Anatomical basis for cell transplantation into mouse seminiferous tubules

Unai Silván^{1,2} and Juan Aréchaga^{1,2}

¹Laboratory of Stem Cells, Development and Cancer, Department of Cell Biology and Histology and ²Biomedical High Resolution and Analytical Microscopy Core Facility, Faculty of Medicine and Dentistry, University of the Basque Country, E-48940 Leioa, Vizcaya, Spain

Correspondence should be addressed to J Aréchaga at Laboratory of Stem Cells, Development and Cancer, Department of Cell Biology and Histology, Faculty of Medicine and Dentistry, University of the Basque Country; Email: juan.arechaga@ehu.es

U Silván is now at Biozentrum, University of Basel, Basel, Switzerland

Abstract

Cell transplantation into the seminiferous tubules is a useful technique for the study of physiological and pathological conditions affecting the testis. However, the precise three-dimensional organization and, particularly, the complex connectivity of the seminiferous network have not yet been thoroughly characterized. To date, the technical approaches to address these issues have included manual dissection under the stereomicroscope, reconstruction of histological serial sections, and injection of contrast dyes, but all of them have yielded only partial information. Here, using an approach based on the microinjection of a self-polymerizing resin followed by chemical digestion of the surrounding soft tissues, we reveal fine details of the seminiferous tubule scaffold and its connections. These replicas of the testis seminiferous network were studied by scanning electron microscopy. The present results not only establish a morphological basis for more precise microinjection into the mouse seminiferous tubules but also enable a more profound investigation of physiological and embryological features of the testis.

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Introduction

Mouse adult testicles are ovoid organs that weight around 150 mg and have an average size of 8.5×5 mm. Gametogenesis occurs inside the seminiferous tubules, which contain the pluripotent germ cell niche, enclosed by Sertoli cells, the basal lamina, and the peritubular myoid cell layer. In this compartment, diploid spermatogonia undergo differentiation into spermatocytes through a complex process (Dym *et al.* 2009). The extraabdominal location of the testicles, together with their vascular heat exchanger and the reflex contraction of the cremaster muscle (that can retract the testes towards the abdomen in response to situations of cold or fear), permits the regulation of testicular temperature, so that it can be maintained constant at a slightly lower temperature than the rest of the body, what is crucial for spermatogenesis in mammals (Liu 2010).

Cell transplantation into the seminiferous tubules was initially developed in the mid 1990s (Brinster & Zimmermann 1994). In these pioneering experiments, cell transplantation was achieved by microinjecting individual seminiferous tubules; however, this method rarely results in the complete filling of the testicular

network. Later on, two improved experimental approaches were described, namely microinjection into the *rete testis* and through the efferent ducts (Ogawa *et al.* 1997). Although these new methods remarkably increase transplantation efficiency, the lack of knowledge of the morphology of the involved structures frequently results in the accidental rupture of the membranes lining them. This invariably leads to the extravasation of the cell solution out of the intratubular compartment and consequently to transplantation failure. We believe that a more precise knowledge of the anatomy of the testis will be very helpful for improving the efficacy of transplantation into the seminiferous tubules.

Using this technique, it was shown that germinal cells from one animal transplanted into the seminiferous tubules of another were able to incorporate into the seminiferous epithelium of the recipient testis and differentiate into fertilization-competent spermatozoa. Until now, several different cell types have been used in transplantation experiments including adult spermatogonia (Brinster & Avarbock 1994, Avarbock *et al.* 1996, Clouthier *et al.* 1996, Dobrinski *et al.* 1999, Ogawa *et al.* 1999, 2000, Li *et al.* 2010), primitive germinal cells and

gonocytes (Jiang & Short 1998), embryonic stem cells (Silván *et al.* 2009, 2010, 2011), bone marrow stem cells (Lue *et al.* 2007), Sertoli cells (Shinohara *et al.* 2003), and seminoma and embryonal carcinoma cell lines (Li *et al.* 2008).

The anatomy of the testis has been extensively studied since the XVII century (De Graaf 1668) mainly in mammals, including humans, using light and electron microscopy. Manual dissection, reconstruction of serial histological sections, and microinjection of dyes have revealed differences in the structures that conform this organ between several mammal species (Roosen-Runge 1961, Dym 1976, Cavicchia & Burgos 1977, Roosen-Runge & Holstein 1978, Wrobel 2001, Wrobel & Schimmel 2001). However, none of these techniques precisely described the three-dimensional architecture of the seminiferous tubule network and of the seminal drainage system of the testis. Nevertheless, a precise knowledge of these networks is required for the detection and characterization of possible abnormalities caused by genetic or acquired diseases that may contribute to infertility (Hsieh *et al.* 2010).

Here, we report a detailed morphological description of the murine testis, based on the microinjection of a self-polymerizing resin into the seminiferous tubules. As all seminiferous tubules and the efferent ducts are interconnected, the resin filled the entire lumen of the testis including its excretory system. Later, following resin polymerization and chemical digestion of the tissues surrounding the cast, a replica of the three-dimensional network of the mouse testis tubules was obtained. Its analysis under the scanning electron microscope, and comparison with histological sections and dye-microinjected samples of the same regions, revealed valuable details of the seminiferous tubule scaffold and its connections with other ducts.

Results

Here, we describe the casts, tracing the same path followed by spermatozoa after their complete differentiation, from the seminiferous tubules to the efferent ducts, paying special attention to the less known structures. All measurements were made directly on the resin casts; only small differences with *in vivo* data are expected.

The bulk of the testis volume is occupied by the convoluted seminiferous tubules (Fig. 1A and B, Supplementary Movie 1, see section on supplementary data given at the end of this article), which have a mean approximate diameter of 110 μm . These tubules do not have blind endings and rarely exhibit bifurcations (Fig. 1C). At high magnification, the tracks left by the tails of immature spermatids can be observed on the surface of the casts, with their heads embedded in the cytoplasm of Sertoli cells (Fig. 1D).

Both ends of the seminiferous tubules, just before entering the *rete testis*, are connected to the straight tubules (*tubuli recti*) showing an abrupt constriction at the level of the junction (Fig. 2A). This narrow communication bridge has an approximate length of 50 μm and a variable diameter that ranges between 15 and 45 μm . However, although the size of all constrictions in a given cast is similar, there is high variability between samples (Fig. 2B and C). Optical microscopy images confirmed that this final portion of the seminiferous tubules has a smaller diameter than the convoluted seminiferous tubules and shows slight thickenings of the peritubular myoid layer (Fig. 2D). After these narrow bridges, the straight tubules recover the same diameter of the convoluted tubules and drain into the *rete testis*.

Corrosion casting of the testis reveals a new view of the so-called *rete testis*, which is not a network ('*rete*' in Latin means 'net') but rather a flat reservoir with an approximate size of 400 $\times$ 600 μm located close to the mediastinum testis, through which the blood vessels penetrate the organ. However, this structure shows some variability in shape and size between specimens (Fig. 3A and B). Standard histological analysis reveals its proximity to the *tunica albuginea*, the slimness of its wall and its location under the vascular pediculum of the testis (Fig. 2D). At high magnification, nuclear imprints of the cells forming the walls of the cavity can be appreciated on the surface of the casts (Fig. 3D). Manual dissection of the corrosion casts under the stereomicroscope allowed us to view the bottom part of the *rete testis* and facilitated the observation of the seminiferous tubules that reach this structure.

The extratesticular *rete testis* (*area cribrosa*) appears as a small vaulted protrusion sticking out from the *tunica albuginea* that connects the intratesticular *rete testis* with the efferent ducts. This structure has an approximate diameter of 130 μm and a height of 200 μm (Fig. 4A) and shows multiple holes of variable sizes, ranging from 20 to 60 μm in diameter. This particular area is hardly interpretable in equivalent histological sections (Fig. 4B).

The efferent ducts (*ductuli efferentes*) emerge from the extratesticular *rete testis*. These channels, which lead spermatozoa to the epididymis, can be easily seen *in vivo* at stereomicroscope low magnification after microinjection of trypan blue dye (Fig. 4C). In turn, corrosion casting offers a detailed image of the duct structure and of their connection with the testis (Fig. 4D). The analyzed testicles had between two to five efferent ducts each, and their shape and diameter was very irregular, with an average of 50 μm . Occasionally, a distended portion of one of the efferent ducts could be seen in both *in vivo* specimens and in corrosion casts (Fig. 4C and D). Histological analysis revealed that these ducts exhibit increasing diameters as they move away from the *rete testis* to the epididymis (Fig. 4E).

Figure 1 Corrosion casts of the murine testis. (A) Lateral overview of a corrosion cast of the whole mouse testis under low-power scanning electron microscopy in which we can recognize (1) the convoluted seminiferous tubules, (2) the straight tubules, (3) the intratesticular *rete testis*, (4) the extratesticular *rete testis*, and (5) the efferent ducts. Some convoluted tubules broke during preparation, due to the fragility of the corrosion cast. The copper wires used as conductive bridges can be observed (*). (B) Cranial overview of another cast of the seminiferous network. (C) The seminiferous tubules appear as a convoluted network of tubes, which rarely bifurcate. (D) At higher magnification, the imprints left by the tails of spermatids on the surface of the resin can be seen (arrows). This is indicative of good filling of the tubules with the resin. Scale bars represent 1 mm in A, 750 μ m in B, 200 μ m in C, and 20 μ m in D.

Discussion

Although cell transplantation into the mouse seminiferous tubules is a widely used technique for the study of germ cell differentiation and germinal cancer invasion processes (Parreira *et al.* 1998, Li *et al.* 2008, 2010, Silván *et al.* 2009, 2010, 2011), the high-resolution anatomy of the seminiferous tubule network and its connections has never been precisely described. This knowledge would facilitate the transplantation technique and more in-depth analysis of the obtained results. In order to overcome this limitation, we have developed a new approach to study in detail the three-dimensional organization of the seminiferous tubule network by microinjecting a self-polymerizing resin into the seminiferous tubules followed by chemical digestion of the surrounding tissues.

The injection of self-polymerizing resins into hollow spaces of organs and tumors is a broadly used technique, especially in the study of blood and lymphatic capillaries

(Ohtani & Ohtani 2008, Silván *et al.* 2009). When employed in the study of testis anatomy, this approach resulted in replicas of the testicular luminal spaces that could be visualized under the scanning electron microscope. The presence of cell imprints on the surface of the obtained corrosion casts is indicative of the complete filling of the seminiferous network with the resin and, thus, minimizes the possibility of artifacts caused by defective casting. As a complement for the interpretation of the casts, we performed microinjection of dyes and routine histological sections of normal murine testis.

There are two types of intratesticular tubules: the 'convoluted' tubules (seminiferous tubules, strictly speaking) and the 'straight' tubules (*tubuli recti*), and the epithelial wear of both is different (Supplementary Figure 2 (see section on supplementary data given at the end of this article); Osman 1978, Osman & Plöen 1978). In the straight tubules, this epithelium has a simple cuboidal form, without germinal cells (Dym 1974), and this characteristic points to a possibly distinct

Figure 2 Connection of straight and convoluted tubules. (A) Corrosion casting of the connection of straight and convoluted tubules reveals a constriction with similar diameter in a given testis, but with high variability between specimens (B and C). This feature corroborates the existence of a possible sphincter mechanism at this level. (D) The same region observed using routine histology exhibits narrowing (white arrowhead) and of a valve-like structure in the region in which the tubules connect with the *rete testis* (black arrowhead). Scale bars represent 100 μ m in A and B and 25 μ m in C and D.

embryological origin of both kinds of tubules, from the mesonephros and the genital ridges respectively. The convoluted seminiferous tubules, lined by the germinal epithelium, represent the vast majority of the testis volume and constitute the location where spermatogenesis takes place. In the corrosion casts, which we obtained, the seminiferous network is seen as a dense bunch of tubules that rarely bifurcate and that have both endings connected to the straight tubules.

In the connection between the two types of tubules, a sudden narrowing of the tubule diameter is found. Interestingly, the diameter of this constriction seems to be subject dependent, with differences of up to 30 μ m between casts. The functional significance of the narrowing of the tubules is not clear. As spermatozoa do not acquire mobility until their maturation in the epididymis (Russell *et al.* 1990), peristaltic contractions of the smooth muscle cell layer surrounding the seminiferous tubules are needed to transport these cells (Wrobel *et al.* 1986, Yamamoto *et al.* 1989). Thus, we speculate that the narrowing may be a sphincter that can open and close to avoid spermatozoa backflow. In fact,

Cavicchia & Burgos (1977) reported that the ligation of the efferent ducts results in a dilation of the *rete testis* and straight tubules, with the effect on the seminiferous tubules being less significant. Later on, Wrobel (2001) suggested two different systems, peritubular contractile cells and vascular plexus, as possible mechanisms to close the tubules. Our histological analysis of this region supports the idea that myoid cells are responsible for a sphincter-like action; however, a complementary valve-like action of the terminal portion of the straight tubules cannot be ruled out (Fig. 2A). Corrosion casts also reveal that the straight and convoluted tubules have similar diameters. The narrow connection between both testis tubules may underlie the less efficient filling of the seminiferous network when microinjection is performed through individual seminiferous tubules (Silván *et al.* 2011).

The exact location where the mammalian urogenital junction (the union between the primitive sex cords of the male genital ridges and mesonephric Wolffian ducts) takes place is an issue of debate (Wrobel 2001). For instance, it has previously been reported that in bovid

Figure 3 The intra- and extratesticular *rete testis*. (A and B) The shape of this 'reservoir chamber' (*lacuna testis*) shows variability between specimens. (C) Light microscopy revealed the position of the intratesticular *rete testis* under the *tunica albuginea* (arrowheads) and its slimness. The connection of several straight tubules can also be observed (asterisks). (D) Cell nuclei imprints from the coating epithelium of a straight tubule ending in the intratesticular *rete testis*, indicative of resin good filling. Scale bars represent 500 μ m in A and B, 100 μ m in C, and 50 μ m in D.

embryos, this event occurs at the level of the extra-testicular *rete testis* (Hinton & Turner 1988, Wrobel & Schimmel 2001). However, histological analysis of adult mice shows dramatic epithelium changes in the region where convoluted and straight tubules are connected (Fig. 2D). This fact suggests that, at least in mice, this area may be the site where the urogenital junction occurs. Nonetheless, further studies during mouse embryogenesis are needed to resolve this question.

Straight tubules (*tubuli recti*) end in a structure termed the *rete testis* (the meaning of *rete* in Latin is net), which in the mouse consists of two portions: one intra- and one extratesticular. Nevertheless, it is known that the location and morphology of this structure varies among species, being 'cavitary' and 'superficial' in rodents (Dym 1974). In fact, corrosion casts and histological images show that the intratesticular portion of the murine *rete testis* is a reservoir-like structure that could be more appropriately named *lacuna testis* (reservoir in English). This structure is located immediately under the tunica albuginea, which makes it easy to use for cell transplantation (Ogawa *et al.* 1997, Silván *et al.* 2011). The most evident function of the *rete testis* is to act as a

collection chamber in which all the seminiferous tubules release spermatozoa on their way out of the testis. An evident advantage of the term *lacuna testis* over *rete testis* is that the seminal fluid produced by all seminiferous tubules is mixed, and thus homogenized, before it continues its journey to the epididymis. Although the shape of the intratesticular *rete testis* is highly variable, its superficial position under the vascular pediculum, together with its generally large size, makes it easy to find. However, the structure is very thin, and if the micropipette is not inserted almost parallel to the surface of the testis, it is possible to pierce the compartment leading to the delivery of the solution in the testis stroma. Thus, when performing transplantation using this path, short and sharp pipettes have to be used and the applied pressure has to be carefully controlled.

Close to the mediastinum testis, where the vascular supply penetrates the organ, the extratesticular portion of the *rete testis* is found. The morphology of this structure is hardly distinguishable using routine histology. In contrast, corrosion casts show it as a small vaulted protrusion located on the testis surface, in a peripheral position to the *lacuna testis*. The extratesticular *rete testis*

Figure 4 The extratesticular *rete testis* and efferent duct connections. (A) Corrosion casting reveals the existence of several holes in the extratesticular *rete testis* (arrowhead). This structure connects the intratesticular *rete* reservoir (white asterisk) with the efferent ducts (asterisk). (B) Histological section at the same level confirms the irregularity of this structure and the vicinity of blood capillaries (asterisk). (C) *In vivo* image of the efferent ducts leaving the *rete testis* filled with trypan blue solution (asterisk: dilated duct). (D) An equivalent area as seen in corrosion casts. Note a similar distension (asterisk) in an efferent duct and the variable sizes of these channels. (E) Histological section at the level of the *rete testis* (asterisk) and the emerging efferent ducts (arrowheads). All scale bars represent 100 μm .

is a pierced structure, although the precise function of its holes remains unclear. One possibility is that these piercings are due to blood capillaries, which warm the seminal fluid before it enters into the collecting ducts. As the interchange of molecules and spermio-phagocytic activity are two functions of the *rete testis* epithelium

(Sinowatz *et al.* 1979), an increased contact surface, just like that resulting from the cavities found in the extratesticular *rete*, may be required. Thus, it is possible that in mice, the larger contact surface resulting from the holes present in this structure subserves the molecular interchange function.

From the extratesticular *rete*, a variable number of efferent ducts emerge. They run in parallel to each other and show very irregular shapes and frequent dilatations or swellings indicative of high flexibility, a functional characteristic that would reduce spermatozoa backflow. In addition to their connecting role, the efferent ducts are responsible for fluid reabsorption and protein synthesis and secretion (Crabo 1965, Jiang & Short 1998). In this regard, it has been suggested that 90% of the fluid passing through these ducts is reabsorbed to concentrate the spermatozoa suspension (Levine & Marsh 1971). Efferent duct walls are lined internally with a single epithelium layer composed of two cell types, ciliated and non-ciliated. When using this path for cell transplantation, one has to take into account the existence of the extratesticular *rete testis* at the end of the ducts. If the micropipette is too deeply inserted and reaches this intricate structure, its lining membrane could be damaged and the cell solution extravasated. Another complication associated with this method is the thickness of the connective walls surrounding the efferent ducts, which makes cannulation difficult.

The results we present here establish the anatomical basis for cell transplantation into the seminiferous tubules and facilitate the localization of the testicular structures involved in the described microinjection approaches. As the laboratory mouse plays a central role in biomedical research, being used as primary model in fields like cancer, aging, diabetes, development, among others, we believe that a more precise knowledge of its testicular morphology is essential for further interpretation of associated pathologies. Moreover, the technique that we report here may also be useful for the characterization of anatomical abnormalities in the testis of animals with acquired or genetic diseases.

Materials and Methods

Borosilicate glass pipettes with an approximate tip diameter of 50 µm were connected to a 10 ml plastic syringe by polyethylene tubing and the whole system was filled with a fresh mixture of Mercor CL-2B resin and Mercor polymerization accelerator (Vilene Med., Tokyo, Japan) at variable proportions. Adult Swiss mice (OF1, IFA CREDO, Barcelona, Spain) were killed and the testes were removed from the abdominal cavity, pinned to a dissection plate, and microinjected with the above resin mixture as described in detail elsewhere (Ogawa *et al.* 1997, Silván *et al.* 2011) until the filling of the seminiferous network was complete. Then, the testes were immersed for 1 h in a 40 °C water bath to accelerate the polymerization of the resin. Subsequently, the organs were submerged in a 5% potassium hydroxide solution that was changed daily until the soft tissues surrounding the testis casts had disappeared. Specimens were washed with abundant distilled water, dehydrated, and mounted on conductive stubs, using thin copper wires as conductive bridges. Finally, samples

were gold coated using a SCD 040 Balzers Union sputter-coater. The testis casts were visualized and photographed using Philips ESEM XL30 and Hitachi S-3400N scanning electron microscopes. For further details on corrosion cast preparation, see the monograph by Aharinejad & Lametschwandtner (1997).

To study the seminiferous network system *in vivo*, we microinjected diluted solutions of trypan blue or bromophenol blue (both at 0.005–0.01% w/v) and viewed the testis using an optical stereomicroscope (Leica MZ Apo). Testes were analyzed as well by standard histological techniques. Briefly, testes were fixed overnight in Bouin's fixative, routinely processed, and embedded in paraffin blocks. Sections (5 µm thick) were stained with hematoxylin and eosin and photographed using light microscopy (Leica, DAS Mikroskop DMR). All protocols and procedures with live animals were carried out in accordance with current European regulations and approved by the Animal Welfare and Ethics Committee of the University of the Basque Country (CEBA/197/2011 and CEBA/168/2010).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-12-0043>.

Declaration of interest

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

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