β3 (PeproTech). The endosialin$^+$ cells were maintained in culture for 4 weeks, and the medium was changed every 3 days. Chondrogenic differentiation was confirmed by Alcian Blue (Sigma-Aldrich) staining and quantitative RT–PCR for chondrogenic lineage-specific genes (primers are listed in Supplementary Table SI).

For LCs differentiation, we modified a previously described method (Ge *et al.*, 2006). In brief, endosialin$^+$ cells were induced in LCs differentiation-inducing medium (DIM) containing phenol red-free M199 (Gibco), 2% FBS (HyClone), 0.5 μM Smoothened agonist (EMD Millipore), 1 ng/ml LH (R&D, Minneapolis, USA), 1 nM thyroid hormone (T3, Sigma-Aldrich), 50 ng/ml insulin-like growth factor 1 (IGF1, PeproTech), 10 ng/ml platelet-derived growth factor AA (PDGF-AA, PeproTech), 1× ITS (Gibco) and 2.5 μM 25-hydroxycholesterol (25-HC, Sigma-Aldrich). By the end of 2 weeks, steroidogenic differentiation was confirmed by immunostaining and quantitative RT–PCR for LCs lineage-specific markers (primers and antibodies are listed in Supplementary Tables SI and SII). The ability of the cells to produce testosterone was assessed after 3 h of incubation with M199 (Gibco) containing 10 ng/ml LH (R&D), 2.5 μM 25-HC (Sigma-Aldrich) and 1× ITS (Gibco). The cell supernatants were collected on the 2nd, 4th, 7th, 10th and 14th days of culture for evaluation of testosterone concentrations. To test whether the testosterone secretion of the cells was regulated by LH, the cells were stimulated with 0.001, 0.01, 0.1, 1, 10 and 100 ng/ml LH (R&D) for 3 h before the supernatants were collected and stored at −20°C until analyses, as reported previously (Wang *et al.*, 2018).

RNA isolation and quantitative RT–PCR

Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. RNA purity was determined by 260/280 ratio (1.87–1.93) and 260/230 ratio (2.18–2.34) using NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Reverse transcription was performed using a High-Capacity RNA-to-cDNA^{TM} Kit (Thermo Fisher Scientific). Quantitative RT–PCR was performed using SYBR PCR Master Mix (Roche) and a Light Cycler 480 Detection System (Roche). To check primers, a melting curve was generated to confirm a single peak and rule out the possibility of non-specific product or primer–dimer formation. GAPDH was amplified as control, and the target gene expression

levels were calculated using the ΔCt methods and expressed as relative expression. The primers are listed in Supplementary Table SI.

Lentiviral vector construction and infection

Lentiviral vectors expressing red fluorescent protein (RFP) driven by the EF-1α promoter were constructed as previously described (Zang *et al.*, 2017). The endosialin$^+$ cells were incubated with the virus overnight, and then the medium was replaced with fresh complete medium. The transfection efficiency was about 60% and RFP-labelled cells were purified by flow cytometry for the subsequent experiments. Before transplantation, over 97% of these cells were RFP and endosialin double positive.

Transplantation of human endosialin$^+$ cells

To investigate the potential of the sorted human endosialin$^+$ cells to differentiate into LCs *in vivo*, we transplanted them into the testes of 3-week-old immunodeficient NCG mice. Approximately 2×10^5 RFP-labelled endosialin$^+$ cells in 15 µl of PBS were injected into the testicular interstitium of each recipient under whole-body anaesthesia using Avertin (Sigma-Aldrich). The control animals received testicular injections of PBS at the same volume. Four weeks after transplantation, the animals were sacrificed after being anaesthetized excessively. Bilateral testes were collected for flow cytometry (Influx Cell Sorter, BD Biosciences) or fixed in 4% paraformaldehyde (PFA) for immunofluorescence analyses.

Flow cytometry analysis of endosialin$^+$ cells

Flow cytometry analysis was used to examine the marker expression of endosialin$^+$ cells. The cells were disassociated, filtered and incubated with monoclonal fluorescent-labelled antibodies against various human antigens, including endosialin, PDGFRα, NGFR and Nestin (antibodies are listed in Supplementary Table SII). Irrelevant isotype-identical antibodies were used as negative controls. The cells were incubated for 20 min with the appropriate antibodies in the dark at room temperature and then detected by flow cytometry (CytoFLEX, Beckman Coulter, Krefeld, Germany). The data were analysed with FlowJo software (BD Biosciences).

Immunofluorescence staining

The cultured cells were fixed with 4% PFA for 10 min at room temperature and rinsed in PBS. For intracellular protein detection, permeabilization was performed with 0.2% Triton X-100 in PBS for 15 min. Cells were incubated with 3% BSA (Sigma-Aldrich) for 30 min at room temperature, and incubated overnight with primary antibodies at 4°C. Following incubation, the cells were rinsed five times with PBS and incubated with secondary antibodies (Invitrogen, Carlsbad, CA, USA) at room temperature for an hour. After being rinsed five times with PBS, the cells were stained with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) for 5 min, after which the DAPI was removed and replaced with mounting medium (DAKO, Glostrup, Denmark). For the negative control, we stained cells in the absence of the primary antibodies (second-antibody-only control). All the primary and secondary antibodies are listed in Supplementary Table SII.

Endosialin$^+$ cells were seeded into 6-well plates at a density of 2×10^5 cells per well and cultured with expansion medium for 7 days to develop into spheres; then the formed spheres were fixed with 4% PFA for 15 min. For intracellular protein detection, permeabilization was performed with 0.2% Triton X-100 in PBS for 15 min. Then, non-specific binding of antibodies was blocked with 3% BSA for 30 min at room temperature, and incubated overnight with the relevant primary antibodies at 4°C. Next, the sections were washed with PBS five times and then incubated with the appropriate secondary antibodies for 45 min at room temperature. The nuclei were counterstained with DAPI for 5 min then the DAPI solution was removed and replaced with mounting medium (DAKO). For the negative control, we stained spheres in the absence of the primary antibodies (second-antibody-only control). All the primary and secondary antibodies are listed in Supplementary Table SII.

Human and mouse testis tissue was fixed with 4% PFA at 4°C for 2 h. The tissues were then dehydrated with 30% sucrose solution in PBS at 4°C for 24 h. Afterwards, the tissues were soaked in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA) for 30 min and then frozen for cryosectioning at 10 µm thickness using a frozen slicer (Leica CM1950, Germany). For intracellular protein detection, sections were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 15 min. Next, non-specific binding of antibodies was blocked with 3% BSA (Sigma-Aldrich) for 45 min at room temperature and then incubated overnight with primary antibodies at 4°C. Then, the sections were incubated with secondary antibodies at room temperature for 45 min in the dark and subsequently incubated for 5 min with DAPI. Finally, the specific fluorescence was visualized and photographed using an LSM800 confocal microscope (Zeiss, Jena, Germany) or a Leica DMi8 microscope (Leica, Wetzlar, Germany). For the negative control, all sections were stained in the absence of the primary antibodies (second-antibody-only control). All the primary and secondary antibodies are listed in Supplementary Table SII.

Hormone measurements

The hormone levels in the cell culture supernatants were measured using a commercially available ELISA kit (R&D) according to the manufacturer's instructions. The absorbance at 450 nm was measured using an ELISA microtiter plate reader (Tecan, Switzerland). The hormone concentrations were evaluated according to a standard curve constructed by plotting the absorbance of each reference standard against its corresponding concentration. The coefficient of variation of this ELISA kit is 2.9–4% for intra-assay precision and 5.6–6.8% for inter-assay precision. The detectable dose of testosterone ranges from 0.012 to 0.041 ng/ml and the mean minimum detectable dose was 0.030 ng/ml.

Karyotype assay

G-band chromosomal analyses were conducted at P20 by DAAN Gene Co., Ltd (Guangzhou, China).

Single-cell sequence analysis

Seurat software (http://satijalab.org/seurat/, R package, v.3.1.0) was used to perform cell clustering analysis on the adult testis scRNA-seq dataset GSM3526588 (Gene Expression Omnibus [GEO]: GSE124263) (Sohni *et al.*, 2019). First, the feature table, barcode table and matrix table were all loaded into R with the Read10X function.

Seurat objects were created, normalized and scaled from these data with the default settings. Specifically, cells for which fewer than 17% of the reads mapped to the mitochondrial genome and fewer than 4000 genes were expressed were retained. Uniform Manifold Approximation and Projection (UMAP) and clustering analyses were performed. Next, we filtered out the cells that had no PDGFRA, or NGFR expression. The remaining cells were then subjected to clustering analysis again. Two separate clusters were found under the pipeline listed in the Guided Tutorial (http://satijalab.org/seurat/vignettes.html). Several markers were used to identify each cell cluster, and a differential gene test was used to find marker genes in each cluster. The genes from the differential test table that were associated with the cell surface (GO:0009986) and had the highest cell cluster proportions were selected.

Statistical analysis

All data are presented as the mean ± SEM and were analysed using SPSS 20.0 software (IBM SPSS Statistics, Armonk, NY, USA). We have performed the Shapiro–Wilk test for normality in each experiment. Statistical differences between samples were assessed with independent-samples Student's *t*-tests or nonparametric tests (Mann–Whitney *U* tests). Differences were considered significant when $P < 0.05$ ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

Results

Expression profile of endosialin in human testes

To re-evaluate whether the cell surface markers PDGFRα and NGFR are distributed specifically in human SLCs, we detected their expression patterns in adult human testes by immunostaining. Consistent with previous findings, some PDGFRα^{+} cells were immunopositive for the LCs markers hydroxysteroid dehydrogenase 3β (HSD3β) (Supplementary Fig. S1A) and cytochrome P450 family 17 subfamily A member 1 (CYP17A1) (Supplementary Fig. S1B). Similarly, an immunofluorescence assay revealed that NGFR was co-expressed with the germ cell markers DEAD-box helicase 4 (DDX4) (Supplementary Fig. S1C) and peanut agglutinin in germ cells (Supplementary Fig. S1D) of human testes. These results confirm that the cell surface markers PDGFRα and NGFR are not adequate markers for the specific identification and isolation of human SLCs.

To identify potential specific cell-surface markers of human SLCs, we analysed the PDGFRA^{+} NGFR^{+} subsets from previously published single-cell sequencing data (Supplementary Fig. S2A and B). Surprisingly, clustering analysis revealed two major subpopulations (Supplementary Fig. S2C). One of them was identified to contain peritubular myoid cells (Cluster 1), and the other was determined to possibly contain putative human SLCs (Cluster 0) (Supplementary Fig. S2D). Furthermore, we examined the expression of cell surface proteins and found that the proportion of endosialin^{+} cells was the highest in Cluster 0 (Supplementary Fig. S2E), indicating that endosialin might be of greatest use for purifying human SLCs.

Subsequently, we examined the expression profile of endosialin in adult human testes to assess whether endosialin could be used as a putative marker of human SLCs. Immunofluorescence detection was performed on testes samples from three donors, and the results confirmed that endosialin was expressed exclusively in the interstitial areas of the testes (Fig. 1). Further examinations revealed that endosialin was present in a subset of cells positive for PDGFRα (Fig. 1A) and NGFR (Fig. 1B). In contrast, endosialin was not co-expressed with the LCs marker CYP17A1 (Fig. 1C), or the endothelial cell marker CD31 (Fig. 1D) or the peritubular myoid cell marker alpha-smooth muscle actin (α-SMA) (Fig. 1E). Moreover, we observed that endosialin^{+} cells did not express the macrophage marker allograft inflammatory factor 1 (AIF1) (Fig. 1F), the Sertoli cell marker SRY-box transcription factor 9 (SOX9) (Fig. 1G) or the germ cell marker DDX4 (Fig. 1H). Collectively, these data indicate that endosialin might be a more specific marker for human SLCs than the currently used molecules.

Isolation and identification of endosialin^{+} cells in vitro

To further study endosialin^{+} cells, human testicular cells were sorted using fluorescence-activated cell sorting (FACS). Endosialin^{+} cells constituted 0.31 ± 0.03% of the total testicular cell population (Fig. 2A). These freshly isolated endosialin^{+} cells were then cultured on plastic dishes in expansion medium at a density of 2×10^{5} cells/ml. One day later, the attached endosialin^{+} cells maintained the typical cellular morphology of SLCs (Fig. 2B). Subsequently, the sorted cells were characterized by immunostaining at the primary passage when they propagated to 50–60% confluence on plastic dishes. They robustly expressed endosialin (99.3 ± 0.32%), PDGFRα (99.7 ± 0.15%), NGFR (98.5 ± 0.21%) and Nestin (99.1 ± 0.27%) (Fig. 2C and D). In addition, the sorted cells were also positive for the transcription factors nuclear receptor subfamily 2 group F member 2 (NR2F2) (98.5 ± 0.66%) and aristaless-related homeobox (ARX) (93.1 ± 0.97%) (Supplementary Fig. S3B), which have been reported to be expressed in SLCs and to be important for testis formation (Miyabayashi *et al.*, 2013; Kilcoyne *et al.*, 2014). In accordance with the *in vivo* results shown in Fig. 1, endosialin^{+} cells showed negligible CYP17A1, CD31, α-SMA, AIF1, SOX9 or DDX4 staining (Supplementary Fig. S3C–J), indicating that the isolated SLC cell population was not contaminated with LCs, endothelial cells, peritubular myoid cells, macrophages, Sertoli cells or germ cells. Overall, these results strongly support the hypothesis that endosialin may be an excellent marker for human SLCs.

It has been reported that SLCs are capable of forming spheres that exhibit continuous proliferation (Jiang *et al.*, 2014). When endosialin^{+} cells were cultured in serum-free expansion medium, most of these cells showed sphere formation capacity and could be passaged every 6–8 days (Fig. 3A). Notably, spheres derived from endosialin^{+} cells could be continuously grown for over 4 months, with cell counts reaching 10^{15} at P20 (Fig. 3B). More importantly, we did not observe any obvious chromosomal elimination, displacement or imbalance after long-term culture (Fig. 3C). There were no signs of tumourigenicity 3 months after endosialin^{+} cells obtained at P20 were injected s.c. into 8-week-old immunodeficient NCG mice, but tumours did grow in mice injected with MA-10 Leydig tumour cells (data not shown).

To examine whether the culture conditions maintained the human SLCs in an undifferentiated state, we analysed the endosialin^{+} spheres by immunofluorescence and flow cytometry assays. Immunofluorescence staining showed that P5 spheres expressed

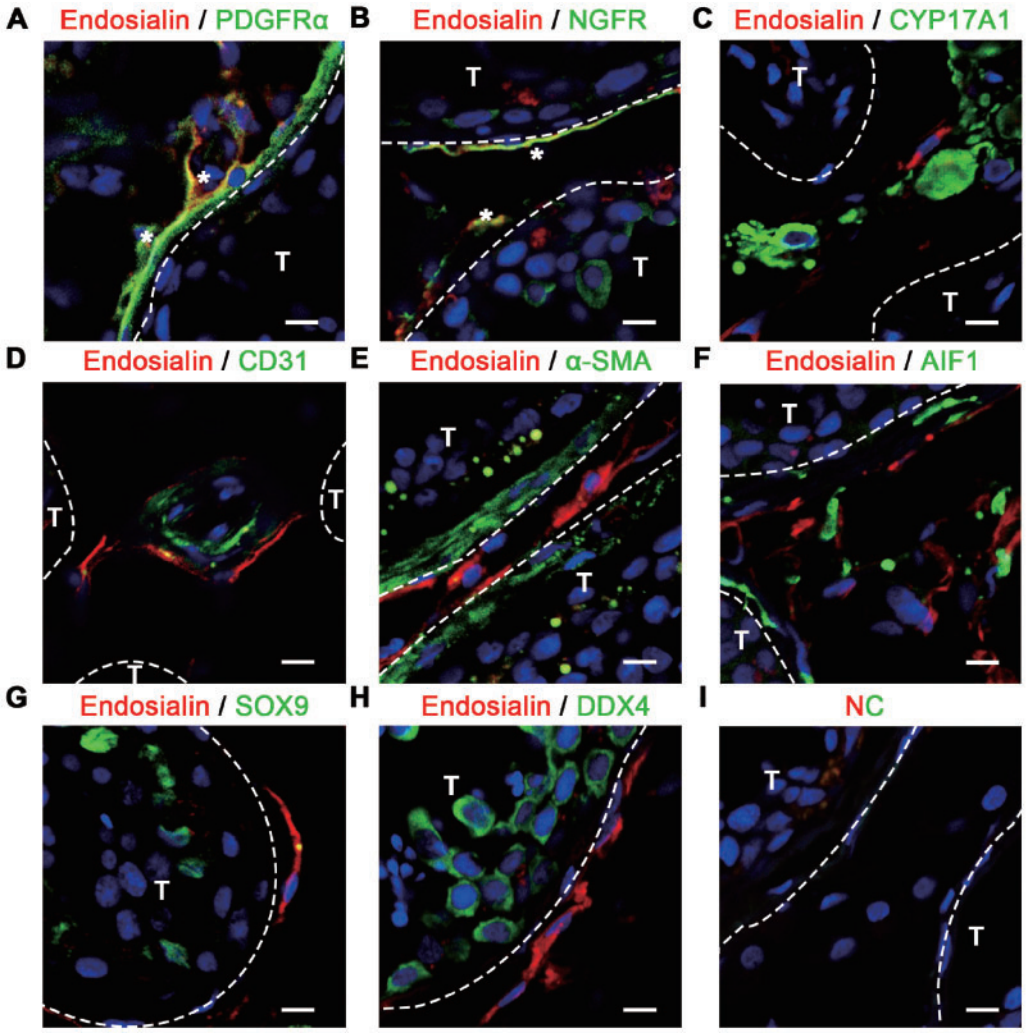

Figure 1. Expression pattern of endosialin in human testes, as shown by immunofluorescence. (**A–C**) Endosialin was co-expressed with the reported SLCs markers PDGFRα (A) and NGFR (B) but did not co-localize with the LCs marker CYP17A1 (C) in the interstitial region. Co-expression is marked with white asterisks in (A) and (B). (**D**, **E**) Endosialin$^+$ cells were located in the perivascular region (endothelial cells are marked with CD31) (D) and peritubular region (peritubular myoid cells are marked with α-SMA) (E) of the human testes. (**F–H**) Endosialin-expressing cells did not express the macrophage marker AIF1 (F), the Sertoli cell marker SOX9 (G) or the germ cell marker DDX4 (H). (**I**) The negative control stained in the absence of the primary antibodies is shown. Nuclei were counterstained with DAPI. T and interstitium were indicated by dotted lines. The scale bars denote 10 μm. The images are representative of results obtained from patient samples (n = 3). AIF1, allograft inflammatory factor 1; CYP17A1, cytochrome P450 family 17 subfamily A member 1; DAPI, 4,6-diamidino-2-phenylindole; DDX4, DEAD-box helicase 4; LCs, Leydig cells; NC, negative control; NGFR, nerve growth factor receptor; PDGFRα, platelet-derived growth factor receptor α; SLCs, stem Leydig cells; SOX9, SRY-box transcription factor 9; T, seminiferous tubules; α-SMA, alpha-smooth muscle actin.

endosialin and the established SLCs markers PDGFRα, NGFR and Nestin (Fig. 3D). The results were confirmed by flow cytometry analysis, in which almost all the cells (>98%) expressed endosialin, PDGFRα, NGFR and Nestin (Fig. 3E). To investigate the self-renewal capacity of these cells, we carried out single-cell sphere formation assays in which single-cell suspensions derived from P5 spheres were seeded into 96-well plates. The seeded single cells divided and formed spheres after 8 days of culture (Fig. 3F). A sphere with a diameter ≥50 μm in a given well was counted as one clone. The clonogenic efficiency of endosialin$^+$ cells was 18.6 ± 4.4%. These results demonstrate that endosialin$^+$ cells can continuously expand and exhibit self-renewal capacity *in vitro*.

Multipotent properties of human endosialin$^+$ cells

Human SLCs have been confirmed to possess multi-lineage differentiation potential *in vitro* (Zhang *et al.*, 2017; Eliveld *et al.*, 2019). To

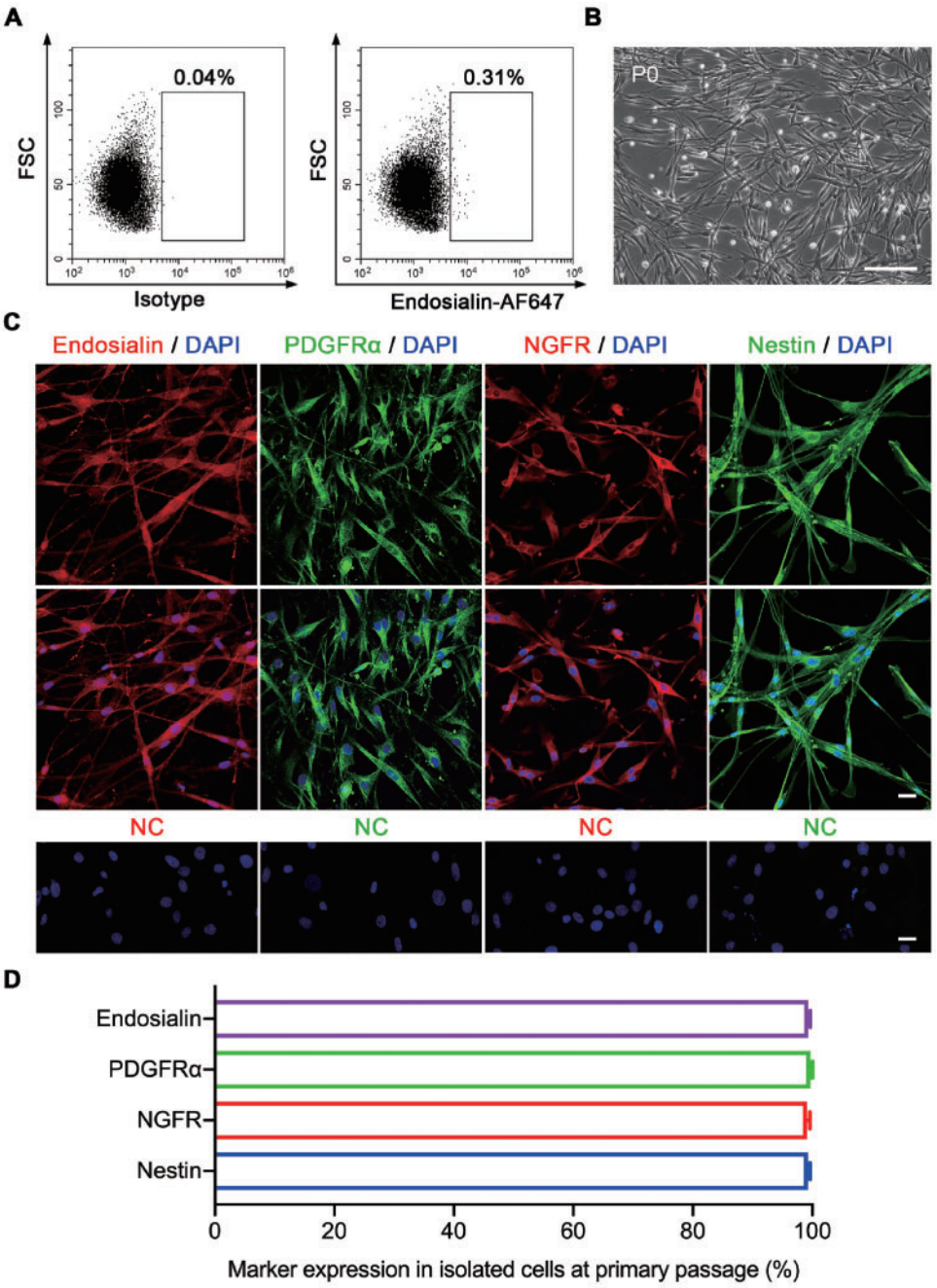

Figure 2. Isolation and identification of endosialin$^+$ cells from human testes. (**A**) Endosialin$^+$ cells were isolated by FACS from adult human testes (n = 4). Left: isotype controls. Right: stained samples. (**B**) Representative phase-contrast micrograph of endosialin$^+$ cells at P0 (n = 4). The scale bar denotes 200 μm. (**C**) Endosialin$^+$ cells expressed the SLCs markers PDGFRα, NGFR and Nestin (n = 3). The nuclei were counterstained with DAPI. NC stained in the absence of the primary antibody. The scale bars denote 25 μm. (**D**) The percentages of endosialin$^+$, PDGFRα$^+$, NGFR$^+$ and Nestin$^+$ cells among isolated cells were analysed. The data are presented as the mean ± SEM (n = 3). FACS, fluorescence-activated cell sorting; FSC, forward scatter; P0, primary culture.

characterize the multipotency of the sorted endosialin$^+$ cells, we induced these cells to differentiate under the defined conditions (Supplementary Fig. S4A). Adipogenesis was confirmed by the presence of lipid-rich vacuoles stained with Oil Red O (Supplementary Fig. S4B). Consistently, the mRNA levels of adipogenic markers, including fatty acid-binding protein 4 and peroxisome proliferator-activated receptor-γ (Supplementary Fig. S4E) were increased. Compared with the undifferentiated cells, the differentiated cells exhibited the deposition

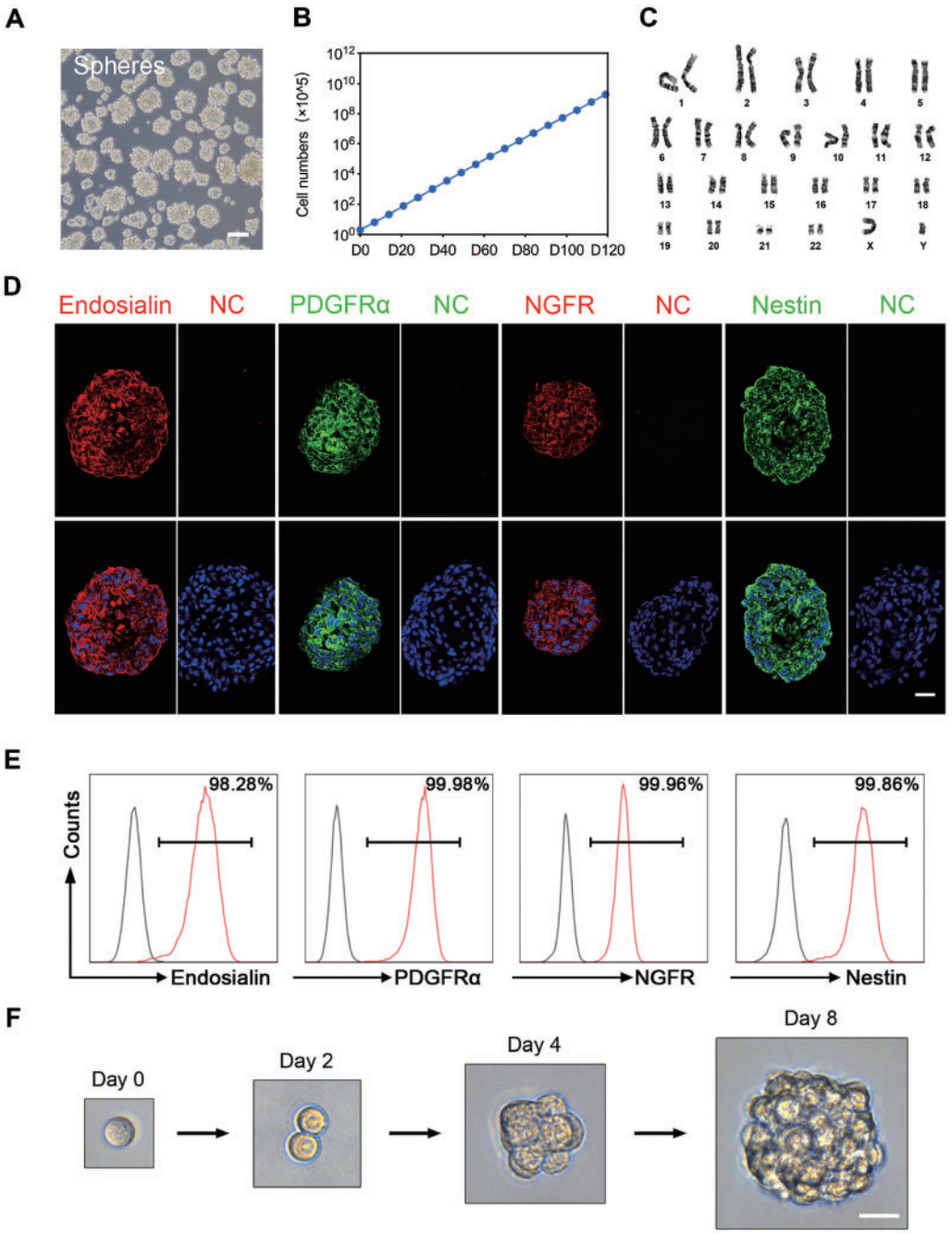

Figure 3. Proliferation and self-renewal capacity of endosialin$^+$ cells. (**A**) Phase-contrast micrograph of floating spheres at P3 growing from human testicular endosialin$^+$ cells in serum-free expansion medium (n = 3). The scale bar denotes 100 μm. (**B**) Proliferation curve of endosialin$^+$ cells cultured in expansion medium over different passages. The initial cell count was 2×10^5. The data are expressed as the mean ± SEM (n = 3). (**C**) Karyotypic stability of expanded endosialin$^+$ cells up to P20 was analysed. (**D**) Immunofluorescence staining for the indicated markers (Endosialin, PDGFRα, NGFR and Nestin) in endosialin$^+$ cell spheres. The nuclei were counterstained with DAPI. NC stained in the absence of the primary antibody. The scale bars denote 25 μm. (**E**) FACS analysis for detection of the indicated markers (Endosialin, PDGFRα, NGFR and Nestin) in endosialin$^+$ cell spheres (n = 3). (**F**) Representative images of a clonal sphere growing from a single cell, as observed using bright-field microscopy (n = 3). The scale bar denotes 25 μm. P3, passage 3; P20, passage 20.

of Alizarin Red-positive mineralized matrix after 2 weeks of induction (Supplementary Fig. S4C). We further found that the expression levels of osteogenic markers, including alkaline phosphatase and runt-related transcription factor 2, were highly upregulated (Supplementary Fig. S4E). Four weeks after chondrogenic induction, the pellets stained intensely stained with Alcian Blue (Supplementary Fig. S4D). In addition, the mRNA expression levels of the chondrogenic genes collagen type 2 alpha 1 chain and aggrecan were also highly upregulated (Supplementary Fig. S4E). Collectively, these results suggest that endosialin$^+$ cells possess multi-lineage differentiation potential.

In vitro differentiation of human testicular endosialin$^+$ cells into LCs

To assess whether endosialin$^+$ cells have the potential to differentiate into functional LCs *in vitro*, we stimulated the cells with DIM (Fig. 4A). After 2 weeks of culture in DIM, the majority of endosialin$^+$ cells exhibited signs of differentiation, as indicated by immunofluorescence staining for proteins involved in testosterone biosynthesis, such as LH receptor (LHCGR), steroidogenic factor 1 (SF-1), steroidogenic acute regulatory protein (STAR) and HSD3β (Fig. 4B). In addition, differentiated endosialin$^+$ cells were examined by quantitative RT–PCR for the LCs markers LHCGR, SF-1, STAR and HSD3β, as well as endosialin and the known SLCs markers PDGFRα, NGFR and Nestin. The expression levels of the indicated genes were compared with those in undifferentiated endosialin$^+$ cells and human testis tissues. As expected, the transcript levels of endosialin, NGFR and Nestin were significantly lower in differentiated endosialin$^+$ cells than in undifferentiated cells (Fig. 4C). However, the expression of PDGFRα was higher in differentiated endosialin$^+$ cells than in undifferentiated cells, in accordance with our previous results (Supplementary Fig. S1A and B). Similarly, upregulation of LHCGR, SF-1, STAR and HSD3β was detected. Culture supernatants were harvested for the determination of testosterone production at the indicated time points. The data showed that testosterone synthesis gradually increased over time after LCs lineage differentiation of endosialin$^+$ cells (Fig. 4D), increasing to approximately 20 ng of testosterone per 1×10^6 cells in 24 h. Furthermore, the testosterone production assay reflected that differentiated endosialin$^+$ cells responded to LH in a concentration-dependent manner, indicating that these testosterone-producing LCs were regulated by upstream gonadotrophin (Fig. 4E). The ability of endosialin$^+$ cells to differentiate into LCs reinforces the idea that endosialin$^+$ cells in human testes are SLCs.

Characteristics of endosialin$^+$ cells in vivo

Having confirmed that the human testicular endosialin$^+$ cells were multipotent, with the ability to differentiate into LCs *in vitro*, we next assessed the *in vivo* properties of endosialin$^+$ cells after xenotransplantation into the testes of immunodeficient NCG mice. To trace the transplanted cells *in vivo*, we cocultured the endosialin$^+$ cells with lentiviruses expressing RFP under the control of the elongation factor 1α promoter. The infected cells stably expressed RFP and proliferated as floating clonal spheres; thus, they were used in the following experiments (Supplementary Fig. S5). We then transplanted the RFP-labelled cells into the testes of eight prepubescent (3 weeks old) mice. To investigate the fate of endosialin$^+$ cells, we harvested the testes for immunofluorescence analysis and flow cytometry assays 4 weeks after transplantation. Immunostaining indicated that the RFP-labelled cells were exclusively located in the interstitial area in the testes (Supplementary Fig. S6). In addition, some RFP-labelled cells were found locating in peritubular (Fig. 5A) and perivascular regions (Fig. 5B; Supplementary Video S1) consistent with the fact that SLCs are located around both seminiferous tubules and blood vessels in adult testes. Furthermore, the transplanted endosialin$^+$ cells were perfused by host vessels, as evidenced by anti-CD31 immunostaining (Fig. 5B; Supplementary Video S1). In addition, $8.34 \pm 1.65\%$ of the RFP-labelled cells were positive for the pan-cell-cycle marker Ki67 (Fig. 5C), indicating the measurable proliferative activity of the transplanted endosialin$^+$ cells.

Previous studies have reported that SLCs can differentiate into LCs after being transplanted into the testes (Ge *et al.*, 2006; Jiang *et al.*, 2014). Surprisingly, approximately 60–80% of the RFP-labelled cells retained expression of endosialin ($72.94 \pm 4.31\%$) (Fig. 6A), indicating that most of the transplanted cells retained their stem cell characteristics. In addition, a small portion of the grafted cells gave rise to LCs, as evidenced by the expression of HSD3β ($10.20 \pm 2.97\%$), CYP17A1 ($8.33 \pm 1.36\%$), STAR ($11.56 \pm 3.71\%$) and CYP11A1 ($10.46 \pm 3.49\%$) (Fig. 6C and D; Supplementary Fig. S7). Nonetheless, almost all of the transplanted cells still expressed PDGFRα ($95.32 \pm 2.47\%$) (Fig. 6B), which has been reported to be expressed either on SLCs or LCs lineages within testes. Furthermore, no signs of tumour formation were detected in any of the animals tested at 4 weeks.

To further confirm the LCs differentiation of the endosialin$^+$ cells *in vivo*, the testes of recipient mice were dissociated into single cells, and RFP-labelled cells were sorted by flow cytometry (Fig. 6E). Subsequently, the RFP-labelled cells before and after transplantation were seeded into separate plastic dishes. After being cultured in M199 supplemented with 10 ng/ml LH and 2.5 μM 25-hydroxycholesterol for 3 h, the supernatants were collected for testosterone quantification. Notably, testosterone production in the post-transplantation cells was 1.232 ± 0.16 ng per 1×10^6 cells in 3 h, while that in the control cells was undetectable.

Taken together, these observations indicate that human testicular endosialin$^+$ cells possess the characteristics expected of SLCs *in vivo*.

Discussion

In this study, we sought to examine whether endosialin can serve as a specific surface marker for the identification and isolation of human SLCs. We first revealed the expression profile of endosialin in adult human testes. Then, we isolated and characterized endosialin$^+$ cells that exhibited persistent proliferative capacity and LCs differentiation potential *in vitro* and *in vivo*. The results collectively suggest that endosialin is an ideal marker for the identification and isolation of human SLCs, which has latent value for further research on human SLCs and advances in cell replacement therapies for future clinical applications related to male hypogonadism.

Transplantation of heterogeneous cell populations might cause safety risks and reduce the effectiveness of treatment, suggesting that increasing purity is a key objective for stem cell research (Tohyama *et al.*, 2013). Previous studies have reported several markers of human

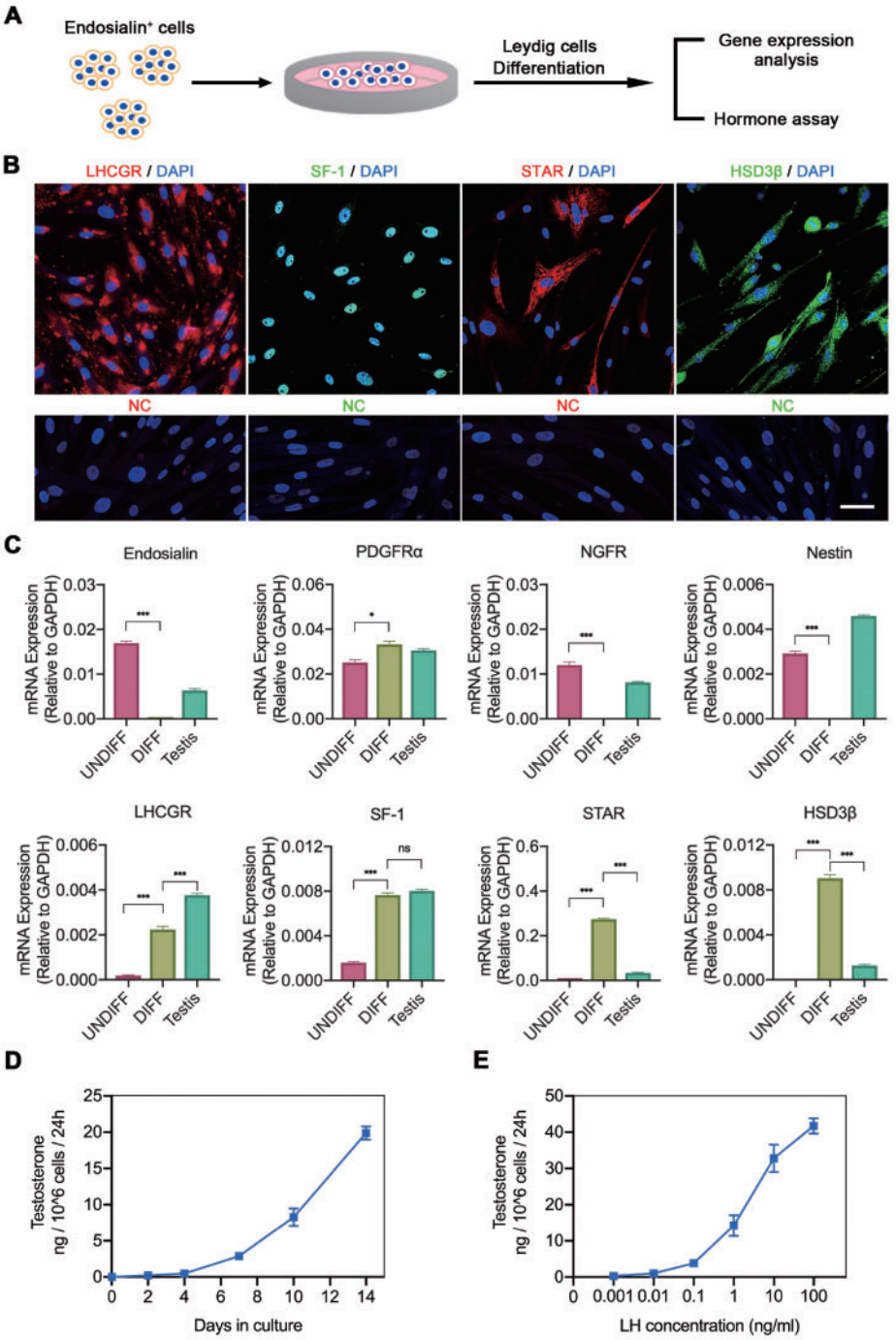

Figure 4. Potential of human testicular endosialin$^+$ cells to differentiate towards LCs lineages. (**A**) Schematic of the experimental procedure used to induce LCs lineage differentiation. (**B**) After 14 days of differentiation in DIM, endosialin$^+$ cells expressed the LCs lineage-specific markers LHCGR, SF-1, STAR and HSD3β, as shown by immunofluorescence (n = 3). The nuclei were counterstained with DAPI. NC stained in the absence of the primary antibody. The scale bar denotes 50 μm. (**C**) DIFF were examined by quantitative RT–PCR analysis of the expression of the indicated markers. The expression levels of each gene were compared with those in UNDIFF and human testis tissue. The data are expressed as the mean ± SEM (n = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns = no significance. (**D**) Testosterone production was analysed by ELISA during LCs lineage induction in DIM. (**E**) Testosterone production of DIFF stimulated by LH increased in a concentration-dependent manner, as analysed by ELISA. The data are expressed as the mean ± SEM (n = 3). DIFF, differentiated endosialin+ cells; DIM, differentiation-inducing medium; HSD3β, hydroxysteroid dehydrogenase 3β; LHCGR, LH receptor; SF-1, steroidogenic factor 1; STAR, steroidogenic acute regulatory protein; Testis, human testis tissues; UNDIFF, undifferentiated endosialin+ cells.

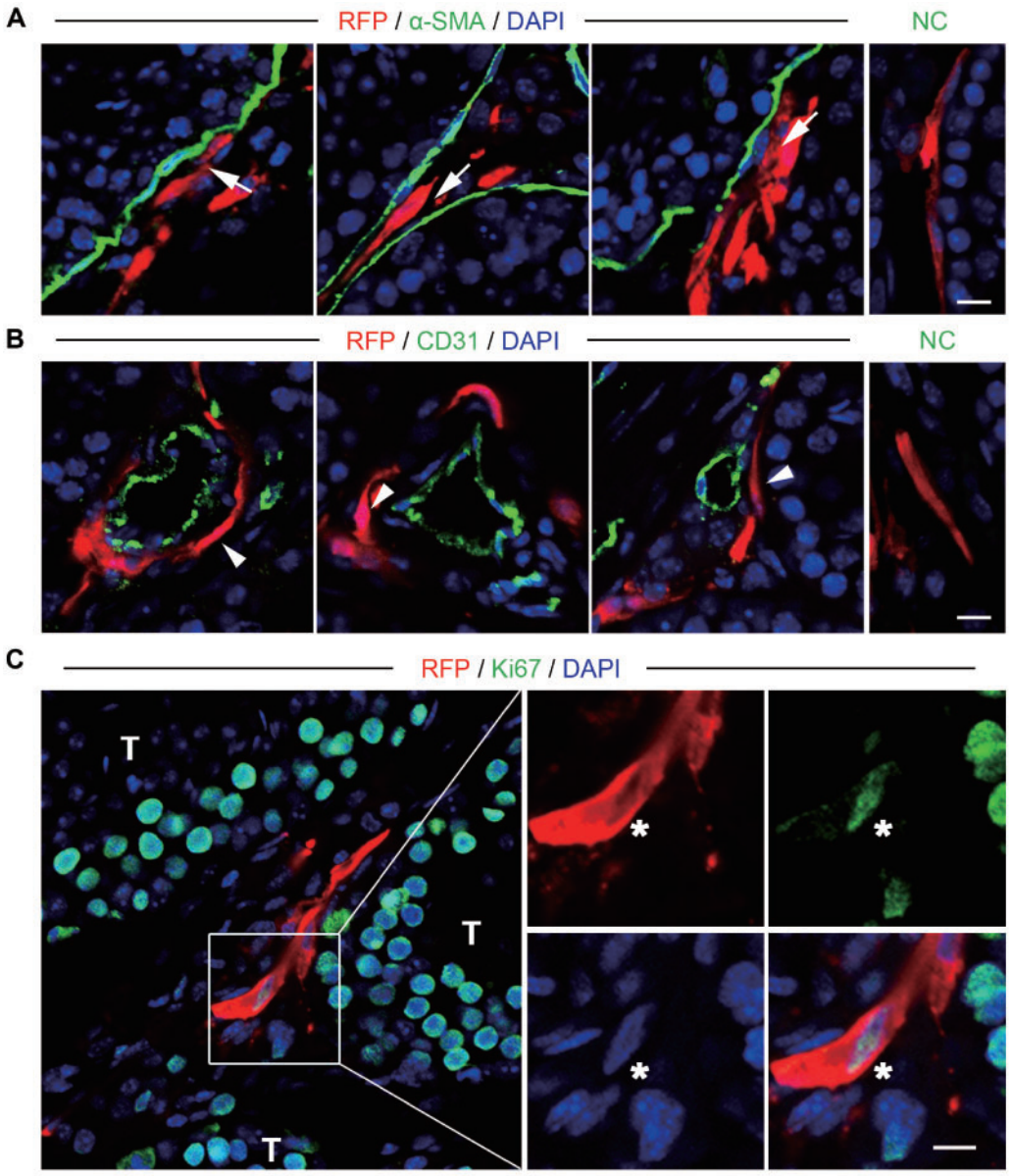

Figure 5. Localization and proliferation of endosialin$^+$ cells after transplantation into the testes of mice. (**A**, **B**) Immunostaining showed the accumulation of RFP-labelled endosialin$^+$ cells in the testicular interstitium tissues of recipient mice. RFP-labelled cells resided in both peritubular (A, white arrows) and perivascular (B, white arrowheads) regions in the testes of mice. Peritubular myoid cells were marked by α-SMA (A), and endothelial cells were marked by CD31 (B). The scale bars denote 10 μm. (**C**) Proliferation of the transplanted cells, as demonstrated by staining for Ki67 (indicated by asterisks). NC stained in the absence of the primary antibody. The scale bar denotes 20 μm. The nuclei were counterstained with DAPI. The images represent the results obtained from recipient mice (n = 3). RFP, red fluorescent protein.

SLCs, including PDGFRα (Eliveld *et al.*, 2019), NGFR (Zhang *et al.*, 2017) and Nestin (Zhang *et al.*, 2017). Although Nestin seems to be exclusively expressed on SLCs in human testes, it cannot be applied for isolation of living human SLCs, since it is an intracellular cytoskeletal protein (Jiang *et al.*, 2014). NGFR is located in germ cells in adult human testes (Jin *et al.*, 2006; Levanti *et al.*, 2006; Lin *et al.*, 2015) and PDGFRα is expressed on LCs (Brennan, 2003; Schmahl *et al.*, 2008); thus, germ cells or LCs could be mistakenly sorted as SLCs if NGFR or PDGFRα is used as a marker for human SLCs isolation. As revealed in our prior study, NGFR$^+$ cells constitute approximately 1.79% of testicular cells (Zhang *et al.*, 2017), while the PDGFRα$^+$ cells in this study accounted for approximately 1.5% (data not shown). Both of these percentages are obviously higher than that of the endosialin$^+$ population (0.3% of testicular cells), to some degree reflecting that the use of either NGFR or PDGFRα may overestimate the proportion of actual SLCs. Consistently, these flow cytometric results were confirmed by

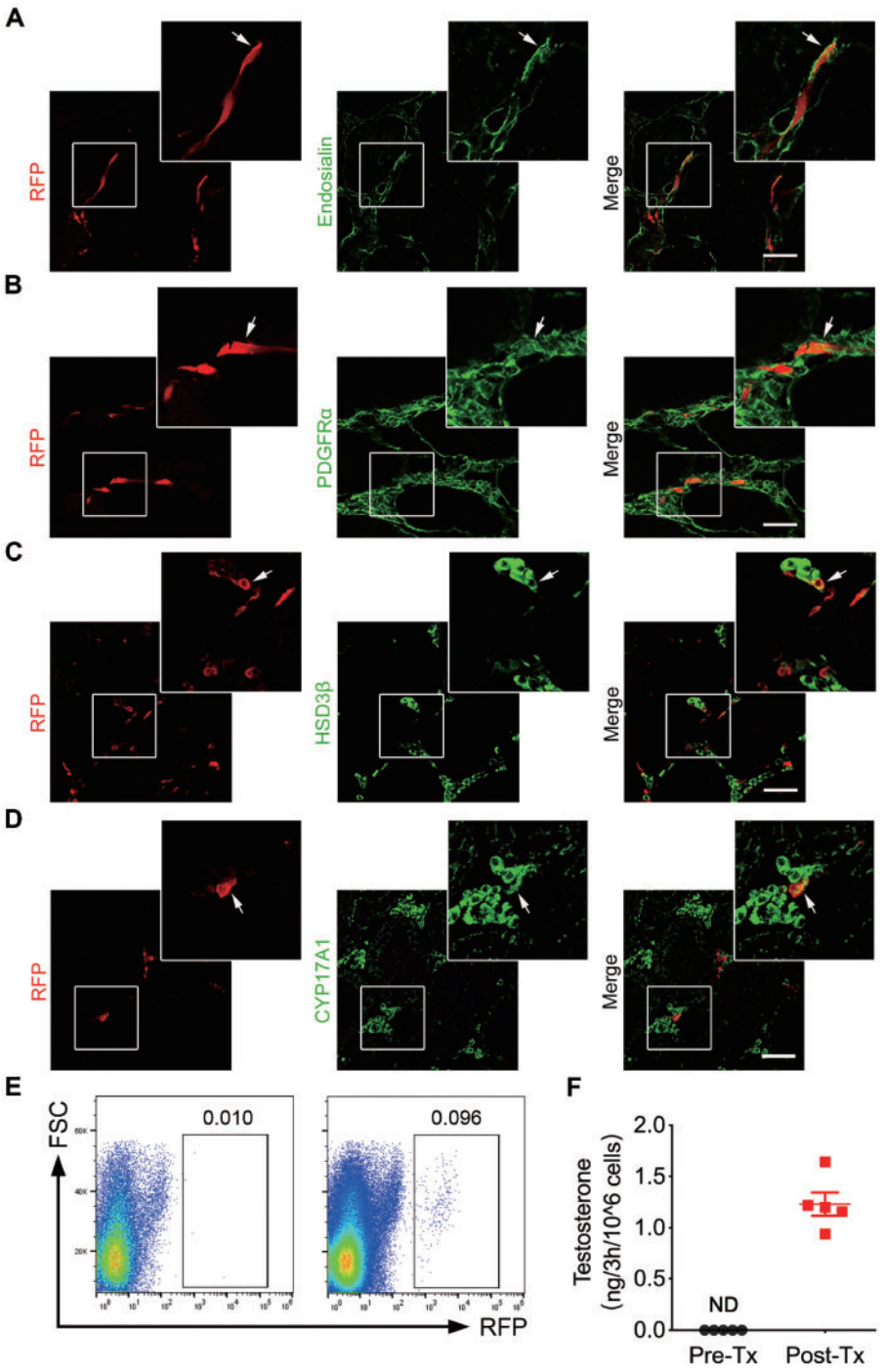

Figure 6. Identification of the transplanted endosialin$^+$ cells in the testes of mice. (**A**, **B**) RFP-labelled endosialin$^+$ cells transplanted into the testes of NCG mice were assessed 4 weeks after transplantation. Immunofluorescence staining showed that some of the engrafted cells retained expression of endosialin (A) and PDGFRα (B) (indicated by arrows). (**C**, **D**) Immunostaining showed that a portion of the transplanted cells differentiated into HSD3β$^+$ (**C**) and CYP17A1$^+$ LCs (**D**; indicated by arrows). The scale bars denote 50 μm. The nuclei were counterstained with DAPI. Representative images obtained from recipient mice are shown (n = 3). (**E**) The images represent the detection and isolation of transplanted RFP-labelled cells from recipient testes in the different groups; left: nontransplantation controls (n = 2); right: RFP-labelled cells transplanted samples (n = 5) as determined by FACS. (**F**) The testosterone produced by sorted RFP-labelled cells 4 weeks Post-Tx and endosialin$^+$ cells Pre-Tx was detected using ELISA. The data are shown as the mean ± SEM (n = 5). NCG, NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Nju; ND, not detectable; Pre-Tx, before transplantation; Post-Tx, after transplantation.

immunofluorescence analysis. As we first illustrated in the current study, endosialin largely overlapped with the former SLCs markers PDGFRα and NGFR in adult human testes. Endosialin$^+$ cells negligibly expressed any of the known markers for other types of testicular cells, including LCs (CYP17A1), endothelial cells (CD31), peritubular myoid cells (α-SMA), macrophages (AIF1), Sertoli cells (SOX9) and germ cells (DDX4). These results suggested that endosialin may serve as a discriminatory cell surface marker of human SLCs, potentially improving the specificity and feasibility of human SLCs isolation.

Given the specificity of endosialin for SLCs, we further determined that endosialin$^+$ cells sorted by FACS exhibited properties of typical SLCs, including proliferation, self-renewal and the potential to differentiate into functional LCs *in vitro* (Ge *et al.*, 2006; Jiang *et al.*, 2014). Here, we found that endosialin$^+$ cells continuously proliferated under cultivation in the expansion medium. Additionally, endosialin$^+$ cells possessed self-renewal ability, inferred by the formation of spherical clones derived from a single cell. Moreover, these cells could differentiate into functional LCs that secreted considerable testosterone in response to LH in a concentration-dependent manner *in vitro*. These self-renewal and differentiation properties reinforce the idea that human testicular endosialin$^+$ cells are SLCs.

According to criteria in previous reports, putative SLCs should have the ability to colonize the interstitium and subsequently replenish LCs after being transplanted *in vivo* (Ge *et al.*, 2006; Zang *et al.*, 2017; Zhang *et al.*, 2017). To examine the *in vivo* characteristics of endosialin$^+$ cells, we chose 3-week-old immunodeficient mice as recipients to avoid interference from immunological rejection. Similar to the findings in earlier reports (Ge *et al.*, 2006; Jiang *et al.*, 2014), transplanted RFP-labelled endosialin$^+$ cells colonized the testicular interstitium and differentiated into functional LCs. Beyond that, we observed that the transplanted cells homed to a specific location in which SLCs usually reside (Li *et al.*, 2016; Kumar and DeFalco, 2018; Chen *et al.*, 2020) and that they were perfused by host vessels, forming an organic whole (Chong *et al.*, 2014). Additionally, the majority of endosialin$^+$ cells retained characteristics of stem cells, as indicated by the expression of the putative SLCs markers endosialin and PDGFRα, which had not been examined for SLCs in previous studies. Only a small proportion of the RFP-labelled cells differentiated into LCs. Meanwhile, the testosterone production of the transplanted cells was not as high as those *in vitro*, in accordance with the low differentiation ratio *in vivo* determined by immunofluorescence assays. A possible explanation for this finding is that the microenvironment in 3-week-old mouse testes was not perfect for the differentiation of human SLCs since SLCs commit to a differentiation pathway to form LCs lineages by postpartum day 11 in mice (Ye *et al.*, 2017; Chen *et al.*, 2020). Surprisingly, although the transplanted endosialin$^+$ cells survived and exhibited measurable cell cycle activity, the retrieved RFP-labelled cells accounted for only 10% of the total transplanted cells 4 weeks after transplantation. We speculate that this might have been due to species differences or immune rejection; these possibilities remain to be thoroughly assessed in future studies. Similarly, even with the use of CD4 antibodies to suppress the immune system, human PDGFRα$^+$ SLCs do not survive over 20 weeks in LH receptor-knockout mouse testes (Eliveld *et al.*, 2019). Given these findings, there is an urgent need to further identify animal models that are optimal for evaluating *in vivo* functions of human SLCs.

Although most of the previous studies focused on LCs differentiation potential after SLCs transplantation (Ge *et al.*, 2006; Jiang *et al.*, 2014; Arora *et al.*, 2019), it has been reported that a subpopulation of LC progenitors derived from foetal gonad could give rise to adult LCs, peritubular myoid cells and so on (Shima *et al.*, 2018). Moreover, we demonstrated that endosialin$^+$ cells showed multipotency with the ability to differentiate into adipocytes, osteoblasts and chondrocytes *in vitro*, which raises the possibility that these cells may be able to give rise to other testicular interstitial cell types. As illustrated by *in vivo* immunostaining data, transplanted RFP-labelled cells did not express the known markers for peritubular myoid cells (α-SMA) or endothelial cells (CD31). These results suggested that the transplanted endosialin$^+$ cell population did not develop into myoid cells or endothelial cells in the present study. However, at this point, we could not rule out the possibility that endosialin$^+$ human SLCs may give rise to other testicular interstitial cell types given the appropriate niche signals, especially in certain testicular disease states. These possibilities remain to be thoroughly assessed in future studies.

Despite the fact that endosialin$^+$ human SLCs have the potential to differentiate into functional LCs *in vitro* and *in vivo*, we admit that this SLCs population might not explain the whole story. Researchers have defined the ancestors of LCs from various perspectives or at different developmental stages. To the best of our knowledge, several cell types besides SLCs have been proposed to differentiate into adults LCs, as follows. First, previous studies have provided evidence that pericytes are the progenitors of the LCs in the testes (Davidoff *et al.*, 2004; Chen *et al.*, 2019; Davidoff, 2019). In the present study, the self-renewal and differentiation properties of endosialin$^+$ human SLCs were highly similar to the characteristics of pericytes (Armulik *et al.*, 2011; Wong *et al.*, 2015). Moreover, a pericyte marker PDGFRβ (Armulik *et al.*, 2011) is co-expressed with endosialin both in testis tissues and in cultured endosialin$^+$ spheres (data not shown), implying that the endosialin$^+$ cell population overlaps with the pericyte population in human testes to some degree. Second, adult LCs are thought to derive from the trans-differentiation of foetal LCs (FLCs) (Prince, 2001). Using lineage tracing of FLCs, Shima *et al.* (2018) suggested that some FLCs undergo dedifferentiation during foetal and neonatal stages and serve as adult LC progenitor cells. Another piece of evidence comes from the observation of expression of NR2F2 in human endosialin$^+$ cells and rodent SLCs (Kilcoyne *et al.*, 2014) since NR2F2 was initially considered a marker for subpopulations of FLCs (van den Driesche *et al.*, 2012; Teerds and Huhtaniemi, 2015). However, we could not analyse the relation between endosialin and FLCs owing to a lack of foetal testicular tissues. Third, LCs are thought to have a mesenchymal origin (Hardy *et al.*, 1989). Several studies have shown that MSCs are present in the testes of both human beings and rodents (Chikhovskaya *et al.*, 2012; Ahmed *et al.*, 2017). Nevertheless, to our knowledge, it has yet to be investigated whether testicular MSCs could be induced into LCs *in vitro* or *in vivo*. We and other groups have demonstrated that PDGFRα$^+$ (Eliveld *et al.*, 2019) and NGFR$^+$ (Zhang *et al.*, 2017) human SLCs are multipotent cells with MSCs properties, similar to endosialin$^+$ human SLCs in the present study. In addition, endosialin$^+$ human SLCs are positive for the MSCs minimal criteria markers CD73, CD90 and CD105 (data not shown). In addition, using lineage tracing models, Liu *et al.* (2016) provided evidence that the GLI1$^+$/CYP17A1$^-$ non-steroidogenic progenitor cells retain their undifferentiated state during the foetal stage and become an adult LC

population in post-pubertal testis. Besides, Kumar and DeFalco (2018) demonstrated that Nestin-expressing cells are adult LC progenitors during normal development. In the present study, endosialin$^+$ cells also expressed GLI1 (data not shown) and Nestin. Nevertheless, to date, the development of LCs from stem cells is still a matter of debate, and it remains unclear whether these distinct cell populations share a common stem cell ontogeny, especially in human. It is anticipated that future studies would resolve these important issues.

The surface protein endosialin has been reported to be expressed in various tissues including muscle (Naylor *et al.*, 2014), brain, thymus (Lax *et al.*, 2012) and liver (Mogler *et al.*, 2015). In the current study, we clarified the expression pattern of endosialin in adult human testes and revealed its superiority over two other markers for the identification and isolation of human SLCs. However, whether endosialin could serve as a surface marker of stem cells in other tissues needs to be further determined. In addition, cell surface proteins usually function as regulators of stem cell fate (Alexander *et al.*, 2016; Tomellini *et al.*, 2019). As previously reported, endosialin can regulate MSCs proliferation and maintain a promigratory phenotype (Naylor *et al.*, 2012), and endosialin expressed on a population of thymic MSCs is involved in controlling thymic growth during postnatal development and infection-dependent thymic regeneration (Lax *et al.*, 2012). In addition, endosialin expression in MSCs is required for wound healing of myofibroblasts (Hong *et al.*, 2019). These findings imply a role of endosialin in tissue remodelling and repair. Moreover, endosialin helps regulate pericyte and hepatic stellate cell proliferation stimulated by PDGF-BB; these processes are blunted by the absence of endosialin, indicating that endosialin expression is necessary for PDGF signalling, at least in these cells (Tomkowicz *et al.*, 2010; Mogler *et al.*, 2015; Wilhelm *et al.*, 2016). Since PDGF-BB is by far the most potent SLCs proliferation stimulator (Li *et al.*, 2016) and endosialin is negligibly expressed once SLCs differentiate into LCs, we suspect that endosialin may play roles in determining the balance between SLCs self-renewal and differentiation. However, the effects of endosialin on SLCs cell biology are not yet fully understood and need to be elucidated in future studies.

In conclusion, we have identified endosialin as a more specific marker of SLCs compared with the currently used molecules. Additionally, we found that endosialin$^+$ cells isolated from human testes show characteristics of SLCs with the ability to undergo extensive proliferation and differentiate into testosterone-producing LCs *in vitro*. Furthermore, transplanted endosialin$^+$ cells appear to colonize the host interstitium, localize to peritubular and perivascular regions, be perfused by host vessels, proliferate measurably and differentiate partially into functional LCs *in vivo*. Thus, the present study identifies a promising discriminatory surface marker for SLCs identification and isolation, which is likely to give insight into the characterization of human SLCs and promote potential clinical applications of cell replacement therapies for male hypogonadism.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

All authors fulfil the criteria for authorship. C.D. and A.P.X. designed the study and revised the manuscript critically. K.X., X.F. and R.D. conducted the experiments, collected and analysed the data. Y.M. and Q.K. participated in interpreting the data. K.X. drafted the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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