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Reassembly of somatic cells and testicular organogenesis *in vitro*

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ABSTRACT

Testicular organogenesis *in vitro* requires an environment allowing a reassembly of testicular cell types. Previous *in vitro* studies using male murine germ cells cultured in a defined three-dimensional environment demonstrated tubulogenesis and differentiation into spermatozoa. Combining scaffolds as artificial culture substrates with testicular cell culture, we analysed the colonization of collagen sponges by rat testicular cells focusing on cell survival and reassembly of tubule-like-structures *in vitro*. Isolated testicular cells obtained from juvenile Sprague Dawley and eGFP transgenic rats were cultured on collagen sponges (DMEM high glucose + Glutamax, 35 °C, 5% CO_2 with or without gonadotropins). Live cell imaging revealed the colonization of cells across the entire scaffold for up to 35 days. After two days, histology showed cell clusters attached to the collagen fibres and displaying signs of tubulogenesis. Clusters consisted mainly of Sertoli and peritubular cells which surrounded some undifferentiated spermatogonia. Flow cytometry confirmed lack of differentiation as no haploid cells were detected. Leydig cell activity was detected by a rise of testosterone after gonadotropin stimulation. Our approach provides a novel method which is in particular suitable to follow the somatic testicular cells *in vitro* an issue of growing importance for the analysis of germ line independent failure of spermatogenesis.

1. Introduction

Testis formation is placed during embryonic development when primordial germ cells arrived at the genital ridges and are enclosed by differentiating Sertoli cells (reviewed in Aponte et al., 2005). Aggregation of somatic Sertoli cells is the first important step in seminiferous cord formation. Sertoli cells have physical contact to the germ cells and regulate the biochemical surroundings which are necessary for their maturation (Griswold, 1998; Russell and Steinberger, 1989). The somatic testicular cell types, *i.e.* Sertoli, myoid peritubular and Leydig cells enable and support spermatogenesis, the process of male gamete maturation. Peritubular cells are smooth-muscle-like, rhythmic contractile and transport immotile spermatozoa, which is important for male fertility (reviewed in Mayerhofer, 2013). Leydig cells stimulate germ cell maturation by testosterone production. Spermatogenesis results from the proliferation and differentiation of undifferentiated spermatogonia which originate from spermatogonial stem cells (SSCs). SSCs self-renew and give rise to differentiating daughter cells which enter the meiotic pathways (Ehmcke et al., 2006; Wistuba et al., 2007). Spermatogenesis in adult rats is initiated when SSCs are induced to spread mitotically and form more differentiated type A_1–A_4 spermatogonia. Afterwards they undergo multiple cell divisions before differentiating into B-spermatogonia. As spermatocytes, the germ cells proceed through meiosis into haploid round spermatids and finally into elongated spermatids (Huckins, 1971; Stukenborg et al., 2010; McLean et al., 2003).

To resemble these complex processes and interactions of testis formation and germ maturation in a culture dish would be a breakthrough offering many putative therapeutic routes, *e.g.* to overcome infertility by providing an effective fertility preservation option in prepubertal patients experiencing germ cell loss due to cytotoxic treatment (Jahnukainen and Stukenborg, 2012). Another option is the analyses of regulatory influences on peritubular cells and other

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; dpp, days post partum; eGFP, enhanced green fluorescent protein; hCG, human chorionic gonadotropin; LH, luteinizing hormone; MCS, methylcellulose system; PAS, periodic acid-Schiff; PFA, paraformaldehyde; r-hFSH, recombinant human-follicle-stimulating hormone; SACS, soft agar culture system; SD, Sprague Dawley rats; SEM, scanning electron microscopy; SSC, spermatogonial stem cell; TBS, Tris-buffered saline; VASA, DDX4, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4.

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somatic cell types and their role in male infertility (Welter et al., 2012).

In studies focusing on testis formation *in vitro* was demonstrated, that somatic Sertoli and peritubular cells organize themselves into cords in response to the underlying nanostructures or the composition of the three-dimensional matrix (Gassei et al., 2006; Pan et al., 2013). Although attempted for almost a century, studies aiming at *in vitro* spermatogenesis so far have failed to reconstruct the entire process in cell culture systems (for review, see Reuter et al., 2012; Huleihel, 2012). Recently, organ culture of mouse testicular tissues resulted in the production of fertility competent sperm (Sato et al., 2011a, 2011b, 2012). Furthermore, various approaches of culture and *in vitro* differentiation of male testicular cells including the germ line have been performed in the past (for review see Hunter et al., 2012; Reuter et al., 2012). In these experiments, novel three-dimensional culture systems like the soft agar culture system (SACS), the methylcellulose system (MCS) (Stukenborg et al., 2008, 2009; Abu Elhija et al., 2011), the cultivation in calcium alginate capsules (Lee et al., 2006a,b) and the cultivation in a three-dimensional collagen gel matrix system, succeeded in inducing spermatocytes to differentiate into spermatids *in vitro* (Lee et al., 2006a,b, 2007). In these approaches, maturation up to morphologically mature spermatozoa appeared independent of the matrix composition as long as three dimensions and structures were offered (Stukenborg et al., 2008, 2009). The gels seemed to support cellular interactions normally taking place in the seminiferous epithelia were thus consistent with organ culture approaches (Stukenborg et al., 2009; Lee et al., 2006a,b). Taken together, the results from studies indicated that the best conditions for germ cell maturation including developmental progress into meiosis and beyond, could be set up when the germ cells are supplemented with somatic testicular cells (Sertoli and Leydig cells) and gonadotropins (follicle stimulating hormone (FSH) and human chorionic gonadotropin (hCG) as a surrogate of luteinizing hormone (LH)) in monophasic three-dimensional matrices (Stukenborg et al., 2009), although the efficiency of gamete production remained limited.

However, apart from germ line failure there is increasing evidence that spermatogenesis requires the correct function and arrangement of somatic cell types (for review see Reuter et al., 2012). In addition to the well known functions of Sertoli and Leydig cells (*e.g.* Wistuba et al., 2007; Stukenborg et al., 2009) for successful spermatogenesis, recently, in particular the so far underestimated peritubular cells were demonstrated to be of importance for correct tubulogenesis and spermatogenic progress (Welter et al., 2012; Mayerhofer, 2013). To date, the culture approaches on testicular tissues and cells were targeted primarily toward the generation of gametes. However, tubulogenesis and the interplay of germ and somatic cells might be of relevance but have not been addressed thus far: Firstly, the majority of attempts focussed solely on germ cells to generate spermatozoa for use in assisted reproductive techniques and/or maintenance of spermatogonial stem cells as a source of pluripotent precursors for use in regenerative medicine and secondly there are as yet no *in vitro* systems available allowing complete reconstitution of the testicular architecture from isolated cell suspensions. Therefore, culture systems allowing both, the separate analysis of germ line maturation, and reconstruction of the spatial relation of the somatic cells *in vitro*, are of interest.

Artificial scaffolds consisting of collagen or gelatine are already used in other areas of regenerative medicine, as they allow oxygen and nutrient supply and display a framework for cell adhesion, proliferation and differentiation of mesenchymal stem cells (Takahashi et al., 2005; Bernemann et al., 2011). Therefore aiming at both the tubule-like rearrangement of the somatic cell types as well as at germ line maintenance, we assumed that the spatial structure and the variable pore sizes of collagen scaffolds may also provide either a framework for testicular cells to build up tubule-like-structures and therefore an option to study the impact of the somatic testicular cells or support complete spermatogenesis *in vitro*, or even both. We intend to proof the principle that collagen scaffolds can be colonized by testicular cells *in vitro* and maintained over substantial culture periods. Furthermore we addressed the question, whether and to which extent tubulogenic rearrangement and germ cell differentiation can take place in such scaffold systems.

2. Materials and methods

2.1. Animals

Testes were obtained from immature (7 days *post-partum* (*dpp*), $n = 37$) Sprague Dawley rats (obtained from JANVIER SAS, Le Genest Saint Isle, France) and enhanced green fluorescent protein transgenic (SD-Tg(CAG-EGFP)CZ-004Osb designated eGFP rats (obtained from the Central Animal Facilities of the Faculty of Medicine, University of Muenster, Germany) which expressed the transgene ubiquitously (Okabe et al., 1997). EGFP rats were obtained from our institutional colony. Animals were maintained under a 12:12 h light dark regimen with access to water and food *at libitum*. In order to control proliferative activity of the cultured cells, we used testicular tissue from aged matched rats that were injected with 5′bromo-2-desoxyuridine (BrdU) 2 h prior to decapitation (50 μL of a 2% solution per g bodyweight; i.p.). All animal experimental procedures were performed in accordance with the German federal law on the handling of experimental animals (LANUV NRW animal license nos. 8.87-50.10.46.08.199 and 8.87-50.10.46.09.053).

2.2. Testicular cell isolation

Testicular cells were isolated on day 7 *pp*. To obtain a single-cell suspension from the tissue, a two-step enzymatic digestion protocol was applied (Wistuba et al., 2002). Testes were removed, decapsulated and dissected with fine scissors. The minced tissue was transferred into culture medium (Dulbecco's modified Eagle medium (DMEM) high Glucose + Glutamax (Gibco-Invitrogen, Darmstadt, Germany) containing collagenase type 1A, (1 mg/mL; Sigma–Aldrich, St Louis, MO, USA) and deoxyribonuclease type I (DNase), (0.5 mg/mL; Sigma–Aldrich). First digestion was performed at 37 °C for 10 min in a shaking water bath. A fraction of tubular fragments and single cells was obtained and separated by sedimentation. The supernatant containing the single cell fraction was removed after 10 min and stored on ice. The tubular fraction was further digested in culture medium containing collagenase type 1A (1 mg/mL; Sigma–Aldrich), DNase I (0.5 mg/mL; Sigma–Aldrich) and hyaluronidase type II (from sheep testes, 0.5 mg/mL; Sigma–Aldrich) (according to Wistuba et al., 2002) in a shaking water bath (37 °C for 20 min). The suspension was then aspirated three times with a pipette and digestion was mechanically continued until the tubules were completely dissociated. Both fractions were combined and washed with medium. Cell numbers were determined microscopically using a Neubauer chamber (Neubauer improved bright line superior, Marienfeld laboratory glassware, Lauda-Königshofen, Germany) and the trypan blue (Sigma–Aldrich) test was used to evaluate the vitality of the single cells (Fig. 1).

2.3. Cell seeding and culture in artificial scaffolds

Scaffolds with a loading capacity of 30 μL (Matricel, Herzogenrath, Germany, 5 × 1.5 mm) were used. Applying the drop-on seeding method (as described by the manufacturer), 40 μL of