Factors driving spermatogonial stem cell fate
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THE EFFECT OF CULTURE MEDIA ON THE PROLIFERATIVE CAPACITY AND TRANSCRIPTION PROFILE OF A SPERMATOGONIAL STEM CELL LINE

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Submitted

Summary sentence: The proliferation of the rat spermatogonial stem cell line GC-6spg increased significantly when cultured in StemPro-medium rather than MEM-based medium and this increase was mediated by ZBTB16, GDNF and EGF.
ABSTRACT

Autotransplantation of spermatogonial stem cells (SSCs) has been suggested as a way to treat infertility in childhood cancer survivors. In order for SSC autotransplantation to be successful, it is crucial that efficient and safe in vitro propagation of SSCs is achieved. However, little is known about the factors that directly stimulate the proliferation of SSCs in vitro and therefore the optimal culture conditions for SSCs have not yet been established. In the current study, we set out to study the effect of two widely used culture media (MEM and StemPro) on proliferation and gene expression in a rat spermatogonial stem cell line (GC-6spg) in order to get more insight into the proliferative activity and associated transcription profile of SSCs in different culture conditions. We demonstrate that StemPro based medium causes a fourfold increase in the proliferation of GC-6spg cells as compared to MEM based medium. Furthermore, culture in StemPro caused a significant upregulation of Zbtb16 and Itgα6, while it induced downregulation of Fgfr2. By using a short hairpin-approach to silence Zbtb16, we demonstrate that Zbtb16 expression directly correlates with GC-6spg proliferation rate. In order to gain insight into the regulatory mechanisms behind StemPro-induced spermatogonial proliferation, we tested the effect of the growth factors GDNF and EGF, present in StemPro but absent in MEM based medium. We found that GDNF and EGF can act synergistically on proliferation, but are only partly responsible for the observed increase in proliferation in StemPro. Furthermore, we show that GDNF and EGF both stimulated GC-6spg proliferation in a Zbtb16 independent manner.

These results shed light on the pathways that are important to trigger proliferation of SSCs in vitro, and can aid in designing the optimal SSC culture media for future clinical use of human SSC propagation and autotransplantation.
INTRODUCTION

Ever since spermatogonial stem cell (SSC) transplantation was developed in 1994, it has been put forward as a possible solution to restore fertility in infertile male childhood cancer survivors. Since then, most studies have focused on developing in vitro culture and transplantation in animal species. In addition, we have recently made an essential step forward towards the clinical use of this technique in humans by establishing a method to propagate human SSCs in vitro. As the number of available SSCs highly influences the efficiency of transplantation, it is crucial to properly culture and propagate SSCs.

Two different culture media are generally used to culture SSCs. One is a Minimum Essential Medium (MEM) based medium, enriched with one or more growth factors thought to be crucial for SSC long-term maintenance and proliferation, such as GDNF, bFGF, EGF and LIF and a feeder layer of STO cells. The other widely used culture medium is StemPro-34®SFM (StemPro). This is a complex medium enriched with the same key growth factors such as described above for MEM.

So far, all studies aimed at elucidating the effect of different culture conditions on SSCs were performed on mixed cell cultures; furthermore, in some cases cell cultures were supported by a cell feeder layer. Therefore, from these studies it is not possible to determine the direct effect of different culture conditions or growth factors on SSC proliferation and gene expression in vitro.

In order to gain insight into the direct effect of the two culture media MEM and StemPro on the proliferation and transcriptional profile of SSCs, we have cultured the single cell cloned rat spermatogonial stem cell line GC-6spg in parallel in MEM or StemPro-based media. Furthermore, we have investigated the effect of two specific growth factors (GDNF and EGF) present in StemPro but absent in MEM on proliferation and expression of selected spermatogonial genes.

MATERIALS AND METHODS

CELL CULTURE

The spermatogonial cell line GC-6spg was routinely cultured in MEM supplemented with nonessential amino acids (100IU/ml), penicillin/streptomycin (100mg/ml), gentamicin (40mg/ml), HEPES (15mM) (all from Life Technologies, Inc., Paisley, UK), sodium bicarbonate (0.12%), L-glutamine (4mM), platelet-derived growth factor-BB (10ng/ml), recombinant human basic fibroblast growth factor (10ng/ml), recombinant human LIF (10ng/ml), forskolin (20μM), 1nM 17β-estradiol, G418 200μg/ml (all from Sigma, St. Louis, USA) and 2.5% FCS (Australian origin 10099141, Invitrogen, Carlsbad, USA). Under these conditions confluent cells were diluted 1:8 each week. To evaluate possible differences in spermatogonial characteristics when cultured in StemPro-34®SFM (StemPro) based medium, GC-6 spg cells were cultured in StemPro-34®SFM (StemPro) (Invitrogen, Carlsbad, USA) supplemented with 0.5% penicillin streptomycin, 1%
FCS (both from Invitrogen), recombinant human basic fibroblast growth factor (bFGF) (10 ng/ml), recombinant human EGF (20 ng/ml), recombinant human GDNF (10 ng/ml) (all from Sigma-Aldrich, St. Louis, USA), recombinant human LIF (10 ng/ml) (Chemicon International Inc, Temecula, California) and G418 (200μg/ml). In this StemPro medium, confluent cells were diluted 1:30 each week.

To investigate proliferation, GC-6spg were cultured for 8 weeks in MEM (MEM group), for 8 weeks in StemPro (StemPro group) or for 4 weeks in StemPro and subsequently for 4 weeks in MEM (StemPro-MEM group). Analysis was performed at week 1, 4, 5 and 8 in culture (fig. S1).

To evaluate the role of GDNF and EGF on the behaviour of SSCs, GC-6spg cells were cultured in MEM enriched with GDNF and/or EGF or in StemPro deprived of GDNF and/or EGF for 1 week.

To block the bFGF pathway, GC-6spg cells cultured in MEM were treated with the FGF inhibitor SU5402 (10μM in DMSO R&D system Minneapolis USA, final concentration of DMSO in culture 0.1% ) for 4, 12, 24, 48, and 168 hours and control cells were cultured in MEM +DMSO.

Cells under all culture conditions were cultured in a humidified incubator at 32°C and 5% CO2, refreshed every 3-4 days and passaged every week.

**Real-time PCR**

Total RNA of cultured GC-6spg cells was isolated using the RNaseasy kit, (Qiagen, Valencia, USA) following the instructions of the manufacturer. cDNA was synthesized using random primers. Q-PCR was performed on a Roche Light Cycler 480 using a primer probe assay (Roche, Almere, the Netherlands), and results were normalized to Rps18. The primer probe details are reported in Table 1. Q-PCR analysis was repeated at least three times in three independent experiments.

**Cell proliferation**

Cell proliferation in different culture conditions was evaluated using the WST-1 cell proliferation assay (ROCHE, Almere, the Netherlands). The same amount of cells was plated in each well of a 96 well plate and cultured for 4 days. The assay was carried out as described by the manufacturer. Briefly, after 4 days of culture, cells were incubated for 1h with a medium containing WST-1 reagent (diluted 1:10), and the optical density (OD) of the medium was evaluated in an ELISA reader (Anthos 2 Biochrom Ltd.) at 450nm with a reference of 620nm.

**Zbtb16 RNA interference**

To investigate the role of Zbtb16 in the proliferation of SSCs, the same amount of GC-6spg cells were plated in each well of a 96 well plate in order to reach a confluency of 50% the next day. Zbtb16 interference was performed with a combination of three different target-specific constructs that encode 19-25nt (plus hairpin) shRNA targeting Zbtb16 or scramble (sc-156168-V and sc-108080 respectively, Santa Cruz biotech). Cells were cultured in MEM
and infected at different time points: 24h before the switch to StemPro medium (condition 1), at the time of the switch (condition 2) and 24h after the switch (condition 3). Infection was carried out overnight in the presence of 8μg/ml Polybrene® (Santa Cruz biotech). As controls, we used cells cultured constantly in standard MEM or StemPro, either not infected or infected with scramble vector. After 48h of culturing in StemPro, proliferation was determined using the WST1 assay. Efficiency of Zbtb16 downregulation was evaluated by Q-PCR and Western analyses.

**Western Blot analysis**

Protein lysates of GC-6spg cells were prepared with RIPA buffer (1% NP40, 0.5% NaDeoxycholaat, 0.1% SDS and 1mM protease inhibitors by ROCHE). Protein concentrations were measured using Bicinonic acid analysis (BCA) methods (Pierce Chemical Co., Rockford USA). Of each protein lysate, 50μg was loaded on a 4-12% gradient gel (NP323BOX, Invitrogen, Carisbad USA). Proteins were blotted on Polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, USA). The membrane was blocked in LICOR blocking buffer (LICOR, Nebrasca, USA) for 1 hour and probed with 1:100 mouse monoclonal ZTBT16 antibody ( SC-28319 Santa Cruz Biotechnology Inc, Santa Cruz, USA). The secondary antibody, rabbit anti mouse IRDye® 800CW (926-32210, LICOR Biosciences, Lincoln, USA), was used with a dilution of 1:15000. As internal standard for loading control, α-tubulin antibody 1:2000 (ms-581-pc, Neomarker, Fremont, USA) was used. Image acquisition and quantification were done with the ODYSSEY Infrared Imaging System (LICOR Biosciences, Lincoln, USA).

**Statistics**

The differences in gene expression levels using the ΔΔCT method or the differences in the OD values of WST1 assay between cells cultured under several conditions were calculated using one-way ANOVA (Graphpad 4, Graphpad Software, San Diego, USA) with Dunnett’s Multiple Comparison post hoc test. A p-value of <0.05 was considered to be statistically significant.
RESULTS

CELL PROLIFERATION IN MEM AND STEMPRO BASED MEDIA

GC-6spg cells cultured in StemPro displayed a fourfold increase in proliferation rate as compared to GC-6spg cells cultured in MEM. When cultured in MEM, confluent cells were passaged 1:8 each week, while in StemPro confluent cells were passaged 1:30 each week. As quantified by the WST-1 assay, GC-6spg cells displayed an sixfold increase in cell number after four days of culture in StemPro as compared to GC-6spg cells cultured for four days in MEM (Figure 1, P <0.001).

Figure 1. Bar chart showing the increase in proliferation rate of GC-6spg cells induced by StemPro medium. Quantification was performed by WST1 kit. Data are plotted as mean ± SEM, p<0.001.

GENE EXPRESSION IN MEM AND STEMPRO BASED MEDIA

Culturing of GC-6spg cells in StemPro for 8 weeks resulted in a significant upregulation of the transcription factor Zbtb16 and the adhesion molecule Itgα6, while the growth factor receptor Fgfr2 was significantly downregulated (Figure 2). This effect was completely reversible, as GC-6spg cells cultured for 4 weeks in StemPro and subsequently for 4 weeks in MEM showed comparable Zbtb16, Itgα6 and Fgfr2 expression levels to GC-6spg cells continuously cultured in MEM. The expression levels of the differentiation marker Kit and the adhesion molecules for early spermatogonia Cdh1 and Itgβ1 (Figure 2A and 2C) were not significantly affected by StemPro, although Cdh1 expression showed a trend towards downregulation when GC-6spg cells were cultured in StemPro based medium for 4 weeks or more.
Figure 2. qPCR: Evaluation of gene expression changes during short- and long-term culture of GC 6-spg cells in StemPro34. Letters represent comparison with significant difference. A) a and g: P<0.05; h: P<0.01; b, c, d, e and f: P<0.001; C) a, b and c: P<0.05; D) a and b: P<0.01; c and d: P<0.05. Data are plotted as mean of three independent samples ± SEM.
Since GC-6spg cells cultured in StemPro showed a consistent downregulation of Fgfr2 with an upregulation of Zbtb16, we checked whether this might indicate crosstalk between the FGF pathway and Zbtb16. However, blocking the bFGF pathway via the bFGF inhibitor SU5402, did not affect the expression of any of the genes studied including Zbtb16 (data not shown).

PROLIFERATION AFTER Zbtb16 INTERFERENCE

Because of the marked increase in Zbtb16 expression in GC-6spg cells cultured in StemPro, we examined further the role of Zbtb16 in SSC proliferation. Silencing Zbtb16 in GC-6spg cells using Zbtb16 specific shRNA constructs resulted in a decrease in Zbtb16 mRNA of about 30% and ZBTB16 protein of about 40% at 24 hours after infection compared to a scramble shRNA, (Figure S2).

Proliferation of GC-6spg was significantly decreased when GC-6spg cells were infected with Zbtb16 shRNA at 0 and 24 hours before switching to StemPro medium compared to GC-6spg cells infected with scramble shRNA (Figure 3, p<0.001 and 0.01 respectively).

![Figure 3. Proliferation of the SSC cell line GC-6 spg after interfering with Zbtb16. A) Zbtb16 interference was performed in three different conditions: 1) 24 hours before, 2) at the time and 3) 24 hours after switching the medium to StemPro-34. B) Significant differences were observed in conditions number 1 and 2, p<0.001 and 0.01 respectively.](image-url)
**Effect of GDNF and EGF on cell proliferation**

We next investigated whether GDNF and/or EGF, present in Stempro medium but absent in MEM, could be responsible for the increased proliferation of GC-6spg. The addition of GDNF or EGF to MEM led to a significantly increase in cell number of 10% and 40%, respectively (Figure 4A, p<0.05 and < 0.001) after 4 days of culture. The addition of both growth factors to MEM increased the proliferation rate in a synergistic way, leading to a 60% increase in cell number after 4 days compared to cells cultured in MEM (Figure 4A, p < 0.001). Alternatively, the removal of GDNF, EGF, or both from StemPro did not significantly affect the proliferation rate (Figure 4B). The proliferation rate of GC-6spg in StemPro with or without GDNF and/or EGF was always higher than of cells cultured in MEM based medium with or without GDNF and/or EGF.

![Figure 4](image.png)

Figure 4. Effect of the growth factors EGF and GDNF (alone or in combination) on the proliferation rate of GC-6spg cells in different media. A) MEM medium, a: p<0.05; b,c,d and e: p<0.001; f: p<0.01. B) StemPro medium: no significant differences were observed between the different media. However, in all cases the proliferation rate was higher than all the MEM-based media. Data are plotted as mean ± SEM of three independent experiments.

**Effect of GDNF and EGF on gene expression**

We found that the addition of GDNF to MEM or the removal of GDNF from StemPro for one week had no effect on expression levels of any of the genes tested (Figure 5A-E). Conversely, the addition of EGF to MEM led to a downregulation of Kit (Figure 5B p<0.05) and Cdh1 (fig. 5E p<0.05), while Zbtb16, Itgα6 and Fgfr2 were not affected (Figure 5A, C and 5D). The removal of EGF from StemPro led to a significant upregulation of Kit (Figure 5B p<0.001) and Zbtb16 (Figure 5 p<0.001 compared to MEM), while no major changes were observed for the other genes tested (fig 5C-E).
When GC-6spg cells were cultured in MEM enriched with both GDNF and EGF, Kit expression, which was already low, was significantly further downregulated (Figure 5B, p<0.05 compared to MEM). No major changes were found in Zbtb16, Itga6, Cdh1 and Fgfr2 expression (Figure 5A,C-E). Removing both growth factors from the StemPro medium resulted in Kit upregulation in GC-6spg, although it remained lower than the expression level in cells cultured in StemPro medium deprived of EGF (Figure 5B p<0.001 compared to MEM and p<0.05 compared to StemPro without EGF). Zbtb16, Itga6, Cdh1 and Fgfr2 levels were not significantly affected in GC-6spg when cultured in StemPro medium deprived of EGF and GDNF compared to complete StemPro (Figure 5 A, C-E).

Furthermore, expression levels of Zbtb16 and Itga6 in all StemPro conditions were significantly higher than in all MEM conditions (Figure 5A,D), while Fgfr2 expression levels were significantly lower (Figure 5C). Kit expression levels were lower in StemPro deprived of EGF or EGF and GDNF when compared to MEM (Figure 5B).

<table>
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<tr>
<th>Gene name</th>
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<th>RV primer</th>
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<td>ctgaaacttctcgggatca</td>
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Table 1. List of the primers used with the corresponding probe number.
Figure 5. qPCR evaluating the effect on expression levels of spermatogonial genes in GC-6spg cells when cultured in the presence of the growth factors EGF and GDNF, alone or in combination, in MEM and StemPro34 medium. A) a and e: p<0.05; b and d: p<0.01; c: P<0.001; B) a, b, f and h: p<0.05; c, d, e and g: p<0.001; C) a and b: p<0.01 c and d: p<0.001; D) a and b p<0.001 c and d: p<0.001; E) a: P<0.05. Data are plotted as mean ± SEM of three independent experiments.
DISCUSSION

In the present study, we found a four-fold increase in proliferation rate of GC-6spg cells cultured in StemPro as compared to GC-6spg cells cultured in MEM. This increase in proliferation in StemPro was accompanied by elevated levels of Zbtb16 and Itgα6 and by a downregulation of Fgfr2. When MEM medium was enriched with GDNF and/or EGF, present exclusively in StemPro, an increase in the proliferation rate of GC-6spg cells was observed, although lower than the proliferation rate induced by StemPro. The increased proliferation after addition of GDNF and/or EGF to MEM was not accompanied by increase in Zbtb16 expression, but by downregulation of Kit and Cdh1.

SSC culture is mostly performed using primary isolated SSCs that always contain somatic feeder cells that seem to be important for SSC propagation. The somatic cells, either testicular or stromal feeder cells, can interact with medium compounds and produce additional factors that can influence SSC behavior, making it difficult to study the direct effects of culture media on SSCs. To study the direct effects of culture media on SSCs, we choose to use the rat SSC cell line GC-6spg. The GC-6spg is a single cell cloned spermatogonial cell line that can be cultured without feeder cells and has SSC characteristics, including expression of several spermatogonial markers such as Oct4, Zbtb16, Itgα6 and Itgβ1, and the capability of homing to the basal membrane after transplantation. Due to these characteristics, the stem cell line GC-6spg is a unique tool to study how culture conditions directly affect proliferation and gene expression of SSCs.

The observed increase in proliferation of GC-6spg cells when cultured in StemPro compared to MEM based medium was associated with an increase in Zbtb16 expression. Furthermore, Zbtb16 interference resulted in a clear reduction in proliferation, indicating a direct link between Zbtb16 and spermatogonial proliferation. It is known that in vivo ZBTB16 expression is restricted to A single (A₁) and A paired (A₃) and small chains of A aligned (A₄) undifferentiated spermatogonia. In cross sections of seminiferous tubules of the Zbtb16 mutant mice a patchwork phenotype is present, with tubules showing either normal spermatogenesis, absence of early germ cells or only Sertoli cells, suggesting a progressive loss of SSCs as a result of reduced proliferation and/or enhanced differentiation. Furthermore, spermatogonia from Zbtb16⁻/⁻ mice are unable to colonize the testis of wild type animals after transplantation, while spermatogonia of wild type animals transplanted in Zbtb16⁻/⁻ mutant mice testis gave rise to normal spermatogenesis, demonstrating that SSCs and not the somatic environment in the testis are impaired in Zbtb16⁻/⁻ mice. It has been shown that ZBTB16 binds to two different regions of the Kit promoter, leading to a ZBTB16-mediated repression of Kit, and thereby suppression of SSCs differentiation. In addition to these findings, our study now shows that ZBTB16 is also directly involved in proliferation of undifferentiated spermatogonia.

Besides Zbtb16, the expression of Itgα6 and Fgfr2 significantly changed when GC-6spg cells were cultured in StemPro compared to MEM based medium. Itgα6 is a member of the integrin
superfamily and it is involved in the binding of SSCs to the basal lamina of the seminiferous tubules, together with \textit{Itg}\textsubscript{$\beta$}1 \textsuperscript{9, 12}. The increased expression levels of \textit{Itg}\textsubscript{$\alpha$}6 suggest a change in the interaction between the SSCs and laminin. The FGF2 receptor and its corresponding growth factor FGF are known to play a role in keeping the SSCs in an undifferentiated state \textsuperscript{11}. The opposite regulation of \textit{Zbtb16} and \textit{Fgfr2} probably underlines different ways of regulating the undifferentiated state of SSCs as our study shows that interfering with the FGF2 pathway by blocking its receptor did not result in changes in \textit{Zbtb16} expression levels. Further research is needed to clarify the precise function of \textit{Fgfr2} on the fate of SSCs.

We examined which growth factors in StemPro medium were responsible for the increased proliferation and associated upregulation of \textit{Zbtb16} and \textit{Itg}\textsubscript{$\alpha$}6 and downregulation of \textit{Fgfr2}. One of the first candidates was GDNF, since it is a well known key factor for SSC selfrenewal \textsuperscript{6, 18, 19}. The role of GDNF in selfrenewal was initially described by using two different animal models. First, the phenotype of GDNF+/- mice showed depletion of SSCs leading to a patched phenotype with Sertoli cell only tubules \textsuperscript{18}. Second, overexpression of GDNF in mice resulted in accumulation of undifferentiated spermatogonia and at later age induced testicular tumor formation \textsuperscript{18, 19}. In vitro studies also showed that SSCs cultured on STO feeder layer with GDNF display increased colonization capacity after transplantation \textsuperscript{11}. When GDNF was added to our GC-6spg cells cultured in MEM, a small but significant increase in proliferation was observed, while we found no significant effect on the expression of the genes tested. Similarly, no effects on cell proliferation or gene expression were observed in GC-6spg cells cultured in StemPro medium deprived of GDNF when compared to those cultured in StemPro.

A second candidate that could explain the differences in proliferation and gene expression between the StemPro based and MEM based medium was EGF. In vivo EGF is expressed in Sertoli cells, pachytene spermatocytes, and round spermatids\textsuperscript{20}. Furthermore, it has been shown that EGF is able to stimulate DNA synthesis in spermatogonia \textsuperscript{21}. In addition, it seems to be a crucial growth factor for SSCs propagation in mixed cell cultures in vitro \textsuperscript{6, 22}. We found that the addition of EGF to MEM culture media resulted in an increase of GC-6spg proliferation rate that was higher than that induced by GDNF, although still not as high as in StemPro based media. In contrast to GDNF, EGF influenced gene expression. More precisely, it resulted in the downregulation of \textit{Kit} and \textit{Cdh1} without increasing \textit{Zbtb16} levels. This suggests that EGF might play a role as a stemness factor by blocking SSC differentiation in a ZBTB16 independent manner. Indeed, we have previously shown that an early step of SSCs differentiation is an increase in expression of \textit{Kit} and \textit{Cdh1} without downregulation of \textit{Zbtb16} \textsuperscript{14}. The fact that EGF acts as a growth factor that maintains the undifferentiated state of SSCs is also suggested by the fact that GC-6spg cells cultured in StemPro deprived of EGF show signs of differentiation by upregulation of \textit{Kit}.

Recently, it has been shown that removing EGF and LIF from StemPro medium does not influence the number of SSCs in culture, as the number of colonies obtained after transplantation was comparable with SSCs cultured in StemPro in the presence of both growth factors\textsuperscript{10}. Since
Effects of medium on SSC proliferation

These cultures were performed on MEFs as a feeder layer, further research is necessary to evaluate the direct role of both growth factors in SSC proliferation. Although, in the present study no differences in the proliferation rate of GC-6spg cells cultured in StemPro deprived of EGF versus cells cultured in complete StemPro, the upregulation of Kit reveals a step towards differentiation.

When investigating the combination of both GDNF and EGF on the behavior of GC-6spg cells in MEM based media, we found that the proliferation rate of GC-6spg cells increased synergistically, suggesting that these factors affect proliferation via independent pathways. The proliferation rate of GC-6spg cells in MEM enriched with both growth factors still remained much lower than that of cells cultured in StemPro. Thus, in addition to GDNF and EGF, other compound(s) in StemPro are important for proliferation of GC-6spg cells. The reason why GDNF and EGF play only a partial role in increasing the proliferation of the GC-6spg cell line is probably because these growth factors were not able to affect the ZBTB16 pathway, which we show is directly associated with proliferation of undifferentiated spermatogonia. Further research is needed to identify the factors that regulate Zbtb16 expression in spermatogonia when cultured in StemPro medium.

We found an increase in proliferation of the rat spermatogonial stem cell line GC-6spg when cultured in StemPro compared to MEM based medium via three independent pathways, mediated through ZBTB16, GDNF and EGF, suggesting they are directly involved in the proliferation of SSCs. It is known that both culture conditions, MEM and StemPro, have the ability to support SSC proliferation as shown by (xeno)transplantation. Recently, a novel culture medium based on MEM/Ham F12, enriched with growth factors GDNF, bFGF and one of the components of StemPro, B27 supplement, is able to support SSC proliferation. Based on proliferation in vitro, this new medium is able to support proliferation with a slightly lower efficiency than StemPro. However, further research, including parallel transplantation experiments, is necessary to determine which culture media and which of the included growth factors, and therefore pathways, will have the highest effect on selfrenewal of SSCs.

In conclusion, the present study showed that GC-6spg cells cultured in StemPro displayed increased proliferative activity as compared to GC-6spg cultured in MEM. This increase was mediated by ZBTB16, GDNF and EGF. Zbtb16 is directly linked to SSCs selfrenewal and the growth factors GDNF and EGF enhance proliferation independent of Zbtb16. Our results shed light on the pathways that are important to trigger proliferation of SSCs in vitro and can aid in designing the optimal SSC culture media for future clinical SSC propagation and autotransplantation.
Chapter 4

ACKNOWLEDGEMENTS

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REFERENCES


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Figure S1. Schematic representation of the time course experiment. Black arrows represent analysed samples.
Figure S2. A) QPCR and B) Westernblot analyses of ZBTB16 expression after shRNA interference.