Novel Markers for Prospective Isolation and Characterization of Stromal, Endothelial and Spermatogonial Progenitor Cells from Adult Human Testis

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Abbreviations

PGCs	Primordial germ cells
TNAP	Tissue non-specific alkaline phosphatase
SSCs	Spermatogonial stem cells
FGFR3	Fibroblast growth factor receptor 3
GFR-α1	GDNF family receptor 1
GPR125	G protein coupled receptor 125
SSEA-4	Stage specific embryonic antigen 4
SSEA-3	Stage specific embryonic antigen 3
ICSI	intra cytoplasmic sperm injection
SUSD2	Sushi domain containing 2
TSCs	Testicular stromal cells
TECs	Testicular endothelial cells
ITGA6	Integrin alpha 6
VLA6	Very late activation antigen 6

TSP180	Tumor surface protein 180
ITGA1	Integrin alpha 1
VLA1	Very late activation antigen 1
ST3GAL2	A 2,3-sialyltranferase
TRA 1-60	Podocalyxin like 1-60
TRA 1-81	Podocalyxin like 1-81
DNA	Deoxyribonucleic acid
5' UTR	5' untranslated region
3' UTR	3' untranslated region
CXCR4	Chemokine (C-X-C motif) receptor 4
CXCL12	Chemokine (C-X-C motif) ligand 12
SMB	Somatomedin B
AMOR	MUC4 and Other Proteins
vWF	von Willebrand factor
PC1	Proprotein convertase subtilisin/kexin type 1
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1
ENPP3	Ectonucleotide pyrophosphatase/phosphodiesterase 3
MUC4	Mucin 4, cell surface associated
CCP	Complement Control Proteins
SCR	Module or Short Consensus Repeats
mAbs	Monoclonal antibodies
PEG	Poly ethylene glycol
FBS	Fetal bovine serum
HAT	hypoxanthine-aminopterine-thymidine
HT	hypoxanthine-thymidine
PB	Peripheral blood
iPS122	Induced pluripotent stem cells 122
PE	Phycoerythrin
FITC	Florescein isothiocyanate
APC	Allophycocyanin
qRT-PCR	Quantitative reverse
STA	Specific target amplification

IFC	Integrated fluid circuit
RT-STA	Reverse transcriptase specific target amplification
TBS	Tris buffered saline
CFSC	Cold fish skin gelatin
DAPI	4',6-diamidino-2-phenylindole
FACS	Fluorescence-activated cell sorting
MACS	Magnetic activated cell sorting
HUVEC	Human vascular endothelial cells
VEGFR2	Vascular endothelial growth factor receptor 2
rhVEGF	recombinant Human vascular endothelial growth factor
rhBFGF	recombinant Human basic fibroblast growth factor
rhGDNF	recombinant Human glial cell derived neurotrophic factor
rhLIF	recombinant Human leukemia inhibitory factor
rhEGF	recombinant Human epidermal growth factor
rhIGF	Recombinant human insulin growth factor
MEM	Minimal essential medium
EDTA	Ethylenediaminetetraacetic acid
TE	Tris EDTA
DIO-AC-LDL	DIO labeled acetylated low density lipoprotein

Summary

The isolation of spermatogonia from adult human testis is hampered by the limited selectivity of available markers. In this study, I evaluated the suitability of combinations of known markers and novel targets for the prospective isolation of spermatogonia from adult human testis. Immunohistochemical studies, multicolor staining and cell sorting of testis samples followed by multiplex PCR and dendrogram cluster analysis revealed that spermatogonia were enriched at high purity in the $CD49f^{+}CD49a^{-}SSEA\text{-}4^{+}SUSD2^{+}$ population as well as in the $CD49f^{+}CD49a^{-}SSEA\text{-}4^{+/-}CD164^{+}$ subsets. In contrast to spermatogonia, testicular stromal cells and testicular endothelial cells were found to be highly enriched in the $CD49f^{+}CD49a^{+}CD144^{-}$ and in the $CD49f^{+}CD49a^{+}CD144^{+}$ subsets, respectively. The delineation was confirmed by the expression of specific stromal and endothelial key markers as well as by the differentiation capacity of each subset. In addition, I identified for the first time two distinct subsets of spermatogonia, distinguished by their $CD49f^{+}SSEA\text{-}4^{+}CD164^{+}$ and $CD49f^{+}SSEA\text{-}4^{-}CD164^{+}$ expression profiles. Multiplex PCR analysis revealed that cells of the $CD49f^{+}SSEA\text{-}4^{-}CD164^{+}$ subset express spermatogonia/germ cell specific markers, whereas the $CD49f^{+}SSEA\text{-}4^{+}CD164^{+}$ subset is enriched in undifferentiated spermatogonia specific markers. The knowledge about the composite phenotype of defined testicular cell subsets and the reliable isolation procedure may contribute to the precise characterization and efficient isolation of spermatogonia from small biopsies and may contribute to the treatment of infertility.

Zusammenfassung

Die Isolierung von Spermatogonien aus adulten menschlichen Hoden ist zur Zeit wegen der begrenzten Zahl und dem Mangel an selektiven Markern nur eingeschränkt möglich. In der vorliegenden Arbeit wurden Kombinationen bekannter Marker und neuer Targets, die für die Isolierung von Spermatogonien geeignet sind, untersucht. Mit Hilfe immunhistochemischer Analysen, Mehrfarbenfluoreszenzanalysen und Zellsortierung von Proben aus Hodengewebe sowie nachfolgenden Multiplex-PCR und Dendrogramm Clusteranalysen konnte gezeigt werden, dass Spermatogonien sowohl in der $CD49f^{+}CD49a^{-}SSEA\text{-}4^{+}SUSD2^{+}$ Population als auch im $CD49f^{+}CD49a^{-}SSEA\text{-}4^{+/-}CD164^{+}$ Subset hochrein isoliert werden können. Außer Spermatogonien konnten aus Hodengewebe auch Stromazellen in der $CD49f^{+}CD49a^{+}CD144^{-}$ Population und Endothelzellen im $CD49f^{+}CD49a^{+}CD144^{+}$ Subset isoliert werden. Die Unterscheidung von Endothelzellen und Stromazellen wurde über den Nachweis der spezifischen Expression von Schlüsselmarkern sowie durch die unterschiedliche Differenzierungkapazität der jeweiligen Zellpopulationen erzielt. Darüber hinaus konnten zum ersten Mal zwei unterschiedliche Populationen von Spermatogonien identifiziert werden, die sich durch ihre differenziellen Expressionsprofile unterscheiden. So konnte über Multiplex PCR-Analysen gezeigt werden, dass $CD49f^{+}SSEA\text{-}4^{-}CD164^{+}$ Zellen Keimzell-spezifische Marker exprimierten, die einem differenzierteren Stadium von Spermatogonien entsprechen, während Zellen im $CD49f^{+}SSEA\text{-}4^{+}CD164^{+}$ Subset Marker exprimierten, die typischerweise bei Spermatogonien im undifferenzierten Stadium vorkommen. Die Kenntnis des genauen Phänotyps von Spermatogonien sowie die hochreine Isolierung dieser seltenen Zellpopulation ist möglicherweise ein wichtiger Baustein bei der Behandlung von Unfruchtbarkeit.

List of our publications

Harichandan A, HJ Buhring. (2011). Prospective isolation of human MSC. Best Pract Res Clin Haematol 24:25-36.

Harichandan A, K Sivasubramaniyan and HJ Buhring. (2013). Prospective Isolation and Characterization of Human Bone Marrow-Derived MSCs. Adv Biochem Eng Biotechnol 129:1-17.

Sivasubramaniyan K, D Lehnen, R Ghazanfari, M Sobiesiak, **A Harichandan**, E Mortha, N Petkova, S Grimm, F Cerabona, P de Zwart, H Abele, WK Aicher, C Faul, L Kanz and HJ Buhring. (2012). Phenotypic and functional heterogeneity of human bone marrow- and amnion-derived MSC subsets. Ann N Y Acad Sci 1266:94-106.

Sivasubramaniyan K, **A Harichandan**, S Schumann, M Sobiesiak, C Lengerke, A Maurer, H Kalbacher and HJ Buhring. (2013). Prospective isolation of mesenchymal stem cells from human bone marrow using novel antibodies directed against Sushi domain containing 2 (SUSD2). Stem Cells Dev

Harichandan A, K Sivasubramaniyan, J Hennenlotter, C Schwentner, A Stenzl, HJ Bühring. (2013). Isolation of adult human spermatogonial progenitors using novel markers. J Mol Cell Biol. (**Accepted**)

1. Introduction

All sexually reproducing organisms arise from fusion of male and female gametes during fertilization. Fertilization results in a new organism of their kind in the form of embryo with a unique combination of genes contributed by both male and female gametes. The embryo develops into multicellular eukaryote, which fundamentally consists of two types of cells, somatic and germ cells. In contrast to the mitotic division of somatic cells, the germ cells also have the capacity to undergo meiosis, essential for cell fate/morphogenesis of the germ cells. This process helps in the development of the gametes. Germ cells are very important for continuation of the species. In case of sexually reproducing organisms only these cells have the capacity to transmit genetic information from one generation to another. Therefore play a significant role in evolution of the species. Hence these cells are sometimes considered to be immortal as they form the link between the generations.

1.1. Germ cells

Germ cells can give rise to a gamete (male/female) in sexually reproducing organism. After fertilization the resulting zygote undergoes several cell divisions and forms the blastula at the 7^{th} cleavage. This stage marks the beginning of gastrulation, during which the single layered cells of the blastula are reorganized into a trilaminar structure known as the gastrula. The gastrula consists of three germ layers known as the ectoderm, mesoderm and endoderm. The germ line development starts from the ectoderm primed epiblast cells of the embryo called primordial germ cells (PGCs). These PGCs are highly positive for tissue nonspecific alkaline phosphatase (TNAP) [1]. The PGCs are embedded in the endoderm which progresses to form the hind gut. These cells migrate behind the hind gut to reach the gonadal ridge and undergo mitotic arrest. At this stage these cells are called gonocytes. These gonocytes are reported to be highly positive for CD117, a receptor for stem cell factor secreted by the neighbouring

cells during its migration to the seminiferous tubules. Shortly after birth, gonocytes differentiate into Type A dark spermatogonia which represents the initial stage of spermatogenesis [2].

Spermatogenesis is a complex process which begins at puberty in humans, 10-13 years after birth [3]. The development of sperm can be divided into three phases: mitotic division of spermatogonia, meiosis of spermatocytes, and spermiogenesis resulting in production of sperm [4,5]. At puberty, type A_{dark} spermatogonia undergo mitotic division and mature to produce type A_{pale} spermatogonia in addition to type A_{dark} cells. Type A_{pale} spermatogonia further replicate by mitosis to form clusters of daughter cells linked to each other by cytoplasmic bridges. While type A_{dark} cells are considered as reserve stem cells, type A_{pale} cells are renewing stem cells and collectively termed spermatogonial stem cells. Type A_{pale} spermatogonia matures into type B spermatogonia, which divides mitotically to produce further type B cells. These cells then mature in a cluster to produce primary spermatocytes (Fig. 1).

1.2. Spermatogonial stem cells (SSCs)

SSCs are undifferentiated germ cells residing in the seminiferous tubule in human adult testis. Several groups have reported multi- and pluripotent differentiation capacity of spermatogonia based on its ability to form teratoma [6-10]. In other studies, pluripotent differentiation capacity of PGCs of the embryo has been reported [10-14]. It was demonstrated that PGCs have the capacity to give rise to cells with a similar differentiation capacity as embryonic stem cells [11,15].

Human SSCs are reported to express the surface markers CD49f, FGFR-3, GFR-a1, GPR-125, SSEA-4 and CD90 [6,16-20]. Of these markers CD49f, CD90 and SSEA-4 were also introduced as selection tools to isolate human SSCs [6,17,18], whereas GFR-a1 and GPR-125

have been proved to be suitable for the prospective isolation of SSC subsets [19,21,22]. However, most of these markers including CD49f and CD90 are not highly specific for SSCs [18] and the number of reports, in which SSEA-4 is used as a selective target for SSC isolation, is limited [17,18].

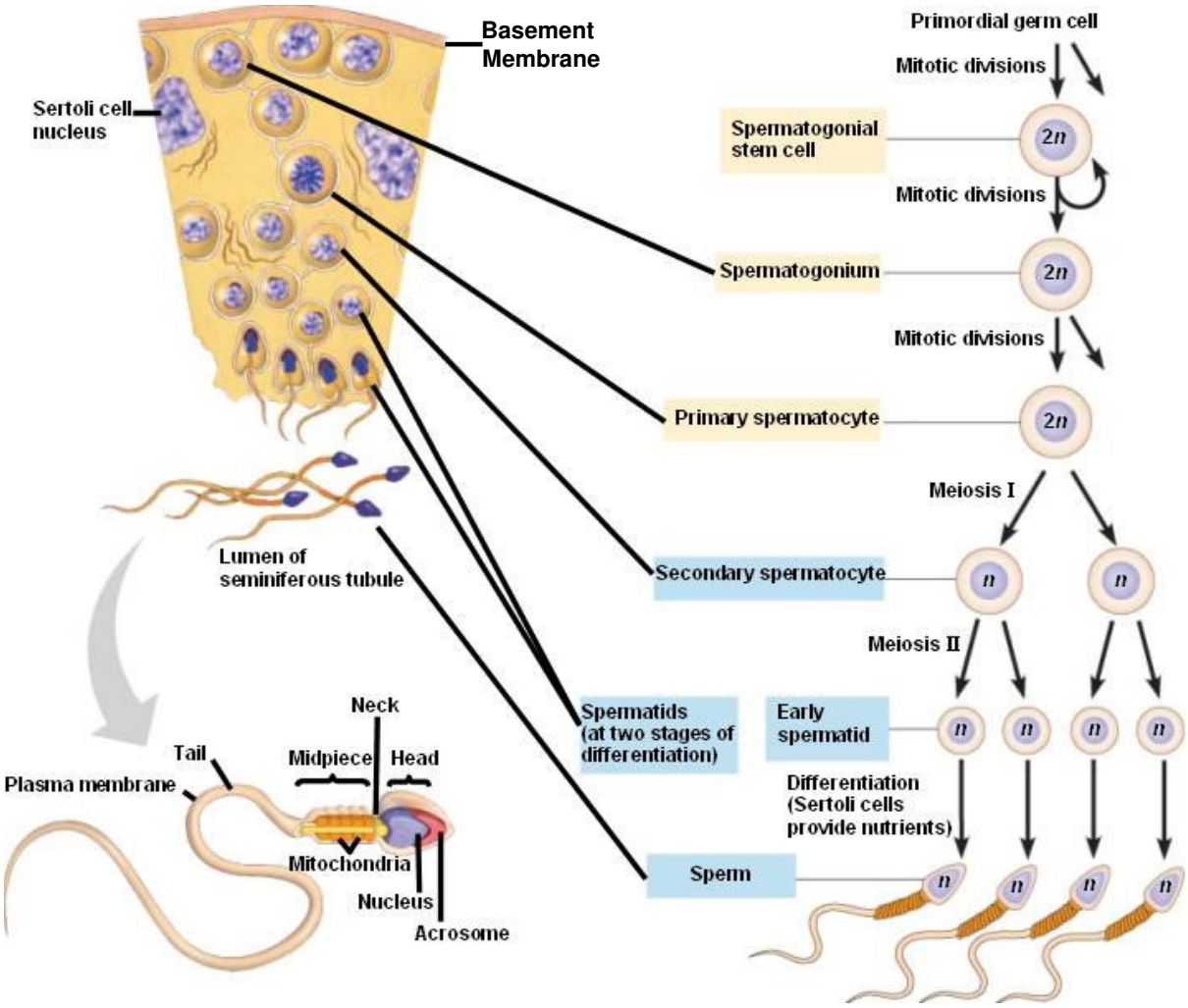


Fig. 1: Schematic representation of spermatogenesis. The SSCs near the basement membrane divide mitotically to maintain their population in the testis. Some SSCs mature into primary spermatocytes. Each primary spermatocyte undergoes meiosis. Meiosis I yields 2 haploid secondary spermatocytes and meiosis II yields 4 equal sized spermatids. The spermatids migrate towards the lumen (central opening). Sertoli cells play an important role in spermatogenesis and produce factors required for maintenance of the SSCs and also for their differentiation. They also supply nutrients for the spermatids which mature into motile sperm in the epididymis. *(Courtesy Biology 1152 Principles of biological sciences)*

A drawback for the isolation and characterization of human SSCs is the limited availability of specimen of normal human testis. The isolation of SSCs is additionally hampered by the low frequency of these cells (approximately 0.02 %-0.03 %).

1.3. Scope of Spermatogonial stem cells in therapy

Different therapies for problem of infertility have been considered, such as testicular transplantation of spermatogonia, tissue grafting, or in vitro culture of tissue fragments, in order to produce sperm for intra cytoplasmic sperm injection (ICSI) [23,24]. Testicular transplantation of spermatogonia restores functional spermatogenesis in mouse models [25]. Regeneration of spermatogenesis after spermatogonia transplantation has been successfully reproduced in other mammalian and primate species [23,26]. Following these studies, the potential application of spermatogonia in regenerative medicine gained importance. In recent studies it has been reported that apart from spermatogonia, sertoli cells, leydig cells, other cell types including myoid/stromal cells and endothelial cells are adhered to the basement membrane (Fig. 2).

In this study, I used a large panel of antibodies with known and unknown specificity to identify more robust markers for the isolation of spermatogonia/spermatogonial progenitors and to characterize additional cell subsets in human testis. The markers identified to be differentially expressed in different cell types of the human testicular tissue that were suitable to isolate these cell types are listed below.

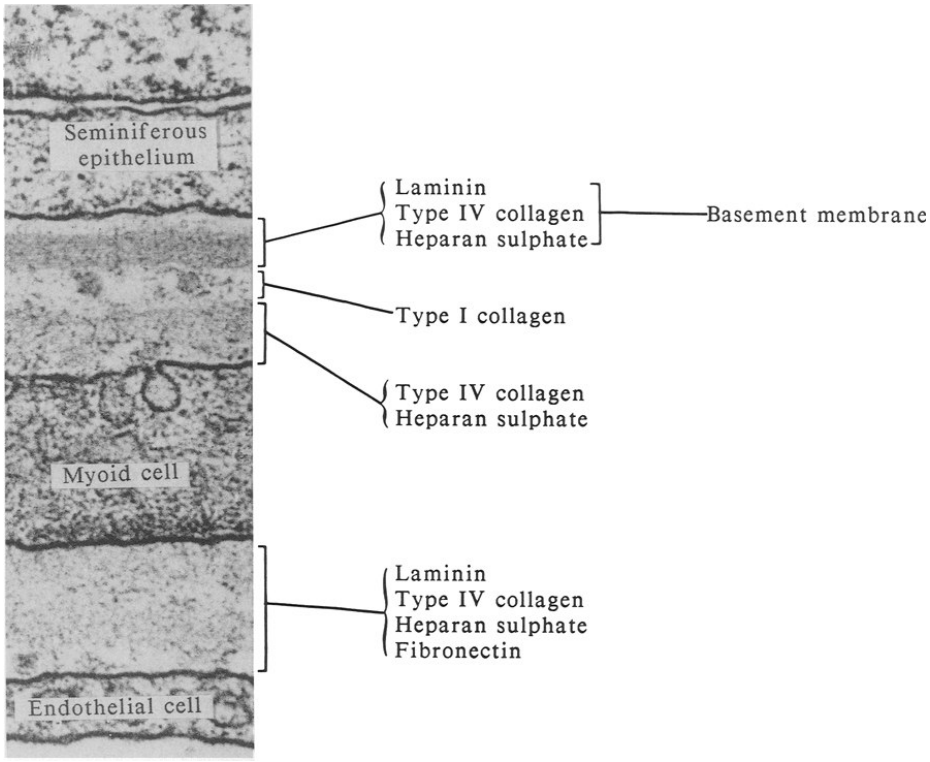


Fig. 2: Schematic representation of distribution of extracellular matrix in testis [27]. Photographic montage of the testicular lamina propria summarizing the distribution of extracellular matrix molecules. The components contained in the different layers have been determined by either indirect immunofluorescent microscopy or by pre embedding peroxidase immunocytochemistry.

1.4. Integrin Alpha 6

Integrin alpha 6 belongs to integrin alpha chain family and is also known as CD49f. It is coded by ITGA6 gene located in 2q31.1 in the human genome. It is a single pass Type I integrin consisting of 7 FG-GAP repeats [UniProtKB; P23229]. It exists in two isoforms. It is predominantly expressed by the epithelial cells and is widely distributed in many tissues. Like all integrins it is a cell surface protein and interacts with a beta chain. It interacts with beta 1 integrin in case of endothelial cells to form TSP 180 or with beta 4 in case of epithelial cells to form VLA-6. It mediates cell adhesion by interaction with extracellular matrix. For instance, TSP 180 acts as a laminin receptor in endothelial cells and VLA-6 acts as a laminin receptor for epithelial cells. It also helps in interaction with other cells by playing a critical role in hemidesmosomes. It also interacts with the cytoskeleton and various signaling molecules in the cell surface mediated cell signaling [UniProtKB; P23229].

CD49f is a stem cell marker present on human embryonic stem cells and induced pluripotent stem cells. It is also reported to play an important role in embryonic development like assisting adhesion of stem cells to laminin for retinal neuron development [28]. It is also reported to be one of the key markers for characterizing and isolating spermatogonial stem cells both from human and mouse [6,29].

1.5. Integrin alpha 1

Integrin alpha 1 belongs to integrin alpha chain family and is also known as CD49a and is coded by ITGA1 gene located in 5q11.2 in the human genome. It is a single pass Type I integrin with 7 FG-GAP repeats and one von Willebrand factor type A domain which is considered to interact with collagen IV [UniProtKB; P56199]. It is reported to be a marker for prospective isolation of mesenchymal stem cells from bone marrow [30]. Like all integrins it is a cell surface protein and interacts with a beta chain. For instance, it interacts with integrin beta 1 to form a dimeric receptor VLA-1. VLA-1 acts as a receptor for both laminin and collagen IV. Similar to CD49f it also interacts with cytoskeleton and various signaling molecules in the cell surface mediated cell signaling.

1.6. Vascular endothelial cadherin

It is also known as Cadherin 5, Type 2 or CD144. It is a cadherin and its function is dependent on Ca^{+2} ions. It is encoded by CDH5 gene located in 16q21.1 in the human genome. It is a single pass Type 1 membrane glycoprotein with five extracellular cadherin domains, single transmembrane region and a highly conserved cytoplasmic tail [UniProtKB; P33151]. It is reported to be a potential marker for prospective isolation of endothelial progenitor cells [31]. Its helps in cell-cell adhesion by homophilic intercellular interaction and plays a critical role in organization of the intercellular junctions and vascular development [32]. It also interacts with alpha catenin in the cytoskeleton

1.7. Stage specific embryonic antigen -4 (SSEA-4)

SSEA-4 is a ganglioside, a molecule composed of a glycosphingolipid with one or more sialic acids linked on the sugar chain (Fig. 3). It is distinguished from globoside by the presence of sialic acid (*N*-Acetylneuraminic acid). It is also known as Monosialosyl globopentaosyl ceramide. It is synthesized from SSEA-3 by the enzyme ST3GAL2 [33]. It undergoes qualitative and quantitative changes during development, differentiation and tumorogenesis [12]. It is reported to play a role in adhesion of cells to the laminin [34].

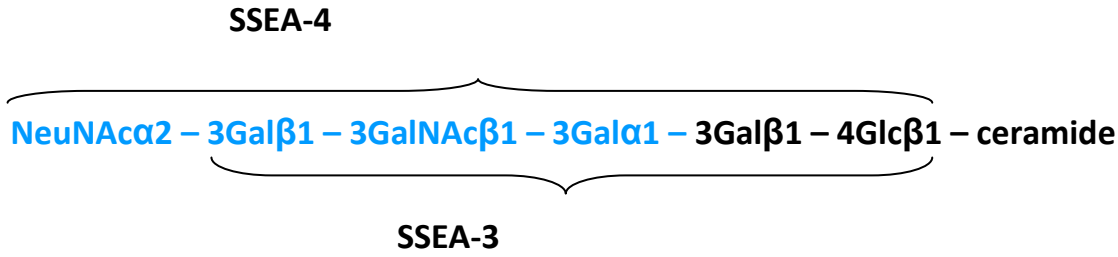


Fig. 3: Structure of SSEA-3 and SSEA-4 [35].

Along with SSEA-3, Tra 1-60, Tra 1-81, SSEA-4 is used routinely to characterize embryonic stem cells [13,35]. It is a stem cell marker. It is also reported as a marker for prospective isolation of spermatogonial stem cells from human adult testis [18].

1.8. Sialomucin core protein 24

This protein is also known as endolyn or CD164. It belongs to a family of mucins or sialomucin glycoproteins. It consists of two mucin domains interrupted by a cysteine rich subdomain [36,37]. The CD164 gene located on human chromosome 6q21 is represented by a 17kb long genomic DNA comprising of 6 exons and 5 introns with splice donor and acceptor sequence. The sequenced 5'UTR, signal peptide and first mucin-like domain are encoded by exon 1, the cysteine rich non-mucin domain by exons 2 and 3, and the second mucin-like domain by exon 4 and part of exon 6. The transmembrane and cytoplasmic

domains, and 3'UTR are all encoded by exon 6 [38]. The 3 isoforms of CD164 are depicted in Fig. 4.

CD164 is reported to take part in two key but opposing roles (adhesive or anti adhesive functions) and glycosylation pattern plays an important role in deciding these functions [39]. Glycosylation provides an external scaffold for adhesion to cells in the microenvironment and extracellular matrix especially to carbohydrate binding proteins like selectins and lectins. It also associates in formation of complex with CXCR4 which helps the cells to interact with CXCL12, thus playing an important role in migration and differentiation [40]. There are many antibodies available that recognize CD164 but antibody produced by clone 67D2 recognizes an epitope which depends on the conformational integrity of the antigen [41]. CD164 plays an important role in regulating the adhesion of $CD34^+$ hematopoietic precursor subset to stroma and acts as a negative regulator of hematopoiesis [36].

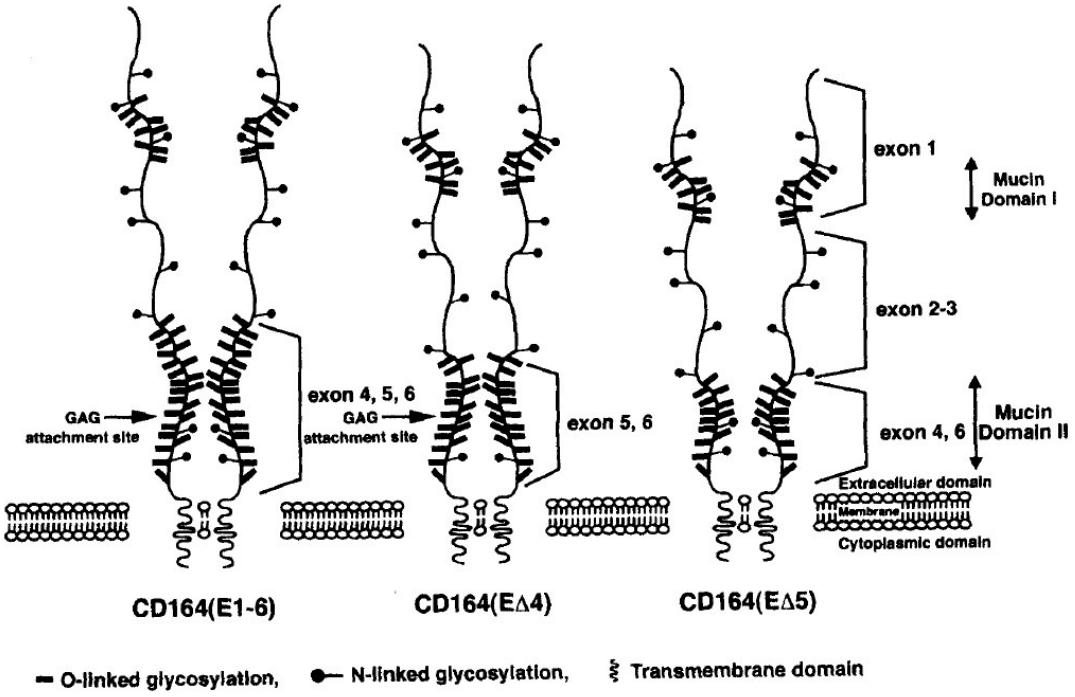


Fig. 4: Schematic representation of the three human CD164 splice variants based on biochemical and nucleotide data [38]. The salient features of these isoforms include (i) the numerous potential sites for o-linked glycan attachment (32 in CD164 (E1-6)and 21 in CD164 (EΔ5, (ii) the variable number of possible N-linked glycosylation sites (9 in CD164 (E1-6 and CD164(EΔ5) or 8 in CD164 (EΔ4) and (iii) the domains encoded by different exons including the two mucin

domains, I and II. A potential GAG attachment site occurs in the CD164 (E1-6) and (EΔ4) splice variants.

1.9. Sushi domain containing 2 (SUSD2)

Sushi domain containing 2 (SUSD2) is a type I transmembrane protein of 820 amino acids consisting of a large extracellular region containing a Somatomedin B (SMB), an Adhesion associated domain in MUC4 and Other Proteins (AMOP), a Von Willebrand factor (vWF), and a Sushi domain (Fig. 5). SMB is a small cysteine-rich serum factor of unknown function, proteolytically cleaved from the N terminus of the cell-substrate adhesion protein vitronectin [42]. Cysteine-rich SMB like domains are found in a number of extracellular proteins including membrane glycoprotein PC1, ENPP-1, ENPP-3 and placental protein 11 [42,43]. AMOP domains are considered to be involved in cell adhesion and represent an important component of MUC4, the most related protein to SUSD2 [44,45]. Similar to SMB domains, AMOP domains contain a high number of cysteine residues. It is supposed that these residues play a role in homodimerization by forming disulphide bridges between adjacent molecules. The vWF domain is found in a variety of plasma proteins, integrins and collagens [46,47]. Proteins containing vWF domains participate in numerous biological events including cell adhesion, migration, homing and signal transduction, and are involved in interactions with a large array of ligands [47]. Sushi domains, also known as Complement Control Proteins (CCP) module or Short Consensus Repeats (SCR), are components of a variety of complement and adhesion proteins [48]. The function of the short cytoplasmic region of SUSD2 is not known. In a recent report, SUSD2 was described to be differentially expressed in skeletal muscle during the development of obesity {Bolton, 2009 131 /id} and to inhibit cellular growth and to reverse tumorigenic phenotypes of cancer cells *in vitro* [49,50]. *The* biological role and molecular mechanism of action of SUSD2 in tumor cells was investigated by analyzing the effect of SUSD2 overexpression in human HT1080

fibrosarcoma and in HeLa cervical carcinoma cells, which resulted in reduced or abrogated tumorigenic features such as anchorage-independent growth, migratory and invasive activity of the cells [49]. Very recently, the MSC marker W5C5 was introduced as a marker for the prospective isolation of highly clonogenic endometrial MSCs, which were able to reconstitute endometrial stromal tissues *in vivo* [51,52]. I identified SUSD2 as the cognate antigen of antibodies W5C5 as well as of W3D5 and introduced this molecule as a novel stand alone marker to isolate human BM-MSCs [53]. In this study, I introduce SUSD2 and CD164 as novel markers to prospectively isolate spermatogonia. In addition, I provide a staining protocol to simultaneously isolate spermatogonia, testicular stromal cells (TSCs) and testicular endothelial cells (TECs) in a single sorting step.

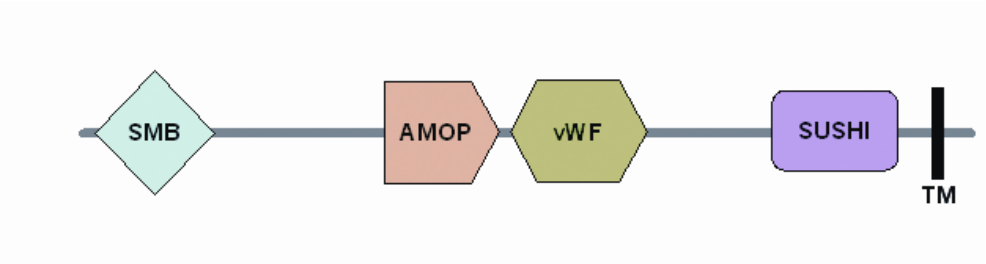


Fig. 5: Domain architecture of the SUSD2 protein [53]. SUSD2 consists of a type I single spanning TM region separating a short cytoplasmic tail with unknown function from a large extracellular region containing SMB, vWF, AMOP, and Sushi domains. SUSD2- Sushi domain containing 2; SMB- Somatomedin B; AMOP- adhesion associated domain in MUC4 and other proteins; vWF- von Willebrand factor, TM- Transmembrane.

2. Aim of the work

There have been numerous studies to identify and isolate spermatogonia/ SSCs which led to the finding that SSCs express CD9, CD90, CD49f, SSEA4, GFRα1 and GPR125. Most of these markers are identified in mice and primates. However the identity of SSCs from adult human testis is poorly understood. Though there are studies indicating CD49f, CD90, SSEA-4, GFRα1 and GPR125 as potential markers for isolation of human SSCs, CD49f and CD90 are not specific and there are only limited reports on SSEA-4, GFRα1 and GPR125 as potential markers for isolating spermatogonia/ spermatogonial progenitors from human adult testis. Further, GFRα1 and GPR125 are expressed only on a subpopulation of SSCs.

In this study, I aim to use a large panel of antibodies with known and unknown specificities to identify more robust markers for the prospective isolation and characterization of human spermatogonia/ spermatogonial progenitors and their subsets. I also aim to isolate and characterize additional cell types in human testis including testicular stromal cells (TSCs) and testicular endothelial cells (TECs).

3. Materials

Table 1. Commercial antibodies

Mouse Anti Human CD9-FITC	BD Biosciences
Mouse Anti Human CD10-FITC	BD Biosciences
Mouse Anti Human CD13-PE	Becton Dickinson Immunocytometry systems
Mouse Anti Human CD15-FITC	BD Biosciences
Mouse Anti Human CD26-PE	Becton Dickinson Immunocytometry systems
Mouse Anti Human CD29-PE	Biolegend
Mouse Anti Human CD34-PE	BD Biosciences
Mouse Anti Human CD44-FITC	BD Biosciences
Mouse Anti Human CD45-PE	BD Biosciences
Mouse Anti Human CD47-PE	BD Biosciences
Mouse Anti Human CD49a-PE	BD Biosciences
Mouse Anti Human CD49b-PE	BD Biosciences
Mouse Anti Human CD49e-PE	BD Biosciences
Mouse Anti Human CD49f-FITC	BD Biosciences
Mouse Anti Human CD51-FITC	BD Biosciences
Mouse Anti Human CD56-FITC	BD Biosciences
Mouse Anti Human CD71-FITC	BD Biosciences
Mouse Anti Human CD73-PE	BD Biosciences
Mouse Anti Human CD90-APC	BD Biosciences
Mouse Anti Human CD105-PE	eBioscience
Mouse Anti Human CD133-PE	Miltenyi Biotec
Mouse Anti Human CD138-FITC	BD Biosciences
Mouse Anti Human CD140b-PE	BD Biosciences
Mouse Anti Human CD144-PE	BD Biosciences
Mouse Anti Human CD146-PE	BD Biosciences
Mouse Anti Human CD151-FITC	BD Biosciences
Mouse Anti Human CD166-PE	BD Biosciences
Mouse Anti Human CD167a-FITC	BD Biosciences
Mouse Anti Human CD200-PE	BD Biosciences
Mouse Anti Human CD271-APC	Miltenyi biotec

Mouse Anti Human CD340-PE	BD Biosciences
Mouse Anti Human TNAP-PE	Miltenyi Biotec
Mouse Anti Human SSEA-3-PE	BD Biosciences
Mouse Anti Human SSEA-3-FITC	BD Biosciences
Polyclonal Goat Anti Mouse Immunoglobulin-PE	DAKO
Polyglobulin (Gamunex 10 %)	Talecris Biotherapeutics
Purified Mouse Anti Human CD49a	BioLegend
Purified Rat Anti Human CD49f	BD Biosciences
Purified Mouse Anti Human CD144	BioLegend
Purified Mouse Anti Human CD164	BioLegend
Purified Rabbit Anti Human DDX4	Abcam
Purified Rabbit Anti Human DAZL	Sigma Aldrich
Alexa Fluor 488 Goat Anti Mouse IgG2a	Life Technologies
Alexa Fluor 488 Goat Anti Mouse IgG1	Life Technologies
Alexa Fluor 488 Goat Anti Mouse IgG2b	Life Technologies
Alexa Fluor 488 Goat Anti Mouse IgM	Life Technologies
Alexa Fluor 488 Goat Anti Mouse IgG	Life Technologies
Alexa Fluor 488 Goat Anti Rabbit IgG	Life Technologies
Alexa Fluor 555 Goat Anti Mouse IgM	Life Technologies
Cy3 Goat Anti Mouse IgG	Life Technologies
Cy3 Goat Anti Rabbit IgG	Life Technologies

Table 2. In-house antibodies with unknown specificities

2E4B4	HEK-6D6	W3C3
6D3H7	HEK-8C6	W3D2
7C5G1	HEK1-2C1	W4A5
9A3G2	HEK4-1A1	W5C4
56A1C2	HEK4-2C6	W5D3
56D2H5	HEK4-2D6	W8C3
56D5A1	HEK5-2B5	IPS-K-1D4C6

57D2B3	HEK5-3D5	IPS-K-3B1A9
58A2D10	HEK5-4A2	IPS-K-3C1D1
59A3B3	HEK5-4C1	IPS-K-3C2D5
67H4D5	HEK7-1C4	IPS-K-3C4A6
BV2A5B6	HEK7-4D1	IPS-K-3C5G8
BV8C2C2	HEK9-2B5	IPS-K-4A2A9
CH1A4D1	HEK9-3C2	IPS-K-4B5G6
CH2A3B5	HEP-B3	T-2B4
CH4D3	HEP-C20	T-3C3
HEK-3D3	W1C3	T-3D4
HEK-3D6	W1D6	T-4C2

Table 3. In-house antibodies with known specificities

Antibody	**Antigen**	**Reference**
1G2	CD105	[54,55]
1B4C3	HER3	
4FR6	CD344	[56]
4G8B3	CD135	[57]
9C4C3	CD326	[58]
16A1	CD140a	
24D2	CD340	[59,60]
28D4	CD140b	[55,59,60]
39D5	CD56	[55,59,60]
43A1	CD34	[61]
48B3	CD167a	[55]
67A4	CD324	[58]
67D2	CD164	[55,59,60]
97A6	CD203C	[62]
97C5	CD10	[54,59]
104D2	CD117	[55]
CH3A4	CD344	[63]
CUB2	CD318	[59,64,65]

W3C4	CD349	[59,66]
W5C5, W3D5	SUSD2	[53]
W6B3	CD133	[55,59,66]
W6D3	CD15	[55]
W7C5	CD109	[67]
W8B2	TNAP	[54,55,59,60]
IPS-K-4A2B8	SSEA-4	[68]
IPS-K-4A4F2	TNAP	[68]
58B1A2, T-1A5, T-4D5, IPS-K-2B6A8, HEK5-1B3	CD276	[manuscript in preparation]

Table 4. List of chemicals and Reagents

Ammonium Chloride	Stem cell technologies
Gelatin	Sigma Aldrich
D^{+}-Glucose	Sigma Aldrich
Ascorbic acid	Sigma Aldrich
DL-Lactic acid	Sigma Aldrich
Sodium azide	Sigma Aldrich
Matrigel	BD Biosciences
Methanol	Prolabo
Para formaldehyde	Sigma Aldrich
Alizarin red S	AppliChem
Oil red O	Sigma Aldrich
Tissue tek OCT	Sakura
Alcian blue	Applichem
Acetic acid	BDH Prolabo
Sucrose	Sigma Aldrich
Triton X 100	AppliChem
Cold fish skin gelatine	Sigma Aldrich
Tween 20	Sigma Aldrich
4’6’-diamidino 2 phenylindole	Sigma Aldrich

Vectaschield hard set mounting medium with DAPI	Vector Laboratories
Sodium chloride	Sigma Aldrich
polyethylene glycol	Sigma Aldrich
hypoxanthine-aminopterine-thymidine	Sigma Aldrich
hypoxanthine-thymidine	Sigma Aldrich
L-Proline	Sigma Aldrich
Dexamethasone	Sigma Aldrich

Table 5. List of basal media

DMEM high glucose	PAA
RPMI 1640	PAA
DMEM-F12 + Glutamax	Gibco
VascuLife basal medium	Lifeline cell technology
Knock out DMEM	Gibco

Table 6. List of ready to use media

NH OsteoDiff	Miltenyi Biotec
NH AdipoDiff	Miltenyi Biotec
Recovery cell culture freezing medium	Invitrogen

Table 7. List of media supplements

Penicillin- Streptomycin	PAA
L-Glutamine	PAA
Non essential amino acids	PAA
β-Mercaptoethanol	Sigma Aldrich
StemPro hESC supplement	Invitrogen
N2 supplement	PAA
Sodium Pyruvate	PAA
MEM vitamins	Sigma Aldrich
Estradiol	Sigma Aldrich

Progesterone	Sigma Aldrich
Monothioglycerol	Sigma Aldrich
MEM amino acids	PAA
ITS supplement	PAA
L-Proline	Sigma Aldrich
Dexamethasone	Sigma Aldrich
Hydrocortisone hemisuccinate	Lifeline cell technology
Heparin sulphate	Lifeline cell technology

Table 8. List of buffers

HANKS BSS with Ca^{2+} and Mg^{2+}	PAA
Phosphate buffered saline without Ca^{2+} and Mg^{2+}	Lonza
Cell direct 2X	Invitrogen
Tris base	Sigma Aldrich
SuperScript ^{TM} III	Invitrogen
RT/ Platinum Taq	Invitrogen
DNA suspension buffer	Teknova

Table 9. List of Sera

Fetal bovine serum	PAA
Goat serum	PAA
Bovine serum albumin	Sigma Aldrich
Human serum albumin	Baxter
Knock Out serum replacement	Gibco

Table 10. List of enzymes

DNAse II	Sigma Aldrich
Dispase II	Roche
Collagenase XI	Sigma Aldrich

Table 11. List of growth factors

Recombinant human basic fibroblast growth factor (rh FGF basic)	PeproTech
Recombinant human glial cell derived neurotrophic factor (rh GDNF)	R&D systems
Recombinant human recombinant leukaemia inhibitory factor (rh LIF)	Sigma Aldrich
Recombinant human epidermal growth factor (rh EGF)	PeproTech
Transforming growth factor beta- 3(TGF-β3)	Sigma Aldrich
Human recombinant insulin like growth factor (rh IGF-1)	Lifeline cell technology

4. Media and stocks solutions

4.1. Complete media: (500 ml)

RPMI 1640 with L-Glutamine : 427.5 ml

Fetal Bovine serum : 50 ml

L-glutamine (200mM) : 5 ml

Penicillin/streptomycin (100x) : 5 ml

MEM amino acids (50x) : 5 ml

Sodium Pyruvate solution 100mM : 5 ml

1-Thioglycerol : 2.5 ml (from stock)

1-Thioglycerol Stock solution – 500 µl in 50 ml RPMI 1640

4.2. Embryonic Stem cell (ES) media

Knock Out DMEM : 400 ml

Knock Out Serum Replacement : 100 ml

L-glutamine (200mM) : 5 ml

Non-essential amino acids (100x) : 2.5 ml

Penicillin-streptomycin (100x)	: 2.5 ml
Human basic fibroblast growth factor	: 5 ng/ml

4.3. Endothelial cell culturing media

VascuLife basal Medium	: 475 ml
rh EGF	: 5 ng/ml
rh FGF basic	: 5 ng/ml
rh IGF-1	: 15 ng/ml
Ascorbic acid	: 50 µg/ml
Hydrocortisone hemisuccinate	: 1 µg/ml
Heparin sulphate	: 0.75 U/ml
L- Glutamine (200mM)	: 10 mM
Penicillin-streptomycin (100X)	: 1 %
Fetal bovine serum	: 2 %

4.4. Human germ cell media: (250 ml)

DMEM/F12 + GlutaMAX-I	: 230 ml
Stem pro hESC supplement	: 5 ml
KnockOut serum replacement	: 2.5 ml
L-Glutamine (200mM)	: 2.5 ml
Penicillin-streptomycin (100X)	: 2.5 ml
MEM Vitamins (100X)	:2.5 ml
N2 supplement (100X)	: 2.5 ml
Non essential amino acids (100X)	: 2.5 ml

β-Mercaptoethanol (14.13M)	: 1.77 µl
D+ Glucose	: 6 mg/ml
Human bovine serum	: 5 µg/ml
Estradiol	: 30 ng/ml
Progesterone	: 60 ng/ml
rh EGF	: 20 ng/ml
rh FGF basic	: 10 ng/ml
rh GDNF	: 8 ng/ml
rh LIF	: 1000 U/ml
Ascorbic acid	: 100 µg/ml
Pyruvic acid	: 30 µg/ml
DL-Lactic acid	: 1 µl/ml

4.5. Incomplete Chondrogenic Induction media: (ICIM)

DMEM High glucose	: 486 ml
L-Glutamine (200mM)	: 10 ml
Sodium Pyruvate (100mM)	: 0.5 ml
IST supplement (100x)	: 5 ml
Penicillin-Streptomycin (100x)	: 5 ml
L-Ascorbic Acid	: 1.75 ml (from stock)
L- Proline	: 1.75 ml (from stock)
Dexamethasone	: 50 µl (from stock)

Stocks:

L-Ascorbic acid	: 50 mmM

L-Proline : 100 mM

Dexamethasone : 1 mM

4.6. Complete Chondrogenic Induction Media: (CCIM)

ICIM : 500 ml

TGF-β3 : 10 ng/ml

Stock:

TGF-β3 stock concentration: 2 µg/ml prepared in 4mM HCL with 1 mg/ml Bovine serum albumin.

4.7. Freezing media

Fetal bovine serum : 90 %

Dimethyl Sulfoxide : 10 %

5. Buffers and stock solutions

5.1. Cell lysis buffer

CellsDirect 2X reaction mix : 5 µl

SuperScript TM III RT/Platinum Taq Mix : 0.2 µl

DNA suspension buffer (TE) : 1.3 µl

5.2. Reverse transcriptase specific target amplification: (reaction mix)

Cell lysate : 6.5 µl

0.2X primer /Probe Mix : 2.5 µl

5.3. 10X Tris buffered saline (TBS): (P^H 7.4) 1 Liter:

Trizma base	: 24.2 g
Sodium chloride	: 80 g
Concentrated HCl	: 17.7 ml

TBS is diluted to 1X by adding 100 ml of 10X TBS to 900 ml distilled water

5.4. Blocking buffer

TBS	: 89.7 %
Normal Goat serum	: 10 %
Triton X 100	: 0.1 %
Sodium azide	: 0.1 %
Cold fish skin gelatine	: 0.1 %

5.5. FACS Buffer

Phosphate buffer saline (without Ca and Mg)	: 500 ml
10 % Bovine serum albumin	: 5 ml
0.1 % Sodium azide	: 2.5 ml

5.6. MACS Buffer

Phosphate buffer saline (without Ca and Mg)	: 500 ml
0.5 M EDTA	: 2 ml
Bovine serum albumin	: 2.5 g

6. Table 12-Consumables and Laboratory equipments

15, 50 ml centrifugal tubes	Greiner Bio-One
Disposable pipettes 1,5,10,25,50 ml	Corning Incorporated
T25, T75, T175 Cell culture flasks	Greiner Bio-One
96, 48, 24, 12, well cell culture plates	Corning Incorporated
6 well cell culture plates	Becton Dickinson Lab ware
96 well U and V bottom micro titer plates	Greiner Bio-One
5 ml Polystyrene round bottom tubes	Greiner Bio-One
48.48 Dynamic Array™ IFC Chip	Fluidigm
40 µm and 100 µm cell strainer	BD Falcon
Nunc cryo tube vials	Thermo Scientific
2x TaqMan universal PCR master mix	Applied Biosystems
GE sample loading reagent	Fluidigm
GE assay loading reagent	Fluidigm

Macilwain Tissue chopper	The Mickle laboratory equipment Co. LTD.
Zeiss observer Z1 AXIO microscope	Carl Zeiss
FACS Canto II Flow cytometer	BD Biosciences
FACS ARIA II Flow cytometer	BD Biosciences
Universal 320R centrifuge	Hettich
VERA safe Biosafety hood	Thermo scientific
Axivert 135 microscope	Carl Zeiss
BioMark™ HD system	Fluidigm

7. Table 13. List of 20x TaqMan gene expression assays for multiplex qRT-PCR (Applied Biosystems)

Gene	**Gene name**	**Species**	**Art.Nr.**
TERT	Telomerase reverse transcriptase	Human	**Hs00972656_m1**
NANOG	Homeobox protein NANOG	Human	**Hs02387400_g1**
POU5F1	OCT4a	Human	**Hs03005111_g1**
LIFR	Leukemia inhibitory factor receptor	Human	**Hs01123581_m1**
SOX2	sex determining region Y-box 2	Human	**Hs01053049_s1**
DDX4	VASA	Human	**Hs00987125_m1**
ZBTB16	PLZF	Human	**Hs00957433_m1**
DAZL	Deleted in azoospermia-like	Human	**Hs00154706_m1**

DPPA3	STELLA	Human	**Hs01931905_g1**
GFRA1	GDNF family receptor alpha-1	Human	**Hs00237133_m1**
GPR125	Probable G-protein coupled receptor 125	Human	**Hs00402930_m1**
DNMT1	DNA (cytosine-5)-methyltransferase 1	Human	**Hs00154749_m1**
TSPY1	Testis-specific Y-encoded protein 1	Human	**Hs00413986_m1**
STAT3	Signal transducer and activator of transcription 3	Human	**Hs01047580_m1**
CDH1	Cadherin-1	Human	**Hs01023894_m1**
FN1	Fibronectin	Human	**Hs00365052_m1**
VIM	Vimentin	Human	**Hs00185584_m1**
FGFR3	Fibroblast growth factor receptor 3	Human	**Hs00179829_m1**
DSG2	Desmoglein-2	Human	**Hs00170071_m1**
KIT	stem cell growth factor receptor	Human	**Hs00174029_m1**
FSHR	follicle-stimulating hormone receptor	Human	**Hs00174865_m1**
SOX9	Transcription factor SOX-9	Human	**Hs00165814_m1**
GATA4	Transcription factor GATA-4	Human	**Hs00171403_m1**
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Human	**Hs02758991_g1**
MMRN1	Multimerin 1	Human	**Hs00201182_m1**
EFEMP2	EGF-containing fibulin-like extracellular matrix protein 2	Human	**Hs00973815_m1**
ESM1	Endothelial cell-specific molecule 1	Human	**Hs00199831_m1**
PECAM1	Platelet endothelial cell adhesion molecule (CD31)	Human	**Hs00169777_m1**
YWHAZ	14-3-3 protein zeta/delta	Human	**Hs00237047_m1**
MAPK1	Mitogen-activated protein kinase 1	Human	**Hs01046830_m1**
18s	18s ribosomal RNA	Human	**Hs03003631_g1**
CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1	Human	**Hs00167984_m1**
HSD3B2	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	Human	**Hs00605123_m1**

Methods

8.1. Tissue preparation and cell isolation

Human testis from 21 patients undergoing transsexual sex reassignment surgery, subcapsular orchiectomy due to prostate cancer or inguinal orchiectomy due to testicular cancer, were obtained after written informed consent and approval of the Ethics Committee of the University of Tübingen (No. 273/2006 and 493/2008A). The age of the patients ranged from 20-76 years. From the surgical specimens a sample of 1-3 cm^3 of testicular tissue was extracted in a sterile manner. After removal of Tunica albuginea, Tunica vaginalis, Vasa efferentia, head and body of epididymis, the tissue was mechanically dissociated with tissue chopper and enzymatically digested in HBSS with Ca^{2+} and Mg^{2+} containing 250 µg/ml of DNAse II, 250 µg /ml of Dispase II and 750 FALGPA units /ml of Collagenase XI for 90 min at 37 ºC. The digest was filtered through 100 µ and 40 µ sieves to remove undigested tissue. The cells were centrifuged at 1000 rpm for 10 min and pellets incubated in ammonium chloride for 10 min on ice for selective lysis of erythrocytes. The cells were washed twice with PBS and maintained on ice for further use.

8.2. Cell culture

8.2.1. *Thawing and freezing of cells*

In this study one malignant pluripotent embryonal carcinoma cell line NTERA-2 (NT2) [69], along with its 2 sub lines NCCIT [70] and 2102Ep [71] were used for flow cytometric analysis of the antibodies listed in table 2 and 3. In addition to this, a seminoma derived cell line TCam-2 was provided by Dr. Sohei Kitazawa, Division of Diagnostic Molecular pathology, Kobe University Graduate School of Medicine, Japan for the antibody screening. The frozen cells of NT2, NCCIT, 2102Ep and TCam-2 cell lines were thawed into 7 ml of complete medium and brought into culture in a T25 flask. The cells were cultured in

complete medium and passaged using trypsin-EDTA in the ratio of 1:3 when 80 % confluent. For freezing, $5x10^6$ cells were resuspended in 1 ml of freezing medium and transferred into 1.5 ml cryovials.

In case of cells derived from the primary testicular tissue, $1\text{-}4x10^6$ cells were resuspended in Recovery cell culture freezing medium, transferred to cryovials and equilibrated for 10 min at 4 ºC. The cryovials were placed in an isopropanol cooling device into a -80 ºC freezer overnight, and transferred to a -140 ºC freezer.

8.2.2 *In vitro expansion of primary testis derived cells*

TSCs were plated and expanded in 0.1 % gelatin coated dishes with Embryonic Stem cell (ES) media [59]. TECs were plated in 1 % gelatin coated dishes with VascuLife cell culture medium. Spermatogonia were plated in germ cell media on a feeder of gamma irradiated CF-1 mouse embryonic fibroblast.

8.3. Generation of spermatogonia reactive monoclonal antibodies (mAbs) using seminoma derived cell line TCam-2

For immunization, 6-8 week old female BALB/c mice were injected three times intraperitoneally with 10^7 TCam-2 cells at 2-week intervals. The spleen was removed 3 days after the final booster. Suspended spleen cells (~10×10^7 cells) and 3×10^7 SP2/0 myeloma cells (German Collection of Microorganisms and Cell Cultures; DSMZ) were mixed, centrifuged, and washed in serum-free RPMI-1640 medium. One milliliter of polyethylene glycol (PEG) was added drop-by-drop to the pellet of the cell mixture that was agitated for 3 min at 37◦C. The resulting hybridoma cells were suspended in 50 ml RPMI-1640 containing 10 % fetal bovine serum (FBS) and hypoxanthine-aminopterine-thymidine (HAT) and plated into four 24 well plates. Culture supernatants from growing hybridoma cells reacting with the cell lines used for immunization but not with peripheral blood (PB) cells were considered to

select the corresponding hybridoma cells. Hybridoma cells secreting antibodies reacting with parental cells used for immunization were cloned by limiting dilution and cultured in the presence of hypoxanthine-thymidine (HT; Sigma-Aldrich). Growing clones were expanded in 75 cm^2 culture flasks and gradual removal of HT was achieved by adding HT-free RPMI-1640 medium.

8.4. Flow cytometric analysis and cell sorting

After blocking of non-specific binding with 10 mg/mL polyglobin (10 min, 4°C), cells were incubated for 15 min, 4°C with either 25 µL of proprietary antibodies or 10 µL (or volume determined by titration) of fluorochrome-conjugated antibodies [59]. Cells stained with conjugates were washed twice in FACS buffer containing PBS supplemented with 0.1 % bovine serum albumin and 2.5 ml of 0.1 % sodium azide and used for flow cytometry. Cells labeled with proprietary antibodies were stained with 10 µl of 1:25 diluted F (ab) 2 fragment of a R-phycoerythrin (PE) conjugated goat anti-mouse antibody for 15 min, washed twice and analyzed by flow cytometry. For combined indirect and direct staining, cells were first labeled with the non-conjugated antibody and then stained with goat anti-mouse secondary antibody for 15 min. Free binding sites of the secondary antibody were blocked by incubating cells for 20 min with 0.05 µg/ml mouse IgG polyclonal antibody prior to counter-staining with conjugated antibodies. Cells were sorted on a FACSAria cell sorter or analyzed on a FACS Canto flow cytometer and FCS express software or FlowJo software. The antibody combinations used for cell sorting, the resulting sorted populations and the cell type present in each sorted population are listed in Table 14.

Table 14. Antibody combinations for the prospective isolation of different cell types from adult human testis

Antibody combinations for sorting	Isolated populations	Cell types present in each population
CD49f-FITC CD49a-Alexa Fluor 647 CD144-PE	$CD49f^{+}CD49a^{-}CD144^{-}$	Spermatogonia
	$CD49f^{+}CD49a^{+}CD144^{-}$	Testicular stromal cells
	$CD49f^{+}CD49a^{+}CD144^{+}$	Testicular endothelial cells
CD49f-PerCP eFluor 710 CD49a-PE SSEA-4-Alexa Fluor 647 CD164-FITC	$CD49f^{+}CD49a^{-}SSEA\text{-}4^{+}CD164^{+}$	Spermatogonia
	$CD49f^{+}CD49a^{-}SSEA\text{-}4^{-}CD164^{+}$	Spermatogonia
	$CD49f^{+}CD49a^{-}SSEA\text{-}4^{-}CD164^{-}$	Sertoli
CD49f-PerCP eFluor 710 CD49a-Alexa Fluor 647 SSEA-4-FITC SUSD2-PE	$CD49f^{+}CD49a^{-}SSEA\text{-}4^{+}SUSD2^{+}$	Spermatogonia
	$CD49f^{+}CD49a^{-}SSEA\text{-}4^{-}SUSD2^{-}$	Spermatogonia Sertoli

8.5. Gene expression analysis by multiplex qRT-PCR

8.5.1. *RNA extraction*

375 cells from each sorted population were resuspended in 6.5 µl lysis buffer containing 5 µl of CellsDirect 2X reaction mix, 0.2 µl of SuperScript TM III RT/Platinum Taq Mix, 1.3 µl of DNA suspension buffer. This mixture was used as lysis and RNA extraction buffer as well. Cell lysates were stored at -80 ºC for further processing. Human umbilical vein endothelial cells (HUVEC) passage 4 were used as positive control for endothelial cells and human fore skin fibroblasts passage 6 were used as positive control for fibroblasts/stromal cells.

8.5.2. *Reverse transcriptase specific target amplification (RT-STA)*

RT-STA was performed for specific set of target genes (Table 13). 2.5 µl of 0.2X Primer /Probe mix containing all the selected primers were added to the 6.5 µl cell lysate. RT-STA was performed on a standard thermal cycler for 18 cycles with the program described in

Table 15. The resulting cDNA was either stored at -20 ºC or immediately used for Multiplex qRT – PCR analysis.

Table 15. Thermal cycling conditions for Reverse transcriptase specific target amplification

			STA-18 cycles	
	RT	**Taq Activation**	**Denaturation**	**Annealing extension**
Temperature	50 ºC	95 ºC	95 ºC	60 ºC
Time	15 min	2 min	15 sec	4 min

8.5.3. *Multiplex qRT-PCR*

The multiplex qRT-PCR was performed using 48.48 Dynamic array integrated fluidic circuit (IFC) on BioMark™ HD System. IFC chip was primed for use by injecting the control line fluid into each of the accumulators on the chip and the chip was placed into an IFC controller for priming using the standard protocol installed for the 48.48 array chips. The chips were used within 60 min after priming.

Prior to analysis, the cDNA obtained from RT-STA was diluted 1:5 with DNA suspension buffer. The sample mix was prepared by mixing 2.7 µl of pre diluted cDNA, 3.00 µl of 2X TaqMan Universal PCR Master mix and 0.3 µl of 20x GE sample loading reagent. The sample control was prepared by mixing 2.7 µl of DNA free water instead of cDNA. For the analysis, 10X assay mix were prepared by mixing individual 20x TaqMan gene expression assays with 2X assay loading reagent (Fluidigm Corporation). Assay controls were prepared by mixing 3 µl of DNA free water and 3 µl of assay loading reagent. Before loading, the sample mix and the assay mix were vortexed and centrifuged. To each sample and assay inlet on the IFC chip, 5 µl of sample mix and assay mix was loaded along with sample and assay

controls respectively. The chip was loaded into the IFC controller and the samples and assays were loaded into the circuits in the IFC chip using the protocol provided by the manufacturer. The chip was then loaded into BioMark™ HD System for Multiplex qPCR analysis to be performed using the standard protocols provided by the manufacturer for 48.48 IFC chip. Sample duplicates were performed for statistical reasons. Relative gene expression and dendrogram cluster analysis were performed using GeneX software.

8.6. Osteogenic, adipogenic and chondrogenic differentiation

TSCs derived from $CD49f^{+}CD49a^{+}CD144^{-}$ sorted population was cultured in NH OsteoDiff or NH AdipoDiff medium [59]. In brief, 2×10^{4} (osteogenesis) or 4×10^{4} (adipogenesis) cells were cultured in 24 well Falcon plates. After 12 days of culture in NH OsteoDiff medium, cells were fixed with methanol (-20°C, 5 min). Calcium deposition in fixed cells (4 % paraformaldehyde, 15 min) was analyzed after staining with 2 % alizarin red S for 10 min at room temperature. The formation of adipocytes was evaluated after 25 days of culture in NH AdipoDiff medium and staining of methanol-fixed cells with oil red O dye for 45 min at room temperature.

For chondrogenic differentiation, 4×10^{5} cells were cultured as a pellet for 4 hours in U-bottom 96 well plate at 37°C in 20 µL of Chondrogenic Induction Medium. After incubation, 400 µL of Chondrogenic Induction Medium supplemented with 10 ng/ml of transforming growth factor β was added. The resulting cell pellets were cultured for 3 weeks, fixed with 4 % Paraformaldehyde, embedded in paraffin and cut into 5 µm thick sections. The dried and deparaffinised sections were incubated with Alcian blue solution for 45 min at room temperature, washed in 3 % acetic acid and embedded. Photographs were taken on a Zeiss Observer.Z1 AX10 microscope.

8.7. Endothelial cell tube formation assay

In vitro Matrigel angiogenesis assay was utilized to assess the tube-formation capabilities of TECs derived from $CD49f^{+}CD49a^{+}CD144^{+}$ sorted cells. Endothelial cell tube formation assay was performed in four well chambered slides coated with 289 µl of chilled Matrigel solution (10 mg/ml) per chamber. The slides were incubated at 37 ºC for 60 min. $2x10^{4}$ cells were plated on top of the Matrigel-coated chambered slides and cultured with 300 µl of VascuLife–VEGF media per chamber at 37°C in a 5 % CO2 humidified atmosphere for 1-2 days. Tube formation was examined using Zeiss Observer.Z1 AX10 microscope.

8.8. Immunohistochemical staining

The tissue was fixed overnight in 4 % paraformaldehyde, equilibrated the following night in 20 % sucrose and frozen in Tissue Tek OCT compound.

Immunohistochemical staining was performed on 5 µm cryosections. The sections were washed 3 times in Tris buffered saline (TBS) for 5 min and blocked for 30 min with TBS containing 0.1 % Triton X 100, 10 % goat serum, 0.1 % sodium azide, 0.1 % cold fish skin gelatin (CFSG). The sections were stained with purified antibodies (Table 1) diluted in staining buffer containing TBS supplemented with 5 % goat serum, 0.1% sodium azide, 0.1 % CFSG for overnight at 4 ºC. The sections were washed 3 times for 5 min with TBS containing 0.05 % Tween 20 (TBST), followed by staining with fluorophore conjugated secondary antibody (Table 1) diluted in staining buffer. The sections were washed 3 times for 5 min with TBST and were mounted with 4'6'- diamidino-2 Phenylindole (DAPI) containing Vectashield hard set mounting medium. The slides were visualized using a Zeiss Observer.Z1 AX10 microscope with ApoTome and AxioVision 4.8 imaging software.

9. Results

9.1. Generation of mAbs Reactive with Human Tcam-2 cells

To identify novel cell surface markers for the isolation of spermatogonia, a large panel of inhouse generated mAbs were screened for reactivity with human testicular derived cells. In addition, novel monoclonal antibodies (mAbs) with specific reactivity against cell surface molecules expressed on human spermatogonia were generated. As there are currently no human spermatogonial stem cell lines available, I used human seminoma derived Tcam-2 cells (kindly provided by Dr. Sohei Kitazawa, Kobe University, Japan) for immunization. This cell line was selected instead of other testis derived cell lines because seminoma constitutes a subtype of human neoplastic testicular germ cell tumors and is uniformly composed of transformed cells that are similar to the pre-spermatogonial stem cells/gonocytes [72,73]. mAb-producing hybridomas were generated by fusion of immune spleen cells with SP2/0 myeloma cells. The resulting hybridoma cells were grown in RPMI 1640 culture medium containing 10 % FCS, antibiotics, and hypoxanthine, aminopterin, and thymidine (HAT). Culture supernatants were screened by flow cytometric analysis on Tcam-2 cells, and positive hybridoma cells secreting antibodies selectively recognizing the Tcam-2 cell line (Fig. 6), but not peripheral blood (Fig. 7), were cloned by limiting dilution. Four such selected hybridomas T-1A5, T-2B4, T-3D4 and T-4D5 were culture expanded and used for further studies.

For expression analysis, cells were first gated on Propidium iodide (PI) negative cells to remove dead cells and cell debris. PI is excluded by viable cells but can penetrate cell membranes of dying or dead cells and intercalate into double-stranded nucleic acids. Next, cells were gated according to size (FSC) and granularity (SSC). In case of peripheral blood, three different populations namely lymphocytes, monocytes and granulocytes were seen

when plotted against FSC vs SSC. Each of these populations were gated separately and analyzed for the reactivity with different antibodies (Fig. 7).

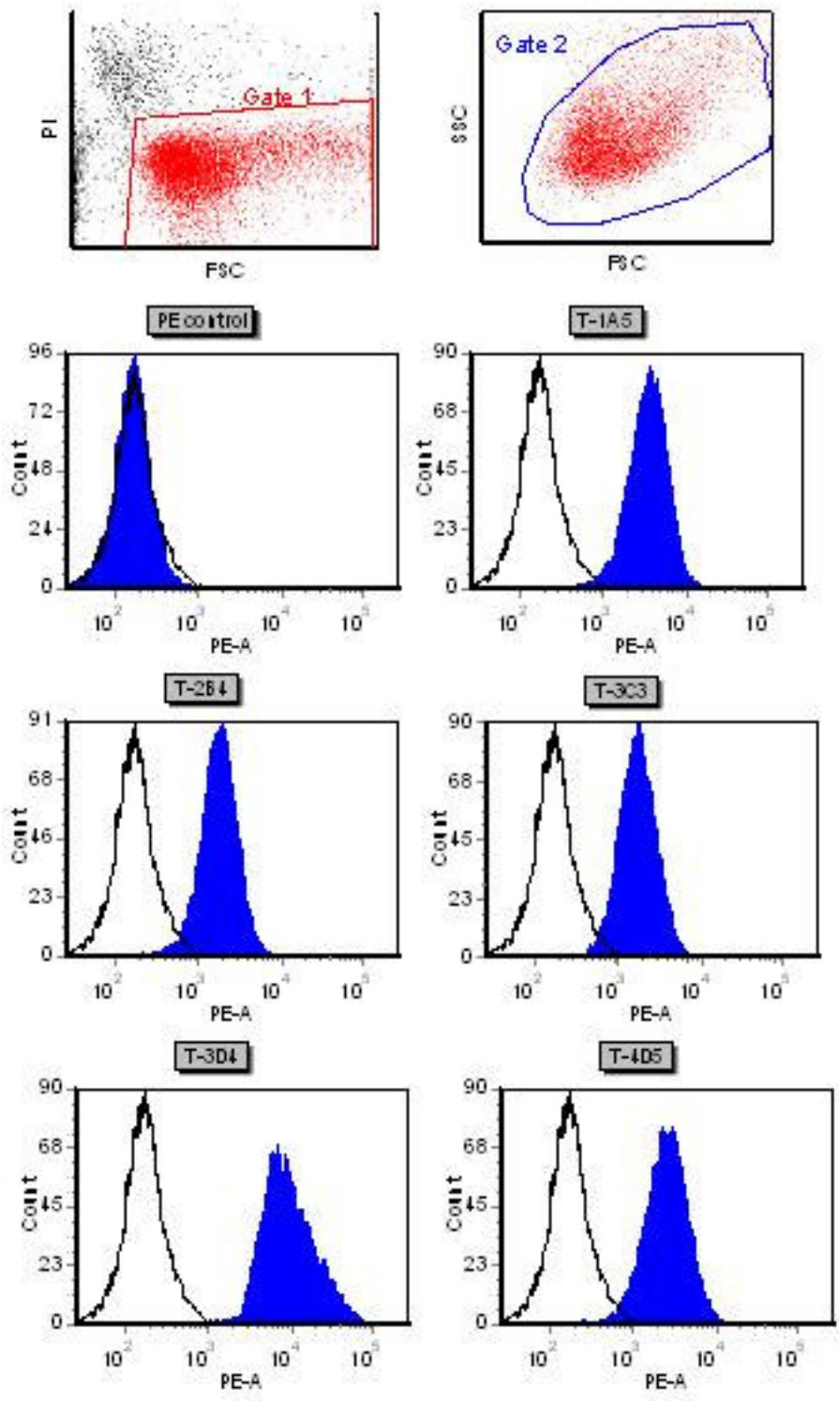
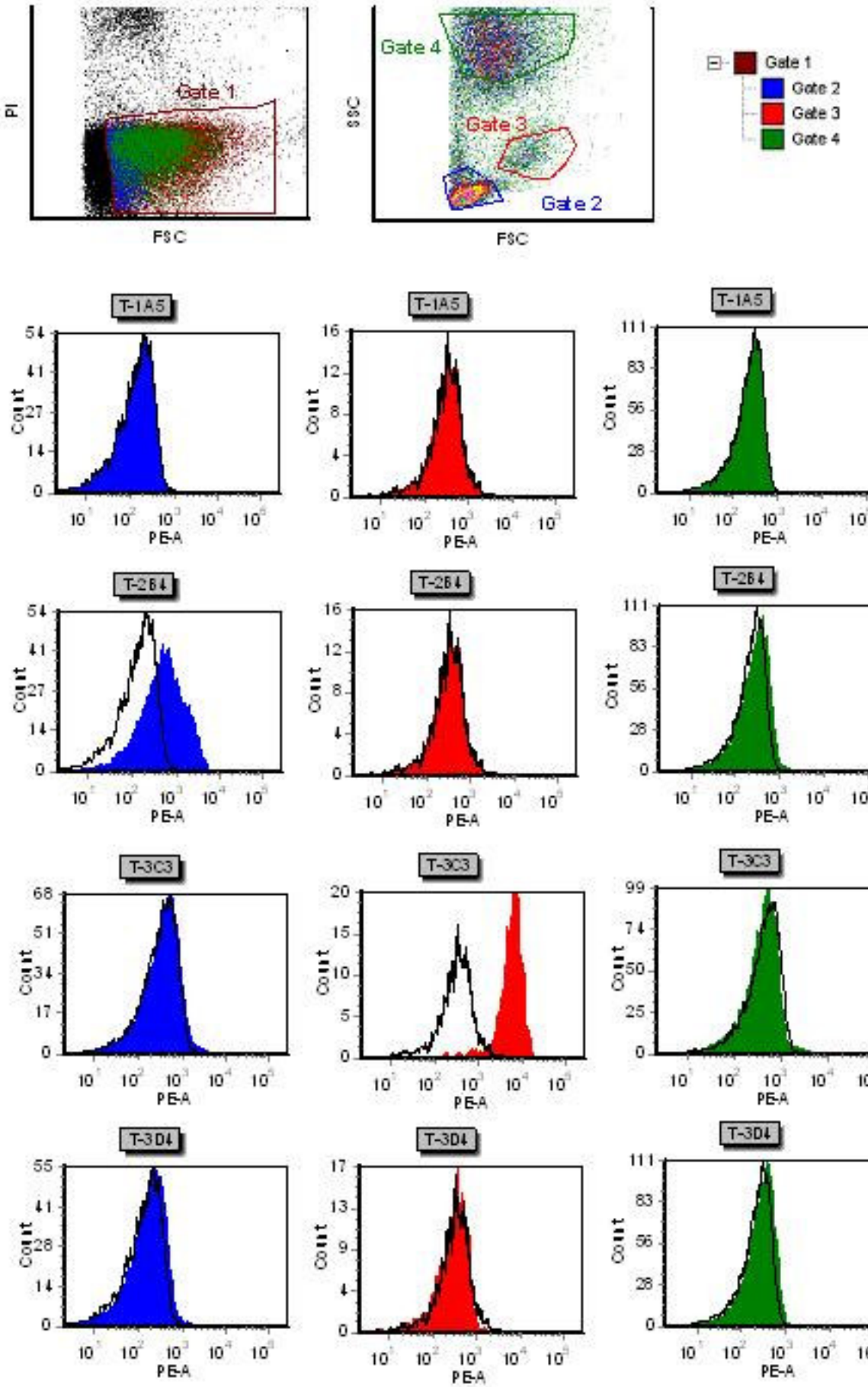


Fig. 6: Reactivity profiles of novel mAbs on Tcam-2 cells. Cells were labeled with the indicated primary antibodies and stained with a secondary PE-conjugated anti-mouse antibody. After washing and labeling with PI, cells were analyzed on a FACSCanto flow cytometer using the FCS express software.


PI
FSC
Gate 1
SSC
FSC
Gate 4
Gate 3
Gate 2
Gate 1
Gate 2
Gate 3
Gate 4
T-1A5
T-2B4
T-3C3
T-3D4
Count
PE-A

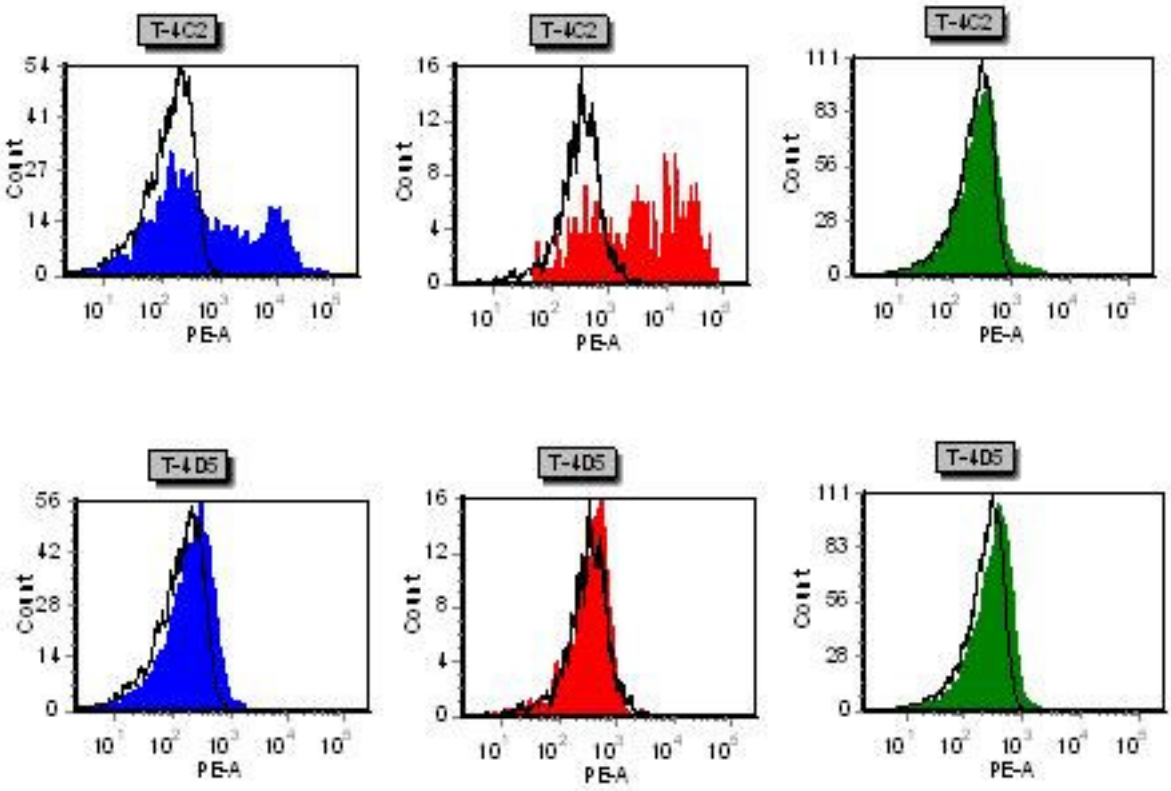


Fig. 7: Display of reactivity profile of Tcam-2 derived mAbs on single color stained peripheral blood cells gated on lymphocytes (gate 2-blue), monocytes (gate 3-red) and granulocytes (gate 4-green). Note the absence of T-1A5, T-2B4, T-3D4 and T-4D5 on the peripheral blood derived cells, the presence of T-3C3 on monocytes and the presence of T-4C2 on a subpopulation of lymphocytes and monocytes.

9.2. Reactivity of monoclonal antibodies with testicular tissue derived cell lines

As primary testicular tissue is difficult to obtain, human testis derived cell lines were initially screened with a large panel of antibodies to select candidate antibodies for the subsequent screening of primary tissue. The human malignant pluripotent embryonal carcinoma cell line NT2, its sublines NCCIT and 2102, as well as the seminoma derived cell line Tcam-2 were analyzed for their reactivity with the antibody panel by flow cytometry (Table 2 and Table 3). The reactivity profiles of these antibodies with different cell lines are listed in Table 15. As these cell lines are derived from testicular cells and resemble either embryonic cells or germ

cells, the information obtained was helpful to identify key markers that can be used to screen primary testicular tissue.

Table 15. Reactivity profiles of antibodies on testicular tissue derived cell lines.

Antibody	NT2	2102Ep	NCCIT	Tcam-2
1G2 (CD105)	-	-	-	-
1B4C3 (HER3)	-	-	-	+
2E4B4	+	-	+	+
4FR6D3B5 (CD344)	-	-	+	-
4G8B3 (CD135)	-	-	-	-
6D3H7	+	+++	+++	+
7C5G1	+	+++	++	++
9A3G2	-	+	(+)	-
9C4C3	+++	+++	+++	+++
16A1 (CD140a)	-	-	-	-
24D2 (CD340)	+	+++	++	++
28D4 (CD140b)	+	-	-	-
39D5D6 (CD56)	-	-	+	-
43A1 (CD34)	-	-	-	-
48B3 (CD167a)	-	-	+	-
56A1C2	+	+	+	++
56D2H5	-	+	+	-
56D5A1	-	-	+	#
57D2B3	-	++	+	-
58A2D10	-	-	-	-
58B1A2 (CD276)	+++	+++	+++	+++
59A3B3	+	+++	++	+
66E2D11	+	-	+	-
67A4 (CD324)	+	++	++	+
67D2 (CD164)	-	-	+	+
67H4D5	-	-	+	-
97A6B3 (CD203c)	-	-	-	-
97C5 (CD10)	-	+	+	++
104D2 (CD117)	+	+	+	++
BV2A5B6	+	++	+	#
BV8C2C2	-	-	++	+
CH1A4D1	-	-	-	+
CH2A3B5	-	-	-	+
CH3A4A7 (CD344)	-	+++	++	+
CH4D3	++	-	-	+++
CUB2 (CD318)	+	++	++	+++
HEK-3D3	+++	++	++	#
HEK-3D6	+++	+++	+++	++
HEK-6D6	-	-	+	-

HEK-8C6-B5	+++	+++	+++	#
HEK1-2C1	-	-	-	-
HEK4-1A1	+	-	++	++
HEK4-2C6	+	+	+++	-
HEK4-2D6	-	-	-	+
HEK5-1B3	+++	+++	+++	+++
HEK5-2B5	+	-	-	-
HEK5-3D5	-	-	-	-
HEK5-4A2	-	-	++	-
HEK5-4C1	-	-	-	-
HEK7-1C4	-	(+)	-	-
HEK7-4D1	+	+++	+++	++
HEK9-2B5	-	+++	+++	+
HEK9-3C2	++	+	++	-
HEP-B3	-	++(+)	++(+)	+
HEP-C20	-	-	-	-
W1C3	+	-	+	-
W1D6C4	-	+	+	+
W3C3	-	-	++	-
W3C4 (CD349)	-	-	-	-
W3D2B10	-	-	-	-
W3D5A9 (SUSD2)	+	+++	+++	+++
W4A5B5	-	+	+++	-
W5C4C5	++	+++	+++	++
W5C5A8 (SUSD2)	++	+++	+++	+++
W5D3B11	-	+	++	-
W6B3C1 (CD133)	++	+	+++	+++
W6D3 (CD15)	-	-	+	-
W7C5F8 (CD109)	-	-	+	+
W7C6F10	++	+++	+++	++
W8B2B10 (TNAP)	++	+++	+++	+++
W8C3B3	-	+	+	-
IPS-K-1D4C6	++	-	-	+++
IPS-K-2B6A8 (CD276)	+++	+++	+++	+++
IPS-K-3B1A9	-	-	-	-
IPS-K-3C1D1	+++	+++	-	+++
IPS-K-3C2D5	+++	+++	++	+++
IPS-K-3C4A6	++	+ (sub)	+	+ (sub)
IPS-K-3C5G8	+++	+++	+	+++
IPS-K-4A2A9	++	+	++	-
IPS-K-4A2B8 (SSEA-4)	+++	+++	++	+++
IPS-K-4A4F2 (TNAP)	+++	+++	++	+
IPS-K-4B5G6	++	+++	-	++
T1A5 (CD276)	+++	+++	+++	+++
T2B4	+++	+++	++	+++
T3D4	++	-	-	+++
T4D5	+++	+++	+++	+++

+ weak reactivity; + (sub) reacts only with a subpopulation of cells; ++ medium reactivity; +++ strong reactivity; # not analyzed.

9.3. Isolation and enrichment of human spermatogonia, testicular stromal cells (TSCs) and testicular endothelial cells (TECs) from human testis.

Recent studies have shown that human SSCs express CD49f [6,18]. As many other cell types in testis associated with laminin in the basement membrane also express CD49f [18], I searched for additional markers which were coexpressed on subpopulations of $CD49f^+$ cells. For identification of these markers, I screened a large panel of antibodies against surface antigens by flow cytometry for their reactivity with $CD49f^+$ cells using single cell suspensions prepared from testicular samples. Unlabelled mAbs were first stained using indirect immunofluorescence (PE) followed by direct staining with CD49f-Alexa fluor 647 mAb, as described in the methods. As both the unlabelled inhouse generated and conjugated CD49f mAbs are generated in mice/ rat, it is very important to remove the excess unbound PE conjugated secondary antibody before staining with CD49f-Alexa fluor 647 mAb, to prevent the binding of PE conjugated secondary antibody to the conjugated CD49f antibody. For this purpose, I incubated the cells with excess of unlabelled mouse IgG before staining with CD49f-Alexa fluor 647 mAb.

As gating strategy, cells were first gated on DAPI negative cells to remove dead cells. DAPI is a fluorescent dye that that binds strongly to A-T rich regions in DNA and can be excited by a violet or UV laser. Intact cell membrane in live cells reduces the efficiency of the dye from entering the cell. Only dead cells and cell debris are stained with the dye. I preferred using DAPI to PI as DAPI works well in violet laser, and leaves the FL3 detector for usage of other antibody conjugates during multi color staining. Next, cells were plotted against FSC vs SSC and analyzed for the reactivity of CD49f and the test antibodies. Fig. 8A and Fig. 8B shows the expression profiles of CD49f and selected markers in testicular tissue.

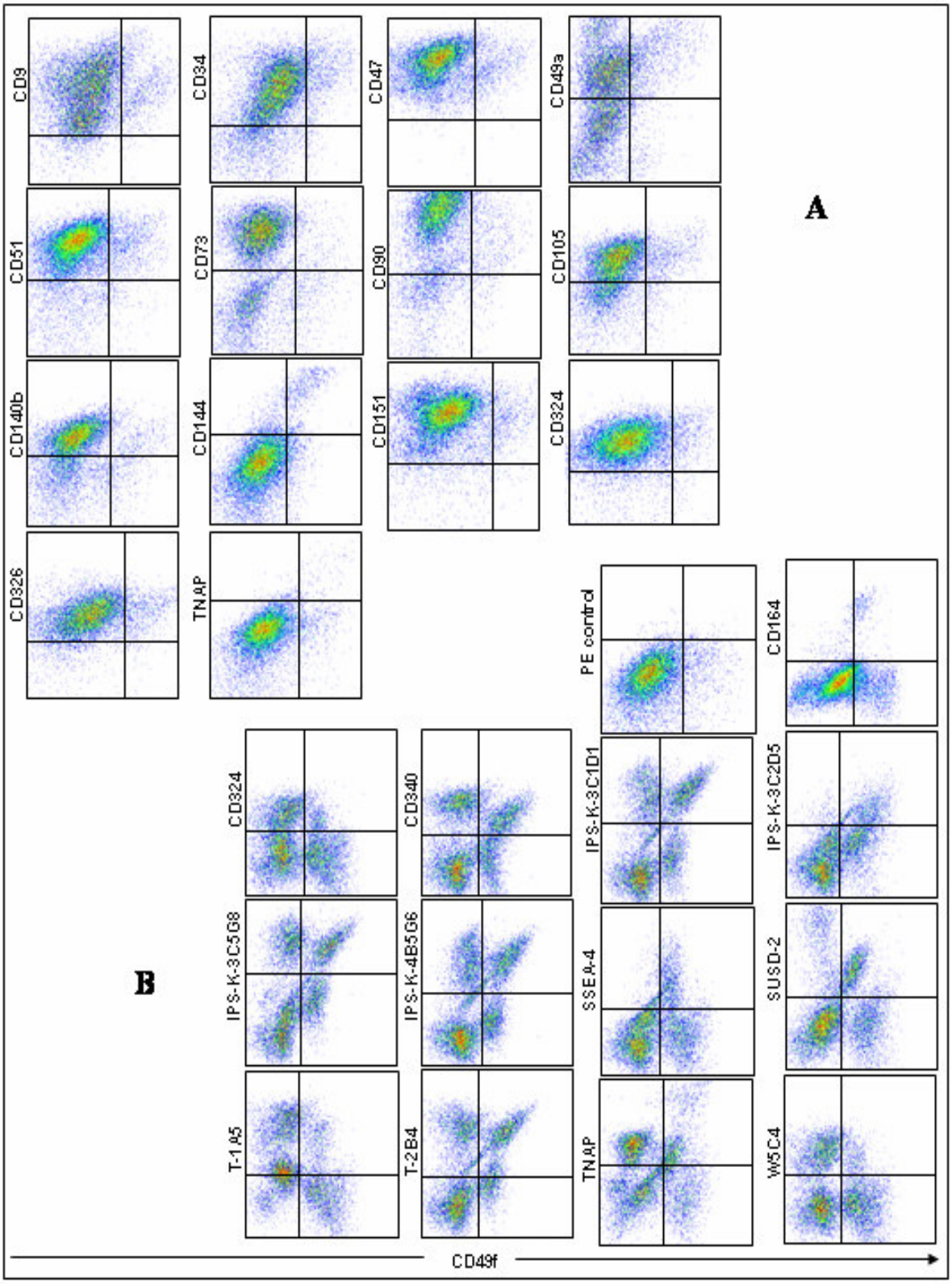


Fig. 8: Reactivity profiles of monoclonal antibodies with CD49f^{+} cells. Testis derived cells were stained with (A) CD49f-FITC or CD49f-Alexa fluor 647 and selected conjugated antibodies (B) unlabelled antibodies using indirect immunofluorescence (PE) staining followed by direct staining with CD49f-Alexa fluor 647, as described in the methods. Cells were analyzed on a FACS Canto flow cytometer and the data were processed using the FlowJo software.

Fig. 9A illustrates the strategy used for separating spermatogonia, TSCs and TECs from the whole testis. Fig. 9B shows that about 8-10 % of testis-derived cells express CD49f. Flow cytometry analysis and FACS revealed that antibodies against CD49a (integrin α1) and CD144 (PCAM1) are suitable to further fractionate the $CD49f^{+}$ population (Fig. 9B). Multicolor staining and FACS of testicular cells showed that spermatogonia are exclusively found in $CD49f^{+}CD49a^{-}CD144^{-}$ population. These cells were morphologically distinct from the other $CD49f^{+}CD49a^{+}CD144^{+/-}$ cells (Fig. 9C) and identified as round cells with a large nucleus to cytoplasm ratio [18](Fig. 9C). Backgating of the $CD49f^{+}CD49a^{-}CD144^{-}$ and $CD49f^{+}CD49a^{+}CD144^{+/-}$ populations revealed that most of the spermatogonia are $CD49f^{dim}$, in contrast to stromal cells which are mostly $CD49f^{bright}$. Hence setting of $CD49f^{+}$ gate is critical to avoid loss of spermatogonia. Further to have viable spermatogonia, it is very important to minimize the time duration between tissue preparation and FACS sorting. Replacing the commercial sheath fluid with PBS (as sheath contains some preservatives such as ethanolamine that may affect the viability) and using lower sample flow rate while sorting helped to increase the viability of spermatogonia. After initiation of culture in germ cell media, spermatogonia formed grape-like clusters (Fig. 9D) as early as 7 days, which could be maintained over a period of 30-120 days. Spermatogonia from older patients failed to proliferate and underwent cell death as soon as a week after plating. For culturing spermatogonia, two different culture conditions in the presence of mouse embryonic fibroblast (MEF) feeder layer or matrigel were tried. It was found that for long term maintenance of spermatogonia, MEF was more suitable. Spermatogonia grew slowly in culture and were passaged every 10–14 days.

In contrast to spermatogonia, TSCs were enriched in the $CD49f^{+}CD49a^{+}CD144^{-}$ subset and TECs in the $CD49f^{+}CD49a^{+}CD144^{+}$ subset. The $CD49f^{+}CD49a^{+}CD144^{-}$ cells (TSCs) were cultured in ES media and gave rise to fibroblast like colonies (Fig. 9D). As $CD49f^{+}CD49a^{+}CD144^{+}$ cells (TECs) failed to proliferate in ES media, they were cultured in

media suitable for endothelial cells growth (VascuLife cell culture media coated with 1 % gelatin). The TECs displayed cobblestone like morphology (Fig. 9D) similar to HUVEC. After 5 or 6 passages, the proliferation of TECs decreased significantly while that of TSCs remained unaltered. TSCs and TECs were passaged every 7 days.

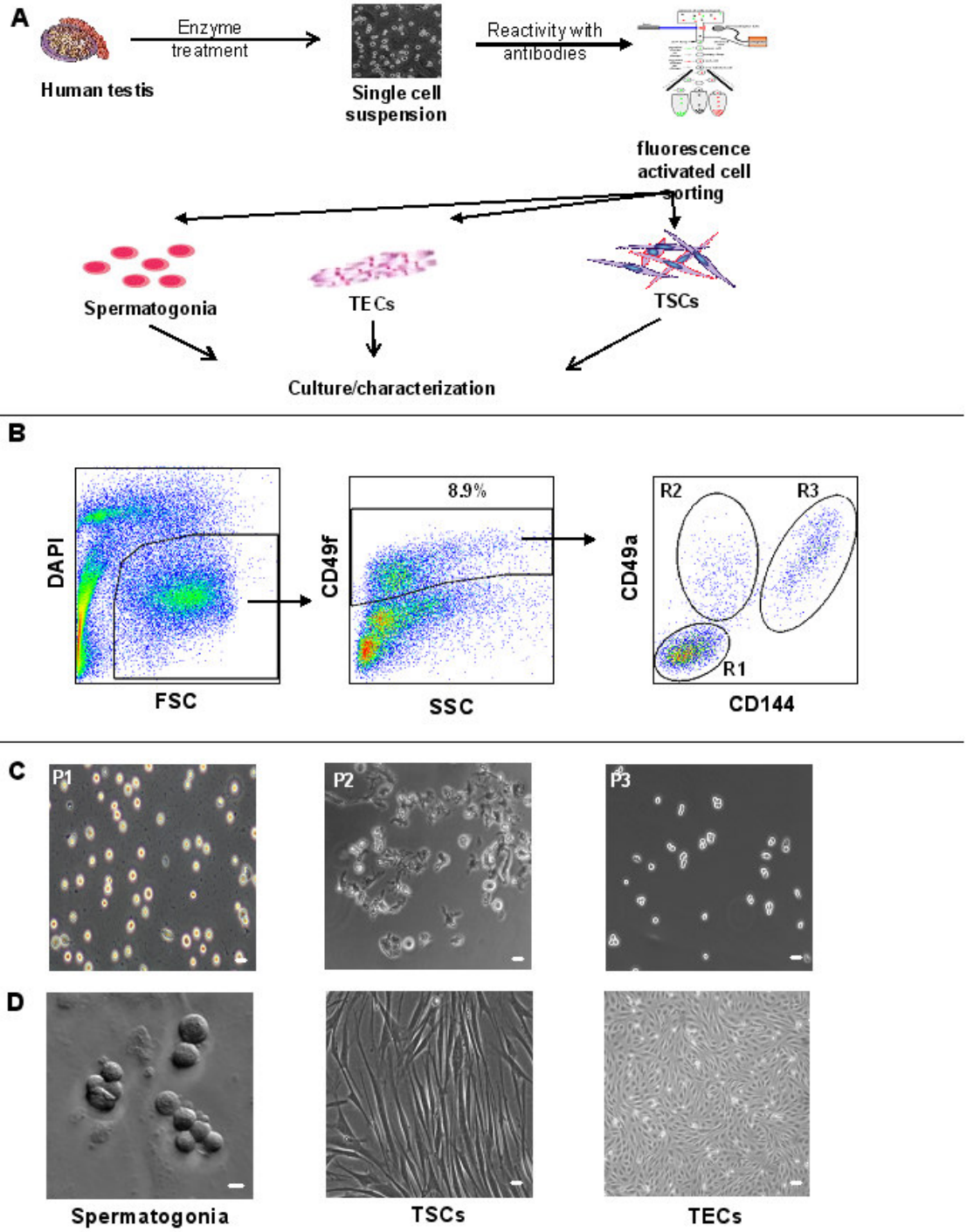


Fig. 9: Isolation of different cell types from adult human testicular tissue. (A) Graphical representation of the strategy used for the isolation of distinct cell type from the human testicular tissue. **(B)** Testis derived cells were triple stained with CD49f-FITC, CD49a-Alexa Fluor 647and

CD144-PE, gated on DAPI-negative live cells, $CD49f^+$ cells and displayed on a CD144 versus CD49a dot plot. Cells were sorted according to the sort windows R1, R2 and R3. Note that in this sample 8.9 % live cells in the testis are $CD49f^+$. **(C)** Morphology of $CD49f^+CD49a^-CD144^-$ (P1), $CD49f^+CD49a^+CD144^-$ (P2), and $CD49f^+CD49a^+CD144^+$ (P3) cells after sorting. Note the presence of round cells with a large nucleus to cytoplasm ratio in P1, large irregularly shaped cells in P2 and small ovoid cells in P3. Scale bars: 10 μm. **(D)** After sorting, cells were cultured in different media conditions as described in the methods. After culturing for 10 days, P1, P2 and P3 gave rise to spermatogonia, fibroblasts (TSCs) and endothelial (TECs) like colonies, respectively. Scale bars: 10 μm.

9.4. Adult testis derived spermatogonia express CD164, SUSD2 and SSEA-4

Although $CD49f^+CD49a^-CD144^-$ population was highly enriched for spermatogonia, contaminating cells were not completely excluded, as verified by morphological analysis of sorted cells. To isolate spermatogonia at higher purity, I searched for additional markers to fractionate the $CD49f^+CD49a^-$ population. Three-color analysis of cells stained with CD49f, CD49a and selected markers is shown in Fig. 10A and Fig. 10B.

Fig. 11A- Fig. 11C shows that the in-house generated monoclonal antibodies against CD164, SUSD2 and SSEA-4 were able to further fractionate the $CD49f^+CD49a^-$ subpopulation. Morphological analysis of the sorted cells showed that spermatogonia were exclusively detected in the $CD49f^+CD49a^-SUSD2^{+/-}SSEA4^+$ and $CD49f^+CD49a^-CD164^+SSEA4^{+/-}$ populations. Among these surface markers, SSEA-4 has been shown to be expressed on the surface of actively dividing SSCs in the adult human testis [17,18]. I therefore used CD49f, CD49a and SSEA-4 in combination with CD164 or SUSD2 for further studies. Fig. 11A and Fig. 11B show that all $SSEA4^+$ spermatogonia co-express CD164 and SUSD2. To our surprise, 55 % of the 7 samples screened contained an additional $CD49f^+CD49a^-SSEA\text{-}4^-CD164^+$ population (Fig. 11C), which also contained cells resembling spermatogonia by morphology. This indicates that not all spermatogonia express SSEA-4 but do express CD164 suggesting that CD164 is a more robust marker for the isolation of spermatogonia.

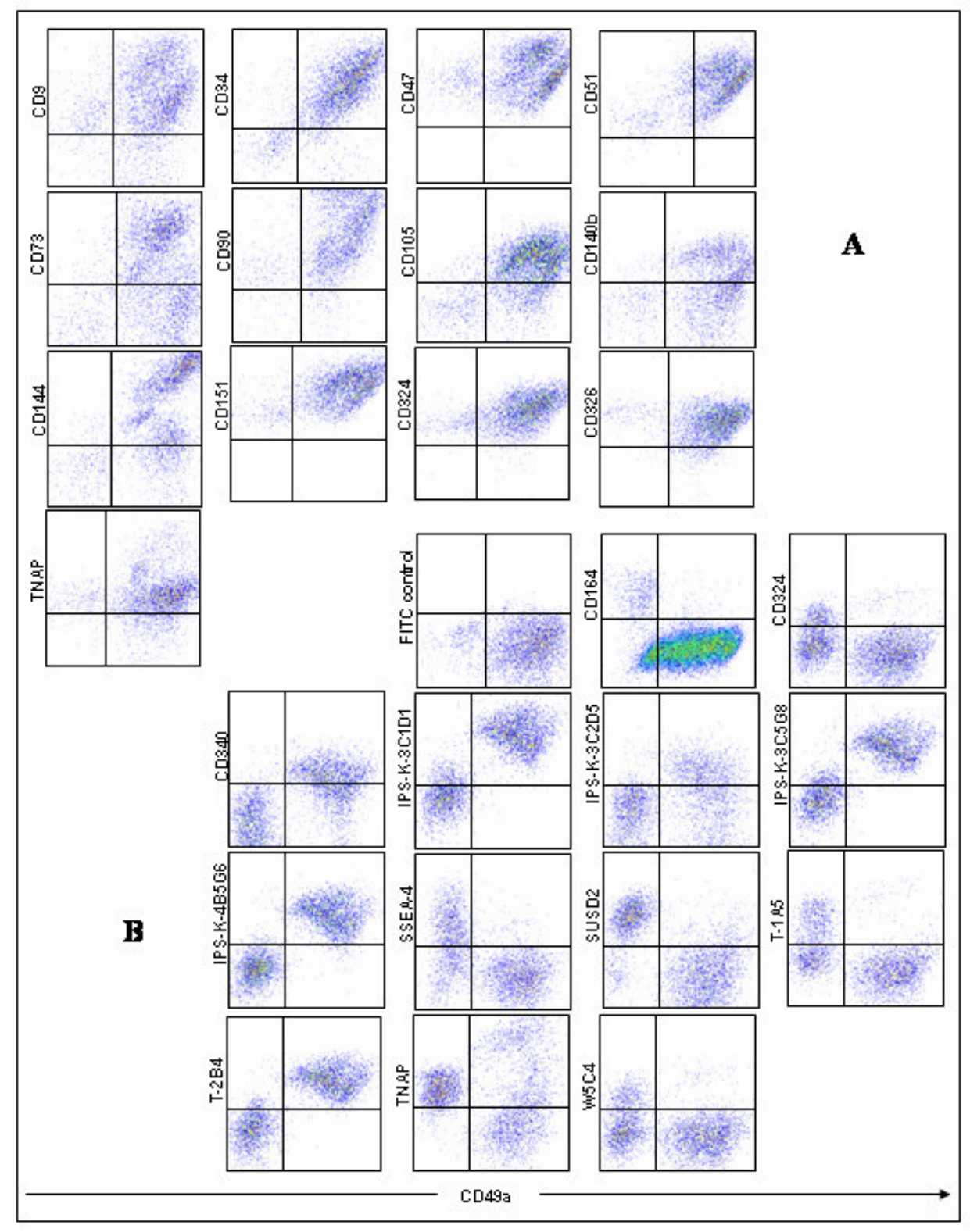


Fig. 10: Reactivity profiles of monoclonal antibodies with CD49f^{+}CD49a^{-} cells. Testis derived cells were stained with (A) CD49f-FITC/CD49f-Alexa fluor 647, CD49a-PE/CD49a-Alexa fluor 647 and selected conjugated antibodies and (B) unlabelled antibodies using indirect immunofluorescence (PE) staining followed by direct staining with conjugated CD49f-FITC, and CD49a- Alexa fluor 647 as described in the methods. Cells were gated on CD49f^{+} cells and analyzed for coexpression of CD49a and selected markers.

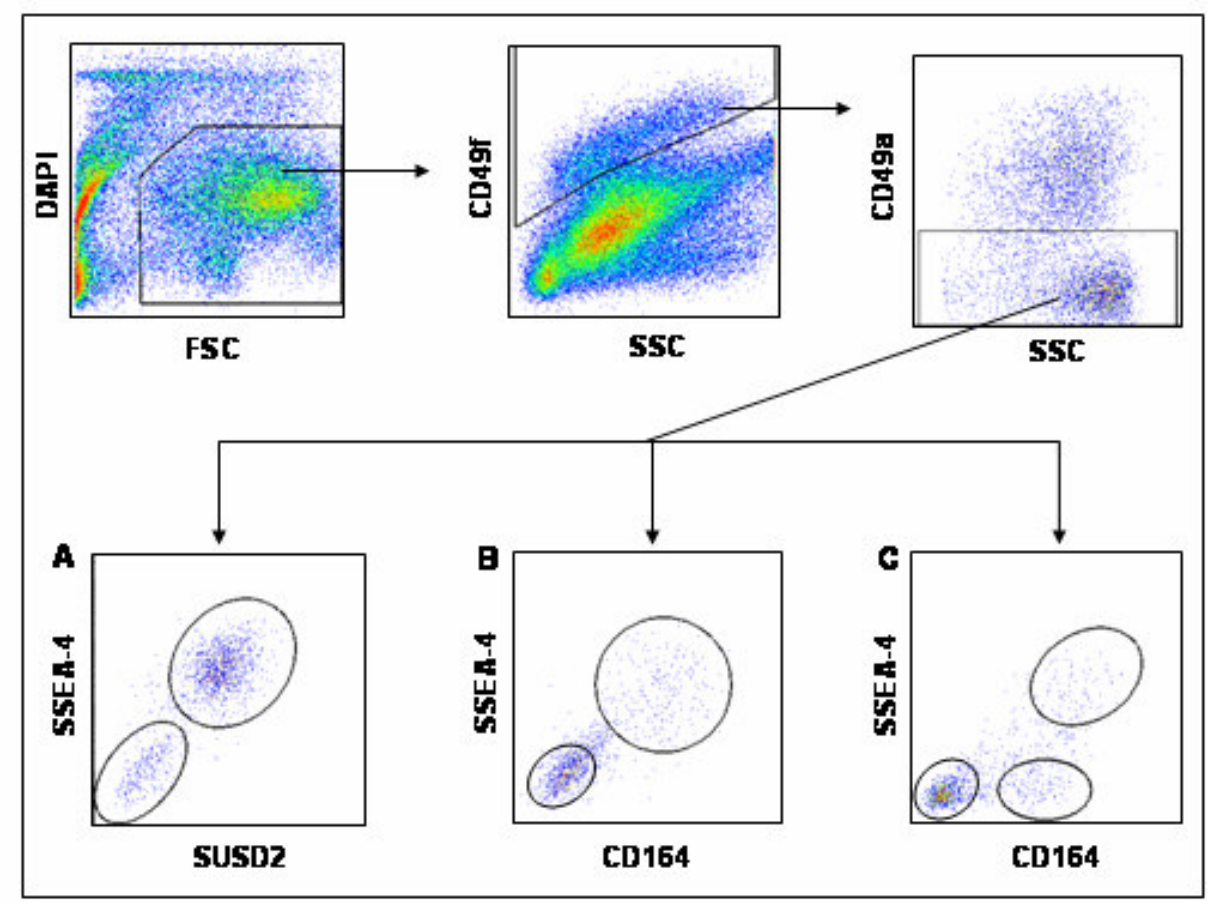


Fig. 11: Coexpression analysis of SUSD2, CD164 and SSEA-4 on spermatogonia. Four color staining of testis cells with CD49f-PerCP eFluor 710, CD49a-PE/APC, SSEA-4-FITC/APC, and CD164-FITC or SUSD2-PE. Cells were first gated on DAPI-negative cells, then on $CD49f^+$ cells followed by gating on $CD49a^-$ cells. Gated cells were analyzed for coexpression of SSEA-4 and SUSD2 or CD164. Note that most of $CD49f^+CD49a^-SSEA4^+$ cells express SUSD2 **(A)** and CD164 **(B)** and that 80 % of the analyzed samples showed an additional $CD164^+SSEA4\text{-}4^-$ population **(C)**.

9.5. $CD164^+$ and $SUSD2^+$ spermatogonia are localized in the basement membrane of seminiferous tubules and express germ cell markers

Immunohistochemical examination of adult human testicular tissue shows that almost all cells adjacent to the basement membrane of seminiferous tubules expressed CD49f (Fig. 12A). CD164 and SUSD2 expression was confined to the cells located adjacent to the basement membrane of the tubules. Apart from the tubular region, some cells in the peri tubular region also expressed SUSD2 (Fig. 12A). CD49a and CD144 were expressed only in the peri tubular region and did not colocalize with VASA within the tubules, indicating that these markers are

not expressed on germ cells but on somatic cells (Fig. 12B). In contrast, germ cell markers including DAZL and VASA were expressed in spermatogonia adjacent to the basement membrane and the differentiating spermatogonia in the luminal part of the tubule. The majority of the cells positive for CD164, SUSD2, and SSEA-4 in the tubule expressed DAZL and VASA indicating a germ line specific expression of these markers. In addition, the SSC-specific marker SSEA-4 colocalized with SUSD2 and a subset of CD164 expressing cells near the basement membrane of the tubule, demonstrating that these markers are expressed on SSCs. As expected, a $CD164^{+}$ subpopulation near the basement membrane did not colocalize with SSEA-4 expressing cells (Fig. 12C- Fig. 12E).

All the immunohistochemical stainings were performed on the cryo and not paraffin sections because our inhouse generated mAbs against SUSD2 and CD164 do not work on paraffin sections. Different tissue processing protocols were tried to achieve optimum staining of the cryosections. Overnight fixation of the tissue in 4 % PFA followed by cryoprotection with 20 % sucrose overnight before embedding of the tissue in OCT gave the best results. The resolution of staining was highly improved after usage of 20 % sucrose. Further, usage of freshly prepared sections for staining helped to decrease the non specific background.

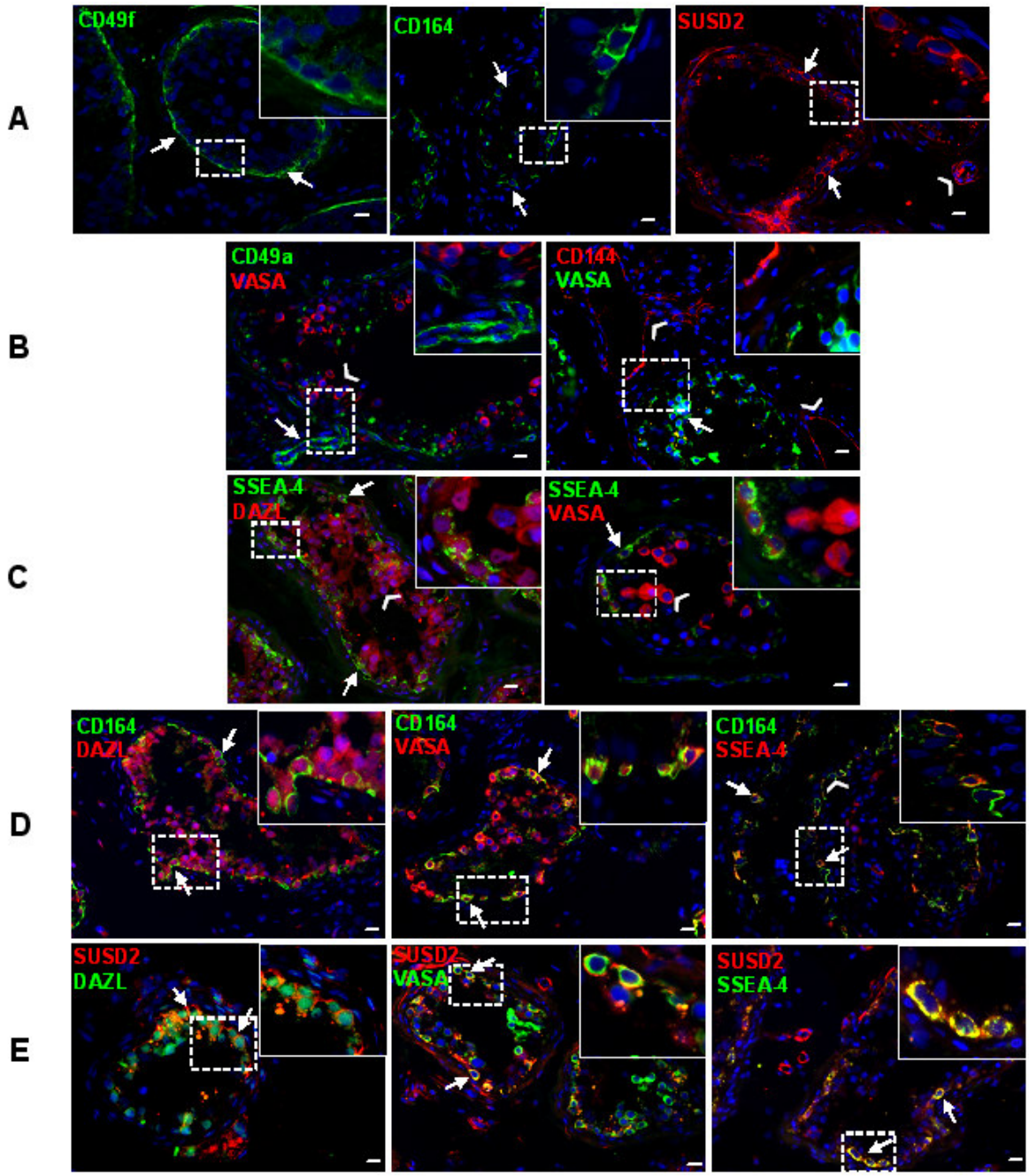


Fig. 12: Localization of selected markers in adult human testis. **(A)** Single color immunohistochemical staining of testicular tissue sections with antibodies against CD49f, CD164 and SUSD2. Note that CD49f is selectively expressed on cells adjacent to the basement membrane (marked by ↑). CD164 and SUSD2 are expressed on a few cells adjacent to the basement membrane (marked by ↑). Additionally, some cells in the peritubular region also express SUSD2 (marked by ›). **(B)** Dual color staining of cells with the germ cell marker VASA and with CD49a or CD144 shows that both CD49a and CD144 are expressed only in the peritubular region (marked by ↑). In contrast, VASA is confined to the intratubular region (marked by ›). **(C)** Colocalization of SSEA-4 and germ cell markers DAZL or VASA revealed that majority of SSEA-4$^+$ cells are DAZL$^+$ VASA$^+$ (marked by ↑), whereas most of cells in the lumen were single positive for either DAZL or VASA (marked by ›). **(D)** and **(E)** Dual color staining with DAZL, VASA or SSEA-4 and CD164 (D) or SUSD2 (E) shows that majority of CD164$^+$ and SUSD2$^+$ cells express DAZL and VASA (marked by ↑). Although the majority of SSEA4$^+$ cells express SUSD2 and CD164 (marked by ↑), there was a population of CD164$^+$SSEA-4$^-$ cells located adjacent to the basement membrane (marked by ›). Scale bars: 10 µm.

9.6. Gene expression pattern of isolated cell populations of testis

To further confirm the identity of each of the sorted populations, I performed gene expression analysis for lineage specific genes by multiplex qRT-PCR. Sorted cells from different populations were resuspended in lysis buffer from the CellsDirect One-Step qRT-PCR Kit and stored at -80°C. The number of cells was maintained constant among different groups of cells and patient samples. As a first step, the RNA in the lysate was subjected to simultaneous specific target amplification (STA) and cDNA synthesis. It is critical to perform STA in order to obtain gene expression data from samples with low cell numbers. Due to the low copy number of individual mRNA targets from smaller samples, STA is necessary to overcome any statistical sampling limitations.

For relative gene expression analysis, GeneX software was used. I initially looked for the most stable reference gene among a set of housekeeping genes including MAPK1, GAPDH, 18S, YHWAZ, CY11A1, HSD3B2, TBP and HMBS. Deciding an appropriate housekeeping gene which is expressed at an optimal stable level in all different populations of cells is important to prevent false interpretation of data. The expression stability was validated using two software program applications, geNorm and NormFinder provided by GENEX5 software package. By using geNorm, I validated the expression stability by calculating M value and compared them pair wise among all tested genes eliminating the genes with highest M-value. The NormFinder program was also used to validate the expression stability of the house keeping genes. Based on these analyses I found MAPK1 to have the highest expression stability and used it as the reference gene in our analysis.

The relative gene expression of the tested genes in all the samples normalized to unfractionated cells is listed in Table 16-19 and displayed as a heat map (Fig. 13A). For performing dendrogram cluster and heat map analysis, I used Ward's algorithm and Euclidean metric measurement to calculate the distance between two closet populations based

on the expression of 30 genes (Table 13). The data displayed in the heat map is color coded so that each data column corresponds to the data file analyzed using the software. High gene expression levels are represented in green and low expression levels in red. The color of each cell in the heat map relates to the expression of the gene in the sample, which the cell corresponds to.

The expression of germ cell specific markers DAZL and VASA was confined to the $CD49f^{+}CD49a^{-}SSEA4^{+}SUSD2^{+}$, $CD49f^{+}CD49a^{-}SSEA4^{+}CD164^{+}$ and $CD49f^{+}CD49a^{-}SSEA4^{-}CD164^{+}$ populations, confirming the germ cell origin of these cells. In addition, these populations were specifically enriched for the spermatogonia-specific markers UTF-1, DSG2, GFR-α1 and GPR125 (Table 16). The expression levels of UTF-1 and GFR-α1, markers specific for undifferentiated spermatogonia, were significantly higher in $CD49f^{+}CD49a^{-}SSEA4^{+}SUSD2^{+}$ and $CD49f^{+}CD49a^{-}SSEA4^{+}CD164^{+}$ spermatogonia than in $CD49f^{+}CD49a^{-}SSEA4^{-}CD164^{+}$ spermatogonia, indicating that the latter subset contains more differentiated spermatogonia. In contrast to spermatogonia, the fibroblast/stromal cell-specific markers fibronectin, vimentin, col1α1 and col1α2 were enriched in human fore skin fibroblasts (positive control) as well as in $CD49f^{+}CD49a^{+}CD144^{-}$ cells (Table 17), confirming the stromal nature of these cells. Similarly, the expression of the endothelial cell specific markers MMRN1, ESM1 and PCAM1 was significantly higher in HUVECs (positive control) and in $CD49f^{+}CD49a^{+}CD144^{+}$ cells than in other cell subsets (Table 18), supporting the endothelial nature of these cells. The Sertoli cell specific markers FSHR and GATA4 were highly enriched in the $CD49f^{+}CD49a^{-}SSEA4^{-}CD164^{-}$ population (Table 19), indicating that these cells may contain sertoli cells. As expected the $CD49f^{+}CD49a^{-}SSEA4^{-}SUSD2^{-}$ population appears to contain a mixture of cells consisting of spermatogonia and sertoli cells as evidenced by their expression of the germ line specific markers DAZL and VASA and the sertoli cell specific markers FSHR and GATA4. This is expected because the use of the marker combination SSEA-4 and SUSD2 results only in the isolation of $SSEA\text{-}4^{+}$

spermatogonia because SUSD2 is not expressed on SSEA-4$^-$ spermatogonia. However, the marker combination of SSEA-4 and CD164 results in the isolation of both sertoli cells (SSEA-4$^-$CD164$^-$) and spermatogonia (SSEA-4$^{+/-}$CD164$^+$). In contrary to primary spermatogonia, the expression profile of cultured spermatogonia derived from CD49f$^+$CD49a$^-$ SSEA4$^+$CD164$^+$ and CD49f$^+$CD49a$^-$SSEA4$^-$CD164$^+$ populations highly resembled each other after 30 days of culture under germ cell culture conditions.

Table 16. Germ cell and stem cell specific gene expression profiles of different cell types from testis as determined by multiplex qRT-PCR

	Relative gene expression										
	CD49f$^+$ CD49a$^-$ SSEA4$^+$ CD164$^+$	CD49f$^+$ CD49a$^-$ SSEA4$^-$ CD164$^+$	CD49f+ CD49a$^-$ SSEA4$^-$ CD164$^-$	CD49f$^+$ CD49a$^-$ SSEA4$^+$ SUSD2$^+$	CD49f$^+$ CD49a- SSEA4$^-$ SUSD2$^-$	HFSF	CD49f$^+$ CD49a$^+$ CD144$^-$	HUVEC	CD49f$^+$ CD49a$^+$ CD144$^+$	Cultured CD49f$^+$ CD49a$^-$ SSEA4$^+$ CD164$^+$	Cultured CD49f$^+$ CD49a$^-$ SSEA4$^-$ CD164$^+$
TERT	0.998 ±0.100	0.805 ±0.003	1.496 ±0.170	1.222 ±0.002	0.561 ±0.093	0.706 ±0.009	0.453 ±0.049	0.070 ±0.001	0.130 ±0.002	0	0
Oct4b	0.888 ±0.05	2.157 ±0.020	27.609 ±3.711	0.795 ±0.019	1.997 ±0.050	12.655 ±0.483	5.099 ±0.397	2.589 ±0.208	5.277 ±0.249	140.241 ±2.363	224.535 ±13.828
Sox2	0.229 ±0.024	0.752 ±0.037	2.831 ±0.017	0.324 ±0.009	1.190 ±0.184	1.174 ±0.067	0.665 ±0.088	0.467 ±0.016	0.499 ±0.037	44.457 ±0.051	56.166 ±0.601
LIN28	1.193 ±0.030	5.096 ±0.242	25.144 ±5.817	1.196 ±0.126	1.190 ±0.191	22.719 ±0.583	7.018 ±0.153	2.979 ±0.087	6.985 ±0.645	111.931 ±1.217	185.612 ±2.207
LIN28B	0.986 ±0.038	0.995 ±0.008	0.248 ±0.025	1.001 ±0.012	1.029 ±0.051	0.006 ±0.000	0.224 ±0.022	0.001 ±0.000	0.006 ±0.007	0	0
DNMT1	0.406 ±0.001	1.696 ±0.461	1.158 ±0.182	0.379 ±0.022	2.954 ±0.052	1.455 ±0.142	0.371 ±0.051	0.423 ±0.023	1.290 ±0.044	0	0
UTF1	3.927 ±0.119	0.014 ±0.012	0.129 ±0.015	4.601 ±0.199	0.016 ±0.001	0.061 ±0.001	0.025 ±0.010	0.006 ±0.000	0.011 ±0.000	16.471 ±0.249	75.994 ±10.662
NANOS	1.595 ±0.019	4.505 ±0.022	32.275 ±4.761	1.567 ±0.103	1.489 ±0.161	14.341 ±1.098	11.615 ±0.017	5.801 ±0.110	4.362 ±0.318	292.452 ±5.531	266.265 ±7.491
VASA	0.497 ±0.005	0.632 ±0.018	0.228 ±0.017	0.676 ±0.004	0.905 ±0.041	0.004 ±0.000	0.001 0.002	0.001 ±0.000	0.009 ±0.000	0	0
PLZF	1.194 ±0.018	0.151 ±0.031	2.822 ±0.580	0.940 ±0.016	0.145 ±0.006	0.552 ±0.007	3.213 ±0.150	0.215 ±0.009	7.574 ±0.077	0	0
DAZL	0.368 ±0.000	2.345 ±0.015	0.501 ±0.042	0.435 ±0.003	3.606 ±0.073	0.053 ±0.001	0.015 ±0.001	0.005 ±0.000	0.020 ±0.000	0	0
GFRa1	3.406 ±0.009	0.125 ±0.014	0.137 ±0.002	3.101 ±0.034	0.103 ±0.001	0.029 ±0.000	0.434 ±0.006	0.003 ±0.000	0.047 ±0.001	0	0
GPR125	1.206 ±0.317	0.433 ±0.360	0.607 ±0.069	1.658 ±0.073	0.075 ±0.003	0.287 ±0.004	0.175 ±0.005	0.072 ±0.018	0.053 ±0.001	0	0
TSPY1	1.697 ±0.010	2.384 ±0.007	0.864 ±0.011	1.621 ±0.019	2.654 ±0.159	0.021 ±0.000	0.390 ±0.008	0.002 ±0.000	0.008 ±0.000	0	0
CD9	0.444 ±0.012	0.578 ±0.009	3.553 ±0.470	0.381 ±0.003	0.477 ±0.021	2.390 ±0.060	1.613 ±0.018	14.890 0.271	9.815 ±0.102	0	0
STAT3	0.359 ±0.018	0.898 ±0.034	1.821 ±0.176	0.218 ±0.021	0.864 ±0.042	1.407 ±0.077	2.562 ±0.008	0.938 ±0.014	11.676 ±0.078	0	0

KIT	0.504 ±0.018	0.860 ±0.020	0.434 ±0.053	0.542 ±0.019	0.770 ±0.010	0.628 ±0.016	0.126 ±0.008	1.004 ±0.015	0.114 ±0.009	0	0
STELLA	0.914 ±0.019	3.600 ±0.131	12.995 ±0.152	0.877 ±0.020	1.489 ±0.063	7.897 ±0.430	4.037 ±0.217	1.146 ±0.023	5.432 ±0.093	29.065 ±0.008	32.851 ±1.612
LIFR	0.194 ±0.002	0.203 ±0.021	1.756 ±0.033	0.221 ±0.000	0.269 ±0.011	0.073 ±0.020	0,166 ±0.000	0.255 ±0.010	21.593 ±0.537	0	0
CDH1	0.618 ±0.035	0.939 ±0.028	1.663 ±0.207	0.832 ±0.002	0.737 ±0.016	0.388 ±0.005	0.341 ±0.104	0.038 ±0.000	0.072 ±0.001	0	0
DSG2	1.376 ±0.022	1.506 ±0.005	0.653 ±0.017	1.128 ±0.067	2.511 ±0.118	0.020 ±0.009	0.180 ±0.026	0.001 ±0.000	1.141 ±0.083	0	0

Table 17. Fibroblast specific gene expression profiles of different cell types from testis as determined by multiplex qRT-PCR

Relative gene expression											
	$CD49f^+$ $CD49a^-$ $SSEA4^+$ $CD164^+$	$CD49f^+$ $CD49a^-$ $SSEA4^-$ $CD164^+$	$CD49f^+$ $CD49a^-$ $SSEA4^-$ $CD164^-$	$CD49f^+$ $CD49a^-$ $SSEA4^+$ $SUSD2^+$	$CD49f^+$ $CD49a^-$ $SSEA4^-$ $SUSD2^-$	HFSF	$CD49f^+$ $CD49a^+$ $CD144^-$	HUVEC	$CD49f^+$ $CD49a^+$ $CD144^+$	Cultured $CD49f^+$ $CD49a^-$ $SSEA4^+$ $CD164^+$	Cultured $CD49f^+$ $CD49a-$ $SSEA4^-$ $CD164^+$
FN	0.382 ±0.004	0.076 ±0.003	2.659 ±0.237	0.329 ±0.005	0.025 ±0.001	35.048 ±0.812	1.719 ±0.030	18.776 ±0.263	15.618 ±0.376	0.139 ±0.017	0.087 ±0.002
VMN	0.033 ±0.000	0.062 ±0.001	2.743 ±0.209	0.010 ±0.001	0.038 ±0.002	17.627 ±0.150	3.201 ±0.050	9.123 ±0.121	9.720 0.176	0.012 ±0.003	0.004 ±0.000
Col1a1	0.018 ±0.001	0.040 ±0.004	2.591 ±0.924	0.014 ±0.001	0,098 ±0.006	5.910 ±1.731	3.408 ±0.515	0.013 ±0.000	0.293 ±0.002	17.141 ±0.259	13.454 ±0.598
Col1a2	0.024 ±0.001	0.004 ±0.000	1.800 ±0.173	0.025 ±0.002	0.025 ±0.000	4.707 ±0.118	4.849 ±0.045	0.015 ±0.000	0.612 ±0.005	0.081 ±0.014	0.042 ±0.010

Table 18. Endothelial cell specific gene expression profiles of different cell types from testis as determined by multiplex qRT-PCR

Relative gene expression											
	$CD49f^+$ CD49a- $SSEA4^+$ $CD164^+$	$CD49f^+$ $CD49a^-$ $SSEA4^-$ $CD164^+$	$CD49f^+$ $CD49a^-$ SSEA4- CD164-	$CD49f^+$ $CD49a^-$ $SSEA4^+$ $SUSD2^+$	$CD49f^+$ $CD49a^-$ $SSEA4^-$ $SUSD2^-$	HFSF	$CD49f^+$ $CD49a^+$ $CD144^-$	HUVEC	$CD49f^+$ $CD49a^+$ $CD144^+$	Cultured $CD49f^+$ $CD49a^-$ $SSEA4^+$ $CD164^+$	Cultured $CD49f^+$ $CD49a^-$ $SSEA4^-$ $CD164^+$
MMRN1	0.007 ±0.000	0.100 ±0.006	0.662 ±0.129	0.007 ±0.000	0.011 ±0.000	0.043 ±0.001	0.027 ±0.003	61.194 ±1.255	1,626 ±0.027	0	0
ESM1	0.051 ±0.014	1.459 ±0.018	0.360 ±0.041	0.100 ±0.027	0.045 ±0.002	0.716 ±0.584	0.049 ±0.000	19.127 ±0.982	45.025 ±1.573	0	0
PCAM1	0.055 ±0.001	0.160 ±0.005	1.496 ±0.555	0.010 ±0.001	0.050 ±0.005	0.057 ±0.001	0.042 ±0.010	42.544 ±0.497	30.085 ±0.365	0	0

Table 19. Sertoli cell specific gene expression profiles of different cell types from testis as determined by multiplex qRT-PCR

Relative gene expression											
	$CD49f^+$ $CD49a^-$ $SSEA4^+$ $CD164^+$	$CD49f^+$ CD49a- $SSEA4^-$ $CD164^+$	$CD49f^+$ $CD49a^-$ $SSEA4^-$ $CD164^-$	$CD49f^+$ $CD49a^-$ $SSEA4^+$ $SUSD2^+$	$CD49f^+$ $CD49a^-$ $SSEA4^-$ $SUSD2^-$	HFSF	$CD49f^+$ $CD49a^+$ $CD144^-$	HUVEC	$CD49f^+$ $CD49a^+$ $CD144^+$	Cultured $CD49f^+$ $CD49a^-$ $SSEA4^+$ $CD164^+$	Cultured $CD49f^+$ $CD49a^-$ $SSEA4^-$ $CD164^+$
FSHR	0.221 ±0.001	0.837 ±0.010	15.662 ±1.778	0.347 ±0.004	1.943 ±0.083	7.392 ±0.097	2.132 0.001	0.732 ±0.005	2.927 ±0.170	0	0
GATA4	0.212 ±0.012	0.204 ±0.006	1.817 ±0.285	0.245 ±0.006	0.355 ±0.032	0.411 ±0.005	4.900 ±0.878	0.041 ±0.000	0.395 ±0.001	0	0

To further explore the relationship among the different sorted populations based on the expression of 30 genes listed in Table 13, I performed dendrogram cluster analysis by using GeneX software applying Ward's algorithm and Euclidean metric measurement. Depending on the branching pattern in the dendrogram, the different sorted populations from the primary testis and cultured spermatogonia derived from the sorted cells can be organized into three main groups namely spermatogonia/germ cells, fibroblasts/stromal cells and endothelial cells (Fig. 13B). As expected, $CD49f^+CD49a^-SSEA4^+SUSD2^+$ and $CD49f^+CD49a^-SSEA4^+CD164^+$ populations were closely related. Also, $CD49f^+CD49a^-SSEA4^-CD164^+$ and $CD49f^+CD49a^-SSEA4^-SUSD2^-$ populations were closely related. In addition, these four populations clustered together as spermatogonia/germ cells. Dendrogram cluster analysis confirms that the $CD49f^+CD49a^-SSEA4^-SUSD2^-$ population also contains spermatogonia, which are closely related to $CD49f^+CD49a^-SSEA4^-CD164^+$ spermatogonia. As already shown in the expression pattern analysis, $CD49f^+CD49a^-SSEA4^-SUSD2^-$ population additionally contains sertoli cells. In contrast, $CD49f^+CD49a^+CD144^-$ cells along with cultured human fore skin fibroblasts clustered together (fibroblast/stromal cells) and $CD49f^+CD49a^+CD144^+$ cells along with HUVEC clustered together (endothelial cells). Surprisingly, cultured

spermatogonia derived from primary spermatogonia clustered with the fibroblasts/ stromal cells, indicating a highly modulating effect of the culture media. The fact that the expression pattern of CD49f^{+}CD49a-SSEA4-CD164- cells appears to be closely linked with fibroblasts supports our view that this population may contain sertoli cells, which are more closely related to stromal cells than to spermatogonia.

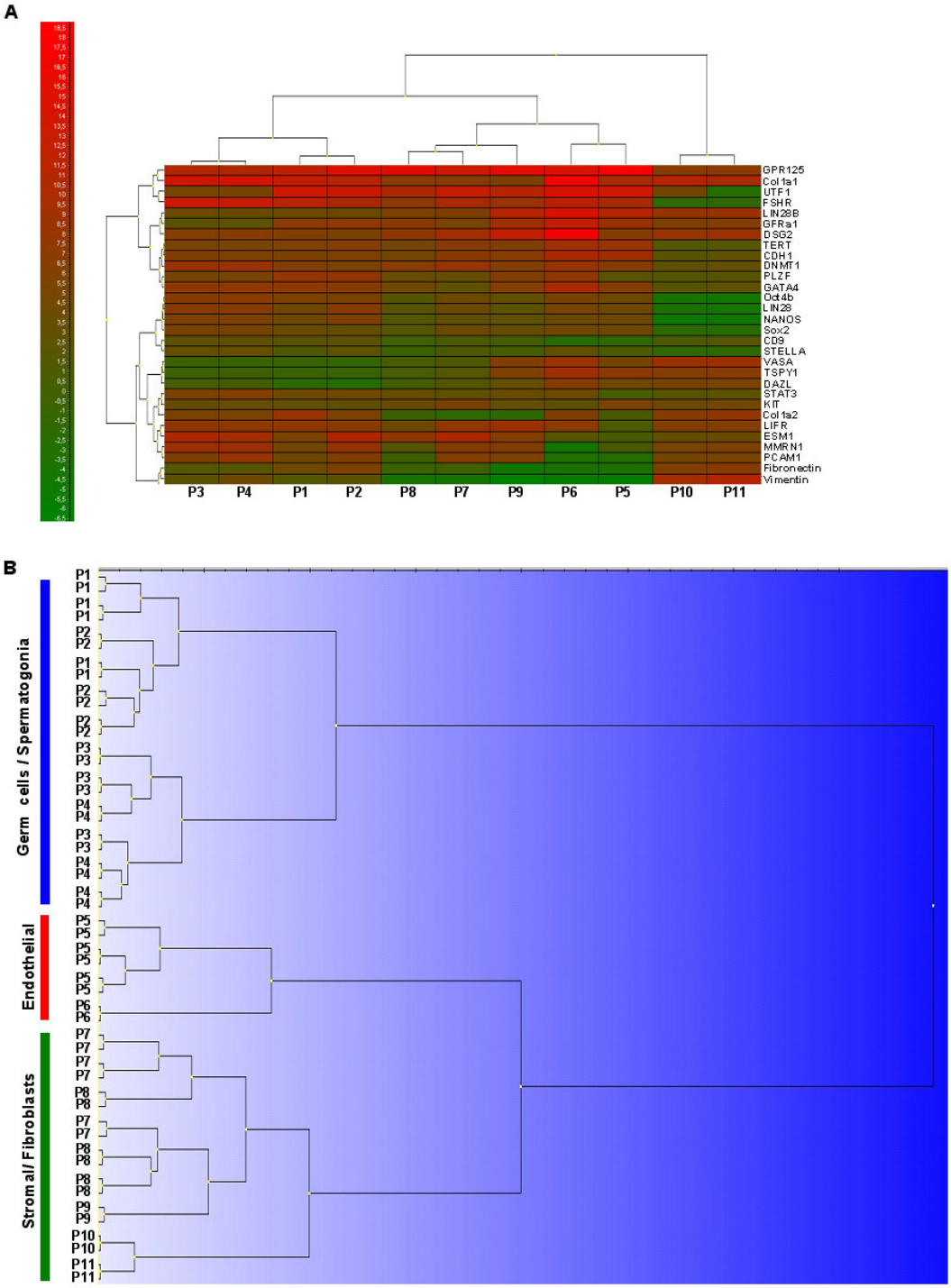


Fig. 13: Relationship of gene expression pattern among different sorted populations from adult human testis. (A) The cluster dendrogram shows the grouping of different sorted populations based on the expression of 30 genes listed in Table 13. The sorted populations clustered into three cell types consisting of germ cells/spermatogonia, endothelial cells and fibroblasts/stromal cells. Shown data are from three biological experiments performed in technical duplicates. (B) Heatmap showing gene clustering and cell heterogeneity of different sorted populations presented in Table 16-19. Gene expression levels of all genes were quantified using MAPK1 as internal standard and normalized to unfractionated cells. Results are presented as ΔΔCt values of one representative biological experiment performed in technical replicates. High gene expression levels are shown in red and low expression levels in green. P1: $CD49f^{+}CD49a^{-}SSEA\text{-}4^{-}CD164^{+}$; P2: $CD49f^{+}CD49a^{-}SSEA\text{-}4^{-}SUSD2^{-}$; P3: $CD49f^{+}CD49a^{-}SSEA\text{-}4^{+}CD164^{+}$; P4: $CD49f^{+}CD49a^{-}SSEA\text{-}4^{+}SUSD2^{+}$; P5: $CD49f^{+}CD49a^{+}CD144^{+}$; P6: HUVEC; P7: $CD49f^{+}CD49a^{+}CD144^{-}$; P8: $CD49f^{+}CD49a^{-}SSEA\text{-}4^{-}CD164^{-}$; P9: Human foreskin fibroblast; P10: cultured $CD49f^{+}CD49a^{-}SSEA\text{-}4^{+}CD164^{+}$; P11: cultured $CD49f^{+}CD49a^{-}SSEA\text{-}4^{-}CD164^{+}$

9.7. Phenotype of primary spermatogonia

To analyze the expression profiles of surface markers on $CD49f^{+}CD49a^{-}SSEA\text{-}4^{+}$ spermatogonia, primary testis cells were stained with CD49f, CD49a, SSEA-4 and a panel of test antibodies (as listed in the methods) The cells were gated on the $CD49f^{+}CD49a^{-}$ population and analyzed for the co-expression of SSEA-4 and the test antibodies. The majority of $CD49f^{+}CD49a^{-}SSEA\text{-}4^{+}$ spermatogonia express CD9 but only a subpopulation expresses CD90 and CD276 (Fig. 14A). On the other hand, they were negative for the stem and tumor cell markers CD133, CD318, CD324, CD340, SSEA-3, TRA-1-60, TRA-1-81, HER3 and c-kit (Table 20). Table 20 shows a list of tested markers, most of which were not expressed on the spermatogonia.

Commercial antibodies	**In-house antibodies**	
	Clone	**Antigen**
CD13	1G2	CD105
CD26	IB4C3	HER3
CD44	4G8B3	CD135
CD45	16A1	CD140a
CD47	24D2	CD340
CD49a	28D4	CD140b
CD49b	39D5D6	CD56
CD51	43A1	CD34
CD71	48B3	CD167a
CD73	67A4	CD324
CD105	97A6B3	CD203c
CD138	97C5	CD10
CD144	104D2	CD117
CD146	CH3A4A7	CD344
CD151	CUB1	CD318
CD166	W3C4	CD349
CD200	W6B3C1	CD133/1
CD271	W6D3	CD15
SSEA-3	W7C5F8	CD109
TRA 1-60	W7C6F10	LAR
TRA 1-81		

Table 20. Markers not expressed on $SSEA\text{-}4^{+}$ spermatogonia. Testis derived cells were stained with CD49f-FITC/CD49f-Alexa fluor 647, SSEA-4-PE/SSEA-4-Alexa fluor 647/ SSEA-4-FITC and selected conjugated antibodies and in-house antibodies using indirect immunofluorescence (PE)

staining followed by direct staining with conjugated CD49f, and SSEA-4 as described in the methods. Cells were gated on $CD49f^+$ cells and analyzed for coexpression of SSEA-4 and selected markers.

9.8. Phenotype of TSCs and TECs

Apart from spermatogonia, two additional $CD49f^+$ populations were identified using CD49a and CD144 as selection markers. After sorting cells of the $CD49f^+CD49a^+CD144^-$ and $CD49f^+CD49a^+CD144^+$ subsets, they were cultured in ES media and endothelial cell culture media, respectively. After 10 days, the cells were analyzed for stromal and endothelial specific markers. Fig. 14B shows that only the cells derived from the $CD49f^+CD49a^+CD144^-$ subset predominantly expressed the mesenchymal/stromal cell specific key markers CD90 and CD200, whereas CD56 and TNAP were expressed in subpopulations of cultured stromal cells. In contrast, cells derived from the $CD49f^+CD49a^+CD144^-$ subset were enriched for TECs, as verified by their selective expression of the endothelial cell-specific key markers CD34, CD31, CD144 and VEGFR2 (Fig. 14C). In conclusion, TSCs are enriched in the $CD49f^+CD49a^+CD144^-$ subset and TECs are exclusively found in the $CD49f^+CD49a^+CD144^+$ subset. This was also confirmed by the selective uptake of Dio-Ac-LDL in TECs but not in TSCs (Fig. 14D).

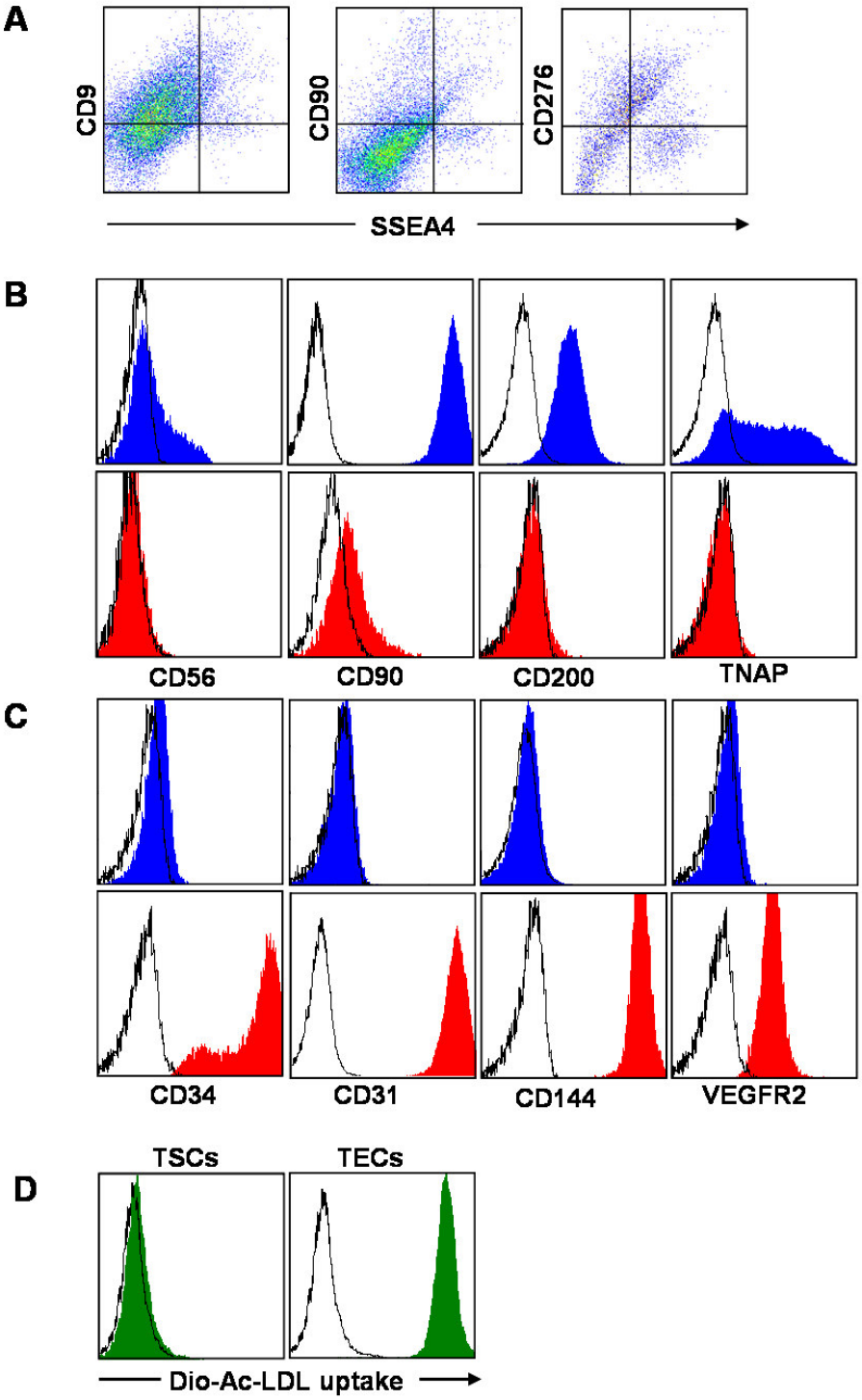


Fig. 14: Phenotype of testis derived spermatogonia, stromal cells and endothelial cells. (A) Testis derived cells triple stained with CD49f-FITC, SSEA-4-APC and selected markers. Cells were gated on the $CD49f^+$ subset and analyzed for coexpression of SSEA-4 and selected markers. The majority of $CD49f^+SSEA\text{-}4^+$ spermatogonia express CD9 but only a subpopulation of cells expresses CD90 and CD276 **(B)** and **(C).** Expression of selected markers on cultured TSCs and TECs at day10 is shown as blue and red histograms, respectively. **(B)** The mesenchymal / stromal cell-specific key markers CD90 and CD200 are predominantly expressed on $CD49f^+CD49a^+CD144^-$ TSCs, whereas CD56 and TNAP are expressed only in a subpopulation of TSCs. **(C)** The endothelial cell specific key markers CD34, CD31, CD144 and VEGFR2 are predominantly expressed on $CD49f^+CD49a^+CD144^+$ TECs. **(D)** Treatment of cultured TSCs and TECs at day 10 with Dio labeled Ac-LDL shows selective uptake of Ac-LDL in TECs but not in TSCs.

9.9. Differentiation capacity of TSCs and TECs

To analyze the differentiation capacity of TSCs and TECs, sorted $CD49f^{+}CD49a^{+}CD144^{+/-}$ cells were expanded until Passage 2 in ES medium or VascuLife cell culture medium. Defined numbers of the resulting TSCs were then induced to differentiate into cells of the osteogenic, adipogenic and chondrogenic lineages. TSCs were able to differentiate into osteoblasts and chondrocytes as verified by Alizarin Red S staining and Alcian blue staining, respectively (Fig. 15A). Surprisingly, TSCs were unable to differentiate into adipocytes, which is in contrast to the capacity of bone marrow derived mesenchymal stromal cells. These cells, which were used as a positive control, were indeed able to differentiate into osteogenic, adipogenic and chondrogenic lineages (Fig. 15B).

The angiogenic differentiation capability of TECs was assessed using an in vitro endothelial tube formation assay. After twenty four hours of induction of tube formation on Matrigel, TECs exhibited capillary like interconnected structures (Fig. 15C). To further confirm the endothelial differentiation of TECs, cells on Matrigel were stained with CD146 and vWF and visualized under a fluorescence microscope. The data presented in Fig. 15C demonstrates significant CD146 and vWF staining of TECs under induction culture conditions, further supporting their endothelial commitment.

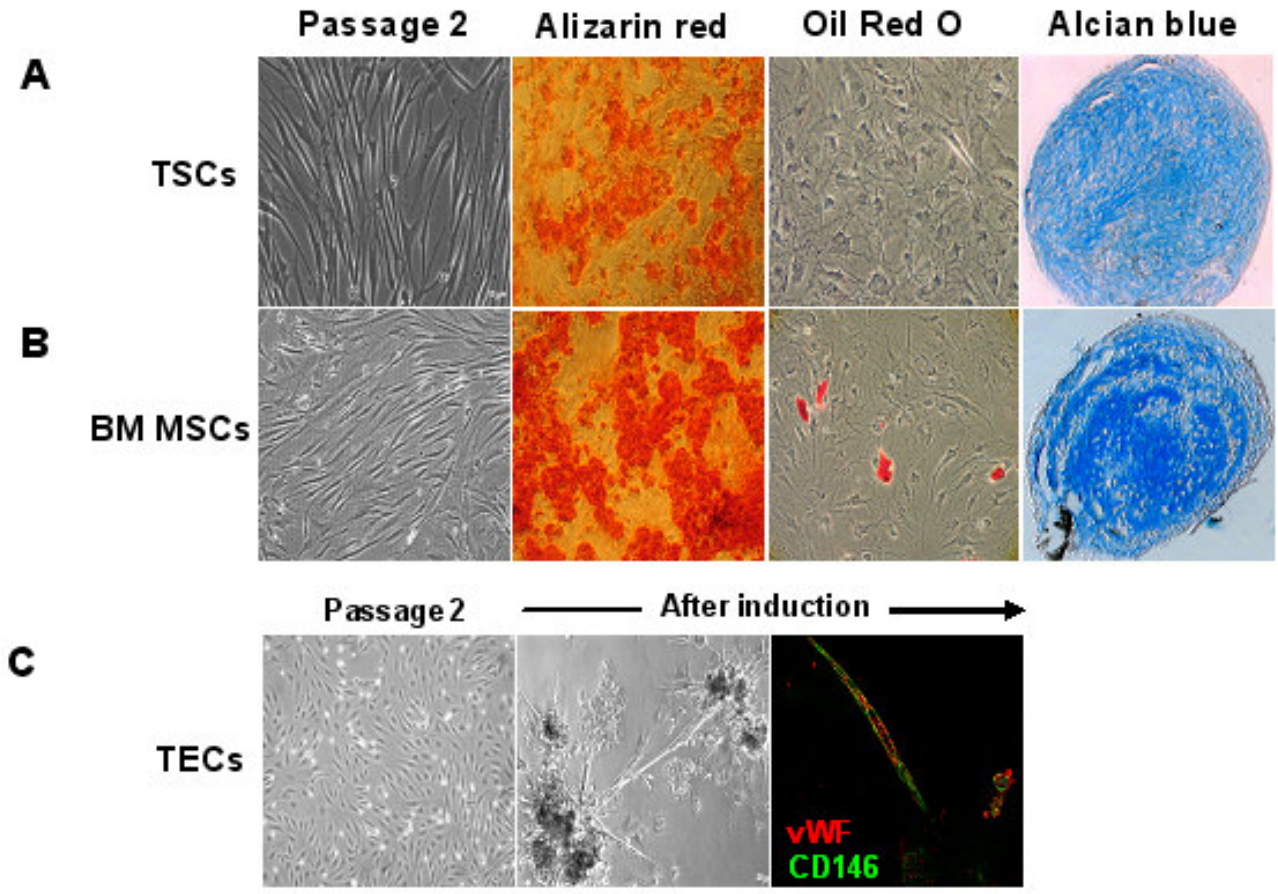


Fig. 15: Differentiation potential of testis derived TSCs and TECs. (A) Expanded TSCs were induced to differentiate into osteogenic, adipogenic or chondrogenic lineages and stained as described in the methods. Photographs were taken on a Zeiss Observer.Z1 AX10 microscope. Note that TSCs (passage 2) were able to differentiate into osteoblasts (alizarin red) and chondrocytes (alcian blue) but not into adipocytes (oil red O). **(B)** In contrast, cultured bone marrow MSCs (passage 2) were able to additionally differentiate adipocytes. **(C)** Morphology of TECs before (left) and 48 hours after induction of angiogenesis (middle, right). The expression of the endothelial associated markers vWF (red) and CD146 (green) was assessed by immunofluorescence microscopy. 4, 6-diamidino-2 phenylindole (DAPI) was used to counter stain cell nuclei (blue). Images are shown at magnification of 10×. Note that TECs developed into capillary tube-like structures and stained positive for vWF and CD146.

10. Discussion

Aim of the current study was to evaluate the most suitable marker combinations for the prospective isolation of spermatogonia from adult human testis by introducing novel targets. I have identified cell-surface markers that serve as valuable tools for the simultaneous isolation of spermatogonia, TSCs and TECs from human adult testis. In a first step, I prepared single cell suspensions from testicular samples and screened a large panel of commercially available and in-house generated antibodies for their reactivity with $CD49f^+$ cells, as CD49f is the most widely used marker for the isolation of SSCs [6,18,29]. However, the $CD49f^+$ population does not contain only spermatogonia but also other cell types including fibroblasts, endothelial cells and smooth muscle cells, which are also attached to the basal lamina [18,74]. To eliminate these undesired cells, I identified CD49a and CD144 as suitable targets to further fractionate the $CD49f^+$ population. As CD49a and CD144 are selective markers of the fibroblast and endothelial compartments [75-77], I expected to separate most of the other contaminating cells from spermatogonia. Using this strategy, I was able to enrich spermatogonia to almost complete purity. Using novel in-house generated monoclonal antibodies against CD164, SUSD2 and SSEA-4, I was able to identify a promising target combination to prospectively isolate spermatogonia. I could demonstrate that all $SUSD2^+$ spermatogonia and a subpopulation of $CD164^+$ spermatogonia coexpress SSEA-4. In line with this finding, Izadyar *et al.* showed that repopulating human SSCs express SSEA-4 [18]. They transplanted MACS purified $SSEA4^+$ SSCs into busulfan treated recipient mouse testis and showed that $SSEA4^+$ SSCs could reconstitute the basement membrane. This suggests that $CD49f^+CD49a^-SSEA4^+CD164^+/SUSD2^+$ cells represent repopulating SSCs in the human testis. But the repopulating efficiency of $CD49f^+CD49a^-SSEA4^-CD164^+$ spermatogonia remains an open question.

The DAZL gene encodes a germ cell specific RNA-binding protein which is essential for spermatogenesis in humans. It has been proposed that DAZL binds to RNA in the cytoplasm of germ cells and controls spermatogenesis [78,79]. VASA is so far the only known gene in mammals whose expression is specific for the germ cell lineage [80,81]. The VASA protein is localized in the cytoplasm of germline cells and is functionally relevant for germline establishment [80]. Our qRT-PCR and immunohistochemical analyses on $CD49f^{+}CD49a^{-}SSEA4^{+/-}CD164^{+}$ and $CD49f^{+}CD49a^{-}SSEA4^{+}SUSD2^{+}$ cells revealed coexpression of VASA and DAZL, indicating a germline lineage origin of these cells.

Desmoglein 2 (DSG2), human telomerase reverse transcriptase (hTERT), undifferentiated embryonic cell transcription factor 1 (UTF1), zinc-finger, BTB-domain containing 16 (ZBTB16; PLZF), and GRR-a1 are reported as specific biomarkers of human spermatogonia [16,21,22,80,82-87]. In particular hTERT, PLZF and GFR-a1 are expressed at high levels in undifferentiated spermatogonia, which decrease upon differentiation [18,82,83,86,87]. In addition, UTF1 expression decreases in human embryonic stem cells upon differentiation [88]. In embryonic stem cells, this gene is responsible for the maintenance of rapid cell proliferation by repressing Arf expression through Dcp1a-mediated mRNA pruning [89]. In accordance to this, $CD49f^{+}CD49a^{-}SSEA4^{+/-}CD164^{+}$ and $CD49f^{+}CD49a^{-}SSEA4^{+}SUSD2^{+}$ spermatogonia expressed these markers. Interestingly, PLZF, GFR-a1 and UTF1 expression was higher in $CD49f^{+}CD49a^{-}SSEA4^{+}CD164^{+}$ spermatogonia compared to $CD49f^{+}CD49a^{-}SSEA4^{-}CD164^{+}$ spermatogonia, indicating that $CD49f^{+}CD49a^{-}SSEA4^{+}CD164^{+}$ spermatogonia are less differentiated. Studies are in progress to investigate the characteristics of both $CD49f^{+}CD49a^{-}SSEA4^{-}CD164^{+}$ and $CD49f^{+}CD49a^{-}SSEA4^{+}CD164^{+}$ spermatogonia and whether $CD49f^{+}CD49a^{-}SSEA4^{-}CD164^{+}$ spermatogonia are derived from $CD49f^{+}CD49a^{-}SSEA4^{+}CD164^{+}$ spermatogonia.

Human SSCs are reported to express CD90 but not ckit [18]. This is in line with the fact that $CD49f^{+}CD49a^{-}SSEA4^{+}$ spermatogonia are positive for CD9, CD90 and CD276 and negative for CD133, CD318, CD324, CD340, SSEA-3, TRA-1-60, TRA-1-81, HER3 and c-kit. However, I could demonstrate that CD9, CD90 and CD276 are not only expressed on spermatogonia, but also on many other cell types in the testis, indicating that none of these markers are highly selective for spermatogonia. It was reported that CD49f, CD90, CD133 and GFR-α1 are suitable markers for the prospective isolation of SSCs by MACS [6]. In our studies, I was unable to detect CD133 on primary spermatogonia. In addition, I observed that spermatogonia are $CD49f^{dim}$ whereas $CD49f^{bright}$ cells consisted of mainly somatic cells. This raises a question whether the selection of spermatogonia by MACS with the single use of CD49f for isolation is sufficient to obtain pure spermatogonia. In addition, it is very likely that many spermatogonia remain in the negative fraction because they express CD49f only at low levels.

In my studies, I observed that cultured spermatogonia derived from primary fractionated spermatogonia showed a significant change in their expression pattern. In line with this, the dendrogram cluster analysis revealed that cultured spermatogonia clustered with fibroblasts. Therefore, it is advisable to perform expression analysis on primary cells and not on their cultured counterparts. Our findings are also of relevance for other cultured cells types such as mesenchymal stem cells or embryonic stem cells.

The existence of spermatogonia in the testis offers clinically relevant options for preservation and restoration of male fertility. New approaches based on male germ cell transplantation and testicular tissue grafting can be applied to generate a limited number of sperm cells and could therefore be considered important new avenues for restoration of fertility [90]. The cause of male infertility can be attributed to disorders of germ cell proliferation and differentiation or somatic cell dysfunction. In addition to this, cryptorchid testis, testicular cancer and cancer

therapy significantly contribute to the cause [90,91]. Although most of the causes are related to genetic disorders, testicular cancer and cancer therapy are reported to be major cause for male infertility.

Treatment regimens for cancer patients involve chemotherapy and radiotherapy which target rapidly dividing cells. Although there is no active production of spermatozoa in the prepubertal testis, the spermatogonia are constantly dividing to populate the growing seminiferous tubules and are thus targets for gonadotoxic treatments [92]. The most common cancer type in children is acute lymphoblastic leukaemia (ALL) and it involves treatment with low risk regimens [92]. Long term follow up of childhood ALL survivors indicates that this treatment does not totally deplete spermatogonia and that spermatogenesis is reinitiated from the surviving stem cell population [92,93]. However, twenty percent of children with ALL exhibit high risk characteristics and, in addition, 20 % of the patients at standard risk experience relapse, which requires intensive therapy with alkylating agents, hematopoietic stem cell transplantation or testicular irradiation. These patients have high risk for long term testicular dysfunction [93]. When these patients want to bank testicular tissue before a more severe therapy, a substantial reduction in spermatogonia may have occurred. Spontaneous recovery of spermatogenesis is possible but this depends on the survival of spermatogonia and their ability to differentiate. Next to cancer patients, patients suffering from blood diseases are often treated with chemo- and radiotherapy as a conditioning therapy for bone marrow transplantation. At present, follow-up data on childhood cancer survivors are scarce. A follow-up study in 51 long term survivors of childhood ALL treated with <10 g/m^2cyclophosphamide indicated that testicular size and semen quality were not altered. However, impaired Leydig cell function was indicated by decreased serum-free testosterone levels. On the other hand, patients who received higher doses of cyclophosphamide or testicular irradiation had no or very few spermatozoa [92]. These patients are at high risk for lifelong sterility and should thus be offered with fertility preservation options.

Several fertility preservation options have been discussed extensively in the literature [90]. The first aim should always be to store the sperm cells. In case the ejaculate does not contain mature sperm cells, testicular sperm extraction might be an option. When the testis is still immature as in case of prepubertal testis, cryopreservation of spermatogonia could be an option. An alternative to conventional freezing is vitrification. Vitrification involves the use of increasing concentrations of cryoprotectants and ultrarapid cooling to avoid ice crystal formation. Recently, the efficiency of vitrification was explored using immature human testicular tissue. The tubular integrity and the proliferation potential of spermatogonia could be maintained [94].

There are different options to cryopreserve spermatogonia; as a testicular cell suspension, as an enriched cell suspension or as testicular tissue. Testicular cell suspensions contain several cell types differing in size, shape and water content, requiring different freezing conditions and media compositions for optimal preservation of viability and functionality. The type of cryoprotectant, its concentration and the cooling rate could all influence cell survival. For spermatogonia transplantation, it is particularly important that the spermatogonia survive the freezing procedure and maintain their functionality. Therefore, protocols have been established focusing on the preservation of spermatogonia [95-98]. Frozen-thawed bovine spermatogonia were able to colonize the mouse testis, although with lower efficiency compared with the fresh population [97]. However, the cryopreservation of testicular tissue might be better, leaving options open to perform testicular tissue grafting or spermatogonia transplantation/ *in vitro* maturation. But it is not clear whether enzymatic digestion of frozen-thawed tissue would result in better viability compared with digestion before freezing. Testicular tissue freezing preserves the cell–cell contacts and requires more permeable cryoprotectants. The best preservation of morphology and hormonal activity was achieved when dimethylsulphoxide (DMSO) was used to freeze adult testicular tissue [99]. The feasibility of the selected protocol was later confirmed using prepubertal testicular tissue [99]. Most of the centres offering testicular tissue banking use a controlled slow freezing protocol with DMSO as cryoprotectant [99,100]. As this method is time consuming and requires expensive equipment, a user-friendly and time-saving uncontrolled slow-freezing protocol was proposed. After having proved the effectiveness in a mouse model [101], the uncontrolled slow-freezing method using 1.5 M DMSO and 0.1 M sucrose was validated using adult human testis tissue [101]. This protocol did not only preserve the seminiferous epithelium and the interstitial compartment at a structural level, but the spermatogonia also maintained the potential to divide. More research is required to confirm these findings using

human prepubertal tissue. As long as there is no efficient way to evaluate the functionality of human testicular tissue after freezing, it is difficult to conclude which of the tested protocols is optimal. It has been shown that cell viability does not necessarily correspond to the functional capacity of the spermatogonia [96].

There is evidence that spermatogenesis in ectopic grafts may not be everlasting. Due to the lack of an excretory system in the grafts, spermatozoa and fluid may accumulate causing damage to the epithelium. Also for intratesticular grafts, it is still an open question whether connections can be established between grafted and endogenous tubules, ensuring a functional excretory system. In contrast to grafting strategies, natural conception might be possible after the injection of spermatogonia into seminiferous tubules. This technique was first described in the mouse almost 20 years ago [25,102] and has since been used widely in both fundamental and translational research. Five years later, spermatogonia injection was proposed as a fertility preservation strategy for the first time [103].

In mouse, efferent duct in the rete testis is the most commonly used route to transplant spermatogonia. This allows the filling of several seminiferous tubules with one single puncture [104]. Goossens et al. experienced that testes from aging donors (aged 40–65 years) were more difficult to inject than the ones from aged donors (aged 65–83 years), because the older testes have less tension [90]. Since testes of boys who were treated with testicular irradiation or with high doses of alkylating agents do not contain differentiated germ cells [105], their testes may have a consistency similar to those from the aged donors. Very recently, rhesus monkey spermatogonia have been used for autologous and allogeneic transplantation. Spermatogonia infusion was performed using ultrasound-guided rete testis injections. Cells were injected under slow constant pressure and chased with saline. After having reached maturity, mature sperm cells could be found in the ejaculate in 60 % of the prepubertal recipients and sperm cells were able to fertilize oocytes in intracytoplasmic sperm injection [23]. This demonstration of functional donor spermatogenesis following spermatogonia transplantation in non-human primates is an important milestone in the translation of spermatogonia transplantation to a clinical setting.

Many paediatric malignancies are capable of metastasizing through the blood, with a potential risk for contamination of the collected testicular tissue. The transplantation of as few as 20 leukemic cells could cause malignant recurrence in rats [106]. In the human, the threshold number of malignant cells able to cause malignant relapse when transplanted to the

testis is unknown. Therefore, it is of immense importance to detect even the slightest contamination of the testicular tissue. In case of contamination, the separation of spermatogonia from malignant cells before transplantation is necessary.

So far, *in vitro* generation of human sperm cells from spermatogonia has not been reported. Late spermatids could be produced from late pachytene spermatocytes and secondary spermatocytes in co-cultures obtained from azoospermic patients. The *in vitro* matured spermatids were microinjected into oocytes, but showed low fertilization rates and chromosomal abnormalities were found in all generated embryos [107]. The initiation of meiosis *in vitro* remains the critical event to achieve full spermatogenesis. More recently, haploid human cells were generated using isolated $CD49f^{+}$ cells from an azoospermic patient [108]. Future research is definitely needed to establish full human spermatogenesis *in vitro*. *In vitro* spermatogenesis could also be an alternative to circumvent the transmission of malignant cells to patients at risk for malignant contamination in their testis, for example boys with leukaemia.

When all the above mentioned obstacles are overcome and when this new technology of spermatogonial transplantation has proved to be safe, still some ethical considerations will remain. One of the first difficulties that will occur is the need to obtain proper informed consent from a prepubertal boy. In this decision, the parents will play a very important role [109]. However, the rights of the child should be considered. Moreover, testicular biopsy is an invasive procedure that may have complications for the child. Therefore, the surgery should be optimally timed with regard to the necessary cancer treatments [110]. Also, the timing of the transplantation of the harvested spermatogonia could be crucial for the physical and mental well-being of the patient. Another ethical concern would be the imprinting status of the haploid products generated by in vitro spermatogenesis. Easley et al. showed that haploid products derived in vitro are epigenetically similar to fertile human sperm on two

loci, but all imprinted genes would have to be examined before this technique could be considered in a clinical setting [111].

Additionally, alternative methods of deriving pluripotent stem cells can circumvent the long standing barriers and ethical concerns in using human embryonic stem cells (hESC). Recent work in mouse have shown that spermatogonia are unipotent *in vivo*, but when removed from their natural environment they spontaneously become pluripotent sharing many features like epigenetic, gene expression and miRNA profiles [8,112-114]. These germ cell derived stem cells also circumvent the serious ethical issues associated with hESC research.

No therapeutic solution is available to restore the compromised fertility of prepubescent boys after cancer treatment. Spermatogonia transplantation is known to have the potential to reconstitute spermatogenesis, enabling the fertility of the recipient. Considering the fact that patients are already preserving testicular tissue and the successful regeneration of spermatogenesis in mice, rats, monkeys, goats, bulls, pigs, sheep, dogs [18,25,26,102,104,115-118] and most importantly in nonhuman primate models [23] makes the spermatogonia transplantation promising. Because some hospitals cryopreserve testicular biopsies from prepubertal boys with cancer, efficient techniques to isolate spermatogonia from small biopsies will favor the clinical translation. For this purpose, CD164 and SUSD2 may serve as attractive targets to prospectively isolate spermatogonia. The cotransplantation of spermatogonia and sertoli cells, which are crucial components of the testicular niche, may additionally increase the efficiency of reconstituting spermatogenesis. For this purpose, I identified a novel marker combination to isolate these cell types. Finally, the knowledge about the composite phenotype of defined testicular cell subsets and their reliable isolation may contribute to a more accurate discrimination between healthy and malignant cells in human testis.

REFERENCES

1. **CHIQUOINE AD. (1954). The identification, origin, and migration of the primordial germ cells in the mouse embryo. Anat Rec 118:135-146.**

2. **Robinson LL, TL Gaskell, PT Saunders and RA Anderson. (2001). Germ cell specific expression of c-kit in the human fetal gonad. Mol Hum Reprod 7:845-852.**

3. **Kolasa A, K Misiakiewicz, M Marchlewicz and B Wiszniewska. (2012). The generation of spermatogonial stem cells and spermatogonia in mammals. Reprod Biol 12:5-23.**

4. **Clermont Y. (1966). Spermatogenesis in man. A study of the spermatogonial population. Fertil Steril 17:705-721.**

5. **HELLER CG, Y Clermont. (1963). Spermatogenesis in man: an estimate of its duration. Science 140:184-186.**

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

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

8. **Izadyar F, F Pau, J Marh, N Slepko, T Wang, R Gonzalez, T Ramos, K Howerton, C Sayre and F Silva. (2008). Generation of multipotent cell lines from a distinct population of male germ line stem cells. Reproduction 135:771-784.**

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

10. **Stevens LC. (1984). Spontaneous and experimentally induced testicular teratomas in mice. Cell Differ 15:69-74.**

11. **Durcova-Hills G, F Tang, G Doody, R Tooze and MA Surani. (2008). Reprogramming primordial germ cells into pluripotent stem cells. PLoS One 3:e3531.**

12. **Muramatsu T. (1988). Alterations of cell-surface carbohydrates during differentiation and development. Biochimie 70:1587-1596.**

13. **Shamblott MJ, J Axelman, S Wang, EM Bugg, JW Littlefield, PJ Donovan, PD Blumenthal, GR Huggins and JD Gearhart. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc Natl Acad Sci U S A 95:13726-13731.**

14. **Stevens LC. (1964). EXPERIMENTAL PRODUCTION OF TESTICULAR TERATOMAS IN MICE. Proc Natl Acad Sci U S A 52:654-661.**

15. **Mise N, T Fuchikami, M Sugimoto, S Kobayakawa, F Ike, T Ogawa, T Tada, S Kanaya, T Noce and K Abe. (2008). Differences and similarities in the developmental status of embryo-derived stem cells and primordial germ cells revealed by global expression profiling. Genes Cells 13:863-877.**

16. **von Kopylow K, H Staege, W Schulze, H Will and C Kirchhoff. (2012). Fibroblast growth factor receptor 3 is highly expressed in rarely dividing human type A spermatogonia. Histochem Cell Biol 138:759-772.**

17. **Kokkinaki M, N Sahibzada and N Golestaneh. (2011). Human induced pluripotent stem-derived retinal pigment epithelium (RPE) cells exhibit ion transport, membrane potential, polarized vascular endothelial growth factor secretion, and gene expression pattern similar to native RPE. Stem Cells 29:825-835.**

18. **Izadyar F, J Wong, C Maki, J Pacchiarotti, T Ramos, K Howerton, C Yuen, S Greilach, HH Zhao, M Chow, YC Chow, J Rao, J Barritt, N Bar-Chama and A Copperman. (2011). Identification and characterization of repopulating spermatogonial stem cells from the adult human testis. Hum Reprod 26:1296-1306.**

19. **He Z, M Kokkinaki, J Jiang, I Dobrinski and M Dym. (2010). Isolation, characterization, and culture of human spermatogonia. Biol Reprod 82:363-372.**

20. **Dym M, M Kokkinaki and Z He. (2009). Spermatogonial stem cells: mouse and human comparisons. Birth Defects Res C Embryo Today 87:27-34.**

21. **He Z, M Kokkinaki, J Jiang, W Zeng, I Dobrinski and M Dym. (2012). Isolation of human male germ-line stem cells using enzymatic digestion and magnetic-activated cell sorting. Methods Mol Biol 825:45-57.**

22. **Grisanti L, I Falciatori, M Grasso, L Dovere, S Fera, B Muciaccia, A Fuso, V Berno, C Boitani, M Stefanini and E Vicini. (2009). Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. Stem Cells 27:3043-3052.**

23. **Hermann BP, M Sukhwani, F Winkler, JN Pascarella, KA Peters, Y Sheng, H Valli, M Rodriguez, M Ezzelarab, G Dargo, K Peterson, K Masterson, C Ramsey, T Ward, M Lienesch, A Volk, DK Cooper, AW Thomson, JE Kiss, MC Penedo, GP Schatten, S Mitalipov and KE Orwig. (2012). Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. Cell Stem Cell 11:715-726.**

24. **Clark AT, BT Phillips and KE Orwig. (2011). Fruitful progress to fertility: male fertility in the test tube. Nat Med 17:1564-1565.**

25. **Brinster RL, MR Avarbock. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci U S A 91:11303-11307.**

26. Kim Y, D Turner, J Nelson, I Dobrinski, M McEntee and AJ Travis. (2008). Production of donor-derived sperm after spermatogonial stem cell transplantation in the dog. Reproduction 136:823-831.

27. Hadley MA, M Dym. (1987). Immunocytochemistry of extracellular matrix in the lamina propria of the rat testis: electron microscopic localization. Biol Reprod 37:1283-1289.

28. de C, I. (1993). The alpha 6 beta 1 integrin is a laminin receptor for developing retinal neurons. Cytotechnology 11 Suppl 1:S41-S43.

29. Shinohara T, MR Avarbock and RL Brinster. (1999). beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 96:5504-5509.

30. Deschaseaux F, F Gindraux, R Saadi, L Obert, D Chalmers and P Herve. (2003). Direct selection of human bone marrow mesenchymal stem cells using an anti-CD49a antibody reveals their CD45med,low phenotype. Br J Haematol 122:506-517.

31. Mutin M, F Dignat-George and J Sampol. (1997). Immunologic phenotype of cultured endothelial cells: quantitative analysis of cell surface molecules. Tissue Antigens 50:449-458.

32. Yang S, J Graham, JW Kahn, EA Schwartz and ME Gerritsen. (1999). Functional roles for PECAM-1 (CD31) and VE-cadherin (CD144) in tube assembly and lumen formation in three-dimensional collagen gels. Am J Pathol 155:887-895.

33. Saito S, H Aoki, A Ito, S Ueno, T Wada, K Mitsuzuka, M Satoh, Y Arai and T Miyagi. (2003). Human alpha2,3-sialyltransferase (ST3Gal II) is a stage-specific embryonic antigen-4 synthase. J Biol Chem 278:26474-26479.

34. Katagiri YU, N Kiyokawa, K Nakamura, H Takenouchi, T Taguchi, H Okita, A Umezawa and J Fujimoto. (2005). Laminin binding protein, 34/67 laminin receptor, carries stage-specific embryonic antigen-4 epitope defined by monoclonal antibody Raft.2. Biochem Biophys Res Commun 332:1004-1011.

35. Kannagi R, NA Cochran, F Ishigami, S Hakomori, PW Andrews, BB Knowles and D Solter. (1983). Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. EMBO J 2:2355-2361.

36. Watt SM, LH Butler, M Tavian, HJ Buhring, I Rappold, PJ Simmons, AC Zannettino, D Buck, A Fuchs, R Doyonnas, JY Chan, JP Levesque, B Peault and I Roxanis. (2000). Functionally defined CD164 epitopes are expressed on CD34(+) cells throughout ontogeny but display distinct distribution patterns in adult hematopoietic and nonhematopoietic tissues. Blood 95:3113-3124.

37. Doyonnas R, CJ Yi-Hsin, LH Butler, I Rappold, JE Lee-Prudhoe, AC Zannettino, PJ Simmons, HJ Buhring, JP Levesque and SM Watt. (2000). CD164 monoclonal antibodies that block hemopoietic progenitor cell adhesion and

proliferation interact with the first mucin domain of the CD164 receptor. J Immunol 165:840-851.

38. **Watt SM, JY Chan. (2000). CD164--a novel sialomucin on CD34+ cells. Leuk Lymphoma 37:1-25.**

39. **Zannettino AC, HJ Buhring, S Niutta, SM Watt, MA Benton and PJ Simmons. (1998). The sialomucin CD164 (MGC-24v) is an adhesive glycoprotein expressed by human hematopoietic progenitors and bone marrow stromal cells that serves as a potent negative regulator of hematopoiesis. Blood 92:2613-2628.**

40. **Forde S, BJ Tye, SE Newey, M Roubelakis, J Smythe, CP McGuckin, R Pettengell and SM Watt. (2007). Endolyn (CD164) modulates the CXCL12-mediated migration of umbilical cord blood CD133+ cells. Blood 109:1825-1833.**

41. **Jorgensen-Tye B, JP Levesque, L Royle, R Doyonnas, JY Chan, RA Dwek, PM Rudd, DJ Harvey, PJ Simmons and SM Watt. (2005). Epitope recognition of antibodies that define the sialomucin, endolyn (CD164), a negative regulator of haematopoiesis. Tissue Antigens 65:220-239.**

42. **Jenne D. (1991). Homology of placental protein 11 and pea seed albumin 2 with vitronectin. Biochem Biophys Res Commun 176:1000-1006.**

43. **Bellacchio E. (2012). In silico analysis of the two tandem somatomedin B domains of ENPP1 reveals hints on the homodimerization of the protein. J Cell Physiol 227:3566-3574.**

44. **Duraisamy S, S Ramasamy, S Kharbanda and D Kufe. (2006). Distinct evolution of the human carcinoma-associated transmembrane mucins, MUC1, MUC4 AND MUC16. Gene 373:28-34.**

45. **Ciccarelli FD, T Doerks and P Bork. (2002). AMOP, a protein module alternatively spliced in cancer cells. Trends Biochem Sci 27:113-115.**

46. **Perkins SJ, KF Smith, SC Williams, PI Haris, D Chapman and RB Sim. (1994). The secondary structure of the von Willebrand factor type A domain in factor B of human complement by Fourier transform infrared spectroscopy. Its occurrence in collagen types VI, VII, XII and XIV, the integrins and other proteins by averaged structure predictions. J Mol Biol 238:104-119.**

47. **Colombatti A, P Bonaldo and R Doliana. (1993). Type A modules: interacting domains found in several non-fibrillar collagens and in other extracellular matrix proteins. Matrix 13:297-306.**

48. **Norman DG, PN Barlow, M Baron, AJ Day, RB Sim and ID Campbell. (1991). Three-dimensional structure of a complement control protein module in solution. J Mol Biol 219:717-725.**

49. **Sugahara T, Y Yamashita, M Shinomi, Y Isobe, B Yamanoha, H Iseki, A Takeda, Y Okazaki, K Kawai, H Suemizu and T Andoh. (2007). von Willebrand factor type D domain mutant of SVS-1/SUSD2, vWD(m), induces apoptosis in HeLa cells. Cancer Sci 98:909-915.**

50. Sugahara T, Y Yamashita, M Shinomi, B Yamanoha, H Iseki, A Takeda, Y Okazaki, Y Hayashizaki, K Kawai, H Suemizu and T Andoh. (2007). Isolation of a novel mouse gene, mSVS-1/SUSD2, reversing tumorigenic phenotypes of cancer cells in vitro. Cancer Sci 98:900-908.

51. Masuda H, SS Anwar, HJ Buhring, JR Rao and CE Gargett. (2012). A novel marker of human endometrial mesenchymal stem-like cells. Cell Transplant

52. Rajaraman G, J White, KS Tan, D Ulrich, A Rosamilia, J Werkmeister and CE Gargett. (2012). Optimization and Scale-up Culture of Human Endometrial Multipotent Mesenchymal Stromal Cells: Potential for Clinical Application. Tissue Eng Part C Methods

53. Sivasubramaniyan K, A Harichandan, S Schumann, M Sobiesiak, C Lengerke, A Maurer, H Kalbacher and HJ Buhring. (2013). Prospective isolation of mesenchymal stem cells from human bone marrow using novel antibodies directed against Sushi domain containing 2 (SUSD2). Stem Cells Dev

54. Sobiesiak M, K Sivasubramaniyan, C Hermann, C Tan, M Orgel, S Treml, F Cerabona, P de Zwart, U Ochs, CA Muller, CE Gargett, H Kalbacher and HJ Buhring. (2010). The mesenchymal stem cell antigen MSCA-1 is identical to tissue non-specific alkaline phosphatase. Stem Cells Dev 19:669-677.

55. Vogel W, F Grunebach, CA Messam, L Kanz, W Brugger and HJ Buhring. (2003). Heterogeneity among human bone marrow-derived mesenchymal stem cells and neural progenitor cells. Haematologica 88:126-133.

56. Wirths S, E Malenke, T Kluba, S Rieger, MR Muller, S Schleicher, vW Hann, F Nagl, F Fend, W Vogel, F Mayer, L Kanz, HJ Buhring and HG Kopp. (2013). Shared cell surface marker expression in mesenchymal stem cells and adult sarcomas. Stem Cells Transl Med 2:53-60.

57. Rappold I, BL Ziegler, I Kohler, S Marchetto, O Rosnet, D Birnbaum, PJ Simmons, AC Zannettino, B Hill, S Neu, W Knapp, R Alitalo, K Alitalo, A Ullrich, L Kanz and HJ Buhring. (1997). Functional and phenotypic characterization of cord blood and bone marrow subsets expressing FLT3 (CD135) receptor tyrosine kinase. Blood 90:111-125.

58. Buhring HJ, T Muller, R Herbst, S Cole, I Rappold, W Schuller, X Zhu, U Fritzsch, C Faul, S Armeanu, A Ullrich, G Klein and H Schmidt. (1996). The adhesion molecule E-cadherin and a surface antigen recognized by the antibody 9C4 are selectively expressed on erythroid cells of defined maturational stages. Leukemia 10:106-116.

59. Battula VL, S Treml, PM Bareiss, F Gieseke, H Roelofs, P de Zwart, I Muller, B Schewe, T Skutella, WE Fibbe, L Kanz and HJ Buhring. (2009). Isolation of functionally distinct mesenchymal stem cell subsets using antibodies against CD56, CD271, and mesenchymal stem cell antigen-1. Haematologica 94:173-184.

60. Buhring HJ, VL Battula, S Treml, B Schewe, L Kanz and W Vogel. (2007). Novel markers for the prospective isolation of human MSC. Ann N Y Acad Sci 1106:262-271.

61. Lanza F, S Moretti, B Castagnari, F Montanelli, A Latorraca, L Ferrari, A Bardi, M Dominici, D Campioni, M Dabusti, N Piva, G Lodi, R Reverberi and G Castoldi. (1999). Assessment of distribution of CD34 epitope classes in fresh and cryopreserved peripheral blood progenitor cells and acute myeloid leukemic blasts. Haematologica 84:969-977.

62. Buhring HJ, PJ Simmons, M Pudney, R Muller, D Jarrossay, A van Agthoven, M Willheim, W Brugger, P Valent and L Kanz. (1999). The monoclonal antibody 97A6 defines a novel surface antigen expressed on human basophils and their multipotent and unipotent progenitors. Blood 94:2343-2356.

63. Buhring HJ, S Treml, F Cerabona, P de Zwart, L Kanz and M Sobiesiak. (2009). Phenotypic characterization of distinct human bone marrow-derived MSC subsets. Ann N Y Acad Sci 1176:124-134.

64. Harichandan A, HJ Buhring. (2011). Prospective isolation of human MSC. Best Pract Res Clin Haematol 24:25-36.

65. Harichandan A, K Sivasubramaniyan and HJ Buhring. (2013). Prospective Isolation and Characterization of Human Bone Marrow-Derived MSCs. Adv Biochem Eng Biotechnol 129:1-17.

66. Battula VL, PM Bareiss, S Treml, S Conrad, I Albert, S Hojak, H Abele, B Schewe, L Just, T Skutella and HJ Buhring. (2007). Human placenta and bone marrow derived MSC cultured in serum-free, b-FGF-containing medium express cell surface frizzled-9 and SSEA-4 and give rise to multilineage differentiation. Differentiation 75:279-291.

67. Giesert C, A Marxer, DR Sutherland, AC Schuh, L Kanz and HJ Buhring. (2003). Antibody W7C5 defines a CD109 epitope expressed on CD34+ and. Ann N Y Acad Sci 996:227-230.

68. Sivasubramaniyan K, D Lehnen, R Ghazanfari, M Sobiesiak, A Harichandan, E Mortha, N Petkova, S Grimm, F Cerabona, P de Zwart, H Abele, WK Aicher, C Faul, L Kanz and HJ Buhring. (2012). Phenotypic and functional heterogeneity of human bone marrow- and amnion-derived MSC subsets. Ann N Y Acad Sci 1266:94-106.

69. Andrews PW. (1988). Human teratocarcinomas. Biochim Biophys Acta 948:17-36.

70. Teshima S, Y Shimosato, S Hirohashi, Y Tome, I Hayashi, H Kanazawa and T Kakizoe. (1988). Four new human germ cell tumor cell lines. Lab Invest 59:328-336.

71. Josephson R, CJ Ording, Y Liu, S Shin, U Lakshmipathy, A Toumadje, B Love, JD Chesnut, PW Andrews, MS Rao and JM Auerbach. (2007). Qualification of embryonal carcinoma 2102Ep as a reference for human embryonic stem cell research. Stem Cells 25:437-446.

72. Dieckmann KP, NE Skakkebaek. (1999). Carcinoma in situ of the testis: review of biological and clinical features. Int J Cancer 83:815-822.

73. Hoei-Hansen CE, E Rajpert-De Meyts, G Daugaard and NE Skakkebaek. (2005). Carcinoma in situ testis, the progenitor of testicular germ cell tumours: a clinical review. Ann Oncol 16:863-868.

74. Hadley MA, SW Byers, CA Suarez-Quian, D Djakiew and M Dym. (1988). In vitro models of differentiated Sertoli cell structure and function. In Vitro Cell Dev Biol 24:550-557.

75. Appleby SL, MP Cockshell, JB Pippal, EJ Thompson, JM Barrett, K Tooley, S Sen, WY Sun, R Grose, I Nicholson, V Levina, I Cooke, G Talbo, AF Lopez and CS Bonder. (2012). Characterization of a Distinct Population of Circulating Human Non-Adherent Endothelial Forming Cells and Their Recruitment via Intercellular Adhesion Molecule-3. PLoS One 7:e46996.

76. Goo YA, DR Goodlett, LE Pascal, KD Worthington, RL Vessella, LD True and AY Liu. (2005). Stromal mesenchyme cell genes of the human prostate and bladder. BMC Urol 5:17.

77. Rider DA, T Nalathamby, V Nurcombe and SM Cool. (2007). Selection using the alpha-1 integrin (CD49a) enhances the multipotentiality of the mesenchymal stem cell population from heterogeneous bone marrow stromal cells. J Mol Histol 38:449-458.

78. Reynolds N, B Collier, K Maratou, V Bingham, RM Speed, M Taggart, CA Semple, NK Gray and HJ Cooke. (2005). Dazl binds in vivo to specific transcripts and can regulate the pre-meiotic translation of Mvh in germ cells. Hum Mol Genet 14:3899-3909.

79. Zeng M, W Deng, X Wang, W Qiu, Y Liu, H Sun, D Tao, S Zhang and Y Ma. (2008). DAZL binds to the transcripts of several Tssk genes in germ cells. BMB Rep 41:300-304.

80. Castrillon DH, BJ Quade, TY Wang, C Quigley and CP Crum. (2000). The human VASA gene is specifically expressed in the germ cell lineage. Proc Natl Acad Sci U S A 97:9585-9590.

81. Zeeman AM, H Stoop, M Boter, AJ Gillis, DH Castrillon, JW Oosterhuis and LH Looijenga. (2002). VASA is a specific marker for both normal and malignant human germ cells. Lab Invest 82:159-166.

82. Achi MV, N Ravindranath and M Dym. (2000). Telomere length in male germ cells is inversely correlated with telomerase activity. Biol Reprod 63:591-598.

83. Costoya JA, RM Hobbs, M Barna, G Cattoretti, K Manova, M Sukhwani, KE Orwig, DJ Wolgemuth and PP Pandolfi. (2004). Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 36:653-659.

84. Kristensen DM, JE Nielsen, NE Skakkebaek, N Graem, GK Jacobsen, E Rajpert-De Meyts and H Leffers. (2008). Presumed pluripotency markers UTF-1 and REX-1 are expressed in human adult testes and germ cell neoplasms. Hum Reprod 23:775-782.

85. von Kopylow K, C Kirchhoff, D Jezek, W Schulze, C Feig, M Primig, V Steinkraus and AN Spiess. (2010). Screening for biomarkers of spermatogonia within the human testis: a whole genome approach. Hum Reprod 25:1104-1112.

86. Wu X, JA Schmidt, MR Avarbock, JW Tobias, CA Carlson, TF Kolon, JP Ginsberg and RL Brinster. (2009). Prepubertal human spermatogonia and mouse gonocytes share conserved gene expression of germline stem cell regulatory molecules. Proc Natl Acad Sci U S A 106:21672-21677.

87. Yamamoto Y, N Sofikitis, K Ono, T Kaki, T Isoyama, N Suzuki and I Miyagawa. (1999). Postmeiotic modifications of spermatogenic cells are accompanied by inhibition of telomerase activity. Urol Res 27:336-345.

88. Tan SM, ST Wang, H Hentze and P Droge. (2007). A UTF1-based selection system for stable homogeneously pluripotent human embryonic stem cell cultures. Nucleic Acids Res 35:e118.

89. Jia J, X Zheng, G Hu, K Cui, J Zhang, A Zhang, H Jiang, B Lu, J Yates, III, C Liu, K Zhao and Y Zheng. (2012). Regulation of Pluripotency and Self- Renewal of ESCs through Epigenetic- Threshold Modulation and mRNA Pruning. Cell 151:576-589.

90. Goossens E, D van Saen and H Tournaye. (2013). Spermatogonial stem cell preservation and transplantation: from research to clinic. Hum Reprod 28:897-907.

91. Lee HD, HS Lee, SH Park, DG Jo, JH Choe, JS Lee and JT Seo. (2012). Causes and classification of male infertility in Korea. Clin Exp Reprod Med 39:172-175.

92. Jahnukainen K, J Ehmcke, M Hou and S Schlatt. (2011). Testicular function and fertility preservation in male cancer patients. Best Pract Res Clin Endocrinol Metab 25:287-302.

93. Nurmio M, V Keros, P Lahteenmaki, T Salmi, M Kallajoki and K Jahnukainen. (2009). Effect of childhood acute lymphoblastic leukemia therapy on spermatogonia populations and future fertility. J Clin Endocrinol Metab 94:2119-2122.

94. Curaba M, J Poels, A van Langendonckt, J Donnez and C Wyns. (2011). Can prepubertal human testicular tissue be cryopreserved by vitrification? Fertil Steril 95:2123-12.

95. Avarbock MR, CJ Brinster and RL Brinster. (1996). Reconstitution of spermatogenesis from frozen spermatogonial stem cells. Nat Med 2:693-696.

96. Frederickx V, A Michiels, E Goossens, G De Block, AC Van Steirteghem and H Tournaye. (2004). Recovery, survival and functional evaluation by transplantation of frozen-thawed mouse germ cells. Hum Reprod 19:948-953.

97. Izadyar F, JJ Matthijs-Rijsenbilt, K den Ouden, LB Creemers, H Woelders and DG de Rooij. (2002). Development of a cryopreservation protocol for type A spermatogonia. J Androl 23:537-545.

98. **Sa R, N Cremades, I Malheiro and M Sousa. (2012). Cryopreservation of human testicular diploid germ cell suspensions. Andrologia 44:366-372.**

99. **Keros V, B Rosenlund, K Hultenby, L Aghajanova, L Levkov and O Hovatta. (2005). Optimizing cryopreservation of human testicular tissue: comparison of protocols with glycerol, propanediol and dimethylsulphoxide as cryoprotectants. Hum Reprod 20:1676-1687.**

100. **Wyns C, M Curaba, S Petit, B Vanabelle, P Laurent, JF Wese and J Donnez. (2011). Management of fertility preservation in prepubertal patients: 5 years' experience at the Catholic University of Louvain. Hum Reprod 26:737-747.**

101. **Baert Y, E Goossens, D van Saen, L Ning, VP in't and H Tournaye. (2012). Orthotopic grafting of cryopreserved prepubertal testicular tissue: in search of a simple yet effective cryopreservation protocol. Fertil Steril 97:1152-1157.**

102. **Brinster RL, JW Zimmermann. (1994). Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 91:11298-11302.**

103. **Bahadur G, D Ralph. (1999). Gonadal tissue cryopreservation in boys with paediatric cancers. Hum Reprod 14:11-17.**

104. **Ogawa T, I Dobrinski, MR Avarbock and RL Brinster. (2000). Transplantation of male germ line stem cells restores fertility in infertile mice. Nat Med 6:29-34.**

105. **Relander T, E Cavallin-Stahl, S Garwicz, AM Olsson and M Willen. (2000). Gonadal and sexual function in men treated for childhood cancer. Med Pediatr Oncol 35:52-63.**

106. **Jahnukainen K, M Hou, C Petersen, B Setchell and O Soder. (2001). Intratesticular transplantation of testicular cells from leukemic rats causes transmission of leukemia. Cancer Res 61:706-710.**

107. **Sousa M, N Cremades, C Alves, J Silva and A Barros. (2002). Developmental potential of human spermatogenic cells co-cultured with Sertoli cells. Hum Reprod 17:161-172.**

108. **Riboldi M, C Rubio, A Pellicer, M Gil-Salom and C Simon. (2012). In vitro production of haploid cells after coculture of CD49f+ with Sertoli cells from testicular sperm extraction in nonobstructive azoospermic patients. Fertil Steril 98:580-590.**

109. **Geens M, d Van, V, G De Block, E Goossens, A Van Steirteghem and H Tournaye. (2007). The efficiency of magnetic-activated cell sorting and fluorescence-activated cell sorting in the decontamination of testicular cell suspensions in cancer patients. Hum Reprod 22:733-742.**

110. **Hovatta O. (2001). Cryopreservation of testicular tissue in young cancer patients. Hum Reprod Update 7:378-383.**

111. **Easley CA, BT Phillips, MM McGuire, JM Barringer, H Valli, BP Hermann, CR Simerly, A Rajkovic, T Miki, KE Orwig and GP Schatten. (2012). Direct**

differentiation of human pluripotent stem cells into haploid spermatogenic cells. Cell Rep 2:440-446.

112. Dihazi H, GH Dihazi, J Nolte, S Meyer, O Jahn, GA Muller and W Engel. (2009). Multipotent adult germline stem cells and embryonic stem cells: comparative proteomic approach. J Proteome Res 8:5497-5510.

113. Zechner U, J Nolte, M Wolf, K Shirneshan, NE Hajj, D Weise, B Kaltwasser, A Zovoilis, T Haaf and W Engel. (2009). Comparative methylation profiles and telomerase biology of mouse multipotent adult germline stem cells and embryonic stem cells. Mol Hum Reprod 15:345-353.

114. Zovoilis A, J Nolte, N Drusenheimer, U Zechner, H Hada, K Guan, G Hasenfuss, K Nayernia and W Engel. (2008). Multipotent adult germline stem cells and embryonic stem cells have similar microRNA profiles. Mol Hum Reprod 14:521-529.

115. Herrid M, J Olejnik, M Jackson, N Suchowerska, S Stockwell, R Davey, K Hutton, S Hope and JR Hill. (2009). Irradiation enhances the efficiency of testicular germ cell transplantation in sheep. Biol Reprod 81:898-905.

116. Honaramooz A, E Behboodi, SO Megee, SA Overton, H Galantino-Homer, Y Echelard and I Dobrinski. (2003). Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. Biol Reprod 69:1260-1264.

117. Mikkola M, A Sironen, C Kopp, J Taponen, A Sukura, J Vilkki, T Katila and M Andersson. (2006). Transplantation of normal boar testicular cells resulted in complete focal spermatogenesis in a boar affected by the immotile short-tail sperm defect. Reprod Domest Anim 41:124-128.

118. Schlatt S, L Foppiani, C Rolf, GF Weinbauer and E Nieschlag. (2002). Germ cell transplantation into X-irradiated monkey testes. Hum Reprod 17:55-62.