Brief exposure of neonatal testis cells to EGF or GDNF alters the regenerated tissue

Awang Hazmi Awang-Junaidi‡, Mohammad Amin Fayaz‡, Savannah Goldstein‡ and Ali Honaramooz‡

Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Correspondence should be addressed to A Honaramooz: ali.honaramooz@usask.ca

‡(A H Awang-Junaidi is now at Department of Veterinary Preclinical Sciences, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Selangor Darul Ehsan, Malaysia)

Abstract

We have previously shown that implantation of testis cell aggregates under the back skin of immunodeficient mice results in de novo regeneration of testis tissue. We used this unique model to investigate the effects of epidermal growth factor (EGF) and glial cell-derived neurotrophic factor (GDNF) on testis cord development. Neonatal piglet testis cells were briefly (<1 h) exposed to either low (L: 0.02 μg/mL) or high (H: 2 μg/mL) doses of EGF, GDNF, or vehicle (control), before implantation in recipient mice. Randomly selected implants were removed from each mouse at 1, 2, 4, and 8 weeks post-implantation. GDNF-L implants showed increased testis cord development over time, and EGF-L implants had increased cross-sectional area. The ratio of regular cords decreased over time in EGF-H and GDNF-H implants and was replaced by a higher ratio of irregular cords in GDNF-H. EGF-L and GDNF-H implants were quickest to display rete testis-like structures. Overall, the lower dose of each growth factor was more effective than its higher dose in improving the implantation outcomes. This is the first comprehensive assessment of these key growth factors on de novo formation (regeneration) of testis tissue.

Lay summary

In recent decades, testicular cancer rates have quadrupled in young men while sperm counts have dropped by half. Both conditions may be related to exposure of fetuses or infants to noxious substances causing disruption of normal testis development. To study the effects of any putative factor on testis development, we established an animal model of testis tissue regeneration. We collected newborn piglet testes after routine castration, used enzymes to completely dissociate testis cells, exposed the cells to two key growth factors (EGF or GDNF), and implanted the cells under the back skin of recipient mice, acting as live incubators. We then examined implant samples after 1, 2, 4, or 8 weeks and assessed testis regeneration. Overall, the high dose of each growth factor had adverse effects on the formation of normal testis. Therefore, this novel implantation model may also be used to study the effects of potentially harmful substances on testis development.

Key Words: growth factors, testis cell implantation, testis cord development, testis tubulogenesis, male reproduction
Introduction

Organogenesis is a complex process that relies on various cellular interactions and coordinated endocrine, paracrine, and autocrine signaling pathways. This process is especially more complicated for the mammalian testis where folding of different germinal layers and extensive migration of both germ cells and somatic cells must result in the compartmentalization of the tissue into testicular cords and an inter-tubular interstitium (Lamb 1993). Testicular/semiferous cords (SC) house primitive germ cells (gonocytes) and immature nursing (Sertoli) cells, encircled by the peritubular myoid cells, while the interstitial tissue contains immature androgen producing (Leydig) cells and the connective tissue components (Ibtisham et al. 2020). Proper testis tissue patterning is imperative for normal reproductive function wherein future spermatogenesis can be adversely affected if the SC are malformed (Lamb 1993). Testis organogenesis starts during the early embryonic stages, and the gradual maturational changes continue postnatally and well into the preadolescence and pubertal periods (Ibtisham et al. 2020). Throughout this time, the developing testis remains susceptible to various exogenous factors, including chemical toxicants or environmental pollutants, which can interfere with key signaling pathways and cause poor testicular cord/tubule formation, impaired spermatogenesis, or altered androgen production (Sharpe 2010, Ibtisham et al. 2020). Since the mid-20th century, sperm counts in men have dropped by up to 60%, especially in industrialized societies (Levine et al. 2017). At the same time, the rate of testicular germ cell cancer in Western nations has increased by ~400% (Richiardi et al. 2004, Batool et al. 2019, Brennet et al. 2019). In both cases, the observed rapid changes cannot be explained by improved detection methods or genetic aberrations alone and are instead thought to be caused by lifestyle choices and/or environmental factors (Sharpe 2010). To test such theories, and to generate potential solutions, it is important to first understand which growth and differentiation factors contribute to the signaling pathways that drive testis development. Given the complexity of performing experimentations during early embryonic stages, especially in non-rodents, the use of robust in vitro or in vivo models of testis development is highly desired (Ibtisham & Honaramooz 2020, Ibtisham et al. 2020). This is especially essential when aiming to decipher the individual role that each putative factor may play in the process of testicular organogenesis.

Several growth factors are believed to play key roles in testis tissue patterning and organogenesis. Epidermal growth factor (EGF), for instance, is predominantly expressed by germ cells in the mature bovine testis (Kassab et al. 2007) and is thought to regulate the proliferation of porcine Leydig cells and their steroidogenesis in vitro (Sordoillet et al. 1991). EGF and its receptor (EGFR) are also involved in the proliferation and differentiation of immature rodents spermatogonia (Wahab-Wahlgren et al. 2003), as also indicated by spermatogenic impairment in EFGR knockout mice (Levine et al. 2000); its potential role in testis cord morphogenesis is not clear. Conversely, glial cell-derived neurotrophic factor (GDNF) is expressed by testis somatic cells (e.g. Sertoli cells) (Meng et al. 2000, Simon et al. 2007) and is thought to aid in establishing the seminiferous tubule stem cell niche (Simon et al. 2007, Hofmann 2008). GDNF may also impart control over the proliferation and differentiation of spermatogonia in a dose-dependent manner (Meng et al. 2000). Interestingly, both EGF and GDNF and their respective receptors are believed to help drive tubulogenesis during embryonic renal development (Taub et al. 1990, Popsueva et al. 2003, Costantini & Shakya 2006). EGF and GDNF have also been used for the maintenance and propagation of cultured spermatogonial stem cells (SSC) (Kanatsu-Shinohara et al. 2003, Kubota et al. 2004a,b, Ryu et al. 2005), but their direct effects on the developing testis have not yet been studied. Further, most of what is known about these growth factors has been discovered using in vitro models, with considerably less data using in vivo models, especially non-rodent species.

Given the various logistic and ethical limitations of working with primate models, pigs have been increasingly used in biomedical research. Being omnivorous and of comparable body and organ size to humans, pigs are also closely related to humans in terms of anatomy, physiology, metabolic regulation, and predisposition to different disorders (H uppertz et al. 2015). In addition, pigs offer a great advantage of having sequences and timing of cellular events that are closer to humans than those of rodent models, especially when it comes to the study and manipulation of testis development and function (Ibtisham et al. 2020). Furthermore, fresh neonatal pig testes can be easily obtained in large quantities following routine castration at swine facilities, which can be used as a consistent source of non-rodent testis tissue and cells (Yang et al. 2010).

We and others have shown that ectopic implantation of testis cell aggregates from neonatal donor piglets into recipient mice results in de novo formation (regeneration) of testis tissue (Dufour et al. 2002, Honaramooz et al. 2007). Similar results have also been obtained using...
other donor species (Dufour et al. 2002, Gassei et al. 2006, Kawasaki et al. 2006, Arregui et al. 2008a). In this testis cell aggregate implantation (TCAl) model, cells obtained by enzymatic dissociation of donor testes are implanted under the back skin of immunodeficient mice. Surprisingly, the heterogeneous population of donor testis cells undergo autonomous rearrangements and form SC that are morphologically comparable to those of the intact testis. Over time, the newly developed testis tissue can even become capable of supporting steroidogenesis and spermatogenesis (Honaramooz et al. 2007).

The unexpected morphogenic capacity of dissociated testis cells to form functional testis tissue suggests that the various extracellular factors involved in testis organogenesis are preserved beyond the early embryonic stages and are retained in the early postnatal period. As such, this unique in vivo culture system offers a promising model for the study and manipulation of the various putative factors involved in testis development, a process that is otherwise difficult to study in situ. Previously, despite the regeneration of a functional testis tissue using this implantation model, the retrieved implants commonly displayed low spermatogenic efficiency, where most SC/tubules lacked germ cells (Honaramooz et al. 2007, Arregui et al. 2008a, Watanabe et al. 2009). The reasons for these observations were unknown, but a low proportion of germ cells in the donor cell populations or subsequent loss of germ cells during development has been proposed as a probable cause (Honaramooz et al. 2007, Arregui et al. 2008a). To address these issues, we have established a three-step enzymatic testis cell isolation protocol that yields ~40% germ cells from donor neonatal pig testes (Yang et al. 2010, Yang & Honaramooz 2011), optimized several aspects of the implantation technique (Awang-Junaidi et al. 2020), and used ultrasound biomicroscopy to monitor the development of implants while in the recipient mice (Awang-Junaidi et al. 2020, Fayaz et al. 2020a,b). As a result, the resultant implants are now consistent, have high spermatogenic efficiency, and their development can be accurately and repeatedly assessed over time in a non-invasive manner (Awang-Junaidi et al. 2020, Fayaz et al. 2020a,b). Hence, the unique ability of enzymatically dissociated neonatal testis cells of various donor species to reassemble and form testicular cords upon implantation under the back skin of recipient mice provides an innovative, accessible, and flexible model to study testis tissue reformation with the convenience of using postnatal donor cells. Taking advantage of these complementary systems, the present study was designed to investigate the potential dose-dependent effects of two important putative growth factors, EGF and GDNF, on the de novo morphogenesis of testis tissue using a porcine animal model of early human testis development events.

**Materials and methods**

**Animals and experimental design**

Donor testes were collected after aseptic castration of 2-week-old piglets (Sus scrofa; n = 75). Recipient mice were immunodeficient intact male mice (Mus musculus), which were anesthetized for implantation and surgical retrieval of implants. Each mouse (n = 7 mice/group, n = 35) received eight subcutaneous cell aggregate implants (n = 56 implants/group, n = 280, each containing ~100 × 10⁶ cells). Within 1 h prior to implantation, cell aggregates were exposed to a designated growth factor and dosage as follows: EGF at 0.02 μg/mL of cell aggregates (EGF-L) or 2 μg/mL (EGF-H), or GDNF at 0.02 μg/mL (GDNF-L) or 2 μg/mL (GDNF-H), or no growth factors (control). Two randomly selected implants were removed from each mouse at 1, 2, and 4 weeks, with the remaining implants retrieved at euthanasia 8 weeks post-implantation (Fig. 1). The growth factors used, EGF (catalog no. 236-EG-200; R&D Systems, Minneapolis, MN, USA) and GDNF (catalog no. 212-GD-101; R&D Systems), were of human recombinant

**Figure 1** Pictorial representation of testis cell aggregate implantation technique (A and B) and implant retrieval (C and D). (A) Testis cell aggregates were injected under the back skin of immunodeficient mice, where each injection site received ~100 × 10⁶ cells in a volume of 0.1 mL. (B) Each mouse received eight subcutaneous implants, four on each side of the midline. (C) Two randomly selected implants were retrieved at 1, 2, and 4 weeks post-implantation, followed by the retrieval of remaining implants at the time of euthanasia at 8 weeks. (D) Individual implants after retrieval.
Testis collection and cell isolation

Donor piglets (Yorkshire-cross; Camborough-22 × Line 65; PIC Canada, Winnipeg, MB, Canada) were from a university-affiliated swine facility (Prairie Swine Center, Saskatoon, SK, Canada). The piglets were castrated aseptically at ~2 weeks of age, and the testes were kept in ice-cold Dulbecco's phosphate-buffered saline (DPBS; catalog no. 20-031-CV; Mediatech, Manassas, VA, USA) containing 1% w/v antibiotics solution (penicillin and streptomycin; catalog no. 20-002-CI; Mediatech) and transported to the lab within 1 h of collection. Each testis was thoroughly rinsed three times with DPBS before excess connective tissues were removed to obtain testicular parenchyma. The parenchyma from different testes was pooled and then divided into 5 equal portions for ease of isolation. Testis cells were then isolated using a three-step enzymatic digestion method established previously by our lab (Yang et al. 2010, Yang & Honaramooz 2011). Each portion was transferred into a 14-mL polypropylene round-bottom tube and thoroughly minced with fine scissors for 10 min, as we have previously described previously (Awang-Junaidi et al. 2020, Fayaz et al. 2020a). The resultant cell pellets were resuspended and stored at 4 °C overnight. We have previously shown that our three-step enzymatic digestion consistently yields ~40% gonocytes (the remaining being mostly Sertoli and Leydig cells (Yang et al. 2010, Yang & Honaramooz 2011).

Cell aggregate preparation, implantation, and retrieval

On the day of implantation, cells were gently resuspended, filtered, and centrifuged at 500 g at 16°C for 10 min. The supernatant was discarded to yield a compact cell aggregate, the volume of which was standardized to 8 mL in a 50-mL conical tube. The designated dose of one of the growth factors was then added (based on treatment, as described above), and the suspension was gently pipetted to ensure homogeneity. For control groups, cell aggregates were mixed with an equal volume of the vehicle (i.e. 0.5 mL of sterile DPBS).

Cell aggregates were then implanted under the back skin of recipient mice within 1 hour, during which time the test tubes containing the cell aggregates were maintained on ice. The recipient mice were double-homozygous severe combined immunodeficient hairless outbred (SHO mice, Crl:SHO-PkdcsidHrhr, strain code 474; Charles River, Montreal, QC, Canada), as we have previously described (Awang-Junaidi et al. 2020, Fayaz et al. 2020a). The mice were housed in sterile Plexiglas micro-isolator individual cages and maintained under controlled photoperiod (lights on 6:00–18:00 h). The mice were handled aseptically, with food and sterile water provided ad libitum. All mice were allowed to acclimatize for 1 week prior to procedures and were ~8 weeks old at the time of implantation. All mice were anesthetized using 5% isoflurane in oxygen for induction and 2% isoflurane in oxygen for maintenance throughout the procedure. Approximately 0.8 mL of the cell aggregates designated to each mouse was aspirated into a 1-mL syringe. The back skin was lifted to create a ‘tent’ (Fig. 1A), the needle (22-gauge) was inserted subcutaneously and 0.1 mL of cell aggregate (~100 × 10⁶ cells/0.1 mL) was injected into each site; four sites were injected on each side of the midline (Fig. 1B).

Two implants were randomly retrieved from each mouse at 1, 2, and 4 weeks post-implantation under the same general anesthesia regimen (Fig. 1C). This was done through a transverse linear skin incision created adjacent to the edge of the selected implant, which was then gently severed from the skin and surrounding subcutaneous tissue. Incisions were closed with metal wound clips (Michel clips; catalog no. 12-460-179; Miltex Inc., York, PA, USA). The remaining implants were retrieved at euthanasia (8 weeks post-implantation), performed using an overdose of isoflurane followed by bilateral thoracotomy to ensure death.

Tissue processing, gross, and histological analyses

Following retrieval, the weight of each implant was recorded, and implants were fixed in Bouin’s solution overnight, rinsed and preserved in 70% ethanol and then processed in an automated tissue processor (Tissue Tek, Sakura Finetek USA Inc, Torrance, CA, USA). Processed implants were then embedded in paraffin blocks, sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin (H&E).

At euthanasia, the remaining implants and vesicular glands were removed. Vesicular gland (VG) weight index was calculated relative to body weight (BW). For histomorphometric analysis of histology slides, each mouse was considered as an experimental unit. From each implant, digital micrographs were captured using a light
Statistical analysis

All data are presented as mean ± s.e.m. Unless otherwise stated, the statistical analyses were initially performed across all groups to compare various outcomes using one- or two-way (time and treatment) repeated measures ANOVA, chi-square tests, or Pearson correlation coefficient analysis, as appropriate. Since EGF and GDNF groups were completely independent of each other, and the high and low doses for each growth factor were 100-fold apart, we then focused on biologically relevant comparisons such as following up on significant interactions as well as comparisons of specific groups of interest. This included comparisons of high and low doses of each growth factor type to each other or to the control. To compare percentages, the data were transformed using the Arcsine function prior to analysis. The level of significance was set at $P < 0.05$. Data were analyzed using the SPSS (Version 22.0, SPSS Inc., Armonk, NY, USA).

Results

Gross evaluations

Recipient mouse body weight and vesicular gland index

Four of the 35 recipient mice died prior to their scheduled time of euthanasia; they were from different groups and died at various time points. The body weight (BW), and vesicular gland (VG) index of the mice, recorded at the time of death or euthanasia (8 weeks post-implantation) did not differ between groups ($P > 0.05$; Table 1).

Implant recovery and weight

We retrieved a total of 244 identifiable implants from the remaining 262 potential implants, including some samples from the mice that died prior to their scheduled euthanasia (Table 1). The overall implant recovery rate was ~93.1%, which ranged from 84 to 98% but did not differ between groups ($P > 0.05$). The effect of time on implant weight was significant for all groups ($P < 0.05$), except EGF-H which only showed a tendency when compared with control ($P = 0.054$; Fig. 2B). The implant weight increased over time in all groups except EGF-H and GDNF-H. The effect of treatment on implant weight was not significant for any group compared with control ($P > 0.05$). Interactions were observed between the effects of time and treatment for implant weight in control vs EGF-H, EGF-L vs EGF-H, and GDNF-L vs GDNF-H ($P < 0.05$; Fig. 2B, E and F).

Table 1  The recipient mouse body weight (BW, in g), vesicular gland (VG) index (% weight of VG/BW) (± s.e.m.), and the relative number of implants retrieved at the time of euthanasia. Each group was compared with the control. High (H) and low (L) doses of the same growth hormone (EGF or GDNF) were compared with each other. BW and VG index data were analyzed using independent t-tests. Implant % was analyzed using the chi-square tests. Data did not differ ($P > 0.05$) among all compared groups.

| Group       | Mouse BW (g) | VG index (%) | No. implant retrieved/total (%) |
|-------------|--------------|--------------|---------------------------------|
| Control     | 32.32 ± 1.2  | 0.69 ± 0.40  | 42/46 (91)                      |
| EGF-L       | 33.62 ± 0.9  | 0.61 ± 0.10  | 53/54 (93)                      |
| EGF-H       | 34.99 ± 1.4  | 0.77 ± 0.04  | 55/56 (98)                      |
| GDNF-L      | 32.60 ± 1.3  | 0.70 ± 0.10  | 47/50 (94)                      |
| GDNF-H      | 31.79 ± 0.5  | 0.78 ± 0.03  | 47/56 (84)                      |
| Total       | 32.86 ± 1.2  | 0.70 ± 0.09  | 244/262 (93.1)                  |

microscope equipped with digital photomicrography (Northern Eclipse Image Analysis software version 7.0; Empix Imaging, Mississauga, ON, Canada). These included a 40× magnification photomicrograph for general observation of the implants and 400× magnification photomicrographs for detailed morphometric analysis (five randomly selected fields). Measurements were made on obtained images using ImageJ software (US National Institute of Health, Bethesda, MD, USA). The evaluations included measuring the implant cross-sectional area at the widest diameter, formation of SC, diameter of SC, and the prevalence of germ cells. The collected data included the total number of recovered implants, implants showing de novo morphogenesis, the level of SC development, and the presence of rete testis-like (RT) structures or fluid-filled cavities (FFC) expressed as their prevalence and percentage (%). Cross-sectional area measurements included the total implant area (mm²), total area occupied by SC (%; relative to total implant area), and relative area of SC by category (i.e., % of regular SC, irregular SC, enlarged SC, and aberrant SC; relative to total SC occupied area). The SC diameters were measured on >50 randomly selected cords per SC type, at each sample timepoint for each treatment group. Two measures from each SC were averaged; this included two right-angle diameters for circular SC cross-sections and two measurements from the smaller diameter of obliquely sectioned SC. The relative number of gonocytes (per 1000 Sertoli cells) in 2-week samples was also counted in five randomly selected fields per slide (at 400× magnification). All the measurements and counting were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).
respectively). The weight of EGF-H implants was greater than EGF-L implants at 1 and 2 weeks and control implants at 2 weeks ($P < 0.05$; Fig. 1E and B, respectively). The weight of implants at 8 week was negatively correlated with the VG index in EGF-H and GDNF-L (Table 2).

**Histological evaluations**

**Implant area**

An effect of time on implant area, measured at the widest cross-section of the samples, was observed in all groups ($P < 0.05$; Fig. 3). Overall, the implant area of 1- and 2-week samples was smaller ($P < 0.05$) than that of 4- and 8-week samples. An effect of treatment on implant area was observed in EGF-L and in GDNF-L vs GDNF-H ($P < 0.05$; Fig. 3A and F, respectively). Among groups, the implant area in EGF-L was greater than that in control at 8 weeks ($P < 0.05$; Fig. 3A). Interactions were observed between the effects of time and treatment for implant area in control vs GDNF-H, EGF-L vs EGF-H, and GDNF-L vs GDNF-H ($P < 0.05$; Fig. 3D, E and F, respectively). The implant area was greater in GDNF-H than that in control at 1 week and greater in EGF-H vs EGF-L at 2 weeks ($P < 0.05$; Fig. 3D and E, respectively). By 8 weeks, the implant area was smaller in GDNF-H than both GDNF-L and control ($P < 0.05$; Fig. 3F and D, respectively). At 8 weeks, positive correlations were found between implant area and implant weight for EGF-H, GDNF-L, and GDNF-H. The correlation was strongly negative in the control ($r = -0.83$; $P < 0.05$). At this same time point, the implant area and VG index were correlated in GDNF-H only ($r = 0.92$; $P < 0.005$; Table 2).

**General development/regeneration of testis tissue**

A total of 215 of 244 retrieved implants (88.1%) displayed at least some degree of *de novo* regeneration of testis parenchyma (Table 3). The overall percentage of implants with regenerated testis tissue ranged from 83 to 93% and did not differ among groups or over time ($P > 0.05$). Typical development observed in implants at 1 week included enclosure within a defined connective tissue capsule and the presence of random cellular arrangements (Figs 4A and 5). In some implants at 1 week post-implantation, the formation of SC was also evident, which first appeared at the periphery of implants. At 2 weeks post-implantation, individual implants typically had both random cellular arrangements and SC formations at the same time (Figs 4B and 5). At 4 weeks, the area covered by random cellular arrangements was reduced and, especially at 8 weeks, was replaced by cordal/tubular structures and occasionally by RT-like formations (Figs 4 and 5).

**Fluid-filled cavities**

FFC were atypical morphological features observed in some implants. FFC were characterized by a space void of cellular organizations (originating from implanted cells) that was filled with fluid or occasionally with red blood cells (Fig. 6G and H). FFC were observed in ~12% (25/215) of all implants displaying regenerated testis tissue across...
Table 2  Relationships among the recipient mouse vesicular gland (VG) index (% weight of VG/body weight), implant weight, implant area, and seminiferous cord (SC) area at euthanasia (8 weeks post-implantation) in different groups of implants. High (H) and low (L) doses of the same growth hormone (EGF or GDNF) were compared with each other. Each treatment group was also compared with the control. Bold font indicates statistically significant values.

| Group/variable | VG index (%) | Implant weight (mg) | Implant area (mm²) | SC area (mm²) |
|----------------|--------------|---------------------|-------------------|--------------|
|                | r | p  | r | p  | r | p  | r | p  |
| Control (n = 5) |   |     |   |     |   |     |   |     |
| VG index       | - | -   | - | -   | - | -   | - | -   |
| Implant weight | 0.02 | 0.98 | - | - | - | - | - | - |
| Implant area   | 0.41 | 0.37 | -0.83 | 0.02 | - | - | - | - |
| SC area        | 0.24 | 0.61 | -0.88 | 0.01 | 0.89 | 0.01 | - | - |
| EGF-L (n = 6)  |   |     |   |     |   |     |   |     |
| VG index       | - | -   | - | -   | - | -   | - | -   |
| Implant weight | -0.57 | 0.19 | - | - | - | - | - | - |
| Implant area   | -0.07 | 0.89 | 0.64 | 0.12 | - | - | - | - |
| SC area        | -0.36 | 0.25 | 0.51 | - | - | - | - | - |
| EGF-H (n = 7)  |   |     |   |     |   |     |   |     |
| VG index       | - | -   | - | -   | - | -   | - | -   |
| Implant weight | -0.79 | 0.04 | - | - | - | - | - | - |
| Implant area   | -0.70 | 0.08 | 0.53 | 0.22 | - | - | - | - |
| SC area        | -0.42 | 0.04 | 0.45 | 0.31 | 0.16 | 0.74 | - | - |
| GDNF-L (n = 7) |   |     |   |     |   |     |   |     |
| VG index       | - | -   | - | -   | - | -   | - | -   |
| Implant weight | -0.86 | 0.01 | - | - | - | - | - | - |
| Implant area   | 0.35 | 0.45 | 0.05 | 0.92 | - | - | - | - |
| SC area        | 0.27 | 0.56 | 0.04 | 0.93 | 0.01 | 0.98 | - | - |
| GDNF-H (n = 6) |   |     |   |     |   |     |   |     |
| VG index       | - | -   | - | -   | - | -   | - | -   |
| Implant weight | 0.64 | 0.12 | - | - | - | - | - | - |
| Implant area   | 0.92 | 0.00 | 0.47 | 0.28 | - | - | - | - |
| SC area        | -0.32 | 0.49 | -0.47 | 0.28 | -0.48 | 0.28 | - | - |

Figure 3  The area (mm²) of implants measured at the widest cross-section in (A, B, E) EGF and (C, D, F) GDNF groups over time. Two doses of each growth factor were evaluated and compared with control; low dose (0.02 μg/mL of cells): (A) EGF-L and (C) GDNF-L, and high dose (2 μg/mL of cells): (B) EGF-H and (D) GDNF-H. The cross-sectional area of implants was compared between doses (low vs high) of (E) EGF and (F) GDNF, respectively. The growth factors were added to the neonatal porcine testis cell aggregates (~100 x 10⁶ cells/implant) before implantation under the back skin of recipient mice (n = 7 per group). The control implants received no growth factors. Implants were retrieved at 1, 2, 4, and 8 weeks post-implantation. Data with letters (a and b) are mean ± s.e.m. Data with different letters (xyz/XY) between treatments differ significantly (P < 0.05). Data with different letters over time differ significantly (P < 0.05).
Table 3 The prevalence of testis tissue regeneration among implants of different groups and over time. Data are the number and % of implants showing de novo morphogenesis of testis tissue among the retrieved samples at each time point. High (H) and low (L) doses of the same growth hormone (EGF or GDNF) were compared with each other. Each treatment group was also compared with the control. Data were compared using the hi-square tests. Data did not differ (P > 0.05) among all compared groups.

| Group   | 1 week | 2 weeks | 4 weeks | 8 weeks | Overall |
|---------|--------|---------|---------|---------|---------|
| Control | 10/13 (77) | 10/11 (91) | 9/10 (90) | 8/8 (100) | 37/42 (88) |
| EGF-L   | 14/14 (100) | 13/14 (93) | 10/13 (77) | 12/12 (100) | 49/53 (93) |
| EGF-H   | 14/14 (100) | 12/14 (86) | 8/13 (62) | 13/14 (93) | 47/55 (86) |
| GDNF-L  | 14/14 (100) | 9/11 (82) | 9/10 (90) | 11/12 (92) | 43/47 (92) |
| GDNF-H  | 9/11 (82) | 9/13 (69) | 10/12 (83) | 11/11 (100) | 39/47 (83) |
| Total   | 61/66 (92.4) | 53/63 (84.1) | 46/58 (79.3) | 55/57 (96.5) | 215/244 (88.1) |

Table 2

| Group | 2 weeks | 4 weeks | 8 weeks | Overall |
|-------|---------|---------|---------|---------|
| Control | 10/11 (91) | 9/10 (90) | 8/8 (100) | 37/42 (88) |
| EGF-L   | 13/14 (93) | 10/13 (77) | 12/12 (100) | 49/53 (93) |
| EGF-H   | 12/14 (86) | 8/13 (62) | 13/14 (93) | 47/55 (86) |
| GDNF-L  | 9/11 (82) | 9/10 (90) | 11/12 (92) | 43/47 (92) |
| GDNF-H  | 9/13 (69) | 10/12 (83) | 11/11 (100) | 39/47 (83) |
| Total   | 53/63 (84.1) | 46/58 (79.3) | 55/57 (96.5) | 215/244 (88.1) |

time points (Table 4). The majority of FFC were present only at 1 week post-implantation; they were present in 37.7% (23/61) of all implants retrieved at this time point. The overall prevalence of FFC dropped at 2 weeks (P < 0.05); they were present in only ~4% (2/53) of implants retrieved at 2 weeks and absent (0%) from all implants retrieved at 4 and 8 weeks (Table 4).

Formation of seminiferous cords

First appearance and prevalence of SC in implants Of the 215 implants displaying regenerated testis tissue, 165 implants (76.3%) also displayed structures resembling SC (Table 5). SC first appeared as early as 1 week post-implantation (earliest), in 14–64% of implants in all treatment groups, including control. At 1 week, implants in EGF-L had significantly fewer SC than implants in EGF-H (14% vs 64%, P < 0.01; Table 5). However, with all time points combined, the overall number of implants containing SC did not differ among groups (P > 0.05). At 4 and 8 weeks, 100% of the retrieved implants contained SC (Table 5).

SC area in implants The area occupied by SC in implants (% relative to the total implant area) increased at each examined time point (i.e. from 2 to 4 weeks, and from 4 to 8 weeks) in all groups (P < 0.05; Fig. 7). An effect of treatment on the SC area was observed for GDNF-L alone, and in GDNF-L vs. GDNF-H groups (P < 0.05; Fig. 7C and F, respectively). The SC area in 2- and 4-week GDNF-L implants was greater than those in both the control and GDNF-H implants (P < 0.05; Fig. 7C and F). A strongly positive correlation was found between SC area and implant area for control (r = 0.89; P < 0.01) and EGF-L implants (r=0.92; P < 0.01). However, SC area in control implants had a strongly negative correlation with implants weight (r = −0.88; P < 0.05; Table 2).

Classification of SC Upon histological examination, implants displayed various SC morphologies which could be classified as follows: (1) regular SC, referring to the normal morphology that is typical of SC in the donor neonatal porcine testis (diameter: 46.8 ± 0.4 µm, range: 34.4–56.2 µm, n = 250; Fig. 6A); (2) irregular SC, with obvious branching, not normally observed in the donor.

Figure 4 Representative histological photomicrographs demonstrating de novo morphogenesis of testis tissue after implantation of porcine testis cell aggregates under the back skin of recipient mice. Each image represents a typical morphology observed at 1, 2, 4, or 8 weeks post-implantation. (A) The morphogenesis at 1 week was characterized by random cellular formations, demarcated in encapsulated areas. Note the initial formation of seminiferous cords starting from the periphery of the implants (yellow arrow). (B) More defined formation of seminiferous cords seen at 2 weeks post-implantation; overall morphology of the cords starting to resemble seminiferous cords in intact testis tissue. The area occupied by the cords also continuously increased. (C) The random cellular formations diminished at 4 weeks, and the implant area was almost entirely occupied by seminiferous cords with formation of rete testis-like structures (white arrow). (D) The testis tissue underwent extensive morphological changes over time. The presence of large and irregularly shaped seminiferous cords become prominent at 8 weeks post-implantation. Also, note the presence of the rete testis-like structures (white box), which initially developed in the center of implants and then branched radially over time (H&E staining, scale bar: 200 µm).
testis (diameter: 147.8 ± 0.8 µm, range: 76.8–239.7 µm, n = 250; Fig. 6B); (3) enlarged SC, with a considerably greater diameter than age-matched regular SC (diameter: 135.8 ± 0.8 µm, range: 63.3–231.4 µm, n = 250; Fig. 6C); and (4) aberrant SC, or SC-like structures completely or partially lined by cuboidal cells (diameter: 94.9 ± 0.6 µm, range: 38.6–199.9 µm, n = 250; Fig. 6D). The relative prevalence of each SC type within implants ranged from 0 to 100% (Table 6).

**Prevalence and area of SC over time** For comparison, the overall prevalence of the regular type of SC was 100% (28/28 implants) at 1 week and 97% (34/35) at 2 weeks, but it dropped (P < 0.05) to 52 and 29% at 4 and 8 weeks post-implantation, respectively. Among groups at 4 weeks, regular SC were less prevalent (0%) in GDNF-H than in control or GDNF-L (both ~78%, P < 0.005; Table 6). The overall area occupied by regular SC also decreased over time (P < 0.05) from 90 ± 3.8% at 2 weeks to 22.98 ± 4.4% at 4 weeks, and 12.50 ± 3.1% at 8 weeks, respectively. At 4 weeks, the area occupied by regular SC was smaller in GDNF-H implants than in control (P < 0.01) or GDNF-L implants (P < 0.01). At 8 weeks, this area was greater (P < 0.05) in EGF-L implants than EGF-H (Table 6).

Irregular SC were first observed at 4 weeks in ~78% of implants (36/46) with no differences in prevalence between groups (P > 0.05; Table 6). At 8 weeks, 100% of SC in GDNF-H implants were classified as irregular, which was greater than the control group at this time (63%; P < 0.05; Table 6). Among individual groups at 8 weeks, the implant area was greater (P < 0.05) in both EGF-H- and
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GDNF-H-treated implants than in their respective low-dose groups (Table 6).

Enlarged SC were first observed at 2 weeks in 20% of implants (7/35). While no enlarged SC were observed in GDNF-L or EGF-H implants, their prevalence ranged between 17% and 50% in other groups (Table 6). At 2 weeks, EGF-L implants had more enlarged SC than EGF-H implants ($P < 0.05$; Table 6). By 8 weeks, 100% of the GDNF-L implants contained enlarged SC, which was greater than that of the control (63%; $P < 0.005$; Table 6).

Aberrant SC first appeared at 4 weeks in 15% of implants (7/46). At this time, only EGF-H (30%) implants had a higher prevalence when compared with the control (0%, $P < 0.05$; Table 6). At 8 weeks, implants of all groups contained aberrant SC, with prevalence ranging from 82 to 100% which did not differ among groups ($P < 0.05$; Table 6).

**Table 4** The prevalence of fluid-filled cavities (FFC) among implants of different groups and over time. Data are the number and % of implants showing FFC among the retrieved samples at each time point. High (H) and low (L) doses of the same growth hormone (EGF or GDNF) were compared with each other. Each treatment group was also compared with the control. Data were compared using the chi-square tests and statistically significant values are in bold.

| Group   | 1 week          | 2 weeks         | 4 weeks         | 8 weeks         | Overall            |
|---------|-----------------|-----------------|-----------------|-----------------|-------------------|
| Control | 2/10 (20)       | 2/10 (20)       | 0/9 (0)         | 0/8 (0)         | 4/37 (11)         |
| EGF-L   | 8/14 (57)       | 0/13 (0)*       | 0/10 (0)        | 0/12 (0)        | 8/49 (16)         |
| EGF-H   | 2/14 (14)       | 0/12 (0)        | 0/8 (0)         | 0/13 (0)        | 2/47 (4)          |
| GDNF-L  | 7/14 (50)       | 0/9 (0)*        | 0/9 (0)         | 0/11 (0)        | 7/43 (16)         |
| GDNF-H  | 4/9 (44)        | 0/9 (0)*        | 0/10 (0)        | 0/11 (0)        | 4/39 (10)         |
| Total   | 23/61 (37.7)    | 2/53 (3.77)*    | 0/46 (0)        | 0/55 (0)        | 25/215 (11.6)     |

* $P < 0.05$ compared with the previous time point (i.e. 2 weeks vs 1 week).

The first analytical approach showed that the overall area occupied by various SC types was different for each treatment group ($P < 0.05$), except EGF-H and control ($P < 0.05$). Regular SC were predominant in EGF-L and GDNF-L implants ($41 \pm 7\%$ and $49 \pm 8\%$ of all SC; $P < 0.05$), whereas irregular and enlarged SC types were predominant in the GDNF-H group ($P < 0.05$; Table 7).

Using the second approach, we found that the overall area occupied by various SC types was different in EGF-H

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**Figure 6** Representative histological photomicrographs demonstrating (A, B, C, and D) different morphologies of seminiferous cords in 8 week implants, (E, F) rete testis-like structures, and (G, H) fluid-filled cavities. Seminiferous cord morphologies were classified based on microscopic appearance as (A) regular, (B) irregular, (C) enlarged, or (D) aberrant seminiferous cords. (E) Rete testis-like structures (arrows) were shown in the center of implants, with (F) a higher magnification of the structures (dashed line). (G) Fluid-filled cavities (dashed line) were atypical morphological structures found in some implants characterized by a space void of cellular organizations (asterisk) filled with fluid or red blood cells, with (H) a higher magnification shown (H&E staining, scale bar: (A, B, C, D) 100 µm, (E, F) 50 µm, and (G, H) 200 µm).
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and GDNF-H groups only (P < 0.05), where irregular SC were the predominant type in both (44 ± 8% and 55 ± 7% of all SC; P < 0.05). The overall area of SC types did not differ in implants of the control, EGF-L, or GDNF-L groups (P > 0.05). While all groups displayed regular SC (6 ± 3% to 2.6 ± 7% of all SC), EGF-H and GDNF-H groups contained less (P < 0.01) than their lower dose counterparts, and EGF-H contained less than control (P < 0.005; Table 7).

Relative number of gonocytes

The number of gonocytes relative to the number of Sertoli cells was calculated in 2-week implants, when gonocytes were morphologically most distinct. The relative number of gonocytes in control was 15 ± 2 per 1000 Sertoli cells. This measure was numerically greater in all treatment groups, but not significantly different from the control or between high and low doses (P > 0.05; 19 ± 3, 19 ± 1, 19 ± 2, 19 ± 4).

Table 5 The prevalence and first appearance of seminiferous cord formations among implants from different groups. Data are the prevalence of SC formations and their first appearance among implants showing de novo morphogenesis of testis tissue at each time point. High (H) and low (L) doses of the same growth hormone (EGF or GDNF) were compared with each other. Each treatment group was also compared with the control. Data were compared using the chi-square tests and statistically significant values are presented in bold. The first appearance of SC was assessed based on their first occurrence (earliest), the midpoint of occurrence (median), and the last occurrence (latest) from all implants retrieved in a single recipient.

| Group   | No. implants with SC/total implants (%) | First appearance (week) |
|---------|----------------------------------------|-------------------------|
|         | 1 week 2 weeks 4 weeks 8 weeks Overall | Earliest Median Latest |
| Control | 3/10 (30) 5/10 (50) 9/9 (100) 8/8 (100) 25/37 (68) | 1 4 4 |
| EGF-L   | 2/14 (14) 10/13 (77) 10/10 (100) 12/12 (100) 34/49 (69) | 1 2 4 |
| EGF-H   | 9/14 (64)* 6/12 (50) 8/8 (100) 13/13 (100) 36/47 (77) | 1 2 4 |
| GDNF-L  | 9/14 (64) 8/9 (89) 9/9 (100) 11/11 (100) 37/43 (86) | 1 2 4 |
| GDNF-H  | 5/9 (56) 6/9 (67) 10/10 (100) 11/11 (100) 32/39 (82) | 1 2 4 |
| Total   | 28/61 (45.9) 35/53 (60.0) 46/46 (100) 55/55 (100) 164/215 (76.3) | 1 2 4 |

**Figure 7** The area occupied by seminiferous cords (SC) relative to the total implant area (%), measured at the widest cross-section of samples over time in (A, B, E) EGF and (C, D, F) GDNF groups. Two doses of each growth factor were evaluated, low dose (0.02 μg/mL of cells): (A) EGF-L and (C) GDNF-L and high dose (2 μg/mL of cells): (B) EGF-H and (D) GDNF-H. The relative SC areas were compared with the control and between the doses (low vs high) of (E) EGF and (F) GDNF. The growth factors were added to the neonatal porcine testis cell aggregates (~100 ×10^6 cells/implant) before implantation under the back skin of recipient mice (n = 7 per group). The control implants received no growth factors. Implants were retrieved at 1, 2, 4, and 8 weeks post-implantation, although the SC area developed in implants retrieved at 1 week was excluded from this analysis. Data are mean ± s.e.m. Data with different letters (a,b) between treatments differ significantly (P < 0.05). Data with different letters (x,y,z) over time differ significantly (P < 0.05).

11P < 0.01 compared with the lower dose.
**Table 6** The prevalence of each type of seminiferous cords (SC) and their relative area among implants of different groups. Data are the prevalence (%) of different types of SC and their relative area (%), compared with the total implant area, measured at the widest cross-section of samples. High (H) and low (L) doses of the same growth hormone (EGF or GDNF) were compared with each other. Each treatment group was also compared with the control. Data are mean ± s.e.m. Data were compared using the chi-square and independent t-tests for the prevalence and area data, respectively. Statistically significant values are presented in bold.

| Group/week | Type of SC | Regular | Area (%) | Irregular | Area (%) | Enlarged | Area (%) | Aberrant | Area (%) |
|------------|------------|---------|----------|-----------|----------|----------|----------|----------|----------|
| Control    |            | 5/5 (100) | 90 ± 10  | 0/5 (0)   | 0        | 1/5 (20) | 10       | 0/5 (0)  | 0        |
|            |            | 7/9 (78)  | 32 ± 9   | 8/9 (89)  | 36 ± 8   | 9/9 (100) | 33 ± 7   | 0/9 (0)  | 0        |
|            |            | 1/8 (13)  | 6        | 5/8 (63)  | 31 ± 12  | 5/8 (63) | 28 ± 9   | 7/8 (88) | 35 ± 12  |
| EGF-L      |            | 9/10 (90)| 78 ± 11  | 0/10 (0)  |           | 5/5 (50) | 22 ± 8   | 0/10 (0) | 0        |
|            |            | 5/10 (50)| 29 ± 11  | 9/10 (90) | 42 ± 10  | 9/10 (90) | 28 ± 7   | 3/10 (30)| 4 ± 1    |
|            |            | 6/12 (50)| 24 ± 9   | 6/12 (50) | 16 ± 7   | 7/12 (58) | 15 ± 6   | 12/12 (100)| 45 ± 10  |
| EGF-H      |            | 6/6 (100)| 100      | 0/6 (0)   | - (−)    | 0/6 (0)  | 0        | 0/6 (0)  | 0        |
|            |            | 5/8 (63) | 10 ± 6   | 4/8 (50)  | 36 ± 14  | 8/8 (100)* | 53 ± 14  | 3/10 (33)* | 1 ± 0.4 |
|            |            | 3/13 (23)| 4 ± 3†   | 11/13 (85)| 49 ± 9†  | 11/13 (84)| 14 ± 4   | 13/13 (100)| 37 ± 10  |
| GDNF-L     |            | 8/8 (100)| 100      | 0/8 (0)   | 0        | 0/8 (0)  | 0        | 0/8 (0)  | 0        |
|            |            | 7/9 (78) | 46 ± 11  | 6/9 (67)  | 36 ± 12  | 9/9 (100) | 18 ± 3   | 0/9 (0)  | 0        |
|            |            | 4/11 (36)| 13 ± 9   | 10/11 (91)| 29 ± 4   | 11/11 (100)* | 27 ± 6   | 10/11 (91)| 31 ± 9   |
| GDNF-H     |            | 6/6 (100)| 92 ± 8   | 0/6 (0)   |          | 1/6 (17) | 8        | 0/6 (0)  | 0        |
|            |            | 0/10 (0)|| 0**†† | 9/10 (90) | 59 ± 10  | 9/10 (90) | 33 ± 10  | 1/10 (10)| 0.30    |
|            |            | 2/11 (19)| 13 ± 9   | 10/11 (91)| 52 ± 10† | 10/11 (91)| 24 ± 8   | 9/11 (82) | 12 ± 3   |

*P < 0.05 compared with the control; **P < 0.01 compared with the control; †P < 0.05 compared with the lower dose treatment; ††P < 0.01 compared with the lower dose treatment.

20 ± 3, and 18 ± 4 for EGF-L, EGF-H, GDNF-L, and GDNF-H, respectively).

**Rete testis-like formation**

Implanted cells also underwent reorganization into structures resembling the RT and efferent ductules, which were observed to be lined by cuboidal epithelial cells (Fig. 6E and F). These structures first appeared in the center of implants and branched out radially over time. Although not significantly different among groups (P > 0.05), RT appeared as early as 2 weeks in EGF-L and GDNF-H implants (Table 8). The median point for RT appearance was 4 or 8 weeks. At 8 weeks (latest), almost all (~98%) were compared with the control; **P < 0.05 compared with the control; †P < 0.05 compared with the lower dose treatment; ††P < 0.01 compared with the lower dose treatment.

**Table 7** The predominant type of seminiferous cords (%) per group, calculated based on two different approaches. Data are the predominant type of seminiferous cords per group, defined as the combined relative area (%) covered by the same type of SC from different time points, measured at the widest cross-section of samples. The two approaches to calculate the values differed based on inclusion of 2-week samples. Data are mean ± s.e.m. High (H) and low (L) doses of the same growth hormone (EGF or GDNF) were compared with each other; independent t-test. Each treatment group was also compared with the control: independent t-test. Statistically significant values are presented in bold.

| Group     | First approach: overall at 2, 4, and 8 weeks | Second approach: overall at 4 and 8 weeks |
|-----------|---------------------------------------------|------------------------------------------|
|           | Regular | Irregular | Enlarged | Regular | Irregular | Enlarged | Regular | Irregular | Enlarged | Regular | Irregular | Enlarged | Regular | Irregular | Enlarged |
| Control   | 0.10    | 36 ± 8    | 26 ± 5    | 26 ± 5  | 13 ± 6    | 0.36    | 20 ± 6    | 33 ± 7    | 31 ± 5    | 17 ± 7    | 0.84    | 26 ± 7    | 28 ± 6    | 21 ± 5    | 25 ± 7 |
| EGF-L     | 0.01    | 41 ± 7a   | 19 ± 5b   | 22 ± 5b | 17 ± 5b   | 0.01    | 6 ± 3b†† | 44 ± 8a   | 29 ± 7ab  | 20 ± 7b   | 0.01    | 7 ± 5b†† | 32 ± 7b   | 23 ± 3    | 17 ± 6 |
| EGF-H     | 0.41    | 27 ± 8    | 34 ± 7    | 23 ± 6  | 16 ± 6    | 0.28    | 28 ± 7    | 32 ± 6    | 23 ± 3    | 17 ± 6    | 0.28    | 7 ± 5b†† | 32 ± 7b   | 23 ± 3    | 17 ± 6 |
| GDNF-L    | 0.00    | 49 ± 8a   | 23 ± 5b   | 17 ± 3b | 12 ± 4b   | 0.00    | 7 ± 5b†† | 32 ± 7b   | 23 ± 3    | 17 ± 6    | 0.00    | 7 ± 5b†† | 32 ± 7b   | 23 ± 3    | 17 ± 6 |
| GDNF-H    | 0.00    | 26 ± 8a   | 43 ± 7a† | 27 ± 6b  | 5 ± 2b    | 0.00    | 7 ± 5b†† | 32 ± 7b   | 23 ± 3    | 17 ± 6    | 0.00    | 7 ± 5b†† | 32 ± 7b   | 23 ± 3    | 17 ± 6 |

*Data with different letters within each group/approach differ significantly (P < 0.05): one-way ANOVA; †P < 0.05 compared with the control; ††P < 0.01 compared with the lower dose treatment; †‡P < 0.01 compared with the lower dose treatment.

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Table 8  The prevalence and first appearance of rete testis (RT) formations among implants from different groups. Data are the prevalence of RT formations and their first appearance among implants showing de novo morphogenesis of testis tissue at each time point. High (H) and low (L) doses of the same growth hormone (EGF or GDNF) were compared with each other. Each treatment group was also compared with the control. Data were compared using the chi-square tests. Statistically significant values are presented in bold. The first appearance of RT was assessed based on their first occurrence (earliest), the midpoint of occurrence (median), and the last occurrence (latest) from all implants retrieved in a single recipient.

| Group     | No. implants with RT/total implants (%) | First appearance (week) |
|-----------|----------------------------------------|-------------------------|
|           | 1 week  | 2 weeks | 4 weeks | 8 weeks | Overall | Earliest | Median | Latest |
| Control   | 0/10 (0) | 0/10 (0) | 6/9 (67) | 7/8 (88) | 13/37 (35) | 4 | 4 | NA |
| EGF-L     | 0/14 (0) | 3/13 (23) | 8/10 (80) | 12/12 (100) | 23/49 (47) | 2 | 8 | 8 |
| EGF-H     | 0/14 (0) | 0/12 (0) | 2/8 (25)† | 13/13 (100) | 15/47 (32) | 4 | 8 | 8 |
| GDNF-L    | 0/14 (0) | 0/9 (0) | 8/9 (89) | 11/11 (100) | 19/43 (44) | 4 | 8 | 8 |
| GDNF-H    | 0/9 (0) | 1/9 (11) | 10/10 (100)† | 11/11 (100) | 22/39 (56) | 2 | 4 | 4 |
| Total     | 0/61 (0) | 4/53 (7.55) | 34/46 (73.9) | 54/55 (98.18) | 92/215 (42.79) | 92/215 (42.79) |

*p < 0.05 compared with the control; †p < 0.05 compared with the lower dose.

The use of 2-week-old piglets as donors of testis cells in the present study was pooled for each group, the prevalence of RT did not differ among groups (P > 0.05), with RT being evident in ~43% of implants. However, at 4 weeks, more implants in GDNF-H group (100%) had RT than in control (67%; P < 0.05), and more EGF-L implants (80%) had RT than its higher dose counterpart (25%; P < 0.05). By 8 wk, 100% of implants retrieved displayed RT in all groups, except the control (88%; Table 8).

Discussion

In the present study, we examined the effects of EGF and GDNF on the de novo morphogenesis of porcine testis tissue using our recently modified testis regeneration model (Awang-Junaidi et al. 2020). This modified injection technique had allowed for the effective manipulation of cell number, accurate placement of implants, and minimized cell leakage which were common issues using the conventional surgical technique (Honaramooz et al. 2007). Under the modified protocol (Awang-Junaidi et al. 2020), ~100 × 10⁴ testis cells were injected per implantation site, which is 2–50 fold higher than the numbers used previously (Arregui et al. 2008a, Watanabe et al. 2009, Dores & Dobrinski 2014). As a result, our overall implant recovery rate was 93% (of 262 implants), which exceeds comparable studies using donor testis cells from piglets (78–82% of 96 implants) (Honaramooz et al. 2007). This high recovery rate of implants alone indicates that our modifications were effective, especially considering our larger sample size (35 recipient mice vs typically ~12 mice) (Honaramooz et al. 2007, Arregui et al. 2008a, Dores & Dobrinski 2014).

The use of 2-week-old piglets as donors of testis cells in the present study was based on our preliminary observation that 1- and 2-week-old donors were equally capable of initiating de novo formation of testicular cords after use in the current cell implantation model. However, the testes of 2-week old piglets being slightly larger provided greater numbers of cells (unpublished data).

Since there had been no prior in vivo study of the effects of EGF or GDNF on testis regeneration, we decided to use two widely different doses for each growth factor. Our low dose of each growth factor (0.02 µg/mL) was selected based on the frequently used amount for in vitro studies (Kanatsu-Shinohara et al. 2003, Kubota et al. 2004a), while our high dose was aimed to be at 100-fold greater than our low dose for general extrapolation to the in vivo situation. This high dose seemed justified considering the brief exposure of growth factors to cell aggregates before implantations and the obvious major differences between the in vitro and in vivo systems. This, for instance, includes the low concentration of growth factors required for the in vitro work to sufficiently affect the cultured cells where the media is in direct and constant contact with only a monolayer of cells for several days at a time. On the other hand, the exponential dilution factor in the animal due to sheer number of cells and in the presence of the circulatory system and various metabolic and excretion mechanisms would have required much higher concentrations to be included.

For the present study, in the absence of previous reports in the literature to be used as a guide, we chose to mix the growth factors and cells immediately prior to their injection. This was done to ensure timely exposure of all individual testis cells to the designated growth factors as they were being implanted. A hypothetical alternative approach, for instance, would have been to inject growth hormones after implantation of cells; however, such
injections would have added unnecessary additional volume to the implants because both cell pellets and growth factors would have required solutions to allow flow during injections. More importantly, post-implantation injections to add a growth factor would not have guaranteed homogenous distribution of the growth factors among all implanted cells. Post-implantation injections of growth factors would also have potentially interfered with the expected migration, interactions, and reorganization of the cells at early implantation stages and losing this critical window of opportunity when every hour or minute may count.

Both growth factors, in at least one dose, showed a significant overall effect on at least one of the evaluated parameters. Within EGF treatments, EGF-L was found to have a significantly positive effect on implant area. Analyzing the data for correlations on the outcomes at 8 weeks post-implantation showed that implant area in the EGF-L group was significantly correlated with SC area. Although this correlation could potentially be due to the proliferative effect of EGF on both germ and somatic cells, as indirectly indicated from results of other studies (Sordoillet et al. 1991, Levine et al. 2000, Wahab-Wahlgren et al. 2003), but in the absence of differences in implant weight, SC area, or gonocyte numbers as compared with the control, such potential effect of EGF-L on testis morphogenesis must be delineated in future studies.

Conversely, GDNF-L had a significantly positive effect on SC area. Measuring SC area is a more reliable criteria than implant area for assessing de novo testis tissue morphogenesis as it excludes non-tubular compartments within the implant. Thus, based on the data for SC alone, we conclude that GDNF-L exposure had a significant effect on the development of SC. Aside from its role in SSC self-renewal (Meng et al. 2000, Kubota et al. 2004b, 2011, Oatley et al. 2007), GDNF has been shown to promote the in vitro proliferation of immature Sertoli cells (Hu et al. 1999, Yang & Han 2010) and in vivo tubulogenesis during non-testicular (kidney) development (Popsueva et al. 2003, Costantini & Shakya 2006). As such, we hypothesize that exposure of the implants to GDNF-L may have enhanced Sertoli cell proliferation and tubulogenesis in our testis regeneration model, giving rise to implants with improved SC development.

The importance of GDNF in these roles is further supported by the finding that the development of specific SC types was enhanced in GDNF-H implants alone. Within the limited available literature on the ectopic TCAI model, detailed examination of SC morphology in de novo testis tissue formation is lacking. Here, we observed that SC can be found in different patterns and morphologies; while regular SC were the predominant type at 2 weeks, enlarged and irregular SC developed and became more prominent at 4 and 8 weeks post-implantation. Considering both approaches for determining the predominant type of SC among groups, we propose that GDNF-H alone enhanced the formation of regular, irregular, and enlarged SC. This is also generally supported by evaluations of the prevalence of different SC types and implant area; by 8 weeks, irregular SC were seen in >90% of the GDNF-H implants and occupied >50% (range 52–59%) of the implant area. The rationale for using two approaches to analyze these data was that at 2 weeks, most of the implants showed regular type of SC, thus statistically, the data were skewed toward the regular type, while disregarding the other types of SC developed at latter stages. To avoid a biased conclusion, we also analyzed the data using a second approach in which we focused on implants at 4 and 8 weeks. The combined analyses (Table 7) suggest that even brief exposure to higher doses of each growth hormone (EGF-H and GDNF-H) can over time adversely affect the formation of regular SC, which would be replaced by a higher ratio of irregular cords in GDNF-H implants. GDNF-H indeed caused complete loss of regular cords at 4 weeks. These observations highlight the value of using the testis cell implantation model in deciphering the effects of various known and unknown factors in testis cords formation.

After observing different patterns of SC formation among the retrieved implants, which had not been previously reported in the literature, we classified these various SC types into regular, irregular, enlarged, and aberrant to be able to compare the results more clearly. While we have provided specific and detailed description and categorization for these SC types, we are unsure of the consequences of such morphological variations for the testis tissue function. For example, whether these ‘non-regular’ cords/tubules would result in discernible abnormal spermatogenesis are to be tested in future studies. In any case, such differences in the morphology of newly developed SC are important observations because they resulted from brief pre-implantation exposure of testis cells to commonly found growth factors, highlighting the potential long-term consequences of short-term exposure of developing testis cells to exogenous factors or pollutants. Of special note are the evidence suggesting that testicular cord malformation/dysgenesis may be an underlying developmental cause for at least some of the observed trends in impaired spermatogenesis and or altered androgen production (Richiardi et al. 2004, Sharpe 2010, Levine et al. 2017, Batool et al. 2019, Brenner
et al. 2019, Ibtisham et al. 2020). Nevertheless, the present study provides a basis for morphological classification and quantification of resultant cords which could be further expanded when examining testis tubulogenesis in future studies.

The development of enlarged seminiferous tubules has not been examined/described previously in the testis regeneration model. However, the closest reference to a somewhat similar observation comes from a related model ‘testicular tissue xenografting’ in which testis tissue fragments (not cell aggregates) are grafted under the back skin of recipient mice where they can fully develop (Honaramooz et al. 2002, Honaramooz 2019, Fayaz et al. 2020a,b, Ibtisham et al. 2020). In the context of these latter studies, occasionally enlarged seminiferous tubules were observed, typically coinciding with the increased size of the seminiferous tubular lumen (Schlatt et al. 2003, Rathi et al. 2005). This was thought to be related to the absence of efferent ducts in the grafts leading to accumulation of the Sertoli cell secretions in the tubular lumens (Schlatt et al. 2003, Arregui et al. 2008a). While many of our implants developed efferent ducts and RT-like structures, they were retrieved prior to their potential development of a tubular lumen, so it could not be determined whether these structures would have contributed to the absorption of such secretions. At this point, even less is known about the reasons for the observed irregular and aberrant SC development in the present study although it is conceivable that irregular SC might result from the less-than-optimal conditions in which the implants are being formed. Additionally, since the observed aberrant SC tend to be proximal to the RT, it could be hypothesized that the hyperplastic RT cells had entrapped some of the neighboring parenchyma undergoing SC development. This is further supported by the presence of cuboidal epithelial lining, typical of RT cells, found surrounding the aberrant SC. Further studies are necessary to examine the causes and potential significance of such alterations in SC development.

In the present study, the FFC were a non-pathological feature found in many implants. These FFC likely develop as a result of the remnant fluid within the cell aggregate suspensions (i.e. from the added media or growth factor diluents). In addition, the needle insertion may have also caused localized bleeding at implantation site, which explains the presence of RBC within some of the FFC observed through histological assessments. The FFC were transient, with high prevalence at 1 week post-implantation, where they were present in ~38% of the samples in multiple groups. The FFC formation and their gradual changes appeared to be irrespective of the growth factor treatment/dose. The absence of these cavities in 4-week samples can be explained by absorption of media/diluents over time and replacement with connective tissue in the center of the implants. Similarly, using ultrasound biomicroscopy, alongside histomorphometric examination of the same implants, we have recently examined this phenomenon further and demonstrated ~30% reduction in the number of FFC from week 1 to 4 post-implantation (Awang-Junaidi et al. 2020, Fayaz et al. 2020a,b).

Male accessory sex glands are highly androgen-dependent and will shrink or expand in response to the decreasing or increasing levels of androgens, respectively. Hence, the relative weight of vesicular glands (VG index) in mice has been measured as a semi-quantitative bioassay for long-term effects of bioactive androgens. This measure provided key evidence on the androgen-production competence of xenogeneic testis tissue fragments from various donor species which were grated into castrated recipient mice (Honaramooz et al. 2002, Schlatt et al. 2003). In the present study, we also calculated the VG indices as a secondary endpoint; however, our recipient mice were not castrated and therefore the circulating testosterone affecting vesicular glands would have come from two potential sources, the recipient mouse own testes or the developing piglet testis implants. As such, no significant differences were observed in the VG indices between groups, although it is worth pointing out the significant correlations observed for GDNF-H implants, indicating a potential effect of GDNF on androgenic development of implants, which warrants further studies including a comparison of implantation results from both castrated and intact recipient mice.

To the best of our knowledge, the present study is the first to report the development of RT-like structures during de novo testis tissue morphogenesis. We observed that these RT-like structures were a frequently occurring feature (present in ~43% of all implants), regardless of treatment. Although there were generally no differences in their prevalence between groups, RT-like structures appeared earlier in EGF-L and GDNF-H implants. The expression of EGF and its receptors (EGFR) have been localized in the straight tubules and RT of bulls (Kassab et al. 2007) and, like GDNF, they might contribute to tubulogenesis in the developing gonad and kidney (Taub et al. 1990, Kassab et al. 2007). Given that previous reports failed to mention the development of RT in implants, it is unclear whether the rete testis formation seen here was due to the inadvertent inclusion of mediastinal cells within the parenchymal...
testis cell isolates or other regenerative mechanisms. This requires further investigation, as does the relationship between RT development and the formation of aberrant SC.

The present study is also the first histomorphometric study to evaluate the survival of gonocytes in TCAI implants. The effect of growth factors on the relative number of gonocytes was evaluated using only 2-week samples because at this time point gonocytes were morphologically most distinctive. Although not significantly different, we found the relative number of gonocytes in all growth hormone-treated implants to be numerically higher than those counted in control implants (15 ± 2 gonocytes per 1000 Sertoli cells). A limitation of previous TCAI studies has been the low number or proportion of germ cells present among donor cells (e.g. 4.5%), resulting in de novo formation of SC/tubules with a limited number of germ cells (Honaramooz et al. 2007, Kita et al. 2007, Arregui et al. 2008b, Zhang et al. 2008, Dores & Dobrinski 2014). In the present study, the implanted testis cells had ~40% gonocytes, although the number of gonocytes examined in implants at 2 weeks was low, ranging from ~1.5% to 2.6% relative to Sertoli cells. This highlights an obvious disconnect between the relative number of gonocytes present at implantation vs that at time of retrieval as early as 2 weeks post-implantation. In a previous in vitro study, we saw somatic testis cells reach full confluency within 1 week, with gonocytes showing relatively less growth (Awang-Junaidi and Honaramooz 2018). Considering this latter observation and the known properties of both growth factors applied, we hypothesize that the observed decrease in gonocytes to be due to a much higher rate of somatic cell proliferation which might have overtaken the number of gonocytes prior to retrieval of the implants. Comparable data from previous studies are lacking but judging from some of their histological images (Honaramooz et al. 2007, Arregui et al. 2008b), the relative number of gonocytes in the present study still appears to be improved. The ideal proportion of gonocytes among donor cells to be implanted requires further investigation.

Interestingly, while the use of a higher dose of EGF and GDNF was not advantageous in the promotion of de novo porcine testis tissue morphogenesis and, in the case of GDNF-H, even hindered implant development, the lower dose of each growth factor provided more favorable conditions for the implantation outcomes. Such differential effects between the two doses for each growth factor may not have been as obvious had the doses were chosen at a closer range to each other. These observations may indicate that the low dose of EGF and GDNF were more biologically relevant to the levels suitable for the developing testis. This reinforces the theory that the expected outcomes of studies using growth factors are not simply a function of their relative dose. Further, we attribute most of our histological findings to the effects that EGF and GDNF exerted on both germ and somatic cell types and on tubulogenesis in the developing gonad. These growth factors appear to play an important role in de novo regeneration of RT- and efferent ductule-like structures.

Conclusions

This is the first comprehensive assessment of EGF and GDNF as important endogenous growth factors, on de novo formation (regeneration) of testis tissue using an implantation model. Overall, our recent modifications to the testis cell aggregate implantation model were proven effective in providing a valuable tool with which to study the complex mechanisms of testis development in vivo. Rather surprisingly, even a brief exposure of pre-implantation testis cells to these growth hormones had long-lasting effects on the implanted cells’ ability to form normal testicular tissue. While some treated implants (GDNF-L) showed increased testis cord development over time, others (EGF-L) had increased cross-sectional area. The number of regular cords decreased over time in implants exposed to high doses of these growth factors which were replaced by a higher ratio of irregular cords (in GDNF-H implants). Yet, some treatments (EGF-L and GDNF-H) could cause earlier formation of RT-like structures. These observations highlight the unique potential of this implantation model in exploring the effects, on early testis cords formation, of other growth factors, hormones, likely chemical toxicants, or putative environmental pollutants using an important animal model.

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
A H A J contributed to conceiving and designing the study, performed experiments and analyses, and wrote first draft of the manuscript. M A F contributed to performing the analyses. S G rewrote some sections, formatted, and revised the final manuscript. A H supervised all work and contributed to conceiving and designing the study, as well as analyses, interpretation of results, writing, and revising the manuscript.

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