

Production of offspring from a germline stem cell line derived from neonatal ovaries

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The idea that females of most mammalian species have lost the capacity for oocyte production at birth^{1–5} has been challenged recently by the finding that juvenile and adult mouse ovaries possess mitotically active germ cells⁶. However, the existence of female germline stem cells (FGSCs) in postnatal mammalian ovaries still remains a controversial issue among reproductive biologists and stem cell researchers^{6–10}. We have now established a neonatal mouse FGSC line, with normal karyotype and high telomerase activity, by immunomagnetic isolation and culture for more than 15 months. FGSCs from adult mice were isolated and cultured for more than 6 months. These FGSCs were infected with GFP virus and transplanted into ovaries of infertile mice. Transplanted cells underwent oogenesis and the mice produced offspring that had the *GFP* transgene. These findings contribute to basic research into oogenesis and stem cell self-renewal and open up new possibilities for use of FGSCs in biotechnology and medicine.

In most mammalian species the production of ovarian oocytes is thought to cease before birth^{1–5}. However, this belief has been challenged by recent research indicating that the female gonad may have regenerative activity in juvenile and adult mice *in vivo*⁶. Subsequently, it has been suggested that both bone marrow and peripheral blood may serve as a source of cells responsible for this regenerative capacity¹¹. Other studies have shown that bone marrow cells, or other normally circulating cells, are not involved in the formation of mature, ovulated oocytes⁷, and that instead of FGSCs, putative thecal stem cells could be isolated from newborn mouse ovaries⁹. Therefore, the presence of FGSCs in mammalian ovaries after birth is still highly controversial^{6–10,12}.

To identify and confirm the presence of FGSCs in mouse ovaries⁶, we located ovarian cells positive for mouse vasa homologue (MVH) protein, which is expressed exclusively in germ cells, using immunohistochemistry. Next, we assessed the proliferative potential of these MVH-positive cells. Adult and 5-day-old female mice were injected with 5'-bromodeoxyuridine (BrdU) and ovaries were collected 1 h later for dual immunofluorescence analysis of BrdU incorporation and

expression of MVH. The results showed the presence of BrdU–MVH double-positive cells in the ovarian surface epithelium, suggesting that they might be FGSCs. Morphological and histological analysis of these BrdU–MVH double-positive cells was performed (Supplementary Information, Fig. S1).

To create mouse FGSC lines, FGSCs were isolated from the ovaries of 5-day-old or adult C57BL/6×CD-1 F1 hybrid mice. Large round or ovoid cells were obtained from 9–12 neonatal (200–300 cells) or 6–8 adult mice (50–100 cells) by two-step enzymatic digestion and immunomagnetic isolation of MVH-positive (MVH⁺) cells^{13,14}. Most of the isolated cells were morphologically similar to freshly isolated type A spermatogonia (SSCs)¹⁵, showing large cell bodies with little cytoplasm, spherical nuclei with slight staining, a large ratio of nuclear plasma and nuclear diameter of 12–20 μm (Figs 1a, 2a; Supplementary Information, Fig. S2d). To determine whether the MVH⁺ cells contained FGSCs, cell proliferation analysis was carried out by adding BrdU to the culture medium. The results show that the isolated MVH⁺ cells consisted of BrdU-positive cells (Figs 1b–d, 2b–d), suggesting that the cells might be FGSCs. BrdU-negative cells were derived from oocytes that had already entered prophase I (Supplementary Information, Fig. S2c). For confirmation, dual immunofluorescence analysis of BrdU incorporation and expression of MVH was performed on isolated cells cultured for more than 14 days with 2 passages. Almost all of these cells were positive for BrdU and MVH (Figs 1e, 2i–m), as oocytes degenerate during culture. After 7 or 8 passages, the isolated cells increased in number, forming clusters (Fig. 1f). Immunofluorescence analysis of BrdU incorporation and DAPI was performed on these FGSCs to confirm that they were undergoing mitosis (Fig. 2e–h). As culturing continued, the clustered cells proliferated and formed compact clusters of cells with blurred cell boundaries. However, these clusters of cells did not resemble embryonic stem (ES) cell colonies. At the time of writing, the FGSCs from neonatal mice (nFGSCs) had been in culture for more than 15 months, had been passaged more than 68 times (5–8 days per passage) and maintained a characteristic morphology similar to that of freshly isolated FGSCs (Fig. 1g). FGSCs from adult mice (aFGSCs) were cultured for more than 6 months and passaged more than 25 times. The proliferation patterns of these FGSCs

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Improved Efficiency of Female Germline Stem Cell Purification Using Fragilis-Based Magnetic Bead Sorting

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The enrichment of female germline stem cells (FGSCs) and the establishment of cell lines are influenced by the efficiency of cell purification. A previous study using mouse vasa homolog (MVH)-magnetic bead sorting for the isolation and purification of mouse FGSCs showed a relatively low efficiency. In this study, we tested 3 further proteins with the aim of improving the efficiency of FGSC purification. Immunofluorescence assays and magnetic sorting were performed using short-type pituitary gland and brain-cadherin (Stpb-c), CD9, and interferon-inducible transmembrane protein 3 (Iftm3, Fragilis), all of which are expressed in germ cells. Although all 3 proteins were expressed in FGSCs, CD9 was unsuitable because of its lack of germline specificity, and Stpb-c was also unsuitable because of the unavailability of an appropriate primary antibody. The efficiency of FGSC purification was remarkably enhanced using the germline-specific protein Fragilis, compared with that using MVH. This new method for the purification of FGSCs may have extensive applications in stem cell studies and clinical research.

Introduction

MAGNETIC BEADS HAVE BEEN widely used for cell sorting in basic and clinical research. However, the identification of suitable specific cell surface markers remains a pivotal problem associated with this technique. Increasing numbers of cell markers have been identified since the upsurge in stem cell research, and this cell sorting technique using magnetic beads facilitates the isolation and identification of new types of stem cells. In a previous study, we identified a type of germ cell with the capacity for proliferation in postnatal ovaries, and subsequently, we isolated and purified these novel germ cells and established a cell line. Following functional characterization both *in vivo* and *in vitro*, these germ cells were called female germline stem cells (FGSCs) [1]. In this earlier study, mouse vasa homolog (MVH) was used as a germline marker for separation of FGSCs from somatic cells using the magnetic bead technique. The *mvh* gene was initially cloned from mouse primordial germ cells (PGCs). Its expression has been subsequently exclusively detected in germline cells [2], from PGCs to postmeiotic spermatids or primary oocytes [3,4], and it has been widely considered to be a germline marker in both vertebrate and invertebrate species [2,5,6]. Using a bioinformatics tool (www.ch.embnet.org/software/TMPRED_form.html), we found that MVH, an RNA helicase of the DEAD-box family [7], has 2 putative transmem-

brane domains. Cells isolated from postnatal ovaries were cultured with MVH primary antibody, and magnetic beads combined with a secondary antibody specific for rabbit immunoglobulin G (IgG) were used to purify the MVH⁺ cells. The magnetic beads spontaneously detached from the cells after several hours of culture and could be removed by the magnetic separator. About 300 MVH⁺ cells were collected from 20 ovaries of 5-day-old mice, but the purification efficiency was relatively low. To optimize the purification of FGSCs, we analyzed 3 other proteins expressed in germline cells: 2 cell surface proteins and 1 cytoplasm protein with transmembrane domains.

CD9 is a tetraspan protein that associates with several $\beta 1$ integrins and plays an important role in sperm-egg binding and fusion [8]. In addition to being located on oocyte membranes, CD9 can also be detected in murine spermatogonial stem cells (SSCs) and embryonic stem cells [9–11]. Short-type pituitary gland and brain-cadherin (Stpb-c) is a member of the cadherin family and contains transmembrane domains and 5 repeats of a cadherin motif in the extracellular domain [12]. This protein and its homeotic protein, long-type pituitary gland and brain-cadherin, are generated by alternative gene splicing. As an important signal molecule, Stpb-c plays a pivotal role in promoting the proliferation of SSCs *in vivo* and *in vitro* [13,14]. Fragilis has been reported to participate in PGC determination and is specifically

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Germline Stem Cells, a Useful Tool for Therapeutic Cloning

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Abstract: Numerous articles have been published on the potential of using embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) in clinical applications. As these types of stem cell are well studied, the research on germline stem cells (GSCs), which hold a huge potential for clinical application and permanent treatment for infertility, is left behind. Besides possessing the characteristics of being able to self-renew and to give rise to differentiated progeny throughout postnatal life, the potential of GSCs to transform into pluripotent status is remarkable but is unexploited. In this review, we summarized development of ESCs, GSCs, iPSCs, and embryonic stem-like (ES-like) cells derived from GSCs, discussed feasibility and the technical hurdles of using these types of stem cells in therapeutic cloning, and finally focused on the comparison of the ESCs, iPSCs and ES-like cells in current as well as potential applications in medicine. Moreover, the prospects of female germline stem cells (FGSCs) and their derived ES-like cells were also discussed as a novel alternative in clinical application.



Keywords: Autologous graft, embryonic stem-like cells, germline stem cells, immuno-compatibility, induced pluripotent stem cells, pluripotency.

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1. INTRODUCTION

As far back as ancient Greek mythologies, the earliest concept of cloning has been recorded. Prometheus was chained to a rock where daily his liver was eaten by an eagle, but was renewed every night. Although it defined as a myth in ancient time, cloning makes it possible in spectacularly advanced stem cell field. As early as in 1908, the term of stem cell was proposed for scientific use by the Russian histologist Alexander Maksimov [1]. After a century's development of stem cell biology, tremendous breakthroughs have been made. Stem cell based therapies provide a therapeutic approach to treat diseases including cardiac diseases [2], bone repair [3], diabetes [4, 5], neurodegenerative disorders [6, 7], and infertility [8]. These achievements bring us closer to the time when majority of human diseases might be cured by therapeutic cloning [9]. Nevertheless, functional stem cells for therapeutic cloning are a rare resource [10], since stem cells are very limited in number and in a metabolically quiescent state in most tissues including the brain [11], bone marrow [12], liver [13], skin [14], ovary [15, 16] and testis [17]. Thus, an alternative source of transplantable stem cells is required.

In 1981, mouse embryonic stem cells were derived from the inner cell mass of blastocyst by Martin Evans, Matthew Kaufman, and Gail R. Martin [18], and seventeen year later human embryonic stem cell (ESC) has been isolated and

maintained *in vitro* by James Thompson's group [19]. It was so well known that ESCs have the remarkable potential to differentiate into other cell types to repair abnormal cells or damaged tissues. Yet in year 2006, a new advanced technology developed by Shinya Yamanaka attracted worldwide attention: a type of pluripotent stem cells artificially derived from adult differentiated somatic cells by induced expression of a set of specific pluripotent genes [20]. It is so amazing how rapid growth of this iPS cell technology during the past eight years.

In 1994, James W. Zimmermann reported that stem cells isolated from donor mouse testes could repopulate sterile testes when injected into recipient seminiferous tubules [21, 22]. Spermatogenesis resulted from donor cells in recipient testes showed normal morphological characteristics with mature spermatozoa production. Later on, immuno-incompatibility has been overcome by spermatogonial stem cells (SSCs) [23, 24], as testicular immune privilege protects immunogenic germ cells from systemic immune attack. Moreover, SSCs do not need to be reprogrammed. Therefore, SSCs might open the door to potential therapeutic applications such as fertility recovery and preservation [25].

One decade after the successful SSCs transplantation, Mito Kanatsu-Shinohara's group first reported the establishment of ES-like cells from neonatal mouse testis germ cells in 2004 [26]. These ES-like cells exhibit similarities to ESCs in cellular and molecular characteristics, except in their genomic imprinting patterns. The ability to derive pluripotent stem cells from the neonatal testis has important implications for germ cell biology and opens the possibility

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Advances in Isolation Methods for Spermatogonial Stem Cells

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Abstract Stem cell research has led to many remarkable achievements in recent years, but progress in the study of spermatogonial stem cells (SSCs) has been relatively slow, partly due to the slow development of techniques for spermatogonial stem cell isolation. The major accomplishments of SSC sorting and identification occurred approximately 10 years ago, and since that time, these techniques have been widely used without major improvements. In this article, we briefly introduce the biological properties of SSCs before reviewing the development of sorting techniques for SSCs in the past decades. We then summarize recent achievements in SSC sorting and finally discuss the advantages and disadvantages of SSC isolation methods, to provide new insight into techniques and research related to spermatogonial stem cells and promote the development of reproductive biology.

Keywords Characterization · Spermatogonial stem cell · Sorting efficiency · Surface markers

Introduction

Spermatogonial stem cells (SSCs), residing on the basement membrane of seminiferous tubule, are a type of germ line stem cells with the potential for self-renewal and differentiation into sperm in the male testes. Due to the success of maintenance

[1] and genetic modification in vitro [2], SSCs present significant potential for the treatment of male sterility, transgenic animal studies and the protection of genetic resources. However, due to the small number of SSCs in the testis (only 0.02–0.03 % of total cells in the mouse testis [3]), enrichment of SSCs with high purity and viability using biologically safe methods is a challenging but pivotal step for SSC-related studies in vitro. Great achievements concerning the isolation and characterization of mouse and human SSCs have been made in the past two decades, which have provided us with a better understanding on SSC biology, presenting many implications for the application of SSCs in reproductive and regenerative medicine. In this review, we summarize the biological characteristics of SSCs, then introduce advances in SSC sorting methods made in recent decades, and we finally systematically discuss the advantages and disadvantages of each method. We hope that this article will provide useful information for germ line stem cell researchers, and we expect that more efficient methods will be developed based on current methods for germ line stem cell isolation.

Biological Characteristics of SSCs

Biological Origin of SSCs

Mouse SSCs arise from primordial germ cells (PGCs), which are derived from epiblast cells during early development [4]. At approximately 8.0 days post coitum (d.p.c), PGCs begin to migrate from the base of the allantois along the hindgut and continue to proliferate during migration, finally reaching the genital ridges at 11.5 d.p.c and forming gonocytes, which are enclosed by Sertoli precursor cells and peritubular cells [5, 6]. These gonocytes show a burst of mitotic activity, followed by arrest in G₀ phase of the cell cycle and then remain mitotically

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Short-type PB-cadherin promotes self-renewal of spermatogonial stem cells via multiple signaling pathways

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Abstract

Stem cells represent a unique population of cells with self-renewal capacity. However, the molecular control of self-renewal and differentiation of stem cells has remained enigmatic. Here, we show that short-type PB-cadherin (STPB-C) promoted self-renewal of spermatogonial stem cells (SSCs) via activating Janus kinase/signal transducer and activator of transcription (JAK-STAT) and phosphoinositide-3 kinase (PI3-K)/Akt, and blocking transforming growth factor (TGF)- β 1 signaling. These data were obtained with varied approaches, including the use of RNA interference (RNAi), SSC cultures infected by STPB-C retroviral vector, bromodeoxyuridine (BrdU) incorporation assay, and other techniques. These findings have important implications for germ cell biology and create the possibility of using SSCs for biotechnology and medicine. They are also critical in understanding tissue homeostasis, the aging process, tumor formation and degenerative diseases.

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Keywords: STPB-C; Self-renewal of spermatogonial stem cells; JAK-STAT; PI3-K/Akt; TGF- β 1 signaling

1. Introduction

Stem cells are characterized by their ability to self-renew and to continuously generate differentiated cells. Uncovering the molecular control of stem cell self-renewal is crucial to the future use of stem cells in regenerative medicine and in understanding tissue homeostasis, the aging process, tumor formation and degenerative diseases [1–3]. To maintain normal spermatogenesis, the processes of self-renewal and differentiation of spermatogonial stem cells (SSCs) must be precisely regulated by intrinsic gene expression in the stem cells and by extrinsic signals, including soluble factors or adhesion molecules from the surrounding microenvironment, the stem cell niche.

A recent study has identified growth factors essential for self-renewal and expansion of mouse SSCs [4]. Glial cell line-derived neurotrophic factor (GDNF) is a key factor in deciding the fate of SSCs [5]. It appears to stimulate self-renewal of mouse SSCs and block their differentiation by acting in a paracrine manner [5,6]. Moreover, it has been found that GDNF-induced cell signaling plays a central role in SSC self-renewal [4]. However, the molecular mechanism underlying SSC self-renewal remains largely unexplored.

Cadherins represent a distinct family of single-transmembrane-domain glycoproteins which are key molecules during development, and serve as specific cell-adhesion molecules acting in a Ca²⁺-dependent manner [7]. Recent findings have demonstrated that DE-cadherin-mediated cell adhesion is essential for maintaining somatic stem cells in *Drosophila* ovaries and for the involvement of cadherins in signaling pathways [8]. In our previous work, we identified a novel adhesion molecule in the testis, short-type PB-cadherin (STPB-C), and found that it plays a critical role in promoting survival of gonocytes, the precursor cells of SSCs, in neonatal rats [9]. Furthermore, we observed that

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Article

Production of transgenic mice by random recombination of targeted genes in female germline stem cells

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Oocyte production in most mammalian species is believed to cease before birth. However, this idea has been challenged with the finding that postnatal mouse ovaries possess mitotically active germ cells. A recent study showed that female germline stem cells (FGSCs) from adult mice were isolated, cultured long term and produced oocytes and progeny after transplantation into infertile mice. Here, we demonstrate the successful generation of transgenic or gene knock-down mice using FGSCs. The FGSCs from ovaries of 5-day-old and adult mice were isolated and either infected with recombinant viruses carrying green fluorescent protein, Oocyte-G1 or the mouse dynein axonemal intermediate chain 2 gene, or transfected with the Oocyte-G1 specific shRNA expression vector (pRS shOocyte-G1 vector), and then transplanted into infertile mice. Transplanted cells in the ovaries underwent oogenesis and produced heterozygous offspring after mating with wild-type male mice. The offspring were genetically characterized and the biological functions of the transferred or knock-down genes were investigated. Efficiency of gene-transfer or gene knock-down was 29%–37% and it took 2 months to produce transgenic offspring. Gene manipulation of FGSCs is a rapid and efficient method of animal transgenesis and may serve as a powerful tool for biomedical science and biotechnology.

Keywords: female germline stem cells, gene-transfer, knock-down, Oocyte-G1, mouse dynein axonemal intermediate chain 2 (*Dnaic2*)

Introduction

Stem cells are a unique cell population with self-renewal potential (Potten, 1992). They often have migratory activity and can be transplanted between animals (Brinster and Zimmermann, 1994; Clouthier et al., 1996). Transplanted stem cells can migrate to a specific position and regenerate self-renewing tissue. Germline cells are unique in that they transmit genetic information from parent to offspring (Meistrich and van Beek, 1993; De Rooij and Russell, 2000). Males retain germline stem cells for spermatogenesis throughout adult life and spermatogonial stem cells (SSCs) can be transfected to produce transgenic offspring via SSC transplantation (Kanatsu-Shinohara et al., 2005, 2006). Female germline stem cells (FGSCs) stop dividing after birth in most mammalian species (Zuckerman, 1951; Borum, 1961; Peters, 1970; McLaren, 1984; Anderson and Hirshfield, 1992). This theory has been challenged with findings from an *in vivo* mouse study suggesting that the female gonad may exhibit unexpected regenerative activity in adulthood

(Johnson et al., 2004). Recently, we reported that an FGSC line from neonatal mice was established and cultured for more than 15 months with normal karyotype and high telomerase activity. FGSCs from adult mice were also isolated and cultured for more than 6 months (Zou et al., 2009). These FGSCs of long-term culture maintained their capacity to produce normal oocytes and fertile offspring after transplantation into ovaries (Zou et al., 2009). Pacchiarotti et al. (2010) also showed the existence of a population of germline stem cells in postnatal mouse ovaries.

Oocyte-G1 is a newly discovered member of the kinesin superfamily of proteins (KIFs). KIFs play a significant role in transport of various membranous organelles and protein complexes on microtubules (Hirokawa, 1998). A previous study showed that *Oocyte-G1* encodes a 997-residue protein with an open reading frame (ORF) of 2994 bp. Northern blot analysis revealed the presence of ca. 3.6-kb *Oocyte-G1* mRNA in the ovary, testis, lung, brain and kidney tissue (Zhang and Wu, 2009). However, the functions of *Oocyte-G1* are not well understood.

In our earlier work, we cloned a novel gene, the mouse dynein axonemal intermediate chain 2 gene (*Dnaic2*), which has an 87% homology with human *DNA2*, a candidate gene for primary ciliary dyskinesia (PCD) (Guichard et al., 2001). Its ORF is 1872 bp encoding a polypeptide of 623 residues. Northern and western

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