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Identification and Pluripotency of Mouse Spermatogonial Stem Cells

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Contents

Chapter 2 Materials and Methods

Chapter 4 Discussion

Zusammenfassung

Spermatogoniale Stammzellen (SSCs) sind in den Samenkanälchen des Hodens lokalisiert und für die Samenproduktion des Mannes verantwortlich. Obwohl viel über die Morphologie und Kinetik der Spermatogonien bekannt ist, so gibt es doch noch Lücken in der Erforschung von spezifischen zellulären, mophologischen und biochemischen Prozessen der SSCs.

Methoden wie die Durchflusszytometrie (FACS), Magnetic Cell Separation (MACS), die Matrix-Selektion und die Selektion aufgrund morphologischer Kriterien sind einige der sehr hiflreichen Techniken, um SSCs aus dem Hodengewebe zu isolieren.

Wir generierten ein einfaches, aber höchst reproduzierbares Protokoll für die Isolierung von SSCs. Es handelt sich um eine Selektion, die auf der Morphologie der Zellen basiert und mit deren Hilfe wir zwei Typen von SSC-Zellen (Typ I und Typ II) selektieren konnten. Wir etablierten die optimalen Wachstumsbedingungen für diese zwei Zelltypen, indem wir Ko-Kulturen mit verschiedenen Mitomycin-C behandelten Feederzellen als Langzeitkultur ausgetestet haben. Damit konnten wir erfolgreich „Typ I-SSCs“ auf embryonalen Fibroblasten der Maus (MEFs) und „Typ II-SSCs“ auf SNL-Fibroblasten (generiert aus STO-Fibroblasten der Maus), aber auch auf primären testikulären Stromazellen der Maus (TSCs) wachsen lassen.

Elektronenmikroskopische Analysen zeigten, dass Typ I-SSCs eine ähnliche Mophologie mit SSCs *in-vivo*, mit einem typischen Kern-/Zytoplasma-Verhältnis aufweisen, während Typ II-SSCs eine unterschiedliche Morphologie in Bezug auf das Kern-/Zytoplasma-Verhältnis besitzen und einen eher kleinen Kern aufweisen.

Die Ergebnisse von Untersuchungen mittels Immunhistologie, FACS und Fluidigm Real Time PCR zeigten deutlich, dass Typ I-SSCs Keimzellmarker exprimieren, während Typ II-SSCs nur partiell das Keimzellmarkerprofil aufwiesen.

Weiterhin konnte man bei Busulfan-behandelten Tieren beobachten, dass transplantierte GFP-positive Typ II-SSCs an der Basalmembran der Hodenkanälchen lokalisiert sind. Ebenfalls wurden GFP-positive Spermien im Nebenhoden gefunden.

Zu den größten Unterschieden zwischen Typ I- und Typ II-SSCs gehört ihre Fähigkeit, sich wieder zu embryonalen Stammzell- ähnliche Zellen (ES- ähnlichen Zellen) zu

reprogrammieren. Dies tritt in einem kritischen Zeitfenster auf, nachdem die Typ I-SSC-Kulturen angelegt wurden.

Obwohl man in verschiedenen Studien ES-ähnliche Zellen aus dem Hoden generieren konnte, so konnte das Zeitfenster, in der sich der Übergang in pluripotente Zellen ereignet, bislang nicht klar definiert werden.

In unseren Experimenten konnten wir das spontane Auftreten von ES-ähnlichen Zellen aus Typ I-SSC Zellen sowohl aus neugeborenen, als auch aus erwachsenen Mäusen beobachten, aber nur während eines speziellen Zeitfensters (Tag 41 bis Tag 125, nachdem die Typ I-SSC Zellkulturen aus neugeborenen und erwachsenen Promotor-Reporter Oct4-GFP transgenen Mäusen angelegt wurden).

Die so erzeugten ES-ähnlichen Zellen reagierten positiv auf Pluripotenzmarker. Diese ließen sich *in-vitro* in alle drei Keimbahnen differenzieren und bildeten komplexe Teratome, nachdem sie in NOD/SCID Mäuse injiziert wurden und produzierten chimäre Mäuse.

Obwohl der exakte Mechanismus der Entwicklung der ES-ähnlichen Zellen aus SSCs noch unklar ist, bieten diese neue Beoachtungen die Möglichkeit, etwas Neues über den Ablauf der Umwandlung der ES ähnlichen Zellen aus Mäusehoden zu erfahren.

Daher bieten diese zwei Zelltypen I und II ein ideales Zellsystem, um sowohl Pluripotenz als auch die *in-vitro* Differenzierung der SSCs zu Spermien zu erforschen. Darüber hinaus findet sich hiermit eine neue Strategie, um SSCs von neugeborenen und erwachsenen Mäusen anhand von morphologischer Selektion zu isolieren.

Abstract

Spermatogonial stem cells (SSCs) are located on the basal membrane in seminiferous tubules of the testis and are responsible for sperm production during the male's life. Although the morphology and kinetics of spermatogonia has been extensively characterized, there is still a lack of specific cellular, morphological and biochemical criteria for SSCs. However, Fluorescence-activated cell sorting (FACS), magnetic-activated cell sorting (MACS), matrix selection and morphology-based selection are some of the useful methods for the isolation of SSCs.

In the current study, we demonstrated that using cell sorting approaches are dispensable for the generation of mouse SSCs cultures. We identified a simple and highly reproducible protocol for the isolation of SSCs with morphology-based selection and successfully established two types of SSCs (Type I and Type II). Using co-culture systems with different mitomycin-C treated feeder layers in long-term culture, we established the optimal conditions for the cultivation and gene expansion of these two types of SSCs. We successfully expanded Type I SSCs on mouse embryonic fibroblasts (MEFs) and Type II SSCs on both SNL and primary testicular stroma cells (TSCs) feeders. Electron microscopic analysis revealed that Type I SSCs display a similar morphology with SSCs *in-vivo* high nucleus/cytoplasm ratio, while Type II SSCs have a different morphology small nucleus/cytoplasm ratio. Immunocytochemistry, FACS and Fluidigm real-time RT-PCR results showed that Type I SSCs clearly express germ cells markers while Type II SSCs only partially express the typical germ cell profile of SSCs. Following the transplantation of Type II SSCs to busulphan-treated NOD/ SCID mice, we observed a localization of GFP-labeled cells in the basal compartment of the seminiferous tubule. Furthermore, GFP labeled sperms were detected in epididymis. Among the most obvious molecular differences between Type I and Type II SSCs were their reprogramming capacity to mouse embryonic stem cells-(ES-) like cells that occurred only on the critical time window after initiation of Type I SSCs culture. Although testis-derived ES-like cells have been obtained in several studies, the time window for the shift to pluripotency was not clearly determined. In our experiments, we observed

that the spontaneous appearance of germline-derived ES-like cells from both neonate and adult Type I cells occurred only during a special time window (41 until 125 days) after initiation of Type I cells from neonate and adult promoter-reporter Oct4-GFP transgenic mice. The generated ES-like cells expressed pluripotency markers, differentiated into all three germ lineages, formed complex teratoma after transplantation in SCID mice and produced chimeric mice. Although the exact mechanism of the development of ES-like cells from GSCs is still unclear, this new information could provide an ideal strategy for scheduling natural conversion mechanisms of ES-like cells from mouse testes. Therefore, the two different types of SSCs could provide an ideal cell system for studying both pluripotency and *in-vitro* differentiation of SSCs to sperm and also provide a new strategy for isolation of SSCs from neonatal and adult mice by morphology-based selection.

Chapter 1 Introduction

1.1. Stem cells

Stem cells are undifferentiated cells that have the ability for self-renewal and differentiation into many different cell types in the body. This potential allows them to differentiate into derivatives of ectodermal, mesodermal and endodermal embryonic germ layers. According to plasticity and developmental versatility, stem cells can be classified into different groups.

Totipotent stem cells are the only type of stem cells that have the potential to develop into every cell type in the body. A combination of sperm cell and an egg cell form a fertilized egg that becomes totipotent stem cells. Totipotent stem cells can specialize into pluripotent stem cells in a few days after development of embryonic cells. Pluripotent stem cells are derived from the inner cell mass of the blastocyst and similar to totipotent stem cells, they can give rise to all tissue types expect extra-embryonic derivatives.

Some types of stem cells have less plasticity potential and they are more differentiated stem cells. These types of stem cells are multipotent stem cells such as mesenchymal stem cells and blood stem cells that can develop into different cell types in the body.

In stem cell classification, multipotent stem cells are in the category of adult stem cells. These cells exist in the adult organs and are able to proliferate and maintain their differentiation potential during *in-vitro* culture [1]. Adult stem cells can replace cells that have died or lost their function. They are undifferentiated cells which are present in differentiated tissue, for example, in mesenchymal, hematopoetic (blood), neural, endothelial, muscle, gastrointestinal and epidermal cells.

A few types of stem cells in adult tissues are unipotent which means that these stem cells are able to differentiate into only one cell type in the body, for example, SSCs and ovarian stem cells that exist in the testis and the ovarian tissue.

1.1.1. Pluripotent stem cells

As mentioned, pluripotent stem cells are undifferentiated cells which have potential for self-renewal and differentiation into many cell types in the body. This ability of pluripotent stem cells allows them to be used in regenerative medicine for cell therapy. Pluripotent stem cells can proliferate and maintain pluripotency during *in-vitro* culture [1].

Several extrinsic growth factors with activation of associated signaling pathways determine the fate of pluripotent stem cells. Proliferation of pluripotent cells occurs in the presence of feeder, growth factors and high concentration of FBS. In the mESCs culture, growth factors such as BMP4 (bone morphogenetic protein 4) and LIF (leukemia inhibitory factor) can replace serum and the feeder cell layer [2]. Observation indicated the LIF and BMP4 signaling pathways promote activation of STAT3 and SMAD1 proteins respectively [2, 3]. In the hESCs culture, it has been demonstrated that BMP4 promotes differentiation into trophoblast cells, while Activin/Nodal and FGF signaling pathways maintain self-renewal and pluripotency of the human cells [4, 5]. In addition to these signaling pathways, activation or inhibition of STAT3, MAP kinase (MEK) and glycogen synthase kinase 3 (GSK3) are important to sustain the pluripotency state [6, 7].

1.2. SSCs as adult stem cells

1.2.1. Development of germ cells and SSCs

Primordial germ cells (PGCs) are specified from pluripotent proximal epiblast cells during fetal development. In the epiblast stage of mouse embryo at about 6 days post coitum (dpc), extraembryonic ectoderm (EXE) and visceral endoderm tissues secrete BMP4, BMP8b and BMP2 signals that influence pluripotent proximal epiblast cells. In response to these extraembryonic signals, some of the cells express fragilis/Ifitm3 and acquire PGC competence. Fragilis encodes E-cadherin protein that is involved in homotypic cell adhesion and cell-cell interaction. At embryonic day 6.25 (E6.25), among fragilis/Ifitm3 positive cells which are in contact with EXE, about 6 cells express Blimp1/Prdm1 and acquire PGC specification. The number of specified cells increases

to 16 in stage E6.5 [8]. In these cells, Blimp1 proteins repress activation of the somatic program allowing the specification of germ cell characters [8, 9]. The specified PGCs start to express some genes such as tissue non-specific alkaline phosphatase (Tnap) [10, 11], stage specific embryonic antigen 1 (SSEA1) [12], Stella [13], Sox-2 [8, 14], Nanog [15] and Prdm14 [14]. PGCs and non-specified competent cells also express mesodermal genes such as Hoxb1, T, Fgf8, and Snail [16]. The development of PGCs continues during their migration from E7.5 to E11.5. At E9.0, PGCs migrate through the primitive streak and merge into the hindgut while freely moving around the cells of the hindgut epithelium. Between E7.5 and E9.5, PGCs undergo an extensive epigenetic reprogramming that mainly consists of a transition from genome-wide suppressive H3K9me2 histone mark to a more permissive mark, the H3K27me3 [17]. This process is apparently coordinated by a protein-binding protein, Mad2l2 [18]. Next, PGCs appear from the dorsal side of the hindgut and migrate towards the developing genital ridges at E9.5. At E10.5, they evolve to form clusters of cells. Finally, at E11.5, most PGCs emerge in the form of a colony in the genital ridge, where they undergo a second round of epigenetic reprogramming to acquire the potential for pluripotency [19-21].

During the move to the genital ridge, some growth factors control PGCs. These include TGF, bFGF, EGF, Interleukins, steel factor, as well as some of the ECM proteins like E-cadherin and b1 integrin.

At E12.5, in both males and females, mitotic cell division of PGCs discontinues and instead the process of sexual differentiation starts to appear. In females, germ cells (GCs) become oocytes in the ovary by entering into the prophase of meiosis I at E13.5, and at E15.5, most of the ovarian GCs transit to the meiotic prophase I [22]. Oocytes pause in E17.5, in the diploten stage of meiosis phase, and resume after birth while surrounded by a single layer of somatic cells and forming a primordial follicle [23].

Most often, male germ cells do not show a tendency to enter the meiosis phase until after birth. Approaching 2.5 dpc, somatic cells in the testis produce meiosis-inhibiting factor that blocks the entry of germ cells into meiosis and their development in the spermatogenic pathway [24]. At the 13.5 dpc, PGCs turn into gonocytes, which become enclosed in testicular cords formed by Sertoli precursor cells and peritubular myoid cells

[25, 26]. Gonocytes can be classified into I-gonocyte or M-prospermatogonia (mitotic prospermatogonia), which are located in the center of the testicular cords; however, their morphology is similar to oogonia in females [27, 28]. M-prospermatogonia continue proliferating until 16.5 dpc of mouse development and give rise to II-gonocytes or T1-prospermatogonia (primary transitional prospermatogonia, as these cells represent the first state of transition between the M-prospermatogonia and the A-spermatogonia) and enter G0 mitotic arrest [26].

During the first week after birth, T1-prospermatogonia migrate to the seminiferous tubules that give rise to T2 prospermatogonia (secondary transitional prospermatogonia, which represent the second state of transition to the A-spermatogonia) and colonize the basement membrane, and can enter into the spermatogenesis pathway during post pubertal life [29, 30].

1.2.2. The SSCs Niche

The testes of mammals are especially responsible for producing both spermatozoa and secretion of male hormones. The reproductive system, the testes in the male mammals, are placed within the scrotum covered by connective tissue called tunica albuginea (capsule). Testes are divided into testicular lobules, which constitute 1-4 seminiferous tubules that are separated by septum. Seminiferous tubules are connected to the Rete testis. The seminiferous tubules release their spermatozoa into Rete testis, which are then conducted to the 7-15 efferent ducts. Each testis is covered by the epididymis and adipose tissue (the epididymal fat body). The spermatozoa migrate to the epididymis for maturation.

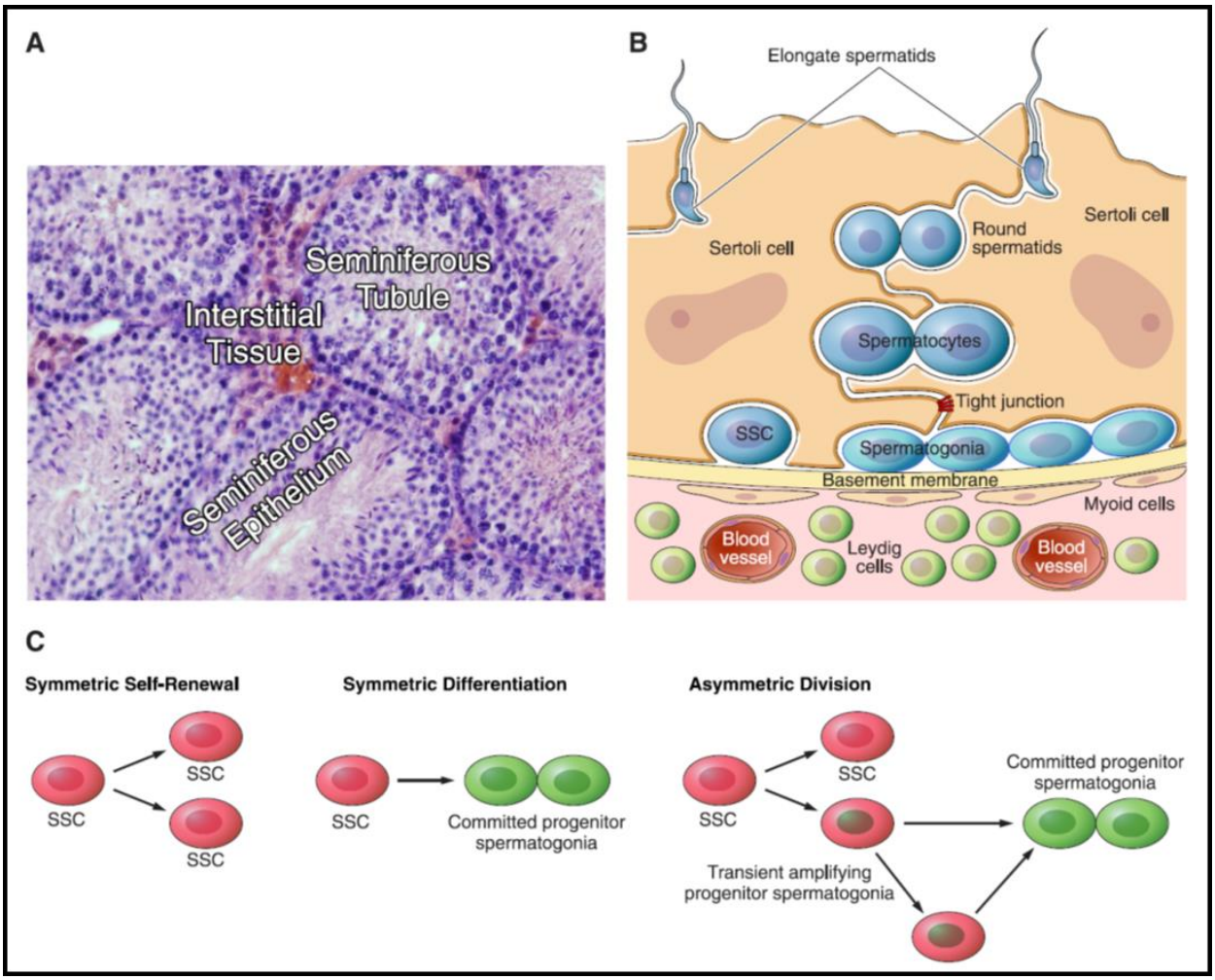

Figure 1.1: Spermatogenesis in the mammalian testes. Figure adapted from reference [31].

SSCs are the adult stem cells of the testis that are involved in spermatogenesis, with a diameter approximately about 14 µm in size, that form after birth and keep their activity throughout a male's life. The number of undifferentiated SSCs are seldom observable in the testis, confirmed by the fact that rate of the SSCs in the adult mouse testis is only 1 in 3000 cells [32]. SSCs reside directly in the germline epithelium above the basal membrane of the seminiferous tubules surrounded by Sertoli cells. In the periphery of the seminiferous epithelium, patches of interstitial tissue containing blood vessels, Leydig cells and macrophages are located (Figure1.1 A and B). These types of somatic cells secrete some factors that control self-renewal and differentiation of SSCs [33].

1.2.3. Division and differentiation

SSCs have the potential for division and differentiation in a cascade to spermatocytes, spermatids and sperm in order to transmit genetic information to the next generation. Differentiation of undifferentiated SSCs to spermatocyte cells is associated with a change in the gene expression profile and the cell morphology that is obvious in the sperm cells.

During the division of undifferentiated spermatogonia in rodents, daughter cells derived from A_s (A-single) spermatogonia can either move away from each other to guide to stem cell renewal or can stay together as a pair of so-called A_{pr} spermatogonia. Finally, this process produces aligned spermatogonia (A_{al}) cells that are connected by intercellular cytoplasmic bridges. Undifferentiated SSCs like A_s, A_{pr} and A_{al} proliferate with two symmetric and asymmetric divisions models (Figure1.1 C) until epithelial stage VII-VIII, in which most of the A_{al} spermatogonia differentiate to A1 spermatogonia without any divisions. During asymmetric division, undifferentiated A_s spermatogonia can convert to two A_s or two progenitor A_{pa} spermatogonia cells. Alternatively, during asymmetric division, A_s spermatogonia can give rise to two A_s or A_t (transient) SSCs [31,34, 35]. The A_{al} spermatogonia, can give rise to several generations of spermatogonia including A1–A4, intermediate, and type B cells [36]. There are usually six generations of differentiated SSCs in mammalians that produce spermatocytes after synchronous division [37, 38]. Cell cycle property and proliferation patterns are altered during differentiation of A_{al} to differentiated A1 spermatogonia [39]. Type B differentiated cells produce spermatocytes, spermatids, and mature sperm.

In primates, the undifferentiated spermatogonial population appears in two types: A_{dark} and A_{pale}. It has been proposed that A_{dark} cells are the reserve spermatogonia and they have very slow cyclin, which are unable to contribute to spermatogenesis pathways and can only convert to A_{pale} cells. In contrast, A_{pale} cells can divide mitotically and give rise to Type B and primary spermatocytes [40, 41].

1.2.4. Isolation of SSCs

Although SSCs do not show any highly specific markers for their enrichment, in general these cells have been isolated by different approaches:

First, by using extracellular matrixes such as laminin and collagen [42, 43]. SSCs express the surface markers a6 and b1 Integrins which bind to laminin [44]. On the other hand, due to a higher affinity of somatic cells for collagen, collagen-binding enrichment of testis somatic cells (Sertoli, Leydig and fibroblast cells) is another practical method for separation of somatic cells from SSCs in testicular cells [42].

Markers	References	Markers	References	Markers	References
A6 integrin(CD49)$^{+}$	[44]	MCAM^{+}	[45]	Laminin^{+}	[44, 46]
B1 integrin(CD29) $^{+}$	[44]	EPCAM^{+}	[47]	Collagen^{-}	[42]
C-Kit^{-}	[48]	GFRa1^{+}	[49, 50]	Gelatin^{+}	[51]
CD24^{+}	[48]	GPR125^{+}	[52]	MHC1^{-}	[48]
CD51^{-}	[53]	CD45^{-}	[54]	Thy-1^{+}	[48]
CDH1^{+}	[55]	CD9^{+}	[56]	Stra8^{+}	[57]
Rhodamine 123	[58]				

Table 1.1: MACS selection of SSCs against different markers

Another strategy for the enrichment of SSCs is by using fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) against a number of different surface markers of α6 (CD49) and β1 (CD29) Integrins [44, 59], CD9 [56], E-Cadherin [55, 60], THY-1(CD90) [61], GFRa1 [50, 62] that are expressed on the cell surface of SSCs (Table 1.1). However, one of disadvantage in this method is the lack of specific molecular surface markers for isolation of SSCs.

The third approach is a morphology-based selection of SSCs after cultivation of total testicular cells on gelatin-coated dishes [49, 51, 63-65]. This method can be more valuable in comparison to other methods due to typical cellular morphology of SSCs (as mentioned above, they have aligned or chain morphology) with somatic cells (cells spread strongly through culture plate). However, combinations of these methods are also used for enrichment of SSCs [42]. In our recent experiment, we isolated human SSCs using CD49f magnetic activated cell sorting and collagen−/laminin+ matrix binding selection from primary testis cultures which were obtained from adult men testis [66]. Although several methods for the enrichment of SSCs exist, there is still a disadvantage for the specific identification of SSCs.

Transplantation of SSCs to the seminiferous tubule of infertile recipients is the strongest technique for identification of SSCs, because only SSCs are capable of regenerating spermatogenesis after transplantation [67, 68].

In the recent years, researchers have been attempting to isolate similar cells with SSC potential from other sources of stem cell types [69, 70].

It has been demonstrated that MSCs have the potential to improve germinal repairing in testicular seminiferous tubules and also recovery of fertility in rat azoospermia models [71, 72]. Similarly, Yazawa et al showed that MSCs might also have the potential to differentiate into Leydig or adrenocortical cells [73]. In addition, mESCs have been shown to give rise to PGCs, sertoli cells and male gametes [69, 74].

1.2.5. Culture of SSCs

The underlying molecular mechanisms regulating proliferation, self-renewal, apoptosis and differentiation of SSCs are not completely understood. For example, various soluble growth factors, different types of basal media, concentrations of FBS and different types of feeder cells have critical roles in the differentiation, proliferation and self-renewal of SSCs during *in-vitro* culture. For example Kubota and colleagues showed that SSCs did not grow on newborn (NB) testis feeder, and in 10% of FBS in the presence of either MEM-a or F10 medium, despite the fact that they could significantly increase on STO feeder and 10% of FBS in presence of MEM-a (not F10 medium) [75].

Under serum-free culture conditions, the number of SSCs gradually decreased after the first week of culture [75]. Shinohara and colleagues presented a defined medium (Stem Pro-34 SFM; Invitrogen, Carlsbad, CA), which is still popular for SSCs cultivation [76]. They derived SSCs from neonate mouse testes and showed that SSCs can be cultivated in the presence of GDNF, FGF2, epidermal growth factor (EGF), LIF and 1% serum on mitomycin C-treated mouse embryonic fibroblasts (MEFs) for long term. It was shown that under a feeder-free culture condition and serum, SSCs can be cultivated for long term, although in the absence of both factors, proliferation of cells is slower than in adherent culture on MEF [43, 77]. For goat SSCs, a low concentration of serum is beneficial for short-term culture, but the expansion of SSCs for long-term culture depends on an increased serum concentration in the culture media [78].

1.2.5.1. Feeder

Although there are some reports on feeder-free culture of SSCs [43, 77], one of the main factors for growing SSCs are feeder layers. So far, different feeder layers were used for the cultivation of SSCs. MEF feeder are currently applied for most of the SSC cultivations [76, 79]. It has been reported that the GPR125 (an orphan adhesion-Type G-protein-coupled receptor) positive testis cells can be cultivated on testicular feeders containing CD34 positive cells [52]. It has been also been shown that the STO (SIM mouse embryo-derived thioguanine and ouabain-resistant fibroblasts) feeder cell line could be considered useful for *in-vitro* cultivation of SSCs [80, 81]. It has also been documented that STO feeder are able to maintain mouse neonate Thy-1 positive cells and bovine testicular germ cells under short-term cultivation conditions [75, 82]. The SNL cell line derived from the mouse fibroblast STO cell line expresses the neomycin resistance and LIF genes abundantly [83]. SNL feeder supports the derivation and maintenance of human induced pluripotent stem (iPS) cells [84]. However, an STO feeder layer could not be useful for self-renewal of rabbit germ cells while the mouse yolk sac-derived endothelial cell (C166) feeder layers could support proliferation of non-rodent SSCs [85]. TM4 or SF7 somatic Sertoli cell lines reduce the *in-vitro* maintenance and stem cell number of mouse male germ line stem cells [86]. Unlike ST2 and PA6 bone marrow stromal cell lines, the OP9 bone marrow stromal cell line has a positive

effecton maintenance of SSCs [86]. Extracellular nanofibrillar matrix could also support the maintenance of mouse neonate SSCs in short term cultivation [87].

1.2.5.2. Growth factors

Soluble growth factors and adhesion molecules play a crucial role for the maintenance of SSCs during cultivation. GDNF is an important growth factor for SSCs cultivation (Figure 1.2). It is a distant member of the transforming growth factor–b superfamily (TGF-B) that was detected for the first time in dopaminergic neurons to promote survival of neuronal cells during activation of cAMP-dependent signaling pathways [88, 89]. GDNF has a vital role in regulation of SSCs in the testis. By binding to a receptor complex which consists of C-Ret (transmembrane tyrosine kinase) and GFRa1 (GDNF family receptor alpha 1) in mammalian testis, GDNF exerts its activity [90]. This molecule promotes self-renewal and proliferation of SSCs during *in-vitro* cultivation and was identified as an essential molecule that inhibits differentiation of SSCs and supports the maintenance of the stem cell pool *in-vivo* [79, 91-93]. Usually, a 10 ng/ml concentration of GDNF is sufficient for SSCs culture, but in some experiments it has been observed that a 100 ng/ml concentration of this protein increased colony formation in the SSCs culture [86, 94]. Similarly, GDNF in combination with soluble growth factors GFRa1 and FGF2 significantly enhanced proliferation and long-term expansion of mouse SSCs in cultivation [79]. In the porcine testicular cells, bFGF decreases the expression level of Nanog and PLZF but it could increase the expression level of Oct-4 and GATA4 [95]. LIF is one of the essential molecules for maintenance of pluripotency in ESCs [96], which enhances survival of Sertoli cells and stimulates the proliferation of quiescent gonocytes and SSCs cells in the mouse testis [79, 97]. Growth factors including FGF, EGF, LIF and steel factor do not affect individually on maintenance of SSCs which cultivated on the STO feeder. However, FIK-2L growth factor (used for cultivation of hematopoietic cells) reduces colony number of SSCs [86]. Growth factors LIF, GDNF, EGF, FGF, IGF and SCF do not have any significant effect on SSC activity individually during the first week of serum-free culture system [75].

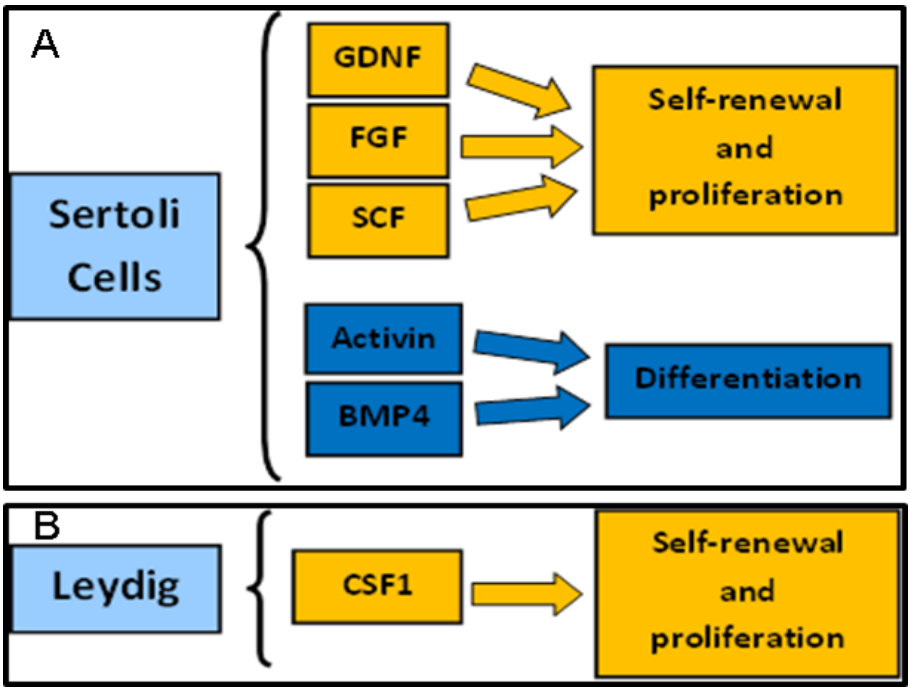

Figure 1.2: Effect of different growth factors from Sertoli cells (A) and Leydig cells (B) on SSCs.

The presence of GDNF, GFRa1 and bFGF (but not LIF) increased the growth of rat SSCs in a short-term culture [98]. Therefore, similar to the mouse SSC culture, a combination of growth factors GFRa1, FGF and LIF in the presence of GDNF supports the proliferation of rat SSCs after 7 days of cultivation [98]. It was reported that LIF, SCF, IGF-1 and Noggin growth factors (an antagonist for bone morphogenetic proteins) have effects on proliferation of mouse SSCs *in-vitro* [79]. In goat SSCs, a combination of growth factors of EGF, FGF, LIF and GDNF maintain the undifferentiated state of SSCs during 2-week cultivation [99]. Activin A and bone morphogenetic protein 4 (BMP4) molecules decrease the maintenance of mouse SSCs (Figure 1.2) [86, 100].

In the rat SSCs, BMP4 increases the expression of the early differentiation marker C-Kit and up-regulates the expression of cell adhesion molecule CDH1 [101]. Both Activin

A and B molecules induce rat spermatogonial proliferation in a co-culture system with Sertoli cells [102].

1.2.6. Regulation of SSCs

Similar to other adult stem cells, SSCs undergo both self-renewal and differentiation, which are controlled by extrinsic factors from the cell niche and by intrinsic gene expression patterns. In the stem cell compartment of the basement membrane of the seminiferous tubules, somatic Sertoli cells exist and surround the base membrane of the undifferentiated SSCs. The development of undifferentiated SSCs to sperm depends on the interaction of SSCs to Sertoli cells and outlying peritubular myoid cells [103]. Sertoli cells determine the fate of SSCs by producing different growth factors (Figure 1.2). Production of glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2) from Sertoli cells promote self-renewal and proliferation of SSCs, while production of activin A and BMP4 regulates differentiation of SSCs (Figure 1.2).

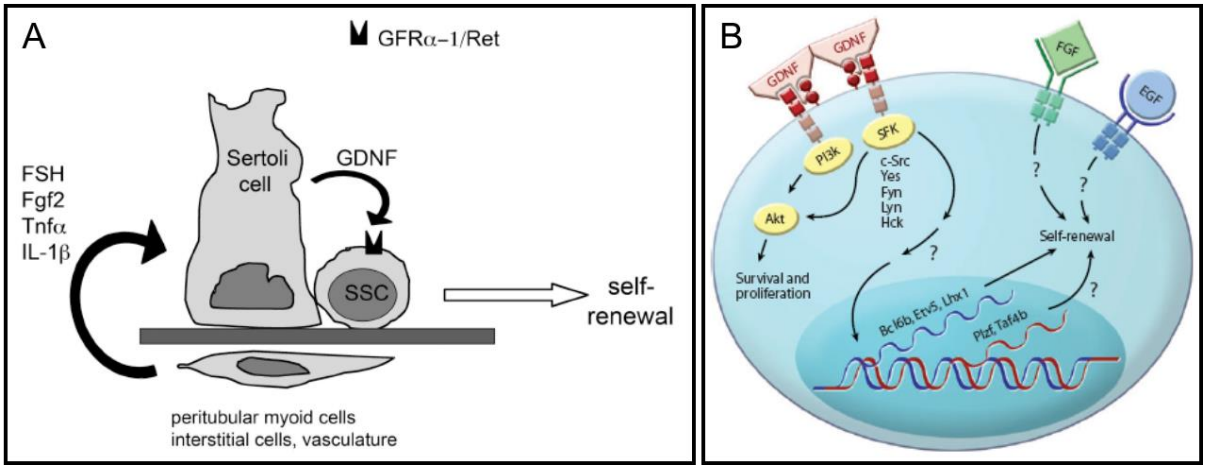

Figure 1.3: Regulation of SSCs. Figure adapted from references A [104], B [6].

FGF2 promotes self-renewal of SSCs by activation of MAP2K1 and upregulation of transcription factors Etv5 and Bcl6b [105].

Major regulators of GDNF concentration are testosterone and the follicle-stimulating hormone (FSH), which are secreted by Leydig cells and the hypothalamus, respectively [106]. SSCs express the receptors for GDNF, c-Ret, and GFRa1 [107]. This effect is mediated by the Akt and Src family kinase (SFK) signaling pathways (Figure 1.3) [80, 108]. In addition, SSCs express the transcription factors PLZF, Taf4b, Bcl6b, Etv5, and Lhx1 that stimulate self-renewal and maintenance of SSCs (Figure1.3). In early spermatogonia, the transcription activity factors Bcl6b, Etv5, and Lhx1 are controlled by GDNF and PLZF, which inhibit the expression of the differentiation receptor c-Kit (Figure 1.3) [109]. Both Activin A and BMP4 reduce the maintenance of SSCs and promote differentiation of these stem cells (Figure 1.2) [86]. Interstitial Leydig and peritubular myoid cells produce CSF1 that increase the proliferation of undifferentiated SSCs (Figure 1.2) [110].

1.2.6.1. Role of different markers on SSCs

As mentioned above, spermatogenesis is regulated by endocrine, paracrine and autocrine growth factors which are produced by sertoli cells, germ cells, peritubular cells, Leydig cells and macrophages. The interactions between growth factors activated different proteins and signaling pathways, which can direct the fate of SSCs. In the below table, we listed most of the popular germ cells proteins which are active during germ cell development (Table 1.2).

Markers	**Property**	**Ref.**
Oct-4	Crucial molecule for SSCs property but not in cell survival.	[111]
VASA	Required for proliferation and differentiation of mouse male germ cells during development	[112]
DAZL	Required for the entry into meiosis in the adult rat testis	[113]
STRA8	Plays a role in the pre-meiotic phase of spermatogenesis	[114]
GFRa1	GDNF receptor is expressed in undifferentiated SSCs and is important for the maintenance of SSCs.	[115, 116]
C-Ret	Ret tyrosine kinase/GFRa1 receptor complex through GDNF signaling activates self-renewal of SSCs	[117]
C-Kit	Positive in the end stage of undifferentiated SSCs and differentiated SSCs	[118]

PLZF, BCL6B, ETV5, LHX1, TAF4B	These transcription factors are required for self-renewal, proliferation and maintenance of SSCs	[60, 119, 120]
Nanog	Expressed in neonate pig testis germ cells but progressively lost with age	[103]
Sall4	There is an antagonism between Sall4 and PLZF to induce expression of Kit. Sall4 is expressed in A_s, A_{pr} and A_{al} SSCs and has overlap with expression of PLZF, Nanog, Kit and GFRa1.	[105, 121, 122]
Lin28 (Tex17)	Expressed in undifferentiated SSCs	[123]
Foxo1	Essential for SSC homeostasis and the initiation of spermatogenesis	[124]
PTEN,TRP53	Suppresses Nanog expression in SSCs	[125]
AKT	Plays a role in self-renewal division of SSCs during activation of phosphoinositide-3 kinase (PI3K-Akt) signaling pathway	[126]
Klf4	Plays a role during spermatogenesis but lack of KLF4 alone does not prevent complete spermatogenesis	[126]
UTF1	Expressed in gonocytes but decreased during further testicular development to a subset of A spermatogonia.	[127]
N-Myc	GDNF with activation of PI3K-Akt signaling and Src kinase up-regulate N-Myc expression	[94]

Table 1.2: Role of different markers during germ cells development.

1.2.7. Transplantation and functionality

Differentiation of undifferentiated SSCs takes place in the adluminal compartment of the seminiferous tubules of the testis. As discussed above, undifferentiated SSCs can be isolated and expanded during *in-vitro* culture. Although SSCs express some of the germ cell markers, they lack any specific marker for identification. Therefore, transplantation is an efficient opportunity to study identification and functionality of SSCs because SSCs in recipient testis have the ability for colonization in the testis and can restore spermatogenesis in infertile mouse models. This functional SSC characterization system was identified for first time by Brinster and his colleagues [67, 128]. This group showed colonization of seminiferous tubules of an infertile recipient mouse model after transplantation of SSCs into testes. The efficiency of colonization of adult testicular cells was higher than neonate testis cells (gonocytes), and donor cells had normal

differentiation spermatogenesis and produced mature sperm [67, 128]. In cultured undifferentiated SSCs, one single SSC has the potential for colonization in recipient testis [129].

The Brinster group demonstrated that ESCs generate tumor in recipient testis and could not replace colonization of testicular cells [67]. The efficiency of transplantation also was not enhanced by an increased number of Sertoli cells [67]. Infertility in recipient animal models can be attained by Busulfan treatment or irradiation. These factors decrease size of testis, deplete endogenous germ stem cells and spermatogenesis in seminiferous tubules of testis. Infertile mouse models can also be generated naturally from W/W^v mutant males. There is mutation in tyrosine kinase domain of KIT which is necessary for proliferation and migration of PGCs during development [130].

Most testis transplantation research was conducted in mouse models. Because of the long time-duration requirement for sexual maturation and also the lack of possible models for primate, xenografting transplantation into mouse has provided a useful method for study of spermatogenesis and infertility in recent years [131-133].

1.2.8. Pluripotency of mouse SSCs

Pluripotent stem cells were achieved by several methods. One technique includes the generation of ES cells from the inner cell mass of an embryo at the blastocyst stage [1, 134]. In humans, this model is associated with major ethical problems. Another approach for the generation of pluripotent cells is by induction of pluripotency genes including Oct4, Sox2, Klf4 and c-Myc into somatic cells, which give rise to a so-called induced pluripotent stem cells (iPSCs) [135, 136]. This technique is a simple and promising method for the generation of patient-specific stem cells, however, the cells are manipulated by extrinsic factors (including, viruses, DNA, RNA, proteins and/or small molecules), which might be somehow problematic in regenerative medicine. Furthermore, the use of oncogenes like c-Myc and Klf4 for the production of iPSCs led to controversy about the medical application of these cells.

One of the promising methods for the establishment of pluripotent stem cells is with SSCs [51, 52, 57, 137]. Recently, generation of ES-like cells from neonatal and adult SSCs has been reported by several researchers [51, 52, 57, 137, 138].

Although SSCs are unipotent stem cells, under specific culture conditions and without any exogenous pluripotency genes they can convert to pluripotent embryonic ES-like cells that can differentiate into a number of cell lineages comprising the three embryonic germ layers [42, 51, 137].

Generation of PSCs from mouse testis cells dates back to 2004 by Kanatsu Shinohara [137] when they generated ES-like cells in SSC culture from two-day-old pups. In these experiments, ES-like cells were shown to be phenotypically similar to mouse ESCs and have the potential for *in-vitro* and *in-vivo* differentiation into various cells types. Furthermore, these ES-like cells contribute to the formation of germline chimeras after being injected into blastocysts. These results proved the potential of pluripotency of the SSCs or gonocytes derived from neonatal testis but the origin of ES-like cells was not clear. Later on, ES-like cells were generated from GFP-marked populations of STRA8-positive [57], GPR125-positive [139] and Oct-4-positive [51] cells in the mouse testis.

Guan et al. demonstrated that SSCs from adult mouse testes could generate multipotent cells *in-vitro* while these cells could be differentiated in three germ layers during *in-vitro* and *in-vivo* condition and also formed germline chimeras after injected into blastocysts [57]. Conrad et al derived human ES-like cell line from enriched human SSCs of the adult testes. In their experiments, human ES-like cells differentiated into three germ layers during under *in-vitro* and *in-vivo* condition and showed molecular characteristics with some similarities to human ESCs [42].

In recent years, it has been demonstrated that chemicals can enhance generation of ES-like cells. In neonate mouse testicular culture, inhibition of glycogen synthase kinase-3 promotes derivation of pluripotent (ES)-like cells [138].

1.3. Aim of the study

In our experiments, in a first step we intended to identify different patterns of gene expression for testis cells which are located in the seminiferous tubule of mouse testis such as undifferentiated SSCs, differentiated SSCs and Sertoli cells (*in-vivo* analysis).

Also during *in-vitro* culture, with morphology-based selection, we wanted to identify different types of cells in the testicular cultures and after characterization we aimed to analyze the best conditions for expansion and long term cultivation of the different cell populations.

Furthermore, in the main part of this study –the generation of pluripotent stem cells- we tried to answer the question which cell population (based on morphology selection) in the testicular culture could show spontaneous reprogramming to a pluripotency state. By several *in-vitro* and *in-vitro* characterizations we intended to compare the pluripotency potential of testis derived ES-like cells in comparison to mESCs and testicular cells.

Chapter 2 Materials and Methods

2.1. Materials

Consumable items include materials, enzymes, chemicals, solutions and growth factors for cell culture, histology, electrophysiology and EM, which are described in detail in the Appendix (Appendix, Tables.1-34). In addition, antibodies used for staining, primers used for PCR, TaqMan gene expression assays for multiplex qRT-PCR and concentration of components in the associated solutions and mediums are described in more detail in the Appendix (Appendix,Tables.1-34).

2.2. Methods

2.2.1. Digestion of testis

All animal care was in accordance with the approval of the Institute for Anatomy and Cell Biology in the University of Heidelberg (Heidelberg, Germany) and the Royan Institutional Review Board and Institutional Ethical Committee (Tehran, Iran). Testis cells were isolated from C57BL/6, 129/Sv mouse strains of 6-day to 6-month old Oct4-promoter reporter GFP transgenic mice after decapsulation and treatment by a one-step enzymatic digestion protocol. After removing of the tunica albuginea, dissociated testicular tissue was placed in a digestion solution (Appendix, Table 10) at 37°C for 8 minutes. Digestion enzymes were stopped with 10% ES cell-qualified FBS and in addition, pipetted up and down to obtain a single cell suspension. After centrifugation, specimens were washed with DMEM/F12, filtered through a 70 µm cell strainer and centrifuged for 10 minutes at 1500 rpm (Figure 2.1). The supernatant was removed and the suspension of testicular cells was plated onto 0.2% gelatine-coated culture dishes.

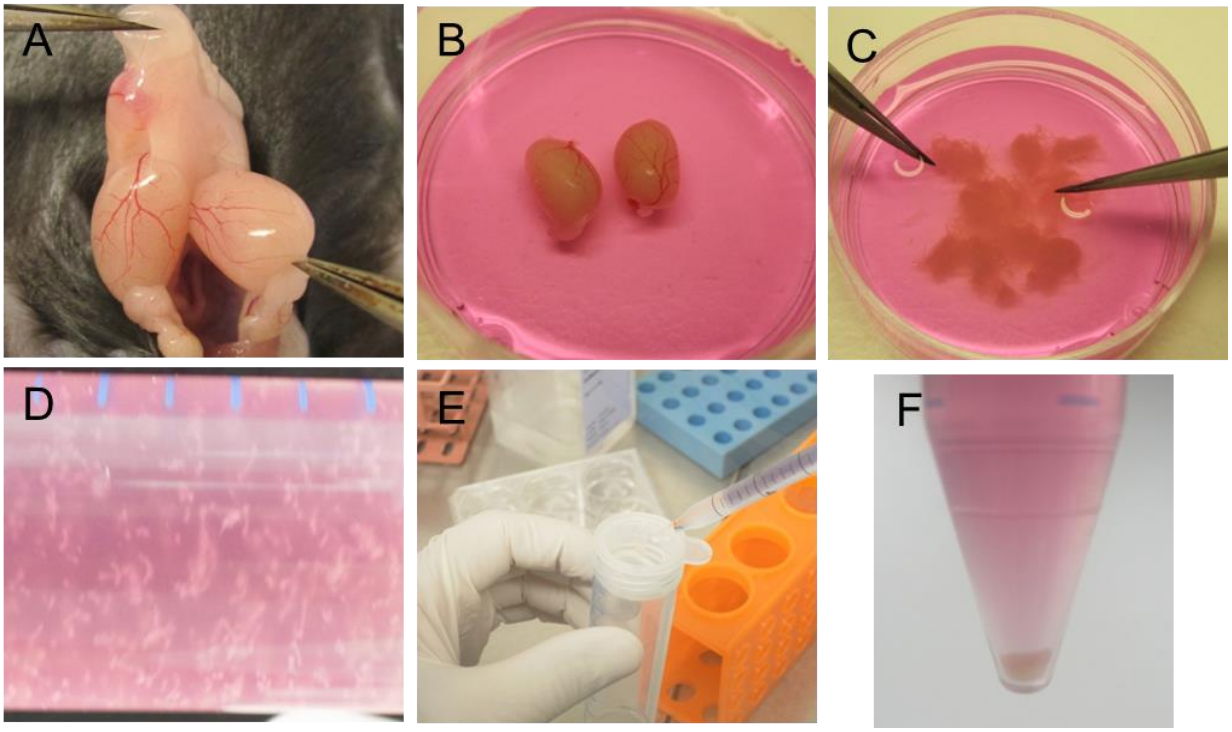

Figure 2.1: The digestion process of the mouse testis was as follows: macroscopic dissection of the testis (A) and (B), mechanical separation of seminiferous tubules after decapsulation of tunica albuginea (C), exposure of separated seminiferous tubules with digestion enzymes (D), separation of tissue pieces with filter mesh (E), ell Testicular cell pellet obtained after centrifuge (F).

2.2.2. Culture of testicular cells

As we mentioned the suspension of digested testicular cells was plated onto 0.2% gelatine-coated culture dishes. Approximately 0.2-0.5 × 10^5 cells plated per 3.8 cm^2 for neonate and 2 × 10^5 cells per 3.8 cm^2 for old mouse. Cells cultured in the mouse GSC (mGSC) medium consisted of stemPro-34 medium, N2-supplement, D+ glucose, bovine serum albumin, L-glutamine, β-mercaptoethanol, penicillin/streptomycin, MEM vitamins, NEAA, estradiol, progesterone, EGF, FGF, GDNF, LIF, ES-FBS, ascorbic acid, pyruvic acid and DL-lactic acid with 37°C and 5% CO_2 in air (protocol for preparation of mGSC medium is mentioned in the appendix, Table 11).

2.2.3. Generation of ES-like cells

Neonate and adult mouse testicular cells were cultivated in GS medium. Type 1 cell colonies were manually picked in primary culture or after subculture of primary supernatants into MEF feeder layer. ES-like colonies were generated from Type I cells in a time window between 40-125 days after initiation of culture (Figure 2.2).

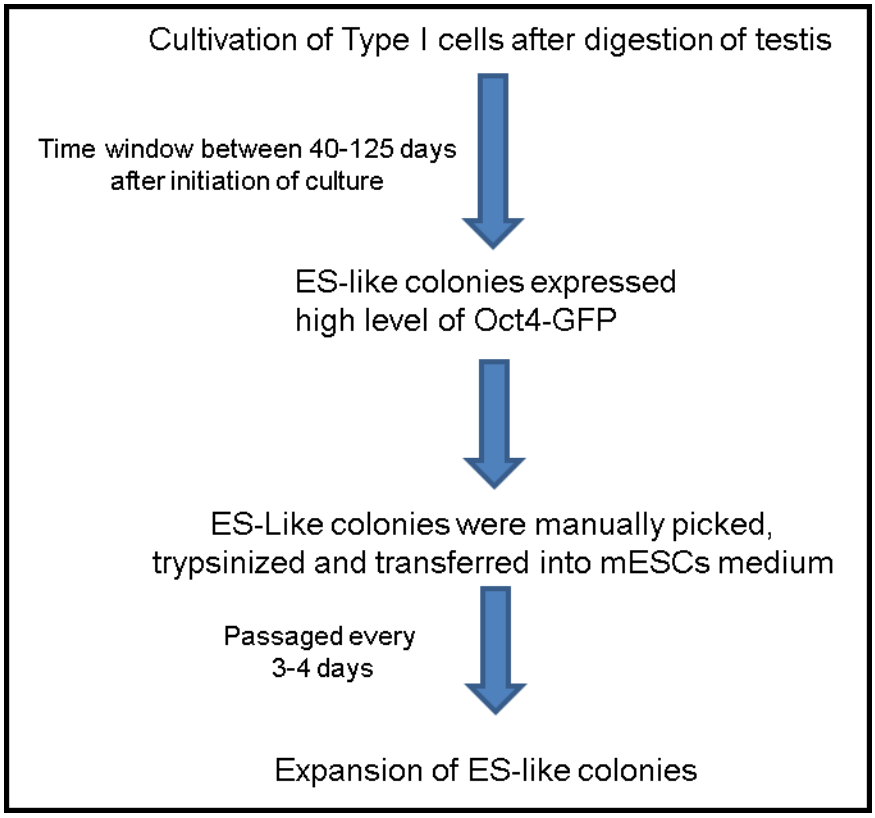

Figure 2.2: Protocol for the generation of ES-like cells from mouse testis:

Single cells of ES-like colonies were obtained after trypsinization under mouse ES medium condition consist of KO-DMEM (or DMEM high-glucose medium), FBS, MEM NEAA solution, L-glutamine, Pen-Strep, mercaptoethanol and LIF (Appendix, Table 12) under MEF feeder layer. ES-like colonies were grown in mESCs media and were passaged every 3-4 days. In the beginning of culture, some of Type 1 cells (especially in neonate mice) expressed low level of Oct4-GFP but this signal was down-regulated after long term culture. During cultivation of Type I cells, we found colonies (rate of

about 33%) which were similar to mESCs (ES-like cells) or cultured epiblast cells that expressed high level of Oct4-GFP about 41-125 days after initiation of culture. Generated ES-like cells were subcultured in mESCs medium (Appendix, Table 12). ES-like cells reached confluence about 4-5 days after initiation of culture. Cells were passaged to a new MEF feeder after washing with PBS and treatment with Trypsin-EDTA for 3min. Trypsin-EDTA was inactivated with 15% of FBS.

2.2.4. Preparation and culture of different feeders

2.2.4.1. SNL feeders

We commercially ordered SNL 76/7 feeder cells (CBA-316) that were established by Dr. Allan Bradley [83]. SNL feeder cells were grown in the present of D-MEM, FBS, MEM NEAA solution, L-glutamine, ß-mercaptoethanol and Pen-Strep (Appendix, Table 13) on T-75 tissue culture flask at 37 °C and 5% CO_2. SNL cells were passaged when the cultured cells reached 90 percent confluence. Two usual techniques, γ-irradiation and mitomycin C (10 mg /ml) treatment, were used for mitotic inactivation (Figure 2.3A).

2.2.4.2. Mouse testicular stromal feeder cells (TSCs)

TSCs were generated from adult mouse testis tissue that was 4-8 weeks old. After digestion of testis, testicular cells were cultured in presence of D-MEM, FBS, MEM NEAA solution, glutamine, ß-mercaptoethanol, Pen-Strep and bFGF (Appendix, Table 14) on T-75 tissue culture flasks at 37 °C and 5% CO_2. TSCs cells were passaged to new culture flasks after reaching 90 percent confluency. In passage, 2-3 TSC cells were used for mitotic inactivation with mitomycin C (10 mg /ml) (Figure 2.3B).

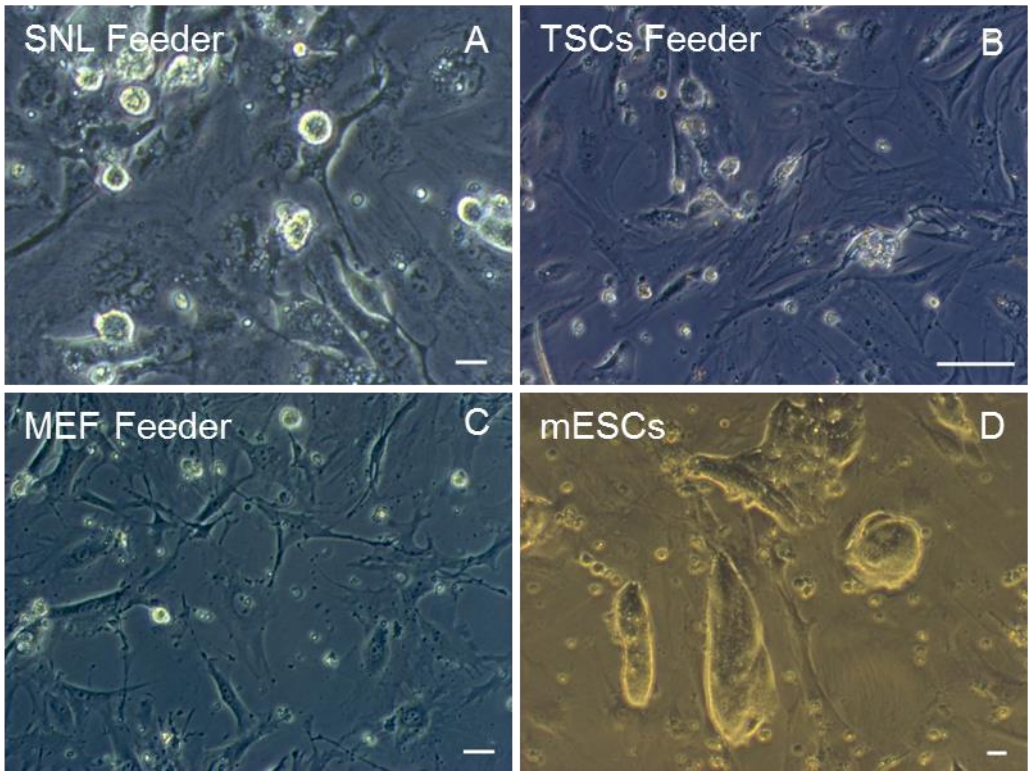

Figure 2.3 Cultivation and morphology of different feeders and mESCs in the associated mediums: SNL (A), TSCs (B), MEF (C), mESCs (D).

2.2.4.3. Mouse embryonic feeder cells (MEF)

Embryos (E13-E14) from a pregnant CF-1 mouse were used for MEF preparation.

Pregnant females were killed by CO_2 asphyxia followed by preparation of placenta and fetal membranes. Embryos were washed with an HBSS buffer and then intestinal and the head of embryos were removed. Subsequently, embryo carcasses were transferred to a new plate with an HBSS buffer and the tissue was minced with a syringe, followed by digestion with the enzyme solutions, trypsin or collagenase-dispase (1mg/ml) for 15-20 min. Digestion enzymes were inactivated with 15% serum and the cells pipetted 10 times to break up the remaining tissue pieces . Digested embryonic cells were grown in the present of D-MEM (or KO-DMEM), FBS, MEM NEAA solution, L-glutamine, Pen-

Strep and ß-mercaptoethanol culture media (Appendix, Table 15) on T-75 tissue culture flasks at 37 °C and 5% CO_2 (Figure 2.3C).

MEF cells were passaged when the cultured cells reached up to about 90 percent confluence. In passage 3-4, MEF cells were used for mitotic inactivation with γ-irradiation or mitomycin C treatment. For mitomycin C treatment, cells were incubated for 3 h with mitomycin C (10 mg /ml) at 37 °C.

2.2.5. Mouse ES and ES-like cells culture

mESCs and ES-like cells were cultured in ES media containing KO-DMEM (or DMEM high-glucose medium), FBS, MEM NEAA solution, L-glutamine, Pen-Strep, mercaptoethanol and LIF (Appendix Table 12). These cells reached confluence about 4-5 days after initiation of culture. mESCs and ES-like cells were passaged to new MEF feeder after being washed with PBS and treated with trypsin-EDTA for 3min. Trypsin-EDTA was inactivated with 15% of FBS (Figure 2.3D).

2.2.6. Freezing and thawing cells

Cell freezing medium which included FBS, DMSO and TSCs medium (Appendix, Table 16) was used to freeze mESCs, ES-like cells, Type I and II cells. Cell plates from Type I and II cells were collected by rinsing while for mESCs and ES-like cells were collected after trypsinization. The cell pellets were resuspended with a small amount of rest culture with gentle shaking. Then 0.5-1 ml appropriate freezing medium was added to each vial, followed by quick transport of the vials into an isopropanol freezing container and put into a -80°C freezer for 24h. The temperature gradually decreased (1°C per minute) in the isopropanol freezing container. After 1 day, frozen vials were transferred to a liquid nitrogen tank. To thaw the cells, they were transferred to prewarmed MEF medium at 37 °C and after centrifugation, plated to special cell culture medium.

2.2.7. Electron microscopy

For electron microscopy, Type I and II cells were fixed as a pellet with paraformaldehyde/ glutaraldehyde in PIPES (Appendix, Table 17). After 5 min, the fixative was renewed. Pellets were then post-fixed in OsO_4/potassium hexacyanoferrate (Appendix, Table 18) for 50 min, rinsed in the solution of uranylacetat buffer and sodium maleate buffer (pH 6.0) (Appendix, Table 19), and block-stained with uranyl acetate (Appendix, Table 20). Following the dehydration through increasing concentrations of ethanol (5 min each, 4 x 20 min), the tissue was Epon-embedded (polymerization at 60 °C, 24 h). Ultrathin sections (50 nm) were examined with a Zeiss EM10.

2.2.8. Flow cytometric analysis

To remove feeder cells, Type 1 cells, mESCs and ES-like cells were collected and incubated for 40 min on gelatine-coated dishes. Staining reaction was performed in staining buffer consisting of PBS supplemented with FBS (Appendix, Table 21). After determining cell viability by trypan blue staining, cells were resuspended in staining buffer and incubated with cell surface primary antibodies-conjugated with fluorochrome (APC) (Appendix, Table 6) for 1h. After washing, flow cytometric analysis was performed with a BD-FACS Calibur Flow Cytometer. (Cells were analysed by Dr. Heike Peterziel, Prof. Angel group, DKFZ, Heidelberg). The experiments were repeated more than 3 times and the acquired data was analyzed using BD CellQuest Pro program.

2.2.9. Immunocytochemistry staining

Cells were cultured in 24 well plates and fixed with 4% paraformaldehyde. After rinsing with PBS, samples were permeabilized with 0.1% Triton/PBS (Appendix, Table 22) and blocked with 1% BSA/PBS (Appendix, Table 23). After removing the blocking solution, the cells were incubated overnight with primary antibodies (Appendix, Table 6). After

rinsing, the process was followed by incubation with species-specific secondary antibodies which were conjugated with different fluorochromes (Appendix, Table 6). Labeled cells were counterstained with 0.2 µg/ml DAPI (4', 6-diamidino-2-phenylindole) for 3 min at room temperature and fixed with Mowiol 4-88 reagent. Omission of each primary antibody in the sample was used as a negative control for all markers. Labeled cells were examined with a confocal Zeiss LSM 700 microscope, and images were acquired with a Zeiss LSM-TPMT camera.

2.2.10. Tissue processing for immunohistochemistry (IMH)

Testis tissue was removed from male animals, which was then washed in PBS and fixed in 4% paraformaldehyde for about 24h at room temperature (RT). Dehydrated testis were embedded in Paraplast Plus. Testis tissue blocks were cut with a microtome at about 10 µm thickness. Sections were mounted on Superfrost Plus slides and stored at RT until used.

All sections were deparaffinized with xylene and rehydrated in an ethanol series prior to staining. After antigen retrieval by heat mediated antigen retrieval (Sodium Citrate Buffer, pH 6 or EDTA, pH 8) (Appendix, Table 24) at 95°C for 20 min, non-specific binding was blocked with serum/Triton X-100 in PBS (Appendix, Table 25) and immunofluorescence staining continued as explained above.

For IMH on cryo-sections, the testis tissue was fixed as described above. Dehydrated testis tissue was rinsed in PBS and incubated in 10%, 20% and 30% sucrose/PBS for 12-18 hours followed by embedding of the tissue in Tissue Tek. Frozen blocks were cut with a cryostat (Leica CM 3050S) in 12-20 µm sections.

The sections were mounted on Superfrost Plus glass slides and stored at -20 °C until analyzed. All frozen sections were dried at RT for 30 min, followed by immunofluorescence staining, which was explained in detail above.

2.2.11. RNA extraction and RT-PCR analysis

For RT-PCR analysis, the expression patterns of different ectodermal, mesodermal and endodermal genes (Appendix, Table 8) were studied after spontaneous somatic differentiation of ES-like cells. In each sample, total RNA of differentiated ES-like cells was extracted by using peq GOLD TriFast reagent. After phenol–chloroform extraction, cDNA was synthesized by using M-MLV Reverse Transcriptase kit. For cDNA synthesis, 2 µg of total RNA were used, reverse transcribed in a volume of 40 µl and finally filled up with water. PCR reactions were performed in single PCR tubes and carried out using a Mastercycler gradient machine (Eppendorf, Germany) in a reaction volume of 50 µl per sample (Components of each PCR reaction is noted in the Appendix, Table 26). The thermal cycling conditions were as follows: initial denaturation at 94°C for 3 minutes, followed by 35 cycles with denaturation at 94°C for 30 seconds, annealing at primer specific temperatures (Appendix, Table 8) for 30 seconds, extension at 72°C for 30 seconds. As last steps, a final extension at 72°C for 10 minutes was performed, followed by a cool down to 4°C. The PCR product was analysed by gel electrophoresis with an agarose gel in TAE (Tris-acetate buffer EDTA) (Appendix, Table 27). The gels were stained with 0.8 µg/ml ethidium bromide and visualized under a UV transillumination imager (INTAS, Germany).

2.2.12. Gene expression analyses on the Fluidigm Biomark system

The Dynamic Array chips (Fluidigm) was used to measure the expression of multiple pluripotency and germ cells specific genes including Oct-4, Nanog, Sox2, Klf4, GDF3, TDGF1, TERT, CDH1, DAZL, KIT, STRA8, RET, NEUROGENIN, EPCAM,TAF4B, TEV5, TBP, PLZF, LHX1, BCL6B and housekeeping gene GAPDH (which was used for normalization) (Appendix, Table 9) with the different Types of cultured cells such as Type I, Type II, TSCs, MEF, ES-like and mESCs. Cells were characterized in very small numbers. In each sample, about 50 cells were manually selected from the different cell cultures with a micropipette or micromanipulator and immediately frozen and stored at -80˚C.Each sample was lysed into special lysis buffer containing different components

(Appendix, Table 28). In a process using reverse transcriptase enzyme, mRNA was reverse transcribed into cDNA, which was sequence-specifically preamplified in a single tube, and the amount of targeted transcripts was quantified using TaqMan real-time PCR on the BioMark Real-Time quantitative PCR (qPCR) system. Reverse transcription was achieved at 50°C for 15 min using reverse transcriptase enzyme, which was inactivated by heating to 95°C for 2 min. cDNA was denatured at 95°C for 15 s. Subsequently, products were pre-amplified at 60°C for 4 min for 14 cycles. The pre-amplified products were diluted up to 5-fold and then analysed with Universal PCR Master Mix and inventoried TaqMan gene expression assays (ABI) in 96.96 Dynamic Arrays on a BioMark System. Each sample was analyzed in two technical replicates. For analysis purposes, data that were missing in the Biomark system were replaced by a Ct of 30 and data were normalized with GAPDH and expression fold change of mRNA compared to MEF feeder cells. Analysis was conducted by GenEx software from MultiD analysis, Excel and SPSS.

2.2.13. Transplantation

Type II cells were transfected with enhanced green fluorescent protein (EGFP) gene (COP-GFP). For site-specific transplantation into the tubuli seminiferi contorti of the testis, approximately 10µl of the donor cell suspension containing 0.5-1x 10^5 SSCs was injected into the rete testis of males SCID NO mice that had been treated with busulfan (44 mg/kg) at 6 weeks of age [67]. Adult recipient mice were anesthetized with Ketamin(100mg/kg) – Xylazine (20mg/kg) solution. Testis were fixed in paraffin and cryo blocks and sections were analysed by confocal microscopy 4-8 week after transplantation.sperm cells were kept at -20^{o}C after isolation from the glandula epididymidis and fixed on slides. Similarly, sperm cells from EGFP mouse samples were isolated from the glandula epididymidis for comparison to our samples. (These experiments were performed in Royan Institute, Tehran, Prof. Baharvand´s group).

2.2.14. Embryoid Body (EBs) formation

ES-like cells were cultured in mESCs medium and after expansion were used for EBs formation. After trypsinization, ES-like cells were incubated on gelatine coated plates for 1h to eliminate somatic cells. About 2-3×10^6 cells were cultured in the medium containing KO-DMEM (or DMEM high-glucose), FBS, MEM NEAA solution, L-glutamine, Pen-Strep and ß-mercaptoethanol (Appendix, Table 29) in low attachment 10 cm^2 bacterial-grade petri dishes. EBs spontaneously formed as a suspension culture in bacterial culture dishes for 3-4 days. Due to the expression of Oct-4-GFP in the ES-like cells, generated EBs also expressed Oct-4-GFP.

2.2.15. Neuronal differentiation

Neuronal differentiation was induced in three stages. Stage 1, which is related to EB formation, is discussed above. Then in stage 2, for generating neuronal progenitor cells, EBs were cultured in the medium containing DMEM-F12, NEAA, L-Glutamin, N2, FGF and RA (Appendix, Table 30) as suspension in low attachment 10 cm^2 bacterial-grade petri dishes for additional 6 days . FBS concentration was decreased to 10% (days 1-2), 5% (days 3-4) and 3% (days 5-6) in the second stage. In stage 3, approximately 10 days after initiation of suspension culture, EBs were plated on poly-L-ornithine coated wells of 6 or 24-well tissue culture plates in the Neurobasal medium contain NEAA, L-Glutamin, B27, N2 and FBS (Appendix, Table 31) for additional 10 days. This condition medium allowed maturation and development of neuronal progenitor cells (Figure 2.4A).

2.2.16. Cardiomyocyte differentiation

Similar to neuronal differentiation, cardiomyocyte differentiation was induced in three stages. Stage one was similar to neuronal differentiation. In stage 2, for spontaneous

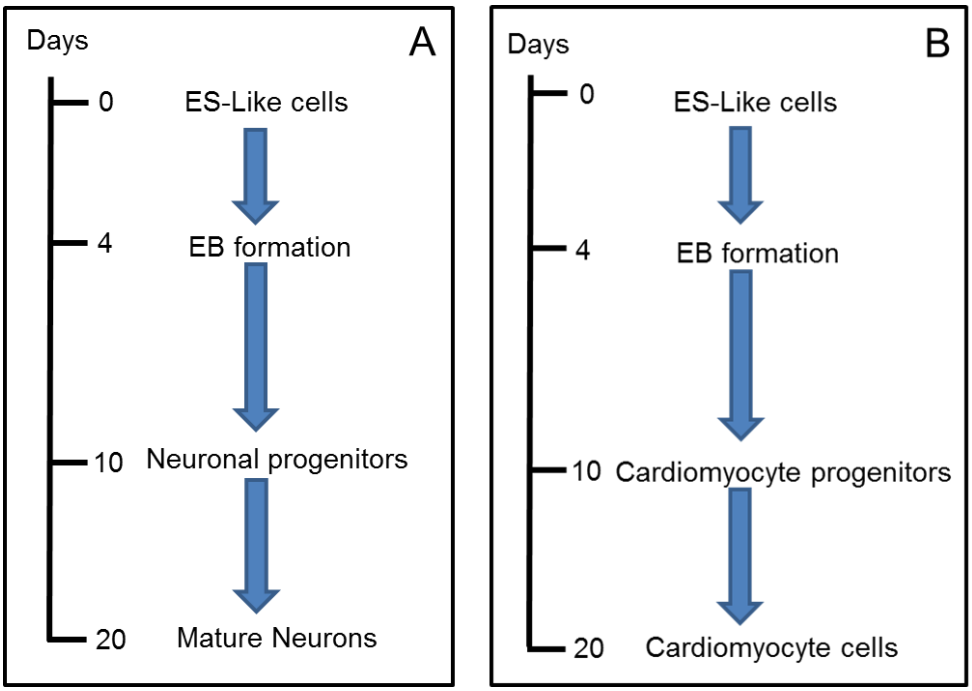

Figure 2.4: Differentiation of mature neurons and cardiomyocyte cells from ES-like cells. According to both differentiation protocols, mature neuron and cardiomyocyte cells were obtained after EBs formation. For neuronal differentiation, EBs were exposure with RA and low concentration of FBS (A), while a high concentration of FBS without RA treatment was used for cardiomyocyte differentiation (B).

mesodermal differentiation, EBs were grown in medium containing DMEM-F12, NEAA, L-Glutamine, N2, FGF and FBS (Appendix, Table 32) as suspension in low attachment 10 cm^2 bacterial-grade petri dishes for a further 8 days. In the final stage, in order to receive beating cells, EBs were plated in1% gelatine-coated wells of 6 or 24-well tissue culture plates under the same medium used in stage 2 (Appendix, Table 32).

Subsequently, the number of beating clusters and the number of beats per minute were counted at the end time of the final stage. In addition, after the EBs were plated, differentiated cells were used for RT-PCR analysis of mesodermal and endodermal lineage genes (Figure 2.4B).

2.2.17. Teratoma assay

After trypsinization of ES-like cells, dissociated cells were replated on 0.1% gelatine coated plates in mESCs medium at 37 °C and 5% CO_2 for about 1 h followed by collecting the supernatants. After centrifugation cells were resuspended in 130 µl PBS and 70 µl matrigel on ice. About 2-3 x 10^6 cells were injected subcutaneously into 7-week old SCID-beige mice and checked 3 times per week for tumor growth. (The experiment was performed in Royan Institute, Tehran).

2.2.18. Electrophysiology

Whole-cell current clamp recording was established using an EPC-9 amplifier and acquired using the Patch Master software. All recordings were made from the somata of visually identified differentiated neurons (Leica DM LFS Dodt-contrast videomicroscopy, 63x water immersion objective). Patch electrodes were pulled from thick-walled borosilicate glass (2 mm OD, 1 mm ID) and were filled with an intracellular solution (Appendix, Table 33).The direct current resistance of the electrodes was 4-6 MΩ. The cells were seeded on glass coverslips (5mm) and transferred to the recording chamber. During recording, the slices were perfused with 34-36°C 1x aCSF extracellular solution buffer (Appendix, Table 34). Data analysis was performed using Igor Pro 6 software (WaveMetrics, Portland, OR). (Patch analysis was performed by Daniel Nunes, Prof. Kuner group, Neuroanatomy Institute, Heidelberg).

2.2.19. Alkaline phosphatase assay

ESCs and ES-like cells were stained with NBT/BCIP in TMN-Puffer, pH 9.5 (NBT:4-Nitro blue tetrazolium chloride, solution; Roche: 11383213001; BCIP: 5-bromo-4-chloro-3-indolyl-phosphate; Roche: 11383221001).

This reaction was stopped using deionized water and fixed with 4% PFA. After washing, samples were embedded with Kaiser's glycerol gelatine and stained cells were examined with a light microscope.

2.2.20. Production of chimeric mice

Generation of chimera was used for the examination of differentiation potential of ES-like cells during *in-vivo*. Blastocysts were collected at 3.5 days post-coitus from superovulated female NMRI mice in the basic M2 medium. About10–15 single-cell ES-like cells were transferred into each blastocyst. Approximately 10 injected embryos were surgically transferred into the uterine horns of in pseudo-pregnant NMRI recipient female mice. Chimera mice were usually identified by coat colour. (This experiment was performed by Dr. Yaser Tahamtani and Behroz Asgari, Prof. Baharvand´s group, Royan Institute, Tehran).

2.2.21. Statistical analysis

All the experiments were replicated at least 3 times. The average expression of genes in groups was calculated, and groups were evaluate using One-way analysis of variance (ANOVA) followed by the Tukey's post-hoc tests (T-Test).

Expression of genes was compared with the non-parametric Mann-Whitney’s test. The variation between groups was considered statistically reliable if a value of $P < 0.01$ was acquired.

Chapter 3 Results

3.1. *In-vivo* and *in-vitro* identification of different types of germ cells of the mouse testis

3.1.1. *In-vivo* identification of germ cells of the mouse testis

3.1.1.1. Germ cells markers are expressed in the mouse testis

In order to discriminate the local expression of different germ cells markers and thereby understand germ cell marker expression patterns during germ cell differentiation in the testis of adult mice, we performed immunohistochemical (IMH) analysis on 10 µm thick sections of the testicular parenchyma.

IMH confirmed the expression of all the known germ cell markers (Oct4, VASA, DAZL, PLZF, N-Myc) which were all expressed in specific cell types and compartments of adult mouse testis sections (Figure 3.1, Table 3.1).

We showed, that in addition to the strong nuclear Oct4 staining in a few SSCs directly in the basement stem cell compartment, the presence of nuclear Oct4 signals with much lower intensity in progenitor cells and spermatocytes above the stem cell layer (Figure 3.1A). In the upper part of the epithelium, the Oct4 signal was down-regulated in spermatids (Figure 3.1A). We observed a cytoplasmic expression of VASA and DAZL protein in cells above the basement membrane through to the middle part of the testicular lumen, not only staining spermatogonia, but also with a lower intense expression in spermatocytes (Figure 3.1B and C). Similar to Oct4, the transcription factor PLZF was detected only in the basal compartment of the testicular tubules (Figure 3.1D). N-Myc protein was expressed only in the middle part of testis luminal region (Figure1E). We also detected cytoplasmic expression for Klf4 which was localized in cells starting from above the basement membrane (data not shown) through to the differentiated part of the seminiferous tubule.

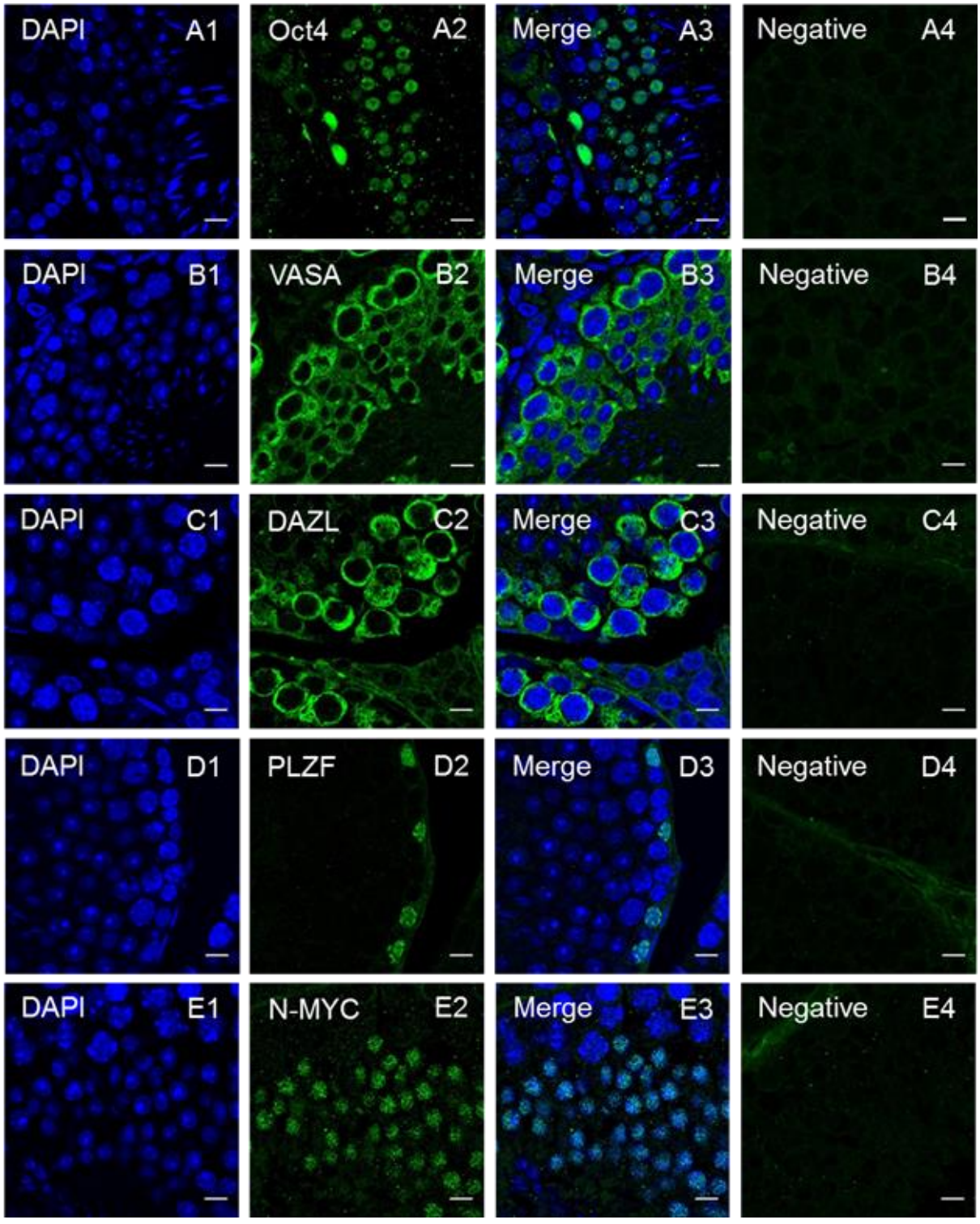

Figure 3.1: Immunohistochemistry documented expression of Oct4 (A1-A4), VASA (B1-B4), DAZL (C1-C4), PLZF (D1-D4) and N-Myc (E1-E4) in adult mouse testis sections (A, B, C). Oct4, PLZF and N-Myc

displayed a nuclear staining, while VASA and DAZL had a cytoplasmatic staining. Only the staining pattern for PLZF was restricted to the SSC stem cell compartment, while Oct4 signals were strongest in the basal stem cell compartment, but also present in the differentiating cells of spermatogenesis. Positive VASA staining signals were weak in the basal compartment and stronger in the differentiating cells. DAZL revealed a more homogeneous staining pattern in SSCs, spermatocytes and spermatids. N-Myc signals were confined to the differentiating cells. Scale bar is 10µm.

3.1.1.2. Co-localization of germ cells markers in the testis section

To answer the question of whether germ cell markers show any co-expression during in-vivo differentiation, we performed co-staining with the different germ cell markers described above.

Our analysis demonstrated a high expression of the germ stem cell marker PLZF and Oct4, which were both localized in cells directly above the basement membrane in the stem cell compartment of testicular tubules (Figure 3.2, Table 3.1). The PLZF signal was present in more SCCs in comparison to Oct4, which was restricted to a lower number of SSCs (Figure 3.2A1-A4). This analysis was quantified for 146 parts of seminiferous tubule from 3 testis sections.

In cross section of 7 week old mouse testis, we observed that all Oct4 positive cells were positive for PLZF cells but in contrast, about 40 percent of PLZF positive cells were positive for Oct4 (Figure 3.2A1-A4).

We also confirmed that PLZF positive cells showed low expression of VASA (Figure 3.2B1-B4) and N-Myc (Figure 3.2C1-C4). These results might confirm that the expression of germ cells markers Oct4, PLZF, VASA, DAZL and N-Myc might be essential during spermatogenesis.

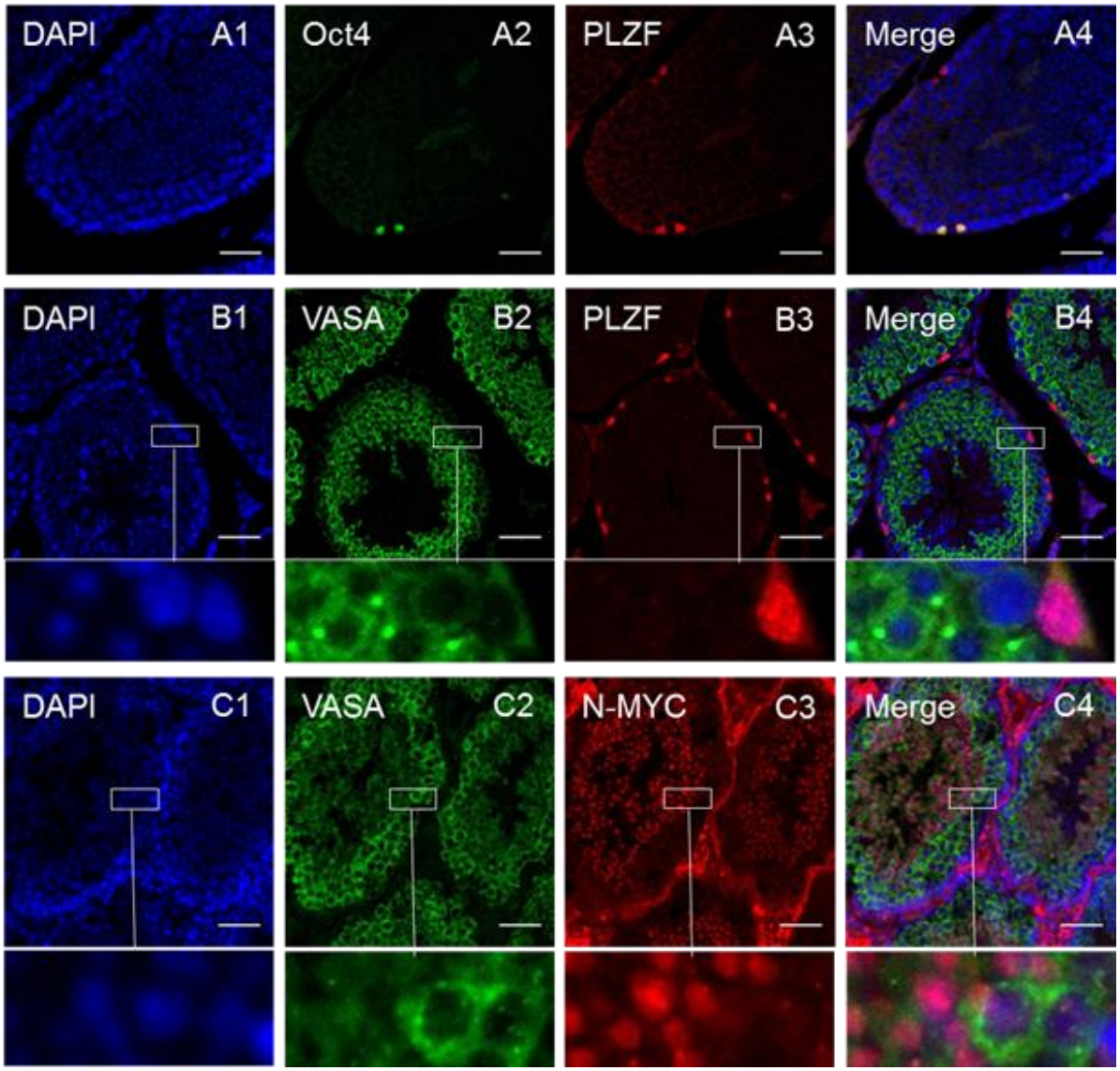

Figure 3.2: All of Oct4 positive cells in the basement membrane of seminiferous tubules were positive for PLZF while only about 40 percent of PLZF positive cells were positive for Oct4 (A1-A4). In the cross section of testis, PLZF positive cells showed low expression for VASA (B1-B4) and VASA positive cells were negative for N-Myc (C1-C4). Scale bars: 50µm.

3.1.1.3. No co-localization of Sertoli cells markers with germ cells markers

To answer the question of whether Sertoli cell markers show any overlap expression with germ cells markers during in-vivo differentiation, we performed co-stainings for

different germ cells markers including DAZL, VASA, N-Myc with sertoli cell markers Sox9 and VIMENTIN.

	Base membrane	After base membrane	Before Sepermatogenesis area	Sepermatogenesis area	Interstitial tissue
PLZF	+++	-	-	-	-
OCT-4	+++	++	+	low	-
VASA	+	+++	+	low	-
DAZL	+	+++	low	-	-
N-MYC	-	-	+++	+	-
KLF4	-	-	+++	+++	-
SOX9	+++	-	-	-	-
Vimentin	+++	+++	+++	+++	+++

Table 3.1: Immunohistochemistry shows distribution and signal density for different markers in the seminiferous tubules of testis.

The expression of Sox9 and VIMENTIN intermediate filament (IFs) proteins was observed in intratubular cells of the 7-week-old mouse testis (Figure 3.3). The nuclear staining of SOX9 was restricted to Sertoli cells, which were co-localized with VIMENTIN-positive cytoplasmatic stainings. This is typical for Sertoli cells bridging the germinal epithelium from the basal stem cell compartment towards the lumen (Figure 3.3, Table 3.1).

We also observed that all Sox9 positive cells were positive for VIMENTIN and negative for germ cells markers DAZL, VASA and N-Myc protein (Figure 3.3B-D, Table 3.1). Sertoli cells regulate the spermatogonial cell functions in the male gonad. Sox9 expression was limited to the Sertoli cells of testis and has been used as a specific marker for the identification of Sertoli cells in rat testis [140].

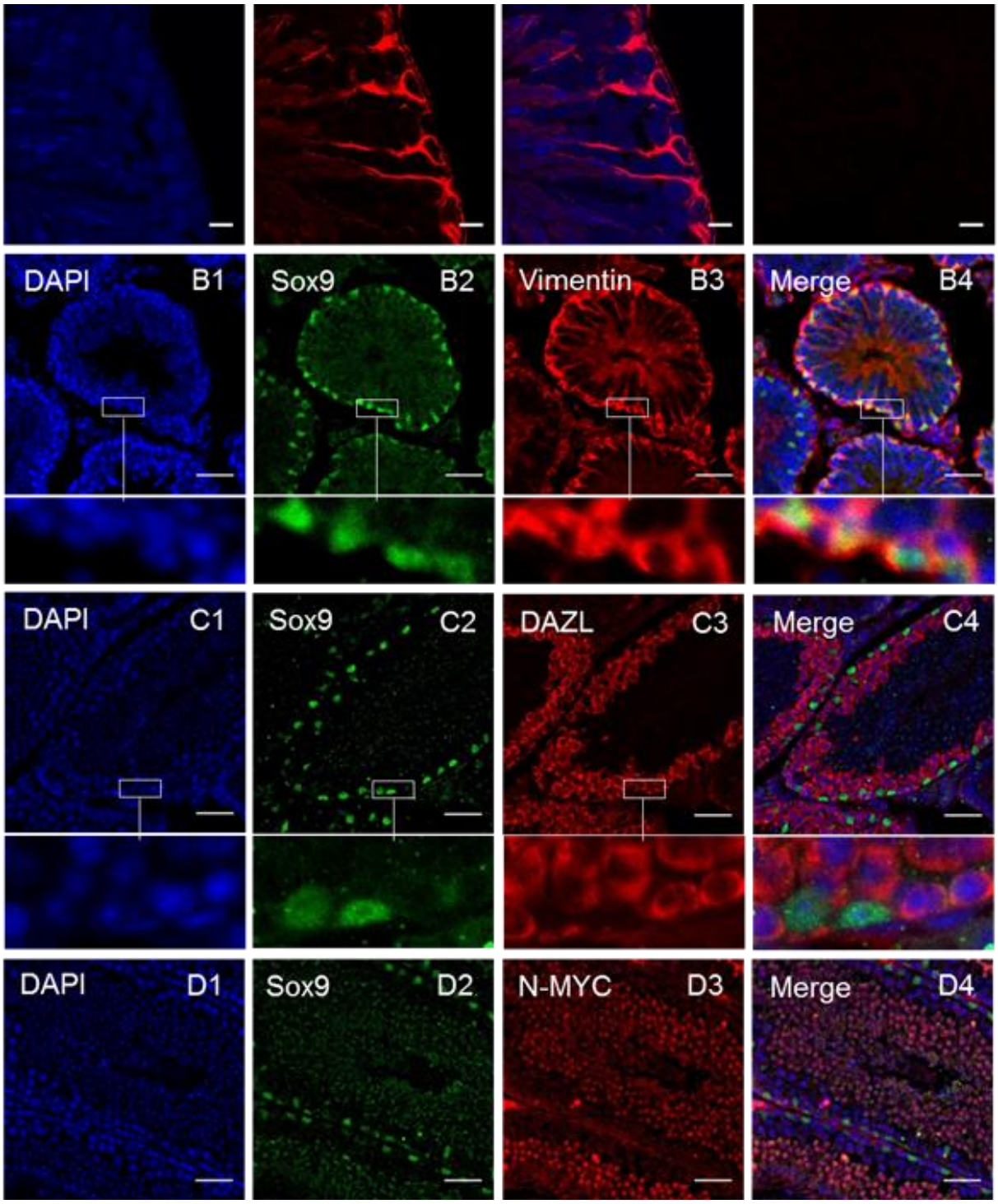

Figure 3.3: Sertoli cells were negative for germ cell markers. Immunohistochemistry showed expression of the Sertoli cell marker VIMENTIN (A1-A4) and Sox9 (B1-B4) in the testis sections. Sox9 positive cells were positive for VIMENTIN (B1-B4) and negative for germ cell markers DAZL (C1-C4) and N-Myc (D1-D4). Scale bars: A 10µm, B-D 50µm.

3.1.2. *In-vitro* identification of mouse germ cells

3.1.2.1. Isolation of different cell populations from testicular cells of Oct4-GFP transgenic mice

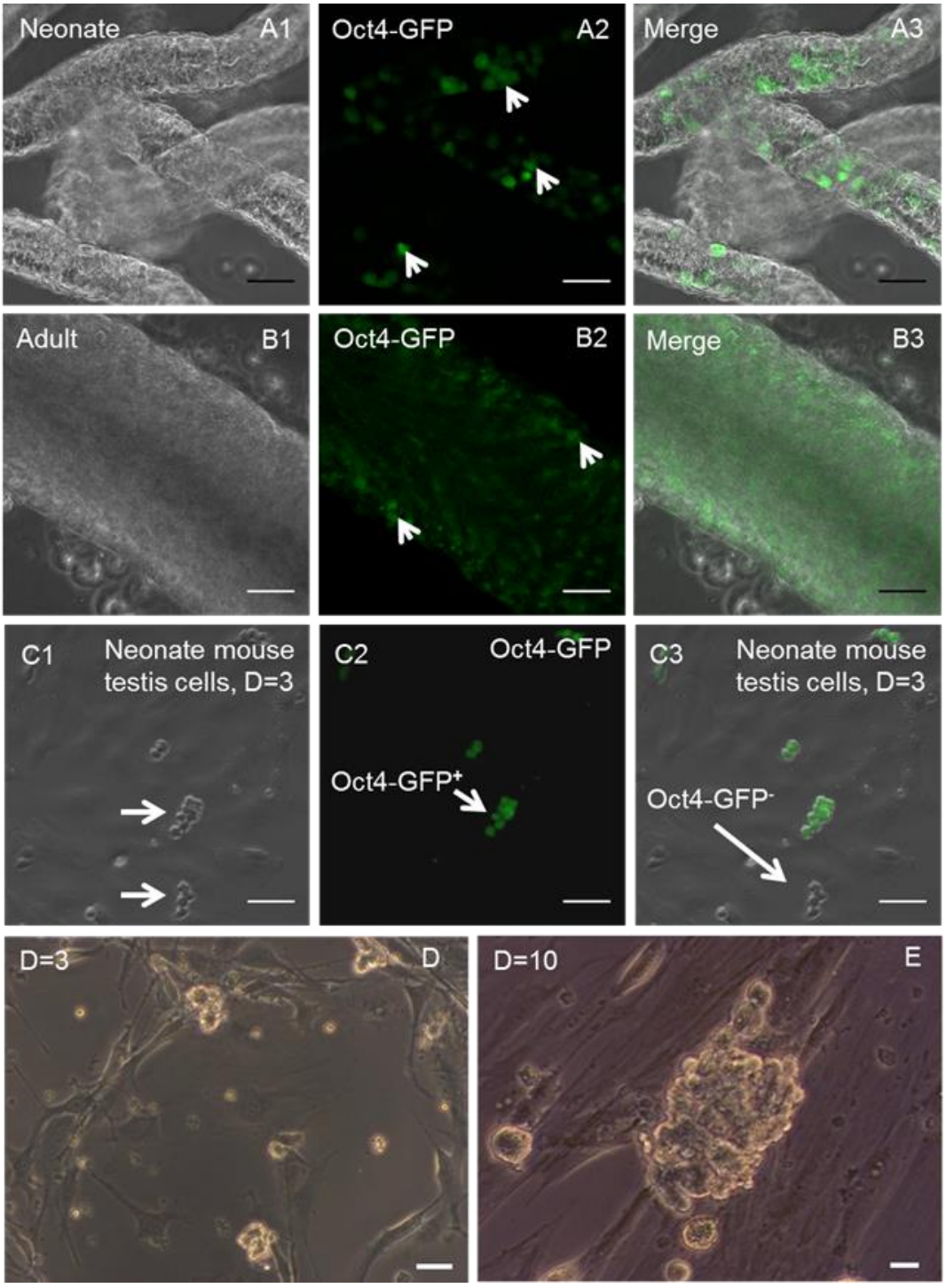

Figure 3.4: Expression of Oct4-GFP in the seminiferous tubule of Oct4-GFP reporter transgenic mouse seminiferous tubules of neonate and adult mouse testis. In the adult mice, the number and signal density of Oct-GFP was lower in comparison to neonate mice (A and B). Typical tightly packed morphology of Type I cells obtained from neonate mouse testicular tissue were observable during initial culture with some colonies positive and some negative for Oct4-GFP (C). Morphology of Type I cells in the adult mouse was similar to neonate GSCs (D). Type I colonies were grown on MEF feeder (E). Scale bar: 50 µm.

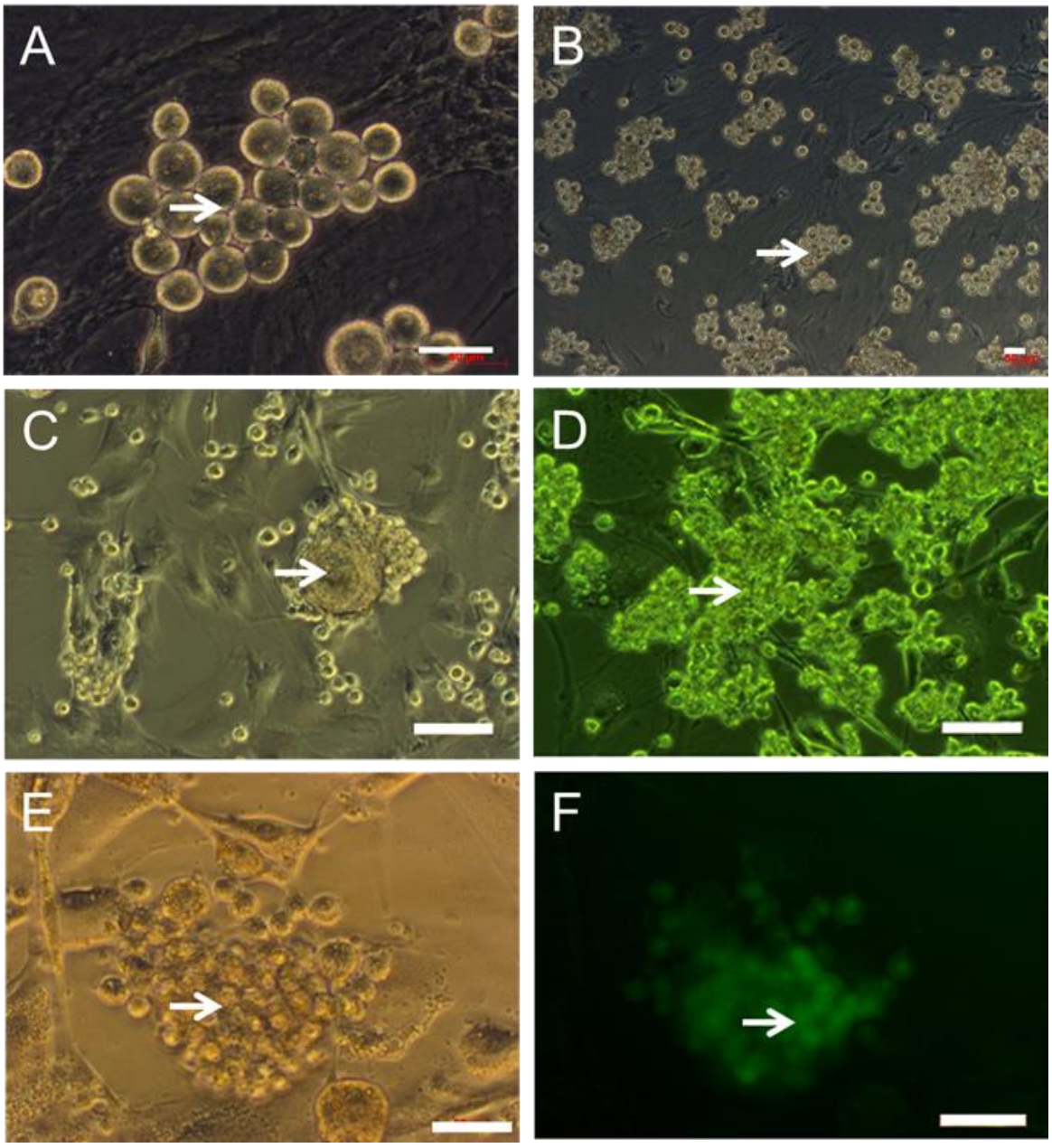

Figure 3.5: Microscopic observation of the expansion of Type II cells on TSCs (A, B) and SNL (D) feeders. Cell clusters were observable under the spermatogonial culture conditions(C). Type II cells were generated from control GFP mice and expanded for long-term culture on SNL feeder (E-F). Scale bar: 50 µm.

We used the Oct4-GFP transgenic mouse model for the isolation of different cell types of the testis. An Oct4-GFP signal was clearly observable in the seminiferous tubules of neonate mouse testis but it was not same in the adult mouse (~6 months old).

In the adult mice testis, the number and signal density of Oct-GFP was lower than in neonate mouse testis (Figure 3.4A and B). After mild digestion of the testicular tissue with a combination of the enzymes collagenase/dispase, the seminiferous tubules were almost separated from each other. A single cell suspension was obtained after further enzymatic digestion and transferred into mGSC medium. Somatic cells started to grow within 2-10 days after the cultures were initiated (both were obtained from neonate and adult mice with mGSC culture medium). During initial culture of both neonate and adult mice, two different morphological populations of germ cells were observable, which will be named Type I and II cells from this point on (Figure 3.4D, E and Figure 3.5).

Type I cells were observable during the initial culture of both neonate and adult mice between 2 or 14 days after initiation of the primary testis cultures with GS medium (Figure 3.4C). Type I cells were round, compact cells with a size of about 12 μm^2 grew in a tightly packed (lumpy colony) formation during long-term culture. In the beginning, some Type I cells expressed low levels of Oct4-GFP (some were negative for Oct4-GFP) (Figure 3.4C), while this signal was completely down-regulated during long-term culture (Figure 3.4D and E). After conversion of these cells to a pluripotent state, the Oct4-GFP signal emerged again (this conversion is explained later in more detail in the section regarding pluripotency). In comparison to Type I cells, Type II cells were usually small round cells with a size about 30 μm^2 which loosely connected to each other. These grew as a flat colony formation, but did not show any three-dimensional growth pattern (Figure 3.5). In primary culture, Type II cells emerged about 2-3 weeks in neonate mice and 3-7 weeks in adult mice. These cells did also not express the Oct4-GFP signal at any time during culture.

Type II cell colonies showed no strong attachment neither between themselves nor with the somatic feeder cells. These cells were easily separated from each other by gentle pipetting.

3.1.2.2. Characterization of different germ cells populations

3.1.2.2.1. Long term culture and expansion of Type I and II cells

As mentioned above, Type I and II cells emerged on primary somatic testicular feeder cells for a short period of time (Figure 3.4 and 3.5).The cells were selected with a micromanipulator or manually hand-picking from primary culture. Different types of feeders such as MEF, TSC and SNL feeder (interrupted cell division with either γ-irradiation or Mitomycin C treatment) were tested for the expansion of Type I and II cells. Our analysis showed that Type I cells disappeared about 2 weeks after initiation of the culture on neonate TSCs (Figure 3.4C1-C3), while the supportive MEF feeder layer provided an ideal environment for long-term expansion of these cells (Figure 3.4D and E).

Similarly, Type II cells grew for more than 5 weeks on neonate TSCs and 10 weeks on old TSCs feeder (Figure 3.5A and B). In some parts of the testicular culture, we also observed aggregation of somatic cells which displayed a strong cell interaction while cell dissociation was difficult (Figure 3.5C). The SNL feeder layer is an immortalized cell line derived from MEF cells transfected with the murine LIF gene [83]. This type of feeder provides an ideal support for the long-term expansion of these cells from neonate and adult mice (Figure 3.5D-F). Both Type I and II cells were cultured in mouse GSCs medium for more than one year and passaged every 3-4 weeks on new feeder cells. Although Type II cells could be expanded on SNL and primary TSC feeders, these feeders were not suitable for cultivation of Type I cells.

3.1.2.2.2. Electron Microscopic analysis of Type I and II cells

Electron microscopic results indicated that unlike Type I cells, Type II cells showed a different morphology compared to *in-vivo* SSCs that are located on the basement membrane of the seminiferous tubule.

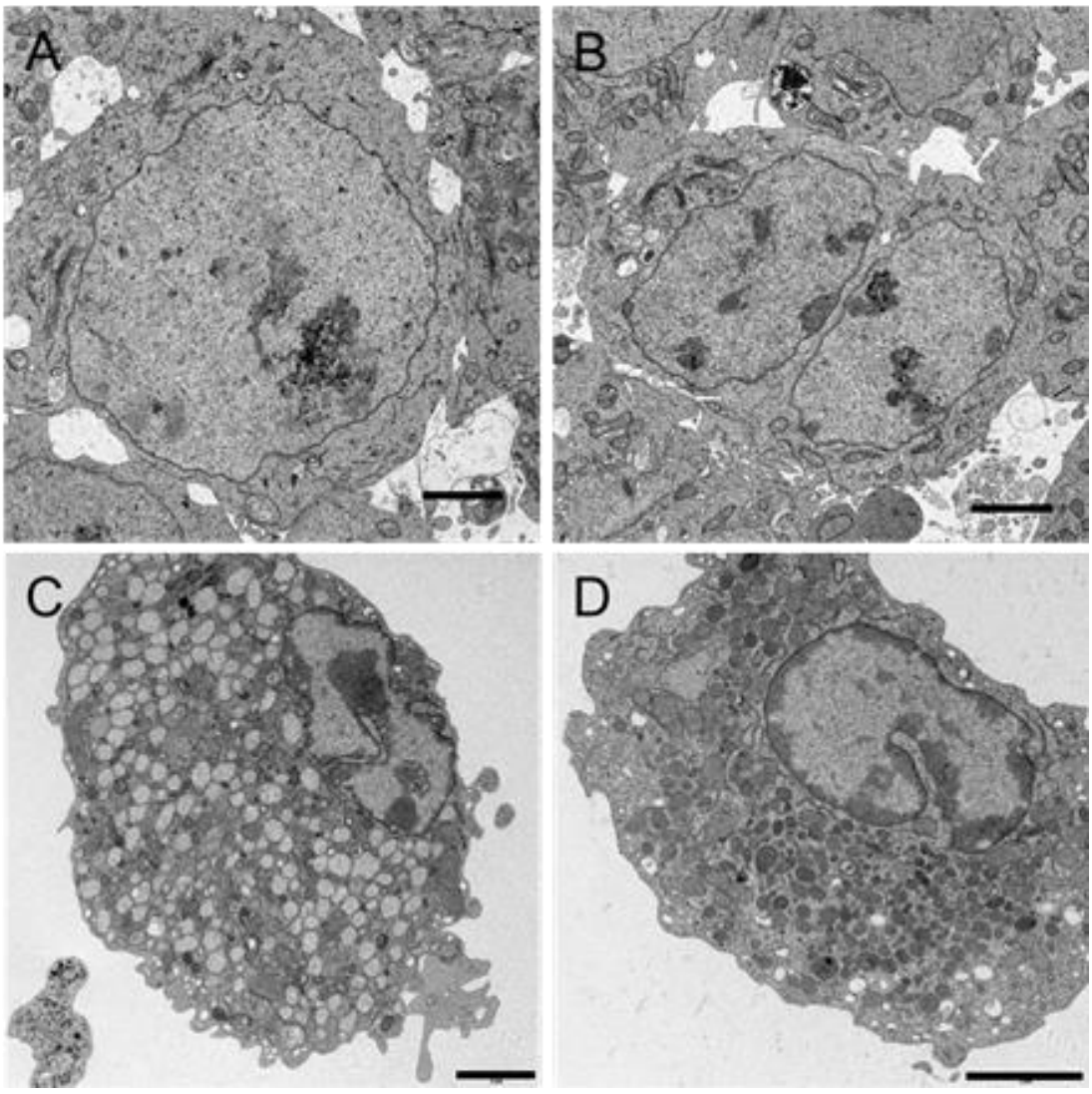

Figure 3.6: Electron microscopic results for Type I (A, B) and Type II (C, D) cells. Nucleus/cytoplasm ratio in the Type I cells was higher than Type II cells. Scale bar: 2 µm.

In contrast, Type I SSCs have a similar morphology in comparison to SSCs *in-vivo* which possess a large nucleus and small cytoplasmic rim (high nucleus/cytoplasm ratio) (Figure 3.6A and B), while in Type II cell's nucleus/cytoplasm ratio was low and they were packed with vesicles and other cell organelles (Figure 3.6C and D).

3.1.2.2.3. Immunocytochemistary of *in-vitro* germ cells

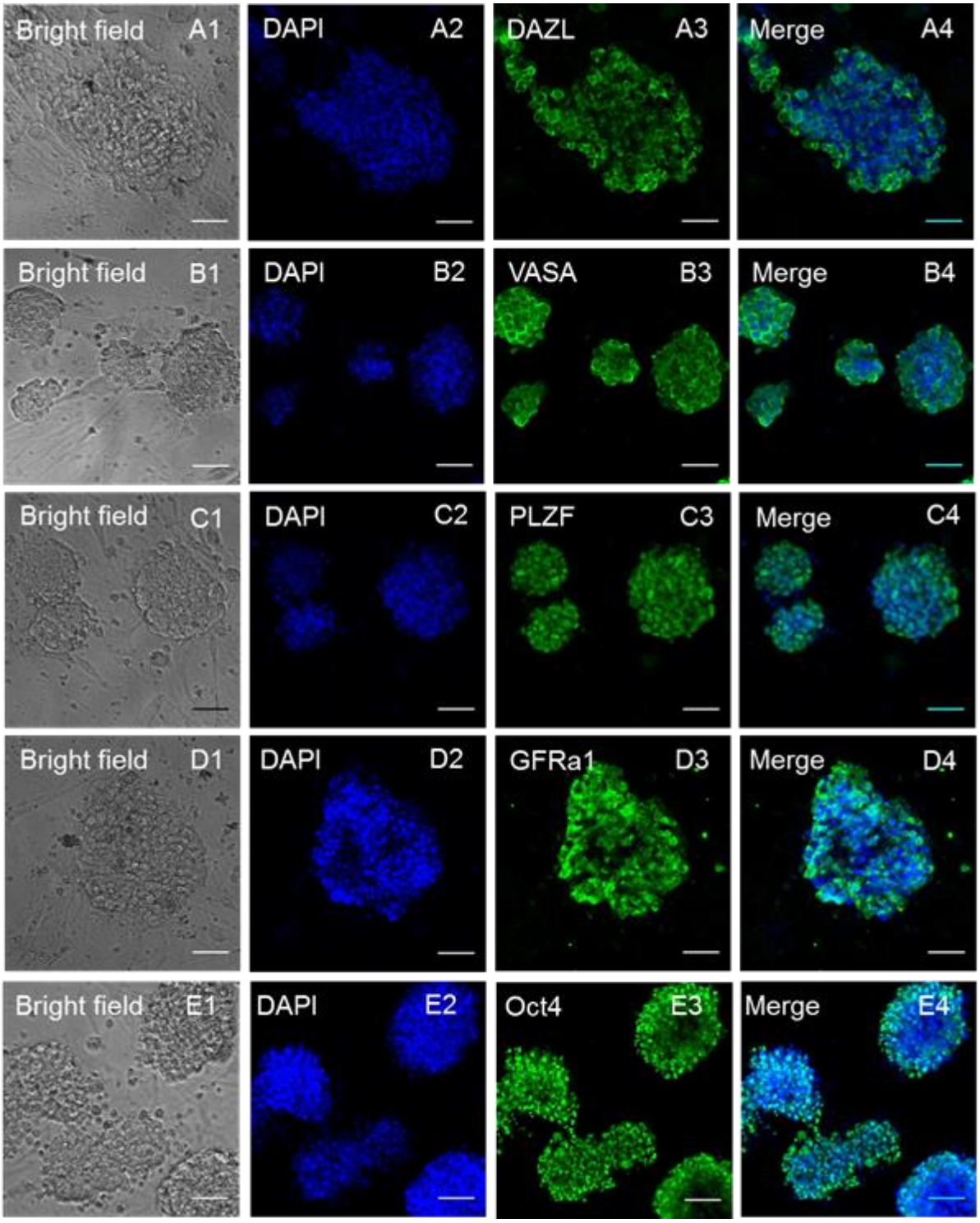

Figure 3.7: Immunocytochemistry results showed that Type I cells express the germ cells markers DAZL (A1-A4), VASA (B1-B4), PLZF (C1-C4), GFRa1 (D1-D4) and Oct4 (E1-E4).

We isolated Type I cells from neonate and adult mouse testis. The cells were passaged about 4 weeks after first isolation in the GS media and MEF feeder.

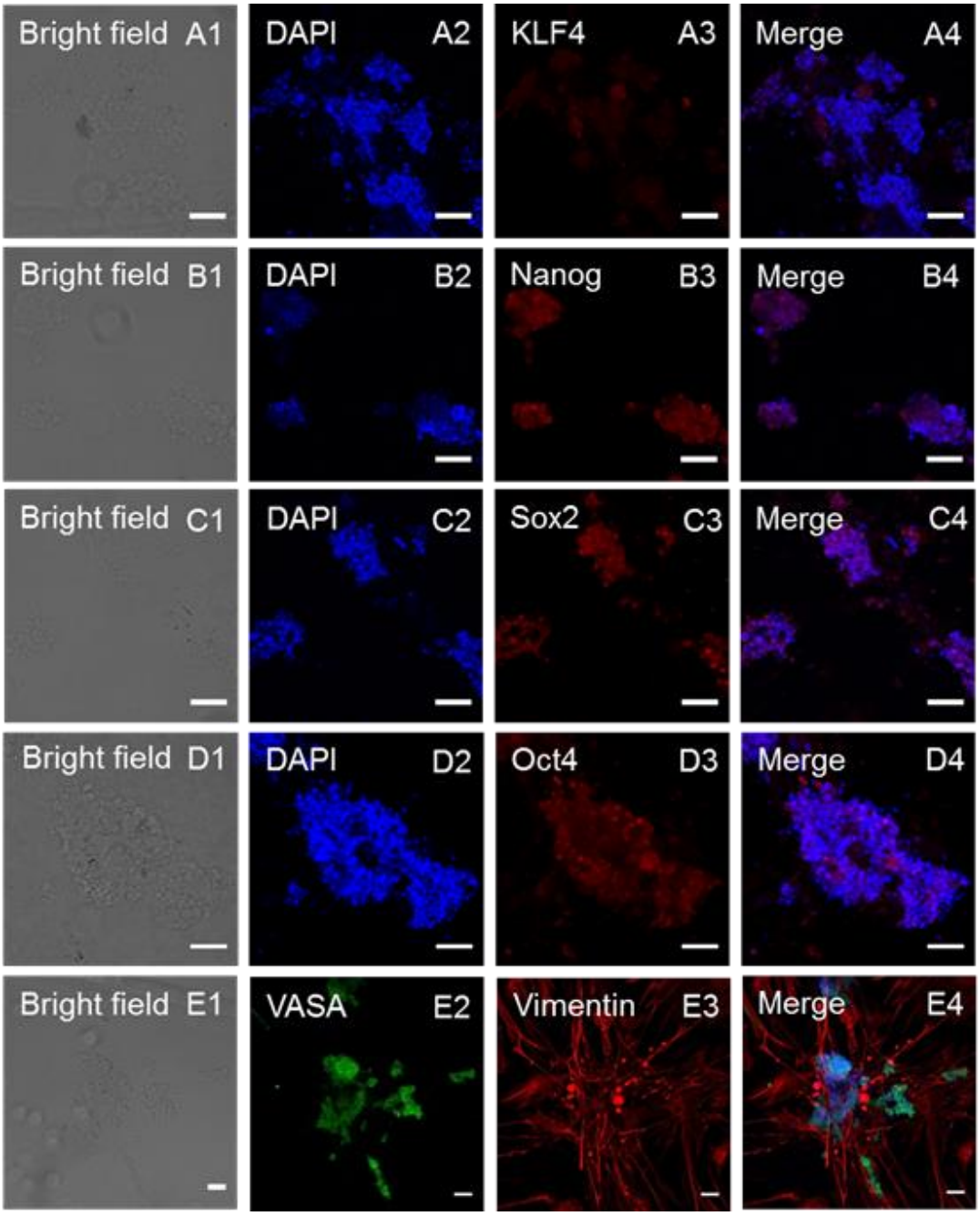

Figure 3.8: Type I cells were negative for Klf4 (A1-A4), Nanog (B1-B4) and Sox2 (C1-C4). Scale bar 50µm.

In passages 3 and 12, Type I cells were cultivated in 24 well plates on cover-slips with MEF feeder cells and used for immunocytochemistry.

Immunocytochemistry analysis showed that Type I cells displayed a high expression for germ cells markers VASA, DAZL, PLZF, GFRa1 and Oct4 (abcam) (Figure 3.7).

On the other hand, they showed low expression for Klf4, Nanog, Sox2, Oct4 (Santa Cruz) and did not express VIMENTIN (confirmed in double staining with VASA) (Figure 3.8, Table 3.2).

Immunocytochemistry results showed that during *in-vitro* culture of PLZF positive Type I cells, these cells were also positive for VASA (Figure 3.7B-C). In contrast, PLZF positive cells in the testis were negative for VASA protein (Figure 3.2B1-B4).

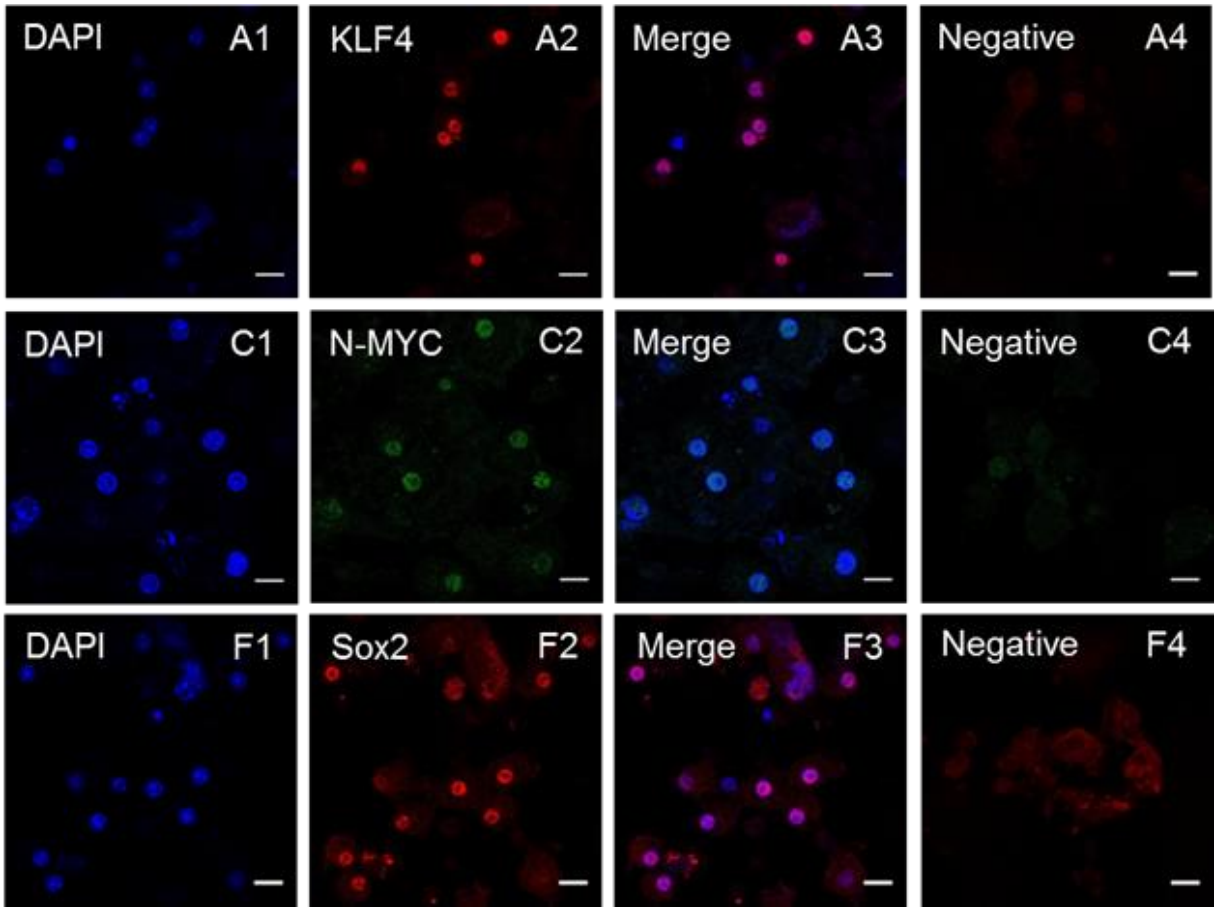

Figure 3.9: Immunocytochemistry showed that Type II cells express Klf4 (A1-A4), N-Myc (B1-B4) and Sox2 (C1-C4).

Immunocytochemistry for Type II cells confirmed that unlike Type I cells, Type II cells were negative for VASA and DAZL, while these cells clearly expressed Klf4 and N-Myc, Oct4 (Abcam) and Sox2 proteins (Figure 3.9, Table 3.2). Oct4 was expressed in Type II cells but this level was lower than in Type I cells. Type II cells also expressed a low level of GFRa1, PLZF, Nanog and Sox9 protein (data not shown).

	Type I	Type II	ES-Like	mESCs	Testis section	TSCs
Oct4	+++	++	+++	+++	+++	-
Nanog	Low	Low	+++	+++	-	-
Sox2	+	+	+++	+++	-	-
Klf4	-	+++	+++	+++	+++	-
N-MYC	+	+++	+++	+++	++	-
PLZF	+++	Low	Low	Low	+++	-
VASA	+++	-	Low	Low	+++	-
DAZL	+++	-	Low	Low	+++	-
GFRa1	+++	Low	ND	ND	+++	-
SOX9	+	Low	Low	Low	+++	Low
Vimentin	-	+++	-	-	+++	+++

Table 3.2: Comparison of immunostaining results for cells Type I, II cells TSCs and testis sections. Type I cells have low expression for Sox9 and Sox2 and in addition, were negative for Klf4, N-Myc and VIMENTIN. Type II cells expressed markers Klf4, N-Myc, VIMENTIN and Oct4. On testis sections, no IMH signals for Nanog and Sox2 were detected.

3.1.2.2.4. Infertile mouse model and transplantation of germ cells into testis

Because SSCs possess no highly selective marker for their identification, the functional characterization of putative SSCs in infertile mice is an ideal model to characterize the ability of the transplanted cells to produce sperm. To induce infertility, the nod SCID mice were treated with busulfan (40mg/kg) for

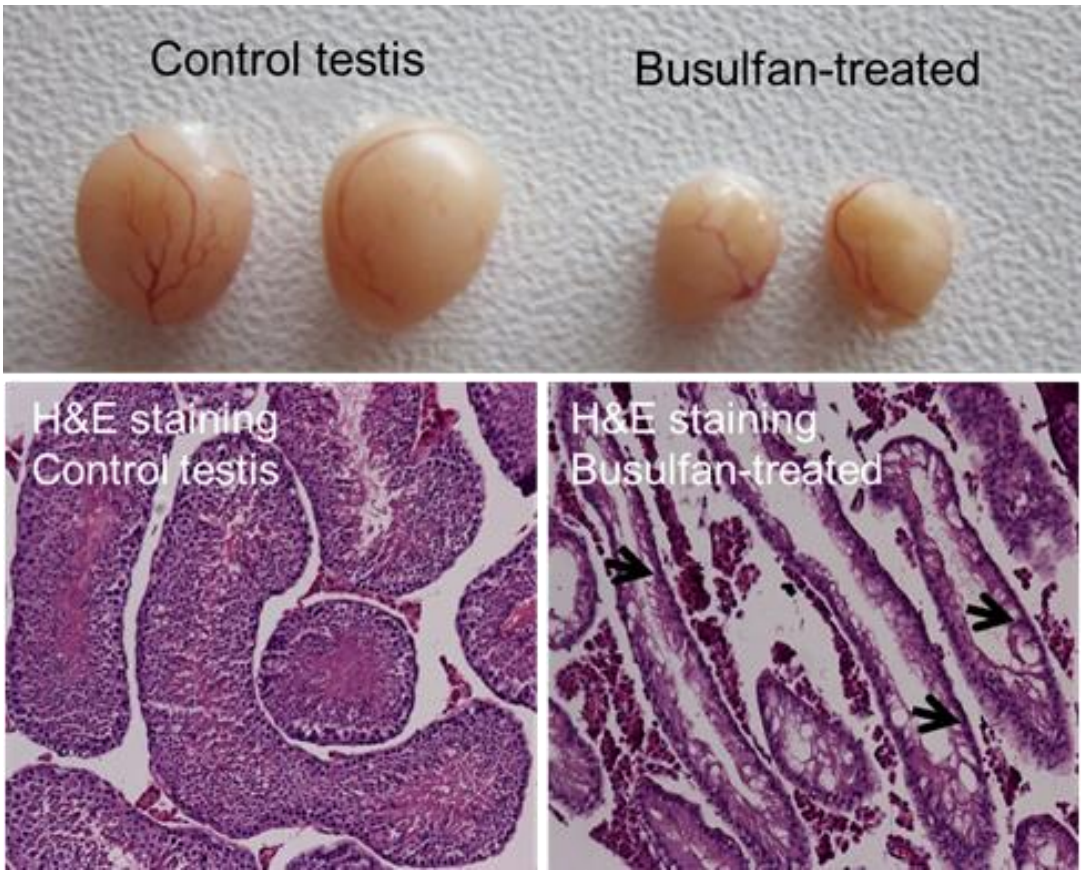

Figure 3.10: Busulfan injection to SCID mouse model. Decreased size of testis (A) and destroyed endogenous spermatogenesis (C) after busulfan injection. Hematoxylin-Eosin staining analysis for normal testis (B) and busulfan injected testis (C). Spermatogenesis was observable in normal testis section (B).

4 weeks, this is selectively toxic for germ stem cells (Figure 3.10). Morphological analysis of the treated mice testis indicated changes in size (testis became much smaller) and histological analysis documented destroyed endogenous germ stem cells in the seminiferous tubules of the testis treated with busulfan (Figure 3.10). In contrast,

Hematoxylin-Eosin staining analysis for cross-sections through seminiferous tubules of normal testis revealed absolutely normal spermatogenesis (Figure 3.10).

As we mentioned above, Type I cells clearly expressed the usual germ cells markers, while Type II cells partially expressed germ cells markers but were positive for N-Myc, Klf4 and Oct4.

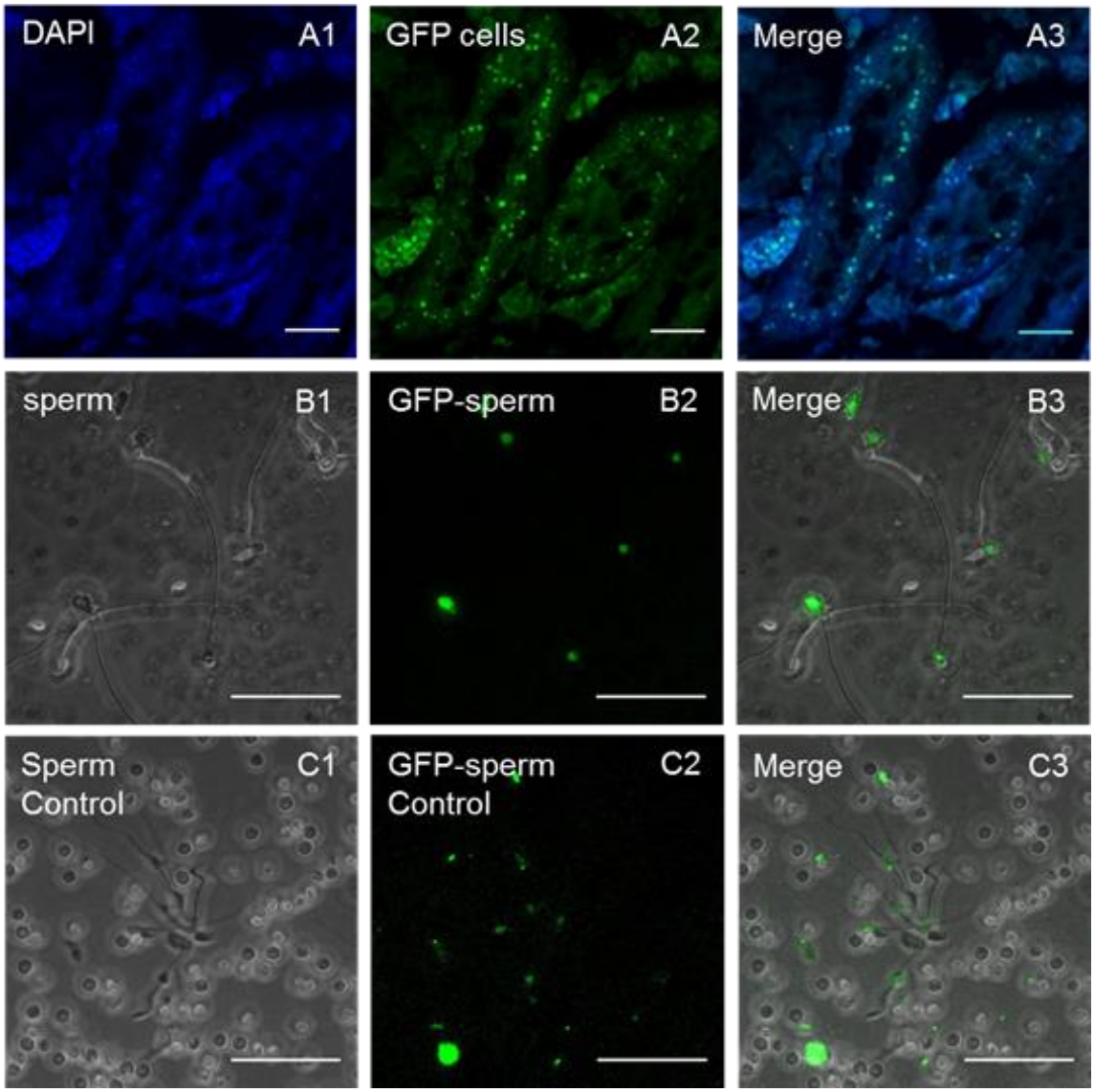

Figure 3.11: Transplantation of Type II cells in testis. About 1month after transplantation of GFP labeled Type II cells, localization of Type II cells in the basal compartment of infertile testis was observable (A1-A3). About 3 months after transplantation of Type II cells, GFP sperm was observable in the epididymis of testis (B1-B3). GFP positive sperms were observed in the epididymis of control testis (C1-C3).

Because Type II cells only had low expression for some of the typical germ cell markers, we analysed the functional potentiality of Type II cells by transplantation of these cells into infertile mouse model. To trace cells after transplantation, we used an actin promoter GFP mouse model ("green mice") or transfected Type II cells with lentiviral particles of COP-GFP construct in order to produce GFP positive sperm.

For this experiment, Type II cells were isolated from "green mice" and used for transplantation after expansion on SNL feeder. We observed that even after about 8 months after cultivation of Type II cells, these cells still expressed GFP on SNL feeder (Figure 3.5E-F).

We diluted about 100 000 Type II cells in 10µl of DMEM and transplanted the cells into the rete testis of the busulfan-treated infertile mouse model. About 4 weeks after transplantation, the histological analysis revealed that that GFP positive Type II cells were located in the basal compartment of seminiferous tubules of the busulfan-treated recipient mouse testis (Figure 3.11A1-A3). About 3 months after transplantation, we observed GFP-positive sperm in the epididymidis of the recipient testis (Figure 3.11B1-B3). No GFP positive cells were detected in the non-transplanted group confirming the specificity of this method.

3.2. Pluripotency of testicular cells

3.2.1. Generation of ES-like cells from testicular culture

Type I and II cells were cultivated in GS media and passaged every 3-4 weeks for long-term culture. As mentioned previously, some of Type I cells expressed low level of Oct4-GFP in the beginning of the culture (Figure 3.4C1-C3) but this expression completely down-regulated during long-term culture (Figure 3.4D and E). In contrast, Type II cells did not express any Oct4-GFP signal at any point during cultivation. We performed more than one year of cultivation (more than 15 successful repeated expansions) for both types of neonate and adult mouse testis cells.

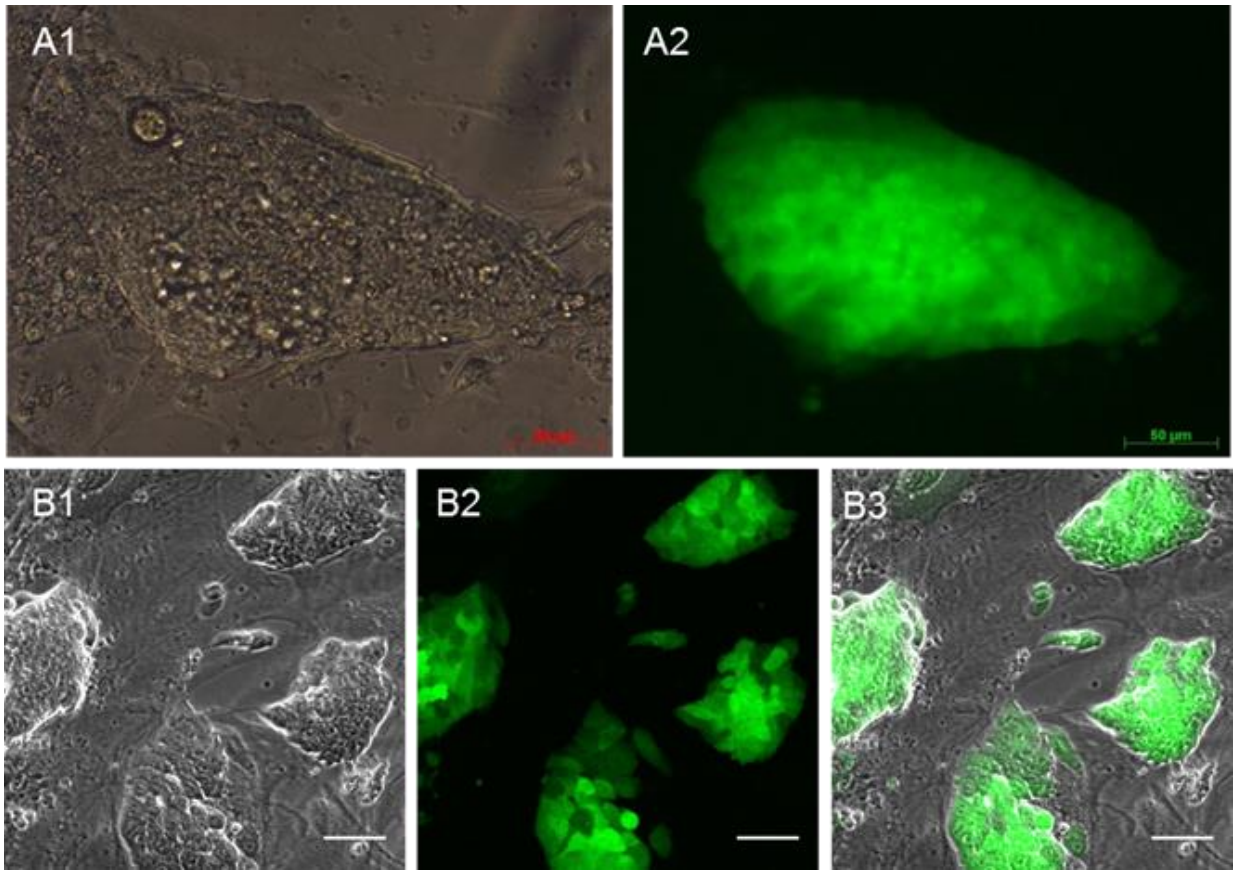

Figure 3.12: Generation and expansion of ES-like cells that express Oct4-GFP. Generation of ES-like cells in the mGSC medium (A1-A2). Expansion of ES-like cells in mouse ESCs medium (B1-B3).

For generation of ES-like cells, we directly isolated Type I and II cells in primary culture or selected the colonies after subculture of supernatants from primary culture (supernatants were collected about two days after initiation of culture from neonate mouse testis). Unlike Type II cells, Type I cells grew and expanded on MEF feeder in mGSC media which contained GDNF, FGF, EFG and LIF (Figure 3.4D and E). During long-term culture for Type I cells, we rarely found colonies that were similar to mESCs (ES-like cells) or cultured epiblast cells that clearly express a high level of Oct4-GFP (Figure 3.12A1-A2). Typical ES-like cell colonies have a packed spindle to round-shaped morphology with smooth borders (Figure 3.12A1-A2). Moreover, as mentioned before, they displayed a high intensity of the Oct4-GFP signal (Figure 3.12A1-A2).

At the same time as the Type I cells were cultured, we cultured Type II cells in mGSC media and MEF feeder. We observed that Type II cells did not expand on MEF feeder and we therefore used SNL feeder for expansion of Type II cells. Under the same culture condition used for Type I cells, we did not observe any Oct4-GFP colonies in Type II cells.

3.2.1.1. Time window for generation of ES-like cells from testis

As we mentioned above, ES-like colonies were generated only in Type I cells. ES-like colonies were successfully established in 5 of 15 experiments (33%). We observed a critical time after initiation of culture (about 40-125 days after initiation of culture) for reprogramming of Type I cells (Figure 3.13). ES-like cell colonies were observed in days 41, 79, 81, 110 and 125 after initiation of culture from the neonate (1-week-old) and adult (7-week-old) mouse testis (Figure 3.13). We did not observe any ES-like colonies after (125 days) and before (40 days) this time period (Figure 3.13). We cultured generated ES-like colonies that express Oct4-GFP under mouse ES medium conditions with DMEM /15% FBS and LIF (Figure 3.12B1-B3). The ES-like colonies were passaged 1:5–1:8 for more than 15 times following trypsin digestion, with an estimated doubling time of 48-72 h. The ES-like cells still expressed Oct4-GFP after long term cultivation (Figure 3.12B1-B3).

The cells preserved their undifferentiated state in multiple passages and demonstrated clonal growth from single cells. The established ES-like colonies were successfully expanded, cryopreserved and thawed with no loss in proliferation or differentiation capacities.

While ES-like colonies could grow and expanded on mESCs medium, we observed Type I cells could not be expanded in this culture condition in 5 experiments (data not shown). As mentioned, Type I cells in the initiation of culture expressed a low level of Oct4-GFP and this signal was lost during long-term culture. It seems that during reprogramming and after conversion of Type I cells to a pluripotent state, the Oct4-GFP signal emerged again.

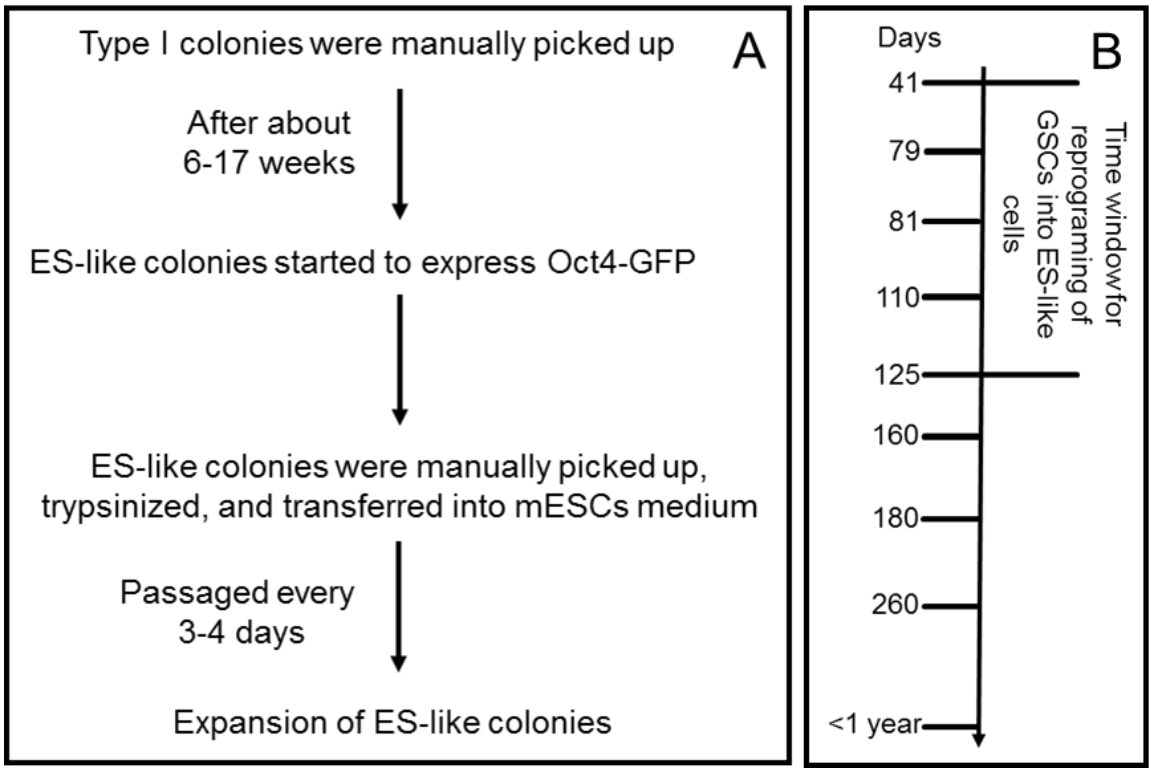

Figure 3.13: Protocol for the generation of ES-like cells from mouse testis.

After trypsinization of ES-like colonies, single cells were obtained from which we cultured new ES-like cell colonies under mouse ES cell medium condition. ES-like cells colonies were grown in mESCs media and were passaged every 3-4 days (A). ES-like cells were only generated in a time window between 40-125 days (in days 41, 79, 81, 110 and 125) after initiation of culture (B).The appearance of ES-like cells was completely independent from a higher density of the cultures as suggested by Ko et al [64].

3.2.1.2. Characterization of ES-like cells

3.2.1.2.1. Gene expression analysis for Type I, II, ES-like cells, mESCs and feeder cells

In our experiments with Fluidigm Real-Time RT-PCR, we examined whether Type I and II cells express germ cells and pluripotency markers and compared the gene expressions patterns between mESCs, ES-like and feeder cells.

As first step, we compared the pluripotency- and germ cell-associated gene expression pattern between Type I and II cells obtained from mice of different ages (after picking up in the initiation phase of culture from neonate, 2, 4, 8, 10 week old mice), ES-like cells,

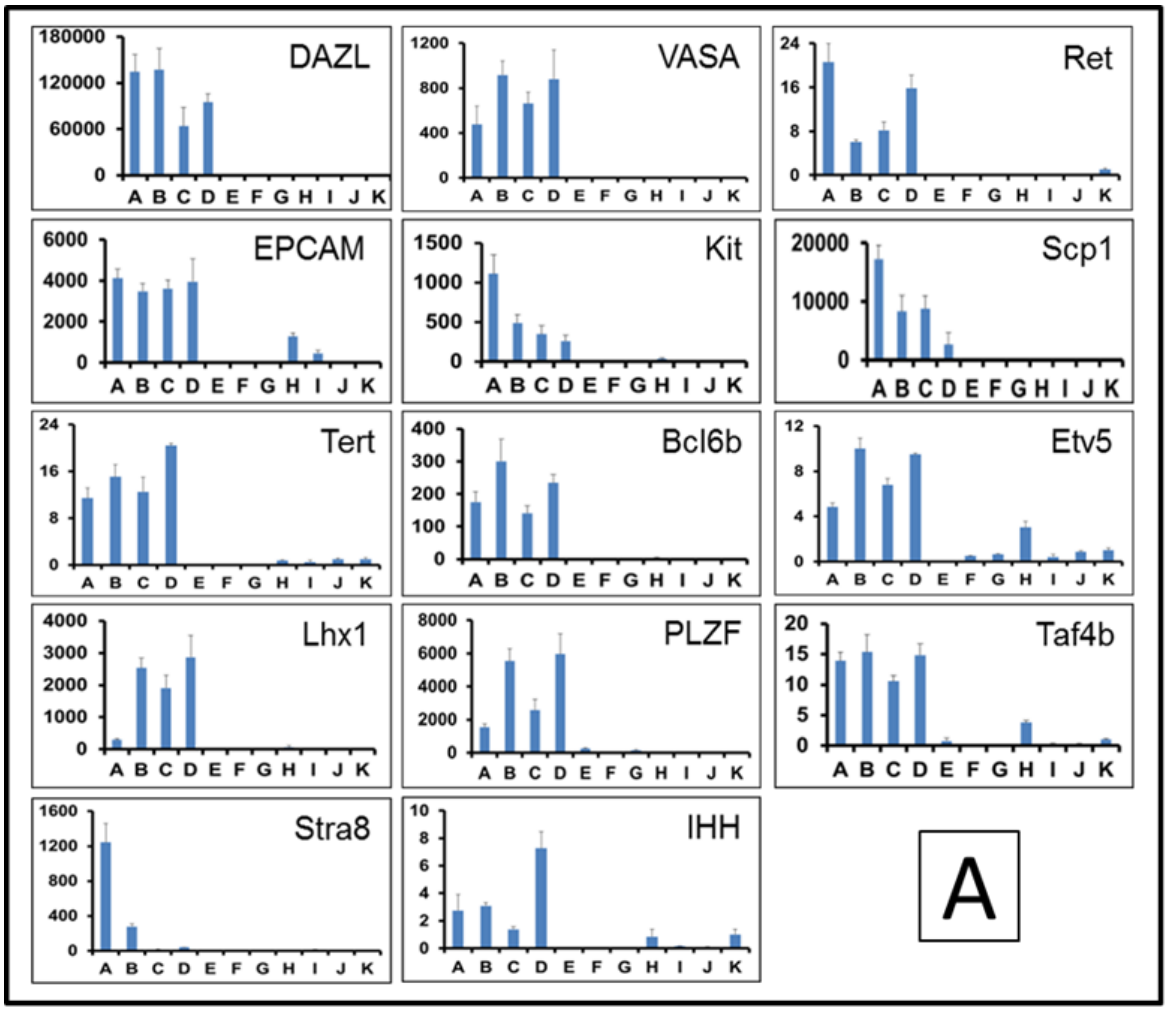
DAZL
VASA
Ret
EPCAM
Kit
Scp1
Tert
Bcl6b
Etv5
Lhx1
PLZF
Taf4b
Stra8
IHH
A
Nanog
Lin28
TDGF1
Dppa5
Nodal
GDF3
Oct4
Sox2
B

Figure 3.14: Fluidigm Real-Time PCR analysis for germ cells and pluripotency markers.
mRNA characterization of germ cell genes between Type I cells (after selection in the initiation of culture=A, neonate mouse=B, 2weeks mouse=C, 4 weeks=D, 8 weeks mouse=D), Type II cells (Neonate mouse= F, 10 weeks mouse=G), ES-like cells=H, mESCs=I, TSCs feeder=J, MEF feeder=K.
(A) RT-PCR analysis showed the expression of DAZL, VASA, Ret, EPCAM, KIT, SCP1, TERT, BCL6B, ETV5, LHX1, PLZF and TAF4B in Type I cells was significantly higher than in ES-like, mESCs, TSCs and MEF while (B) the expression of some pluripotency markers such as Nanog, LIN28, TDGF1, DPPA5, NODAL and GDF3 in the ES-like significantly was higher than Type I, TSCs and MEF cells. There was no significant regulation for genes Oct4 and SOX2 in Type I, ES-like and mESCs, while these genes were significantly were higher than TSCs and MEF. Y-axis shows expression fold change of mRNA in comparison to MEF feeder cells. Significance difference between ES-like and ESCs with Type I, Type II and MEF cells is at least P<0.01, t-test.

mESCs, TSCs and MEF (Figure 3.14). We observed that the expression of DAZL, VASA, Ret, EPCAM, Kit, Scp1, Tert, Stra8, IHH and the transcription factors Bcl6B, Etv5, Lhx1, PLZF and Taf4B in Type I cells was significantly higher than in the other groups ($p<0.01$) (Figure 3.14A). We also confirmed that the expression of pluripotency markers such as Nanog, Lin28, TDGF1, Dppa5 and Nodal was significantly down regulated in Type I and II cells and up regulated in the ES-like cells ($p<0.01$) (Figure 3.14B). We did not observe any significant regulation between Type I, II and ES-like cells for Oct4, Sox2 and GDF3 gene expression, while these genes showed a higher significant expression than feeder cells ($p<0.01$) (Figure 3.14B).

3.2.1.2.2. Expression of surface markers in Type I, II, ES-like, mESCs and testis cells

We used flow cytometry analysis to investigate the expression of some of the known surface germ cells markers including CD9, CD29, CD49, GFRa1, CD117 in Type I, II, ES-like cells, mESCs and testis cells (Figure 3.15).

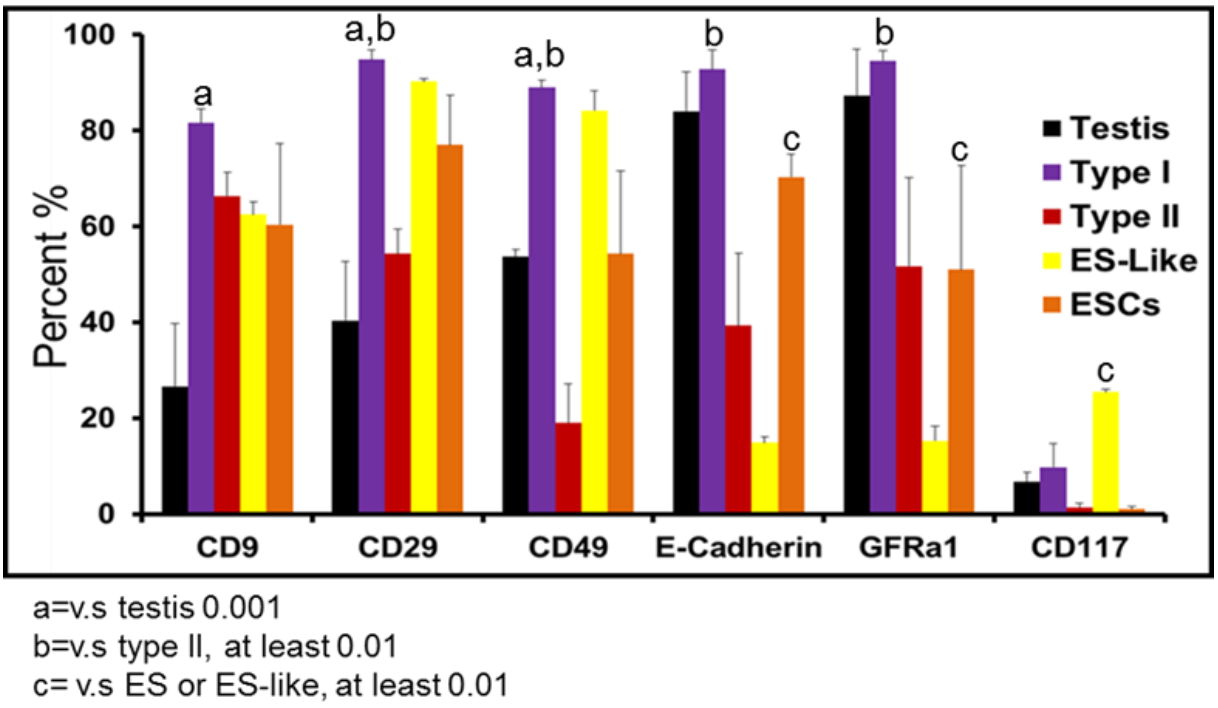

Figure 3.15: Flow cytometry analysis for some surface germ cells markers in cells Type I, Type II, ES-like cells and mESCs. Expression of CD9, CD29, CD49, E-Cadherin, GFRa1 and CD117 in Type I cells is higher than in Type II cells. The expression of E-Cadherin and GFRa1 was down-regulated in ES-like cells in comparison to Type I and II cells.

In this experiment, Type I and II cells were isolated after expansion on feeders in mGSC medium. mESCs and ES-like cells colonies were used after expansion in ES cell standard medium. Testis cells were directly isolated from adult mice (5, 6 and 7 weeks) after enzymatic digestion.

Flow cytometry analysis in Type I cells demonstrated that expression of CD9, CD29 and CD49 was significantly higher than in testis cells ($p<0.01$) (Figure 3.15) and the expression of CD29, CD49, E-cadherin and GFRa1 was significantly higher than in Type II cells ($p<0.01$) (Figure 3.15). Similarly, the expression of CD9 in Type II cells was significantly higher than in testis cells while the expression of CD49, E-Cadherin, GFRa1 and CD117 in testis cells was significantly higher than in Type II cells (Figure 3.15).

We did not observe any significant difference for markers CD9, CD29, CD49 between Type I cells and ES-like cells, while the expression of GFRa1 and E-Cadherin in Type I cells and testis cells was significantly higher than in mESCs and ES-like cells ($p<0.01$) (Figure 3.15). In addition, we did not observe any significant difference for markers CD9, CD29, CD49 between ES and ES-like cells, while the expression of GFRa1 and E-Cadherin in the ESCs was higher than in ES-like cells. We confirmed that C-Kit expression in the ES-like cells was significantly higher than in all other groups ($p<0.01$) (Figure 3.15).

3.2.1.2.3. Embryoid Body (EBs) formation of ES-like cells

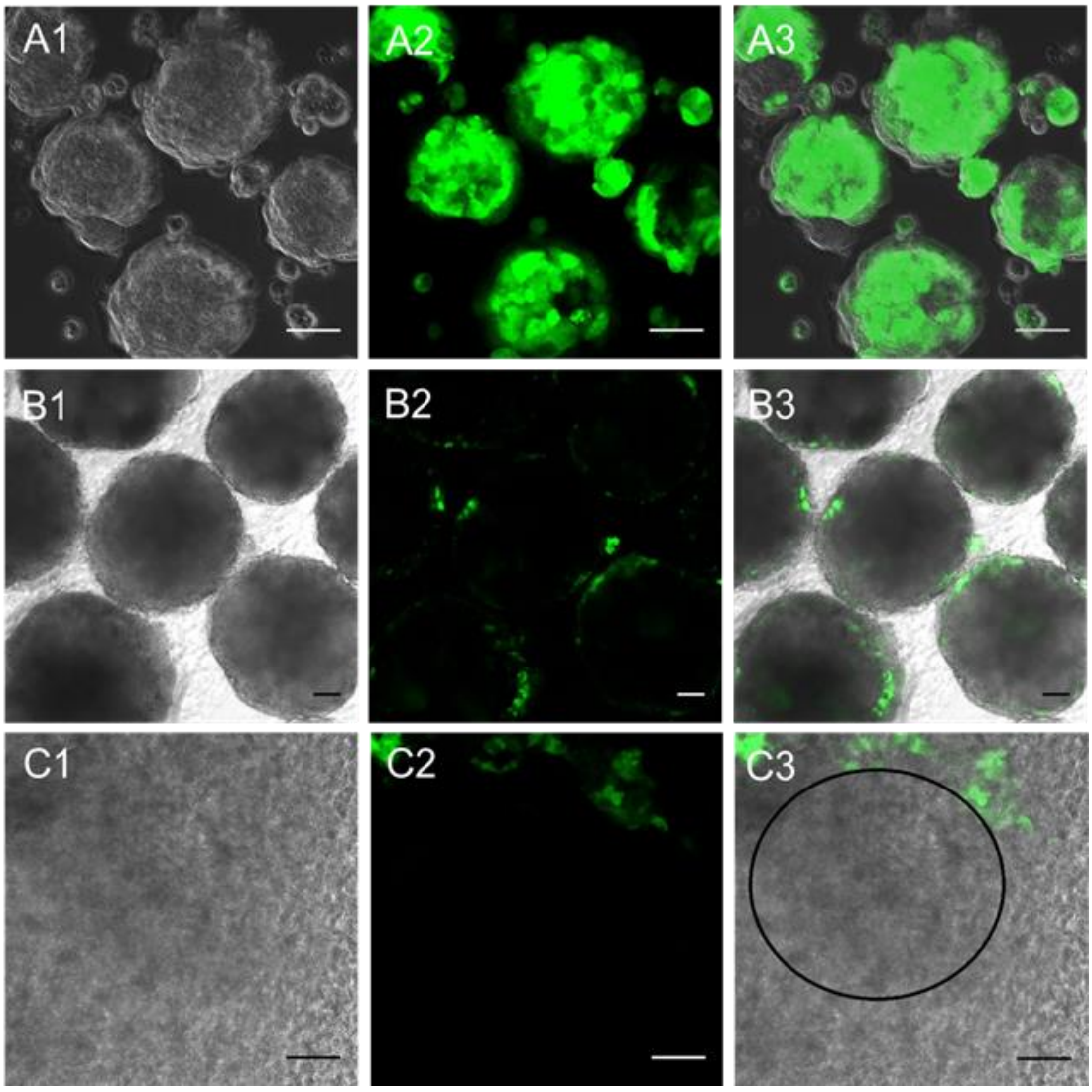

Figure 3.16: *In-vitro* differentiation of ES-like cells into EBs. Expression of Oct4-GFP in EBs from ES-like cells which were generated from Oct4-GFP transgenic mouse testis (A1-A3). Down-regulated expression of Oct4-GFP after treatment with 3µM RA (B1-B3). Beating differentiated-ES-like cell areas were completely negative for Oct4-GFP (C1-C3).

We used spontaneous differentiation during EBs formation to demonstrate the pluripotency of ES-like cells, and also performed direct differentiation to neuronal and cardiomyocyte phenotypes. Similar to mESCs, ES-like cells could form EBs in low attachment bacterial dishes (Figure 3.16). EBs formed during 4 days in mESCs media without LIF and expressed high levels of Oct4-GFP (Figure 3.16A1-A3). For further neuronal differentiation, EBs were cultured in the presence of retinoic acid (RA) and low concentration of FBS for 6 days (Figure 3.16B1-B3) , while for cardiomyocyte differentiation EBs were cultured in a high concentration of FBS. We observed down regulation of Oct4-GFP expression in the EBs, about 2 days after initiation of the differentiation with RA (Figure 3.16B1-B3). Similarly, we showed beating differentiated-ES-like cell areas which were completely negative for Oct4-GFP (Figure 3.16C1-C3).

3.2.1.2.4. *In-vitro* neuronal differentiation of ES-like cells (ectodermal lineage)

In order to promote directed differentiation of ES-like cells into a neural fate, we cultured EBs in the presence of 3µm RA and low percentage of FBS.

For neuronal differentiation, EBs were cultured in neurobasal medium with B27 and N2 supplement on poly-L-ornithine coated dishes. Neuronal morphology was observable

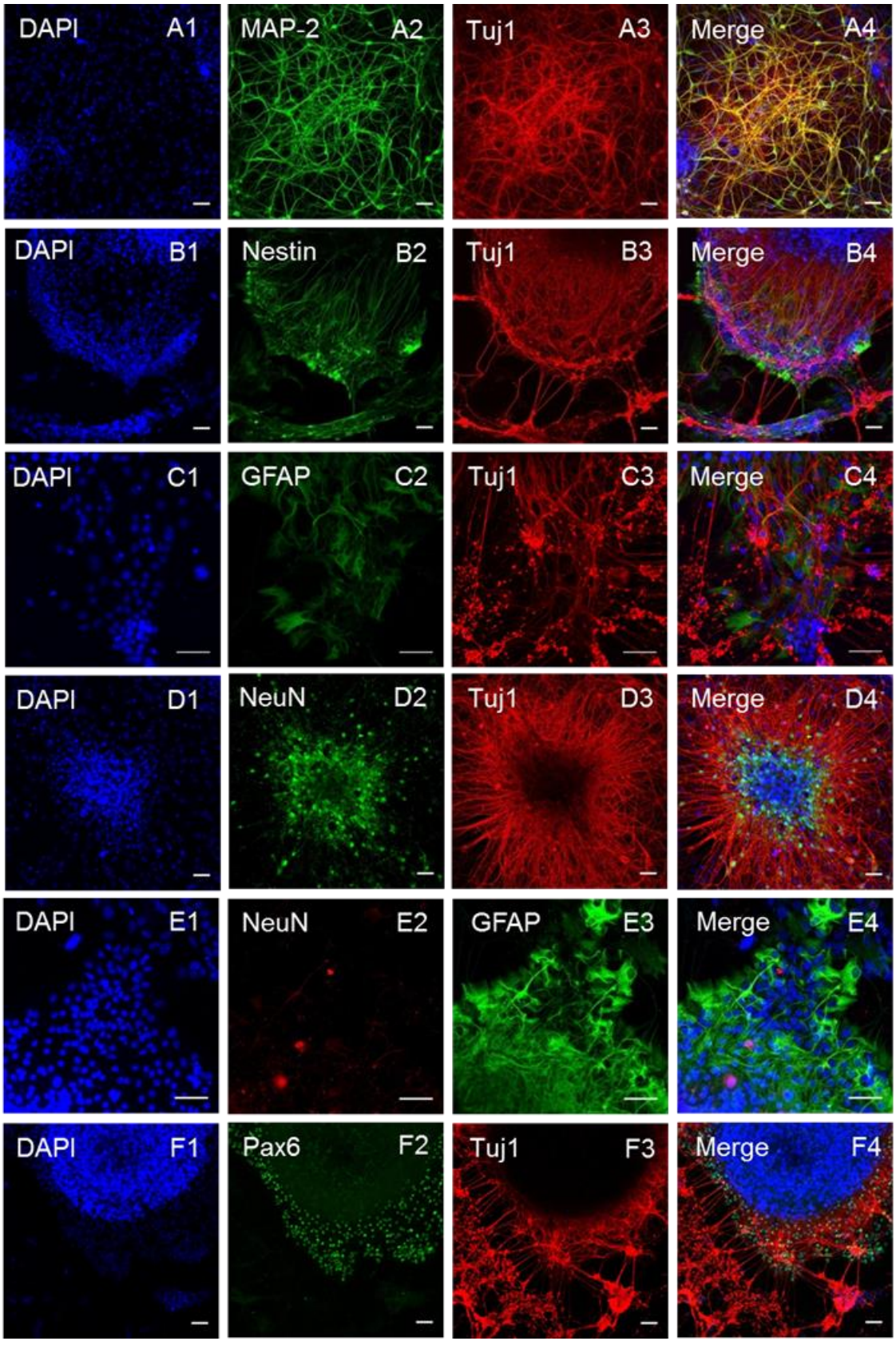
DAPI A1
MAP-2 A2
Tuj1 A3
Merge A4
DAPI B1
Nestin B2
Tuj1 B3
Merge B4
DAPI C1
GFAP C2
Tuj1 C3
Merge C4
DAPI D1
NeuN D2
Tuj1 D3
Merge D4
DAPI E1
NeuN E2
GFAP E3
Merge E4
DAPI F1
Pax6 F2
Tuj1 F3
Merge F4

Figure 3.17: *In-vitro* differentiation of ES-like cells to a neuronal fate after RA treatment. After differentiation, ES-like cells expressed markers Map-2, Tuj1, NeuN, Pax6 and GFAP (A, B, C). We observed that Map-2 positive cells were positive for Tuj1 (A1-A3) and the transcription factors Pax6 and NeuN (B1-B3 and C1-C3). A few GFAP positive cells expressed NeuN (D1-D3).

about 3 days after plating and about 10 days after plating immunofluorescence staining of the typical neuronal markers such as Map-2 (Figure 3.17A2), Tuj1(Figure 3.17A3-D3 and F3), Nestin (Figure 3.17B2), glial fibrillary acidic protein GFAP (Figure 3.17C2), NeuN (Figure 3.17D2 and E2) and the neuronal transcription factor Pax-6 (Figure 3.17F2). With double staining immunocytochemistry, we observed that Tuj-1 positive cells were also positive for Map-2 (Figure 3.17A1-A4), Nestin (Figure 3.17B1-B4), NeuN (Figure 3.17D1-D4) and Pax6 (Figure 3.17F1-F4), while GFAP positive cells were negative for Tuj1 (Figure 3.17C1-C4) and NeuN (Figure 3.17E1-E4). As mentioned, RA induces neuronal differentiation and suppresses Oct4 expression in the EBs during neuronal differentiation (Figure 3.16B1-B3).

3.2.1.2.5. *In-vitro* differentiation of ES-like cells into endodermal and mesodermal lineage (cardiomyocyte/beating cells)

For mesodermal and endodermal lineage differentiation, ES-like cells were used for EB formation in a high concentration of FBS serum. After plating of differentiated EBs, cells were used for analysis of mesodermal and endodermal differentiation by mRNA expression (Figure 3.18A) and functional cardiomyocyte analysis (Figure 3.19). About 5-10 days after plating of EBs, we observed areas with contractions of beating cells (cardiomyocyte cells) (Figure 3.16C1-C3) which started in the late stage of cultured EBs. We measured the number of beating areas in each well of a 6-well plate and the number of beating contractions per minute in each beating area. We observed about 14 (±3) beating areas per well of the 6-well plate and 87 (±36) beating contractions per minute. As mentioned before, ES-like cells expressed Oct4-GFP and we observed down-regulation of Oct4-GFP during cardiomyocyte differentiation. The beating area was completely negative for Oct4-GFP (Figure 3.16C1-C3). RT-PCR analysis revealed that EBs and differentiated cells expressed genes typical for vascular, epithelial and

hepatic or myogenic cells, early mesoderm and cardial-differentiated cells, while differentiated neuronal EBs were negative for MyoD, Myf5, AFP and mESCs and ES-like cells were negative (or showed only low expression) for PEPCAM1, Myf5, MyoD, Islet1 and FLK1 (Figure 3.18A) .

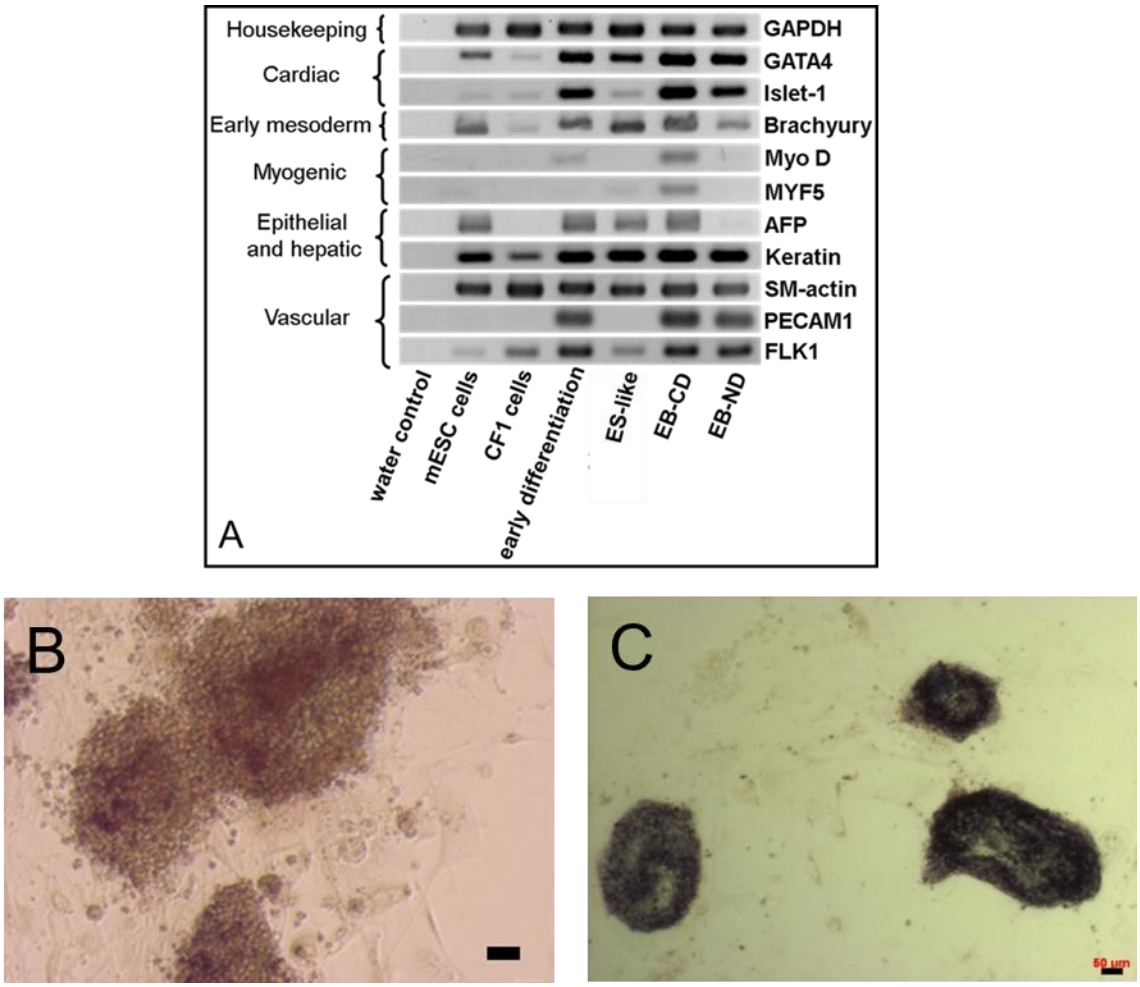

Figure 3.18: RT-PCR and alkaline phosphatase analysis:

RT-PCR analysis for expression of mesodermal and endodermal lineage genes from mESCs, mGPS, CF-1 MEF cells and early spontaneous differentiated EBs (6-10 days after plating of cardiomyoctes EBs), EB formation of cardiomyoctes (EB-CD) cells in day 10, EBs formation for neuronal cells (EB-ND) in day 10 and water control (A).Type I cells were partially positive for alkaline phosphatase (B) while ES-like cells were positive for alkaline phosphatase (C). Scale bare 50µm.

3.2.1.2.6. ES-like cells are positive for pluripotency markers and alkaline phosphatase

As we mentioned above, we isolated Type I cells from Oct4-GFP transgenic mice during a critical time-window after initiation of culture and converted these cells to ES-like cells that express high level of Oct4-GFP.

ES-like cells were passaged 1:5-1:8 more than 15 times following trypsin digestion. We observed that the ES-like cells maintained their undifferentiated state which demonstrated the effectiveness of clonal growth from single ES-like cells under MEF feeder layer and LIF cytokine conditions. In order to characterize the undifferentiated state of ES-like cells, we performed alkaline phosphatase (AP) staining (Figure 3.18 B and C) and immunocytochemistry for pluripotency markers Oct4 from Abcam (Ab), Oct4 from Santa Cruz (SC), Nanog, Sox2 and Klf4 (Figure 3.19).

While Type I cells were partially positive for AP which display a more faint staining, ES-like cells showed a similar intense staining for AP in comparison to mESCs (Figure 18B and C).

In our experiment, we observed that Type I cells were positive for Oct4 (Ab) and negative for Oct4 (SC) while both proteins were expressed in ES-like cells. This observation might confirm different isoforms of Oct4 protein in mouse cells. Similarly, while Type I cells were negative for Nanog and Klf4 or showed a low expression of Sox2, ES-like cells clearly expressed these pluripotency markers (Figure 3.19).

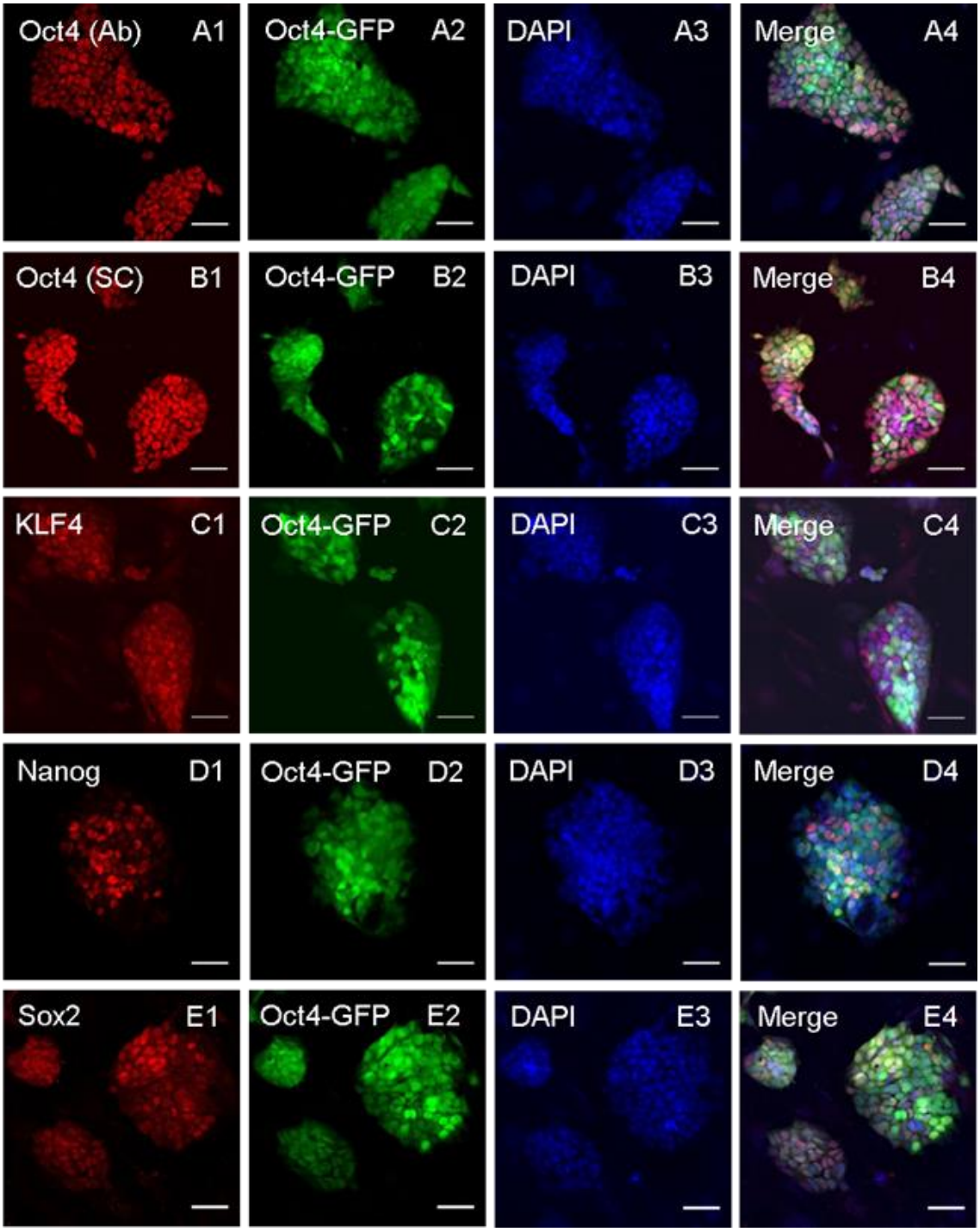

Figure 3.19: Immunocytochemistry results showed that ES-like cells express pluripotency markers Oct4 from Abcam (A1-A4), Oct4 from Santa Cruz (B1-B4), Klf4 (C1-C4), Nanog (D1-D4) and Sox2 (E1-E4).

3.2.1.2.6. Electrophysiology analysis

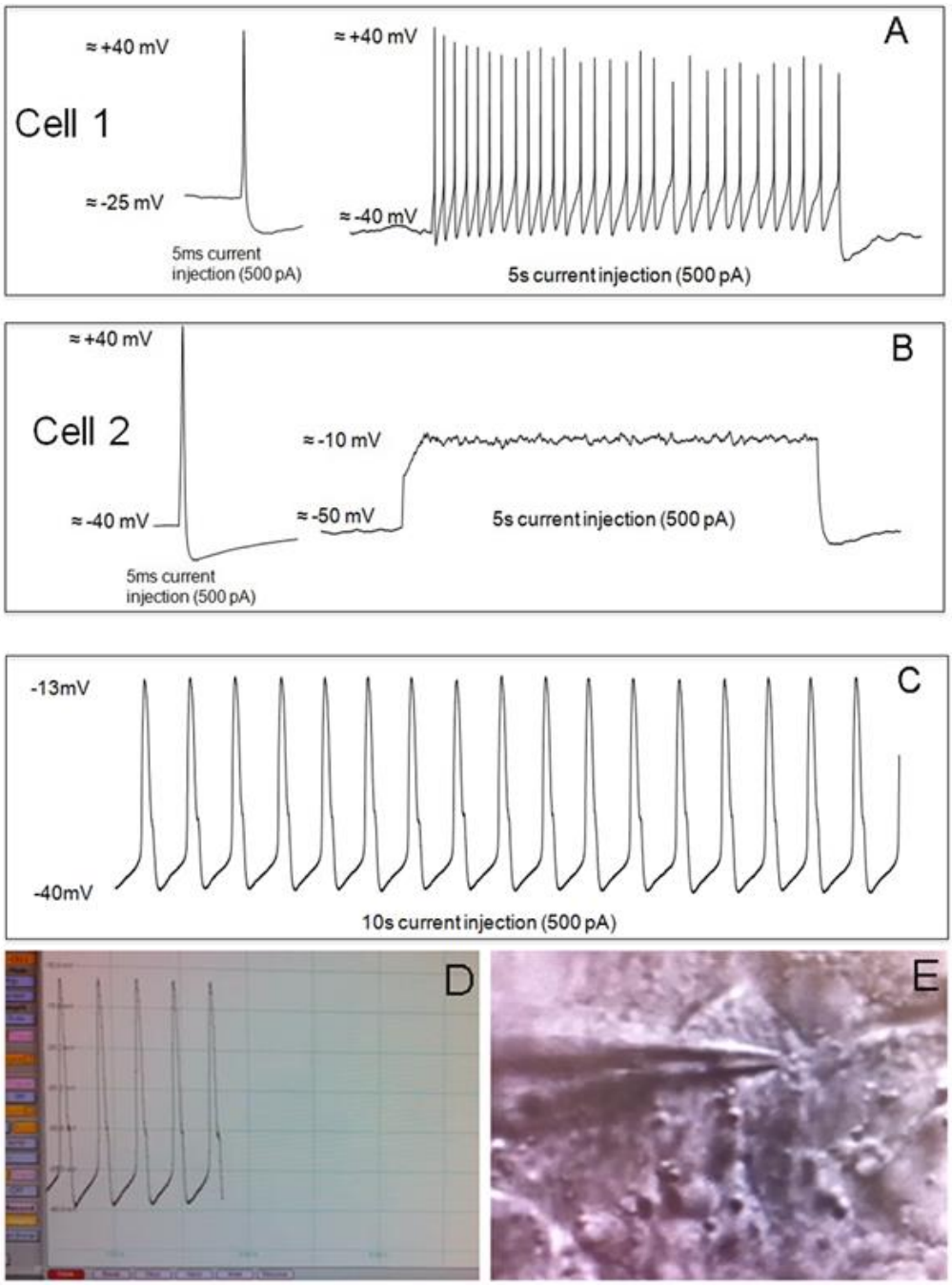

Figure 3.20: Electrophysiology analysis for differentiated neurons. Whole-cell current clamp recordings of differentiated neuronal (A and B) and cardiomyocyte cells (C). Recording visualization on the oscilloscope in the beating area (D). A cardiomyocyte patched in the beating area (E).

In order to promote directed differentiation of ES-like cells into the neural and cardiomyocyte fate, isolated EBs from ES-like cells differentiated in the presence of RA and FBS respectively.

After plating of EBs in tissue culture plates, neuronal morphology and beating areas were clearly observable and electrophysiological analysis of the differentiated cells was performed.

We analyzed the generation of action potentials in differentiated neuronal cells (Figure 3.20A and B). Upon a brief current pulse of 5ms, all cells (n=6) could fire an action potential, long current injections of 5s resulted in two types of activity.

There were either cells that fired continuously, like cell 1 (Figure 3.20A, 2 cells out of 6 cells recorded) or cells that would not fire, like cell 2 (Figure 3.20B, 4 cells out of 6 cells recorded). Interestingly, after action potential generation, the recorded cells gave rise to a strong hyperpolarizing current. Moreover, the resting membrane potential was very variable between the cells (Figure 3.20).

In addition, the pacemaker activity was analyzed by whole-cell current clamp for differentiated cardiomyocyte. It was observed that in the beating regions, the cells performed a rhythmic action potential generation over time, with constant amplitude (Figure 3.20C, D and E).

3.2.1.2.8. Tumor formation in SCID mouse after injection of ES-like cells

The development of teratoma formations is one of the most important functional methods for the characterization of pluripotency in stem cells studies.

In our study, the pluripotency of ES-like cells was verified by subcutaneous injections of about $2x10^6$ ES-like cells to five nod SCID mice. About 4 weeks after transplantation of the ES-like cells, we could observe teratoma in all five nod scid recipient mice and no teratoma were observed in the control group (just transplanted matrigel) (Figure 3.21). Hematoxylin-Eosin staining for teratoma showed many highly differentiated structures of ectodermal, mesodermal and endodermal origin in these encapsulated tumors. All three germ layers from ES-like cells derived teratoma are shown (Figure 3.21).

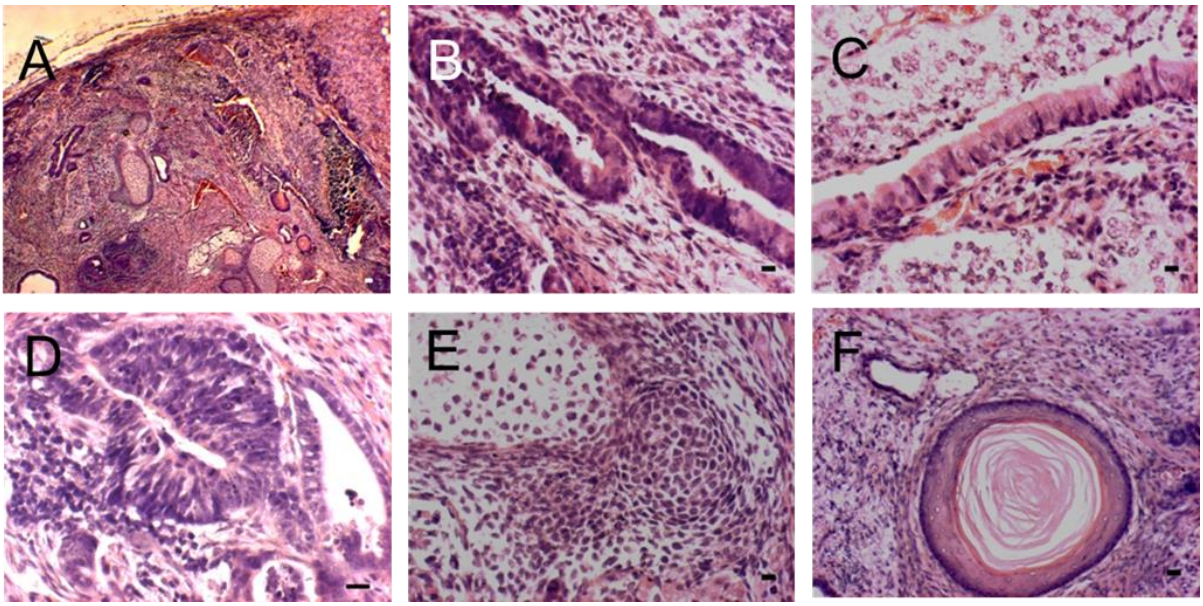

Figure 3.21: Teratoma formation in SCID mouse after transplantation of ES-like cells.
Teratoma formed after the transplantation of GPS cells into SCID mice (A). Ectormal and endodermal epithelium were observable, here a crosswise truncated lumina with gut epithelium and goblet cells is shown (B). Respiratory epithelium of endodermal origin (C). Example of an early neuronal rosette (D). Structures for mesodermal origin, early cartilage formation and other more unorganized connective tissues were observable (E). Structures of keratinizing epithelium were clearly observable (F).

3.2.1.2.9. Chimera formation after injection of ES-like cells into blastocyst

To answer the question whether ES-like cells are similar to ESCs contributing to embryo development *in-vivo*, we investigated chimera formation by injection of GSCs into mouse blastocyst. About 10–15 single ES-like cells were selected by a micromanipulator (Figure 22A) and injected into 3.5 day old mouse blastocysts of the embryo (Figure 3.22B and C).

Injected embryos were transferred into the uterus of pseudo pregnant recipient females and gestated for 18 days. The resulting pups were analyzed for chimera formation by coat color (Figure 3.22D). In some of the pups, coat color exhibited patches of coat color from the host embryo, but also patches from the respective injected ES-like cell clone.

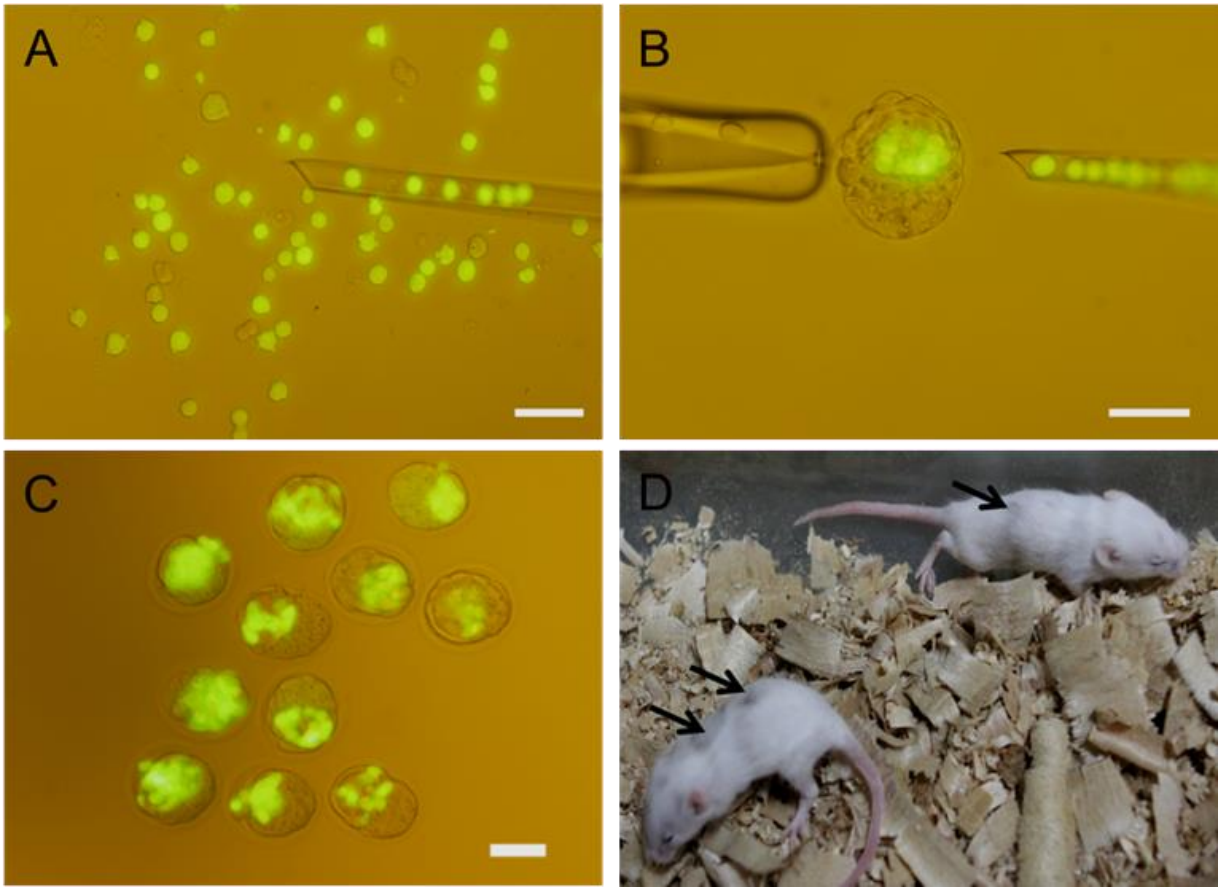

Figure 3.22: Generation of chimeric animals. About 10–15 single ES-like were ollected cells by a micromanipulator. (A) Injection of ES-like cells into blastocyst (B and C), identification of chimera mice by coat color (D).

Chapter 4 Discussion

4.1. *In-vivo* and *in-vitro* identification of germ cells by analysis of different germ cells markers

4.1.1. Oct4 expression in the germ cells

During early development, Oct4, a 352 amino acid protein, is expressed in pluripotent ESCs, blastomeres and in the inner cell mass of the blastocyst [141, 142]. After gastrulation, the Oct4 is down-regulated in trophectoderm and primitive endoderm but maintained in PGCs [142]. PGCs maintain the expression of Oct4 until the initiation of the spermatogenesis in males or oogenesis in females [142]. Our study provides evidence for the expression of Oct4-GFP in the seminiferous tubules of Oct4-GFP transgenic testis throughout life. We observed that the numbers and signal density of Oct4-GFP in the seminiferous tubule of the neonate mouse testis was higher than in adult mouse testis (~6 month old). The reduction of Oct4 expression during age may be related to a reduction of gonocytes and undifferentiated SSCs pool in the testis of neonates and older animals. On the other hand, we observed that the enhancement of Oct4 expression in the neonate testis is detectable not only in undifferentiated SSCs but also in T1-prospermatogonia. In addition, a low OCT-4 signal is also observable in spermatocytes (Figure 3.1A1-A4).

4.1.1.1. Different isoforms of Oct4 in germ cells

With alternative splicing and alternative translation, three mRNA isoforms of Oct4 genes including Oct4A, Oct4B and Oct4B1 and four protein isoforms including Oct4A, Oct4B-190, Oct4B-265, and Oct4B-164 are present in the human cells [143, 144]. Oct4A is a transcription factor that confirmed the stemness and pluripotency state of ESCs [145-148]. Oct4B is mainly localized in the cytoplasm of non-pluripotent cells which cannot maintain ES cell self-renewal and reduced in non-pluripotent stem cells [148]. During co-expression of Oct4A and Oct4B, activation of transcriptions by Oct4A was not repressed by Oct4B and in parallel overexpression of Oct4B was not activated by Oct4A

[148]. Two variants of the Oct4 gene including Oct4A and Oct4B were identified in mouse cells [149] . Oct4A exists only in one variant, which is the main regulator of pluripotent stem cells, while three isoforms of Oct4B include Oct4B-247aa, Oct4B-190aa and Oct4B-164aa were detected in somatic cells of the mouse [149].

Oct4 expressed in undifferentiated SSCs is required for maintenance of SSCs during *in-vitro* culture and is also important for colonization activity following cell transplantation [109, 150]. Although the expression of the Oct4A and B pattern have been shown in human testis [151], there is no evidence of different variants of Oct4 in the mouse testis.

In our study, we used the Oct4 antibody from Abcam (ab19857) and Santa Cruz (SC-8628) to identify germ cells and pluripotent stem cells. For antibody generation,Santa Cruz used peptide from N-terminal and Abcam again C-terminal of Oct4 protein.

Warthemann et al demonstrated that Oct4 from Abcam (ab19857) is common to both Oct4A and Oct4B, while the Santa Cruz antibody (Sc-5279) is directed against Oct4A [152].

In the testis section, we observed expression of the Oct4 gene in the seminiferous tubule of mouse testis (with the Abcam, but not from Santa Cruz antibody). We detected that the level of Oct4 expression in the SSCs population, was higher than in differentiated spermatogonial cells.

This result confirms down-regulation of Oct4 expression during spermatogenesis. This could be related to the production of different differentiation factors produced by sertoli cells in the two different compartments surrounded and separated by these cells, the stem cell and the differentiation compartment [153].

With immunocytochemistry, we did not detect Oct4 expression using the Santa Cruz antibody in the testis section or *in-vitro* in Type I and Type II cells. However, we detected Oct4 in generated ES-like and mESC cells using this antibody.

Oct4 mRNA was observable in ES-like cells, Type I, Type II cells and also in low levels in somatic feeders. In the primary culture of isolated SSCs from Oct4-GFP transgenic mice Oct4 was expressed in low level in the Type I cells but not in Type II cells.

4.1.2. PLZF

The transcription factor PLZF has an essential role in the maintenance and self-renewal of the SSC pool in the testis [119]. The functional importance of PLZF was shown in Zfp145–/– mice (Zfp145-null mice) were spermatogenesis is lost.

In addition, PLZF is expressed during embryogenesis and regulates limb and axial skeletal patterning [154]. PLZF has genetic interaction with Gli3 (GLI-Kruppel family member 3) and Hox5 genes during limb development [155, 156].

In the testis section, just a few cells in the basement membrane of seminiferous tubule expressed PLZF. It has been demonstrated that PLZF expression is restricted to As, Apr and Aal SSCs [157].

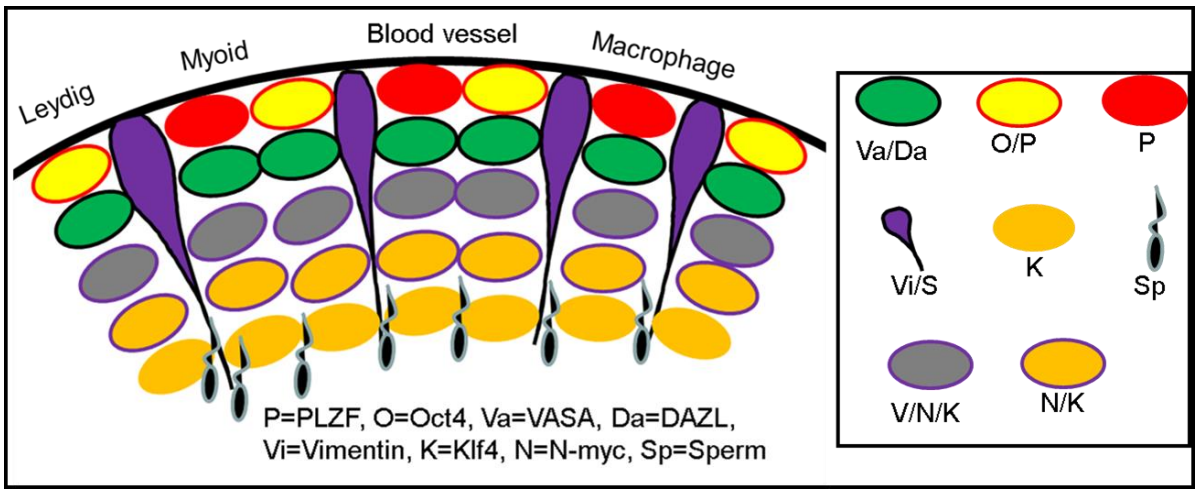

Figure 4.1: Schematic illustrations for expression of different germ cells markers in the seminiferous tubule

PLZF is co-expressed with Oct4 in undifferentiated mouse spermatogonial cells [157]. Similarly, Filipponi et al. observed both $PLZF^{+}$-$Oct4^{+}$ and $PLZF^{+}$-$Oct4^{-}$ cells in testicular germ cells. They suggested that $PLZF^{+}$-$Oct4^{+}$ cells are As spermatogonia and $PLZF^{+}$-$Oct4^{-}$ cells are Ap spermatogonia [109]. In our experiments, about 40 percent of cells in the cross section of 6 week-old mouse testis were $PLZF^{+}$-$Oct4^{-}$. Unlike Oct4, we confirmed that localization of PLZF was restricted to the basement membrane of seminiferous tubules. Until now, the mechanisms of down regulation of PLZF during spermatogenesis were not fully understood.

We observed that all PLZF and Oct4 positive cells showed low expression for DAZL and VASA protein (Figure 4.1).

In germ cells, PLZF represses both kit expression and expression of a reporter gene which is under the control of the kit promoter region [109] .

Hobbs et al identified an antagonism between Sall4 and PLZF. They demonstrated that Sall4, with inhibition of PLZF, induces the expression of c-kit and promotes postnatal spermatogonial progenitor cells (SPCs) [105].

In prostate cancer cell lines, it has been demonstrated that PTEN suppresses and phosphoinositide 3-kinase (PI3K) inhibitor treatment increases PLZF expression [158].

4.1.3. VASA and DAZL

In the adult testis, VASA protein was expressed in the cytoplasm of spermatocytes and round spermatids [159]. It is essential for the proliferation and differentiation of male germ cells during development [160].

Male mouse homozygotes for VASA homolog gene (Mvh) produce no sperm or premeiotic germ cells [160].

Similarly DAZL is a cytoplasmic protein which is expressed in germ cells. In adult rat testis, a weak DAZL signal was detected in the spermatogonia, intermediate and also in primary spermatocytes [161]. Highest intensity for DAZL protein expression was detectable between stages IV and VIII during the spermatogenic cycle and first meiotic division [161].

In the seminiferous tubule of the adult mouse testis, we observed DAZL protein only in cells located above the basement membrane and a decrease of VASA protein expression during differentiation to sperm. These results confirmed that VASA and DAZL proteins, although they were not expressed in undifferentiated SSCs (they were negative in PLZF and Oct4 positive cells), were expressed in progenitor SSCs and down-regulate during differentiation into sperms (Figure 4.1).

In-vitro culture of Types I and II cells, we observed that Type I cells clearly expressed VASA and DAZL but Type II cells had a very low expression signal for VASA, but were completely negative for DAZL. We also confirmed that VASA and DAZL positive cells in Type I cells were completely negative for VIMENTIN and clearly expressed several

germ cells markers such as PLZF. These results might confirm that the gene expression pattern in SSCs, which exist in the testis niche *in-vivo*, might be different in *in-vitro*. For example, unlike *in-vivo* SSCs in the testis, VASA and DAZL positive cells during *in-vitro* culture were positive for PLZF and Oct4 proteins.

4.1.4. Klf4 and N-Myc

In the cross sections of mouse testis, we found cytoplasmic expression for Klf4 by IMH. Klf4 expression started in a cell layer above the basement membrane compartment until differentiated cells of testis that are responsible for spermatogenesis while N-Myc proteins were expressed only in the middle part of tubular lumen.

In the mouse testis section, we observed that $Oct4^{+}$, $PLZF^{+}$, $Sox9^{+}$ and interstitial cells were negative for Klf4 and N-Myc proteins.

During *in-vitro* culture, we confirmed that Type I cells were negative for Klf4 but were positive for N-Myc, while Type II cells expressed both Klf4 and N-Myc protein.

Undifferentiated spermatogonial cells in the testis section were negative for Klf4 and N-Myc, while differentiated cells in the testis were positive for both markers. Similar to testis sections, Type II cells were positive for both markers while Type I cells were negative for Klf4 but positive for N-Myc protein. In testis sections, N-Myc positive cells were negative for DAZL and VASA, while this gene expression pattern was same for Type II cells but not for Type I cells (N-Myc expression) during *in-vitro* culture.

Although Type I cells were negative for Klf4 but we observed mRNAs expression similar to mESCs. This result might confirm that in Type I cells, mRNA for Klf4 were abolished for protein translation by an unknown mechanism.

Klf4 has also been shown to be an important transcription factor for the regulation of pluripotent cells [136]. Klf4 accompanied by the genes Oct4, Sox2 and C-Myc, are the main transcription factors for a generation of iPSCs from somatic cells [135, 136]. Beher et al showed that Klf4 is expressed in high levels in the nucleus and cytoplasm of round spermatids, postmeiotic germ cells and leydig cells, but not in sertoli cells [162], both in the human and mouse testis. These results suggested the importance of Klf4 during spermatogenesis. Further analysis has shown that deletion of Klf4 in germ cells did not

prevent complete spermatogenesis and mutant mice had normal testes and fertility [163].

Klf4 is known to be involved for transition of a normal fetal germ cell to a germ cell tumor. It has been reported that mRNA expression for Klf4 gene in seminoma testis was higher than in normal testis [164].

Depending on the activation of different signalling pathways, Klf4 can act as an oncogene (inactivation of the cyclin D1 proto-oncogene and the cyclin-dependent kinase inhibitor p21) or tumor-suppressor (directly suppress the expression of p53 promoter) [165, 166].

Myc proto-oncogene protein has been demonstrated to be involved in many cellular functions and regulations such as cell proliferation, apoptosis, metabolism activity and DNA synthesis [167, 168]. Myc activity was essential for ESC maintenance by controlling the LIF/STAT3 self-renewal pathway [169]. Similarly, C-Myc and N-Myc proteins control cellular functions such as proliferation, differentiation and survival in HSC (hematopoietic stem cell) [170]. In stem cells and neuroblastoma tumor, N-Myc protein can regulate the expression of a number of pluripotency genes such as LIF, Klf2, Klf4 and LIN28b [171]. N-Myc has been shown to be able to block differentiation in medulloblastoma cells and promote progression to tumors [172].

There is little evidence concerning N-Myc expression for spermatogenesis and its signalling pathway in the SSCs. Braydich-Stolle et al showed that Src kinase with activation of the PI3K/Akt signalling pathway could up-regulate N-Myc expression in SSCs [173]. In neural stem cells, N-Myc protein can be replaced by insulin-like growth factor signalling (IGF) pathways in a mouse model of sonic hedgehog-induced medulloblastoma [174].

4.1.5. Sox9 and VIMENTIN

Sertoli cells produce different cytokines and thereby can regulate SSCs functions in the male gonad. In testis sections, Sox9 expression was limited to the Sertoli cells and its use in combination with VIMENTIN is a highly specific marker for identification of sertoli cells in testis [175].

In the present study, we observed nuclear expression of Sox9 protein only in cells in connection to basement membrane of seminiferous tubules that were negative for Oct4, PLZF, VASA, DAZL, N-Myc and Klf4, but which were positive for VIMENTIN.

The Sox9 transcription factor is a member of the Sox gene family, similar to Sox8 and Sox10, and plays a critical role during embryogenesis for cellular differentiation of various tissues such as sertoli cells of the testis [176], neural crest cells [177, 178] and chondrocyte differentiation [179, 180].

The Sox9 gene also plays a crucial role for vertebrate sex determination which are regulated by SRY gene [181]. Fgf9 and Wnt4 proteins are expressed in the gonads of both sexes as antagonistic signals for sex determination [182].

During early testis development, FGF9 is important for the proliferation of a population of cells which give rise to sertoli progenitors and for localization of FGF receptor (FGFR2) in the nucleolus of early differentiated sertoli cells [183]. These events overlap with Sry expression and the nucleus localization of Sox9 during the earliest stage of sex determination (about 11.0 and 11.2 dpc).

Experiments showed that Sox9 has been used for identification of sertoli cells in transplantation studies [175]. In the testis section, sertoli cells contain intermediate filaments (IFs) which expand throughout the whole cytoplasm [184]. IFs were also found in interstitial cells, such as peritubular-myoid cells and leydig cells [185, 186].

It has been shown that the distribution of VIMENTIN filaments in sertoli cells is related to the cycle of seminiferous epithelium and also depends on the location of the spermatid heads [187].

Busulphan treatment modified expansion of VIMENTIN filaments in the sertoli cells which alter structural integrity of the seminiferous epithelium [188]. Alterations in the distribution of VIMENTIN filaments in the sertoli cells are related to apoptosis of germ cells [189]. VIMENTIN is also expressed during the maturation phase of spermatids of germ cells where the distribution of IFs are organized differently within sertoli cells [190].

In our experiment, we also observed low expression of VIMENTIN in the differentiated SSCs of testis and that unlike Type I cells, Type II cells during *in-vitro* culture clearly expressed the VIMENTIN marker.

Although Type II cells were positive for VIMENTIN, in comparison with Sertoli cells, they had a different morphology, low expression of Sox9 and only partial expression of germ cells markers. In addition to these results, Type II cells expressed N-Myc and Klf-4, which are negative in sertoli cells.

4.2. Identification, culture and expansion of SSCs

4.2.1. Expression of different surface markers

While some surface markers were used for the identification of SSCs, no highly specific markers for the identification and isolation of SSCs have been identified until now.

In our experiments we observed a high expression of surface markers CD9, CD29 and CD49, E-cadherin and GFRa1 in the Type I and II cells, while low expression of GFRa1 and E-Cadherin was observed in the ES-like cells.

Analysis confirmed that E-cadherin cell adhesion protein is a highly specific surface marker which can be used for enrichment of mouse SSCs [191, 192] although it might be expressed only by a small sub-population of undifferentiated SSCs [193].

E-cadherin is critical for the maintenance of pluripotency of mESCs and inhibition of E-cadherin promotes differentiation to epithelial cells [194, 195]. E-cadherin-mediated cell-cell contact is important during reprogramming and for efficiency of iPS cells [196]. E-cadherin can be replaced with exogenous Oct4 during somatic cell reprogramming [194] (For more about the importance of E-cadherin effect, see review [197]).

Similarly, surface marker THY1 (CD90) has been shown to be a conserved marker for undifferentiated SSCs in bulls [61, 198]. Some of the integrin subunits such as alpha 6 (CD49) and beta1(CD29) are expressed on the surface of SSCs and can also be used for the enrichment of SSCs [42, 199] but they are not specific for SSCs and can also be expressed by somatic cells in the testis [200].

Also the CD9 surface molecule has been used for enrichment of SSCs in the testis of mice and rats [201]. After xenotransplantation of enriched CD9-positive cells from human testis, colonization was higher in comparison with unsorted human testis cells [202].

It has been shown that the activation of LIF/STAT3 pathway induces the expression of CD9, which is important for maintenance of undifferentiated ESC and in addition of anti-CD9 antibodies reduced colony formation and cell viability [203].

GFRa1 receptor is another important cell surface marker which is expressed in undifferentiated SSCs in rodents and used as a protein for sorting of undifferentiated SSCs [107, 204]. As we mentioned before (chapter 1), GFRa1 receptor by activation of the Ret signalling pathway stimulates self-renewal and proliferation of SSCs [205].

The C-kit proto-oncogene surface molecule is expressed in differentiated SSCs [206]. It has been shown that the C-Kit protein is expressed in low level in the undifferentiated Type A SSCs while level of C-kit expression is enhanced when undifferentiated spermatogonia are shifted into A1 cells [206]. For example, the expression of the melanoma cell adhesion molecule (MCAM) was recently used as another factor for enrichment of SSCs [45].

4.2.2. Morphological selection

As we mentioned above, diverse gene expression patterns are present in the different testis cells. For this reason, scientists used different approaches for the isolation of SSCs by extracellular matrixes, FACS, MACS, transgenic animals or morphology based selection. Here, we describe for the first time the characterization of two different types of testis cell population (we named them Type I and Type II cells) during *in-vitro* culture. These two types of cells were different in isolation, morphology, culture conditions and gene expression. We believe that Type I cells might be undifferentiated but Type II cells seem to be differentiated SSCs in evaluation and comparison with *in-vivo* testicular cells.

In the present study, cell separation was achieved by morphological based selection to purify/enrich two types of cells from the somatic cells of the mouse testis. The two types of cells were easily distinguishable from each other and from testicular somatic cells (Figures 3.4 and 3.5).

We observed the growing of Type I cells that ceased after long term in primary culture and needed MEFs feeder for expansion while Type II cells could grow in primary culture with somatic feeders.

Differences in growing conditions of the two cell populations in primary somatic cells might be related to:

1- Existence of different somatic cell populations, 2- Difference in production (or concentration) of different cytokines which are produced by the different primary somatic cells. We also observed that long term culture and expansion of Type II cells only occurred on SNL feeders but not on MEF feeder.

As we mentioned before, SNL feeders are one type of MEF feeder which produce LIF in the culture media and are suitable for human iPSC culture [207]. There are some reports that show that LIF individually does not have any effect on SSCs during short term culture, but in combination with other growth factors could support proliferation of SSCs [208-211] (see introduction part). It has been shown in human amniotic epithelial cells that a high expression of LIF maintains mouse SSCs in an undifferentiated state [212]. In monkey ESCs, LIF enhances germ cell differentiation (increased high level of germ cell marker expression) [213].

The question why Type II cells grow on primary TSCs and SNL feeder but Type I cells could emerge on primary TSC feeder and be expanded on MEF feeder, could be related to different cytokines which are secreted from the different primary testicular cells such as sertoli cells, leydig cells and myoid cells.

Because we used a low concentration of serum in GS culture media, the kinetic growth of TSCs was not fast. Especially in old mice the kinetic grow on TSCs was much slower than those from neonate SSCs. Therefore the condition was ideal for Type II cells (instead of high growing of TSCs). Unlike with human cells, in the mouse testis, growing of TSCs was limited only to a few passages (about 1-2) after initiation of culture in mGSCs medium [65] and it was not clear which population of TSCs dominated other types of cells. Therefore productions of cytokines might have been different in the culture media.

Because of variant molecular properties and character, Type I and II cells react differently to these culture conditions. The exact mechanisms were not clear yet but Type I and II cells require MEF and SNL feeder for more expansion. Similar with neonate mouse GS cells in Shinohara experiments [76], neonate and adult Type I cells had a low rate of growing on primary passage on MEF feeder (4-6 weeks) and higher

rate of growing (3-4 weeks) during further passages but this rate of growing for Type II cells in every passage was the same (3-4 weeks).

4.3. Spermatogonia and somatic cells potential for Type I and II cells

We performed different characterization and analysis for Type I and II cells to show spermatogonial potential of both Types of cells. With morphology analysis, both types of cells have single, pair and aligned morphology which is typical morphology for undifferentiated and progenitor spermatogonial cells *in-vivo* [34, 35]. While somatic cells and sertoli cells have adherent morphology [214] , Types I and II cells cultured as colony formation.

Another characteristic for Types I and II cells in comparison with somatic cells was rate of growth. Unlike human testicular cells, growth of TSCs ceased a few weeks after cultivation in GSCs medium but both Types of cells had more than 1 year in cultivation in GSCs medium.

Electron microscopic analysis showed big nucleolus and small cytoplasm for Type I cells. High ratio of nucleolus to cytoplasm is an important property of undifferentiated SSCs in testis [215]. In contrast we observed a low ratio of nucleolus to cytoplasm in Type II cells [216].

As we mentioned, mRNAs and protein analysis confirmed Type I cells clearly, while Type II cells partially expressed typical germ cells markers.

	Type I	Type II	sertoli cells (*in-vivo*)	Somatic cells
Cell morphology	single and aligned chains morphology	single and aligned chains morphology	adherent monolayer culture	adherent monolayer culture
Electron microscopic	high nucleus/cytoplasm ratio	low nucleus/cytoplasm ratio	NT	NT
Limitation for	Unlimited (more	unlimited (more	NT	About 2

growing	than 1year)	than 10 months)		passage
Transplantation	NT	sperm	NT	NT
Germ cells markers expression	express typical germ cells markers	partially express germ cells markers	negative	negative
Sox9 expression	low	low	positive	negative
VIMENTIN	negative	positive	positive	positive
Klf4 expression	negative	positive	negative	negative
Pluripotency potential	positive	negative	NT	negative

Table 4.1: Comparison of Type I, II, sertoli and somatic cells

Since Type II cells were partially positive for germ cell markers and also expressed some TSC cells markers such as VIMENTIN, we considered the functional property of Type II-GFP cells by transplantation to an infertile mouse model. We observed localization of Type II cells in the basal compartment of seminiferous tubule of infertile mice and GFP-sperms. These results showed that both Types of cells might be SSCs but not somatic cells with different lineage in testis.

4.4. Application of germ line stem cells

4.4.1. Pluripotency of germ cells

Although SSCs expressed some genes of pluripotency, due to the heterogeneity of SSCs in the testis, it is not clear from which type or population of testicular cells, ES-like cells were generated from. In our experiment, we generated two different cell populations from testis cells and reprogramming only occurred in the Type I cell population and not in the Type II cells.

In the pluripotent stem cells, the expressions of core transcriptional genes include Sox2, Nanog and Oct4.

These transcriptional factors control the expression of different lineage-specific genes and sustain maintenance of pluripotent cells from differentiation [147].

We showed expression of pluripotency markers (although they were not at the same level with mESCs) which were expressed in Types I and II cells. We confirmed that both cell types showed low expression of Nanog and Sox2. We also observed that Type I cells were negative for Klf4.

It has been shown that germ cells expressed all of the different Yamanaka markers at the mRNA level, while mRNA for Sox2 was not translated into a protein [217]. It has been documented that blastocysts with a deficiency in Sox2 are unable to shape a pluripotent inner cell mass (ICM) with loss of pluripotency state [218]. The Nanog transcription factor maintains self-renewal in mESCs during independent mechanism of LIF/STAT3 signaling pathway [219]. In mESC deletion of Nanog causes loss of pluripotency state and differentiation into extraembryonic endoderm cell lineage [219].

During the initiation of culture, Type I cells expressed Oct4 mRNA but down-regulated this gene completely during long term culture until they shifted to ES-like cells which restored mRNA expression of Oct4 in same level as mESCs. In the pluripotent ESC, a special level of Oct4 mRNA expression was essential for the undifferentiated state of pluripotency [146]. With the overexpression of Oct4 mRNA until about two-fold caused loss of pluripotency state and differentiation into primitive endodermal cells and mesodermal cells whereas 50% repression of Oct4 supported dedifferentiation into trophectoderm cells [146]. It has been shown that Oct4 suppresses neural ectodermal differentiation and promotes mesododermal differentiation while Sox2 transcription factor inhibits mesododermal differentiation and enhances neural ectodermal differentiation [220].

The transcription factor Klf4 is able to cooperate with both Oct3/4 and Sox2 by activating a number of genes, including Klf4 itself and Lefty1. In contrast, over-expression of Klf4 inhibits differentiation of ESCs [221].

LIN28 was expressed in Type I cells at the same level as mESCs but was negative in Type II cells. LIN28 is a specific marker which is expressed in undifferentiated mouse

SSCs [222] but recent reports confirmed that LIN28 might not be present in adult human testes while a high expression was observed in human testicular germ cell tumors [223]. The reprogramming mechanism and the establishment of ES-like cells from SSCs are currently not completely understood. However, it is apparent that the age of animals, the mouse strain, culture conditions with growth factors, cell density of SSCs during culture, the time period after initiation of culture and the length of culture and typical population of testis cells all affect the reprogramming process [51, 52, 57, 137, 138]. In addition, some controversial challenges exist for pluripotency and multipotency of ES-like cells (especially in ES-like cells which generated from human testis) [224-226].

We demonstrated that conversion of SSCs to ES-like cells is a rare event which occurs spontaneously only between about 40-125 days after initiation of culture and not thereafter. As we mentioned in Table 4.2, ES-like cells might have a similar potential for teratoma formation and making chimera animal as mESCs.

Shinohara et al generated ES-like cells about 4-7 weeks after initiation of culture in the neonate mouse SSCs, but the origin of ES-like cells was not clear. In order to answer this question Guan et al used Stra8GFP$^+$ [57], while Seandel et al used GPR125GFP$^+$ [52] reporter mice for generation of ES-like cells. The established cell lines by this groups were not grown clonally (initial germ cell population grown from a single cell) and ideal ES morphology (transitional morphophology for Seandel et al.). Ko et al showed that induction of pluripotency in Oct4 GFP transgenic mice was related to a special number of SSCs (1000-4000) and length of culture (2-4 week) but this process did not occur in our experiments for Type I and II cells in all experiments conducted.

Age	Enrichment of cells	Culture medium	Characterization	ES-like generation	Rate	References
Neonate mouse	Supernatants in primary culture after 1 day	Stem pro Medium with GDNF, FGF,EGF,LIF, 2%FCS	Gene expression, teratoma, chimera, EBs formation and *in-vitro* differentiation to embryonic lineage	Spontaneously 4-7 week after initiation of culture	19%	[137]
4-6 Week	Stra8-GFP	Different	Gene expression,	Spontaneo	27%	[57]

mouse	mouse model,	condition with DMEM,15% FCS, GDNF, LIF,MEEF	teratoma, EBs formation, *in-vitro* and in-vivo differentiation to embryonic lineage	usly 4-7 days		
3week to 8 month mouse	Isolation of GPR125 cells and culture on TSCs Feeder	Stem pro Medium with GDNF, FGF,EGF,LIF, 2%FCS	Chimera and teratoma formation, *in-vitro* and *in-vivo* differentiation to embryonic lineage	after 2 week, transitonal colony	-	[52]
5-8 week	Low expression of Oct4 positive cells	Stem pro Medium with GDNF, FGF,EGF,LIF, 2%FCS	Gene expression, chimera and teratoma formation, *in-vitro* and *in-vivo* differentiation to embryonic lineage	After 2 week in 4000 cells per each well	-	[51]
Neonate mouse	Testicular culture on gelatine	DMEM,GDNF, FGF,EGF,LIF, 2%FCS	Gene expression, chimera , EBs formation, *in-vivo* differentiation to embryonic lineage	Using small molecule, After 2 week,	-	[138]
Neonate and adult	Morphology selection	Stem pro Medium with GDNF, FGF,EGF,LIF, 2%FCS	Gene expression, EBs formation, *in-vivo* differentiation to embryonic lineage	40-110 day after initiation of culture	33%	Azizi, in the prep aratio n

Table 4.2: Generation of ES-like cells from testis under different conditions

In the pluripotent stem cells, two phases of naive and prime pluripotency states have been suggested by Nichols et al [227].

Naive pluripotent cells, such as early epiblast stem cells or ESCs, could make teratoma formation and chimera formation, whereas prime pluripotent stem cells similar to late epiblast cells or post-implantation epiblast cells could create teratoma formation but

could rarely make any chimeric animals [227]. ES-like cells which were generated from Seandel and Guan et al groups might have been prime pluripotent cells.

4.4.2. Infertility applications

Infertility treatment is another important aim for application of germline stem cells.

Infertility in primates and non-primates might be related to genetic deficiencies, therapeutic reasons or toxic agents from the environment [228].

In patients undergo chemotherapy or radiotherapy treatment especially in young prepubertal and adolescent cancer patients, preservation of fertility by restoring SSCs and transplanting them after treatment is a reasonable approach [229]. Similarly, hormone applications have been shown for preservation and restoration of fertility after cancer treatment [230].

Because SSCs do not really possess specific marker for their identification, transplantation of labelled candidate cells into infertile models and tracking of spermatogenesis is a very powerful technique for the identification of SSCs.

In our experiments, we observed that Type II cells partially expressed germ cells markers and therefore we assessed the functional properties of Type II-GFP cells by transplantation into an infertile mouse model. About one month after transplantation, we observed localization of Type II cells in the basal compartment of the seminiferous tubule of an infertile mouse, also in addition to GFP positive spermatids. About three months after transplantation, we observed GFP- positive sperms in the epididymis.

In conclusion, we generated two different cell populations under germ stem cell conditions *in-vitro*. These two SSC populations have different potential for clinical treatments. If Type II cells actually display differentiation potential for SSCs, they would be one step further than undifferentiated SSCs and therefore *in-vitro* differentiation to sperm might be much easier than starting from undifferentiated SSCs.

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Appendix

Consumable items include materials, enzyme, chemical, solutions, growth factors, antibody, primers used for PCR, concentration of components in the associated solutions and mediums have been described with more detail in the appendix

Table 1: Materials for cell culture

Materials	**Company**	**Materials**	**Company**
T25 and T75 Cell culture flaske	Greiner	Cell Culture Dishes	Greiner
Cell culture plate: 6-well, 12-well, 24-well,	Greiner	Microcentrifuge tube	Plastibrand
Falcon tubes: 15 and 50ml	Greiner	Serological Pipets	Greiner
Bottletop Filters	Millipore	Cell Culture Scrapers	Greiner
Freezing cryovial	Nunc Thermo	Cover glass	Deckglaser
Millex Syringe	BD	Cell strainer: 40µm and 70 µm	BD
Syringe	BD	Bacterial dishes for suspension culture	Greiner
Isopropanol freezing container	Nunc Thermo		

Table 2: Enzymes and chemicals for cell culture

Title	Company	Title	Company
Collagenase IV	Sigma-Aldrich	Dispase	Sigma-Aldrich
DNAse	Sigma-Aldrich	Trypsin-EDTA	Gibco
Mitomycin C	Roche	Gelatine	Sigma-Aldrich
Matrigel			

Table 3: Solutions for cell culture

Solutions	Company	Solutions	Company
StemPro-34 medium	Invitrogen	DMEM/F12	PAA
HBSS buffer with Ca++ and Mg++	PAA	DMEM high-glucose	Invitrogen
KO-DMEM	Invitrogen	PBS	PAA
Penicillin/Streptomycin	PAA	β-mercaptoethanol	Invitrogen
Trypan blue	Invitrogen	DMSO	AppliChem
poly-L-ornithine	Sigma-Aldrich		

Table 4: Growth factors for cell culture

Growth factors	Company	Growth factors	Company
FBS and ES-cell qualified FBS	Invitrogen	StemPro-34 supplement	Invitrogen
D+ glucose	Sigma-Aldrich	N2-supplement	Invitrogen

L-glutamine,	PAA	Progesterone	Sigma-Aldrich
Bovine serum albumin	Sigma-Aldrich	MEM vitamins	PAA
Non-essential amino acids	PAA	FGF	Sigma-Aldrich
Estradiol	Sigma-Aldrich	Pyruvic acid	Sigma-Aldrich
EGF	Sigma-Aldrich	LIF	Millipore
DL-lactic acid	Sigma-Aldrich	Ascorbic acid	Sigma-Aldrich
BSA	Invitrogen	L-glutamine	PAA
RA	Sigma-Aldrich		

Table 5: Consumable items used for Histology, Electrophysiology and EM

Titles	**Company**	**Titles**	**Company**
DNAse	Promega	PIPES	Serva Electrophoresis
Paraformaldehyde	Applichem	Glutaraldehyde	Serva Electrophoresis
Uranylacetat	Serva Electrophoresis	Potassium hexacyanoferrate	Serva Electrophoresis
Sodium maleate buffer	Serva Electrophoresis	OsO_4	Serva Electrophoresis
Epon-embedded	Serva Electrophoresis	Paraplast Plus	Langenbrinck

PBS	Invitrogen	Triton	Merck Millipore
DAPI	Sigma	BSA	Sigma
Tissue Tek	Sakura	Superfrost Plus glass slides	Langenbrinck
Xylene	VWR Chemicals	Sodium Citrate Buffer	Riedel-de Häen
EDTA	Merck Millipore		

Table 6: Antibody used for staining

Antibody	**Species**	**Company**	**Cat. number**	**Concentration**
c-Myc	ms mono IgG1	Invitrogen	460603	1:500
CD 29-APC	mouse	MACS	130-096-356	1:11
CD 49f -APC	rat IgG 2a	R&D Systems	FAB13501A	10 µl (0,1 µg)
CD 9-APC	rat IgG 2a	R&D Systems	FAB 5218A	10 µl (0,1 µg)
CD 117-APC	mouse	MACS	130-091-729	1:11
DAZL	ms mono IgG1	AbD Serotec	MCA 2336	1:100
E-Cadherin-APC	rat IgG 2a	R&D Sytems	FAB7481A	10 µl (0,1 µg)
GFAP	rab poly	Dako	Z0334	1:400
GFAP	ms mono	Chemicon	MAB 3402	1:400
Cop-GFP	rab poly	Evrogen	AB 501	1:15.000

GFP (4B10)	ms mono	Cell Signalling	2955	1:100
GFR α1	rab poly	Sigma	SAB 4501166	1:50
Klf4	rab poly	Cell Signaling	4038	1:100
MAP 2	ms mono	Sigma	M 1406	1:200
MAP 2	ms mono	Sigma	G 3893	1:200
Nanog	rab poly	Abcam	ab80892	1: 100
Nanog	rab poly	Abcam	ab 21624	1:100-400
Nestin	ms mono	Abcam	ab 22 035	1: 500
Nestin	ms mono	BD	556 309	1: 100
Neu N	mouse mono	Chemicon	MAB 377	1: 100
N-Myc	mouse mono	Abcam	ab 16898	1: 50
Oct 4	rab poly	Abcam	ab19857	1:200
Oct3/4 (C-10)	ms mono	Santa Cruz	SC -5279	1:100
PLZF (2A9)	ms mono	Calbiochem	OP128	1:100
Sox2	rab poly	Abcam	ab 15830	1:100
Sox2	ms mono	Abcam	ab 75485	1:100
Sox9	rab poly	Linaris	PAK0058	1:50
ß-Tubulin III	ms mono	Covance	MMS-435-P	1:500
ß-Tubulin III	ms mono	Sigma	T8660	1:400
VASA (DDX4)	rab	Abcam	ab 13840	1:100
VASA	goat poly	R&D System	AF 2030	1:100

VIMENTIN	mouse mono IgM	Sigma Aldrich	V2258	1:100
VIMENTIN	mouse mono	Dako	M 0725	1:100

Table 7: Materials and solutions used for RNA analysis

Items	**Company**	**Items**	**Company**
TE buffer	Prepared in the Lab	M-MLV Reverse Transcriptase kit	(Promega, Germany)
Direct 2× Reaction Mix	Invitrogen	RT/Taq Superscript III	Invitrogen
dNTP mix	Promega	Ethidium bromide	Roth
$MgCl_2$	Invitrogen	1x AmpliTaq 360 DNA Polymerase	Applied Biosystems
Peq GOLD TriFast reagent	Peqlab, Erlangen, Germany	RT-PCR primer	Sigma
qRTPCR primer	Applied Biosystems		

Table 8: Primers used for PCR

Name	**Sequence**	**Product size**	**Annealing TM (°C)**
Brachyury	F: GCTGTGACTGCCTACCAGAATG R: GAGAGAGAGCGAGCCTCCAAAC	231	58
Gapdh	F:GCAGTGGCAAAGTGGAGATT R: TCTCCATGGTGGTGAAGACA	249	56
Gata4	F:CTGTCATCTCACTATGGGCA R: CCAAGTCCGAGCAGGAATTT	257	56
α-	F:CCCACCCTTCCAGTTTCC	150	59

Feteoprotein	R: TCGTACTGAGCAGCCAAGG		
SM actin	F:ACTACTGCCGAGCGTGAGATT R: GTAGACAGCGAAGCCAAGATG	449	59
Islet1	F:ACGTCTGATTTCCCTGTGTGTTGG R: TCGATGTGGTACACCTTAGAGCGG	275	63
Keratin18	F:TTGTCACCACCAAGTCTGCC R: TTTGTGCCAGCTCTGACTCC	213	63
Myf 5	F:GGAGATCCTCAGGAATGCCATCCC R:TGCTGTTCTTTCGGGACCAGACAGG	178	67

Table 9: List of TaqMan gene expression assays for multiplex qRTPCR

Gene	**Gene name**	**Species**	**Assay ID**
DAZL	deleted in azoospermia-like	mouse	Mm00515630_m1
Ddx4 or VASA	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	mouse	Mm00802445_m1
Ret	Ret proto-oncogene	mouse	Mm00436304_m1
Epcam	epithelial cell adhesion molecule	mouse	Mm00493214_m1
Kit	kit oncogene	mouse	Mm00445212_m1
Scp1	synaptonemal complex protein 1	mouse	Mm01297993_m1
Tert	telomerase reverse transcriptase	mouse	Mm01352136_m1
Bcl6b	B cell CLL/lymphoma 6, member B	mouse	Mm00455914_m1
Etv5	ets variant gene 5	mouse	Mm00465816_m1
Lhx1	LIM homeobox protein 1	mouse	Mm00521776_m1

ZBTB16 or PLZF	zinc finger and BTB domain containing 16	mouse	Mm01176868_m1
Taf4b	TATA box binding protein (TBP)-associated factor	mouse	Mm01254136_m1
Stra8	stimulated by retinoic acid gene 8	mouse	Mm01165142_m1
IHH	Indian hedgehog	mouse	Mm00439613_m1
Nanog	Nanog homeobox	mouse	Mm02384862_g1
Lin28a	lin-28 homolog A (C. elegans)	mouse	Mm00524077_m1
TDGF1	teratocarcinoma-derived growth factor 1	mouse	Mm00783944_g1
Dppa5	developmental pluripotency associated 5	mouse	Mm01171664_g1
Nodal	nodal	mouse	Mm00443040_m1
Gdf3	growth differentiation factor 3	mouse	Mm00433563_m1
Pou5f1 or Oct4	POU domain, class 5, transcription factor 1	mouse	Mm03053917_g1
Sox2	SRY (sex determining region Y)-box 2	mouse	Mm00488369_s1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	mouse	Mm99999915_g1

Table 10: Components in the digestion solution of testis

Name	**Concentration**	**Name**	**Concentration**
Collagenase IV	0.5 mg/ml	Dispase	0.5 mg/ml
DNAse	0.5mg/ml	HBSS buffer	Up to volum

Table 11: mGSC medium

Name	Concentration	Name	Concentration
StemPro-34 medium	Up to volume	D+ glucose	6 mg/ml
N2-supplement	1%	L-glutamine	1%
Bovine serum albumin	5 µg/ml	β-mercaptoethanol	0.1%
Penicillin/streptomycin	1%	Non-essential amino acids	1%
MEM vitamins	1%	Progesterone	60 ng/ml
Estradiol	30 ng/ml	Fibroblast growth factor	10 ng/ml
Epidermal growth factor	20 ng/ml	Leukemia inhibitory factor	100 U/ml
Glial-derived neurotrophic factor	8 ng/ml	Ascorbic acid	100 µg/ml
ES-cell qualified FBS	1%	DL-lactic acid	1 µl/ml
Pyruvic acid	30 µg/ml		

Table 12: mESCs and ES-like medium

Name	concentration	Name	concentration
KO-DMEM	Up to volume	MEM NEAA solution	1%
FBS	15%	L-glutamine	1%
Pen-Strep	1%	ß-mercaptoethanol	0.1%
LIF	1000 U/ml		

Table 13: SNL feeder medium

Name	Concentration	Name	Concentration
D-MEM with 4500 mg/L glucose	Up to volume	FBS	10%
MEM NEAA	0.1 mM	L-glutamine	2 mM
Pen-Strep	1%		

Table 14: TSCs feeder medium

Name	Concentration	Name	Concentration
D-MEM with 4500 mg/L glucose	Up to volume	FBS	20%
MEM NEAA	0.1 mM	L-glutamine	2 mM
Pen-Strep	1%	bFGF	10ng

Table 15: MEF feeder medium

Name	Concentration	Name	Concentration
D-MEM with 4500 mg/L glucose (or KO-DMEM)	Up to volume	FBS	10%
MEM NEAA	0.1 mM	L-glutamine	2 mM
Pen-Strep	1%		

Table 16: Components in the freezing media

Name	Concentration	Name	Concentration
TSCs medium	70%	FBS	20%
DMSO	10%		

Table 17: Components in the fixative solution for EM

Fixative solution for EM	Concentration	Name	Concentration
Paraformaldehyde	3 %	PIPES	0.1 M
Glutaraldehyde	3%		

Table 18: Components in the post fixative solution for EM

Post-Fixative solution for EM	Concentration	Name	Concentration
OsO_4	1%	Potassium hexacyanoferrate	1.5%

Table 19: Components in the washing buffer for EM

Washing buffer for EM in (pH 6.0)	Concentration	Name	Concentration
Uranylacetat buffer	1 %	Sodium maleate	0.2 M

Table 20.Blocking buffer for EM:

Blocking buffer for EM	Concentration
Uranyl acetate	1%

Table 21: Staining solution in the Flow cytometry

solution	Concentration	solution	Concentration
FBS	3%	PBS	97%

Table 22: Permeabilization solution for immunocytochemistry

solution	Concentration	solution	Concentration
Triton	0.1 %	PBS	99.9 %

Table 23: Blocking solution for immunocytochemistry

solution	Concentration	solution	Concentration
BSA	1 %	PBS	99. %

Table 24: Antigen retrieval solution for immunohistochemistry

Solution	Concentration	Solution	Concentration
Sodium Citrate Buffer in pH 6	10 mM	EDTA in pH 8	1 mM

Table 25: Blocking solution for immunohistochemistry

Blocking solution for IMH	Concentration	Blocking solution for IMH	Concentration
FCS serum	10%	PBS	89.7 %
Triton	0.3 %		

Table 26: Components in the PCR reaction

PCR reaction	Concentration	PCR reaction	Concentration
$MgCl_2$	1.5 mM	dNTP	0.4 mM
Forward primer	0.5 mM	Revers primer	0.5 mM
AmpliTaq 360 PCR buffer	1.25 U (or 1x)	cDNA	1 µl

Table 27: Solution for gel electrophoresis

Solution	Concentration	Solution	Concentration
Agarose gel	2.5%	TAE (Tris-acetate buffer EDTA)	97.5%

Table 28: Components in the lysis buffer for multiplex qRTPCR

Lysis buffer solutions	Concentration	Lysis buffer solutions	Concentration
Cells Direct 2× Reaction Mix	5.0 µl	TE buffer	1.3 µl

0.2× assay pool	2.5 µl	RT/Taq Superscript III	0.2 µl

Table 29: Medium for EBs formation

Components	Concentration	Components	Concentration
KO-DMEM	desired volume	MEM NEAA solution	1%
FBS	15%	L-glutamine	1%
Pen-Strep	1%	ß-mercaptoethanol	0.1%

Table 30: Medium for differentiation into neuronal progenitor cells

Components	Concentration	Components	Concentration
DMEM-F12	desired volume	NEAA	1%
FBS	10%, 5% and 3%	L-glutamine	1%
Pen-Strep	1%	FGF	10ng/ml
N2	1%	RA	3µM

Table 31: Medium for maturation and development of neuronal progenitor cells

Components	Concentration	Components	Concentration
Neurobasal medium	desired volume	NEAA	1%
FBS	10% and 5%	L-glutamine	1%
Pen-Strep	1%	B27	2%
B27	2%	N2	1%
RA	3µM		

Table 32: Endodermal and mesodermal differentiation medium

Components	Concentration	Components	Concentration
DMEM-F12	desired volume	NEAA	1%
FBS	10% and 5%	L-glutamine	1%
Pen-Strep	1%	FGF	10ng/ml
N2	1%		

Table 33: Intracellular Solution buffer for electrophysiology

Intracellular Solution **Jack Special (low chloride) pH=7.2 with KOH**	**Concentration**	**Intracellular Solution** **Jack Special (low chloride) pH=7.2 with KOH**	**Concentration**
K-Gluconate	135mM	Na-phosphocreatine	10mM
HEPES	10mM	KCl	4mM
Mg-ATP	4mM	Na-GTP	0.3mM
mOsm	295 mM		

Table 34: Extracellular Solution buffer for electrophysiology

Extracellular Solution **1x aCSF (artificial Cerebrospinal Fluid**	**Concentration**	**Extracellular Solution** **1x aCSF (artificial Cerebrospinal Fluid**	**Concentration**
NaCl	125mM	KCl	2.5 mM
$NaHCO_3$	25mM	NaH_2PO_4	1.25 mM
$CaCl_2$	2 mM	$MgCl_2$	1 mM
Glucose	25mM		

Abbreviation

µL	Microliter
µM	Micromolar
Mm	Milimolar
RPM	Revolutions per minute
h	Hour
min	Minute
Ca^{+2}	Calcium ion^{+2}
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DMEM	DMEM Dulbecco's Modified Eagle Medium
DNTPs	Deoxynucleotide
e.g	For example
et al	And other authors
$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGF2	Fibroblast Growth Factor-2
EGF	Epidermal growth factor
GDNF	Glial cell line-derived neurotrophic factor
STAT3	Signal transducer and activator of transcription 3
SMAD1	SMAD family member 1
BMP8-b	Bone morphogenetic protein 8b)
BMP2	Bone morphogenetic protein 2
Cadherins	Calcium-dependent adhesion
HOXB1	Homeobox B1
FGF8	Fibroblast growth factor 8
Dpc	Days post coitum
E	Embryonic day

EXE	Extraembryonic ectoderm
SSEA1	Stage specific embryonic antigen 1
H3K9me2	Histone H3 dimethyl Lys9 antibody
MAD2L2	Mitotic arrest deficient 2-like 2
PGCs	Primordial germ cells
SSCs	Spermatogonial stem cells
FACS	Fluorescence-activated cell sorting
MACS	Magnetic-activated cell sorting
MEFs	Mouse embryonic fibroblasts
TSCs	Testicular stroma cells
EM	Electron microscopic
RT-PCR	Reverse transcription polymerase chain reaction
BMP4	Bone morphogenetic protein 4
LIF	leukemia inhibitory factor
MEK	MAP kinase
GSK3	Glycogen synthase kinase 3
Tnap	Tissue non-specific alkaline phosphatase
SSEA1	Stage specific embryonic antigen 1
ECM	Extracellular markers
As	A-single
Apr	A-pair
Aal	A-Aligned
At	Transient
GFP	Green fluorescent protein
MSCs	Mesenchymal stem cell
MEM-a	Minimum Essential Media Alpha
GPR125	An orphan adhesion-Type G-protein-coupled receptor
STO	SIM mouse embryo-derived thioguanine and ouabain- resistant fibroblasts
TGF-B	Transforming growth factor–b superfamily
Camp	Cyclic adenosine monophosphate
GFRa1	GDNF family receptor alpha 1

SCF	Stem cell factor
IGF-1	Insulin-like growth factor-1
FSH	Follicle-stimulating hormone
SFK	Src family kinase
IPSCs	Induced pluripotent stem cells
PIPES	Piperazine-N,N′-bis (2-ethanesulfonic acid)
DAPI	4', 6-diamidino-2-phenylindole
IMH	Immunohistochemistry
TAE	Tris-acetate buffer EDTA
EGFP	Enhanced green fluorescent protein
EBs	Embryoid Body
RA	Retinoic Acid
NMRI	Naval Medical Research Institute
T-Test	Tukey's post-hoc tests
ES-like	Embryonic stem cell-like
MESCs	Mouse embryonic stem cells
HESCs	Human embryonic stem cells
C57BL/6	C57 black 6
H and E	Hematoxylin-Eosin
MGSCs	Mouse germline stem cells
Zfp145$^{-/-}$mice	Zfp145-null mice
Gli3	GLI-Kruppel family member 3
SPCs	Spermatogonial progenitor cells
PI3K	Phosphoinositide 3-kinase
Mvh	Mouse homozygotes for VASA homolog gene
SRY	Sex-determining region Y
FGFR	FGF receptor
Ifs	Intermediate filaments
MCAM	Melanoma cell adhesion molecule
ICM	Inner cell mass

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