REPRODUCTION
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Regeneration of Leydig cells in ectopically autografted adult mouse testes

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Abstract

Ectopic autografting of testis tissue is a promising approach for studying testicular development, male germline preservation and restoration of male fertility. In this study, we examined the fate of various testicular cells in adult mouse testes following ectopic autografting at 1, 2, 4 and 8 weeks post grafting. Histological examination showed no evidence of re-establishment of spermatogenesis in autografts, and progressive degeneration of seminiferous tubules was detected. Expression of germ cell-specific proteins such as POU5F1, DAZL, TNP1, TNP2, PRM1 and PRM2 revealed that, although proliferating and differentiating spermatogenic germ cells such as spermatogonia, spermatocytes and spermatids could survive in autografts until 4 weeks, only terminally differentiated germ cells such as sperm persisted in autografts until 8 weeks. The presence of Sertoli and peritubular myoid cells, as indicated by expression of WT1 and ACTA2 proteins, respectively, was evident in the autografts until 8 weeks. Interestingly, seminal vesicle weight and serum testosterone level were restored in autografted mice by 8 weeks post grafting. The expression of Leydig cell-specific proteins such as CYP11A1, HSD3B2 and LHCGR showed revival of Leydig cell (LC) populations in autografts over time since grafting. Elevated expression of PDGFRA, LIF, DHH and NEFH in autografts indicated *de novo* regeneration of LC populations. Autografted adult testis can be used as a model for investigating Leydig cell regeneration, steroidogenesis and regulation of the intrinsic factors involved in Leydig cell development. The success of this rodent model can have therapeutic applications for adult human males undergoing sterilizing cancer therapy.
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Introduction

Grafting of immature testicular tissue into immunodeficient mice is a successful strategy for growing immature testicular tissue and retrieving differentiated germ cells with fertilization capacity (Honaramooz *et al.* 2002). Successful generation of sperm from xenografts of immature testes from mice, hamsters, monkeys (Schlatt *et al.* 2002), bulls (Oatley *et al.* 2005), pigs, goats (Honaramooz *et al.* 2002), cats (Snedaker *et al.* 2004), buffaloes (Reddy *et al.* 2012) and mouse hosts has already been reported. Previous studies have shown that xenografted testis tissue from sexually mature donors is less successful than that from sexually immature donors (Arregui *et al.* 2008). Moreover, complete spermatogenesis has been reported to be absent in the xenografts that contained post-meiotic germ cells at the time of grafting in all species analyzed to date, and most of the mature donor grafts regressed or were found to contain degenerated tubules (Schlatt *et al.* 2002, 2006, Geens *et al.* 2006, Arregui *et al.* 2008, Abrishami *et al.* 2010). Spermatogenesis was arrested at meiosis in grafts from mature horse (Rathi *et al.* 2006), dog (Abrishami *et al.* 2010) and photoregressed hamster testes (Schlatt *et al.* 2002). Survival of only seminiferous tubules with Sertoli cells was seen in mature bull and monkey donor grafts; however, complete spermatogenesis was observed when the donor graft tissue was not completely mature (Arregui *et al.* 2008). No germ cell differentiation was observed in adult human testes xenografted onto mouse hosts (Geens *et al.* 2006, Schlatt *et al.* 2006).

Testis cryopreservation and autografting offer a valuable option for restoring fertility in cancer patients who undergo cancer therapy that leads to subsequent sterilization. Testis autografting has been shown to be a feasible option for production of sperm in monkeys (Jahnukainen *et al.* 2012). However, autologous immature testis grafts in the marmoset matured to meiosis, but normal serum testosterone level was not restored (Wistuba *et al.* 2006). In addition, autologous transplantation of testis tissue to the greater omentum and abdominal wall without vascular anastomosis was not viable in rats (Nunes *et al.* 2013). The fate of testicular cells of adult mouse testes following ectopic autografting is so far unreported.

The objective of this study is to examine the fate of testicular germ and somatic cells in ectopically autografted adult mouse testes. Our results indicate that

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Leydig cells regenerated *de novo* in the autografted adult testes, restoring the serum testosterone level.

Material and methods

Testis tissue autografting

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India. Adult *Balb/c* mice (10 weeks old; $n=27$) were anesthetized with ketamine (0.1 mg/kg body weight (BW)) and xylazine (0.5 mg/kg BW) in sterile physiological saline. The mice were castrated through a ventral medial incision in the abdomen and the testes were removed; subsequently, the peritoneum and skin were sutured closed using absorbable sutures (Ethicon, www.novartis.com). During the same surgery, each mouse received two incisions (~5 mm) on each side of the back (four incisions total). Each collected testis was weighed and then cut in half, and each half was inserted through each incision. The other testis was treated similarly. All four incision sites were sutured closed and the mice were then allowed to recover and returned to their cages.

Recovery and analysis of autografts

At 1, 2, 4 and 8 weeks post grafting, the mice were killed by CO_2 inhalation. The grafts were recovered, weighed and fixed in Bouin's solution followed by three changes in 70% ethanol before being processed for histology. The testes from 10-week-old mice ($n=5$) were also collected as the control and then fixed and processed for histology. After processing, the tissues were embedded in paraffin, sectioned (7 μm thick), stained with haematoxylin and eosin (H&E), dehydrated, mounted in Vectamount (Vector Laboratories, www.vectorlabs.com) and observed under a Zeiss Axioplan 2 microscope (Carl Zeiss AG, www.zeiss.de). The tubules that did not contain distinct cell types were considered to be degenerated. The grafts were classified as degenerated when no tubules were found and non-degenerated even if it contained a single seminiferous tubule with spermatogenesis. In grafts which had seminiferous tubules were observed, all tubules were analyzed and classified either as degenerated or non-degenerated.

The percentage of recovered grafts was calculated for all mice in which grafts were found, and it was also recorded when no grafts were recovered. The percentage of seminiferous tubules and degenerated tubules within a graft were also calculated.

Autografted mouse analysis

Seminal vesicles from all autografted mice were weighed for assessment of the bioactive circulating testosterone level (Honaramooz *et al.* 2002, Reddy *et al.* 2012). Blood was collected from autografted mice at the time when being killed by cardiac puncture, and serum was subsequently collected by centrifugation. Blood and average weight of seminal vesicles from intact age-matched male mice (*Balb/c*; $n=20$, 5 mice for each collection time point) were taken as controls. Serum samples were analyzed for testosterone concentration using a commercial kit (CAN-TE-250; Diagnostics Biochem Canada, Inc., www.dbc-labs.com).

CYP11A1, HSD3B and PRM2 immunostaining of autografts

The autografted testes sections were stained with cytochrome P450, family 11, subfamily A (CYP11A1) and hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2 (HSD3B2) antibodies to examine the expression of Leydig cell-specific proteins. The autografts were also stained with protamine 2 (PRM2) antibody to confirm the existence of sperm. Adult testes were also stained as positive controls. Primary and secondary antibodies were prepared diluted in PBS with 1% BSA (Sigma, www.sigmaaldrich.com). Briefly, after de-paraffinization and rehydration, the sections were blocked with 10% foetal bovine serum (Gibco, www.invitrogen.com) and 3% BSA in PBS for 30 min; incubated with rabbit anti-CYP11A1 (1: 100; Millipore, www.millipore.com), goat anti-HSD3B2 (1:30) or goat anti-PRM2 antibody (1:20; both from Santa Cruz Biotechnology, Inc., www.scbt.com) overnight at 4 °C; washed several times with PBS; incubated with 3% H_2O_2 for 10 min; washed three times with PBS, incubated with goat anti-rabbit or rabbit anti-goat HRP-conjugated secondary antibody (1:200; Calbiochem, www.calbiochem.com) for 30 min at 37 °C, rinsed three times with PBS, incubated for 3–5 min in DAB Substrate Kit (Vector Laboratories) according to the manufacturer's instructions, rinsed thoroughly in distilled water; and mounted. For negative controls, the rabbit/goat isotype control antibodies (Santa Cruz Biotechnology, Inc.) were used instead of primary antibodies at the same concentration, but all other procedures remained the same.

Western blotting analysis of autografts

Total proteins from the autografts were extracted upon homogenization by sonication in a dissolving buffer (7 M urea, 2 M thiourea, 4% CHAPS, 18 mM Tris–HCl, 14 mM Tris–Base, 0.2% Triton-X and 50 mM dithiothreitol). Single-strength ProteCEASE-50, EDTA-free protease inhibitor (G-Biosciences, www.gbiosciences.com) was added to the dissolving buffer before protein extraction. The lysed samples (50 μg) were subjected to electrophoresis in 12% SDS–polyacrylamide gel. The gels were transferred onto PVDF membranes (Millipore). The membranes were blocked with Starting Block (TBS) blocking buffer (Thermo Scientific, www.piercenet.com) for 1 h at room temperature. The blocked membranes were incubated with one of the following primary antibodies: proliferating cell nuclear antigen (PCNA; 1:1000) or CYP11A1 (1:1000) from Millipore; deleted in azoospermia-like (DAZL; 1:5000); leukemia inhibitory factor (LIF; 1:1000); desert hedgehog (DHH; 1:10 000); neurofilament, heavy polypeptide (NEFH; 1:10 000); POU class 5 homeobox 1 (POU5F1; 1:1000) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000) from Thermo Scientific; and actin, alpha 2, smooth muscle (ACTA2; 1:10 000); luteinizing hormone/choriogonadotrophin receptor (LHCGR; 1:200);

HSD3B2 (1:200); platelet-derived growth factor receptor-a (PDGFRA; 1:400); Wilms tumour 1 (WT1; 1:200); transition nuclear protein 1 (TNP1; 1:200); transition nuclear protein 2 (TNP2; 1:200) or PRM1 (1:200) from Santa Cruz Biotechnology, Inc. The membranes were then washed with TBS-T and incubated with goat anti-rabbit, goat anti-mouse or rabbit anti-goat HRP-conjugated secondary antibody (1:10 000; both from Thermo Scientific) in TBS-T for 1 h at room temperature. After washing with TBS-T, immunoreactivity was detected by chemoluminescence using a C-DiGit Blot Scanner (LI-COR Biosciences, www.licor.com) against SuperSignal West Femto chemiluminescent substrate (Thermo Scientific), and the generated signal was analyzed using a densitometer. To control protein loading on the gels, the membranes were further probed with GAPDH antibody.

Statistical analyses

The results are presented as mean ± S.E.M. The statistical analyses were performed by ANOVA. Significant differences between the means were determined by analyzing the data using the Fisher's Protected Least Significant Difference (PLSD) test. The level of significance was set at $P<0.05$. In all figures, data are presented as mean ± S.E.M. Bars with asterisks (*) and bars with different letters are significantly different.

Results

Grafts recovery, graft weight and histological analysis

The autografted testicular tissues were identified and recovered from the dorsal skin at various time points. Although the recovery of grafts until 4 weeks post grafting was not significantly different (Table 1; $P>0.05$), the recovery at 8 weeks was significantly reduced ($P<0.05$).

Testis autografts were weighed at each collection time point to assess the proliferation of testicular somatic cells and progression of spermatogenesis. Average graft weight did not significantly differ before grafting (T0) and 1 and 2 weeks post grafting (Fig. 1A; $P>0.05$). However, the weight of grafts collected at 4 and 8 weeks was significantly lower than that at T0 as well as at 1 and 2 weeks post grafting ($P<0.05$). The weight of grafts collected at 4 weeks was higher than that at 8 weeks, but this difference was not statistically significant ($P>0.05$).

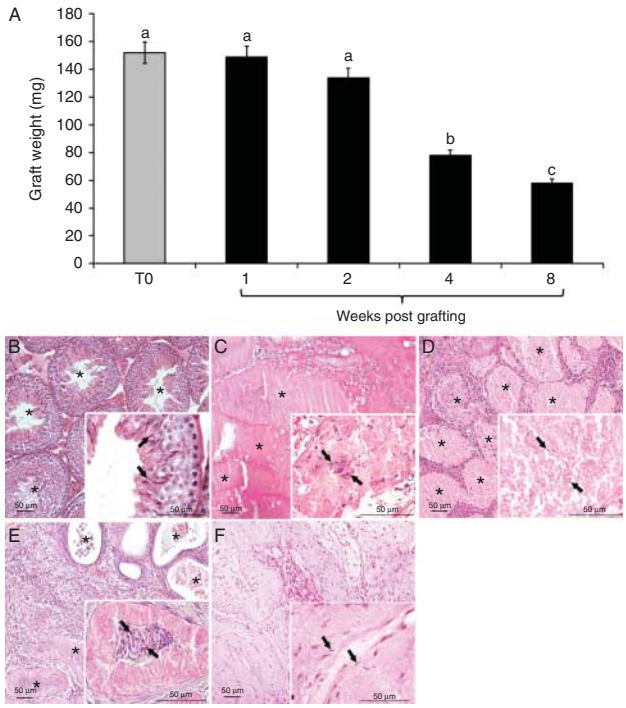

Figure 1 Graft weights and histological evaluation of grafts. (A) Average weight of autografted testis tissue showed significant reduction at 4 and 8 weeks post grafting. T0, testicular weight before grafting as a representation of starting material. Haematoxylin and eosin staining of (B) 10-week-old testis tissue before grafting and (C) 1 week, (D) 2 weeks, (E) 4 weeks and (F) 8 weeks post grafting. Note the progressive degeneration of seminiferous tubules (*) in the grafts over time since grafting. At 8 weeks post grafting, no visible tubules are observed. Insets show magnified region of a section in which sperm are indicated by arrows. Data represent mean ± S.E.M. Bars with different letters are significantly different at $P<0.05$. Scale bar = 50 µm.

Histological evaluation of 10-week-old mice testes showed intact seminiferous tubules with spermatogenesis in 98.9% of tubules (Fig. 1B). A prominent lumen was present in seminiferous tubules, and spermatozoa were the most advanced germ cells. Histological examination of graft tissues showed varying degrees of tubular degeneration during the grafting period. Grafts collected at 1 week contained almost all degenerated tubules (Fig. 1C); however, in one graft, a few intact tubules (12.4%) were detected (Table 1). In grafts collected at 2 weeks, greater degenerative changes were evident in the tubules (Fig. 1D). In grafts collected at 4 weeks (Fig. 1E), degeneration was extensive with loss of most tubules and at 8 weeks, no intact tubules were observed in the autografts (Fig. 1F). The autografts showed progressive loss of sperm cells with increase in collection time. At 8 weeks, a few sperm could be detected in 0.2–0.5% of degenerated tubules (Fig. 1F).

Table 1 Experimental autograft data.

Graft period (weeks)	No. of mice autografted and analysed	No. of autografts recovered[a] (%)	No. of autografts with non-degenerated tubules[b] (%)
1	7	100 (28/28)^{A}	3.5 (1/28)
2	6	95.8 (23/24)^{A}	0 (23/23)
4	7	96.4 (27/28)^{A}	0 (27/27)
8	7	75.0 (21/28)^{B}	0 (21/21)

Different superscript letters (A and B) indicate significant difference within the column ($P<0.05$).
[a]Total grafts removed divided by the total number of grafts grafted.
[b]Percentage of recovered grafts with intact seminiferous tubules.

Serum testosterone and seminal vesicle weight

The serum testosterone level of grafted mice until 4 weeks was significantly lower than that of age-matched control mice (Fig. 2A; $P<0.05$). At 8 weeks, the serum testosterone level of grafted mice was similar to that of age-matched controls ($P>0.05$). Similarly, the average weight of seminal vesicles recovered from grafted mice until 4 weeks was significantly lower than that from age-matched control mice (Fig. 2B; $P<0.05$). At 8 weeks, average weight of seminal vesicles of grafted mice did not differ from that of age-matched controls ($P>0.05$).

CYP11A1, HSD3B2 and PRM2 immunostaining of autografts

Immunohistochemical analysis with the Leydig cell-specific markers CYP11A1 and HSD3B2 facilitated identification of Leydig cells in grafts (Fig. 3). The steroidogenic enzymes CYP11A1 and HSD3B2 showed restricted immunolocalization in the Leydig cells and were present as well-defined clusters in the interstitial space of the 10-week-old mouse testes (Fig. 3A and G). The CYP11A1 and HSD3B2 staining was localized in the cytoplasm of stained cells. Fewer cells were positive for CYP11A1 and HSD3B2 in the grafts collected at 1 week

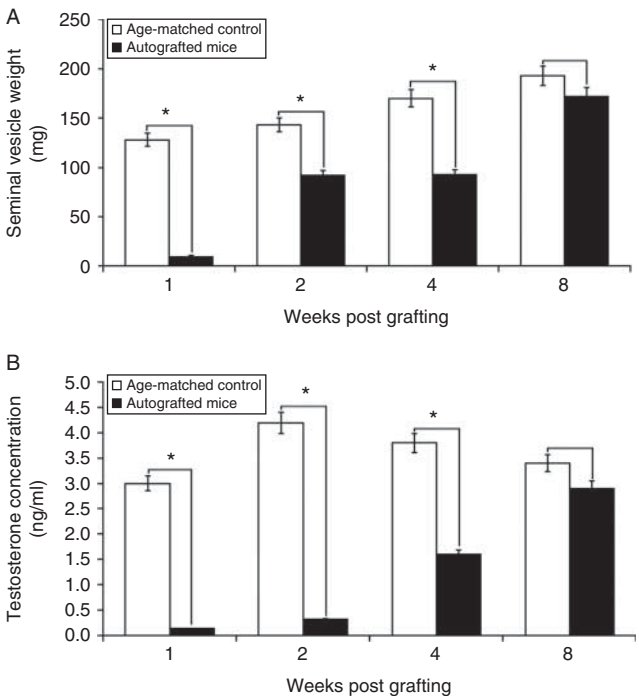

Figure 2 (A) Seminal vesicle weights and (B) serum testosterone autografted recipients and age-matched control mice at 1, 2, 4 and 8 weeks post grafting. Note that the seminal vesicle weight and serum testosterone are significantly lower in autografted mice than that in age-matched control mice at all collection time points except at 8 weeks post grafting. Data represent mean ± S.E.M. (*$P<0.05$).

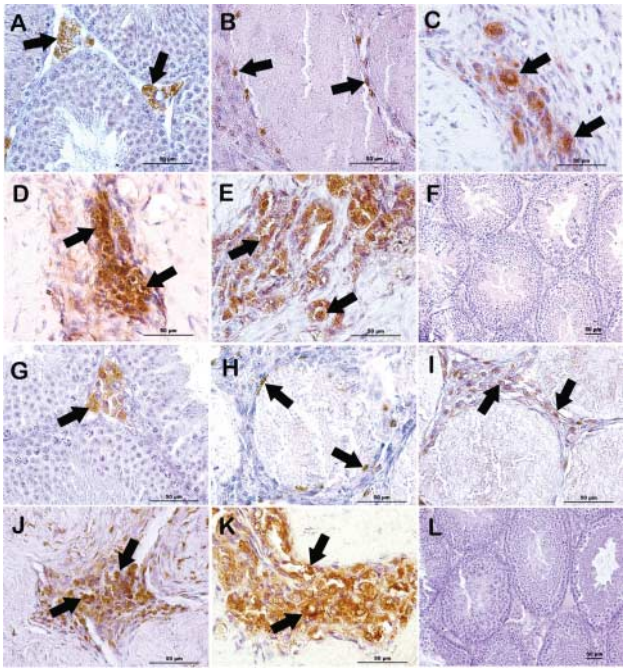

Figure 3 Identification of Leydig cells in autografts by expression of the Leydig cell-specific proteins (A, B, C, D, E and F) CYP11A1 and (G, H, I, J, K and L) HSD3B2. Stained cells are indicated by arrows. Both markers showed Leydig cell-specific staining in 10-week-old mouse testis tissue, (A) CYP11A1 and (G) HSD3B2. Fewer Leydig cells were found in grafts collected at 1 week (B and H) and 2 weeks (C and I). At 4 weeks (D and J) and 8 weeks (E and K) post grafting, Leydig cells are abundantly present in the grafts. Note that the staining intensity of both markers progressively increased with increase in graft collection time. In negative controls (F and L), where primary antibodies were omitted and sections were incubated with isotype control antibodies, no positive cells are present. Scale bar = 50 μm.

and the stained cells were localized in peritubular region (Fig. 3B and H). However, CYP11A1- and HSD3B2-positive cells progressively increased in the grafts collected at 2 weeks (Fig. 3C and I), 4 weeks (Fig. 3D and J) and 8 weeks (Fig. 3E and K). Interestingly, the staining intensity for CYP11A1 was stronger than that for HSD3B2 in the sections at all collection times. No CYP11A1- and HSD3B2-positive cells were present in negative control sections, in which the isotype control antibodies were used instead of primary antibodies (Fig. 3F and L).

The presence of sperm in autografts was evaluated by PRM2 immunostaining. PRM2 staining in 10-week-old mouse testis sections was detected in elongated spermatids and sperm (Fig. 4A). In autografts collected at 1 week (Fig. 4B), 2 weeks (Fig. 4C) and 4 weeks (Fig. 4D), PRM2-positive cells could be identified in 50–85% of the degenerated tubules. In the autografts collected at 8 weeks, PRM2-positive sperm were detected in 1–2% of the degenerated tubules (Fig. 4E). Most PRM2-positive sperm appeared fragmented as indicated by their smaller size. In negative control sections in which isotopic control antibody was

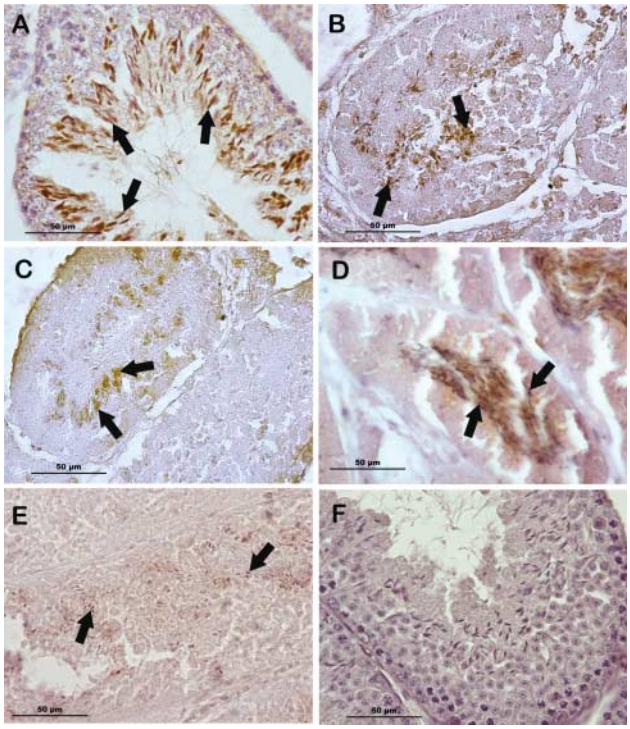

Figure 4 Immunostaining of autografts with PRM2 antibody for identifying sperm. PRM2-positive cells are indicated by arrows. (A) PRM2-positive elongated spermatids and sperm in a 10-week-old mouse testis section. In autografts collected at (B) 1 week, (C) 2 weeks and (D) 4 weeks post grafting, PRM2-positive sperm can be seen in the degenerated tubules. In grafts collected 8 weeks post grafting (E), many fragmented sperm that are PRM2-positive can be seen. In negative control (F), where primary antibody was omitted and section was incubated with an isotype control antibody, no positive cells are present. Scale bar = 50 μm.

used instead of primary antibody, no PRM2-positive cells were present (Fig. 4F).

Western blotting analysis of autografts

The expression of several testicular cell-specific and Leydig cell regeneration signalling-specific proteins was analyzed in the grafted testes at each collection time point by western blot analysis (Fig. 5A). The expression levels of the Leydig cell-specific proteins CYP11A1, HSD3B2 and LHCGR progressively increased with increased collection time of grafts (Fig. 5B). The expression levels of CYP11A1, HSD3B2 and LHCGR proteins were significantly higher in grafts collected at 2 and 4 weeks than in those collected at 1 week (Fig. 5B, $P<0.05$). Interestingly, CYP11A1, HSD3B2 and LHCGR expression increased to 35-, five- and 3.5-fold respectively, in grafts collected at 8 weeks ($P<0.05$). The expression of cell proliferation-specific protein PCNA level was elevated in grafts collected at 1 week (Fig. 5B). Thereafter, PCNA expression level gradually decreased in grafts with increase in collection time and was lowest at 8 weeks ($P<0.05$). The expression of PDGFRA, a stem Leydig cell (SLC)-specific protein, was elevated in grafts at 1 week, reached its peak level at 2 weeks and thereafter, and progressively declined at 4 and 8 weeks (Fig. 5C; $P<0.05$). Interestingly, PDGFRA expression was absent from adult testes. Similar to PDGFRA, NEFH expression was raised elevated in grafts collected at 1 week, peaked at 2 weeks, and then a significant gradual decrease was observed at 4 and 8 weeks (Fig. 5C; $P<0.05$). Like PDGFRA, NEFH expression was absent in adult testes. The expression of DHH protein was elevated in grafts collected at 1 week and reached its peak level at 2 weeks (Fig. 5C; $P<0.05$). Thereafter, DHH expression significantly lowered in grafts collected at 4 and 8 weeks ($P<0.05$). The expression of LIF protein was high in grafts collected at 1 week, declined twofold at 2 weeks, and was absent from grafts collected at 4 and 8 weeks (Fig. 5C; $P<0.05$). The expression of the Sertoli cell-specific protein WT1 progressively increased in grafts over time since grafting (Fig. 5D; $P<0.05$). The expression of the ACTA2 protein was present in grafts collected at 1 week, peaked significantly in grafts collected at 2 weeks, and thereafter declined in autografts collected at 4 and 8 weeks (Fig. 5D; $P<0.05$). The expression of the germ cell-specific proteins POU5F1, DAZL, TNP1 and TNP2 was present in grafts collected at 1, 2 and 4 weeks (Fig. 5E). However, at 8 weeks, their expression was absent from grafts (Fig. 5E; $P<0.05$). Interestingly, POU5F1 protein expression level showed a twofold increase in grafts collected at 4 weeks compared with autografts collected at 1 and 2 weeks ($P<0.05$). The expression level of PRM1 protein was low at 1 week and significantly raised in grafts collected at 2, 4 and 8 weeks (Fig. 5E; $P<0.05$).

Discussion

This study explores the fate of various cell types in adult testes following ectopic autografting. A significant number of grafts were recovered until 1, 2 and 4 weeks post grafting. However at 8 weeks, the number of recovered grafts was significantly reduced. The weight of collected grafts was also found to be significantly declined at 4 and 8 weeks. Histological examination of grafts showed a progressive disintegration of the testicular architecture and extensive degeneration of seminiferous tubules as time since grafting increased.

Degeneration of graft testicular tissue could have possibly occurred because of the absence of the natural scrotal environment and exposure to hyperthermic conditions at the ectopic site of grafting. The adult testes of species with high daily sperm production such as pig and goat after xenografting to mice recipients showed a complete degeneration of tubules and grafts (Arregui *et al.* 2008). However in species such as bull and monkeys where daily sperm production is low, xenografted adult testes had either degenerated tubules or

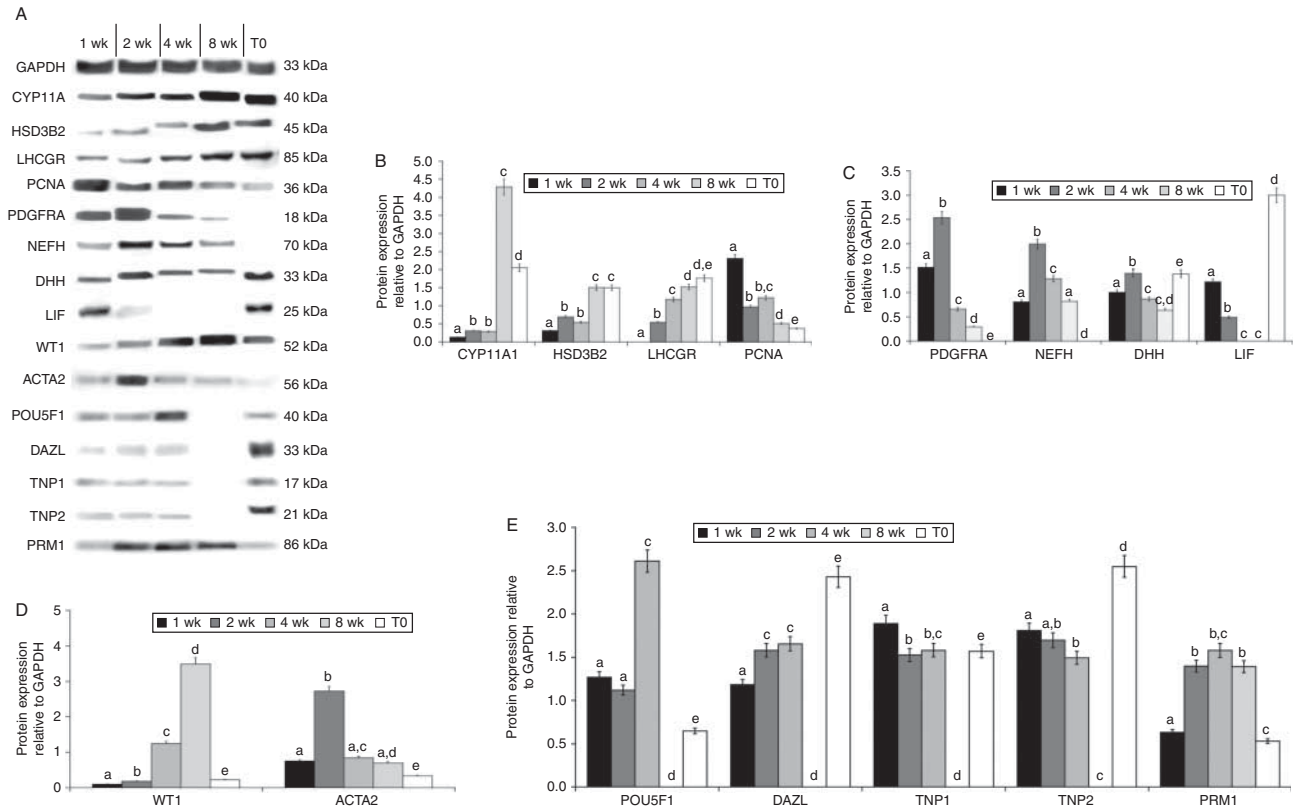

Figure 5 Expression of Leydig cell-, germ cell-, Sertoli cell- and cell signalling-specific proteins in autografted testes at 1, 2, 4 and 8 weeks post grafting by western blotting analysis. T0, protein expression in 10-week-old mice before grafting as a representation of starting material. Representative western blotting (A) and densitometry analysis of CYP11A1, HSD3B2, LHCGR and PCNA (B), PDGFRA, NEFH, DHH and LIF (C), WT1 and ACTA2 (D), POU5F1, DAZL, TNP1, TNP2 and PRM1 (E). Data represent mean ± S.E.M. Bars with different letters are significantly different for a given protein at $P<0.05$.

tubules that contained only Sertoli cells (Arregui *et al.* 2008). In rare cases, tubules with spermatogonia without meiotic spermatogenic cells were also present. Degenerated tubules or tubules that contained only Sertoli cells were also observed in adult human testis (in which, daily sperm production is low) after xenografting (Geens *et al.* 2006). As mice testis also has efficient spermatogenesis with high sperm production, extensive degeneration of autografts was expected in this study. The adult testis tissue grafts are also highly sensitive to ischaemia (Schlatt *et al.* 2002) and have a high demand for oxygen because of ongoing spermatogenesis (Schlatt *et al.* 2010). Ischaemic conditions could have contributed to extensive degeneration of grafts that led to significant loss in graft weight and complete atrophy of several grafts that may have affected their recovery. These findings are consistent with those of previous studies, which reported regression of adult grafts or extensive sclerosis following ectopic xenografting (Schlatt *et al.* 2002, 2006, Geens *et al.* 2006, Arregui *et al.* 2008). Seminiferous tubules with complete spermatogenesis were only detected in one graft, which was collected at 1 week. This rare graft could have survived the initial period of hypoxia, leading to the persistence of seminiferous tubules with spermatogenesis. However, by 2 weeks and thereafter, seminiferous tubules could not be detected in any graft. Therefore, we conclude that spermatogenesis cannot be revived in ectopically autografted adult testes.

As the grafted testes showed extensive degeneration, it was difficult to identify testicular cells. Therefore, to evaluate the existence of various testicular cell types in grafts, expression of several testicular cell-specific proteins was examined by western blotting analysis. The presence of Sertoli cells in the grafts was ascertained by the expression of a transcription factor, WT1. WT1 expression in Sertoli cells is initiated early in foetal life and continues thereafter, and it is primarily localized to the nucleus throughout all phases of life (Mackay 2000). WT1 is localized in Sertoli cells in both embryonic (Gao *et al.* 2006) and adult mice (Sridharan *et al.* 2007). WT1 protein expression showed progressive increase in grafts over time since grafting. This could be due to degeneration of other testicular cell types in autografts that may have led to net increase in the amount of proteins of surviving Sertoli cells. Nonetheless, this indicates that Sertoli cells were rather resilient to ischaemic damages induced as a result of ectopic grafting. Surprisingly, Sertoli cells survived despite

extensive degeneration of seminiferous tubules in the autografted testes. This finding is in agreement with earlier studies which reported that Sertoli cells could be detected in xenografts following extensive degeneration and sclerosis of seminiferous tubules (Schlatt *et al*. 2006, Arregui *et al*. 2008). It remains elusive and therefore requires further validation to determine whether the Sertoli cells survived or originated *de novo* in the grafts.

ACTA2 protein expression could be detected in grafts at all collection time points; however, it was significantly higher at 2 weeks. ACTA2 is expressed by the peritubular myoid cells and vascular tissues in testes. Peritubular myoid cells not only provide structural integrity to the tubule but also take part in the regulation of spermatogenesis and testicular function (Maekawa *et al*. 1996). Several studies have found that Leydig cells are generated by transdifferentiation of pericytes (PCs)/vascular smooth muscle cells (VSMCs), as determined by morphological and immunohistological analyses (Davidoff *et al*. 2004, 2009). The presence of CYP11A1 and HSD3B2-positive cells in peritubular location in autografts collected at 1 week in this study supports previous findings. ACTA2 is co-expressed in nestin-positive PCs/VSMCs and freshly regenerated Leydig cells after experimentally induced Leydig cell elimination in adult animals (Davidoff *et al*. 2004). The high expression of ACTA2 at 2 weeks is suggestive of perivascular origin of Leydig cells in the grafts.

The presence of germ cells in grafts was evaluated by examining proteins expressed at different stages of germ cell differentiation. Although expression of POU5F1, DAZL, TNP1 and TNP2 proteins was present in grafts collected at 1, 2 and 4 weeks, their expression was absent at 8 weeks. Only the expression of PRM1 was present in grafts collected at all-time points. The expression of POU5F1 in postnatal mouse testes is restricted to spermatogonial stem cells (SSCs; Imamura *et al*. 2006, Western *et al*. 2010). DAZL is expressed in spermatogonia, spermatocytes and round as well as elongated spermatids (Reijo *et al*. 2000). The protein TNP1 and TNP2 belongs to nuclear transition protein family (Yelick *et al*. 1991), and these are expressed in round and elongated spermatids (Wykes *et al*. 1995, Adham *et al*. 2001).

PRM1 and PRM2 are arginine-rich proteins synthesized in late-stage spermatids (Balhorn 2007), and thus have been used as markers for spermatid differentiation (Lee *et al*. 1995, Zhao *et al*. 2004). The transition proteins or histones are replaced by PRM in mature sperm nuclei (Balhorn *et al*. 1984, Lewis *et al*. 2004). The results from this study indicate that proliferating and differentiating spermatogenic germ cells, such as spermatogonia, spermatocytes, as well as round and elongated spermatids, are sensitive to ischaemic insults and are not able to survive until 8 weeks in grafted testes. However, terminally differentiated germ cells such as sperm survived until 8 weeks as indicated by PRM1 and PRM2 protein expression in autografts. This corroborates the results of an earlier report in which sperm were observed in otherwise completely sclerotic tubules and without the presence of other spermatogenic cells in adult mouse testes that were grafted onto nude mice (Geens *et al*. 2006). Although sperm were observed in very few tubules in sections that were stained with H&E, a higher number of tubules had PRM2-stained cells in autografts collected at 8 weeks post grafting. This could be because PRM2 immunostaining is more sensitive to the presence of sperm. The stained sperm appear fragmented due to extensive degeneration of tubules. This suggests that, despite extensive degeneration of seminiferous tubules, some sperm can still survive and some sperm-specific proteins are expressed in autografts at 8 weeks post grafting. However, it remains unclear as to how the sperm survived in grafts in which there is complete degeneration of seminiferous tubules.

A consistent increase in the serum testosterone level and seminal vesicle weight was observed in autografted mice with increased time since grafting onto the host; however, they differed from the age-matched control until 4 weeks. At 8 weeks, serum testosterone level and seminal vesicle weight of grafted mice were not different from that of the age-matched control. This indicates that, by 8 weeks, autografted testes were able to produce sufficient testosterone to restore normal serum level and seminal vesicle weight, which are similar to the results of the age-matched control mice. The results from earlier studies in rodents showed restoration of seminal vesicle weight in mice (Boyle *et al*. 1975) and serum testosterone level in rats (Miragem *et al*. 2009) following testis autografting, which are consistent with the findings if this study. Although serum testosterone level and seminal vesicle weight of autografted mice were restored by 8 weeks, it remains unclear whether this was due to survival of Leydig cell populations in ectopically autografted testes or *de novo* regeneration of Leydig cells.

Despite extensive degeneration of seminiferous tubules in grafts, the presence of interstitial cells was evident. Although there were very few steroidogenic enzyme-specific protein-expressing cells (e.g. CYP11A1 and HSD3B2) in grafts at 1 week, their number increased as collection time increased post grafting. CYP11A1 and HSD3B2 are Leydig cell-specific markers in mouse testes (Davidoff *et al*. 2004). The western blotting analysis also substantiates the loss of Leydig cell populations in autografts at 1 week. Testosterone level and seminal vesicle weight until 4 weeks were significantly lower than that of the age-matched controls. These findings indicate degeneration of the original Leydig cell population in grafts after ectopic transplantation because of the hypoxic damage during the ischaemic period immediately following grafting. Interestingly, translation of cell-signalling proteins pertaining to Leydig cell regeneration was significantly higher in grafts collected at 1 and 2 weeks.

PDGFRA is reported to be expressed in foetal and neonatal rat testes of SLC populations (Ge *et al*. 2006). Its expression level was significantly higher in grafts collected at 1 and 2 weeks, but showed a significant decline thereafter. PDGFRA and its ligand, platelet-derived growth factor-a (PDGFA) are required for development of Leydig cell (Ge *et al*. 2006). In PDGFRA-null mice, differentiation of foetal Leydig cells is disrupted (Brennan *et al*. 2003). The expression of PDGFRA in autografts indicates the presence of SLC/progenitor populations that may have contributed to Leydig cell regeneration in a manner similar to that reported in rats following ethane dimethanesulfonate (EDS) treatment (Davidoff *et al*. 2004, O'Shaughnessy *et al*. 2008).

DHH expression was present in grafts at all collection time points; however, a surge in expression was observed at 2 weeks. DHH is a cell signalling molecule produced by Sertoli cells, and disruption of spermatogenesis and loss of adult-type Leydig cells are reported in DHH-null mice (Clark *et al*. 2000, Pierucci-Alves *et al*. 2001, Yao *et al*. 2002). DHH signalling triggers Leydig cell differentiation by upregulation of CYP11A1 expression because it acts as a positive regulator of steroid-producing Leydig cell differentiation from foetal to adult testes (Yao *et al*. 2002). In the absence of DHH and PDGFA, adult Leydig cells (ALCs) fail to develop, which can also be related to the failure of stem cell development (Clark *et al*. 2000, Gnessi *et al*. 2000, Park *et al*. 2007). However, high translation of DHH and PDGFRA proteins at 2 weeks in grafts indicates an abundance of SLCs.

LIF, a pleiotropic cytokine produced predominantly by the peritubular myoid cells of the seminiferous tubules (Dorval-Coiffec *et al*. 2005) is expressed in Sertoli cells and spermatogonia (Piquet-Pellorce *et al*. 2000). LIF and PDGFA stimulate SLC proliferation (Arregui *et al*. 2008). Interestingly, LIF expression peaked at 1 week, whereas PDGFRA expression peaked at 2 weeks in this study. It is therefore likely that LIF and PDGFA signalling in SLCs occurs at different time points; moreover, LIF withdrawal stimulates embryonic stem cell differentiation (Ward *et al*. 2004). The absence of LIF in grafts at 4 and 8 weeks indicates SLC differentiation. As we did not examine expression of the LIF receptor, it is possible that LIF activity may have persisted at later time points in the grafts. As LIF is known to control the proliferation and survival of SSCs, including primordial germ cells and gonocytes (De Felici & Dolci 1991, Pesce *et al*. 1993, Piquet-Pellorce *et al*. 2000), the absence of LIF expression in grafts at 4 and 8 weeks could be an indication SSC loss. A recent study has shown that selective inhibition of PDGFR signalling in human mesenchymal stem cells leads to upregulation of POU5F1 and NANOG expression (Ball *et al*. 2012). In this study, PDGFRA expression showed an inverse correlation with POU5F1 expression in grafts. A similar signalling pathway could possibly be involved in the regeneration of Leydig cells in autografted testis.

LH stimulates testosterone synthesis in Leydig cells by binding to specific, high-affinity receptors (LHCGR) that are present on the surface of Leydig cells (Dufau 1988). LHCGR protein expression progressively increased in grafts over time since grafting, which is a pattern similar to CYP11A1 and HSD3B2 protein expression in this study. LHCGR receptor signalling is essential for Leydig cell differentiation (Teerds *et al*. 2007) and Leydig progenitor cells (LPCs) possess very few LH receptors (Shan & Hardy 1992). The lowered expression of LHCGR in grafts collected at 1 and 2 weeks indicates the existence of LPCs. Progressive increase in LHCGR expression in grafts with increasing collection time demonstrates increased tropic stimulation of the developing Leydig cells.

NEFH is a type IV intermediate filament protein that is expressed in the neuronal/glial lineage (Doetsch 2003). LPCs begin to express not only steroidogenic enzymes such as CYP11A1, but also a number of neuronal antigens including NEFH (Davidoff *et al*. 2009). Newly formed Leydig cells acquire neuroendocrine characteristics that are required for maturation of Leydig cells in adult testes (Middendorff *et al*. 1993, 1996, Bakalska *et al*. 2002, Davidoff *et al*. 2004). A weak expression of NEFH was present in grafts at 1 week in this study. Interestingly, NEFH expression peaked in grafts at 2 weeks and then declined at 4 and 8 weeks. These findings are consistent with those of an earlier report that revealed transient elevated expression of NEFH after EDS exposure in rats localized to the vascular Leydig cell progenitor, which coincided with their transition from proliferative to transformation activity (Davidoff *et al*. 2004). A similar signalling pathway in autografted testes may be involved, although this idea requires further validation.

PCNA is a cyclin protein that is expressed in proliferating cells (Almendral *et al*. 1987). In testes, PCNA expression is detected in the mitotically proliferating spermatogonia, but not in spermatocytes that have just entered meiosis (Chapman & Wolgemuth 1994, Liang *et al*. 2001). In later stages of meiotic prophase, PCNA is again observed in spermatogenic cells, in particular the zygotene and pachytene spermatocytes (Chapman & Wolgemuth 1994). In this study, autografted testes showed substantial degeneration of tubules. Therefore, proliferation of germ cells can be ruled out. The high expression of PCNA protein in the grafts at 1, 2 and 4 weeks could be due to the presence of proliferating SLCs and PLCs, which have high mitotic activities. The decline in PCNA expression in autografts collected at 8 weeks indicate the appearance of ALCs, which do not usually proliferate (Keeney *et al*. 1988).

In conclusion, ectopically autografted adult testes undergo extensive degeneration with loss of spermatogenic cells, but terminally differentiated germ cells, such

as spermatozoa, survive. Peritubular myoid and Sertoli cells also survived in autografts. Leydig cells regenerated *de novo*, restoring the serum testosterone level and seminal vesicle weight in autografted mice. This study describes a novel model system for studying Leydig cell regeneration in mice. As mouse Leydig cells are resistant to the cytotoxic effect of EDS (Kerr *et al.* 1987), the testis autograft model system can be used for investigating Leydig cell regeneration, steroidogenesis and regulation of the intrinsic factors involved in Leydig cell development. This model could also provide insights into overcoming testosterone deficiency and Leydig cell dysfunction in vertebrate species.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H Makala, L Pothana, S Sonam and A Malla performed the research and analyzed the data; S Goel designed the research study and wrote the paper.

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