Dual Age-dependent function of *KLF4* in Wnt regulation linked with *TERT* and *Ctnnb1* in two differentiated and undifferentiated populations of SSCs

Kiana Sojoudi
Amol University of Special Modern Technologies
Hossein Azizi (h.azizi@ausmt.ac.ir)
Amol University of Special Modern Technologies
Thomas Skutella
University of Heidelberg

Article

Keywords: Klf4, Tert, Wnt/β-catenin, Ctnnb1, differentiation

Posted Date: March 10th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1426870/v1

Abstract

KLF4 is a transcription factor involved in proliferation, differentiation, apoptosis and somatic cell reprogramming; thus, it has a critical role in accurate spermatogenesis. Currently, how KLF4 regulates the Wnt cascade has been discussed and suggested that this connection be related to TERT and Ctnnb1, but its regulatory mechanisms are still very vague. Contrary to the data of many researchers who have identified KLF4 as a factor in maintaining stemness, we have stated that KLF4 is a differentiating factor that its expression increases during spermatogenesis. Furthermore, we show how the regulatory loops of KLF4, TERT and Ctnnb1 through the Wnt pathway is age-dependent. Data collected during immunohistochemistry, immunocytochemistry and Fluidigm real-time RT-PCR besides in silico analyzes indicate that the performance of KLF4 is context-dependent.

1. Introduction

Spermatogenesis is an essential process in maintaining male fertility, in which Spermatogonial stem cells (SSCs) that are the germ stem cells of the seminiferous epithelium in the testis and known as unipotent stem cells undergo a series of differentiation processes and eventually give rise to mature Spermatozoa. Therefore, SSCs maintain spermatogenesis throughout the mammalian reproductive life by self-renewal or differentiation during meiosis divisions (1–3). Past studies have shown that some factors are crucial for the normal process of spermatogenesis in vivo. Therefore, various investigations were done to find suitable markers of germ cells to advance new treatments against infertility (4). Krüppel-like factor 4 (Klf4) is one of the factors that its expression and effects on spermatogenesis is very controversial.

Krüppel-like factor 4 (KLF4), a gut-enriched Krüppel-like factor (GKLF) or epithelial zinc finger (EZF), which located in the cell nucleus, was first identified and characterized by Shields et al. and isolated from the NIH 3T3 library (5). The mouse Klf4 gene locus mapped on chromosome 4B3, which encoded a polypeptide with 53 kb molecular weight, and its amino acid sequence is 91% similar to humans (6). Klf families are characterized by three highly conserved Cys2His2 (C2H2) type zinc-finger motifs in its carboxy terminus for DNA binding and contradistinction between KLF4, and other KLF members are due to the N-terminal transcription activation domain for protein interacting, transcription inhibition zone, and nuclear localization signal (NLS) (7). The presence of the transcriptional activation domain, which is rich in proline and serine, and the existence of the transcriptional repression domain are the reasons for the dual function of the Klf4 as an activator and inhibitor of gene transcription (5, 7). Hence, klf4 plays an essential role in determining the fate of cells because it can regulate various physiological and biological processes such as proliferation, differentiation and apoptosis, neuroinflammation, oxidative stress, which depending on the gene targeted, Klf4 can activate or inhibit transcription so that the result of its performance depends on the context and may play quite the opposite roles under different circumstances (8). Also, for the first time, Yamanaka and Takahashi have shown that Klf4 can collaborate with other reprogramming factors to convert somatic cells to induced pluripotent stem cells (iPSC) and prevent stem cell differentiation. This has led to a broad perspective in the treatment of diseases such as immune diseases and vascular diseases(9). The importance of other Yamanaka factors in embryonic stem cells

(ES cell) self-renewal is well known, but the function of Klf4 has not been well investigated in previous studies (9).

According to Chen et al., has been reported that KLF4 expression is correlated with the up-regulation and down-regulation of many genes involved in the cell cycle arrest and in promoting cell proliferation and differentiation. One of these genes associated with the expression level of Klf4 is telomerase reverse transcriptase (TERT) (10).

The TERT gene localized on the short arm of chromosome 5 (5p.15: 33) a megabase distance from the 5p end, with 16 exons and 15 introns, including instructions that lead to the codification of an essential component of the enzyme called telomerase (11). The ribonucleoprotein telomerase consists of two principal components, which form the stabilizing complex of telomerase activity and are crucial for genome durability. This complex contains one enzymatic component, TERT, which proves the catalytic activity of the telomerase, and an internal telomerase RNA template called TERC. TERC provides a template for creating the repeated sequence of DNA; meanwhile, TERT adds the new DNA segment to chromosome ends (12, 13). It has been shown that the TERC gene can be pervasively expressed in several cells of organisms. In contrast, the expression of the TERT gene is severely repressed in most somatic cells of the body, which leads to the inactivation of telomerase (14). This telomerase complex controls the length of the telomere. Telomere is the physical terminal of linear chromosomes that consists of a non-coding sequence. Various studies have shown that stem cells commonly contain longer telomere sequences and gradually shorten as the cell divides along the differentiation pathway. Moreover, if the telomere does not expand, the cell proliferation capacity is limited by the continuous erosion of the telomeres, which leads to cellular dilapidation (12, 13, 15).

Additionally, one of the most characteristic features in cancer cells is the reactivation or re-expression of telomerase, and its high expression remarkably increases the number of cancer stem cells (CSC) and self-renewal in prostate cancer (16). The cell senescence response due to telomere shortening is a mechanism for suppressing cancer .(13)

Gene Sequences of the TERT have shown that the promoter region of this gene contains numerous binding motifs for transcription factors, including the MYC oncogene. These findings indicate that the presence of transcription factors is required for the regulation of TERT transcription (11).

Klf4 expression pattern and its association with other genes, including TERT, and its involvement in cellular signaling pathways still raises several questions. Furthermore, contemporary findings exhibit many contradictions that exacerbate these ambiguities of the exact function of klf4. In this paper, we examine the expression pattern of klf4 during spermatogenesis, focusing on its connection with other genes, including TERT and CTNNB1. These findings have dramatically increased our understanding of molecular mechanisms regulating spermatogenesis and have opened up new opportunities for infertility treatment.

2. Material And Methods

2.1 Isolation and culture of testicular cells: All animal care was performed according to guidelines of the Amol University of Special Modern Technologies (Amol, Iran). The testes of 7-week-old male C57BL / 6 mice were placed in saline solution of phosphate buffer containing 2% BSA and 0.1% Triton X100 after isolation from the animal. Seminiferous testicular tubules were then separated from the testicular capsule and, After removing the tunica albuginea, were divided into smaller pieces in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA). After that it were placed in a digestion solution, including collagenase IV (0.5 mg/mL, Sigma), DNAse I (0.5 mg/mL, Sigma), and Dispase I (0.5 mg/mL, Roche) in HBSS buffer with Mg + + and Ca++ (PAA) at 37°C for 8 minutes. Single-cell suspension obtained by enzymatic digestion of testicular tissue was washed by DMEM / F12 (Invitrogen, USA), then centrifuged at 1500 rpm for 10 minutes after passing through a 70 µm nylon filter. The supernatant was removed, and the remaining cells were kept in the air at 37 ° C at 5% CO2 so that the culture medium was changed every third day. This media contained StemPro-34 medium, 1% L-glutamine (PAA, USA), 6 mg/ml D + glucose (Sigma Aldrich, USA), 1% N2-supplement (Invitrogen, USA), 1% penicillin/streptomycin (PAA, USA), 5 µg/ml bovine serum albumin (Sigma Aldrich, USA), 0.1% s-mercaptoethanol (Invitrogen, USA), 30 ng/ml oestradiol (Sigma Aldrich, USA), 60 ng/ml progesterone (Sigma Aldrich, USA), 100 U/ml human LIF (Millipore), 1% MEM vitamins (PAA, USA), 8 ng/ml GDNF (Sigma Aldrich, USA), 1% non-essential amino acids (PAA, USA), 10 ng/ml FGF (Sigma Aldrich, USA), 20 ng/ml epidermal growth factor (EGF, Sigma Aldrich, USA), 30 µg/ml pyruvic acid (Sigma Aldrich, USA), 1% ES cell qualified FBS, 100 µg/ml ascorbic acid (Sigma Aldrich, USA) and 1 µl/ml DL-lactic acid (Sigma Aldrich, USA) (17, 18).

2.2. Fluidigm Biomark system gene expression analyses: The expression of genes associated with pluripotency and germ cells, including KLF4 (Mm00516104_m1), TERT (Mm01352136_m1), and CTNNB1 (Mm00483039_m1), was analysed utilising dynamic array chips (Fluidigm). The housekeeping gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Mm99999915_g1), was selected for normalisation of data in different cultured cell types. With the help of a micromanipulator (Narashige Instruments), testicular cultured cells were manually selected. Henceforth the selected cells were lysed with a lysis buffer solution containing 1.3 µl TE buffer, 9 µl RT-PreAmp Master Mix, 0.2 µl R.T./Taq Superscript III (Invitrogen, USA), 2.5 µl 0.2× assay pool and 5.0 µl Cells Direct 2× Reaction Mix (Invitrogen, USA). Using TaqMan real-time PCR on the BioMark Real-Time quantitative PCR (qPCR) system to quantified the targeted transcripts. Two technical replicates were concocted to analyze each sample and by using Excel and GenEx applications, the Ct values were determined (19).

2.3. Immunocytochemical staining: In this experimental study, Testicular cells were fixed with 4% paraformaldehyde (PFA)/phosphate buffered saline (PBS) and permeabilized with 0.1% Triton/PBS solution. Testicular cells were blocked with 1% BSA/PBS and followed by incubation with primary antibodies for Klf4 and TERT. Then, we used secondary antibodies specific for incubation fluorochrome species, and the labeled cells were nuclear-counterstained treatment with 0.2 µg/ml of 4', 6-diamidino-2-phenylindole (DAPI) for 3 minutes at room temperature and fixed with Mowiol 4–88 reagent (Sigma,

USA). The labeled cells were examined with a confocal microscope (Zeiss LSM 700) and images were obtained using a Zeiss LSMTPMT (18, 20).

2.4. Tissue processing for immunohistofluorescence staining: Mouse testis tissue was picked up after decapsulation of tunica albuginea, washed with PBS and fixed in 4% paraformaldehyde. Subsequently dehydrated tissue was located in Paraplast Plus and cut with a microtome device at 10 µm thickness. Sections from the testis's tissues were mounted on Hydrophilic Plus slides and stored at room temperature until use. During the process of immunohistofluorescence staining, samples were washed with xylene and gradually replaced with water in ethanol before staining. For the tissue, antigen retrieval was performed by heat-induced epitope retrieval at 95˚C for 20 minutes. The non-specific binding site of tissue samples was blocked with 10% serum/0.3% Triton in PBS. As explained above, the experiment of immunofluorescence staining for these samples was continued (18).

2.5. Construction of the Protein-Protein interactive (PPI) network: Search Tool for the Retrieval of Interacting Genes/Proteins database (STRING) is a database that can search for all known and predicted interactions between proteins, including physical interactions and functional associations; then generate the PPI network consisting of all these proteins and all the interactions between them. STRING app (version 11.5, https://string-db.org/) in Cytoscape Software (version 3.8.2) was used to generate a PPI network. We used STRING: PubMed query as our data source to generate a network between these fist 200 proteins in Mus musculus organism involved in "self-renewal". The minimum confidence score cut-off was set 0.40. The network was created, designed and organized on the Cytoscape platform and subsequently we constructed subnetworks of the first neighbors' nodes with KLF4 and TERT.

2.6. Functional and pathway enrichment analysis: We create a network of some selected genes of the "self-renewal" network and to seek potential functions and the biological roles of them, we have performed functional gene enrichment analysis using the STRING Enrichment analysis in the Cytoscape platform. We were selected a number of functional enrichments related to our laboratory data without considering a specific FDR value.

Statistical analysis: The experiments were repeated at least three times. The average genes expression in all groups was calculated and the groups were evaluated utilizing one-way analysis of variance (ANOVA), continued with the Tukey's post-hoc tests (t Test) and compared with the non-parametric Mann-Whitney's test. The difference among groups was considered statistically if $P < 0.05$. PPI networks were analyzed based on relevant databases or online data analysis tools.

Ethical statement: In the current investigation, animal experiments were approved (It.ausmt.rec.1400.04) by the Ethics Committee of Amol University of Special Modern Technologies.

3. Results

3.1. Immunohistochemistry and Immunocytochemistry of KLF4 in SSCs: In this current experiment, using immunohistochemical (IMH), the localization of Klf4 was determined in the seminiferous tubules. It is observed that the high expression of KLF4 is in the differentiation process, but undifferentiated cells in the basal section of the mouse seminiferous tubules do not express KLF4 or rarely express it. Therefore, the expression of KLF4 increases during the spermatogenesis process (Figure-1). In the next step, we

examined the expression of KLF4 in two differentiated and undifferentiated spermatogonia populations by immunocytochemical (ICC). Differentiated and undifferentiated spermatogonia cells were isolated after enzyme digestion and generated cells cultivated in the presence of above-mentioned growth factors. Characterization of the isolated SSCs was conducted as described in our former study (18). Confocal scanning UV-laser microscope in ICC analysis exhibited an extremely higher expression of KLF4 in differentiated cells while undifferentiated cells exhibited very lower expression of KLF4 (Figure-2). Subsequently we focused on the Ki67, an indicator for dividing cells and a marker of cells in all phases of the cell cycle except G0 (21). We compared its expression with the KLF4 expression pattern in differentiated and undifferentiated SSCs. Confocal scanning UV-laser microscope in ICC analysis exhibited the similar expression of KLF4 and Ki67. Both of them have higher expression in differentiating cells than undifferentiated cells (Figure-3).

3.2. Performance of the Fluidigm real-time RT-PCR for KLF4, TERT, CTNNB1: Quantitative mRNA expression by Fluidigm real-time RT-PCR for the KLF4 gene indicated significant expression ($P<0.05$) in passage 0 of undifferentiated cells of neonate mice compared to passage 10 (Figure-4, A). However, Fluidigm real-time RT-PCR analysis did not show any significant expression in passage 0 of differentiated cells of the neonate, two weeks, three weeks, eight weeks, and twelve weeks mouse (Figure-4, B). Significant high mRNA expression of KLF4 has also been shown with increasing age of mice (Figure-4, C) ($P<0.05$). Also, the Fluidigm real-time RT-PCR was performed to discover the difference of TERT mRNA level in differentiated and undifferentiated population, which showed a significant expression ($P<0.05$) of TERT in undifferentiated SSCs as opposed to differentiated SSCs both in passage 0 (Figure-5, A). In addition to further confirm the fidelity of our results we compared the expression of TERT between the differentiated adult SSCs and neonate SSCs both in passage 0. The results indicate a higher significant expression ($P<0.05$) of TERT in neonate than adult SSCs (Figure-5, B). We continued Fluidigm real-time RT-PCR for CTNNB1 gene and discover the difference mRNA expression of CTNNB1 between neonate and adult undifferentiated SSCs and also between neonate and adult differentiated SSCs (Figure-6, A and B), and also the difference between differentiated and undifferentiated neonate SSCs and mESCs (Figure-6, C). Results shows a significant ($P<0.05$) increase of CTNNB1 mRNA during differentiation process (Figure-6, C), and a significant ($P<0.05$) higher expression in neonate differentiated SSCs than adult (Figure 6, B).

3.3. Identification of proteins involved in self-renewal in Mus musculus and extraction subnetworks: An interactive network of first 200 genes involved in "self-renewal" of SCSs was successfully constructed, concluding 7210 edges (Figure-7). Network nodes represent proteins (splice isoforms or post-translational modifications are collapsed, i.e., each node represents all the proteins produced by a single, protein-coding gene locus.) and edges represent protein-protein associations. Associations are meant to be specific and meaningful, i.e., proteins jointly contribute to a shared function. The more lines the more valid associations. We have shown the TERT edges in red and the KLF4 edges in bold black (Figure-7). After identifying the first neighbors' nodes of the KLF4 and TERT protein, we constructed subnetworks of them. The KLF4 subnetwork includes 120 nodes (Figure-8) and the TERT subnetwork includes 73 nodes (Figure-9).

3.4. Gene set enrichment analysis: By STRING enrichment analysis in the Cytoscape, we have identified enriched biological processes associated with our selected 16 genes that we had selected randomly and have interactions with KLF4, TERT, and CTNNB1. According to the purpose of our experiment, we have selected some GO biological processes, and also, we use Wiki Pathways and TISSUES databases to evaluate molecular functions and cellular locations (Figure-10, Table-1).

4. Discussion

Microscopic imaging during immunohistochemical analysis shows a significant expression of KLF4 in the central part of the seminiferous tube containing differentiated cells, while in the basal membrane there is no expression. In addition, images from confocal microscopy during the immunocytochemical analysis of two differentiated and undifferentiated populations of SSCs also indicate that KLF4 is a differentiating factor and its increased expression leads to differentiation of SSCs cells and spermatogenesis. While the results of Yamanaka's experiments and many other researchers after him have shown that KLF4 is essential for somatic cell reprogramming and, in intersection with other proteins, regulates ES cells self-renewal and pluripotency (9, 22). Peilin Zhang et al. Also stated that Klf4, the same as Oct4, NANOG, and Sox2, is highly expressed in ES cells, while its expression decreases dramatically during differentiation (23). Studies by Yan Sun et al. On the association of KLF4 with NANOG have also shown that NANOG expression levels are directly controlled by KLF4, which prevents differentiation in ES cells, and on the other hand KLF4 itself is directly activated by Oct4 and Stat3 and the overexpression of these factors increased the amount of Klf4 transcripts .(24) The results obtained from Fluidigm analysis show a significant decrease in KLF4 expression in passage 10 of undifferentiated SSCs in comparison to passage 0, which indicates that KLF4 expression has decreased with increasing self-renewal and proliferation. Similar to the results of our experiments, Dandan Yang et al. has found that during the knockout of KLF4 in the testis of Chlamys farreri, the spermatogenesis process was disrupted and it has even been associated with the change of gender from male to female, proving the crucial role of KLF4 in differentiation and spermatogenesis (25). Kit-Ling Sze et al. Has shown that KLF4 is involved in the activation of some proteins involved in tight junction, so it appears to regulate the movement and translocation of germ cells to cross the blood-testis barrier (26). Deletion of KLF4 has also been shown to impair Sertoli cell morphology during puberty in mice, although it does not impair fertility (27-29). From another perspective, we can point to the oncogenic role of KLF4 in cancer, which has been reported to be required for the maintenance of cancer stem cells (CSCs) in breast ductal carcinoma and prostate cancer. It also sustains and develops tumors in oral squamous carcinoma, and head and neck cancer (30). However, decreased klf4 expression in gastric, lung, and colon cancers has also been reported, and KLF4 expression levels are lower in cancer cells than in adjacent non-cancerous tissues, and its expression is inversely related to tumorigenesis, which supporting the role of KLF4 as a tumor suppressor. A combination of these studies proves that, KLF4 can play a dual role depending on different cellular contexts and types of cancer. This behavior is similar in other factors such as TGF-β (27, 30).

The complex telomerase activity is dependent on TERT, which has RNA-dependent RNA polymerase (RdRP) activity, and its gene expression is required to maintain or reactivate telomerase. As a result, it is

one of the momentous factors in regulating mitotic progression and the characteristics of stem cells as well as cancer stem cells (11, 31). To ensure that TERT is silenced in most normal cells but expressed in a timely manner and a proper place in a small group of cells such as SSCs, many factors, indirectly or directly, regulate TERT transcription in collaboration with transcription factors or other regulatory elements in a context-dependent manner; Therefore, the TERT promoter can have multiple and dynamic outputs in response to a variety of signaling paths (14, 32, 33). Recent studies have shown that WNT signaling is associated with TERT due to the cofactor role of TERT for β-catenin (Ctnnb1) (11). The TERT-BRG1 association has been shown to be involved in modulating transcription of Wnt target genes such as b-catenin (34). Wnt signaling is one of the essential growth, development, differentiation, stemness regulatory cascades and plays a critical role in fetal development and maintenance of cellular homeostasis. Hence, its mechanism is highly conserved in terms of evolution. However, its improper regulation is also associated with various inflammatory-related cancers (35). The canonical Wnt pathway is β-catenin-dependent, although it can also be non-β-catenin-dependent, which is called the non-canonical signaling pathway (36). β-catenin is the major nuclear agent in the WNT signaling pathway, and if it is present in the cytoplasm freely and not attached to E-cadherin, it will be destroyed by a complex consisting of Axin, APC, glycogen synthase kinase-3b (GSK3b) and casein kinase 1a (CK1a) that does not allow β-catenin to accumulate (37). Initiation of Wnt signaling leads to the accumulation and nuclear transport of the stable β-catenin that could affect transcriptional activators or the expression of transcription factors (37, 38). The Wnt/β-catenin signaling pathway has been shown to induce the differentiation of pluripotent embryonic stem cells into mesoderm and endoderm progenitor cells, which during this process, β-catenin is increased nuclear and cytoplasmic that leads to the activation of transcription of proteins such as cyclin D1 and c-Myc. These two proteins play a crucial role in controlling the cell cycle's G1 to S phase transition. And since cell proliferation is directly related to stem cell differentiation, the Wnt cascade is active during differentiation, and the Ctnnb1 is considered a differentiating factor (39-41). In this case, according to the results of our experiments, it seems that KLF4 is aligned with Ctnnb1, and both have increased expression during the differentiation process of SSCs. So, under these circumstances, the effect of Wnt signaling on TERT expression and telomerase activity is indirect, in which the c-Myc transcription factor directly controls TERT transcription and is a transcriptional stimulus of TERT expression, which is also regulated by Wnt/β-catenin signaling (38, 42). But extensive studies on this cascade have shown that the Wnt pathway connects to TERT in a slightly different way. Nusse Roel et al. (43) has identified the Wnt pathway as a main cascade in maintaining and regulating self-renewal and pluripotency in embryonic stem cells. In addition, Peter Wend et al. had endorsed the Wnt pathway support in the formation and maintenance of cancer stem cells (44). On the other hand, according to data collected by Hoffmeyer et al., by the β-catenin deficiency in ESCs and adult stem cells, TERT expression decreases dramatically; therefore, Stem cells have a higher expression of TERT because they have higher β-catenin expression. Their findings demonstrate the importance of β-catenin expression and the activation of the Wnt pathway to maintain stemness. The authors also showed that β-catenin binds to the TERT promoter so that in vivo β-catenin expression directly increases TERT transcription. Therefore, c-Myc no longer plays a role in this regulatory process. According to these studies, Ctnnb1 expression levels directly affect TERT expression and telomerase activity. On the other

hand, it has been suggested that TERT expression also affects Ctnnb1 expression reciprocally, and klf4 also cooperates in this regulatory loop (45, 46). According to the evidence obtained in our experiments, the expression of KLF4 protein increases during differentiation, so if we consider that the Wnt pathway is active in stem cells, then during the differentiation, the expression of genes involved in this signaling must be decreased. So, it seems that as KLF4 increases, the Wnt signaling pathway shuts down. Now the question arises: How does KLF4 affect the Wnt signaling pathway, and what is the KLF4 regulatory role?

Guang-Jun Zhang et al. has found that MiR-92a activates Wnt signaling during colorectal cancer. MiR-92a does this stimulation directly by downregulating KLF4 and promoting stem cell-like properties in CSCs. This finding also confirms the anti-cancer role of KLF4. And it appears that KLF4 suppressed the Wnt pathway by inhibiting the β-catenin transactivating domain in CRC cells (47). Many studies have also shown that KLF4 can bind to the TERT promoter and control TERT expression levels in cooperation with β-catenin (38, 45, 46). Thus, the findings generally suggest that the regulation of TERT expression requires two central transcriptional regulators, KLF4 and β-catenin, to ensure that there is not too much that may allow cancer cells to grow or lead to a lack of differentiation of SSCs, and not too little, which may lead to the destruction of stem cells in the body.

5. Conclusion And Future Perspective

In summary, our study demonstrates the pattern of KLF4 expression during differentiation and its regulatory function and connection with TERT and Ctnnb1 expression levels along the Wnt/β-catenin signaling pathway. Due to the significant role of telomerase in tumorigenesis and the development of stem-like cells features, it can be used for therapeutic or diagnostic purposes. Also, due to its role in anti-ageing properties, which is a very controversial topic currently, an accurate understanding of its regulatory mechanisms can be helpful in regenerative medicine. Various complex interactions are involved in the process of differentiation, proper spermatogenesis and thus reproduction, and one of the most interesting signaling pathways is the Wnt cascade due to its dual behavior during testicular development. Our findings could provide a better understanding and broader perspective to the scientific community to better understand molecular mechanisms during testicular development and related abnormalities, leading to discovering or optimizing existing methods for infertility treatment.

Declarations

Acknowledgements

This research was supported by faculty of biotechnology of Amol University of Special Modern Technologies, Islamic Republic of Iran and the University of Heidelberg, Institute for Anatomy and Cell Biology III, Department of Neuroanatomy, Germany (According to the memorandum of understanding (MOU) agreement).

Ethical statement

The animal experiments: approved by the ethical committee of Amol University of Special Modern Technologies (It.ausmt.rec.1400.04).

Authors' Contributions

Kiana Sojoudi: Carried out and designed the experiment, designed the bioinformatics data, assembly of data, data analysis and edited the final document; Hossein Azizi: Carried out and designed the experiment; Thomas Skutella: Provided critical feedback and data analysis. The authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Funding

This research was supported by the Centre for International Scientific Studies and Collaboration (CISSC), Ministry of Science, Research and Technology and memorandum of understanding (MOU) agreement between the Institute for Anatomy and Cell Biology in the University of Heidelberg and Amol University of Special Modern Technology.

Data Availability Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

References

1. Corbineau S, Lassalle B, Givelet M, Souissi-Sarahoui I, Firlej V, Romeo PH, et al. Spermatogonial stem cells and progenitors are refractory to reprogramming to pluripotency by the transcription factors Oct3/4, c-Myc, Sox2 and Klf4. Oncotarget. 2017;8(6):10050.
2. Azizi H, Asgari B, Skutella T. Pluripotency potential of embryonic stem cell-like cells derived from mouse testis. Cell Journal (Yakhteh). 2019;21(3):281.
3. Niknejad P, Azizi H, Sojoudi K. POU5F1 Protein and Gene Expression Analysis in Neonate and Adult Mouse Testicular Germ Cells by Immunohistochemistry and Immunocytochemistry. Cellular Reprogramming. 2021;23(6):349–58.
4. Azizi H, NiaziTabar A, Mohammadi A, Skutella T. Characterization of DDX4 gene expression in human cases with non-obstructive azoospermia and in sterile and fertile mice. Journal of Reproduction & Infertility. 2021;22(2):85.
5. Cheng Z, Zou X, Jin Y, Gao S, Lv J, Li B, et al. The role of KLF4 in Alzheimer's disease. Frontiers in cellular neuroscience. 2018:325.
6. Shi Y, Ai W. Function of KLF4 in stem cell biology. Pluripotent Stem Cells. 2013.

7. Schuetz A, Nana D, Rose C, Zocher G, Milanovic M, Koenigsmann J, et al. The structure of the Klf4 DNA-binding domain links to self-renewal and macrophage differentiation. Cellular and Molecular Life Sciences. 2011;68(18):3121–31.
8. Wang X, Xia S, Li H, Wang X, Li C, Chao Y, et al. The deubiquitinase USP10 regulates KLF4 stability and suppresses lung tumorigenesis. Cell Death & Differentiation. 2020;27(6):1747–64.
9. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. cell. 2006;126(4):663–76.
10. Chen X, Whitney EM, Gao SY, Yang VW. Transcriptional profiling of Krüppel-like factor 4 reveals a function in cell cycle regulation and epithelial differentiation. Journal of molecular biology. 2003;326(3):665–77.
11. Yuan X, Larsson C, Xu D. Mechanisms underlying the activation of TERT transcription and telomerase activity in human cancer: old actors and new players. Oncogene. 2019;38(34):6172–83.
12. Arish N, Petukhov D, Wallach-Dayan SB. The role of telomerase and telomeres in interstitial lung diseases: from molecules to clinical implications. International Journal of Molecular Sciences. 2019;20(12):2996.
13. Colebatch AJ, Dobrovic A, Cooper WA. TERT gene: its function and dysregulation in cancer. Journal of clinical pathology. 2019;72(4):281–4.
14. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu C-P, Morin GB, et al. Extension of life-span by introduction of telomerase into normal human cells. science. 1998;279(5349):349–52.
15. Brenner KA, Nandakumar J. Small molecules restore telomeres in patient stem cells. Trends in pharmacological sciences. 2020;41(8):506–8.
16. Vinagre J, Almeida A, Pópulo H, Batista R, Lyra J, Pinto V, et al. Frequency of TERT promoter mutations in human cancers. Nature communications. 2013;4(1):1–6.
17. Azizi H, Hamidabadi HG, Skutella T. Differential proliferation effects after short-term cultivation of mouse spermatogonial stem cells on different feeder layers. Cell Journal (Yakhteh). 2019;21(2):186.
18. Azizi H, Conrad S, Hinz U, Asgari B, Nanus D, Peterziel H, et al. Derivation of pluripotent cells from mouse SSCs seems to be age dependent. Stem cells international. 2016;2016.
19. Conrad S, Azizi H, Skutella T. Single-cell expression profiling and proteomics of primordial germ cells, spermatogonial stem cells, adult germ stem cells, and oocytes. Stem Cells: Biology and Engineering. 2017:77–87.
20. Azizi H, Skutella T, Shahverdi A. Generation of mouse spermatogonial stem-cell-colonies in a non-adherent culture. Cell Journal (Yakhteh). 2017;19(2):238.
21. Okada S, Kuroki K, Ruiz CA, Tosi AJ, Imamura M. Molecular histology of spermatogenesis in the Japanese macaque monkey (Macaca fuscata). Primates. 2021;62(1):113–21.
22. Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, et al. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nature biotechnology. 2008;26(1):101–6.

23. Zhang P, Andrianakos R, Yang Y, Liu C, Lu W. Kruppel-like factor 4 (Klf4) prevents embryonic stem (ES) cell differentiation by regulating Nanog gene expression. Journal of Biological Chemistry. 2010;285(12):9180–9.
24. Sun Y, Liu J, Wang B, Liu X, Du X, Liu Y, et al. Klf4 could be activated by Pou5f3/Stat3 to induce the expression of Nanog in Japanese flounder. Gene. 2019;687:56–63.
25. Yang D, Zhang Z, Liang S, Yang Q, Wang Y, Qin Z. A novel role of Krüppel-like factor 4 in Zhikong scallop Chlamys farreri during spermatogenesis. Plos one. 2017;12(6):e0180351.
26. Sze KL, Lee WM, Lui WY. Expression of CLMP, a novel tight junction protein, is mediated via the interaction of GATA with the Kruppel family proteins, KLF4 and Sp1, in mouse TM4 Sertoli cells. Journal of cellular physiology. 2008;214(2):334–44.
27. Ghaleb AM, Yang VW. Krüppel-like factor 4 (KLF4): What we currently know. Gene. 2017;611:27–37.
28. Yan R-G, Yang Q-L, Yang Q-E. E4 transcription factor 1 (E4F1) regulates sertoli cell proliferation and fertility in Mice. Animals. 2020;10(9):1691.
29. Godmann M, Gashaw I, Katz JP, Nagy A, Kaestner KH, Behr R. Krüppel-like factor 4, a “pluripotency transcription factor” highly expressed in male postmeiotic germ cells, is dispensable for spermatogenesis in the mouse. Mechanisms of development. 2009;126(8–9):650–64.
30. Qi X-t, Li Y-l, Zhang Y-q, Xu T, Lu B, Fang L, et al. KLF4 functions as an oncogene in promoting cancer stem cell-like characteristics in osteosarcoma cells. Acta Pharmacologica Sinica. 2019;40(4):546–55.
31. Wu K-J, Grandori C, Amacker M, Simon-Vermot N, Polack A, Lingner J, et al. Direct activation of TERT transcription by c-MYC. Nature genetics. 1999;21(2):220–4.
32. Liu T, Yuan X, Xu D. Cancer-specific telomerase reverse transcriptase (TERT) promoter mutations: biological and clinical implications. Genes. 2016;7(7):38.
33. Sahm F, Schrimpf D, Olar A, Koelsche C, Reuss D, Bissel J, et al. TERT promoter mutations and risk of recurrence in meningioma. JNCI: Journal of the National Cancer Institute. 2016;108(5).
34. Yuan X, Xu D. Telomerase reverse transcriptase (TERT) in action: cross-talking with epigenetics. International journal of molecular sciences. 2019;20(13):3338.
35. Silva-García O, Valdez-Alarcón JJ, Baizabal-Aguirre VM. Wnt/β-catenin signaling as a molecular target by pathogenic bacteria. Frontiers in Immunology. 2019:2135.
36. Flores-Hernández E, Velázquez DM, Castañeda-Patlán MC, Fuentes-García G, Fonseca-Camarillo G, Yamamoto-Furusho JK, et al. Canonical and non-canonical Wnt signaling are simultaneously activated by Wnts in colon cancer cells. Cellular Signalling. 2020;72:109636.
37. Lybrand DB, Naiman M, Laumann JM, Boardman M, Petshow S, Hansen K, et al. Destruction complex dynamics: Wnt/β-catenin signaling alters Axin-GSK3β interactions in vivo. Development. 2019;146(13):dev164145.
38. Ghareghomi S, Ahmadian S, Zarghami N, Kahroba H. Fundamental insights into the interaction between telomerase/TERT and intracellular signaling pathways. Biochimie. 2021;181:12–24.

39. Inestrosa NC, Varela-Nallar L. Wnt signalling in neuronal differentiation and development. Cell and tissue research. 2015;359(1):215–23.
40. Muroyama Y, Kondoh H, Takada S. Wnt proteins promote neuronal differentiation in neural stem cell culture. Biochemical and biophysical research communications. 2004;313(4):915–21.
41. Tokue M, Ikami K, Mizuno S, Takagi C, Miyagi A, Takada R, et al. SHISA6 confers resistance to differentiation-promoting Wnt/β-catenin signaling in mouse spermatogenic stem cells. Stem Cell Reports. 2017;8(3):561–75.
42. Shen Q, Yu W, Fang Y, Yao M, Yang P. Beta-catenin can induce hair follicle stem cell differentiation into transit-amplifying cells through c-myc activation. Tissue and Cell. 2017;49(1):28–34.
43. Nusse R, Fuerer C, Ching W, Harnish K, Logan C, Zeng A, et al., editors. Wnt signaling and stem cell control. Cold Spring Harbor symposia on quantitative biology; 2008: Cold Spring Harbor Laboratory Press.
44. Wend P, Holland JD, Ziebold U, Birchmeier W, editors. Wnt signaling in stem and cancer stem cells. Seminars in cell & developmental biology; 2010: Elsevier.
45. Hoffmeyer K, Raggioli A, Rudloff S, Anton R, Hierholzer A, Del Valle I, et al. Wnt/β-catenin signaling regulates telomerase in stem cells and cancer cells. Science. 2012;336(6088):1549–54.
46. Greider CW. Wnt regulates TERT—putting the horse before the cart. Science. 2012;336(6088):1519–20.
47. Zhang G-J, Li L-F, Yang G-D, Xia S-S, Wang R, Leng Z-W, et al. MiR-92a promotes stem cell-like properties by activating Wnt/β-catenin signaling in colorectal cancer. Oncotarget. 2017;8(60):101760.

Tables

Table 1. Name, color and details of each function specified in Figure 10.

Category	Color	Term number	Description	FDR value
Go Process		GO:0007346	regulation of mitotic cell cycle	5.79E-6
Go Process		GO:0008285	negative regulation of cell population proliferation	2.38E-5
Go Process		GO:0030111	regulation of Wnt signaling pathway	0.0023
Go Process		GO:0045595	regulation of cell differentiation	2.63E-10
Go Process		GO:0045596	negative regulation of cell differentiation	2.94E-11
Go Process		GO:0008134	transcription factor binding	1.72E-6
Go Process		GO:0043067	regulation of programmed cell death	2.4E-4
WikiPathways		WP723	Wnt signaling pathway and pluripotency	5.67E-8
WikiPathways		WP1763	pluriNetWork; mechanisms associated with pluripotency	9.32E-17
TISSUES		BTO:0000556	germ layer	1.32E-12
TISSUES		BTO:0001086	embryonic stem cells	1.13E-10
Go Process		GO:0030154	cell differentiation	7.09E-10
Go Process		GO:0042981	regulation of apoptotic process	0.0018

Figures

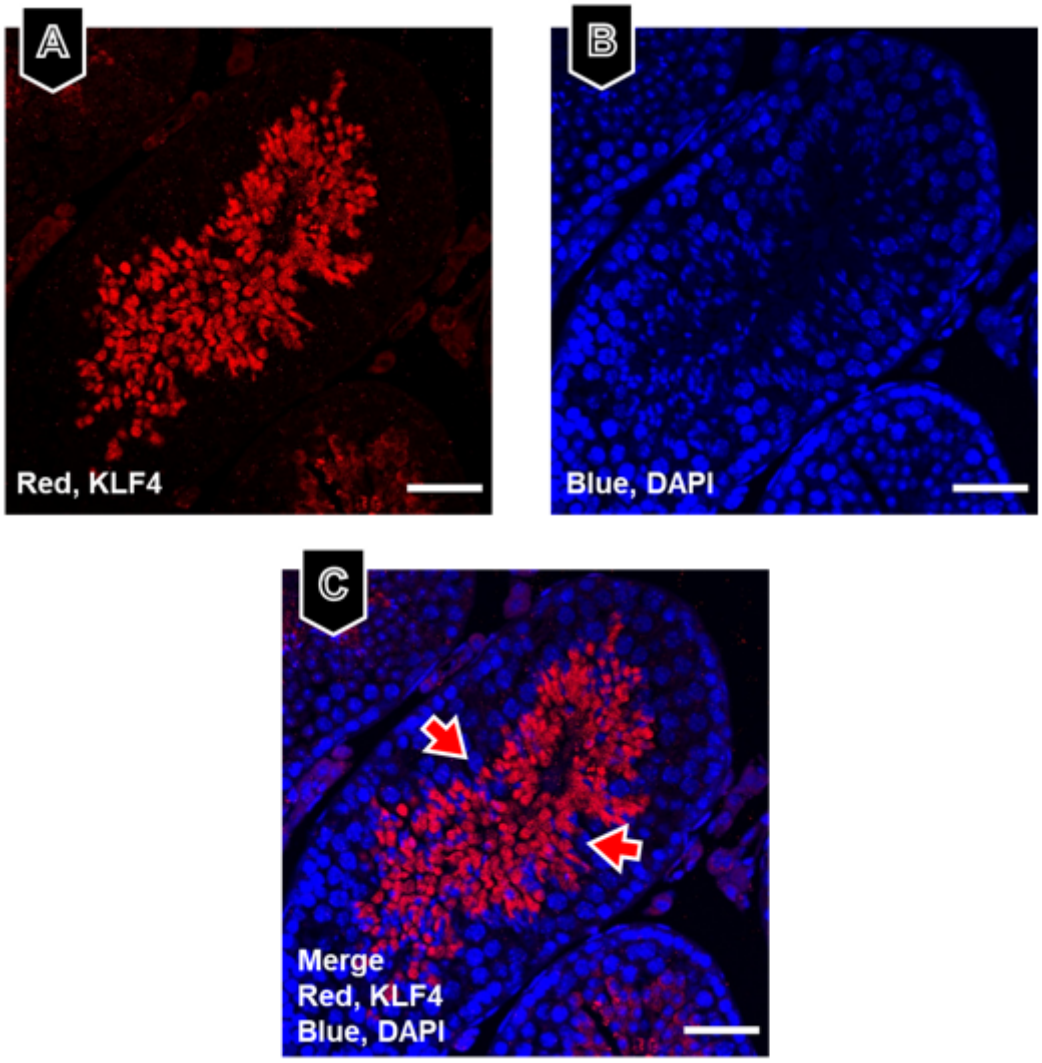

Figure 1

Immunohistochemistry (IMH) analysis of KLF4 expression in the seminiferous tubules of mice (scale bar: 50 µm). The high expression of Klf4 in the central parts of seminiferous tubules showing the expression of KLF4 up-regulated during differentiation of SSCs into sperm cells. **(A)**; Blue for 4', 6-diamidino-2-phenylindole (DAPI) was used to stain nuclear DNA **(B)**; Representation of the merged images **(C)**.

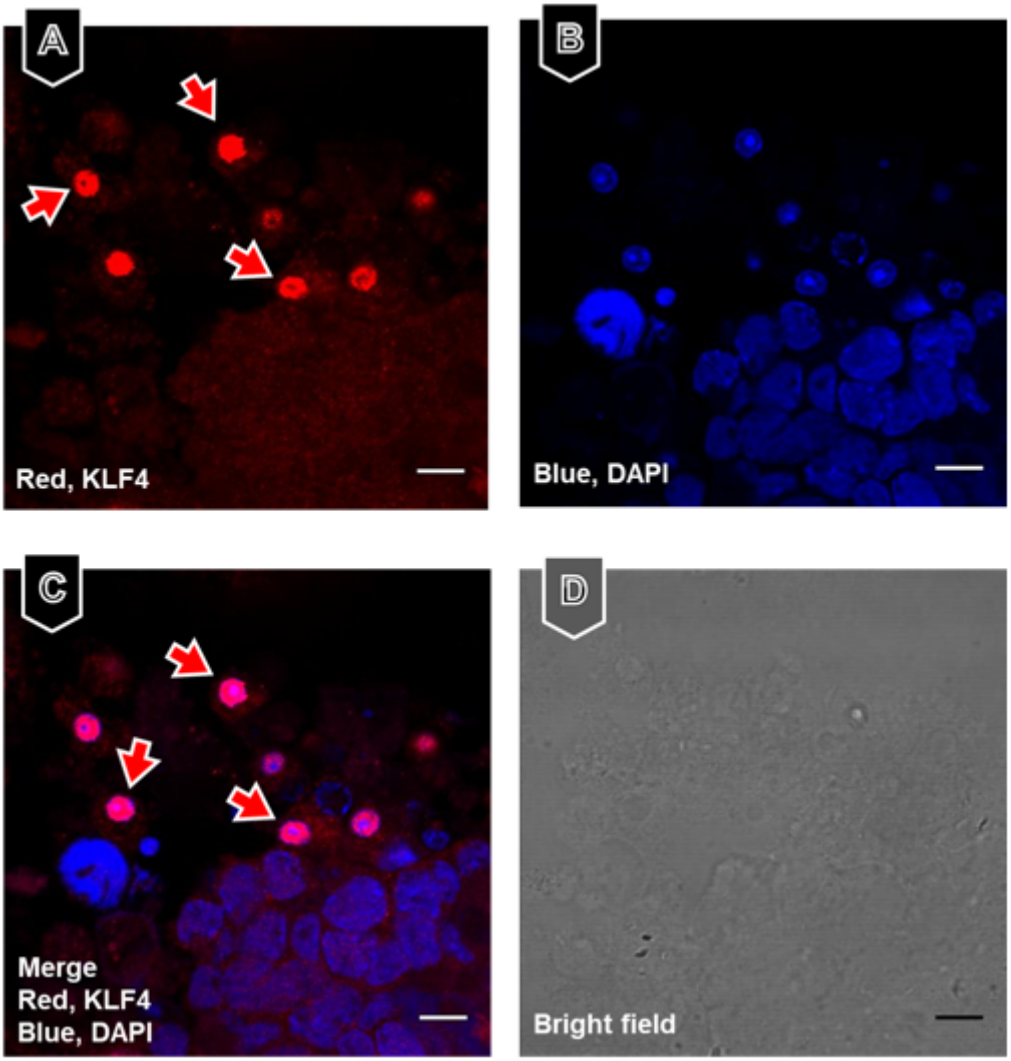

Figure 2

The difference of expression of KLF4 in two populations of spermatogonia by immunocytochemical (ICC) analysis In-vitro (scale bar: 50 µm). Red arrows indicate differentiated cells. Red fluorescence shows KLF4 which have high expression in differentiated cells **(A)**; Blue shows DAPI **(B)**; Representation of the merged images with DAPI. KLF4; Red and DAPI; Blue **(C)**; Bright field **(D)**.

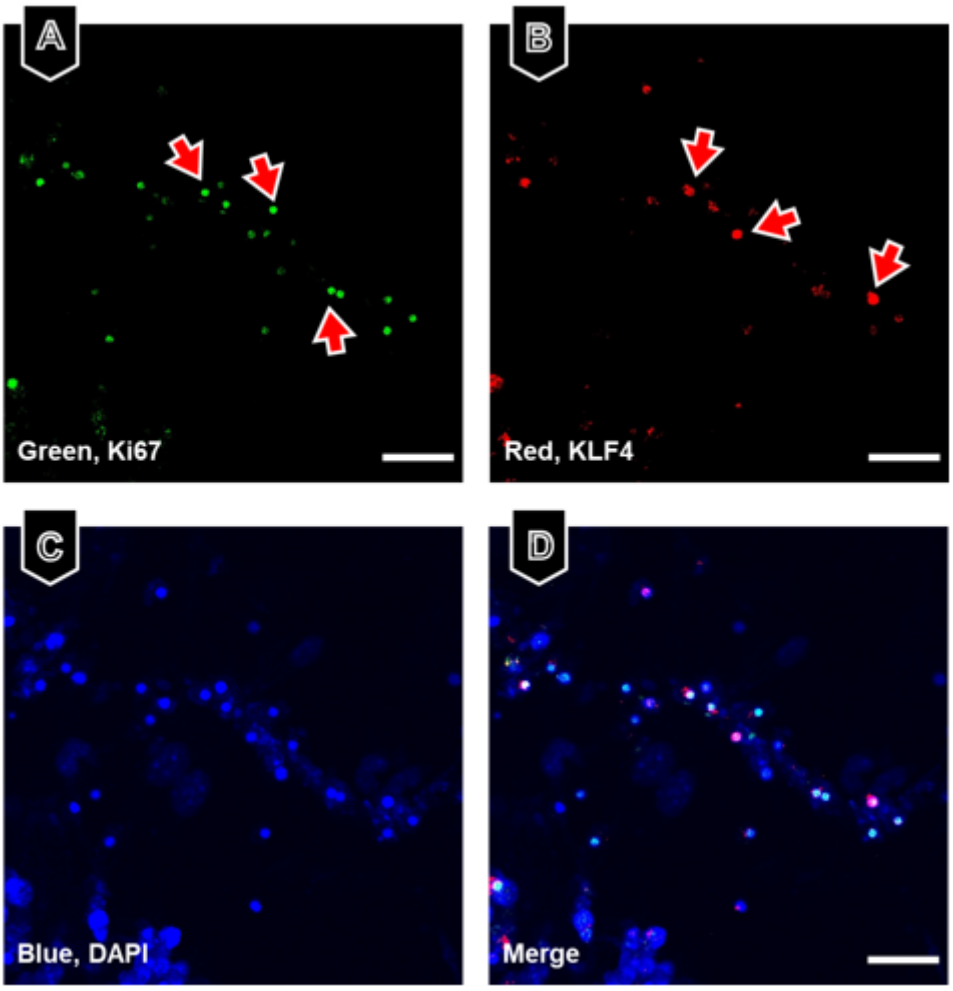

Figure 3

The difference of expression of KLF4 and Ki67 in two populations of spermatogonia by immunocytochemical (ICC) analysis In-vitro (scale bar: 50 µm). Red arrows indicate differentiated cells. Green fluorescence shows Ki67 **(A)**; Red fluorescence shows KLF4 **(B)**; Blue shows DAPI **(C)**; Representation of the merged images **(D)**.

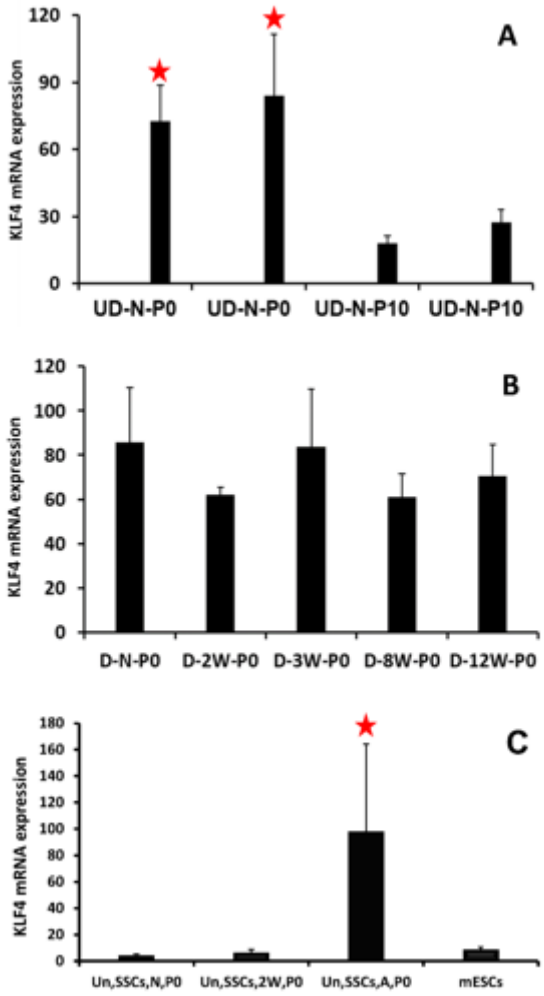

Figure 4

Fluidigm real-time RT-PCR analysis for KLF4 expression. Significant decrease of the expression of KLF4 in neonate mouse undifferentiated spermatogonia passage 10 in comparison to passage 0 by Fluidigm real-time PCR analysis **(A)**; Not seeing a significant difference in passage 0 of differentiated spermatogonia of neonate, two weeks, three weeks, eight weeks, and twelve weeks mouse **(B)**; Significant increase in KLF4 mRNA expression with increasing age of mice in passage 0 **(C)** ($P<0.05$).

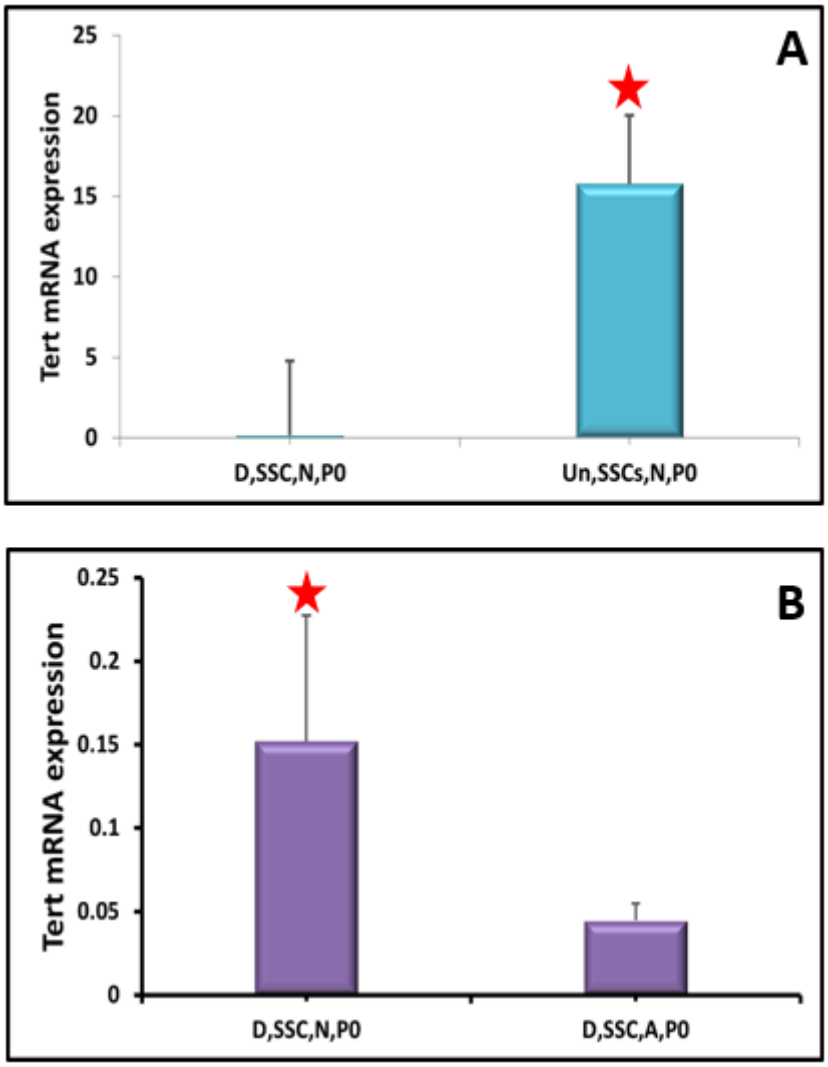

Figure 5

Comparison of TERT mRNA expression in different population and different ages. Significant decrease (P<0.05) of TERT in differentiation process **(A);** significant (P<0.05) higher expression level of TERT in neonate SSCs than Adult SSCs **(B)**.

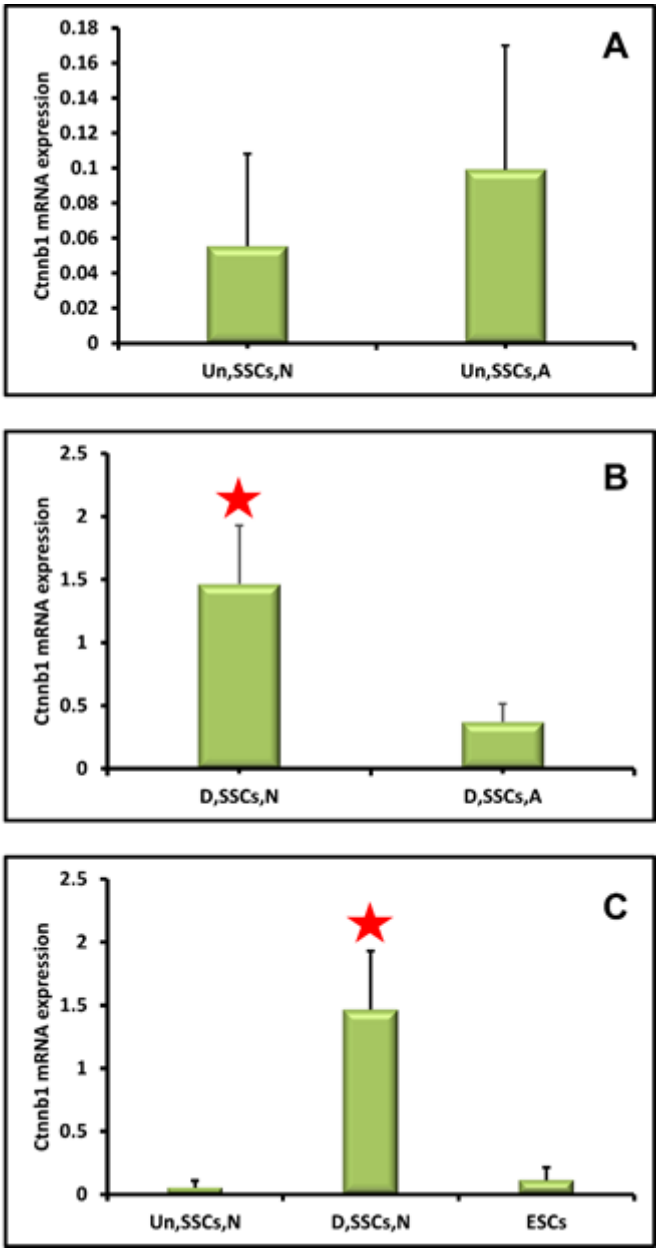

Figure 6

Comparison of CTNNB1 mRNA expression in different population and different ages. All samples are in passage 0. Insignificant higher expression of CTNNB1 in adult undifferentiated SSCs than neonate SSCs **(A)**; a significant higher expression of CTNNB1in adult differentiated SSCs than neonate SSCs **(B)**; And a significant increase of CTNNB1 during differentiation ($P<0.05$) **(C)** ($P<0.05$).

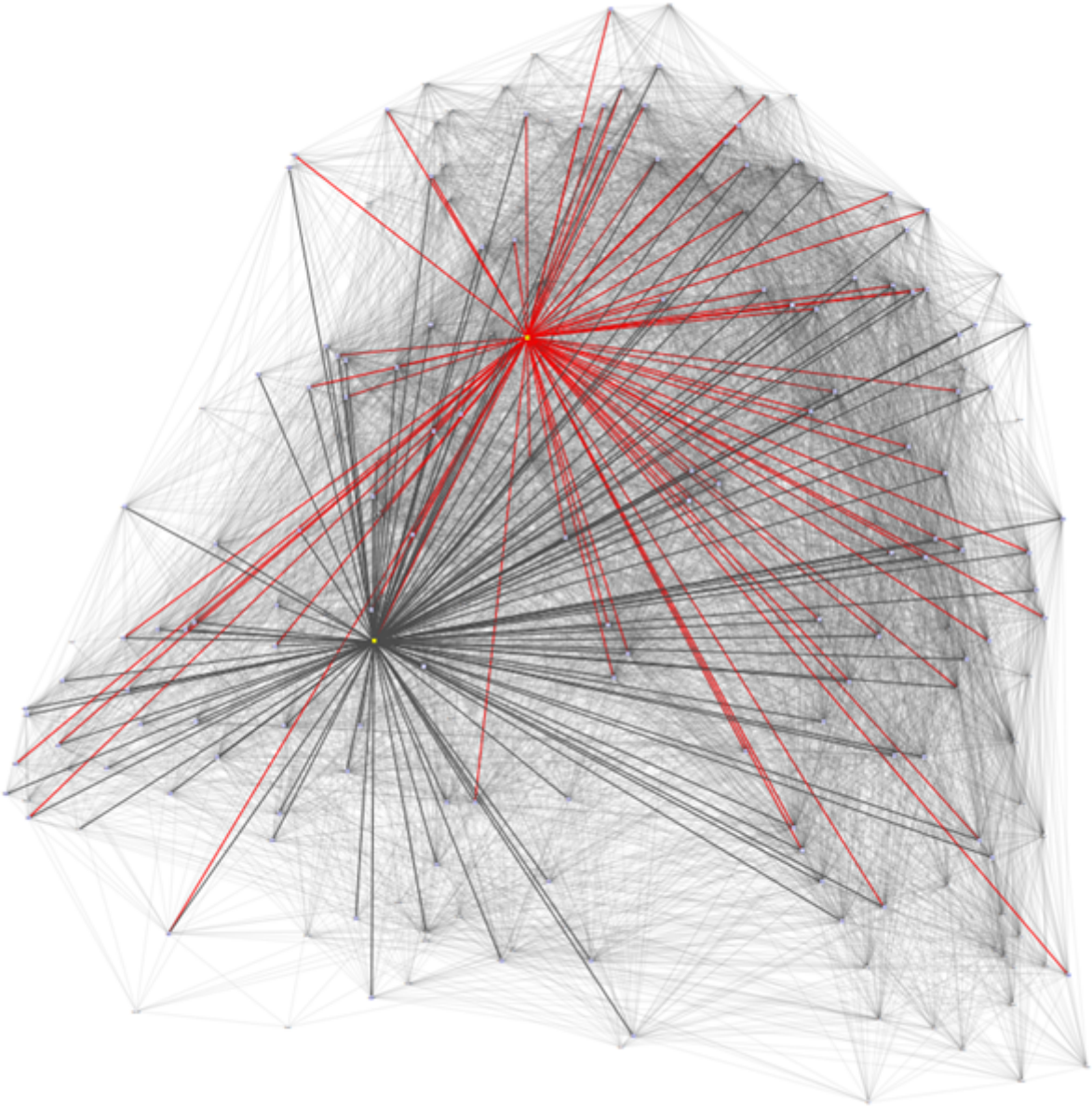

Figure 7

STRING functional gene analysis using Cytoscape. The first 200 genes involved in the self-renewal**.** Each nodes represents a protein and Edges indicate protein–protein interactions, with line thickness being indicative of evidence strength for a predicted interaction. Red edges represent first direct interactions of TERT and bold black edges represent first direct interactions of KLF4 (confidence score cutoff >0.40).

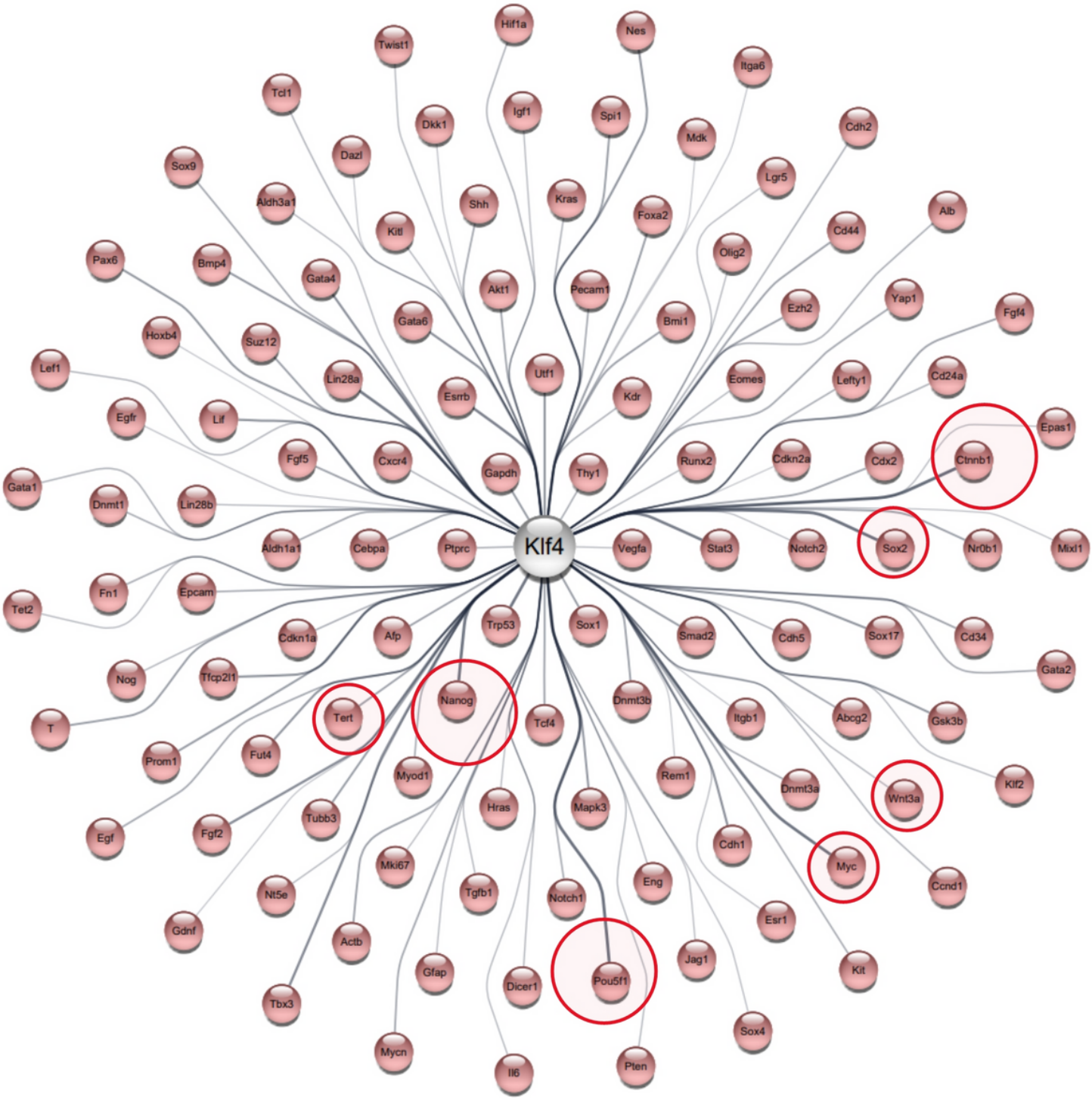

Figure 8

The interactions between the first neighbors' nodes of KLF4. The subnetwork extracted from the main network of self-renewal. With line thickness being indicative of evidence strength for a predicted interaction (confidence score cutoff >0.40).

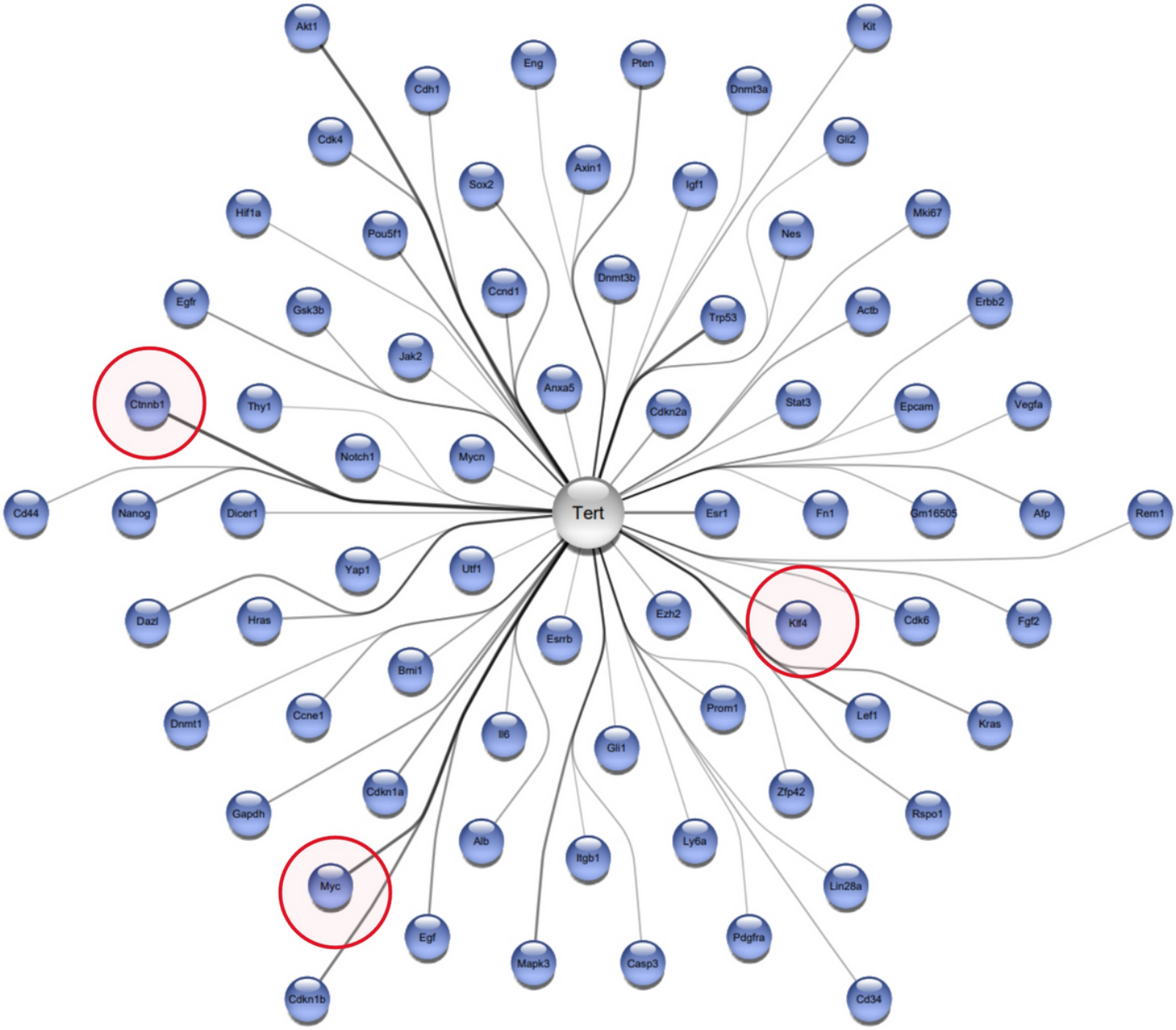

Figure 9

The interactions between the first neighbors' nodes of TERT. The subnetwork extracted from the main network of self-renewal. With line thickness being indicative of evidence strength for a predicted interaction (confidence score cutoff >0.40).

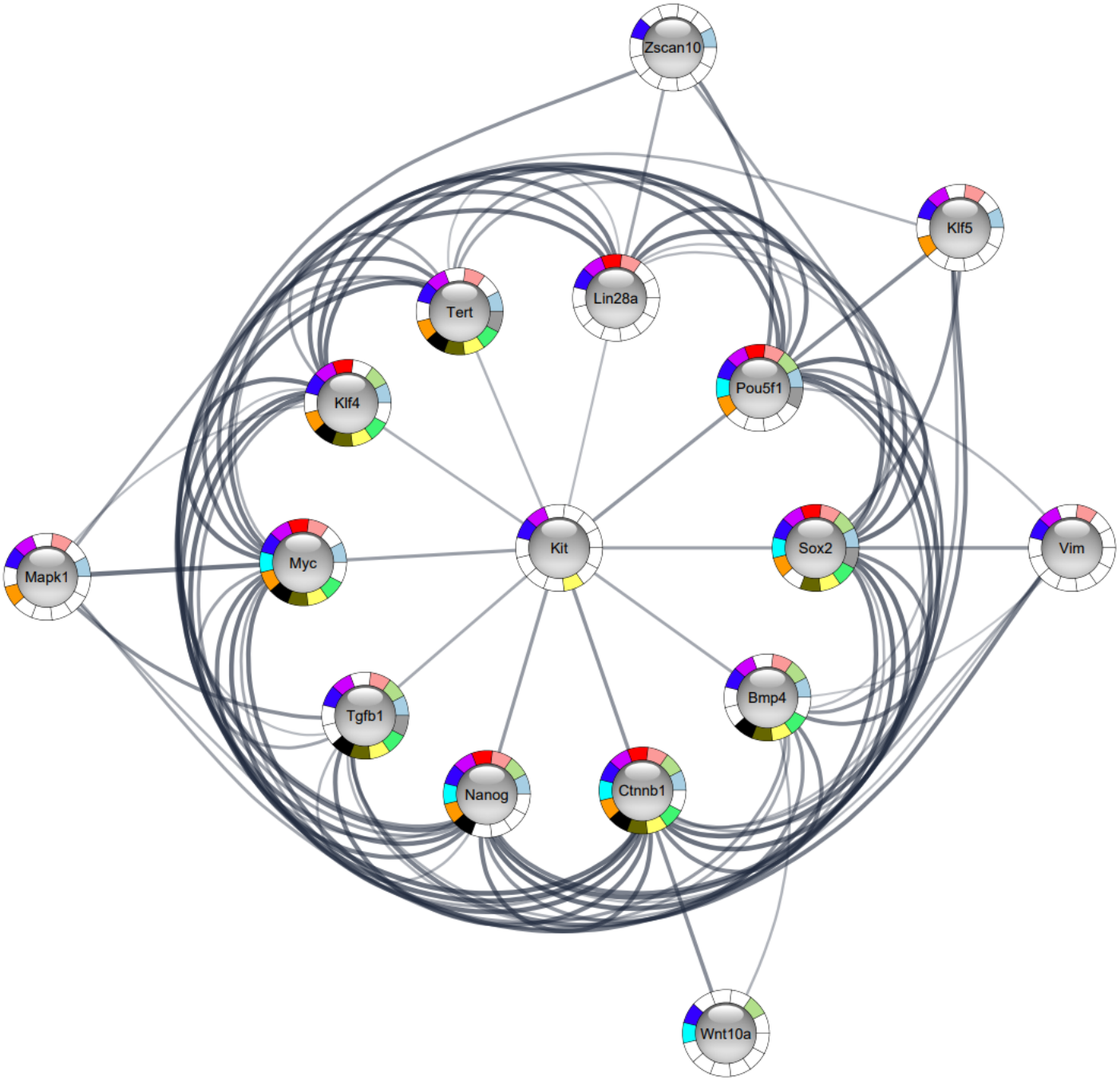

Figure 10

The functional enrichment analysis of selected genes. Different colored parts of the circles refer to the related biological processes and with line thickness being indicative of evidence strength for a predicted interaction.