

# De Novo Morphogenesis of Seminiferous Tubules From Dissociated Immature Rat Testicular Cells in Xenografts

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**ABSTRACT:** Testicular development is initiated with the differentiation of Sertoli cells in the embryonic gonad. The aggregation of Sertoli cells is crucial for the generation of testicular cords and thus for the first sign of male gonadal development. To date, functional testicular tissue has not yet been generated in vitro. The objective of this study was to explore the de novo morphogenesis of testicular tissue from isolated postnatal rat testicular cells using a combination of in vitro culture and ectopic xenografting. Immature rat testicular cells were cultured in either a 2-dimensional (laminin-coated cover-glass) or a 3-dimensional (extracellular matrix gel) culture system. Whereas testicular cells cultured on laminin showed a slow morphogenetic cascade resulting in cord formation after about 10 days of culture, cells cultured on extracellular matrix gel assembled to a network of cordlike structures within several hours after plating and formed spherical cell aggregates at day 3. Further progression of the morphogenetic cascade was not obtained in either

the 2- or the 3-dimensional culture system. In contrast, structures resembling immature testicular tissue were obtained after xenografting of extracellular matrix gel-enclosed spherical testicular cell aggregates. The grafts were vascularized and contained elongated seminiferous tubules. Histologic analysis revealed the presence of a basement membrane, a histologically normal interstitium containing putative Leydig cells, the establishment of tubule lumen, and the integration of few putative spermatogonia into the seminiferous epithelium. We conclude that immature rat testicular cells carry the full potential to generate all somatic components of a testis in xenografts, thus opening fascinating pathways to study testicular organogenesis.

Key words: Xenografting, testis, Sertoli cells, spermatogonia, organogenesis.

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Testis differentiation in the rat becomes evident at embryonic day 15.5, when seminiferous cords become visible within the gonadal ridge as a result of Sertoli cell aggregation (Clermont and Huckins, 1961). Testicular morphogenesis is dependent on and initiated by expression of the SRY gene, whose downstream functions include changes in gonadal cell proliferation and mesonephric-gonadal cell migration, Sertoli cell differentiation, and testis cord formation (for review, see Ross and Capel, 2005). Because of their pivotal role for testicular function (Griswold, 1998), Sertoli cells have been studied extensively in vitro and in vivo, and various strategies to achieve in vitro generation of differentiated germ cells have been followed (Sofikitis et al, 2005).

The components of the basal lamina (eg, laminin and collagen) and reconstituted extracellular matrix have been widely used in culture and were found to promote Sertoli cell differentiation and germ cell differentiation, in some cases through the pachytene stage of meiosis (Hadley et al, 1985, 1990). Here, a new model combining in vitro culture in extracellular matrix gel and subsequent grafting of the generated spherical cell aggregates is introduced. Ectopic grafting has become a valid model to induce spermatogenesis in immature testicular tissue (Honaramooz et al, 2002). Subcutaneous transplantation of small pieces of testis tissue in immunodeficient hosts initiates complete spermatogenesis in fresh and cryopreserved neonatal and prepubertal testicular tissue from pig, goat, monkey, hamster, and mouse (Honaramooz et al, 2002, 2004; Schlatt et al, 2002, 2003).

We hypothesized that xenografted testicular cords, which were reconstituted in vitro from single-cell suspensions of immature rat testicular cells, would grow and differentiate into fully functional seminiferous tubules and would evoke a de novo morphogenesis of extratubular testicular components, including blood vessels and Leydig cells. This experimental strategy offers a novel method for the study of testis development.

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## Material and Methods

### Cell Preparation and Culture

For testicular cell isolation, 7-day-old CD1 rats were obtained from The Jackson Laboratory (Bar Harbor, Me). Animal husbandry and all procedures were in compliance with the University of Pittsburgh Guidelines for the Care and Use of Laboratory Animals.

Single-cell suspensions of testicular cells were prepared by sequential enzymatic digestion (Schlatt et al, 1996). In brief, testes were decapsulated and the seminiferous tubules were first digested with 1 mg/mL collagenase I (C-2674; Sigma Chemical Co, St Louis, Mo) and 5 µg/mL DNase (No. 104132, 15 U/mL; Roche Applied Science, Indianapolis, Ind) in Dulbecco minimum essential medium (DMEM; 4.5 g glucose/mL). Isolation of seminiferous tubular fragments from interstitial cells was achieved by repeated sedimentation at unit gravity. In a second digestion step, tubule fragments were incubated with 1 mg/mL collagenase I and 5 µg/mL DNase in combination with 1 mg/mL hyaluronidase (H-3506; Sigma) until a single-cell suspension was achieved, which was washed and resuspended in low-glucose DMEM (1 g glucose/L) supplemented with nonessential amino acids and antibiotics. Total cell numbers were assessed using a bright-line hemacytometer (No. 3100; Hausser Scientific, Horsham, Pa).

Approximately  $1 \times 10^6$  cells per well (24-well plates) were plated on laminin-coated glass coverslips ( $3.3 \mu\text{g}/\text{cm}^2$ ) and incubated at 35°C in an atmosphere containing 5% CO<sub>2</sub> and 100% humidity. For extracellular matrix cultures,  $1 \times 10^6$  rat cells were plated on 250 µL reconstituted extracellular matrix gel (Matrigel™, No. 354234, diluted 1:1 with DMEM; BD Biosciences, Bedford, Mass). Extracellular matrix gel is a complex mixture of extracellular basement membrane components and also contains growth-promoting soluble factors (Kleinman et al, 1982). At 3, 10, and 15 days of culture, the cell culture medium was exchanged against medium containing 100 µM bromodeoxyuridine (BrdU; No. B5002, proliferation label; Sigma) for the last 2 hours of culture. The cultures were then fixed in Bouin fixative for 15 minutes and later transferred to 70% ethanol.

### Live Imaging of Cell Cultures in Extracellular Matrix Gel and Quantitative Analysis

For live imaging of cultures in extracellular matrix gel, pictures were captured with an Olympus IX81 microscope (Olympus, Melville, NY) equipped with a Retiga EXI FAST camera (Olmaging, Burnaby, British Columbia, Canada).

The spatial arrangement of spherical cell aggregates was analyzed at day 3 of culture using bright field micrographs obtained on an Olympus SZX12 dissecting microscope. Because this arrangement appeared to be highly nonrandom, the number of neighboring aggregates was determined as well as the distance to each of these neighbors. Five micrographs, each covering a culture area of  $9.86 \text{ mm}^2$ , were taken from each of 12 culture wells. A total number of 300 colonies were evaluated (5 colonies per micrograph, 25 colonies per well).

Some spherical cell aggregates were fixed in 2.5% glutaraldehyde and 2% formalin in 0.1 M sodium cacodylate buffer for histologic evaluation.

### Grafting Procedure and Graft Recovery

Adult male nude mice (strain: nu/nu), obtained from Charles River Laboratories (Wilmington, Mass), served as hosts for the xenografting. Some hosts were castrated, and others were left intact. Castration was performed under anesthesia at the time of grafting. Castration of hosts was performed to assess the influence of elevated follicle-stimulating hormone and luteinizing hormone serum levels on graft differentiation, compared to testosterone influence in intact hosts. For grafting, extracellular matrix gel that contained spherical cell aggregates was injected subcutaneously in the back of the host using an 18-gauge injection needle. Four to 6 injections (250 µL each) were applied per animal. Aggregates containing rat testicular cells only were grafted in 15 hosts; 13 of 15 hosts survived. Seven of the surviving hosts were castrates and 6 were intact. As controls, 2 additional hosts (1 castrate and 1 intact) were grafted with extracellular matrix gel without cell aggregates (100% survival rate). Grafts were allowed to develop for up to 8 weeks. Host mice received an intraperitoneal injection of BrdU at 100 mg/kg and were killed 2 hours later by exsanguinations under deep anesthesia. Grafts were removed from the inner surface of the back skin. Each host's body weight, seminal vesicle weight, graft weight, and testes (if present) weight were recorded. Grafts were fixed in Bouin fixative overnight and then transferred to 70% ethanol.

### Histology and Immunohistochemistry

Cultures on coverslips were stained for  $\alpha$ -smooth muscle actin (a marker of peritubular cells, Tung and Fritz, 1990), using an anti- $\alpha$ -smooth muscle actin antibody (A2547, dilution 1:3000; Sigma) as primary antibody. Cultures were also stained using a monoclonal mouse antibody against BrdU (diluted 1:50 in Tris-buffered saline containing 0.1% bovine serum albumin; Biomed, Foster City, Calif). Primary antibody detection employed horseradish peroxidase-conjugated secondary antibody (A9044, dilution 1:400; Sigma) and diaminobenzidine (DAB; SigmaFast™, D4168; Sigma). Morphologic differences between the 3 time points (3, 10, and 15 days) were noted. Grafts and spherical cell aggregates were embedded either in resin (Technovit 7100; Heraeus Kulzer, Hanau, Germany) or in paraffin. Technovit-embedded samples were sectioned to 2 or 4 µm and stained with the periodic acid-Schiff reagent method followed by hematoxylin counterstaining. Paraffin-embedded samples were sectioned to 7–10 µm and an antibody against cytochrome p450 side chain cleavage enzyme was used to detect putative Leydig cells (No. AB1244, dilution 1:500; Chemicon International, Temecula, Calif). A horseradish peroxidase-coupled secondary antibody and SigmaFast DAB (see above) were employed for visualization.

### Image Acquisition and Statistical Analysis

Samples were analyzed using a Nikon Eclipse E800 fluorescence microscope (Nikon, Melville, NY) with attached digital

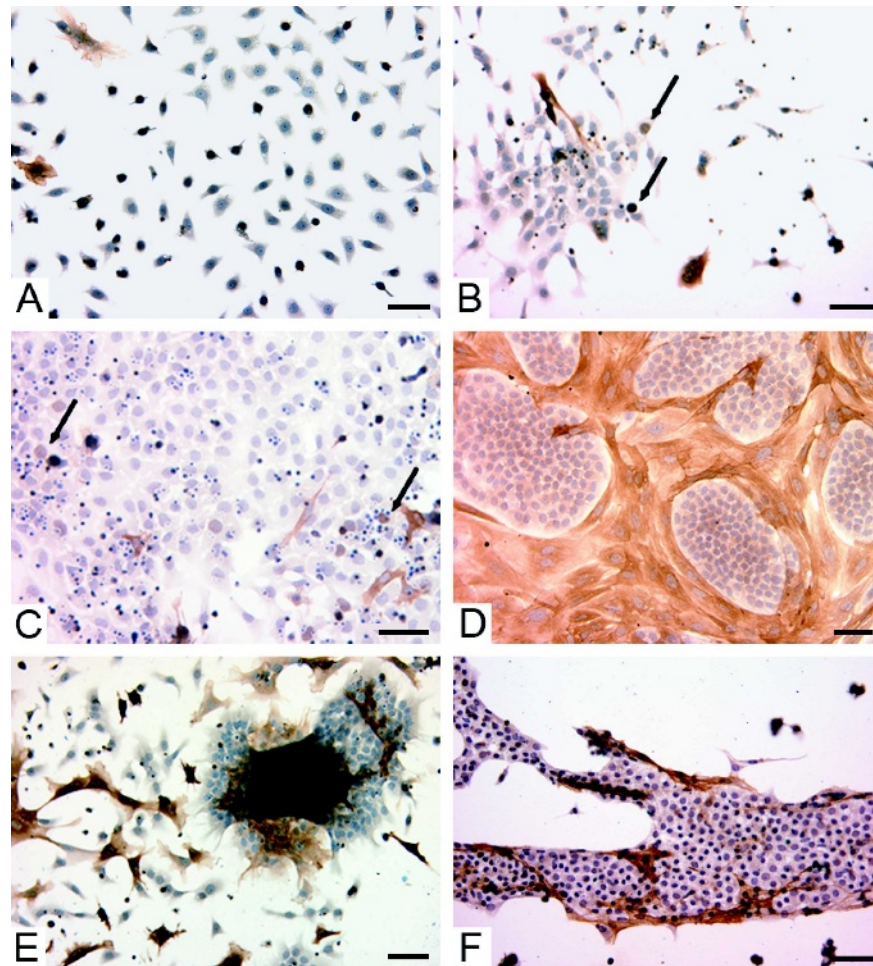


Figure 1. Representative micrographs of testicular single-cell cultures on laminin-coated coverslips. To determine mitotic activity, cultures were incubated with bromodeoxyuridine (BrdU) 2 hours prior to fixation. Immunohistochemistry was performed to detect BrdU (proliferation label, arrows) and  $\alpha$ -smooth muscle actin (marker for myoid peritubular cells) using diaminobenzidine as chromogen (brown) and counterstaining with hematoxylin (blue). (A) Single, spindle-shaped Sertoli cells, 3 days after plating. (B) Single-layered plaque of Sertoli cells, 3 days after plating. (C) Complete Sertoli cell monolayer, 3 days after plating. (D) Islands of Sertoli cells enclosed by strands and whirls of peritubular cells, 10 days after plating. (E) Multilayered mound of Sertoli cells, 15 days after plating. (F) Multilayered cordlike structure of Sertoli cells, accompanied by peritubular cells, 15 days after plating. Scale bars = 50  $\mu$ m.

camera (Olympus, Melville, NY). All images were acquired digitally using MagnaFire Software (Optronics, Goleta, Calif). Statistical analysis was performed using StigmaStat 3.1 (Systat Software Inc, Point Richmond, Calif).

## Results

### *The Morphologic Cascade in 2-Dimensional Cultures*

The single-cell suspensions cultured on laminin-coated coverslips showed a morphologic cascade as described earlier for Sertoli cells with some contamination of peritubular cells (Tung and Fritz, 1980; Schlatt et al, 1996). They first formed an incomplete monolayer (Figure 1A and B), then a complete monolayer (Figure 1C). Later, islands of aggregating Sertoli cells

enclosed by strands of peritubular cells (day 10; Figure 1D) and finally cordlike structures and mounds composed of densely packed cells were observed (Figure 1E and F).

### *Enhanced Morphogenesis in 3-Dimensional Extracellular Matrix Gel*

Primary testicular cells seeded on extracellular matrix gel aggregated 3–24 hours after plating (Figure 2 and video sequence in supplemental information). Initially, elongated cordlike aggregates of testicular cells were formed 4 hours after plating (Figure 2A). Thereafter, the cells migrated into irregular-shaped cell aggregates with radial cytoplasm processes (Figure 2B). After 3 days of culture, sphere-shaped aggregates were ob-

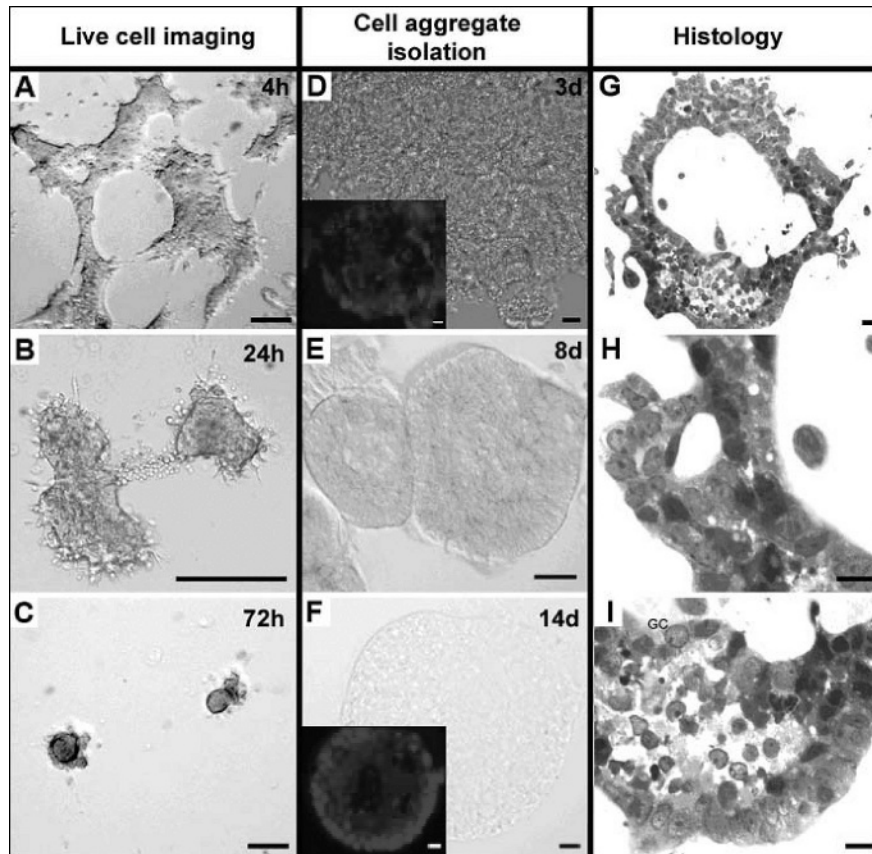


Figure 2. Testicular single-cell cultures in extracellular matrix gel. (A–C) Cordlike structures after 4 hours (A) condense to form increasingly dense cell aggregates with time (B, C). Scale bars = 100  $\mu$ m. (D–F) Cell aggregates at day 3 disintegrate during isolation. Inset: 4',6'-diamidino-2-phenylindole (DAPI). Scale bars = 50  $\mu$ m. Cell aggregates isolated at day 8 (E) and day 14 (F) are more robust. Scale bar = 50  $\mu$ m. Inset: DAPI. Scale bars = 10  $\mu$ m. (G–I) Histology of cross-sections from Sertoli cell aggregates at day 3 of culture. Sertoli cells show epithelial organization with basement membrane character (G, H). Few cells resembling germ cell (I) phenotype were observed within the cell aggregates. GC indicates germ cell. Scale bars = 10  $\mu$ m. The live cell imaging video sequence supplied as supporting material provides a documentation of the aggregation process starting at 4 hours and ending at 24 hours after plating the testicular cells onto matrix gel.

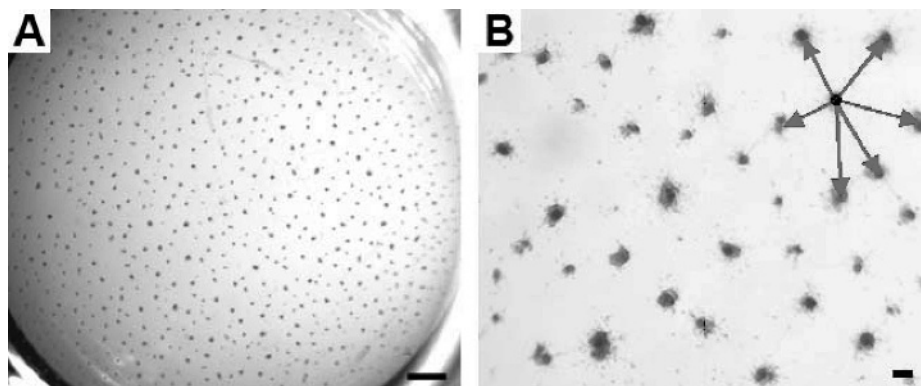


Figure 3. Documentation of the regular pattern resulting from testicular cell aggregation. Spherical cell aggregates formed a highly regular hexagonal array in matrigel. (A) Low magnification overview of the culture well 3 days after plating. Scale bar = 1 mm. (B) High magnification of the same culture. Note the highly organized patterning of the cell aggregates. Arrows indicate determinations of distances to neighboring aggregates. Scale bar = 100  $\mu$ m.

*Hosts and grafts\**

Days	Host	n (Hosts)	BW, g	SVW, mg	n (Grafts)	GW, mg
14	Castrate	3	22.33 ( $\pm$ 3.51)	39.00 ( $\pm$ 27.00)	18	15 ( $\pm$ 6.68)
	Intact	2	28.50 ( $\pm$ 0.71)	253.50 ( $\pm$ 7.78)	9	11.09 ( $\pm$ 5.84) <sup>†</sup>
	Total	5	N/A	N/A	27	13.52 ( $\pm$ 6.65)
	Castrate control (ECM only)	1	29.00	13.00	6	7.00 ( $\pm$ 2.00)
	Intact control (ECM only)	1	30	522	4	10.50 ( $\pm$ 4.65)
28	Castrate	4	27.25 ( $\pm$ 2.63)	25.00 ( $\pm$ 13.09)	25	15.20 ( $\pm$ 8.32)
	Intact	2	33.00 ( $\pm$ 1.42)	409.00 ( $\pm$ 117.38)	13	14.36 ( $\pm$ 6.96)
	Total	6	N/A	N/A	38	14.94 ( $\pm$ 7.92)
47	Intact	1	29	173	3	20.00 ( $\pm$ 6.24)
56	Intact	1	32	215	6	32.00 ( $\pm$ 17.10) <sup>†</sup>

\* Grafts were recovered after 14, 28, 47, and 56 days from castrated and intact hosts that either had been grafted with  $6 \times 250 \mu\text{L}$  ECM containing spherical cell aggregates derived from immature rat testicular single-cell suspensions or had received ECM only as controls. Values are shown as mean  $\pm$  SD. BW indicates body weight; SVW, seminal vesicle weight; GW, graft weight; and ECM, extracellular matrix gel.

<sup>†</sup> Statistically significant difference among groups carrying intact grafts ( $P < .05$ ; Mann-Whitney rank sum test).

served (Figure 2C). A highly regular hexagonal arrangement of the aggregates was established (Figure 3). Each aggregate was surrounded by  $5.66 \pm 0.07$  neighboring aggregates in an average distance of  $452 \pm 5.4 \mu\text{m}$ .

Histologic analysis of spherical cell aggregates revealed that the cells were organized in multilayered epithelia containing different cell types; some aggregates also showed a lumen (Figure 2G and H). Morphogenetic differentiation of these aggregates arrested after day 3 of culture. Although no structural change was noted at later time points, an increasing stability of these aggregates with regard to mechanical or enzymatic disturbance was observed with time (compare Figure 2D with Figure 2E and F). Occasionally, putative germ cells (spermatogonia) with ovoid nucleus and high nuclear/cytoplasm ratio were observed in the aggregates (Figure 2I).

*Advanced Testicular Differentiation After Grafting*

Xenografted fragments were recovered from intact and castrated hosts after 2 and 4 weeks. Details about hosts and grafts are presented in the Table. Compared to a nongrafted control mouse, the hosts had larger seminal vesicles, although those were smaller than in intact hosts. On macroscopic observation during dissection, the grafts were located along dorsal subcutaneous blood vessels (Figure 4A). Vascularization of grafts was well established at all analyzed time points (Figure 4B and C). The grafts could easily be dissected from the back skin of the mice and almost always contained clearly visible elongated tubulelike structures (Figure 4B and C).

Most grafts were recovered after 2 and 4 weeks from a total of 11 hosts (Table). No notable differences between grafts retrieved from castrates or intact animals were observed. Additionally, 1 single intact host was

sacrificed after 47 days. Its 3 grafts had an average weight of  $20.00 \pm 6.24$  mg. Another single intact host was killed after 56 days. Six grafts with an average weight of  $32.00 \pm 17.10$  mg were recovered (Table). After injection of extracellular matrix gel containing no cells, 6 grafts (mean weight:  $7.00 \pm 2.00$  mg) were recovered from the castrated control host, and 4 grafts (mean weight:  $10.50 \pm 4.65$  mg) were recovered from the intact control host (Table). None of these control grafts contained any tubular structures, but they were encapsulated in a mesenchymal cell layer.

Histology of the testicular grafts showed a progressive morphologic differentiation with time after grafting (Figure 5). The grafts contained seminiferous tubules resembling an immature developmental pattern. A morphologically normal interstitial compartment with blood vessels and different interstitial cell types was observed (Figure 5E). Putative Leydig cells recognized as P450scc-positive cells were observed in the interstitial space (Figure 6), confirming that androgen-producing cells are present in the grafts at the later time points.

Few putative germ cells were located at the basement membrane (Figure 5F). The nuclei of these cells were oval and their morphology resembled spermatogonia. Further differentiation of germ cells was not observed. Sertoli cells were intensely proliferating in grafts at the 6-week time point (Figure 5C) indicating that seminiferous tubules are still growing even 6 weeks after xenografting.

**Discussion**

The single-cell suspension of immature testicular cells, which we obtained as a result of our 2-step digestion protocol, contained mainly Sertoli cells. Comparable

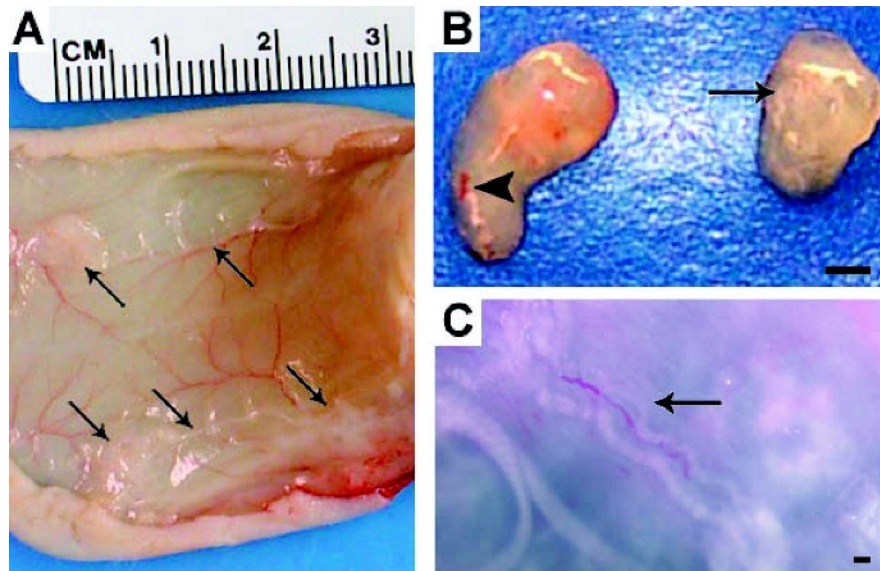


Figure 4. **(A)** Inner surface of the back skin of a recipient. Grafts are situated along the subcutaneous blood vessels (arrows). **(B)** Grafts recovered after 4 weeks showing elongated cordlike structures (arrow) and blood vessels (arrowhead). Scale bar = 1 mm. **(C)** Blood capillary accompanying a tubule within a graft recovered after 8 weeks. Scale bar = 100  $\mu$ m.

cell preparation and culture procedures are routinely used for the study of Sertoli cell physiology and initially contain small fractions of peritubular cells, germ cells, and cells originating in the interstitium. Because of the high rate of proliferation of peritubular cells, those may become more prominent after extended culture periods, especially in cultures employing media rich in fetal calf serum. Our current and previous (Schlatt et al, 1996) data, however, indicate that using serum-free conditions the cultured cells are highly enriched for Sertoli cells.

Extracellular matrix gel was used as a culture matrix for immature testicular cells because of its ability to support cord formation, as shown by others (Hadley et al, 1985). Cell differentiation and the formation of tight junctions, in addition to the secretion of extracellular matrix components, most likely contributed to the increased aggregate stability, because we observed higher resistance to mechanical handling of the aggregates after prolonged periods of culture. These results correspond with earlier reports of cell aggregates generated from immature testicular cells that revealed progressive establishment of Sertoli-Sertoli junctional complexes (Erickson et al, 1980; Hadley et al, 1985). Our spherical cell aggregates could be maintained in culture for up to 5 weeks, when they finally disintegrated. The restoration of spherical cell aggregates suggests that immature rat testicular cells recapitulate their initial morphogenetic program during testis formation in the indifferent gonad (Ross and Capel, 2005). These experiments revealed that cells aggregate spontaneously and quickly when they are in contact with extracellular

matrix gel. The aggregates form a highly regular pattern in the extracellular matrix gel, each aggregate having the same number of neighboring aggregates at very constant distances. This very regular pattern indicates the presence of morphogenetic gradients. The agents forming these gradients may *in vivo* potentially be involved in the control of testis cord formation.

However, the cellular composition derived from the single-cell suspension appeared not to be able to establish a coordinated basal lamina at the time of aggregate formation, which may be one reason for the limited developmental potential of tubulelike structures in matrix gel. In the spherical cell aggregates derived in our study, we observed partial differentiation into epithelial cells, although a typical basement membrane and a well-established cord with an adluminal and a basal compartment were rarely established. We speculate that formation of structures resembling seminiferous tubules is arrested at this stage of development because the aggregates do not contain appropriate proportions of different cell types or are missing an environmental input. It is known that mesonephric cell migration plays an important role during cord formation in the embryonic gonad. Thus, it seems likely that this pivotal environmental input cannot be provided by cells and signals contained in the culture system alone, and therefore the establishment of a typical cordlike appearance is not supported here. Alternatively, the aggregates might need support from blood vessels, which also cannot be generated in an *in vitro* environment. We have therefore taken the next

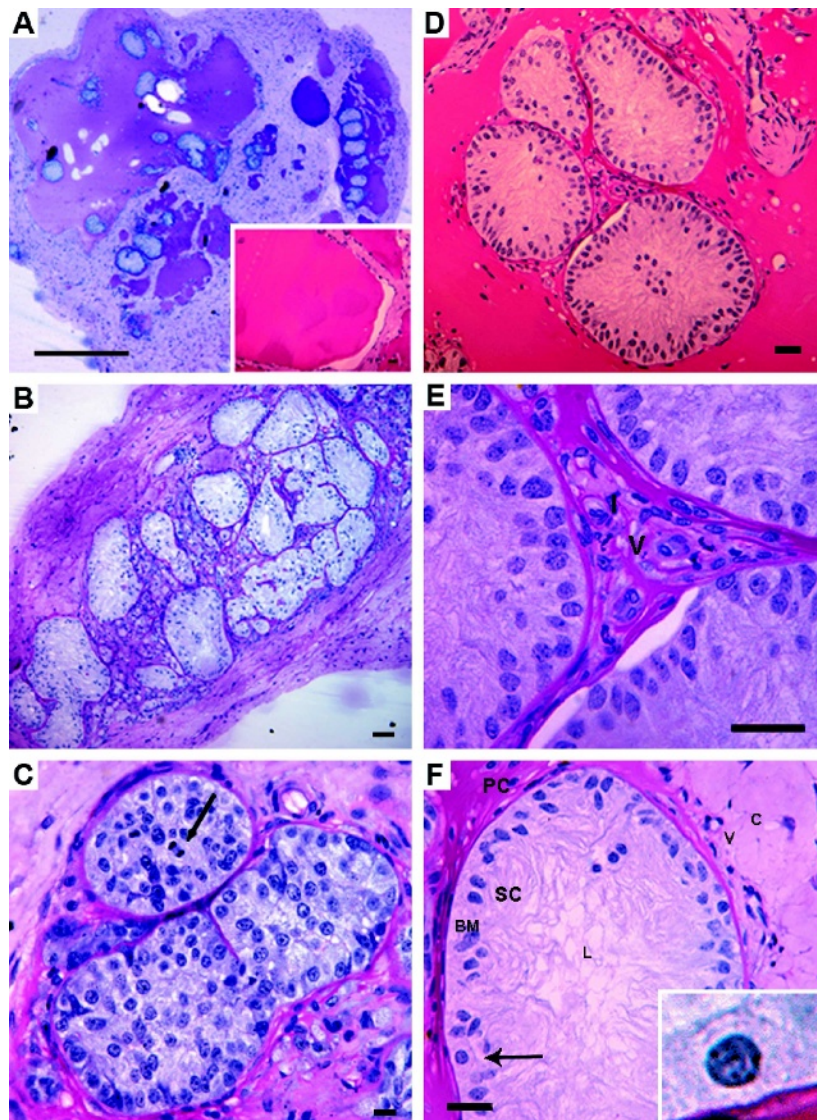


Figure 5. Graft histology. **(A)** Cross-section through the grafted tissue 2 weeks after grafting. Scale bar = 500  $\mu$ m. Inset: extracellular matrix gel grafted without Sertoli cell aggregates (2 weeks). Scale bar = 50  $\mu$ m. **(B, C)** Cross-section through the grafted tissue 6 weeks after grafting. Scale bar = 50  $\mu$ m in B, 10  $\mu$ m in C. Occasionally, proliferating Sertoli cells are seen (**C**, arrow). **(D)** Cluster of seminiferous tubules in grafts 2 weeks after grafting. Scale bar = 50  $\mu$ m. **(E)** Higher magnification of **(D)**. A normal interstitial space is observed. Peritubular cells are aligned along the tubule. SC indicates Sertoli cell; PC, peritubular cell; I, interstitial cells; and V, blood vessel. Scale bar = 50  $\mu$ m. **(F)** Differentiated tubule containing germ cells (arrow, inset) and a tubule lumen (L) in a graft 4 weeks after grafting. C indicates mesenchymal infiltration of outer layer of matrigel. Scale bar = 50  $\mu$ m.

step and xenografted the extracellular matrix gel-embedded fragments ectopically into nude mice to determine a potential further development of the spherical cell aggregates. Earlier studies have shown long-term development of xenografted Sertoli cells (Dufour et al, 2003).

The findings from our grafting experiments showed that the spherical aggregates employed for grafting had grown into large tissues. Moreover, the presence of blood vessels within recovered grafts indicated the potential of testicular cells to induce angiogenesis in the same manner as observed in vivo (Takayama and

Tomoyoshi, 1981) and indicated that blood vessel formation is a crucial step for further development of the grafted cells.

All grafts (except cell-free controls) contained significant lengths of seminiferous tubules, and many hallmarks of testicular differentiation were established: seminiferous tubules had a morphologically normal basement membrane, Sertoli cells showed a polarized epithelial differentiation, and a lumen was formed (Table). This list reveals that many aspects of somatic rat testicular development occurred in the xenografted tissue, and that after grafting, the spherical cell

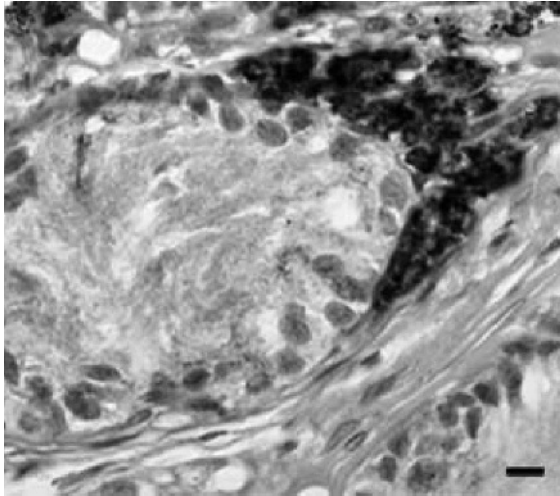


Figure 6. Immunohistochemical staining of p450scc on paraffin-embedded graft tissue. Interstitial cells in grafts are positive for p450scc in a graft after 6 weeks of grafting. Scale bar = 10 mm.

aggregates underwent morphogenetic changes leading to seminiferous tubules in xenografts.

Androgen production by the grafts appeared to be low during the first weeks after grafting, leading to seminal vesicle weights in castrate hosts only slightly above the weight normally observed in castrates (Table; see also Schlatt et al, 2003). However, even the release of a low amount of androgen is an additional indicator supporting our assumption that the p450scc-positive cells detected in the interstitium of the grafts at the same time points are indeed functional Leydig cells.

Our experiments show that immature testicular cells carry the full potential to create all somatic components of the rat testis. It appears that the initial processes of Sertoli cell aggregation and epithelial differentiation can occur in a 2-dimensional or a 3-dimensional culture system, but that any further progression is arrested under in vitro conditions. Further progression of the morphogenetic cascade, however, can be initiated when the environmental conditions change. Support from ingrowing blood vessels or the migration of mesenchymal precursors into the tissue fragments could be potential triggers for further growth and differentiation. Our combined in vitro culture/grafting approach will be highly relevant for further exploration of testicular morphogenesis.

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