Factors driving spermatogonial stem cell fate
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SUMMARY, CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH
**SUMMARY AND CONCLUSIONS**

In the last decade, the treatment of childhood cancer has greatly improved and nowadays 1 out of 250 adults is a long term survivor of childhood cancer. Once adult, there is high probability that these individuals are confronted with one of the major side effects linked to anti-cancer treatments, namely infertility. For prepubertal boys with cancer, there are currently no means to preserve their fertility, since cryopreservation of semen is not yet possible. Consequently, these boys are doomed to remain childless.

The development of a strategy to restore fertility in these infertile childhood cancer survivors is eagerly awaited. The theoretical way to solve this important issue is to store a testicular biopsy before anti-cancer treatment, and when infertility has manifested itself, to thaw the biopsy, to propagate spermatogonial stem cells (SSCs) from this biopsy and to autotransplant these SSCs to the testis. Indeed, animal models have clearly shown that SSCs can be propagated in vitro and, upon transplantation, are able to re-establish full spermatogenesis. In order for this technique to be successful, extensive and controlled propagation of SSCs is necessary as the number of SSCs in a small prepubertal testis biopsy is low and the transplantation efficiency is estimated to be only 5-10%. In light of this future clinical use of SSCs for the treatment of infertile childhood cancer survivors, it is crucial to understand and possibly control the proliferation and differentiation of SSCs in vitro. Unfortunately, our knowledge on the optimal culture conditions in terms of medium, growth factors and temperature is limited. This is mainly due to the fact that there are very few SSCs within the testis and so far there is no unique molecular SSC marker, and therefore no technique, to isolate SSCs.

In our laboratory, we previously established a rat spermatogonial cell line called GC-6spg that is an excellent tool to investigate the effect of different culture conditions and growth factors on SSC proliferation and differentiation.

In this thesis, we aimed to gain insights into the proliferation and differentiation of SSCs in different culture conditions in terms of medium, growth factors and temperature by using the GC-6spg cell line. This research provide us with important information on the pathways that are important in the process of proliferation and differentiation of SSCs in vitro and can aid in designing the optimal SSC culture media for future clinical applications.
In chapter 2, we investigated the effects of the growth factor BMP4 on the GC-6spg cell line. BMP4 is expressed by Sertoli cells in mice until postnatal day 7, while at later ages it is predominantly expressed in spermatogonia and spermatocytes. BMP4 is thought to stimulate SSC differentiation based on the fact that isolated spermatogonia from adult mice cultured in the presence of BMP4 give rise to a lower number of colonies upon SSC transplantation compared to cells cultured in the absence of BMP4. It was previously unclear whether BMP4 stimulates SSC differentiation in a direct or an indirect manner and via which pathways, as all culture systems that have been used thus far consisted of a mixture of testicular cells and in some studies a cellular feeder layer.

In our study, we provided evidence that BMP4 can directly induce SSC differentiation. GC-6spg cells express all BMP4 receptors and exposure to BMP4 induces downstream SMAD phosphorylation and upregulation of KIT, a marker for differentiating spermatogonia. Using a microarray pathway analysis, we were able to disclose which pathways were involved in the early steps of spermatogonial differentiation. We found that, among others, adherens junctions, focal junctions, gap junctions, cell adhesion molecules (CAMs) and molecules regulating the actin cytoskeleton, were significantly regulated. This study therefore suggests that adhesion pathways are important in the first steps of differentiation, most likely to ensure the migration of SSCs out of their vascular associated niche to a random distribution along the basal membrane of the seminiferous tubules.

We next focused in chapter 3 on the effects of the growth factor Activin A on the GC-6spg cell line. Previous studies provided only indirect evidence that Activin A induces SSC differentiation and, as for BMP4, all previous studies investigated the effects of Activin A in mixed culture systems.

Using the same experimental approach used in Chapter 2, we were able to show that GC-6spg cells cultured in the presence of Activin A upregulated their expression of KIT. Using microarray pathway analyses, we found seven pathways that were significantly affected by Activin A and four of them were pathways involved in adhesion processes, namely adherens junctions, cell adhesion, leukocyte transendothelial migration and the phosphatidylinositol signaling system. This study confirmed the significance of adhesion pathways in the early step of SSC differentiation. In addition and in contrast to BMP4, the expression levels of genes involved in cell cycle regulation are highly affected by Activin A suggesting a possible role of Activin A in the synchronization of the mitotic germ cell cycle with the cycle of the seminiferous epithelium.

Taken together, the studies described in chapter 2 and 3 suggest that both BMP4 and Activin A cause initiation of differentiation of SSCs by changing their adhesion properties (Figure 1).

The effect on the expression of specific genes was similar but not identical between BMP4 and Activin A. The main difference between the pathways triggered by Activin A and BMP4 is that BMP4 affects the focal adhesion pathway that includes genes such as Itga6 and Itgb1,
Summary, Conclusions and Implications for Future Research

and the regulation of actin cytoskeleton pathway, whereas Activin A does not. Proteins belonging to the focal adhesion pathway are involved in the interaction between cells and the extracellular matrix \(13\). In particular, integrins are capable to bind to several matrix compounds, such as laminin \(14\), that are present in the basal lamina \(15\). We suggest that the differences and similarities in terms of gene expression between Activin A and BMP4 indicate two different modes of inducing the migration of differentiating spermatogonia along the basal lamina and later towards the lumen of the seminiferous tubule.

An additional difference is that Activin A also appears to affect cell cycle regulation and synchronization of differentiating spermatogonia within the cycle of the seminiferous epithelium while BMP4 does not. Indeed, \(Cdkn1a\) and \(Bhlhe40\), known to be expressed in spermatogonia, were found in the top 20 of the most differentially expressed genes upon Activin A-induced differentiation and are suggested to be involved in this process \(16-18\).

Taken together, our data indicate that BMP4 and Activin A are involved in early differentiation of SSCs by affecting the focal adhesion pathway for binding to the basal lamina and the cell cycle pathway to synchronize the cell proliferation to the cycle of the seminiferous epithelium, respectively. The changes in various adhesion pathways may be related to the enhanced migratory behavior in vivo in stages VII-IX in the epithelial cycle. In these stages, differentiating late Aal and A1 spermatogonia migrate from a vascular orientated niche to a random distribution in the seminiferous tubules of A2, A3 and A4 spermatogonia \(19\).

Besides providing an insight in the early steps of SSC differentiation, these results are also important for designing the optimal in vitro culture conditions for SSCs. Cell adhesion is an important phenomenon in germ cell development and each germ cell type has its own specific cell adhesion properties related to its niche. In vitro, SSCs show proliferation when cultured on a feeder layer of somatic cells or on a matrix of laminin \(10, 20-22\), which apparently also serves for the right environment for maintenance of their stem cell character. Our results indicate that it is important to avoid alteration of cell adhesion properties of SSCs in vitro as this induces differentiation and therefore loss of stem cell capacity.

In Chapter 4, we describe the effects of different culture media on the proliferation of SSCs. The culture of SSCs from several species is mainly performed using two different media. One is a Minimum Essential Medium (MEM), 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 \(21, 23\). The other widely used culture medium is StemPro-34®SFM (StemPro), first described for the culture of mouse SSCs on MEF feeder cells \(24\). StemPro is a complex medium enriched with the same key growth factors as described above for MEM \(25\).

Our study showed that SSCs cultured in StemPro based media proliferate four times faster than when cultured in a MEM-based medium. As expected, SSCs cultured in StemPro or MEM-based media show a different gene expression pattern. Among the genes tested, two were highly affected: \(Zbtb16\) was highly upregulated and \(Fgfr2\) was highly downregulated in GC-
6spg cells cultured in StemPro compared to GC-6spg cells cultured in MEM based medium. Although we found no evidence of a cross talk between these two pathways, we were able to directly link ZBTB16 expression with the proliferation rate of GC-6spg cells by showing that reducing the level of ZBTB16 caused a decrease in proliferation.

We next examined whether GDNF\textsuperscript{21, 26, 27} and/or EGF\textsuperscript{21, 28, 29} present in StemPro medium were/was responsible for the increased proliferation and associated upregulation of Zbtb16 and Itga6 and downregulation of Fgfr2. When GDNF was added to 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 any of the genes tested. No effect on cell proliferation or gene expression was observed in GC-6spg cells cultured in StemPro medium deprived of GDNF when compared to cells cultured in StemPro with GDNF. 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 did influence gene expression. More precisely, it resulted in the downregulation of Kit and Cdh1 without affecting Zbtb16 levels. This suggests that EGF might play a role as a stemness factor by blocking SSC differentiation in a ZBTB16 independent manner. The hypothesis that EGF acts as a growth factor that maintains the undifferentiated state of SSCs is further supported by the fact that GC-6spg cells cultured in StemPro deprived of EGF show signs of differentiation as they upregulate the expression of Kit. The proliferation rate of GC-6spg cells in MEM enriched with one or both growth factors increased but 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 cells 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.

In Chapter 5, we investigated the effect of temperature on SSC selfrenewal. Although in physiological conditions testicular temperature is between 2 to 8°C lower than body temperature, most (mixed) culture systems for spermatogonia operate at 37°C, regardless of the medium used.

We found increased proliferation when GC-6spg cells were cultured at 37°C. The addition of GDNF and/or EGF to MEM based medium caused a further increase in proliferation.

Among the genes tested, only Zbtb16 was significantly downregulated at 37°C. Moreover, it was further downregulated when GC-6spg cells were cultured at 37°C in MEM medium enriched with both GDNF and EGF. In the same medium conditions, but at 32°C, no effect on Zbtb16 expression was observed. The Zbtb16 downregulation at 37°C suggests that, besides increasing their proliferation, cells are triggered to enter the differentiation pathway. In support of this notion, we also found that, while EGF and GDNF at 32°C induced Kit downregulation, at 37°C Kit downregulation did not occur most likely due to the downregulation of Zbtb16. From these results we can conclude that the proliferative activity of SSCs at 37°C is higher, while stemness seems to be maintained better at 32°C.

Taken together, the studies in chapter 4 and 5 indicate that there are distinct effects of culture media and temperature on the fate of SSCs. In terms of maximizing proliferation, SSCs can best be cultured in StemPro with GDNF and EGF. In order to optimally maintain stemness, SSCs can best be cultured at 32°C in MEM in the presence of GDNF and EGF.

In terms of clinically applying SSC autotransplantation, the results of this thesis suggest it would be optimal to provide SSCs with the appropriate extracellular matrix and to use two different culture conditions in two different phases of in vitro culture: StemPro in the amplification phase, and MEM including EGF and GDNF at 32°C in the phase prior to transplantation.
FUTURE RESEARCH

In light of the future clinical use of SSCs for the treatment of infertile childhood cancer survivors, it is crucial to be able to control the proliferation and differentiation, i.e. the fate, of SSCs. In this thesis we identified growth factors, media and specific culture conditions necessary for SSC proliferation in vitro and at the same time the maintenance of their stemness. Such information is important in light of the establishment of SSC proliferation for efficient autotransplantation in subfertile childhood cancer survivors.

It is important to note that the results of the studies described in this thesis are based on molecular analyses. It is evident that functional analyses of the effects of different culture conditions should also be performed. The functional test to demonstrate spermatogonial stem cell activity is transplantation of the cultured cells to the testis of recipient mice. Thus, in order to provide a definite answer on the effect of all the conditions described in this thesis on the proliferation and stemness of SSCs, parallel transplantation experiments need to be carried out.

Besides the functional analysis to be carried out, the experiments also need to be conducted using a human rather than a rat SSC cell line although some of the mechanisms described in this thesis are likely to be common for SSCs in all species including human. Unfortunately the only human SSC culture system available is a mixed cell culture which precludes the analysis of direct effects of culture conditions on SSCs. It is therefore crucial that a pure human SSC cell line is established. This could be conducted in a similar manner as we previously used to generate the GC-6spg cell line, namely transfection with SV40 large T antigen. Ideally the expression of SV40 should be controlled so that an effect of SV40 itself can be ruled out.

In the seminiferous epithelium, all steps of differentiation have to occur in a defined moment. The way germ and somatic cells acquire this synchrony is far from being understood. Our work has identified Cdkn1a and Bhlhe40 as being regulated by Activin A. We suspect that these genes could play an important role in the synchronous development of germ cells and are necessary for proper germ cell development. Therefore, further studies, such as germ cell specific knock outs of these two genes, should be conducted.

Besides being important in light of culturing SSCs in vitro for future autotransplantation, the changes observed in cell adhesion pathways during early SSC differentiation are also of interest in the search for causes of male infertility. Future studies should aim at finding the “adhesome” of SSCs and Sertoli cells and define the spatiotemporal expression of the major adhesion proteins. The study of the regulation of adhesion proteins in SSCs from infertile men could reveal insights into the etiology of male infertility and as such requires further study.
REFERENCES


