International Journal of Genetics and Molecular Biology Vol. 3(2), pp. 39-44, January 2011
Available online at http://www.academicjournals.org/ijgmb
ISSN2006-9863 ©2011 Academic Journals

Full Length Research Paper

Optical microscope gross characterization of rat neonatal testicular germline stem cell expansion *in vitro*

Osunkwo, U. A.[1]*, Ezeunala, Mercy N.[2], Akuodor, G. C.[1], Giwa-Amu, Judith Osunkwo[2], Damaris, A.[3], Duru, M. O.[1], Idris-Shehu, Maryam[1], Odo, Eucheria[1], Ugwu, Theresa C.[1], Nwalozie, C.[1], Mgbemere, Chidimma[1] and Agonsi, Chinyere[1]

[1]Stem Cell Research Laboratory, Department of Pharmacology and Toxicology, National Institute for Pharmaceutical Research and Development, Idu, Abuja, Nigeria.
[2]Department of Microbiology and Biotechnology, National Institute for Pharmaceutical Research and Development, Idu, Abuja, Nigeria.
[3]Department of Medicine, National Hospital, Abuja, Nigeria.

Accepted 21 January, 2011

Neonatal testicular germline stem cell suspension prepared from twenty 3-5 day old albino rats were incubated in normal saline or in NC-01in triplicate groups. A control group of saline alone (without cell suspension) was also prepared. The cell-containing groups and control were incubated for 21 days at room temperature, stained with 50% Mezo (organic) and visually studied daily with a view to histological characterizing the changes in cellular aggregation, morphology and coloration during incubation. Images of dense oval cells and cellular developmental transformation into mixed populations of these testicular cells showed clearly identifiable patterns of clump formation. Saline alone (control) did not show the presence of any cells during the period of incubation. *In vitro* expansion of neonatal germline stem cells within 21 days of incubation was associated with multiple divisions into numerous structurally similar cells and transformation into clusters of multi-colored component rod-like cells. A many-fold increase in the number of dense oval cells was observed in the NC-01 culture medium than in the normal saline. Using these morphological criteria and functional characteristics, we suggest a many-fold increased presence of rat neonatal spermatogonial stem cells capable of cellular division in NC-01 culture medium than in normal saline.

Key words: Rat, neonatal germline stem cell, Mezo stain, cell suspension, NC-01.

INTRODUCTION

Neonatal testicular stem cells originating from the mouse (Kanatsu-Shinohara et al., 2005; Kanatsu-Shinohara et al., 2007) or rat (Munsie et al., 1997; Ravindranath et al., 1997; Orwig et al., 2002; van Bragt et al., 2008) are thought to be the main occupants of this tissue within the first few days of life. In the first 3-7days postpartum, seminiferous tubular type A spermatogonia have been reported to renew themselves and to differentiate. This spermatogonial stem cell proliferation and subsequent differentiation forms the basis of spermatogenesis and perpetuity of species in adult life (De Rooij and Russell, 2000; De Rooij and Van Pelt, 2003). Conservation of self renewal signaling (Ryu et al., 2005) and genetic properties for producing rats (Shinohara et al., 2006) following interspecies spermatogonial transplantation between mice and rats has been reported.

The population of early A spermatogonia in rat neonatal testis has also been reported to strongly express the pluripotency marker, undifferentiated embryonic cell

*Corresponding author. E-mail: ucheosunkwo@yahoo.com. Tel: +234 803 329 6137.

transcription factor 1 (van Bragt et al., 2008). While studying stem cell and niche development in the postnatal rat testis, Ryu et al. (2003) reported a many-fold increase in stem cell activity during neonate to adult testicular development, and also that testicular spermatogenic colonies of similar size were produced in neonate, pup and adult donors. Stem cell activity during testicular development is associated with stimulation of primary cultures of rat type-A single spermatogonia to develop into chains of aligned spermatogonia at the 8-, 16-, and 32-cell stages and has resulted in the identification of neuregulin as a factor required for formation of aligned spermatogonia (Hamra et al., 2007). Again, a soluble factor promoting proliferation of spermatogonial stem cells has been reported to be the glial cell line-derived neurotrophic factor (GDNF) (Kubota et al., 2004). A combination of these factors during testicular development is associated with formation of densely packed clumps of cells which continuously proliferated and were identified as spermatogonial stem cells (Kubota et al., 2004; Kubota and Brinster, 2006).

In the report of Orwig et al. (2002), morphological identification of pseudopod and round gonocyte populations in neonatal rat testis cell suspensions is suggested to provide a powerful tool indicating a nearly pure population of male germ-line stem cells. Thus, these cells will make a rich source of stem cell population that can be used for various scientific reasons including manipulation of cellular genetic mechanisms involved in self-renewal and differentiation, and possibly mechanisms of safety and efficacy of drugs. Another possibility of finding suitable culture media that encourages stem cellular transformation *in vitro* at room temperature as well as defining the temporal relationships between expanding neonatal testicular germline stem cells exists. However, there seem to be an inadequate characterization of rat testicular neonatal germline stem cell alterations in cellular aggregation, morphology and coloration during *in vitro* expansion. Furthermore, a temporal relationship may exist between cells and their transformations during neonatal testicular germline stem cell expansion. For this reason, we adapted the optical microscope for digital interface to further examine rat testicular neonatal germline cell suspensions for their characteristic morphological properties during *in vitro* cell expansion and development.

MATERIALS AND METHODS

Preparation of neonatal testicular germline stem cells for histological examination

Three to five day old male albino rats weighing 4.7 – 5.0 g were exsanguinated and their abdominal and scrotal regions were immediately swabbed using 70% ethanol before opening of the lower abdominal region to dissect and to expose the tiny bulb-like testis. This was carefully dissected out under sterile conditions and separated from other attaching tissue. The testis was ruptured into Petri dish containing normal saline or NC-01 (a newly formulated room temperature culture medium for spermatogonial stem cells). There-after, testicular cell suspension samples taken from the Petri dish were placed on glass slides and stained with 50% Mezo (organic stain) for 15 min and then decolorized using 70% ethanol in water. Glass slides were subsequently rinsed with distilled water and air-dried for 10 – 15 min. Neonatal testicular germline stem cells in saline, NC-01 or saline alone control on glass slide was examined under an optical microscope with X40 or X100 (oil immersion) eye piece interfaced with a camera and computer output for higher than normal magnification and better resolution. The photomicrographs were digitally stored or printed out on glossy photographic paper.

Incubation of neonatal testicular germline stem cells suspension

Neonatal testicular germline stem cells extracted from 3-5 day old albino rats were used to prepare cell suspensions and these were incubated in varying test solutions at room temperature in the dark for 21 days. One drop of the incubated samples was withdrawn for histological examination. The control group consisted of normal saline alone in Petri dishes and this was incubated under conditions similar to those used for Petri dishes containing neonatal testicular germline stem cells. Room temperature (25 ± 3°C) was approximately similar for all the days of incubation. Samples of incubated cell suspensions as well as those from the control group were examined daily at about the same time of the day for alterations in cellular aggregation, morphology and coloration.

Examination of neonatal testicular germline stem cell expansion in vitro

Neonatal testicular germline stem cell suspension samples or saline alone control on glass slide was histological examined daily for 21 days. Saline alone (control) or cellular suspension sample was taken daily and placed on to glass slides and prepared for histological examination as described above. In addition, care was taken during daily examination of cell culture samples and control to minimize contamination with extraneous cellular materials. The cells were clearly identifiable under the microscope and these were manually counted on the slide-view. The cell number estimate in each slide view was compared with others and used as observations representative of seminiferous tubular cellular content. Images of dense oval cells characteristic of neonatal testicular germline stem cells suspension and changes in cellular aggregation, morphology and coloration during *in vitro* developmental transformation were observed.

RESULTS AND DISCUSSION

Self renewal and proliferation of dense oval cells during incubation

One drop of cellular suspension examined histologically before incubation showed the presence of some dense oval cells in a slide view (Figure 1A) and each dense oval cell was characterized by a fine capsular luster. Histological examination of a slide view of one drop of cellular suspension in saline alone on day 3 and on day 6 (Figure 1B) or in NC-01 on day 3 and on day 6 (Figure 1C) of

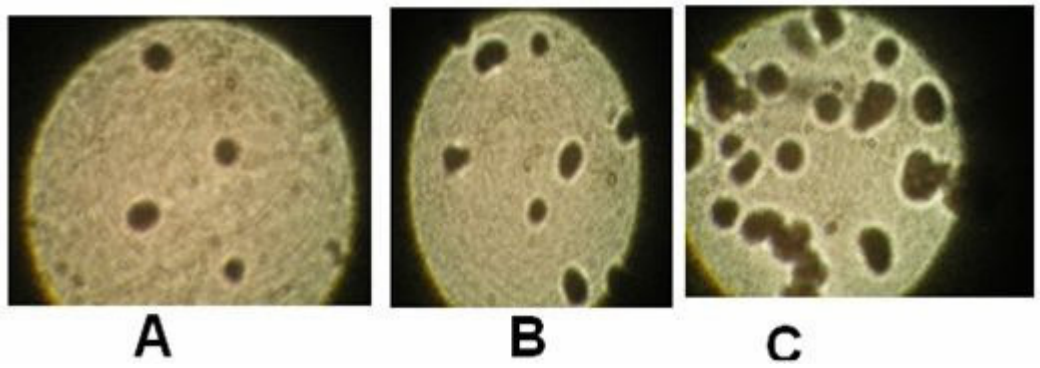

Figure 1. Photomicrographs showing (A) Some dense oval cells prior to incubation in culture medium; (B) self renewal and proliferation of dense oval cells in saline alone on day 3 or on day 6; (C) self renewal and proliferation of dense oval cells in NC-01 on day 3 or on day 6 of incubation. Images of cell proliferation are similar for days 3 and 6.

incubation showed larger number of the dense oval cells. There were many more cells in NC-01 than in saline (Figure 1C).

Clump formation from dense oval cells during incubation

An aggregation of cells in saline alone (Figures 2A, 2B and 2C) or in NC-01 (Figure 2D) that are smaller in size than their predecessor cells was observed during the 14 days of incubation. This clump formation within 14 days resulted in the presence of many clumps of cells showing three identifiable morphologies (Figures 2A, 2B and 2C). Cells of the same clump possessed similar morphology.

Transformation into dense clusters of multi-colored component rod-like cells

Histological examination within 14 days of incubation showed that the aggregated cells in saline alone (Figure 3A) or in NC-01 (Figure 3B) that are smaller in size than their predecessor cells also possessed variations in cellular component coloration when compared with observations within day 6 of incubation. In summary, neonatal testicular germline stem cell expansion *in vitro* was characterized by an observable progressive self renewal and proliferation into dense oval cells within three or six days of incubation and subsequently exhibited transformation into dense clusters of multicolored component rod-like cells within fourteen days of incubation in suitable culture medium at room temperature. However, by twenty one days of incubation, only a scanty number of single oval cells that have lost their capsular luster were present.

The results of this study showed that neonatal testicular germline stem cell expansion *in vitro* was characterized by an observable progressive self renewal and proliferation into dense oval cells during a period of incubation in saline or in NC-01 and at room temperature. In the rat, germline stem cells have been reported to be the major occupants of neonatal testicular tissue cell suspension in the first 3-7 days after litter (van Bragt et al., 2008; Munsie et al., 1997; Ravindranath et al., 1997; Orwig et al., 2002) and the seminiferous tubular type A spermatogonia of the cell suspension are known to renew themselves and to differentiate. Differentiation of germ cells is known to take place in the seminiferous tubules of the testis where a population of diploid stem-cell spermatogonia lying on the basement membrane of the tubule repeatedly undergoes self-renewal to produce progeny cells that initiate the process of cellular differentiation and eventually generating mature spermatozoa (Ogawa et al., 1997). De Rooij (1988) has suggested that this process is determined by many factors in deciding fate of a single spermatogonial stem cell (it could be self-renewal, proliferation or differentiation).

The observation of progressive self renewal and proliferation of testicular germline stem cells into dense oval cells during a period of incubation in our study was therefore consistent with observations in these previous reports (van Bragt et al., 2008; Munsie et al., 1997; Ravindranath et al., 1997; Ogawa et al., 1997). Self renewal and proliferation of dense oval cells in saline or in NC-01 during the 21 day incubation observed in our studies represent known spermatogonial stem cell activities originating from testicular tubular basement membrane. It may suffice to agree with the suggestions in the report of Kanatsu-Shinohara et al. (2008) that in our saline or NC-01 culture medium at room temperature, the major constituents of this germline cell suspension would be spermatogonial cells which can now be subjected to long-term *in vitro* expansion and that the postnatal testis has become a rich source of pluripotent stem cells. This report further indicates that an opportunity exists in the

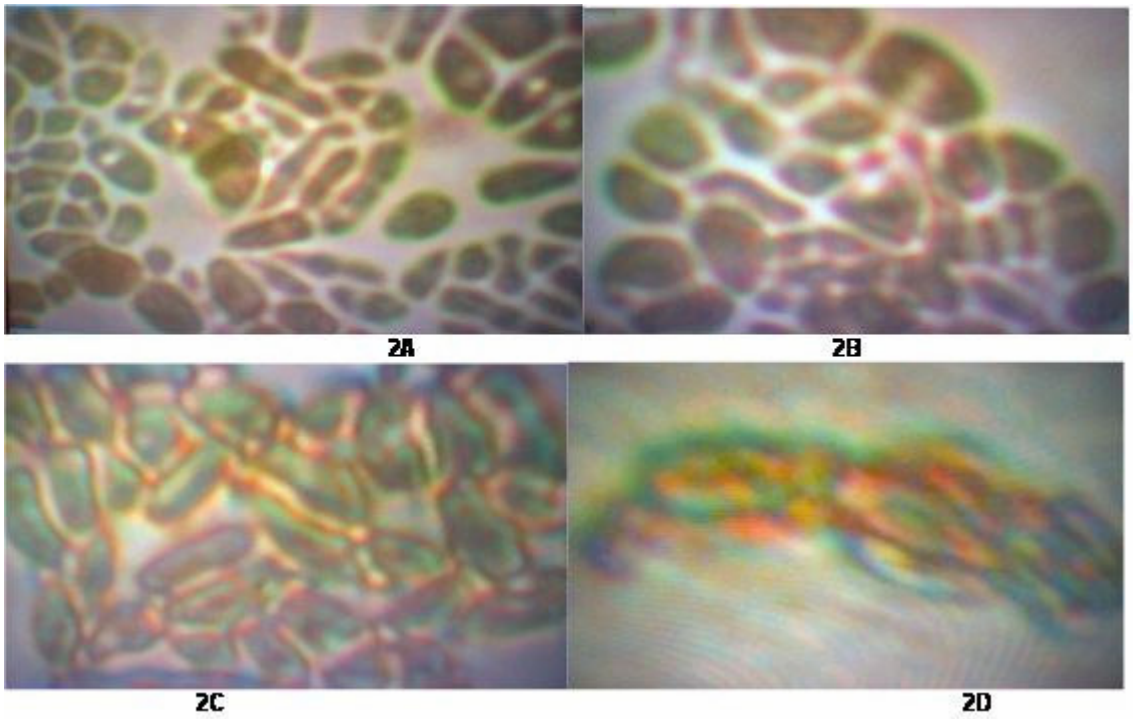

Figure 2. Photomicrographs showing three different clumps of smaller sized cells in saline alone (Figures **2A, 2B and 2C**) or in NC-01 (Figure **2D**) during 14 days of incubation.

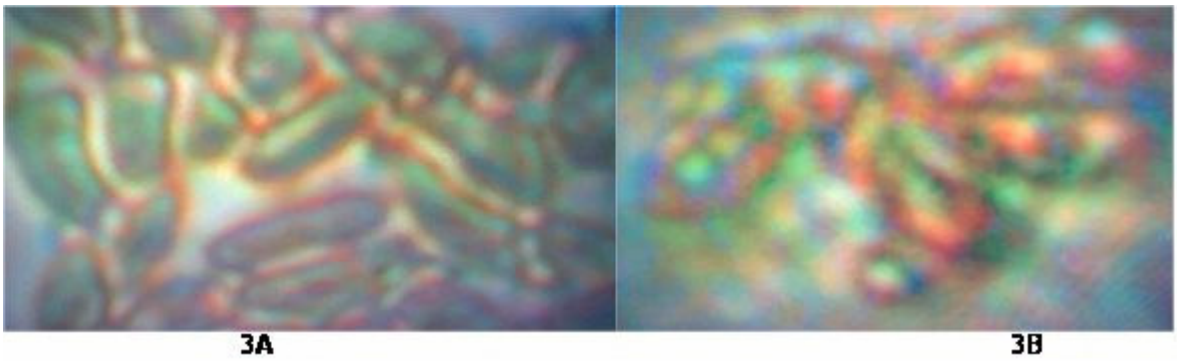

Figure 3. Photomicrographs showing dense clusters of multi-colored component rod-like cells in saline alone (Figure **3A**) or in NC-01 (Figure **3B**) that transformed from dense oval cells and their clumps of smaller sized cells.

utilization of this long-term *in vitro* expansion of spermatogonial stem cells for the study of the mechanisms of self-renewal division and differentiation as well as the regulation of pluripotency following genetic manipulation at the molecular level (Kanatsu-Shinohara et al., 2008). During the period of incubation, we observed that the dense oval cells at the beginning of our studies trans-formed into an aggregate of cells that are smaller in size than their predecessor cells. This clump formation resulted in the presence of many clumps of cells each with similar cell morphology.

This type of cell aggregation to form clumps has previously been observed and described as cell clusters (Kubota et al., 2004, Kanatsu-Shinohara et al., 2005; Kubota and Brinster, 2006). In a recent report, Yeh and colleagues (2007) suggested that spermatogonial stem cells generated three-dimensional structures of aggregated germ cells (clusters) *in vitro* within six days in a culture system and that each cluster which originated from a single cell represented a reliable short-term assay technique to detect spermatogonial stem cells. Although we did not study the generation of three-dimensional structures during germ cell aggregation, our observation of a reduction in cell size secondary to formation of cell

aggregates and the similarity in cell aggregate morphology is worthy of note. Furthermore, Yeh et al. (2007) have suggested that functional spermatogonial stem cells can be determined semi-quantitatively by simply counting the number of germ cell clusters formed in an *in vitro* culture system within one week. It is therefore plausible to imagine that our findings of a reduction in cell size secondary to formation of cell aggregates as well as the similarity in cell aggregate morphology are central points in the germline stem cell expansion *in vitro*. To buttress this view is the report that transplanted cell clusters gave rise to regeneration of spermatogenesis in recipient animals and this was taken as evidence that such clusters contained spermatogonial stem cells (Kubota et al., 2004; Kubota and Brinster, 2006) which were capable of repopulating deficient testicular tissue and re-starting spermatogenesis. Results of our study also reveal transformation of dense oval cells into clusters of multi-colored component rod-like cells during the period of incubation. The different colors that we found to be present in histological samples may suggest the presence of varying sub-cellular components and or macromolecular aggregates that we have not been able to determine at the present time. However, Kolthur-Seetharam et al. (2008) have suggested that the continuous differentiation of spermatogonial stem and progenitor cell population, as part of spermatogenesis, is uniquely characterized by the germ cell-specific expression and function of paralogues of components of the general transcription machinery which play critical but mechanistically distinct roles in spermatogenesis.

This diversity of expression during continuous differentiation may explain our observation of transformation of dense oval cells into clusters of multi-colored component rod-like cells. Observation of multi-colors and an analysis of multi-color expression in a mixed population of spermatogonial stem cells have previously been reported by Shimizu et al. (2006). These scientists used Hoechst 33342 dye and pyronin Y staining in their studies whereas we used the organic Mezo stain. Shimizu and colleagues, on the basis of staining results, categorized a new subpopulation of murine testicular spermatogonial stem cells lacking expression of Oct4 (a pluripotency marker) and this newly described sub population was thought to be in the G0 quiescent state (Shimizu et al., 2006) of the cell cycle. Our multi-color observation which may suggest the presence of varying sub-cellular components or factors distinguishing sub populations of germline stem cell suspension seems to be in agreement with the findings reported by Shimizu et al. (2006). A need still arises for further studies to adequately interpret our observation. Derivation of pluripotent cells from spermatogonial stem cells have been reported (Guan et al., 2006; Kossack et al., 2009).

Spermatogonial stem cell-derived stem cells have been reported to express embryonic stem cell markers and differentiated into the three primary germ layers, and this derivation is thought to involve reprogramming of endogenous spermatogonia in culture, a case for which has been reported for human multipotent germline cells obtained from a testis biopsy (Kossack et al., 2009). Similarly, the generation of human adult germline stem cells from testicular biopsies which differentiated into various types of somatic cells of all three germ layers has been reported (Kossack et al., 2009; Conrad et al., 2008). These investigators therefore suggested that there exists a potential to generate human adult germline stem cells from human testis biopsies thereby by passing the ethical and immunological challenges inherent in human embryonic stem cell research. Characterization of testicular germline stem cell expansion *in vitro* as we have carried out in the present report may contribute in realizing the generation of adult germline stem cells in the near future (Amabile and Meissner, 2009; Geoghegan and Byrnes, 2008; Rooij and Mizrak, 2008).

REFERENCES

Amabile G, Meissner A (2009). Induced pluripotent stem cells: current progress and potential for regenerative medicine. Trends Mol. Med. 15: 59-68.

Conrad S, Renninger M, Hennenlotter J, Wiesner T, Just L, Bonin M, Aicher W, Buhring HJ, Mattheus U, Mack A, Wagner HJ, Minger S, Matzkies M, Reppel M, Hescheler J, Sievert KD, Stenzl A, Skutella T (2008). Generation of pluripotent stem cells from adult human testis. Nature 456: 344-349.

De Rooij DG (1988). Regulation of the proliferation of spermatogonial stem cells. J. Cell Sci. Suppl. 10: 181-194.

De Rooij DG, Russell LD (2000). All you wanted to know about spermatogonia but were afraid to ask. J. Androl. 21: 776-798.

De Rooij DG, Van Pelt AMM (2003). Spermatogonial stem cell biology. ARBS Annu. Rev. Biomed. Sci. 5: 105-114.

De Rooij DG, Mizrak SC (2008). Deriving multipotent stem cells from mouse spermatogonial stem cells: a new tool for developmental and clinical research. Development 135: 2207-2213.

Geoghegan E, Byrnes L (2008). Mouse induced pluripotent stem cells. Int. J. Dev. Biol. 52: 1015-1022.

Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W, Hasenfuss G (2006). Pluripotency of spermatogonial stem cells from adult mouse testis. Nature 440: 1199-1203.

Hamra FK, Chapman KM, Nguyen D, Garbers DL (2007). Identification of neuregulin as a factor required for formation of aligned spermatogonia. J. Biol. Chem. 282: 721-730.

Kanatsu-Shinohara M, Narumi O, Iwano T, Lee J, Kazuki Y, Inoue K, Miki H, Takehashi M, Shinya T, Shinkai Y, Oshimura M, Ishino F, Atsuo O, Takashi S (2005). Genetic and epigenetic properties of mouse male germline stem cells during long-term culture. Development. 132: 4155-4163.

Kanatsu-Shinohara M, Kimiko I, Narumi O, Hiromi M, Shosei Y, Shinya T, Jiyoung L, Atsuo O, Takashi S (2007). Leukemia inhibitory factor enhances formation of germ cell colonies in neonatal mouse testis culture. Biol. Reprod. 76: 55-62

Kanatsu-Shinohara M, Takehashi M, Shinohara T (2008). Brief history, pitfalls and prospects of mammalian spermatogonial stem cell research. Cold Spring Harb Symp Quart Biol. [Epub ahead of print].

Kolthur-Seetharam U, Martianov I, Davidson I (2008). Specialization of the general transcriptional machinery in male germ cells. Cell Cycle 7: 3493-3498.

Kossack N, Meneses J, Shefi S, Nguyen HN, Chavez S, Nicholas C, Gromoll J, Turek PJ, Reijo-Pera RA (2009). Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. Stem Cells. 27: 138-149.

Kubota H, Avarbock MR, Brinster RL (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells.Proc. Natl. Acad. Sci. USA. 101: 16489-16494.

Kubota H, Brinster RL (2006). Technology insight: in vitro culture of spermatogonial stem cells and their potential therapeutic uses. Nature Clin. Pract. Endocrinol. Metabol. 2: 99-108.

Munsie M, Schlatt S, deKretser DM, Loveland KL (1997). Expression of stem cell factor in the postnatal rat testis. Mol. Reprod. Dev. 47: 19-25.

Ogawa T, Arechaga JM, Avarbock MR, Brinster RL (1997). Transplantation of testis germinal cells into mouse seminiferous tubules. Int. J. Dev. Biol. 41: 111-122.

Orwig KE, Ryu BY, Avarbock RM, Brinster RL (2002). Male germ-line stem cell potential is predicted by morphology of cell in neonatal rat testes. Proc. Natl. Acad Sci. USA. 99: 11706-11711.

Ravindranath N, Dalal R, Solomon B, Djakiew D, Dym M (1997). Loss of telomerase activity during during male germ cell differentiation. Endocrinology. 138: 4026-4029.

Ryu BY, Orwig KE, Avarbock RM, Brinster RL (2003). Stem cell and niche development in the post natal rat testis. Dev. Biol. 263: 253-263.

Ryu B-Y, Kubota H, Avarbock RM, Brinster RL (2005). Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. Proc. Natl. Acad. Sci. 102: 14302 – 14307.

Shimizu Y, Motohashi N, Iseki H, Kunita S, Sugiyama F, Yagami K (2006). A novel subpopulation lacking Oct4 expression in the testicular side population. Int. J. Mol. Med. 17: 21-28.

Shinohara T, Kato M, Takehashi M, Lee J, Chuma S, Nakatsuji N, Kanatsu-Shinohara M, Hirabayashi M (2006). Rats produced by interspecies spermatogonial transplantation in mice and in vitro microinsemination. Proc. Natl. Acad. Sci. USA. 103: 13624-13628.

Van Bragt MP, Roepers-Gajadien HL, Korver CM, Bogerd J, Okuda A, Eggen BJ, de Rooiji DG, van Pelt AM (2008). Expression of the pluripotency marker UTF1 is restricted to a subpopulation of early A spermatogonia in rat testis. Reproduction. 36: 33-40.

Yeh JR, Zhang X, Nagano MC (2007). Establishment of a short-term in vitro assay for mouse spermatogonial stem cells. Biol. Reprod. 77: 897-904.