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Testicular organoid generation by a novel *in vitro* three-layer gradient system

João Pedro Alves-Lopes*, Olle Söder, Jan-Bernd Stukenborg

Department of Women's and Children's Health, NORDFERTIL Research Lab Stockholm, Pediatric Endocrinology Unit, Q2:08, Karolinska Institutet and Karolinska University Hospital, SE-17176 Stockholm, Sweden

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ABSTRACT

A system that models the testicular microenvironment and spermatogonial stem-cell (SSC) niche *in vitro* has not been produced yet. Here, we developed and characterized a novel three-dimensional multilayer model, the Three-Layer Gradient System (3-LGS), which permits the generation of rat testicular organoids with a functional blood-testis barrier (BTB) and germ cell establishment and proliferation. The model is unique as regards the formation of cellular organizations that more closely represent the *in vivo* germ-to-somatic cell associations *in vitro*. Moreover, we also verified the roles of retinoic acid (RA), IL-1α, TNFα and RA inhibitors in germ cell maintenance and BTB organization *in vitro*. Treatment with RA was beneficial for germ cell maintenance, while IL-1α and TNFα were observed to impair the formation of testicular organoids and germ cell maintenance. Taking in account our characterization and validation results, we propose the 3-LGS as a new platform to investigate the SSC niche *in vitro* and to search for novel unknown factors involved in germ cell proliferation and differentiation. Moreover, we suggest that this model can be used in other scientific fields to study organogenesis and development by the generation of organoids.

1. Introduction

The testis has two main roles in male organisms: production of gametes and synthesis of androgens. The seminiferous tubules are composed of an internal epithelium formed of Sertoli cells, needed for germ cell differentiation, and external layers of smooth muscle-like cells known as peritubular cells. A network of thigh junctions (composed by zonula occludens-1/tight junction protein 1 (Zo-1/TJP1) and occludin proteins) between adjacent Sertoli cells forms the blood-testis barrier (BTB) and compartmentalize the seminiferous tubules in basal (below the BTB) and adluminal compartment (above the BTB) [1,2]. Moreover, the seminiferous tubules are supported by the interstitial tissue, which supplies oxygen, nutrients and important paracrine and endocrine hormones [3].

An *in vitro* system to model the testicular microenvironment has been tried for the last few decades. One of the first approaches applied was germ cell culture or co-culture on a monolayer of feeder cells in a two-dimensional (2D) fashion using standard culture dishes [4,5]. Although germ cells were co-cultured in close contact with feeder cells, such as Sertoli cells, it seems that the three-dimensional (3D) organization present *in vivo* is essential for germ cell proliferation and differentiation *in vitro* [6]. These observations led researchers to explore 3D culture approaches in order to closely model the testicular microenvironment *in vitro*. The formation of *de novo* seminiferous tubule-like structures was demonstrated when pre-pubertal primary testicular cells from rats and piglets were cultured into a supportive matrix [7] and/or grafted under the skin of immunodeficient mice [8,9]. Other researchers obtained similar structures when pre-pubertal mouse testicular cells were allowed to form cellular pellets which were then cultured on top of agar blocks [10]. Furthermore, spermatogenesis was recapitulated *in vitro* when pre-pubertal mouse testicular cells were cultured into agar and for 31 days [11–13].

An alternative 3D approach is to culture small testicular tissue samples placed on top of an agar block [14–16]. The principle of this method was already described in the 20th century [17]. However, it was not until 2011 that Sato and colleagues for the first time reported the birth of healthy mice after intra-cytoplasmic sperm injection using *in vitro*-produced sperm [18]. Recently, this system has been used by our group to differentiate rat spermatogonia into round spermatids [15]. Although *in vitro* production of functional

* Corresponding author.
E-mail address: joao.lopes@ki.se (J.P. Alves-Lopes).

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sperm has been achieved in mice, a lot of basic understanding regarding testicular physiology remains to be studied, namely aspects related with the regulation of the spermatogonial stem-cell (SSC) niche in rats and humans. Furthermore, none of the previous models give a completely controlled *in vitro* platform to follow and influence testicular development and spermatogenesis. Thus a model which permits replication of testicular organogenesis and functionality is importantly needed.

In this work we aimed to develop and characterize a system that allows the reorganization of dissociated rat testicular cells into organoids that more closely represent the *in vivo* germ-to-somatic cell associations *in vitro* (Fig. 1). The Three-Layer Gradient System (3-LGS) permits the generation of testicular organoids with a functional BTB and germ cell establishment and proliferation. This model represents a new platform to explore, exclusively *in vitro*, the role of novel factors as well as the importance of distinct somatic testicular cells in the SSC niche.

2. Material and methods

2.1. Animals

Testes from 5–8-, 20- and 60-day *post partum* (d*pp*) Sprague Dawley male rats were collected and enzymatically dissociated in order to obtain total testicular cell suspensions. Animals were delivered by Charles River (Sulzfeld, Germany). All the experimental procedures were carried out in agreement with ethics permit number 280/14 accorded by Karolinska Institutet Ethics Committee for Experimental Laboratory Animals.

2.2. Sequential digestion

Rat testes were sequentially digested in order to obtain total testicular cell suspensions. Briefly, per experiment, 8 testes from 5–8-d*pp* rats, 4 testes from 20-d*pp* rats and ¼ testes from 60-d*pp*

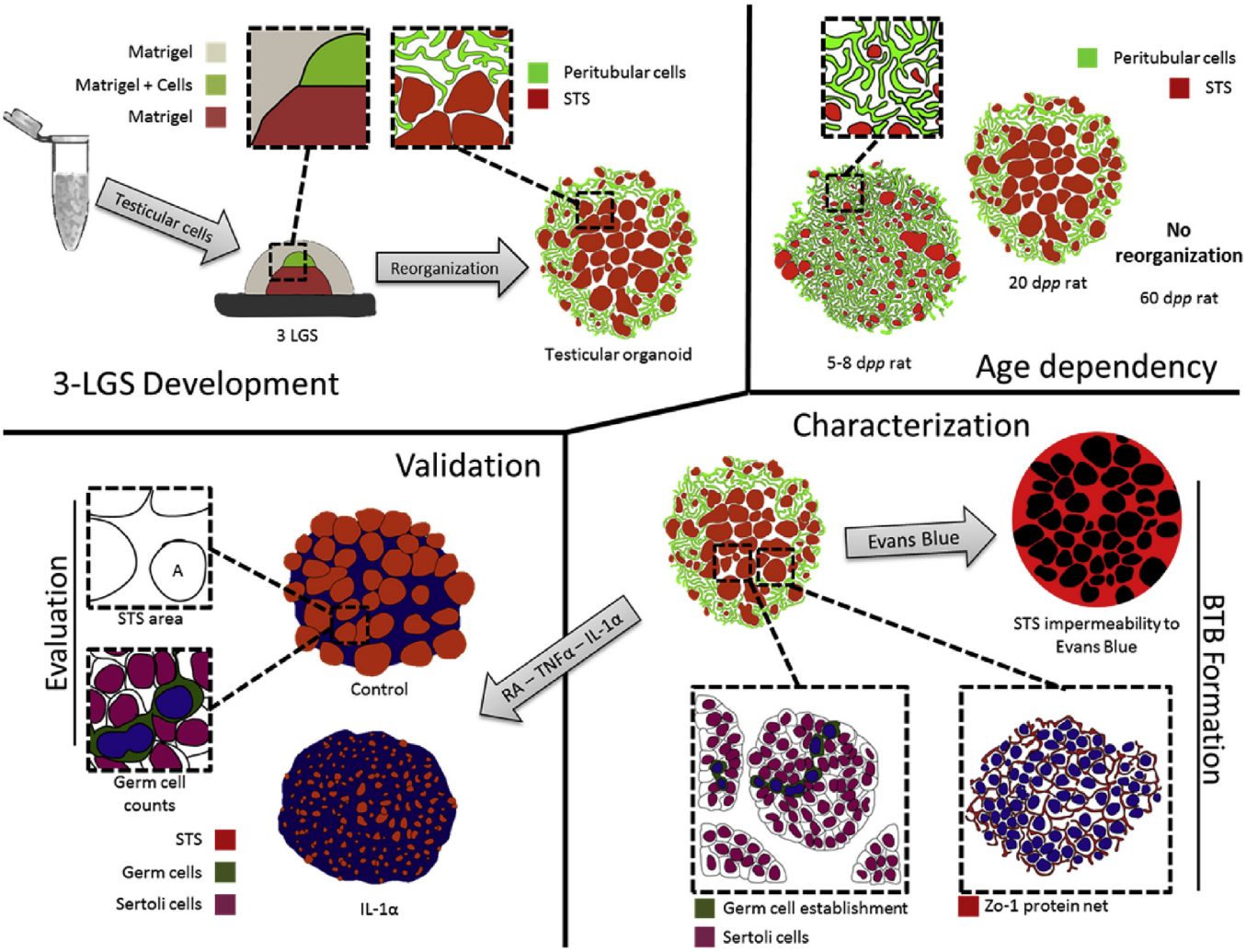

Fig. 1. Schematic overview of development, characterization and validation of the 3-LGS as a model to generate testicular organoids. During 3-LGS development, 20-day *post-partum* (20-d*pp*) rat testicular cells were combined with Matrigel and placed between two layers of that same material without cells. This arrangement led to cellular reorganization into spherical-tubular structures (STSs) and testicular organoid formation. Differences in colony organization were identified when testicular cells from different rat maturational stages were used to generate organoids. Characterization of 20-d*pp* rat testicular organoids showed that STSs are mainly formed of Sertoli cells and that germ cells can establish and proliferate on these structures. A functional blood-testis barrier was also displayed by STS impermeability to Evans Blue and detection of tight junction proteins. System validation was performed by verifying the roles of endogenous factors (RA, IL1-α and TNFα) and exogenous RA inhibitors (HX 531 and ER 50891) in germ cell maintenance and blood-testis barrier organization. These factors, with known effects *in vivo* on the testicular microenvironment, displayed similar influences on the 3-LGS organoids *in vitro*.