Testicular Morphogenesis

Comparison of *In Vivo* and *In Vitro* Models to Study Male Gonadal Development

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ABSTRACT: The organogenesis of a functional testis is the basis for male fertility and perpetuation of each species. In mammals, testicular development is dependent on two crucial events during embryonic and pubertal development. First, primary sex determination is initiated by expression of the *Sry* gene on the Y chromosome and directs the primordial gonad toward testicular development rather than ovarian differentiation. The male pathway comprises highly regulated cell differentiation of somatic cells within the gonadal primordium, as well as migration of mesonephric cells and primordial germ cells, ultimately leading to the formation of testis cords. These cords present the earliest visible sign of male gonadal differentiation. Second, during puberty immature Sertoli cells cease to proliferate and differentiate into their postmitotic, adult phenotype. The maturation of the Sertoli cells is pivotal for initiation and maintenance of spermatogenesis. The regulation of the two separate functions of Sertoli cells—during testis development and in spermatogenesis—are poorly understood. In this review, different models that have been used to study embryonic gonadal development and testicular maturation are compared. *In vivo* models, organ, and cell culture systems are discussed as regards their applicability to study testicular organogenesis. Then, a new tissue engineering approach is presented that mimics male embryonic gonadogenesis and that offers novel ways to study early testicular differentiation, as well as Sertoli cell maturation and spermatogonial stem-cell niche formation.

KEYWORDS: Sertoli cell maturation; testicular development; 3-dimensional cell culture; spermatogenesis; xenografting

EMBRYONIC TESTICULAR DEVELOPMENT

The bipotenial gonadal primordium is a peculiar organ system in mammals, as it has the potential to develop into two different organs: testes or ovaries.

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The fate of the primordium is mainly depending on the Y-encoded gene *Sry*, which initiates testicular rather than ovarian differentiation. Upon expression of *Sry* in the XY gonad, a cascade of cellular and morphogenetic events has been described. Most dominantly, proliferation of coelomic epithelial cells, testis-specific vascularization, and migration of mesonephric cells into the gonad occur as immediate changes after *Sry* expression. As a result, the earliest morphogenetic sign of testicular differentiation becomes evident in rats between E13.5 and E14.5, when seminiferous cords become visible within the gonadal ridge.[1,2] (For a review, see Ross and Capel, 2005.[3])

Model Systems to Study Embryonic Testicular Development

The study of embryonic testicular development has employed a large variety of *in vivo* models, including studies on wild-type mice, knockout mutant mice with impaired testicular development, and transgenic mutant mice.

In addition, *in vitro* organ cultures of genital ridges and embryonic testis have provided valuable tools to investigate testis cord formation, and these tools were especially helpful in determining the importance of mesonephric cell migration into the gonad.

In Vivo *Studies*

Studying wild-type gonadal development in rodents yielded understanding of the cellular and morphogenetic events leading to testis differentiation. Early studies by Albert Jost showed that in rabbits, the removal of the primordial gonads drives the female developmental pathways in XX as well as XY individuals.[4] These experiments postulated that the differentiation of the testis is mandatory for a male phenotype development, and the absence of this androgen-driven process results in a female phenotype.

Since the discovery of *Sry* in 1990,[5,6] extensive research effort has further provided insight into several signaling pathways that regulate gonad differentiation. However, our knowledge about downstream targets of *Sry* is still incomplete. An immediate pathway that is thought to foster male development on *Sry* expression is *Sox9* (Sry-box containing gene 9). *Sox9* is expressed in the urogenital ridge later than *Sry*, and moreover, follows the same spatial expression pattern as SRY.[7,8] Although expressed in both XX and XY gonadal ridges at first, *Sox9* is upregulated in XY gonads at E11.5, shortly after onset of *Sry* expression at E10.5. In contrast, in XX gonads *Sox9* expression is downregulated.[9] Interestingly, the occurrence of SRY-negative, SOX9-positive cells has been demonstrated,[10] suggesting that Sertoli cells are being recruited during later stages of testis differentiation. The participation of prostaglandin D2 (PGD2) as a paracrine factor secreted by Sertoli cells and able to induce SOX9

phosphorylation and nuclear import has been reported recently.[11–13] The presence of active SOX9 transcription factor in turn initiates further Sertoli cell differentiation. Once formed, Sertoli cells orchestrate the cellular and morphogenetic events leading to primary sex determination, most likely by formation of the testis cords and by regulating the differentiation of the other somatic testis cell lineages such as steroidogenic Leydig cells, and peritubular myoid cells. Taken together, there is now a great deal of evidence that *Sry* uses both cell-autonomous and non-cell-autonomous mechanisms to ensure the differentiation of an adequate number of Sertoli cells that are needed for testicular development.[14,15]

Transgenic Mouse Models and Knockout Mutants with Altered Testicular Development

In vivo studies have provided insight into the cellular and morphogenetic mechanisms of sex determination in the embryo. In addition, a wide range of transgenic models and knockout mutant mice have been analyzed in terms of their testicular phenotype. These investigations further clarified the role of many factors thought to be involved in testicular differentiation.

When ectopically expressed in XX mice, *Sox9* has been shown to substitute for *Sry* and to induce testis differentiation in XX gonads.[16] Further, the essential role of *Sox9* in testis cord formation was confirmed using targeted deletion of *Sox9* in XY gonads.[17] In this model, *Sox9*-deficient XY gonads showed impaired testis cord formation and up-regulation of female-specific markers *Bmp2* and follistatin.

Another signaling molecule that has been shown to be important for testicular development is fibroblast growth factor 9 (FGF9). The male-specific proliferation of coelomic epithelial cells is pivotal for testis cord formation, and it has been reported that *Fgf9* may play a role in mediating these events.[18] In *Fgf9* mutant knockout mice, an imbalance in male and female littermates has been described, with an incidence of male-to-female sex reversal in as many as 85–100% of $Fgf9^{-/-}$ XY embryos.[19] In these mice, the proliferation rate of coelomic epithelial cells was significantly decreased in $Fgf9^{-/-}$ XY mice.

The specialized vascularization of the testis presents another hallmark of early male gonadal development that is not occurring in XX embryos. Migration of vascular endothelial cells is crucial for the formation of blood vessels within the testis to support this fast growing organ, as well as allowing for the transport of testosterone to nongonadal target tissues. The family of platelet-derived growth factors (PDGF) and its receptors PDGFRα and PDGFRβ have been shown to be present in the embryonic gonad of both sexes. However, at E12.5 PDGFRα is up-regulated in XY embryos, but not in XX embryos, suggesting a role in testicular development rather than ovarian differentiation.

This hypothesis is further supported by the finding that in male *Pdgfr-alpha* knockout mice, the testicular vascularization is disturbed and shows a highly irregular phenotype.[20] Further, testicular cord formation is also impaired in this knockout model. Whether PDGF plays a role in vascularization and cord formation, or if the two phenotypes account for one another is still to be clarified. In contrast, deficiency of *Pdgfr-beta* did not result in a severe testicular phenotype in knockout mice.[21]

The lack of a severe testicular phenotype was also reported for knockout mice deprived of several other growth factors, including the HGF receptor MET (formerly named c-met),[22] and the high-affinity neurotropin receptors NTRK1 (formerly named trkA) and NTRK3 (formerly named trkC).[23] In *Ffg2* null mice, only a mild global phenotype was observed.[24] Interestingly, the aforementioned growth factors have been shown to act as morphogens in organ culture experiments (see later in this chapter).

Beyond doubt, *in vivo* models offer the best approach to study organogenesis and development of any organism. In this respect, the gonad primordium offers an important source to study general aspects of organ development. Because of its bipotential character, gain-of-function and loss-of-function mutations will directly affect tissue fate and organ development.

The complexity of the whole animal, and the experimental effort to maintain, manipulate, and analyze organs and cells *in vivo*, however, demand additional approaches, such as organ and cell cultures, to study in more detail specific windows of cell differentiation and the molecular mechanisms mediating testicular physiology.

Organ Cultures of Embryonic Gonads

In vitro organ cultures of embryonic gonads and mesonephri provided powerful tools to study the mechanisms involved in testis cord formation. The role of mesonephric cell migration into the developing testis has been demonstrated to be crucial for testis cord formation, using cocultures of mouse embryonic mesonephros and gonad or whole genital ridges.[25] In these experiments, the ability of embryonic mesonephri and gonads to induce cord formation in XX and XY gonads was found to be stage-specific, and the gonads only showed cord forming ability during a narrow time window (E11.5–E12.5). Similar organ cultures have been used to identify molecular factors involved in cord formation. A number of growth factors has been found to induce testis cord formation in these organ-culture experiments, including VEGF,[26] HGF,[27] PDGF,[28] and neurotrophin-3.[29] Findings from *in vivo* studies indicating a role for FGF9 in coelomic cell proliferation and cord formation (see earlier) could be confirmed using rat embryonic gonads in organ culture.[30] Moreover, FGF2 and FGF9 were shown to mediate mesenchymal-epithelial transition by modulating the composition of the basal lamina, the specialized testicular basement

membrane surrounding testicular cords that is reconstituted by cooperative Sertoli cells and peritubular cells. In these studies, the authors also show a dominant effect of FGF2 over FGF9.

A common signal transduction pathway for these growth factors is the phosphotidylinositol 3-kinase pathway. Using a specific PI3K inhibitor on embryonic gonads in organ culture inhibited cord formation,[31] suggesting that PI3K is a major player during testis cord formation.

Gonadal organ-culture systems offer the possibility to study molecular factors and cell–cell communication in a defined environment, without the complexity of the whole animal, while the testicular architecture is preserved.

However, decreased steroidogenic activity and increased necrosis in organ cultures using agar-coated grids make it difficult to study steroidogenesis and to maintain cultures over an extended period of time.[32] Alternatively, floating organ cultures have been used and shown to be a better approach, since Sertoli cell, Leydig-cell, and germ-cell growth and development were not negatively affected and appeared to be similar to the *in vivo* situation.[33] Using floating cultures and a synthetic serum-free medium in organotypic cultures, Livera *et al.* showed that in mouse and rat fetal and neonatal gonads the morphofunctional development of Leydig, Sertoli, and germ cells could be sustained for up to 10 days (mouse) and 21 days (rat), respectively.[34] However, fewer Leydig, Sertoli, and germ cells differentiated *in vitro* than *in vivo*, and after 10 days of culture, Sertoli and peritubular layers were disorganized. The lower number of Leydig cells might account for the decreasing testosterone production in organ cultures, which is opposite an increasing testosterone level *in vivo*. In these cultures, gonocytes could be maintained for up to 14 days *in vitro*, although in far lower numbers than *in vivo*. Gonocytes resumed mitosis and differentiated into spermatogonia in both mouse and rat gonadal explants. This observation is of particular interest for the development of an *in vitro* system capable of supporting spermatogenesis, since germline stem cells and germ cells have been found to be extremely difficult to culture.[35] Maintenance of the testicular architecture and cell–cell contacts in organ cultures offers a great advantage, since all principal testicular cell types were shown to be sustained in a more efficient way than in primary cell cultures.

Taken together, somatic and germ cells in fetal and neonatal testis explants develop in a comparable manner to the *in vivo* situation, and rat testis cultures have been shown to be more efficient than testis explants from mice. However, organ cultures using adult testicular tissue to investigate spermatogenesis and Sertoli–germ cell communication have not been successfully applied. Therefore, a range of *in vitro* cell culture systems has been employed to study the regulation of adult Sertoli cells via FSH, testosterone, and paracrine signals. In the next chapter we will review the current knowledge about Sertoli cell maturation, and we will discuss the application of different culture systems applied for postnatal and adult Sertoli cells.

SERTOLI CELL MATURATION IS REQUIRED FOR THE INITIATION OF SPERMATOGENESIS

The proliferation of Sertoli cells during prepubertal life leads to an elongation of the immature seminiferous cords.[36] In addition, the formation of the basal lamina, the determination of Sertoli cell polarity, and the establishment of the blood–testis barrier are crucial steps during the formation of the seminiferous cords and tubules in ontogenesis.[2] Primordial germ cells are first present in the center of the cords as gonocytes. Depending on the species, at different times of life the gonocytes migrate to the basement membrane to become spermatogonial stem cells. The continuously growing testicular cords provide an increasing number of niches for spermatogonial stem cells.[37] As gonocytes and to a lesser extent as spermatogonial stem cells, male germline stem cells are quiescent during fetal and prepubertal life due to the presence of the surrounding Sertoli cells that appear to maintain the inactive state.[38] During the onset of puberty, coinciding with the cessation of proliferation, Sertoli cells mature and gain unique characteristics that allow the support, nourishment, and protection of spermatogonial stem cells and differentiating germ cells.

In Vitro *Systems to Study Sertoli Cell Maturation*

For the study of Sertoli cell maturation, *in vitro* culture systems present a commonly used approach to study biochemical and genomic effects of hormones and growth factors on Sertoli cell proliferation, metabolism, and differentiation. *In vitro* Sertoli cell culture has become increasingly popular over the last 20 years, and many hallmarks of Sertoli cell function, such as the secretion of androgen binding protein, transferrin and inhibin were identified during the 1970s and 1980s in pioneering work using Sertoli cells in culture.[39] In this review, we can only highlight some of the many important discoveries derived from Sertoli cell cultures, and we will mainly focus on studies investigating Sertoli cell maturation at the onset of spermatogenesis.

Primary Sertoli Cell Cultures

Proliferation and differentiation of Sertoli cells can be characterized by the complement of proteins expressed during each stage of Sertoli cell development. During the proliferative phase of Sertoli cell development until the onset of puberty, Sertoli cells express anti-Mullerian hormone (AMH). Hormonal regulation of *Amh* mRNA expression was shown in cell cultures derived from 2-day-old rats.[40] In these studies, thyroid hormone (T3) negatively stimulated *Amh* mRNA expression. Cultures treated with T3 showed a more dramatic decrease in *Amh* mRNA levels during 4 days of culture than

controls. Interestingly, the authors also observed a dose-dependent increase in mRNA expression of the Sertoli cell differentiation marker inhibin in the same T3-treated cultures. Follicle-stimulating hormone was shown to have a similar effect on *Amh* expression *in vitro* as T3, and T3 and FSH where further shown to have an additive effect on *Amh* expression in cultured Sertoli cells. Taking into account that follicle-stimulating-hormone receptors (FSHR) become more abundant during the start of spermatogenesis, results derived from these *in vitro* studies suggest that an increased FSH sensitivity induces Sertoli cell proliferation, and simultaneously contributes to the down-regulation of AMH expression in cooperation with T3. Using similar Sertoli cell cultures from 5- and 20-day-old rats, Arambepola *et al.* also showed that T3 and FSH increased androgen receptor (AR) mRNA expression and had a synergistic effect on *Ar* mRNA expression.[41] Together, T3 and FSH could regulate both *Ar* up-regulation and *Amh* down-regulation, thus progressively leading to Sertoli cell maturation. Other differentiation markers for Sertoli cells that were identified using cultivated Sertoli cells derived from 20-day-old rats include transferrin and androgen binding protein (ABP).[42,43]

Although there are many more reports on molecular factors that regulate Sertoli cell function *in vitro*, actions of one of the major regulators of spermatogenesis, testosterone, cannot be reliably studied *in vitro*. Investigations of androgen action on Sertoli cells using *in vitro* models provided only limited information. Most culture systems were found to be unresponsive to androgens under culture conditions, or showed only very low responses,[44,45] most likely due to decreased AR expression in the Sertoli cells.[46] Further, contaminating peritubular myoid cells or Leydig cells might influence the experiments performed on primary Sertoli cell cultures, and careful isolation and characterization of the cell preparation is needed for adequate interpretation of the results yielded from primary Sertoli cells.

In most studies, investigators isolated Sertoli cells from immature postnatal animals, despite evidence that Sertoli cells function optimally in the adult testis with complete spermatogenesis. Immature Sertoli cells are less differentiated than their adult counterparts, and therefore retain mitotic activity and show a better adaptation to culture conditions. These features are favorable for the *in vitro* culture of Sertoli cells, but careful interpretation of results obtained from such culture systems is crucial, as their status differs from mature Sertoli cells. In addition, the isolation of more mature Sertoli cells (e.g., 15–20 days postpartum (dpp) in the rat) has to be evaluated critically, because of the possibility of enriching the cell isolate specifically for nondifferentiated Sertoli cells that might be residing in the testis at the time of isolation. It appears possible that once in culture, only the nondifferentiated Sertoli cells can be propagated, whereas more differentiated Sertoli cells could potentially become diluted during the culture period. Thus, it can be difficult to interpret results obtained from these cultures in terms of their relevance in mature, differentiated Sertoli cells.

Immortal Sertoli Cell Lines

To circumvent the shortcomings of primary cell cultures, the derivation of a number of immortal Sertoli cell lines has been sought (for a Review, see Roberts[47]). The mouse SK11 cell line, derived from 10-day-old animals, was recently reported to express AR and ERβ, as well as retaining the phenotype of murine Sertoli cells.[48] These results suggest that the SK11 cell line may be suitable to complement investigations regarding androgen actions on Sertoli cells. However, in another report it was shown that SK11 cells as well as SK9 cells do not bind to spermatids *in vitro*,[49] rendering both cell lines unsuitable for studies on the impact of steroids on spermatogenesis, and other aspects of Sertoli cell–germ-cell interactions. In agreement with that, other frequently used murine Sertoli cell lines, such as the TM4 line,[50] which has been shown to be FSH responsive,[51] and the SF7 line, were shown to be unable to maintain spermatogonial stem cells in coculture experiments.[52]

Furthermore, transformed cell lines often show low genomic stability, and most reported cell lines are aneuploid and show chromosome translocations,[53] which may be associated with functional alterations.

The alterations in immortalized Sertoli cell lines mentioned previously have to be taken into consideration when interpreting results obtained from *in vitro* culture experiments. In addition, most transformed Sertoli cell lines were generated from postnatal animals, thus investigations face the same complications as discussed for primary Sertoli cell cultures in terms of data interpretation.

Conventional Two-dimensional Sertoli Cell Culture

In vivo, Sertoli cells possess a highly specialized epithelial character, with tight junctions separating the basal compartment from the adluminal compartment. The reconstitution of a basement membrane, a specialized extracellular matrix deposited by Sertoli and peritubular cells that surrounds each seminiferous tubule, provides structural cues for Sertoli cells that might facilitate Sertoli cell polarization. Therefore, in primary Sertoli cell cultures, the components of the basement membrane (e.g., laminin, collagen) and reconstituted extracellular matrix have been investigated in culture. They were found to promote Sertoli cell differentiation and germ-cell differentiation, in some cases through the pachytene stage of meiosis.[54–56] Thus, using coated culture surfaces has an advantage over uncoated tissue culture plates in that it provides better adhesion for Sertoli cells, and it creates a culture system that resembles the basal lamina of the seminiferous tubules. However, most culture systems used for either primary Sertoli cells or Sertoli cell lines are 2-dimensional systems and do not mimic the complex architecture of the seminiferous tubule, and Sertoli cell

polarization is not established to the degree observed *in vivo*. The tendency of Sertoli cells in culture to aggregate and to form cordlike structures has been reported for various coated culture surfaces. In these cultures, Sertoli cells follow a distinct morphogenetic cascade within 10 days of culture on laminin-coated culture dishes (FIG. 1A and 1B).[57,58] The aggregation of Sertoli cells into cordlike structures and mounds closely resembles the events leading to testis cord formation *in vivo*. The fact that Sertoli cells prefer to aggregate with each other and with contaminating peritubular cells, rather than growing in a flat, fibroblast-like monolayer, mirrors the close relation between Sertoli cell architecture, Sertoli cell function, and tissue integrity. Therefore, it is arguable that the creation of a Sertoli cell culture system that shows a structural resemblance to the testicular environment would be desirable.

NOVEL APPROACHES: *IN SITU* MODELS TO STUDY TESTICULAR DIFFERENTIATION: XENOGRAFTING AND TISSUE ENGINEERING MODELS FOR BASIC RESEARCH APPLICATIONS

To investigate testicular differentiation and maturation, researchers have at their disposal a wide variety of *in vivo* and *in vitro* models. Here, we add to this repertoire a new model that combines 3-dimensional *in vitro* culture and ectopic xenografting into immunodeficient nude mice.

Three-dimensional Cell Culture

The importance of the extracellular matrix for tissue development and homeostasis has become evident in recent years. The finding that the extracellular matrix provides structural cues crucial for tissue maintenance and function, and creates a reservoir for soluble factors has drawn much attention. In the testis, the basement membrane presents a specialized extracellular matrix produced by Sertoli and peritubular myoid cells. It has been described previously in the rat model that nidogen, perlecan, laminin, and collagen IV are present in the seminiferous tubule basement membrane and have been shown to be important for Sertoli cell polarization and seminiferous-cord formation.[59–61]

Reconstituted extracellular matrix gel (Matrigel) has been widely used for culturing primary testicular cells and Sertoli cell lines, as well as other epithelial cells.[62,63] The immortalized Sertoli cell line SF7 has been reported to form hollow tubules when cultured on Matrigel.[64] These observations are in accordance with the previously described tendency of primary Sertoli cells to aggregate *in vitro* and to form testicular cordlike structures when cultured on an adequate substratum.

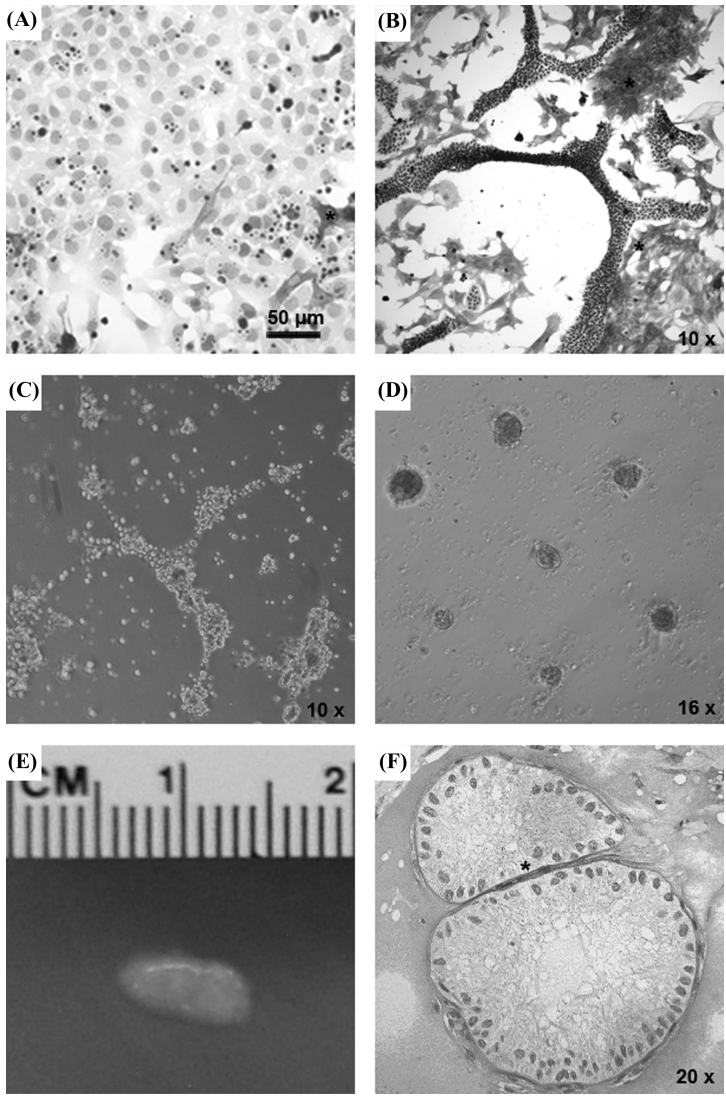

FIGURE 1. Cord formation of Sertoli cells in different culture systems. (A) Two-dimensional Sertoli cell culture. Sertoli cells form monolayers after 3 days in culture. Few peritubular myoid cells are present in cultures (*). (B) Ten days after plating, Sertoli cells form cordlike structures. Note the increased abundance of peritubular cells in culture (*). (C) Three-dimensional culture system. Sertoli cells migrate into cordlike structures after 4 hours in culture. (D) After 3 days of culture in Matrigel, Sertoli cells form compact spherical aggregates. (E) Xenograft containing tubule-like structures after removal from host after 12 weeks. (F) Cross sections through xenograft. Sertoli cells arranged in single-layered epithelia. In cooperation with peritubular myoid cells, a basement membrane was established (*).

We have recently developed an improved 3-dimensional culture system consisting of Matrigel for the *de novo* generation of testicular cordlike structures.[57] Primary testicular cells were derived from immature rats and seeded on a 300-μm layer of concentrated Matrigel. Sertoli cells rapidly aggregated, upon plating, and formed cordlike aggregates within 4 hours (FIG. 1C). Thereafter, the cells migrated into irregular-shaped cell aggregates with radial cytoplasm processes. After 3 days of culture, sphere-shaped aggregates were observed and arranged throughout the Matrigel in a highly regular hexagonal pattern (FIG. 1D). Spheres were organized in multilayered epithelia that contained different cell types; some aggregates also showed a lumen. Occasionally, putative germ cells (spermatogonia) with an ovoid nucleus and high nuclear/cytoplasm ratio were observed in the aggregates. However, we also found that the morphogenetic cascade of Sertoli cells was arrested after 3 days in culture, and Sertoli cells did not further mature morphologically, even after extended culture periods. Usually, cordlike structures disintegrated after 5 weeks of culture.

Compared to 2-dimensional culture on tissue culture plates, morphogenesis was greatly advanced in 3-dimensional culture, indicating that immature Sertoli cells retain the potential to form testicular cords *in vitro* when cultivated in an appropriate culture system. We suggest that the 3-dimensional character of the culture system facilitates Sertoli cell migration, aggregation, and polarization in a manner similar to that of the microenvironment during embryonic testis cord formation. Thus, the model presented offers a new tool to study primary Sertoli cells *in vitro* by mimicking, at least in part, the complex tissue structure of the developing testis *in vivo*. We propose that our model will help to gain insight into testis development that could not be achieved in conventional 2-dimensional cultures because of the previously cited constraints of such cultures.

Xenografting of Testicular Tissue and Testicular Cords Derived In Vitro

In recent years, ectopic xenografting has become a valid model to induce spermatogenesis in immature testicular tissue.[65] 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.[65–68] This model has also been used for toxicological studies on nonhuman primate testicular tissues[69] and studies on the influence of irradiation and cryopreservation of nonhuman primate testes.[70,71] To study testicular development, this tool could be used to cultivate gonadal tissues from knockout mutant mice with fetal or neonatal lethality. It is possible to study the influence of various defects, testis-specific and non-testis-specific, on testicular organogenesis and spermatogenesis in tissues that otherwise would not be available, or that would never reach maturity.

We further employed the concept of xenografting to find out whether testicular cordlike structures derived from 3-dimensional Sertoli cell cultures would continue the differentiation of testis cords when introduced to the physiological environment of a host. The lack of pivotal environmental input—as it occurs during embryonic testis development through extensive mesonephric migration and the establishment of the testis-specific vasculature—could be one reason for arrested tubule-formation in 3-dimensional culture. We therefore hypothesized that the limitations of cell and tissue culture models could be overcome by xenografting, and that Sertoli cells could be maintained for a longer period of time when compared to 3-dimensional cell culture. In our experiments, we xenografted Matrigel patches that contained tubule-like structures after 10 days of *in vitro* culture. Matrigel patches were subcutaneously injected under the back skin of immunodeficient nude mice. The formation of testis-like structures was followed up to 12 weeks. The findings from our grafting experiments showed that the spherical aggregates employed for grafting grow into larger tissues (FIG. 1E). Moreover, the presence of blood vessels within recovered grafts indicated the potential of testicular cells for inducing angiogenesis in the same manner as observed *in vivo*,[72] and indicated that blood vessel formation is a crucial step for testis cord formation and outgrowth. Histological analysis revealed seminiferous tubules (normal basement membrane, fluid-filled lumen, epithelium composed by Sertoli cells, few spermatogonial, but no meiotic germ cells, and peritubular cells) and the presence of an interstitial compartment in xenografts (FIG. 1F). In preliminary experiments, the differentiation of Sertoli cells in xenografts was monitored using RT-PCR analysis. Expression of Sertoli cell differentiation markers (e.g., *Ar, Fshr,* and transferrin) as well as Sertoli cell immaturity markers (e.g., *Amh*, cytokeratin 18) was detected in grafts after 4 to 12 weeks. The preliminary data indicate the concurrent presence of differentiated and immature Sertoli cells, respectively. Therefore, further studies are ongoing in our laboratory to characterize the developmental state of Sertoli cells in xenografts. To this end, the immunohistochemical detection of the steroidogenesis marker P450 scc in cross sections may indicate the maturation of the interstitial compartment and the presence of functional Leydig cells. Taken together, our observations were in agreement with recent reports about the generation of functional testis tissue from crude isolations of neonatal pigs,[73] where complete spermatogenesis was established in xenografts. Xenografting of immature Sertoli cells from various species could be applied in the future to study testicular development and spermatogenesis.

Finally, *in situ* approaches to generate testicular tissue offer exciting new ways to study testicular morphogenesis that can overcome some of the limitations of other model systems. In addition, using 3-dimensional culture systems similar to the one described here might also add to our understanding of testis cord formation and Sertoli cell maturation. Ultimately, besides contributing to the field of gonadal development, an easily accessible *in vitro* or *in situ*

culture system capable of supporting spermatogenesis would be of great interest for the study of germ-cell differentiation and spermatogonial stem-cell niche formation.

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