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Analysis of the intratesticular control of spermatogenesis by *ex-vivo* approaching

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Abstract: Spermatogenesis involves the realization of a particular genetic program which requires a specific environment ("niche"). Multiplication, differentiation and apoptosis of male germ cells are finely regulated by pituitary hormones (mainly LH and FSH), and by a complex network of factors originating from both the somatic cells and the germ cells of the testis. It is becoming clear that hormones and intra-testicular regulatory factors can compensate, at least in part, for the absence of some hormones or factors including FSH and LH or androgen receptors. Since, most of the growth factors, cytokines and neurotrophins produced within the testis are widely expressed in the organism, the attempts to understand their role in spermatogenesis by "classical" knock-out strategies have been often disappointing. Therefore an important aspect of our previous work was to settle and characterize carefully two systems of cocultures of testicular germ cells with somatic cells in bicameral chambers. For instance, we showed for the first time that the whole meiotic step could be performed *in vitro* in a mammalian species (the rat). Moreover, all our data indicate that our co-culture systems enable to highlight mechanisms pertinent to the physiological processes. Sperm parameters have been deteriorated considerably during the past 4-5 decades. There is now evidence that chemical exposure is at least partly responsible for these testicular diseases. If a large number of environmental pollutants are able to affect male fertility and to exert carcinogenic effects, their cellular and molecular mechanisms are still unidentified. The cultures in bicameral chambers that we settled can be used to study the effects of a toxicant when added in the basal compartment of the culture chamber, which appears relevant to the *in vivo* situation. Taken together our results indicate that our *in vitro* culture systems, which allow screening for the effect of biological activity of different physiological factors, can be also helpful to study that of any chemicals on both survival and multiplication/differentiation of somatic and/or spermatogenic cells on a relatively long time period.

Key words: spermatogenesis, germ cell culture, testicular regulations, testicular toxicology

Introduction

Spermatogenesis involves the realization of a particular genetic program that requires a specific environment ("niche"). Multiplication, differentiation and apoptosis of male germ cells are finely regulated by pituitary hormones, mainly LH and FSH [1], and by a complex network of factors originating from both the somatic cells and the germ cells of the testis [2], as well as by direct communications between supporting somatic Sertoli cells and germ cells [3].

It is becoming clear that hormones and intra-testicular regulatory factors can compensate, at least in part, for the absence of some hormones or factors including FSH [4-6] and androgen [7-10], or luteinizing hormone [11] receptors. Since most of the factors produced within the testis are also widely expressed in other organs, the attempts to understand their role in spermatogenesis by conventional knockout (KO) strategies have been limited. Some mice die shortly

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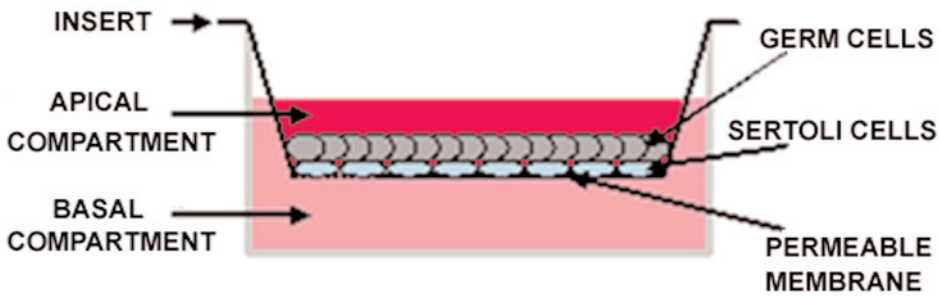

Fig. 1. Culture system of germ cells together with Sertoli cells, in bicameral chambers.

after birth (*e.g.* KO of insulin-like growth factor-I, IGF-I, [12]; transforming growth factor β (TGF-β) [13]; nerve growth factor (NGF) [14], or they do not exhibit any particular phenotype on male fertility (*e.g.* KO of IGF-II, IGF-binding protein-2 to -6 (IGFBP-2 to -6) [15]. Moreover, even if for some of these factors produced by, and acting on, testicular cells, KO models or spontaneous genetic defects have allowed to understand their role on the early steps of spermatogenesis (*e.g.* stem cell factor (SCF) [16], Glial cell line-derived neurotrophic factor (GDNF) [17], their action, if any, on later steps of spermatogenesis could not be studied since invalidation of these genes results in an early blockade of the spermatogenic process. In addition, ubiquitous factors (such as IGF-I, TGF-β) may be expressed by several cell types in the testis and their role can change during development and spermatogenesis [18].

Physiological studies in *ex-vivo* models

Therefore, an important aspect of our work, over the past twelve years, was to settle and characterize carefully two systems of co-cultures of testicular germ cells with somatic cells in bicameral chambers (Fig.1), in a defined culture medium, which maintain the blood testis barrier (Figs. 2, 3) and enable to study the regulations involved in the mitotic phase and the meiotic phase of spermatogenesis, and the early steps of spermiogenesis over a 4-week culture period in the rat. In the first system, elutriated spermatogonia/early spermatocytes, pachytene spermatocytes or round spermatids are cultured on a monolayer of Sertoli cells, whereas in the other system, small pieces of seminiferous tubule segments are seeded [19-21]. In these systems, it is indeed possible to study: 1) the physiology of the blood-testis barrier; 2) survival / death of somatic and/or germ cells, proliferation of Sertoli cells and spermatogonia; 3) the course of meiotic divisions (key stage of spermatogenesis during which genetic recombinations occur); 4) the cytogenetic features of germ cells; 5) the expression of specific genes in the germ cells or Sertoli cells; 6) the peptide profiling of the culture supernatant/cultured cells.

An important aspect of the results obtained using *in vitro*/*ex-vivo* models is their relevance to the *in vivo* physiological situation. The germ cell/Sertoli cell coculture systems that we settled [19-21] have been carefully validated from the physiological point of view, on many aspects, over the last twelve years. To our knowledge, there is no other system of culture of male germ cells and Sertoli cells which has been so carefully and extensively validated.

We were the first to show that the whole meiotic process could occur *in vitro* in a mammal, the rat [22]. This was ascertained by cytological and cytometrical analyses and by the expression of germ cell specific genes. We showed subsequently that the development of the meiotic step, *in vitro*, in the testis of pubertal rats was close to what happens *in vivo* when considering the changes in the cell populations of different ploidy, the gene expression of germ cells, the kinetics of differentiation of BrdU-labeled early or middle pachytene spermatocytes and the levels of apoptosis in the different cell populations, even if the rate of *in vitro* differentiation of BrdU-labeled spermatocytes slowed down when reaching the stage of middle pachytene spermatocyte [23]. More recently, we also demonstrated that there was no significant difference between the percentages of leptotene, zygotene, pachytene, and diplotene stages in 42 day-old rats and on day 16 of culture of testes from 23 day-old rats (Fig. 4), indicating a similar development *in vivo* and *in vitro* (Geoffroy-Siraudin *et al* submitted).

Our findings also showed that FSH and testosterone have positive and somewhat overlapping effects on the meiotic divisions and the post-meiotic expression of a germ cell-specific gene (transition protein1; TP1), effects which cannot be related solely to their ability to reduce germinal cell apoptosis [24].These results have been recently confirmed by others using KO mouse models [8,9,25].

Recently we have reported that NGF and its two receptors are similarly expressed *in vivo* and under our culture conditions and that both NGF and TGF-β are able to regulate negatively the second meiotic division of rat spermatocytes [26, 27]. More recently we have demonstrated that NGF and TGFβ have a totally

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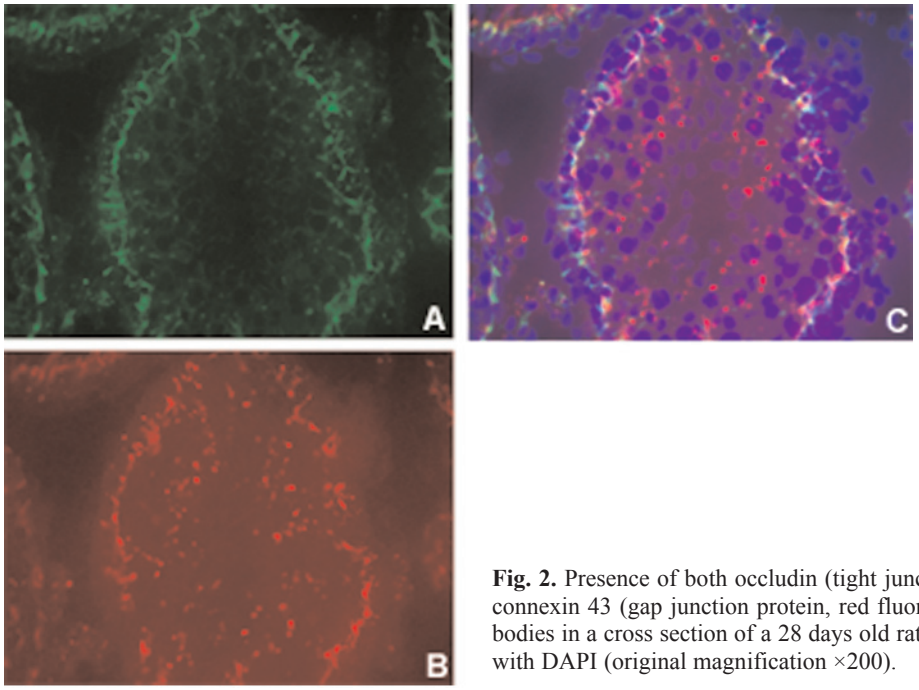

Fig. 2. Presence of both occludin (tight junction protein, green fluorescence) (**A**) and connexin 43 (gap junction protein, red fluorescence) (**B**), detected with specific antibodies in a cross section of a 28 days old rat testis. (**C**) merge; the nuclei were stained with DAPI (original magnification ×200).

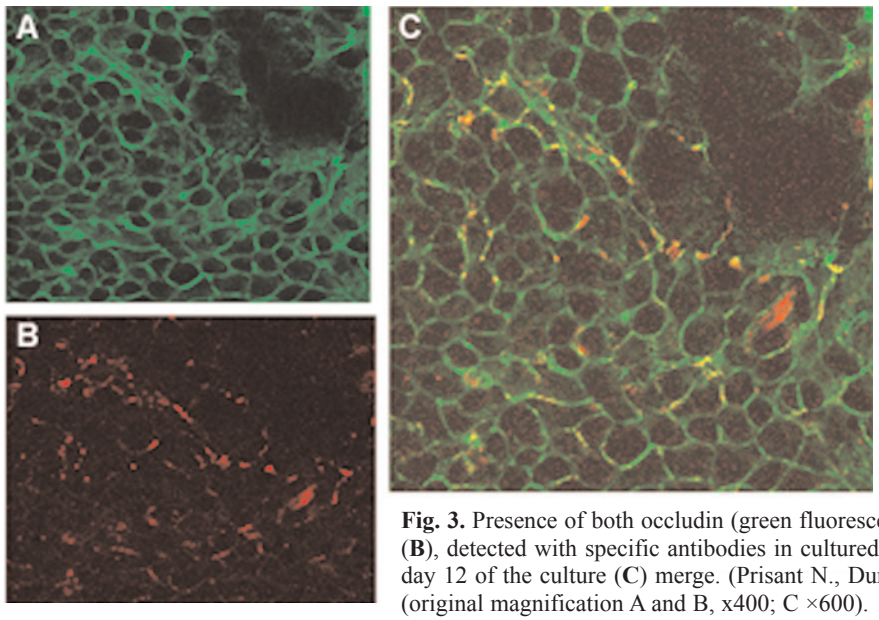

Fig. 3. Presence of both occludin (green fluorescence) (**A**), and connexin 43 (red fluorescence) (**B**), detected with specific antibodies in cultured seminiferous tubules from 20days old rats at day 12 of the culture (**C**) merge. (Prisant N., Durand P. Segretain D., Pointis G. unpublished). (original magnification A and B, x400; C ×600).

redundant (without additivity) effect on this step (Perrard MH and Durand P 2009 in press). This result offers an explanation to the study of Ingman and Robertson [28], with null mutant mice for TGF-β1, claiming that their data conflicted with conclusions from *in vitro* culture experiments that TGFβ1 might act to impair spermatocyte progression through the second meiotic division [26]. Indeed, these authors did not observe a change at this level in their mouse model, but it must be emphasized that male gametes synthesize TGFβ1 with greatest abundance detected in spermatocytes and early round spermatids [29], *i.e* precisely, the same germ cell types which synthesize β-NGF [27]. Therefore, it is reasonable to speculate that the absence of effect of TGFβ1 KO, reported by these authors, was due to the redundant effect of β-NGF on that step.

We have shown that *in vitro* meiotic progression of rat spermatocytes requires mitogen-activated protein kinases of Sertoli cells and close contacts between the germ cells and the Sertoli cells [30]. Now, connexin 43 is detected between spermatocytes and Sertoli cells in our cultures. These results fit with a recent study [31] showing that replacement of connexin 43 by connexin

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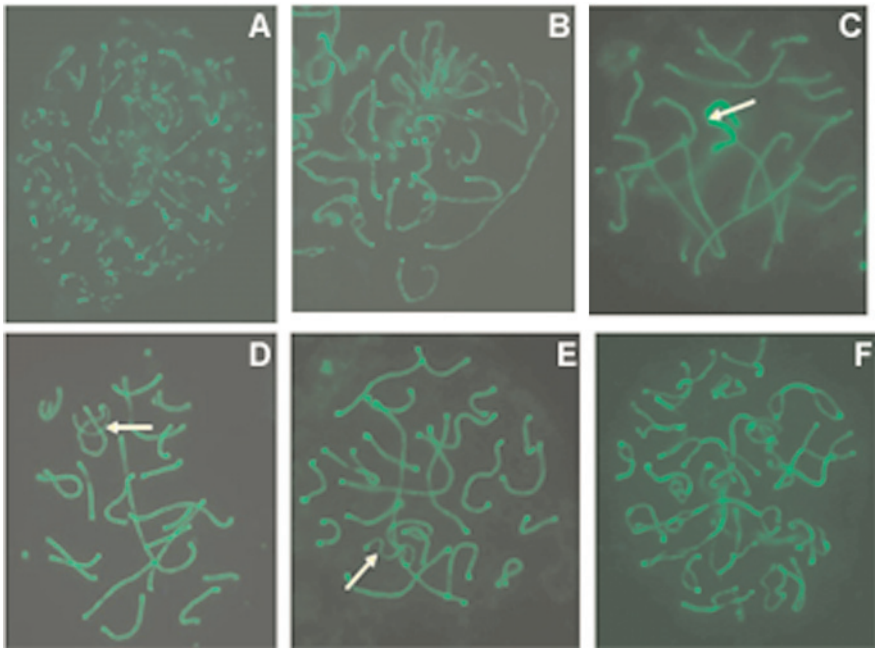

Fig. 4. The different sub-stages of meiotic prophase I in seminiferous tubule cultures: an anti-SCP3 antibody was used to reveal axial elements and lateral elements of synaptonemal complexes. Arrows show different configurations of the sex chromosomes. (**a**) leptotene spermatocyte; (**b**) zygotene spermatocyte; (**c, d, e**) pachytene spermatocyte; (**f**) diplotene spermatocyte. (Geoffroy-Siraudin C, Durand P, Guichaoua MR unpublished) (original magnification ×1000).

26 in transgenic mice impairs spermatogenesis leading to the absence of germ cells beyond spermatocytes type I. Other studies in our laboratory demonstrated that both meiotic divisions are blocked by pharmacological inhibitors of MPF [32], as could be expected [33]. On the mitotic step of spermatogenesis, we have got results consistent with a role of GDNF in inhibiting the S-phase entrance of a large subset of differentiated type A spermatogonia, together with an enhancing effect of the factor on a small population of undifferentiated (stem cells) spermatogonia [34]. Actually, these studies fit quite well with the results of *in vivo* studies [17,35-37]. Further, we have recently shown that Cx43 gap junctions between Sertoli cells participate in the control of Sertoli cell proliferation and that Cx43 gap junctions between Sertoli cells and spermatogonia are indirectly involved in germ cell number increase by controlling germ cell survival rather than germ cell proliferation [38]. Similar results were obtained by the observation of Sertoli cell conditional Cx43 KO mice demonstrating that Cx43 is essential for control of Sertoli cell proliferation and differentiation [39,40].

Taken together, the above data reinforce the view that our co-culture systems enable to highlight mechanisms pertinent to the physiological processes.

Assessment of toxicological potency of chemicals

Sperm parameters have been deteriorated considerably during the past 4-5 decades. There is now evidence that chemical exposure is at least partly responsible for these testicular diseases. If a large number of environmental pollutants are able to affect male fertility and to exert carcinogenic effects, their cellular and molecular mechanisms are still unidentified. The cultures in bicameral chambers that we settled should be a major methodological breakthrough in assessing toxicological potency of chemical compounds on a relatively long time period and enabling the study of many aspects of their mechanism of action while reducing the number of animals needed. In these culture systems, testicular germ cells from one rat allow to perform ten to twenty different assays. It is noteworthy that our systems of culture in bicameral chamber allow studying the effects of a toxic substance added to the basal compartment of the culture chamber, and thus mimic what could happen *in vivo* in the testis. Indeed, the cellular junctions, between Sertoli cells and between Sertoli cells and germ cells, which are essential for spermatogenesis, are maintained or rebuilt in our systems of culture (see above). Therefore, before reaching the germ cells, in our systems, the toxic compounds must cross the barrier structured by Sertoli cells (principal component the blood-testis barrier). By contrast, in "conventional" cultures wells, a compound may be toxic to differentiated germ cells because it is placed directly in contact with these cells, whereas *in vivo*, it may not have access to the compartment of the seminiferous tubules where this population of germ cells is located. Furthermore, our cultures can be analyzed by cell physiology, cytology, biochemical and molecular biological approaches allowing the determination of the mechanisms responsible for the gonadal toxicity or carcinogenic effect of organic or mineral compounds and of nanoparticles. It should be emphasized that knowledge of the mode of action of the molecules studied can help to determine whether the obser-

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vations made in animals are transferable to humans, which remains a major problem in this type of study.

Our co-culture systems can also serve as an original tool for rapid screening of molecules for therapeutic purposes in order to improve a failing male fertility. The use of a cell population exposed (lot) or not (control group) to the substance allows to get rid of much of the variability between animals that are encountered in testing *in vivo* and to optimize the power of the tests. In addition, the possibility of rather long term cultures of the cells will allow, most often, testing for the reversibility of the observed effects. Recently, we compared cultures of normal, and irradiated by gamma rays, germ cells. In spermatocytes for non-irradiated cultures, the comet assay revealed the presence of breaks of DNA, whose number decreased during the culture, indicating the involvement of mechanisms of DNA repair associated with meiotic recombination. In irradiated cells, the development of DNA strand breaks was heavily modified. Thus, our model is able to detect genotoxic lesions and / or abnormal DNA repairing [41].

By using these cultures, we have observed that chromium at low concentrations (1 µg/L) severely alters the synaptonemal complexes of the meiotic cells, and decreases by 40% the number of round spermatids formed in culture (Geoffroy-Siraudin *et al* submitted).

Taken together these results indicate that our *in vitro* culture systems, which allow screening for the effect of biological activity of different physiological factors, can be also helpful to study that of any chemicals (either alone or in combination with others) on both survival and multiplication/differentiation of somatic and/or spermatogenic cells on a relatively long time period.

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