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Development of an In Vivo Model to Study Testicular Morphogenesis

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ABSTRACT: We have developed an in vivo model to examine testicular cord formation by isolated Sertoli and myoid cells when implanted under the kidney capsule of severe combined immunodeficient (SCID) mice. Neonatal porcine Sertoli (92.5% ± 3.5%) and myoid (2.2% ± 0.7%) cellular aggregates were transplanted underneath the kidney capsule of SCID mice. Grafts were removed between 0 and 60 days post-transplantation and examined histologically for the progressive development of structures resembling testicular cords. Aggregates began to reorganize by day 3, and cord structures were present at day 7 post-transplantation. These structures became larger and more defined as the time progressed after implantation. To localize Sertoli and peritubular myoid cells, grafts were immunostained for the Sertoli cell proteins, vimentin, DNA transcription factor GATA-4, and Müllerian inhibiting substance (MIS), as well as for a myoid cell protein, smooth muscle alpha-actin. In the "seminiferous" epithelial layer, the Sertoli cells were arranged with their nuclei along the basal edge adjacent to the peritubular myoid cells that were surrounding the tubules. Moreover, the expression of MIS mimicked that during porcine testicular development, suggesting the Sertoli cells were developing normally. In addition, proliferating cell nuclear antigen (PCNA) was detected in the Sertoli cells at all time points, indicating the proliferation of Sertoli cells in the grafts, which is consistent with Sertoli cell proliferation prior to puberty in the native porcine testis. These results suggest that the specific factors required for cord formation and prepubertal development are inherent in the transplanted cells. Moreover, we have developed a novel in vivo transplantation model to study seminiferous cord formation and prepubertal development.

Key words: Sertoli cell, peritubular myoid cell, cord formation, severe combined immunodeficient mice, testis development.

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Proper development of the testis is critical to attain maximal reproductive capacity. While much is known about spermatogenesis, the factors directly regulating testicular development are still not well understood. The first embryological process to distinguish the male and female gonad is the formation of seminiferous cords in the male. During cord formation, Sertoli cells aggregate with germ cells, begin to differentiate, and become polarized (Magre and Jost, 1980, 1991; Jost et al, 1981). Furthermore, cells from the mesonephros migrate into the male gonad and surround the aggregating Sertoli and germ cells (Buehr et al, 1993; Tilmann and Capel, 1999). It is presumed that these migrating cells are preperitubular myoid cells and that this process is required in order for cords to form.

It is believed that interactions between peritubular myoid and Sertoli cells are important for the formation of tubules and the development of a proper testicular environment. Using an in vitro culture system, Tung and Fritz (1980) demonstrated that when Sertoli and peritubular myoid cells isolated from pubertal rats are cocultured, they aggregate and form structures resembling germ cell-depleted seminiferous tubules. This process of tubulogenesis required the presence of both cell types since Sertoli or peritubular cells cultured alone were unable to form tubule structures (Tung and Fritz, 1980).

In this study, we have developed a novel in vivo transplantation model in which dissociated neonatal porcine Sertoli and myoid cells reaggregate and form seminiferous cords following implantation under the renal subcapsular space of immunoincompetent severe combined immunodeficient (SCID) mice. We have also extensively characterized the morphological changes that occur during the formation of these cords. Further study and experimental application of this in vivo model may provide valuable insight into intrinsic factors and mechanisms involved in testicular development, possibly by manipulating isolated testicular cells in vitro prior to implantation to observe the effect of specific factors on cord formation and development in vivo.

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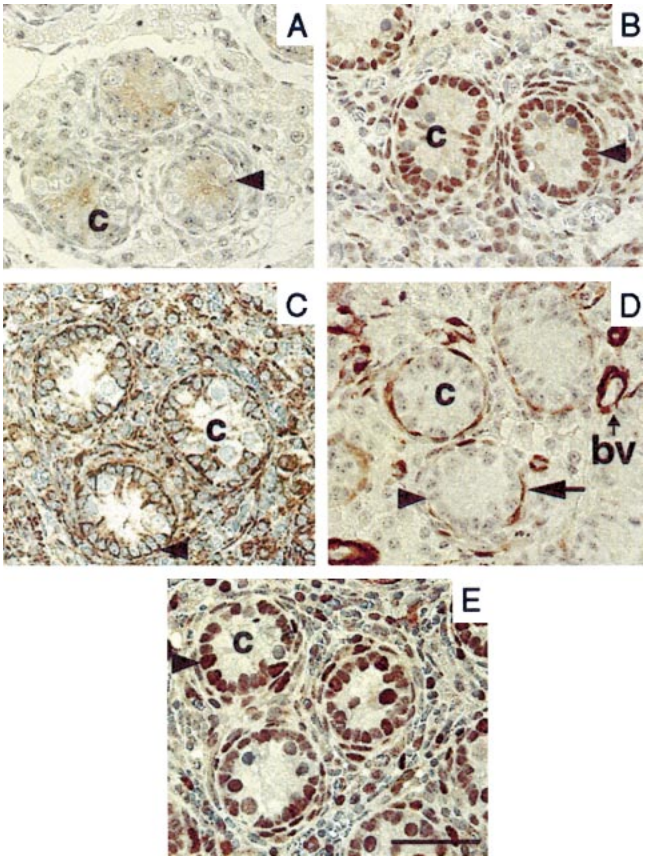

Figure 1. Testicular sections from neonatal pigs aged 1–3 days were immunostained with antibodies against Müllerian inhibiting substance (MIS) **(A)**, DNA transcription factor GATA-4 **(B)**, and vimentin **(C)** to identify Sertoli cells, smooth muscle alpha-actin (D) to identify myoid cells, and proliferating cell nuclear antigen (PCNA) **(E)** to identify proliferating cells. 3′3-Diaminobenzidine Hcl (DAB) was used as chromogen for **(A)**, **(B)**, and **(D)**. Aminoethyl carbazole (AEC) was used as chromogen for **(C)** and **(E)**. Note: c indicates cord; arrowhead, Sertoli cell nucleus; arrow, myoid cell; and bv, blood vessel. Bar in **(E)** = 50 μm for **(A–E)**.

Methods

Animals

Male Landrace-Yorkshire neonatal pigs (1–3 days old) were used as testis donors. Male SCID mice (Taconic Farms), 6 to 8 weeks old, were used as recipients. Animal experimentation was conducted according to the guidelines of the Canadian Council of Animal Care.

Sertoli Cell Isolation

Neonatal porcine Sertoli cells (NPSCs) were isolated using a technique similar to that previously described for rat Sertoli cells (Korbutt et al, 1997). Briefly, 1- to 3-day-old male Landrace-Yorkshire neonatal pigs were anesthetized with Halothane, and testicles were surgically removed and placed in 50-mL conical tubes containing cold (4°C) Hanks balanced salt solution supplemented with 0.25% (wt/vol) fraction V bovine serum albumin (Sigma Chemical Company, St Louis, Mo). The testes were cut into 1-mm fragments with scissors, digested for 10 minutes at 37°C with collagenase type V (2.5 mg/mL; Sigma), and then washed 3 times with Hanks balanced salt solution. The tissue was resuspended in calcium-free medium supplemented with 1 mM ethyleneglycoltetraacetic acid (EGTA) and further digested with trypsin (25 μg/mL; Boehringer Mannheim, Laval, Canada) and DNase (4 μg/mL, Boehringer) for 10 minutes at 37°C. The digest was passed through a 500-μm nylon mesh, washed with Hanks balanced salt solution, and cultured in nontreated petri dishes (15-cm diameter) containing 60–80 $\times$ 10^6 cells and 35 mL of Ham F10 media supplemented with 10 mmol/L D-glucose, 2 mmol/L L-glutamine, 50 μmol/L isobutylmethylxanthine, 0.5% bovine serum albumin, 10 mmol/L nicotinamide, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated neonatal porcine serum. Cells were incubated for 48 hours at 37°C to allow the formation of Sertoli cell aggregates (100- to 300-μm diameter).

Transplantation of Sertoli Cell Grafts

Prior to transplantation, the purity and number of Sertoli cells were determined. Specifically, we assessed the number of vimentin-positive Sertoli cells and smooth muscle alpha-actin-positive peritubular myoid cells in a representative aliquot after dissociation of the cell aggregates using techniques previously described for islet dissociation (Korbutt et al, 1996). The dispersed cell suspension was allowed to attach to Histobond adhesive microscope slides (F.G.R. Steinmetz Inc, Surrey, BC, Canada), fixed with Bouin solution for 30 minutes, washed with 70% ethanol, and immunostained using the Sertoli cell marker vimentin (Korbutt et al, 1997) or the myoid cell marker smooth muscle alpha-actin (Tung and Fritz, 1990). In each preparation, a minimum of 500 single cells were counted to assess the proportion of vimentin-positive Sertoli cells and smooth muscle alpha-actin-positive myoid cells.

In addition, to determine the number of cells transplanted in each recipient, 3 representative aliquots of the cell suspension were measured for total cellular DNA content using a Hoefer DyNa Quant 200 fluorometric assay (Amersham Pharmacia Biotech, San Francisco, Calif). Aliquots were washed with citrate buffer (150 mmol/L NaCl, 15 mmol/L citrate, and 3 mmol/L EDTA, pH 7.4), resuspended in TNE buffer (10 mM Tris, 0.2 mM NaCl, and 1 mM EDTA, pH 7.4), and sonicated. Aliquots of 10 μL were assayed in triplicate by diluting them in 2 mL of assay solution (0.1 μg/mL Hoechst 33258 in 1$\times$ TNE) and measuring fluorescence (365 nm excitation/460 nm emission). A 6-point (0–500 ng/mL) DNA standard curve was generated using calf thymus DNA. For transplantation, aliquots consisting of 11 $\times$ 10^6 porcine testicular cells (6.6 pg DNA/cell) were aspirated into polyethylene tubing (PE-50), pelleted by centrifugation, and gently placed under the left renal subcapsular space of Halothane-anesthetized SCID mice (Korbutt et al, 1997).

Assessment of Tubule Formation Posttransplantation

Removal of the graft-bearing kidneys was performed for morphological analysis at 0, 3, 7, 10, 20, 30, 40, and 60 days after transplantation ($n \geq 3$/time point, except $n = 2$ at 60 days). The graft-bearing kidneys were immersed in Z-fix and embedded in paraffin. After deparaffinization and rehydration, antigen retrieval was performed by heating slides for 15 minutes in 0.01 M

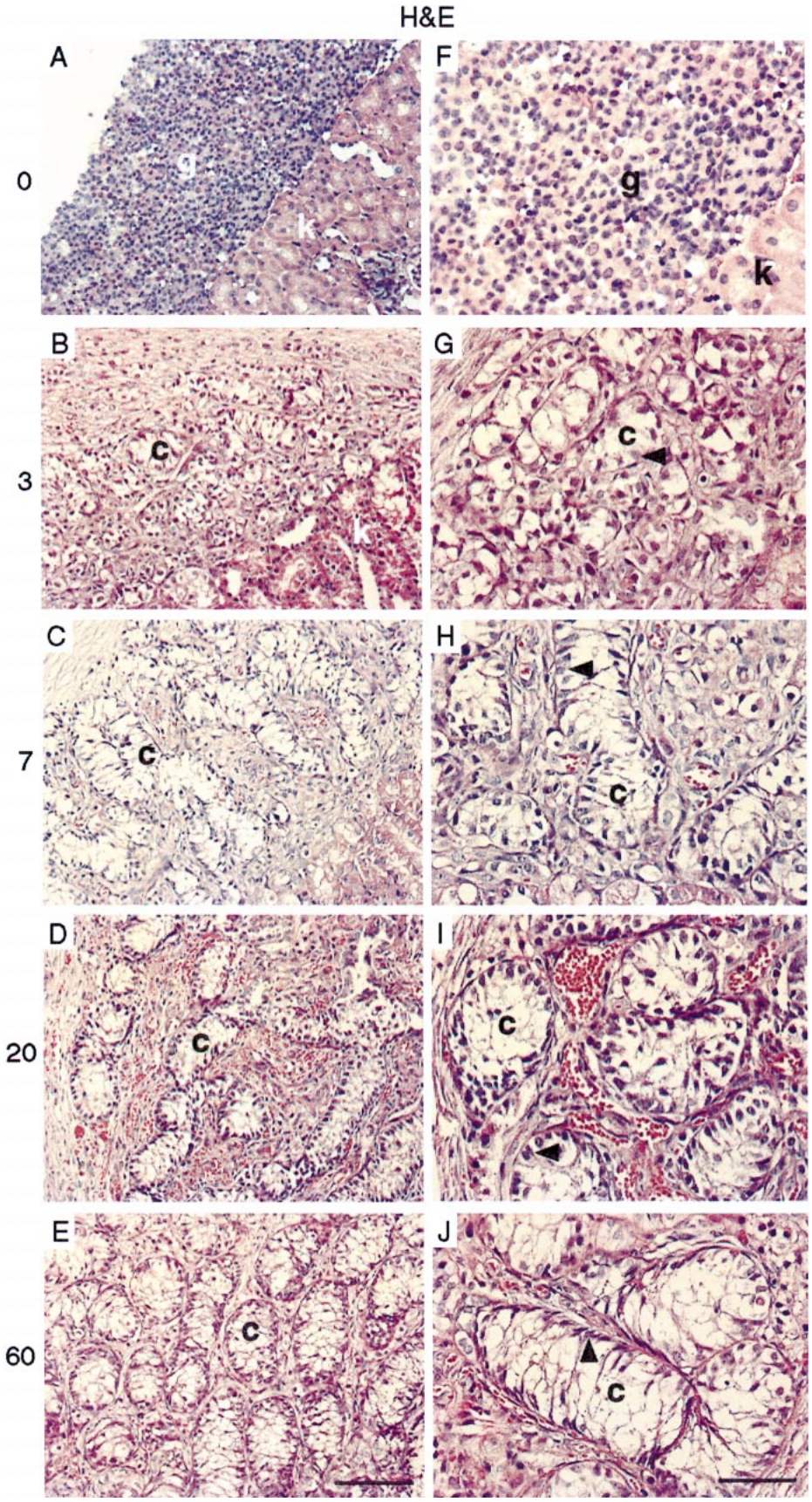

Figure 2. Formation of cords by porcine testicular cells in grafts underneath the kidney capsule of severe combined immunodeficient (SCID) mice. Grafts were removed at 0 **(A, F)**, 3 **(B, G)**, 7 **(C, H)**, 20 **(D, I)**, and 60 **(E, J)** days posttransplantation and stained with hematoxylin and eosin. Note: g indicates graft; k, kidney; c, cord; and arrowhead, Sertoli cell nucleus. Bar in **(E)** = 100 μm for **(A–E)**; bar in **J** = 50 μm for **(F–J)**.

sodium citrate buffer (pH 6.0) in a microwave at full power. Tissue sections were then immunostained as described previously (Korbutt et al, 1997; Akmal et al, 1998; Dufour and Kim, 1999). Consecutive sections were incubated with 10% hydrogen peroxide to quench endogenous peroxidases, blocked with nonspecific serum, and incubated with primary antibody for 30 minutes. After incubation with primary antibody, sections were incubated with the appropriate biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, Calif) for 20 minutes, followed by peroxidase-streptavidin, substrate-chromogen (3′3-diaminobenzidine Hcl [DAB] or aminoethyl carbazole [AEC]), and then stained with hematoxylin (Zymed Laboratories Inc, San Francisco, Calif). The primary antibodies used were as follows: mouse monoclonal anti-vimentin (1:100; Dako, Carpinteria, Calif), mouse monoclonal anti-proliferating cell nuclear antigen (anti-PCNA, 1:50; Dako), mouse monoclonal anti-smooth muscle alpha-actin (1:50; Dako), goat polyclonal anti-Müllerian inhibiting substance (anti-MIS) (1:100; Santa Cruz Biotechnology, Santa Cruz, Calif), and goat polyclonal anti-DNA transcription factor GATA-4 (1:50; Santa Cruz Biotechnology). Positive controls included sections of 1- to 3-day-old neonatal pig testes in which immunoreactivity for MIS, GATA-4, and vimentin was localized to Sertoli cells, smooth muscle alpha-actin to peritubular myoid cells, and PCNA to proliferating cells (Figure 1). Negative controls, which showed no staining, consisted of sections incubated without primary antibody.

Results

Graft Preparation

Porcine testicular cells were isolated from neonatal testes as single cell suspensions, cultured 2 days to allow the formation of cellular aggregates, and then implanted underneath the kidney capsule of SCID mice. Before transplantation, the composition of these cellular aggregates was determined by assessing the proportion of immunoreactive vimentin-positive Sertoli cells and smooth muscle alpha-actin-positive peritubular myoid cells (Tung and Fritz, 1990; Korbutt et al, 1997). In 3 independent preparations, the aggregates were shown to contain 92.5% plus or minus 3.5% Sertoli cells and 2.2% plus or minus 0.7% myoid cells. The remaining cell population (ie, $<6\%$) is likely composed of germ cells, Leydig cells, testicular mast cells, and fibroblasts.

In Vivo Cord Formation

In previous studies, we observed that testicular cells formed tubulelike structures when cotransplanted with pancreatic islets of Langerhans underneath the kidney capsule of mice (Suarez-Pinzon et al, 2000). To examine the formation of these structures, NPSCs were implanted underneath the kidney capsule of immunoincompetent SCID mice. Between 0 and 60 days after transplantation, NPSC grafts, which were easily identifiable underneath the kidney capsule, were examined histologically for the presence of cord formation and development. At the time of transplantation, the NPSCs were randomly distributed underneath the kidney capsule (Figure 2A and F); however, by 3 days posttransplantation, the NPSCs had organized into clusters forming precursors to cords (Figure 2B and G). After 7 days posttransplantation, cords similar to those found in germ cell-depleted seminiferous tubules were evident (Figure 2C and H). Moreover, it was clear that the Sertoli cells were arranged with their nuclei along the basal edge of the tubules (Figure 2H). With progression of time, the cords developed further, becoming more defined and larger (Figure 2D, E, I, and J). No evidence of a lumen in the center of the cords was detected at any of the time points analyzed. This is in agreement with the lack of a lumen in the native porcine testis until after 90 days of age (Kosco et al, 1989). A consistent progression of cord formation was observed for all grafts at each time point.

Epithelial Organization

Immunohistochemistry with multiple antibodies was performed to identify Sertoli and myoid cells. MIS is only expressed in the cytoplasm of Sertoli cells in the male (Figure 1A) (Munsterberg and Lovell-Badge, 1991). It is known to cause regression of the müllerian ducts during embryogenesis, thus preventing differentiation of the oviducts, uterus, and upper vagina of the female reproductive tract (Munsterberg and Lovell-Badge, 1991). In porcine Sertoli cells, MIS activity is low at birth, increases to maximal levels between 10 and 19 days, and then declines to low levels by 60 days of age (Tran et al, 1981). This decrease is suggested to be an indication of testicular maturation in the porcine testis. To identify Sertoli cells, the antibody against MIS was used to immunostain sections from the grafts. An examination of MIS-immunostained sections revealed the presence of cords, which had begun to develop 3 days after implantation (Figure 3B and G). MIS was clearly present in the cytoplasm of Sertoli cells for at least 60 days (Figure 3B through E, G through J). While not quantitative, the levels of MIS did, however, appear to increase after 3 days posttransplantation (Figure 3B and G) and then decrease by day 30 after implantation (Figure 3D and I), with further declining levels present at 40 (data not shown) and 60 days posttransplantation (Figure 3E and J). This pattern is similar to the MIS expression in the prepubertal porcine testis, suggesting the transplanted cells may be developing along their normal pathway.

GATA-4, a transcription factor thought to be involved in the gonadogenesis of the testis, is present in Sertoli and Leydig cell nuclei in testicular sections (Figure 1B) (McCoard et al, 2001a,b) as well as in other tissues such as the kidney and lymphocytes. Immunolocalization of GATA-4 in sections from the grafts identified Sertoli cells that were initially randomly distributed (Figure 3K) and

that then began to organize into small discernible circles (Figure 3L) and finally became aligned on the basal side of the cords that resemble the seminiferous tubules present in the Sertoli cell-only or germ cell-depleted testis (Figure 3M through O) (Chakraborty, 1993).

Immunostaining was also performed using an antibody against smooth muscle alpha-actin to identify peritubular myoid cells (Figure 4A through E). Smooth muscle alpha-actin is known to be present in smooth muscle cells of blood vessels and in testicular myoid cells (Figure 1D) (Tung and Fritz, 1990). Smooth muscle alpha-actin-positive cells were randomly dispersed throughout the grafts at the time of transplantation (Figure 4A). Within 3 days after transplantation, although there were still disorganized regions, some myoid cells had begun to circle the Sertoli cells (Figure 4B). The majority of smooth muscle alpha-actin-positive cells were localized around the cords by 10 to 40 days posttransplantation (Figure 4C through E).

Smooth muscle alpha-actin-positive cells were also detected surrounding newly formed blood vessels (Figure 4C and E). No vessels were present in the grafts at the time of transplantation; however, by day 3 posttransplantation, new vasculature was evident, with more blood vessels forming between days 3 and 10 posttransplantation (Figure 4C). These vessels were surrounded by a layer of smooth muscle alpha-actin-positive cells (Figure 4C through E). This induction of vessel formation is most likely to supply nutrients to the grafted tissue and may be induced by the Sertoli cells, which are known to produce potential angiogenic factors (Griswold, 1993; Skinner, 1993).

At higher magnification (Figure 4F through I), it was evident that the Sertoli and peritubular cells in the more mature grafts (40 days) were arranged almost exactly like the seminiferous epithelial layer in prepubertal porcine testicular cords (Tran et al, 1981). GATA-4 immunostaining clearly showed that as the Sertoli cells matured, they became polarized, with their nuclei aligning the basal edge of the tubule and their cytoplasm extending toward the center of the tubules (Figure 4G). Moreover, the peritubular cells, as identified with a smooth muscle alpha-actin antibody, were surrounding the tubules, located adjacent to the Sertoli cell nuclei (Figure 4I).

Cellular Proliferation

Gross morphological analysis at the time of graft removal indicated that there was marked growth of the transplanted tissue as the cords developed. Porcine Sertoli cells in the native testis have been shown to proliferate during postnatal testicular development until puberty, which occurs between 100 and 120 days of age in the pig (Tran et al, 1981; Franca et al, 2000). To determine whether the grafted Sertoli cells are proliferating, serial sections were immunostained for vimentin (Figure 5A through E) and PCNA (Figure 5F through J). Vimentin is an intermediate filament protein that was demonstrated to be present in the cytoplasm of Sertoli cells (Figure 1C) (Korbutt et al, 1997). PCNA is involved in DNA replication and has been shown to be localized to proliferating cells (Bravo et al, 1987; Prelich et al, 1987; Hall et al, 1990). Immunostaining of the transplanted tissue with an anti-vimentin antibody showed vimentin localized to the cytoplasm of Sertoli cells within the tubules and also in the interstitial cells (Figure 5A through E). Immunostaining with an anti-PCNA antibody showed that Sertoli cells were positive for PCNA, indicating that they were proliferating throughout 0 to 60 days posttransplantation (Figure 5F through J, data not shown). In addition, some interstitial cells were also proliferating (Figure 5H through J).

Discussion

When Sertoli and myoid cellular aggregates isolated from the testes of neonatal pigs were implanted underneath the kidney capsule of immunoincompetent SCID mice, the cellular aggregates reorganized into cords resembling seminiferous tubules from Sertoli cell–only or germ cell–depleted testes (Chakraborty, 1993). Using antibodies against Sertoli cell–specific and peritubular cell–specific proteins, we demonstrated that Sertoli and peritubular cells were initially randomly distributed. However, the Sertoli cells were aligned next to each other, while the peritubular myoid cells migrated and surrounded the polarized Sertoli cells, forming distinct cords by 7 days after transplantation.

While the process by which cords form under the kidney capsule is not identical to the formation of cords in the embryonic testis, it is strikingly similar. During cord formation, cells from the mesonephros migrate into the male gonad and interact with the aggregating Sertoli and germ cells (Magre and Jost, 1980, 1991; Jost et al, 1981; Buehr et al, 1993; Tilmann and Capel, 1999). It is presumed that these migrating cells are peritubular myoid cells. Consistent with these results, the myoid cells in the graft also migrated and surrounded the aggregating Sertoli cells. As the graft matured, the myoid cells were juxtaposed to Sertoli cell nuclei that were present on the basal region of the cords. The Sertoli cells in normal seminiferous tubules have this relationship with peritubular cells and also are polarized, with their cytoplasm extending into the apical region of the tubular structures (Russell et al, 1990). The cellular organization of the cords in the grafts is similar to the morphological arrangement of cells found in Sertoli cell-only or germ cell-depleted seminiferous tubules of the testis (Chakraborty, 1993).

Other studies have demonstrated the formation of tubulelike structures in vitro (Tung and Fritz, 1980; Hadley

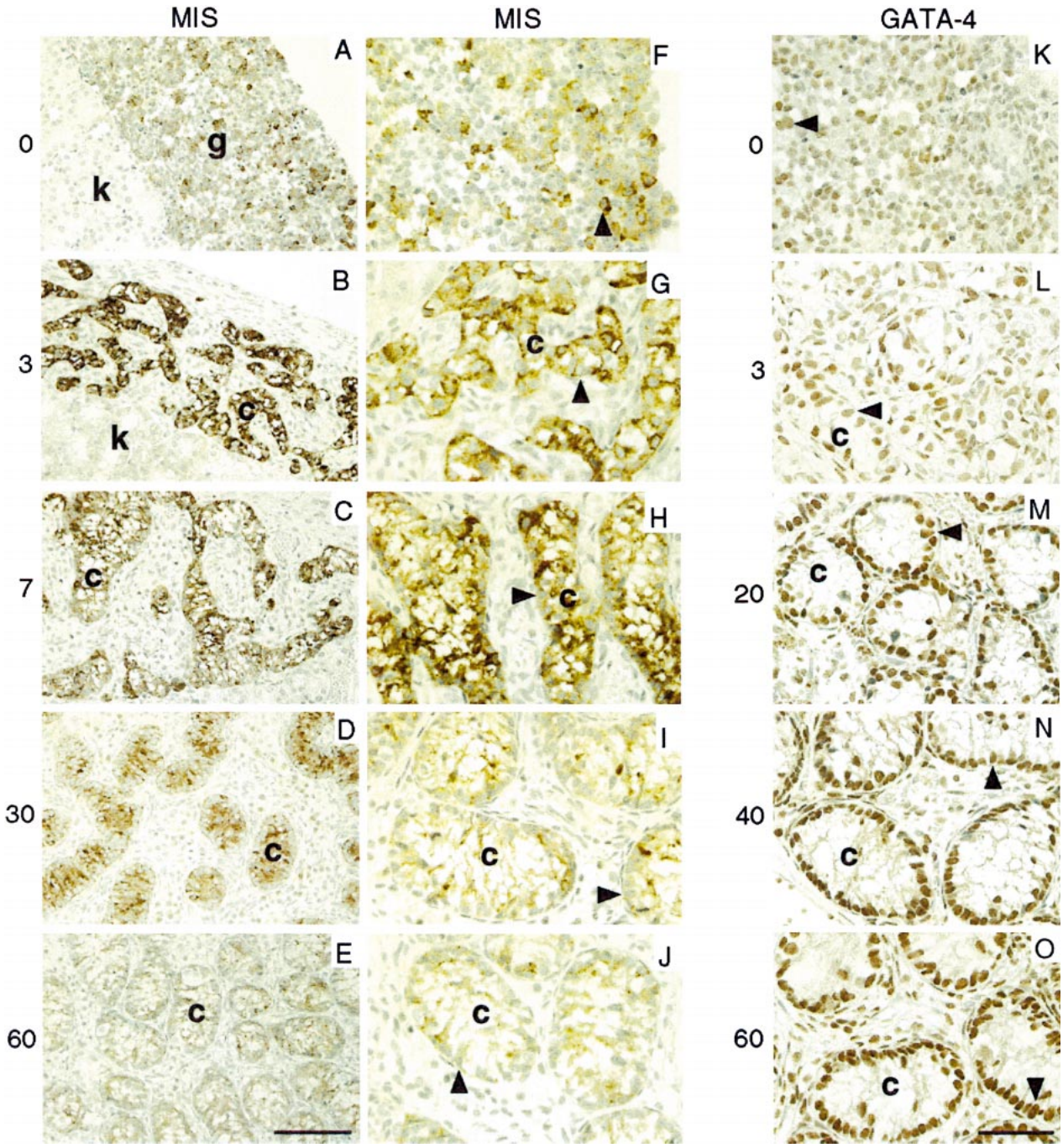

Figure 3. Immunolocalization of Sertoli cells in grafts from severe combined immunodeficient (SCID) mice. Grafts were removed at 0 **(A, F, K)**, 3 **(B, G, L)**, 7 **(C, H)**, 20 **(M)**, 30 **(D, I)**, 40 **(N)**, and 60 **(E, J, O)** days posttransplantation and then fixed and immunostained with antibodies against Müllerian inhibiting substance (MIS) **(A–J)** or DNA transcription factor GATA-4 **(K–O)** to identify Sertoli cells. 3′3-Diaminobenzidine Hcl (DAB) was used as chromogen. Note: g indicates graft; k, kidney; c, cord; and arrowhead, Sertoli cell nucleus. Bar in **(E)** = 100 μm for **(A–E)**; bar in **(O)** = 50 μm for **(F–O)**.

et al, 1985); however, to our knowledge, our transplantation model is the first description of an in vivo model. Using an in vitro culture system, Tung and Fritz (1980) demonstrated that pubertal Sertoli/peritubular cocultures are able to aggregate and form structures resembling germ cell-depleted seminiferous tubules. These in vitro studies have demonstrated that mesenchymal–epithelial (myoid–Sertoli) cell interactions are important for the deposition of the extracellular matrix components that are required for tubule formation (Tung and Fritz, 1987). This process of tubulogenesis required the production of laminin by Sertoli cells, since anti-laminin antibodies prevented the

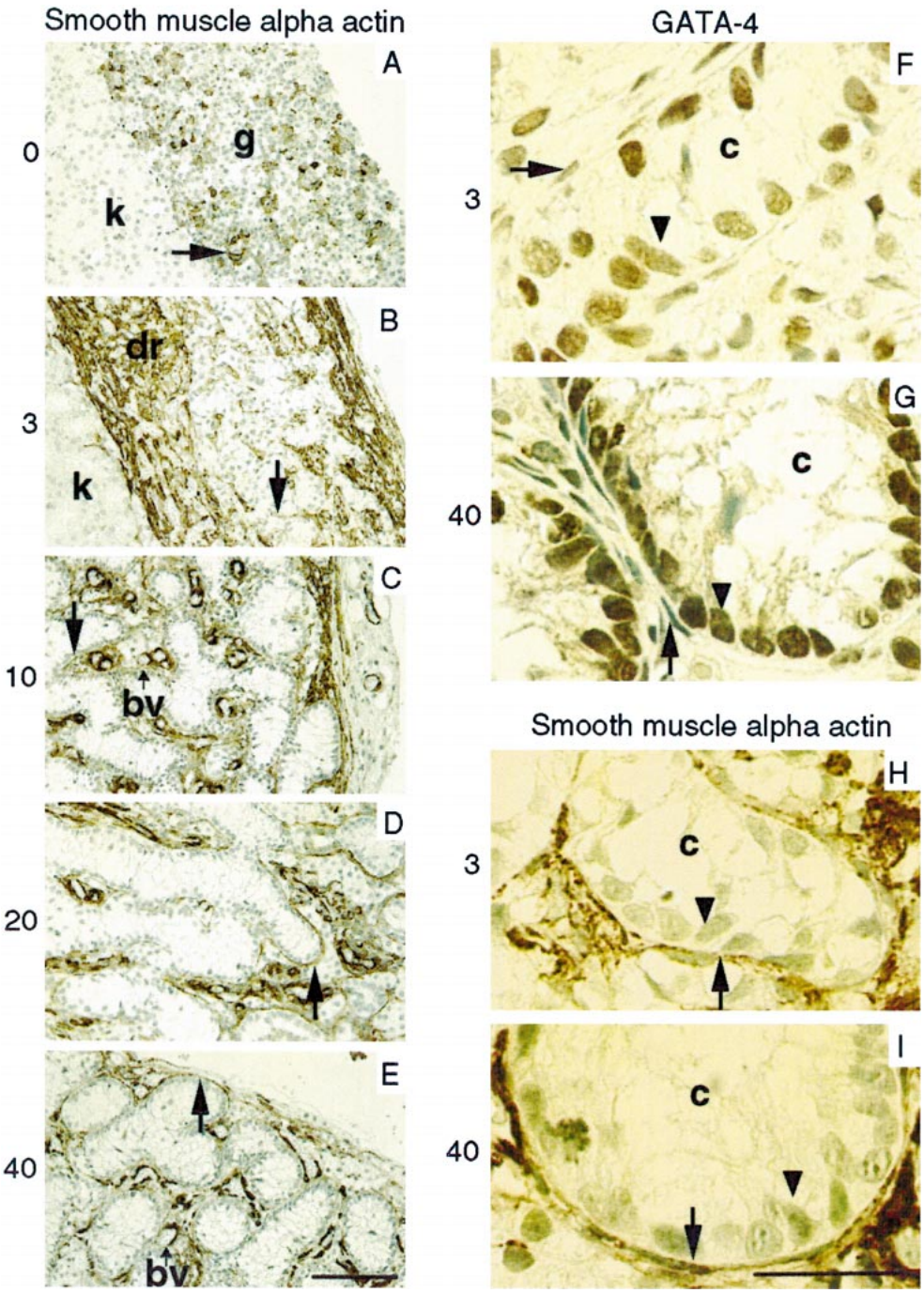

Figure 4. Immunolocalization of peritubular myoid and Sertoli cells in the cords. Grafts were removed at 0 **(A)**, 3 **(B, F, H)**, 10 **(C)**, 20 **(D)**, and 40 **(E, G, I)** days posttransplantation and then fixed and immunostained with antibodies against smooth muscle alpha-actin **(A–E, H, I)** to identify myoid cells or DNA transcription factor GATA-4 **(F, G)** to identify Sertoli cells. 3′3-Diaminobenzidine Hcl (DAB) was used as chromogen. Note: g indicates graft; k, kidney; dr, disorganized region; arrow, myoid cell; bv, blood vessel; c, center of cord; and arrowhead, Sertoli cell nucleus. Bar in **(E)** = 100 μm for **(A–E)**; bar in **(I)** = 30 μm for **(F–I)**.

formation of tubules (Hadley et al, 1990; Tung and Fritz, 1994). These factors required for tubule formation in the in vitro system have since been shown to be required for cord formation during embryonic testis development (Buehr et al, 1993; Marinos et al, 1995; Tilmann and Capel, 1999). Our in vivo model provides an excellent alternative model to study the cellular factors, secretory products, and mechanisms responsible for interaction of cells during cord formation in the testis. In contrast to in vitro models, the present in vivo model allows for a more

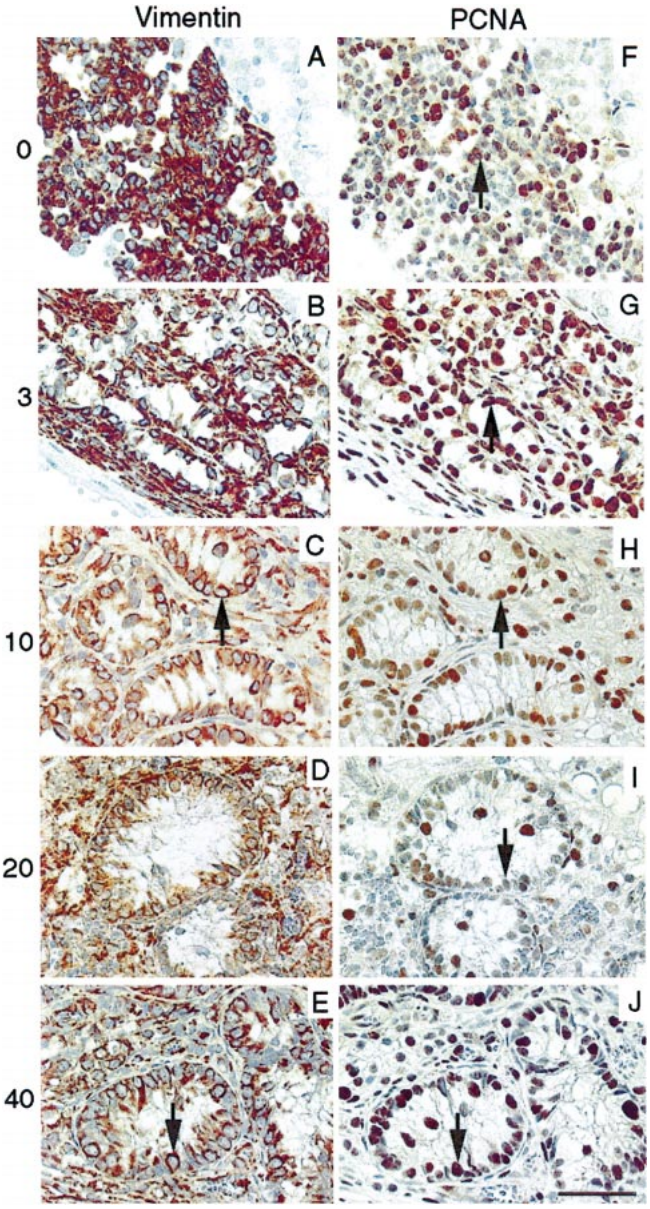

Figure 5. Cellular proliferation of Sertoli cells in porcine testicular grafts from severe combined immunodeficient (SCID) mice. Grafts were removed at 0 **(A, F)**, 3 **(B, G)**, 10 **(C, H)**, 20 **(D, I)**, and 40 **(E, J)** days posttransplantation and then fixed and immunostained with antibodies against vimentin **(A–E)** or proliferating cell nuclear antigen (PCNA) **(F–J)**. Aminoethyl carbazole (AEC) was used as chromogen. Arrow indicates proliferating Sertoli cell. Photomicrographs **(A–E)** are from serial sections to **(F–J)**, respectively. Bar in **(J)** = 50 μm for **(A–J)**.

physiological environment, thereby permitting the testicular cells to interact with other endogenous systems.

Similar to the in vitro system (Tung and Fritz, 1980), the Sertoli and myoid cells within the graft appear to direct the formation of cords, indicating that the developmental signals required for cord formation are intrinsic characteristics of the Sertoli and myoid cells. In addition, we have observed similar structures in other transplant sites such as the subcutaneous space, the epididymal fat pad, and the omental pouch (data not shown). These results suggest that it is not the renal environment but rather the cells within the graft that direct the formation of cord structures.

Johnson et al (1996a,b) have developed a transplantation model in which they transplant the entire testis into the pinna of the ear. In this model, they demonstrate that the Sertoli cells are able to develop normally, although this development is slightly delayed (Johnson et al, 1996b). They even report instances of complete spermatogenesis in some of the tubules; however, most of the tubules were devoid of germ cells (Johnson et al, 1996b). They further demonstrate the effects of hypophysectomy, sex of the host, and number of transplanted testes on Sertoli cell proliferation and circulating hormone levels (Johnson et al, 1996a). These studies indicate that transplantation of the testis is a valid model to study testicular development and validate our in vivo model for the study of testicular morphogenesis. Furthermore, our model will be useful because it allows the isolated cells to be manipulated in vitro prior to transplantation to examine the outcome during testis development.

GATA-4, a member of the GATA transcription factor family, has been shown to be important in cell differentiation and organ development (Simon, 1995; Kuo et al, 1997; Molkentin et al, 1997) as well as in the up-regulation of MIS in the testis (Viger et al, 1998; Tremblay and Viger, 1999; Wantanabe et al, 2000). In our model, GATA-4 was detected in the transplanted Sertoli cells at all time points analyzed, which is consistent with its role in testicular development. In the normal testis, it is expressed in Sertoli cells prior to cord formation, throughout puberty, and into adulthood, suggesting it may be involved in testis development (McCoard et al, 2001a,b).

The presence of MIS in the grafts also may be important since MIS is thought not only to regulate regression of the Müllerian ducts but also to be a marker of Sertoli cell function during testis differentiation (Vigier et al, 1987; Behringer et al, 1990). MIS is expressed in the gonads in a sexually dimorphic manner (Munsterberg and Lovell-Badge, 1991); it is present in Sertoli cells after cord formation, continues to be expressed throughout embryonic development, and then declines to low levels after birth (Tran et al, 1977). In pigs, MIS is again upregulated to high levels between postnatal days 10 and 19 and steadily declines to low levels after 60 days (Tran et al, 1981). MIS expression in the transplanted Sertoli cells was similar to that in the native porcine testis, suggesting the Sertoli cells in this in vivo model are developing as in the prepubertal testis.

Sertoli cell proliferation in the grafts also paralleled that in the native testis (Orth, 1982; Franca et al, 2000). During testicular development, Sertoli cells proliferate maximally in the embryo, continue to proliferate at birth, and cease to

divide after puberty (Orth, 1982; Franca et al, 2000), which is around 100–120 days of age in the pig (Tran et al, 1981). This growth is essential for male fertility since the number of Sertoli cells defines the sperm capacity of the adult testis. Consistent with these results, transplanted neonatal Sertoli cells in the grafts were found to proliferate normally at all time points after transplantation up to 60 days. Many factors can regulate testis growth, including follicle-stimulating hormone, epidermal growth factor, calf serum, nerve growth factor, neurotropin-3, and transforming growth factor-α (Cupp et al, 1999a,b, 2000; Levine et al, 2000). It remains to be seen if the factors that regulate growth in the native testis are the same factors that regulate growth in our in vivo model system. In order to study testicular growth with this model, Sertoli cells engineered to produce specific inhibitors to growth factors believed to be required for Sertoli cell proliferation can be transplanted and the grafts examined for the effect. This model would allow for similar studies in cord formation and other developmental processes.

In summary, it is remarkable that transplanted porcine Sertoli and myoid cells were able to reorganize into cords similar to the cellular organization in the seminiferous tubules from Sertoli cell-only or germ cell-depleted testes after implantation underneath the kidney capsule of SCID mice. Equally interesting is the expression of GATA-4 and MIS as well as the proliferation of the Sertoli cells, which mimicked the expression patterns and growth of Sertoli cells in the native porcine testis. These results suggest the transplanted testicular cells were able to develop normally in this ectopic site, independent of the local environment and even across species barriers (ie, pig to mouse). Therefore, this in vivo model of cord formation and prepubertal development represents a novel system to study morphogenesis and growth during prepubertal testicular development. In addition, prepubertal testicular development in the pig is delayed when compared to the rodent models normally studied, allowing a more detailed analysis of the changes that occur during prepubertal development.

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