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Research Article

A Study of Dual Function and Expression Pattern of *Klf4* between Two Different Processes of Differentiation and Maintenance of Pluripotency in Spermatogonial Stem Cells

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Krüppel-like factor 4 (*Klf4*) is a transcription factor involved in proliferation, self-renewal, differentiation, apoptosis, and somatic cell reprogramming; thus, it has a critical role in accurate spermatogenesis. In this study, we examine the different protein localizations of *Klf4* in the mouse testis and in two populations of spermatogonial stem cells (SSCs), including differentiated and undifferentiated cell types. We show that in contrast to its function during spermatogenesis in the adult testis, *in vitro Klf4* is a factor in maintaining stemness in different SSC populations. Furthermore, we suggest that its functions could be age-dependent. Bioinformatics analysis in the Cytoscape platform was conducted to highlight pathways and networks of *Klf4* interactions. Our results support the different functions of *Klf4* dependent on the age and differentiation state of SSCs.

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 cells are 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. Chronologically, spermatogonia stages are either undifferentiated A-type or differentiating. GSCs are undifferentiated A_{single} (As) spermatogonia [1, 2]. Daughter cells formed from undifferentiated As (A_{single}) spermatogonia in mice may move to direct stem cell renewal or give rise to additional undifferentiated A-type spermatogonia, which go through differentiation stages. The consecutive stages of undifferentiated spermatogonia proliferate synchronously as A_{paired} (Apr) spermatogonia in clusters of 2 germ cells and $A_{aligned}$ (Aal) spermatogonia in clusters of 4, 8, or 16 germ cells. Undifferentiated A spermatogonia changes into differentiating spermatogonia, which then undergoes six more rounds of synchronous interconnected division to become A1, A2, A3, A4, intermediate, and ultimately B spermatogonia. Spermatocytes, spermatids, and mature sperm are produced by type B differentiated cells. Past studies have shown that some factors are crucial for the normal process of spermatogenesis in vivo. Therefore, various investigations were performed to identify suitable markers of germ cells to advance new treatments against infertility [1, 3]. Krüppel-like factor 4 (*Klf4*) is one of the factors that its expression and effects on spermatogenesis are very controversial.

Klf4, a gut-enriched Krüppel-like factor or epithelial zinc finger, which is located in the cell nucleus, was first identified, characterized, and isolated from the NIH 3T3 library [4, 5]. For the first time, Yamanaka and Takahashi [6] have shown that *Klf4* can collaborate with other reprogramming factors (Oct3/4, Sox2, and Myc) to convert somatic cells to induced pluripotent stem cells and prevent stem cell differentiation. This has led to a broad perspective in the treatment of diseases such as immune diseases and vascular diseases. The importance of other Yamanaka factors in embryonic

stem cells (ESCs) self-renewal is well known, but the function of *Klf4* has not been well investigated in previous studies. After that, several studies mention *Klf4* as a stemness factor that is strongly expressed in undifferentiated stages and leads to the preservation of pluripotency and also self-renewal in ESCs [7]. A number of recent investigations have shown that *Klf4* has a twofold function in the induction of pluripotency, first suppressing differentiation markers and then promoting the expression of pluripotency genes. The leukemia inhibitory factor/signal transducer and activator of the transcription 3 (LIF/STAT3) pathway was shown to regulate *Klf4* independently. However, there are few *in vivo* functional investigations on the role of *Klf4* in stem cells, and the ones that do exist often find significant inconsistencies between the roles that *Klf4* plays [7, 8]. According to the findings of this research, *Klf4* may either stimulate or repress transcription, and it can either operate as an oncogene or a tumor suppressor, depending on the gene that it is interacting with and the environment in which it is found so that the result of its performance depends on the context and may play quite the opposite roles under different circumstances. It seems that 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 [9]. Hence, *Klf4* plays an essential role in determining the fate of cells because it can regulate various physiological and biological processes such as proliferation, apoptosis, neuroinflammation, and oxidative stress [10].

The *Klf4* expression pattern in SSCs and its association with other genes, its association with other genes, and its involvement in cellular signaling pathways still raise several questions. Furthermore, contemporary findings exhibit many contradictions that exacerbate these ambiguities of the exact function of *Klf4*. Here, we examine the expression pattern of *Klf4* during spermatogenesis and differentiation stages and show its connection with other genes during differentiation in mice. These findings have dramatically increased our understanding of molecular mechanisms regulating spermatogenesis and have opened up new opportunities for infertility treatment [11].

2. Materials and Methods

2.1. Isolation and Culture of Testicular Cells. In the current investigation, animal experiments were approved (It.ausmt. rec.1400.04) by the Institutional Animal Care and Ethics Committee of Amol University of Special Modern Technologies. All animal care was performed according to the guidelines of the Amol University of Special Modern Technologies (Amol, Iran). The testes of three neonates and adult male C57BL/6 mice were placed in a saline solution of phosphate buffer containing 2% BSA and 0.1% Triton ×100 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 was 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 min. The single-cell suspension obtained by enzymatic digestion of testicular tissue was washed by DMEM/ F12 (Invitrogen, USA), then centrifuged at 1,500 rpm for 10 min 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% CO_2 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 estradiol (Sigma-Aldrich, USA), 60 ng/ml progesterone (Sigma-Aldrich, USA), 100 U/ml human leukemia inhibitory factor (LIF) (Millipore), 1% MEM vitamins (PAA, USA), 8 ng/ml GDNF (Sigma-Aldrich, USA), 1% nonessential 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). All experiments were performed with mycoplasma-free cells. Identification of undifferentiated and differentiated spermatogonia was done according to the methods mentioned in our previous studies [12, 13].

2.2. Tissue Processing for Immunohistochemical Staining. Mouse testis tissue was picked up after decapsulation of the tunica albuginea, washed with phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde (PFA). 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 min. The nonspecific 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 [13, 14].

2.3. Immunocytochemical (ICC) Staining. In this experimental study, testicular cells were fixed with 4% PFA/PBS and permeabilized with 0.1% Triton/PBS solution. Testicular cells were blocked with 1% BSA/PBS and followed by incubation with primary antibodies. After 29 rinsings, the process was followed by incubation with species-specific secondary antibodies, which were conjugated with different fluorochrome, and the labeled cells were nuclear-counterstained treatment with 0.2 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) for 3 min 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 [13, 14].

2.4. Fluidigm Biomark System Gene Expression Analyses. The expression of *Klf4* (Mm00516104_m1) was analyzed utilizing

dynamic array chips (Fluidigm). The housekeeping gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Mm99999915_g1), was selected for normalization 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 0.2x assay pool and 5.0 μl Cells Direct 2x Reaction Mix (Invitrogen, USA). Using TaqMan real-time PCR on the BioMark Real-Time quantitative PCR system to quantify the targeted transcripts. Two technical replicates were concocted to analyze each sample, and by using Excel and GenEx applications, the Ct values were determined [14].

2.5. Construction of the Protein–Protein Interactive (PPI) Network and Identification of Hub Proteins. 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. PPI for *Klf4* was predicted by STRING app (version 11.5, https://string-db.org/) in Cytoscape Software (version 3.9.2) on the basis of evidence sources such as text mining, experimental evidences, databases, coexpression, neighborhood, gene fusion, and cooccurrence. We used STRING: PubMed query as our data source to generate a network between the first 50 genes that associated with *Klf4*. *Mus musculus* was selected as an organism. The edge score was calculated on the basis of molecular action and nodes with a confidence score of more than 0.400. The network was created, designed, and organized on the Cytoscape platform. Then, we clustered this network using clusterMaker2 (version 2.3.3). clusterMaker2 is a Cytoscape app that unifies different clustering, filtering, ranking, and dimensionality reduction algorithms along with appropriate visualizations into a single interface. We used molecular complex detection (MCODE) network cluster algorithms. The MCODE algorithm finds highly interconnected regions in a network. The cluster finding parameters were adopted, such as a degree cutoff of 2, a node score cutoff of 0.2, a kappa score (*K*-core) of 2, and a max depth of 100, which limits the cluster size for coexpressing networks. Subsequently, the first neighbors' nodes with *Klf4* were selected for further network construction and analyses. *Cytohubba* plugin that is implemented in Java, based on the Cytoscape API, was used to calculate node importance in a biological network on the basis of different criteria like maximal clique centrality (MCC), the density of maximum neighborhood component, maximum neighborhood component, degree, edge percolated component (EPC), bottleneck, eccentricity, closeness, radiality, betweenness, stress, and clustering coefficient. Nodes were ranked on the basis of EPC to predict hub nodes from the PPI network. EPC predicted the global connectivity properties of the PPI network.

2.6. Functional and Pathway Enrichment Analysis. To seek potential functions and the biological roles of the first neighbors' nodes with *Klf4*, we have performed functional gene enrichment analysis using the STRING Enrichment analysis in the Cytoscape platform. We selected a number of functional enrichments (Table 1) related to our laboratory data without considering a specific FDR value.

Table 1: Network graph properties as calculated by CytoHubba EPC algorithm.

Rank	Name	Score (EPC)
1	Klf4	14.721
2	NANOG	6.203
3	KLF2	6.097
4	ERAS	6.087
5	GATA4	6.002
6	SOX2	5.994
7	FGF2	5.993
8	FUT4	5.988
9	ESRRB	5.981
10	STAT3	5.978
11	UTF1	5.961
12	TBX3	5.948
13	BMP4	5.94
14	CDH1	5.908
15	DPPA4	5.885
16	NR5A2	5.868
17	TAGLN	5.858
18	LIN28A	5.856
19	AFP	5.838
20	FBXO15	5.837
21	PO5F1	5.815
22	MYCL	5.809
23	PROM1	5.808
24	MYC	5.769
25	GAPDH	5.765
26	EGF	5.763
27	CD44	5.756
28	TUBB3	5.721
29	DPPA5A	5.718
30	ACTB	5.688
31	FGF4	5.683
32	SOX17	5.669
33	LIF	5.655
34	GATA6	5.653
35	TFCP2L1	5.653
36	CTNNB1	5.647
37	NES	5.647
38	GDF3	5.633
39	TCF4	5.619
40	CDX2	5.603
41	PRRX1	5.552
42	TRP53	5.481

2.7. Identification of Predicted miRNAs and lncRNAs of Klf4. To identify predicted miRNAs that affect *Klf4*, four different

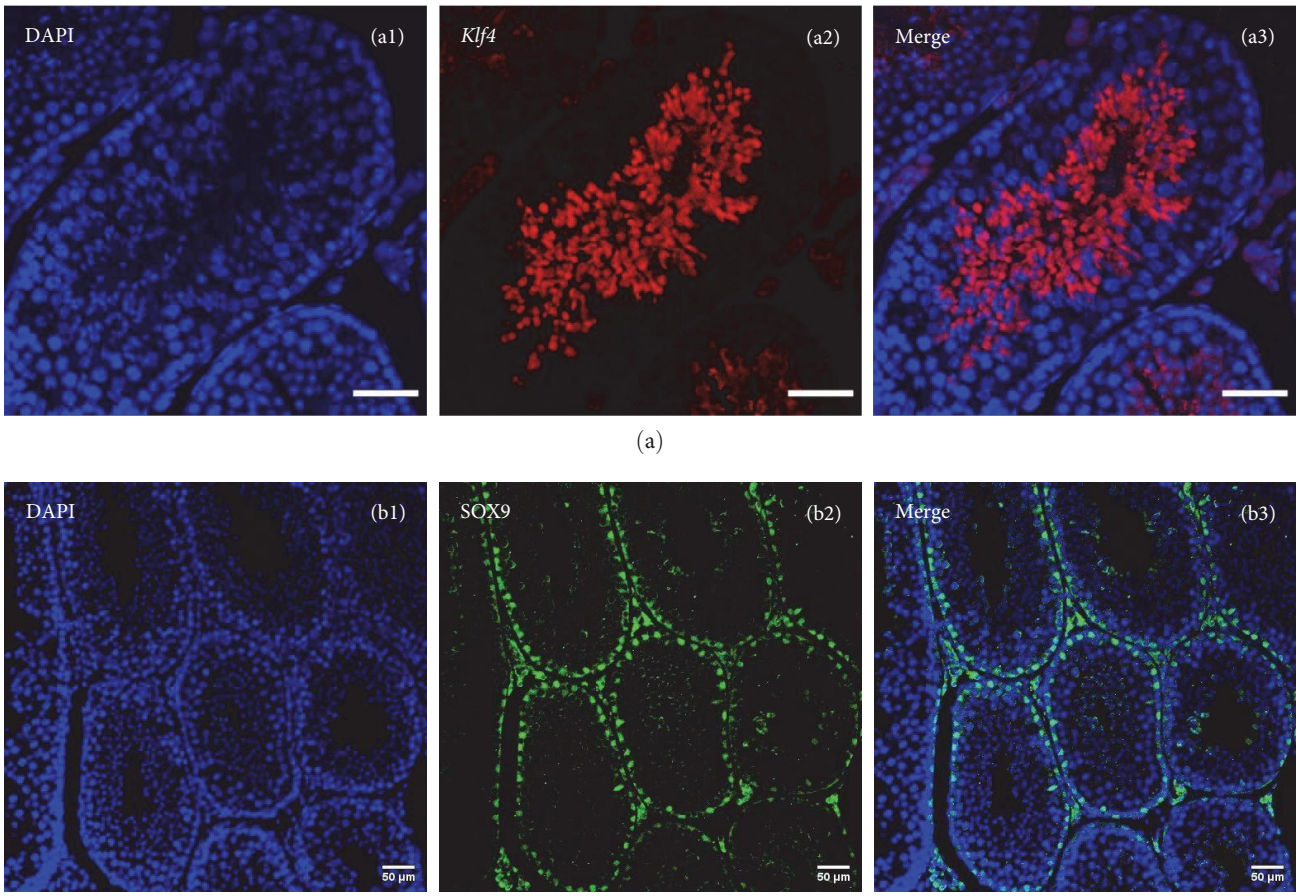


Figure 1: (a, b) Immunohistochemistry (IMH) analysis of *Klf4* expression in the seminiferous tubules and comparing it with SOX9: (a1) blue for 4′,6-diamidino-2-phenylindole (DAPI) was used to stain cell nuclear; (a2) red fluorescence shows the *Klf4* expression pattern, which has a high expression in the central parts of seminiferous tubules that means the expression of *Klf4* is upregulated in the adluminal compartment during spermatogenesis; (a3) representation of the merged images (scale bar: 100 μm); (b1) blue shows DAPI; (b2) green fluorescence shows the expression of SOX9, which shows the location of Sertoli cells; (b3) merge image of Sertoli cells marker with DAPI (scale bar: 50 μm).

databases were used, including TargetScan (https://www.targetscan.org/vert_80/; Whitehead Institute for Biomedical Research, Cambridge, UK; accessed on July 2023), miRDB (https://mirdb.org/cgi-bin/search.cgi; The Department of Pharmacology, University of Illinois at Chicago; Version 6.0; accessed on July 2022), RNAInter (http://www.rnainter.org/; Department of Bioinformatics, Southern Medical Universit; version 4.0; accessed on July 2023), and NPInter (http://bigdata.ibp.ac.cn/npinter4; Key Laboratory of RNA Biology, Institute of Biophysics, Chinese Academy of Sciences; version 4.0; accessed on July 2023). We looked for miRNAs that were common among all four databases. The regulatory link between miRNA and mRNA was used to build the miRNA–mRNA regulatory network. Moreover, we predicted the first 30 lncRNAs that affect *Klf4* expression using RNAInter (http://www.rnainter.org/; Department of Bioinformatics, Southern Medical Universit; version 4.0; accessed on July 2023). The regulatory link between lncRNA and mRNA was used to build the lncRNA–mRNA regulatory network. Both regulatory networks were visualized using Cytoscape software (version 3.9.2).

2.8. Statistical Analysis. The experiments were repeated at least three times. The data were statistically analyzed using the software Statistical Package for the Social Sciences (SPSS) version 20.0. After examining the results of the normality test on the data obtained from the Fluidigm test, we concluded that the gene expression data were not normally distributed; therefore, nonparametric tests such as the Kruskal–Wallis test and then one-way analysis of variance were used to measure its significance (equal variances not assumed: Tamhane's T2 and Dunnett's T3 tests). The variation between groups was considered statistically reliable, if a value of $p < 0.05$ had been acquired. Networks were analyzed based on relevant databases or online data analysis tools.

3. Results

3.1. Immunohistochemical Analysis of Klf4 in Seminiferous Tubules. In this particular investigation, immunohistochemistry (IMH) techniques were used in order to identify the specific localization of *Klf4* inside the seminiferous tubules (Figure 1(a1)–1(a3)). During the process of spermatogenesis,

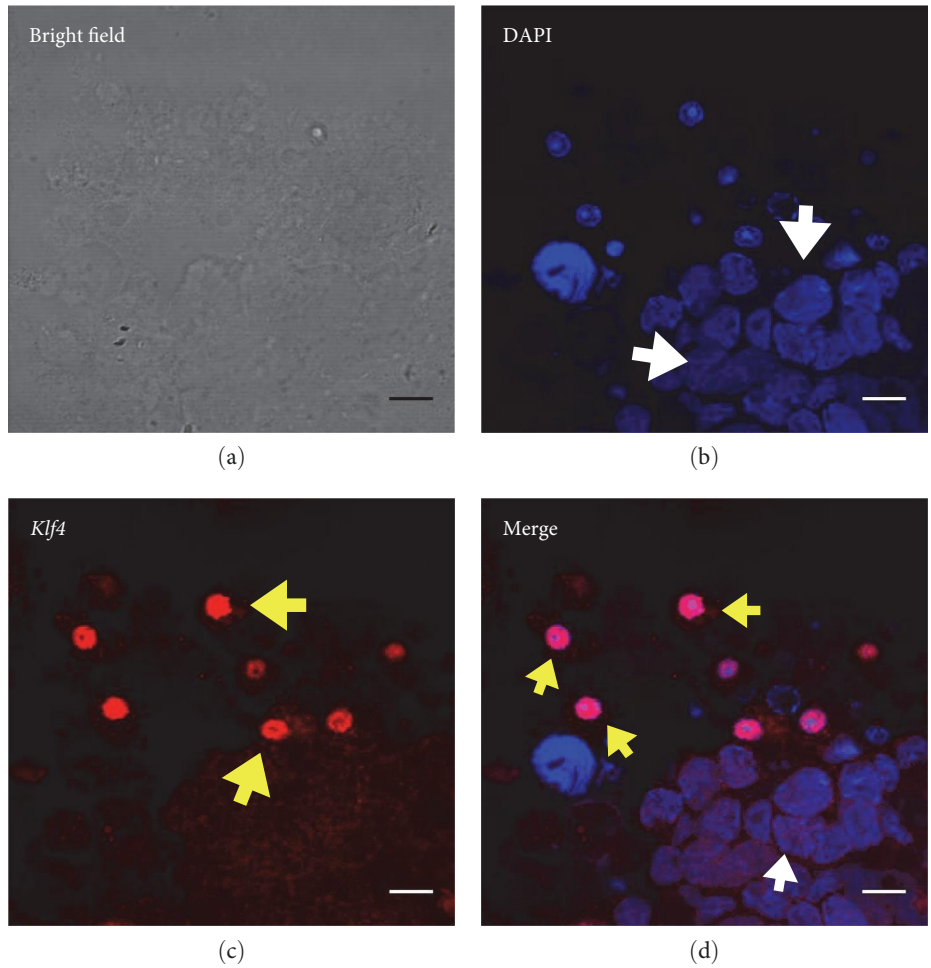


Figure 2: The difference of the protein expression pattern of *Klf4* in two populations of spermatogonia by immunocytochemical (ICC) analysis in vitro. Wight arrows indicate undifferentiated SSCs population, and the yellow arrows indicate differentiated SSCs population: (a) the bright field image of two populations of SSCs; (b) blue for 4′,6-diamidino-2-phenylindole (DAPI) was used to stain cell nuclear; (c) red fluorescence shows the expression pattern of *Klf4*, which has sharp expression in differentiated cells but a low expression in undifferentiated population of SSCs; (d) representation of the merged images with DAPI (scale bar: 50 μm).

it is noticed that there is an increase in the protein expression of the *Klf4* toward the adluminal compartment. It seems that during the differentiation phase, *Klf4* expression is at its highest in postmiotic round spermatids; However, the basal cells in the seminiferous tubules either lack entirely the expression of *Klf4* or express it in a highly restricted manner. Also, we compare the *Klf4* expression pattern with the expression pattern of the Sertoli cell marker, including SOX9. By comparison with the SOX9, it has been shown that the *Klf4* is not expressed in somatic cells of the seminiferous tubule, including Sertoli cells (Figure 1(b1)–1(b3)).

3.2. Comparison of Klf4 Expression in Two Differentiated and Undifferentiated Spermatogonia Populations. We performed an ICC analysis to determine the protein expression level of *Klf4* in two populations of spermatogonia: one differentiated and one undifferentiated. After enzyme digestion, both differentiated and undifferentiated spermatogonial cells were separated, and the resulting cells were cultured in the presence of the aforementioned growth factors. After several days of culture of undifferentiated spermatogonial colonies in the culture medium, we added some differentiated spermatogonial cells to them. These cells can be easily recognized based on their morphology. The characterization of the isolated SSCs was carried out in the same manner as was detailed in our previous work [14]. Using a confocal scanning UV-laser microscope to do an ICC analysis revealed that differentiated cells had an exceptionally high level of *Klf4* expression, while undifferentiated cells had a much lower level of *Klf4* expression (Figure 2). Also, we examined these two cell populations separately in different cultivation environments (Figure 3). In the following, we subjected SSCs differentiated in MEF culture medium to co-staining two factors, *Klf4* and *Vimentin*. In our previous articles, we have introduced *Vimentin* as a marker to identify differentiated SSCs [15]. Microscopic images of the differentiated SSC population show that Klf4 and Vimentin are strongly expressed in these cells. In addition, *Vimentin* is also expressed in fibroblastic cells related to the culture medium (Figure 4).

Subsequently, we focused on the Ki67, an indicator for dividing cells and a marker of cells in all phases of the cell

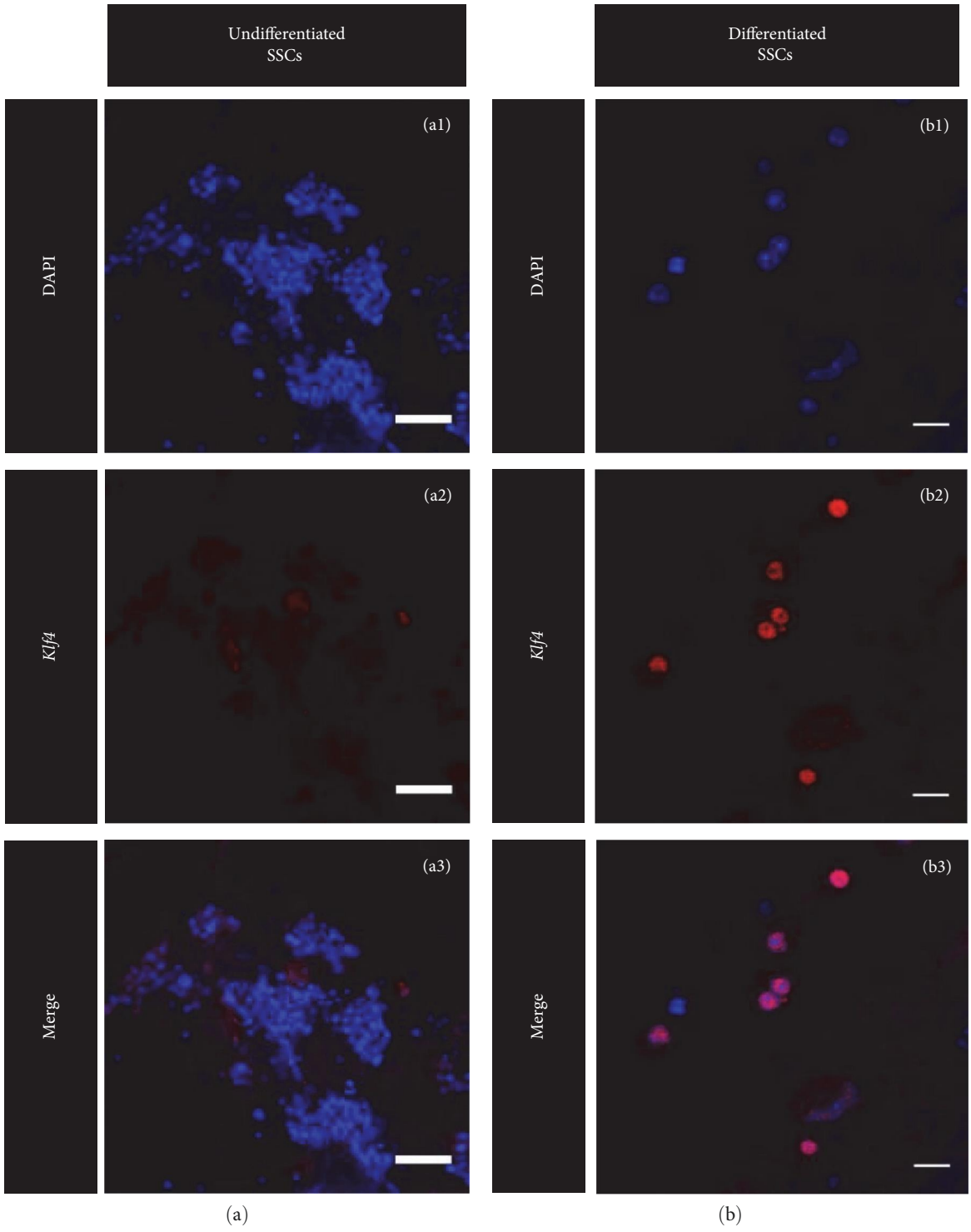


Figure 3: (a, b) Comparison of the *Klf4* expression pattern in the two cell populations of SSCs: (a1–a3) shows the *Klf4* pattern in the undifferentiated population of SSCs, and (b1–b3) shows the *Klf4* expression pattern in the differentiated population of SSCs; (a1 and b1) blue for 4′,6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus of cells; (a2 and b2) red fluorescence indicates the expression pattern of *Klf4* that has higher expression in the differentiated population than in the undifferentiated population of SSCs; (a3 and b3) representation of the merged images with DAPI (scale bar: 50 μm for b1–b3 and 100 μm for a1–a3).

cycle except G0 [16]. We observed that the expression of Ki67 in differentiated stages due to cell division is higher than in undifferentiated cells. We compared its expression with the *Klf4* expression pattern in differentiated and undifferentiated spermatogonia. Confocal scanning UV-laser microscope in ICC analysis exhibited a similar expression of *Klf4* and *Ki67*. Both of them have higher expression in differentiating cells than undifferentiated cells (Figure 5).

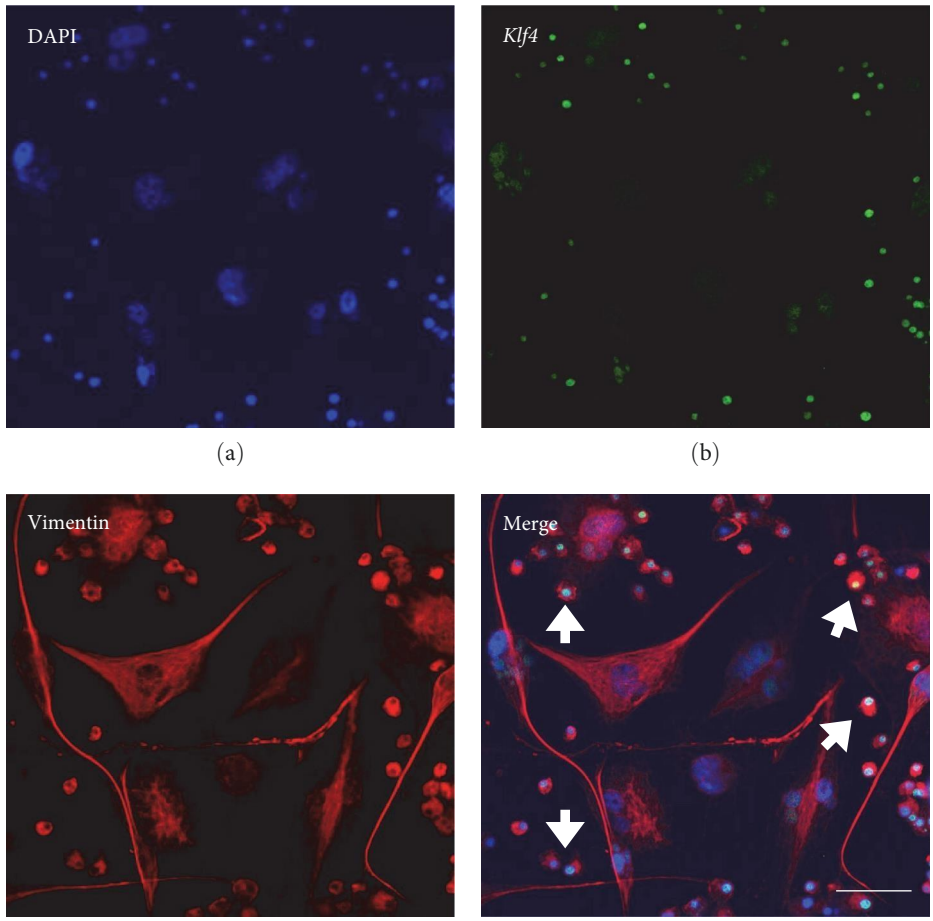


FIGURE 4: Comparison of expressions of *Klf4* and *Vimentin* in differentiated populations of spermatogonia by immunocytochemical (ICC) analysis in vitro. Arrows indicate differentiated SSCs. The expression pattern of *Klf4* and Vimentin in SSCs are the same, and *Vimentin* is also expressed in MEF cells in culture medium: (a) blue shows DAPI; (b) green fluorescence shows *Klf4*; (c) red fluorescence shows *Vimentin*; (d) representation of the merged images (scale bar: 100 μm).

3.3. Fluidigm RT-PCR Analysis for Klf4 Expression. Quantitative mRNA expression by Fluidigm real-time RT-PCR for the *Klf4* gene indicated significant differences between undifferentiated and differentiated populations and between adult and neonate populations (Figure 6(a)). In general, the differentiated spermatogonia populations have been demonstrated to have higher RNA expression levels. When comparing the spermatogonia of adult mice, a significant difference can be seen between two differentiated and undifferentiated populations, and the expression of *Klf4* is found to be higher in the differentiated population; however, this difference is not significant when comparing the SSCs of neonatal mice. On the other hand, in the population of differentiated spermatogonia, the expression of *Klf4* in adult mice is significantly higher than in neonate mice. But precisely the opposite is shown in the population of undifferentiated spermatogonia. By changing the scale of the graph, we have also demonstrated that undifferentiated spermatogonia in neonate mice exhibit a high and significant expression of *Klf4* compared to undifferentiated spermatogonia from adult mice (Figure 6(b)). On the other hand, Fluidigm real-time RT-PCR for the *Klf4* gene indicated significant expression in passage 0 of undifferentiated cells of neonate mice compared to passage 10 (Figure 6(c))

3.4. PPI Networks and Enrichment Analysis. PPI data of *Klf4* and the first 50 proteins interacting with it were retrieved from STRING v10.5 (Figure 7(a)). Cytoscape is a flexible, extensible platform with several plugins that provide new visualization tools and enhance the program's capacity for network research. Cytoscape makes it access the graphical representation of a network and annotate the interactome with various types of data, such as results from large-scale, genome-wide research and information on the functions of individual proteins. The nodes in a network may be scored and ranked using data from a number of Cytoscape plugins. The STRING database identified 675 edges for *Klf4* PPI with a confidence score above 0.400. These predictions were based on various sources of evidence, including text mining, experimental data, databases, coexpression, neighborhood, gene fusion, and cooccurrence. The network represents a group of proteins that work in conjunction to carry out a specific function. PPIs with a STRING score below 0.4 were excluded due to their low confidence level. The network

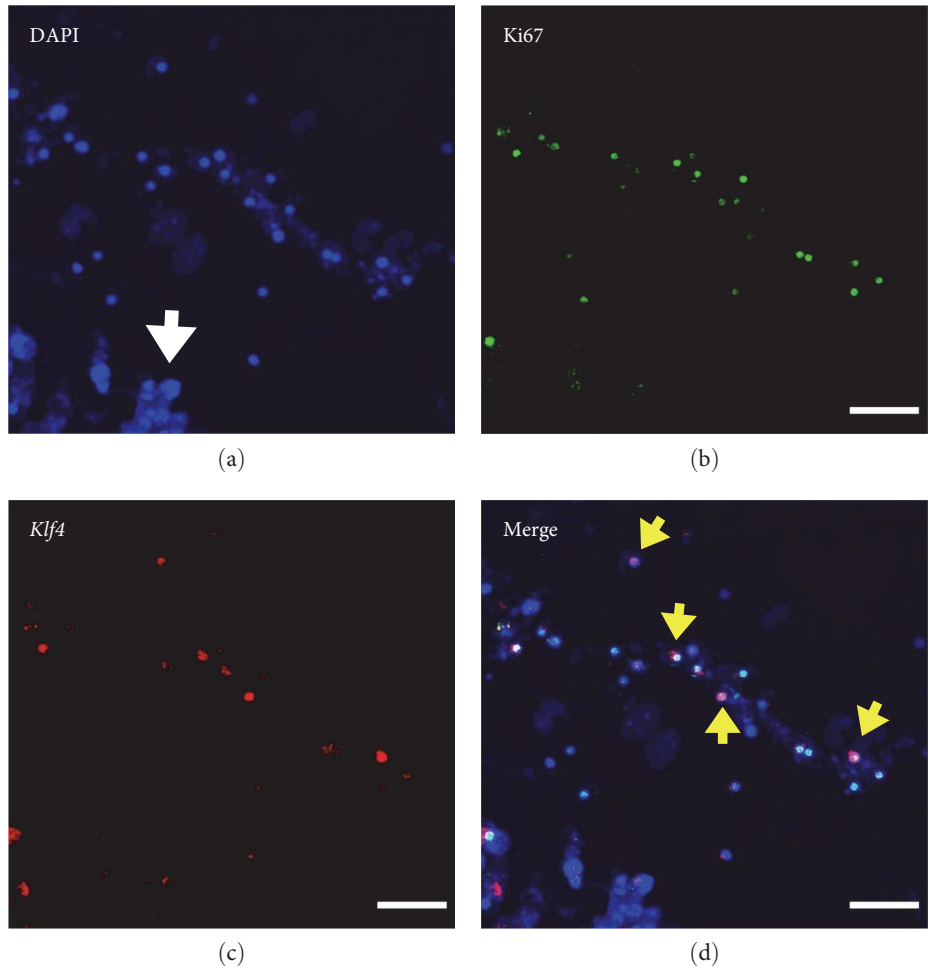


Figure 5: Comparison of expressions of *Klf4* and Ki67 in two populations of spermatogonia by immunocytochemical (ICC) analysis in vitro. Yellow arrows indicate differentiated cells and white arrows show undifferentiated SSCs. The expression pattern of *Klf4* and Ki67 is the same, and both of them are more expressed in differentiated cells than in undifferentiated cells: (a) blue shows DAPI; (b) green fluorescence shows Ki67; (c) red fluorescence shows *Klf4*; (d) representation of the merged images (scale bar: 100 μm).

nodes represent proteins, with splice isoforms and posttranslational modifications being collapsed into a single node. Edges in the network represent significant PPIs that are intended to be specific and meaningful, indicating that the proteins work together to achieve a shared goal. The more lines, the more valid the associations. We have shown the *Klf4* as a yellow node (Figure 7(a)). Using the Clustermaker2 app and the MCODE algorithm, we have shown that there are two important clusters in this network. Clusters are highly interconnected regions in a network, like protein complexes and parts of pathways (Figure 7(b)). After identifying the first neighbors' nodes of *Klf4*, we constructed subnetworks of these hub-genes, including 42 nodes and 41 edges. We use cytoHubba plugins to score all proteins in the yeast protein interaction network by EPC method (Figure 8(a) and Table 1). CytoHubba plugins provide a simple interface to analyze a network with eleven scoring methods. We have composed the cytoHubba plugin in order to make it simpler for biologists to do network analysis and make use of additional network characteristics. Researchers are able to search and explore the network, as well as extract user-interesting subnetworks, with the assistance of the upgraded node retrieving feature included in the cytoHubba control panel. A method for anticipating the main cluster structure inside a graph is provided by the network of edge percolation in networks. Based on non-local characteristics of the network, such as short path length, the percolation method calculated the correlation score between two nodes of a network that had the potential to be linked even after the removal of certain edges. In a PPI network, one protein's direct or indirect effect on another may be explained biologically by correlation calculated using the EPC method. With the predicted hub proteins of the network having high biological relevance and being more evolutionarily conserved than other proteins, each node of the PPI network was colored from highly essential (red) to essential (yellow) according to the EPC scores of the nodes (Figure 8(a) and Table 1). In addition, by STRING enrichment analysis in the Cytoscape, we have identified enriched biological processes associated with *Klf4*

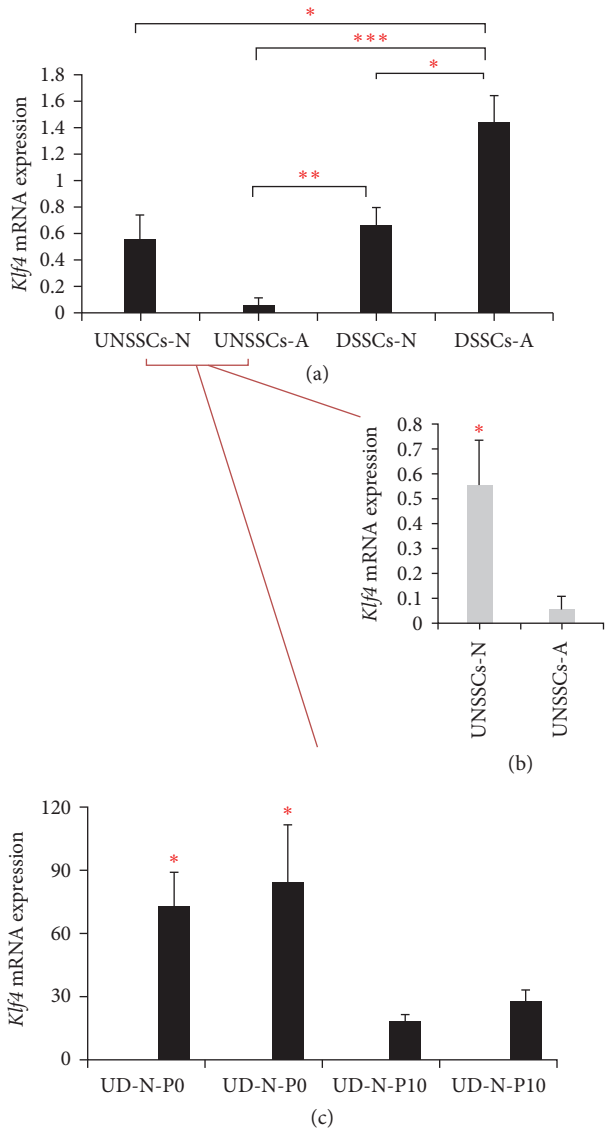


Figure 6: Fluidigm real-time RT-PCR analysis for *Klf4* expression. *Klf4* mRNA expression increases during differentiating. (a) Differentiated SSCs of adult mice (DSSCs-A) have significantly higher expression of *Klf4* than undifferentiated SSCs of adult mice (UDSSCs-A) and also have significantly higher expression of *Klf4* than differentiated and undifferentiated SSCs of neonate mice (DSSCs-N and UDSSCs-N). In addition, differentiated SSCs of neonate mice have a significantly higher expression in comparison with undifferentiated SSCs of adult mice. In the neonate mice, there is no significant difference between differentiated and undifferentiated stages of SSCs. (b) By decreasing the scale of the graph, undifferentiated SSCs of neonate mice show a significantly higher expression of *Klf4* than undifferentiated SSCs of adult mice. (c) Significant decrease of the expression of *Klf4* in neonate mouse undifferentiated spermatogonia passage 10 (UD-N-P10) in comparison to passage 0 (UD-N-P0) by Fluidigm real-time PCR analysis (*<0.05, **<0.01, ***<0.001).

Hub-genes. According to the purpose of our experiment, we have selected some GO biological processes, and we use KEGG pathways and TISSUES databases to evaluate molecular functions and cellular locations (Figure 8(b) and Table 2).

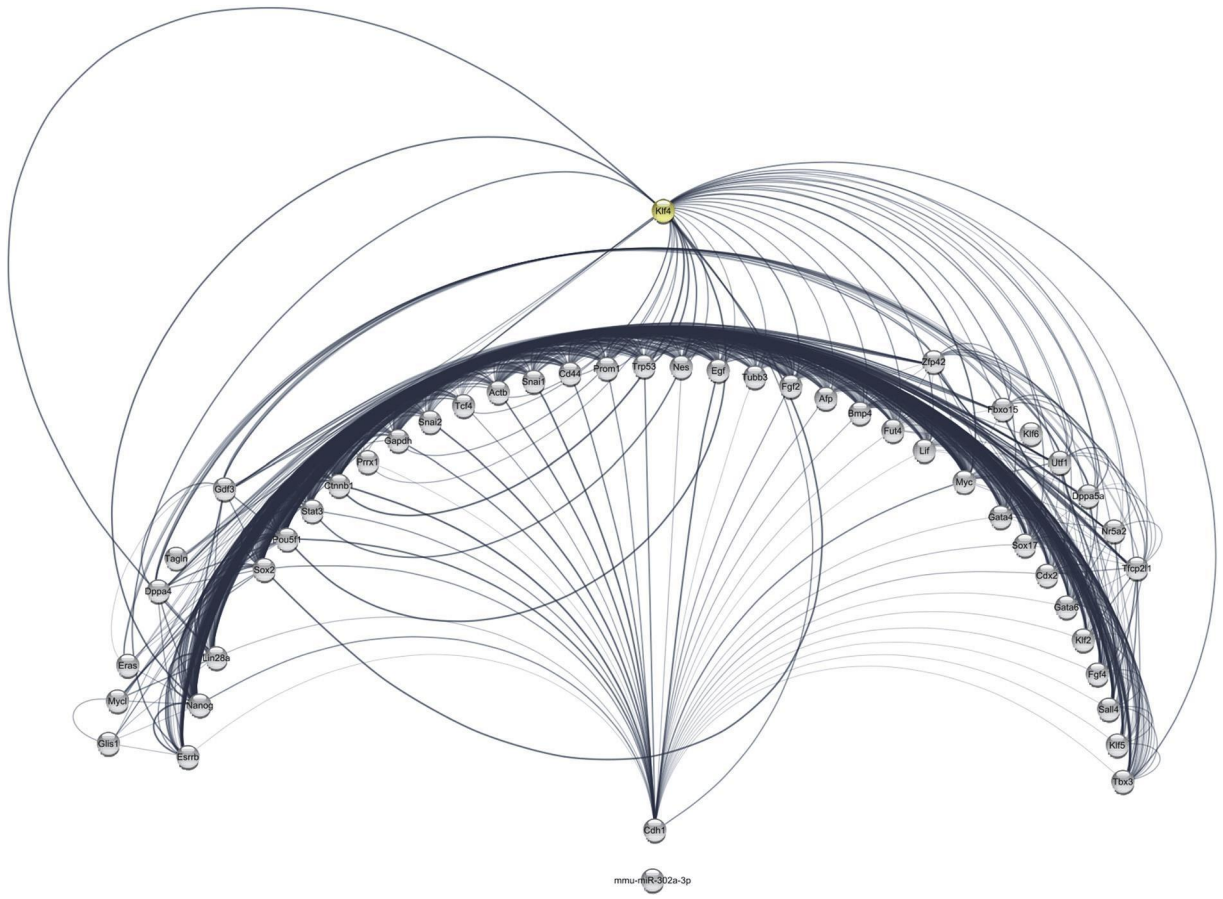
3.5. Construction of the miRNA–lncRNA–Protein Network. Using four different databases (TargetScan, NPInter, miRDB and RNAInter), we have identified miRNAs that have a regulatory effect on *Klf4* and visualized them in the Cytoscape platform. A total of 15 miRNAs have been predicted in common in all databases (Figure 9). In addition, using RNAInter, we found and predicted the first 30 lncRNAs that have a regulatory effect on *Klf4* (Figure 8). Each of these miRNAs and lncRNAs can change the expression pattern of *Klf4* depending on the context.

4. Discussion

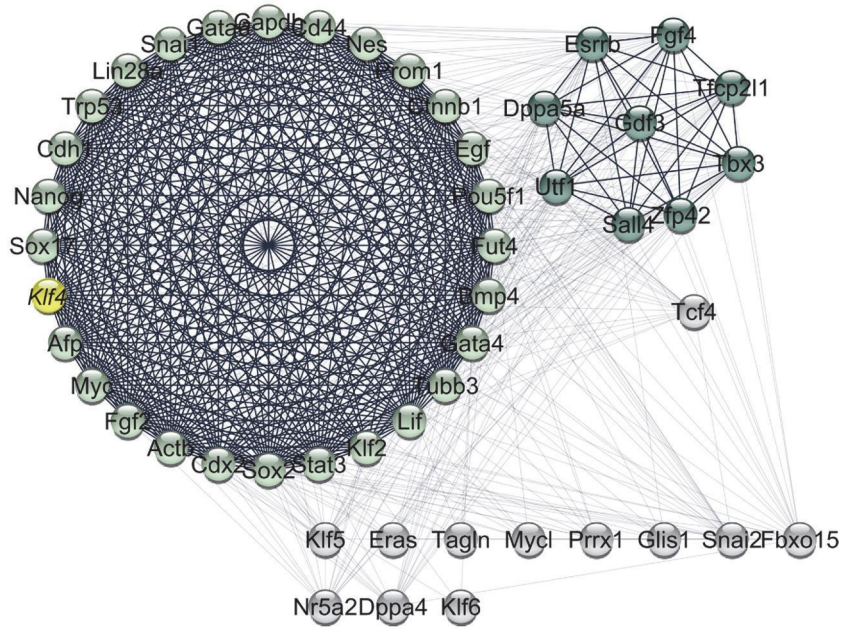
During an immunohistochemical examination, microscopic imaging reveals a considerable expression of *Klf4* in the adluminal parts of the seminiferous tubule that contains differentiated cells, including round spermatids; however, there is no expression in the basal membrane, or its expression is very low. In addition, images obtained through confocal microscopy during immunocytochemical analysis of both isolated and culture-differentiated and undifferentiated populations of SSCs indicate that the expression of *Klf4* increased during the differentiation of SSCs cells. So, it seems that *Klf4* is a differentiating factor. While the findings of Yamanaka's experiments have shown that *Klf4* is necessary for the reprogramming of somatic cells, it also, in association with other proteins, is responsible for the positive regulation of the self-renewal and pluripotency of ES cells [6, 8]. Zhang et al. [17] also claimed that *Klf4*, in addition to Oct4, NANOG, and Sox2, is highly expressed in ES cells; however, its expression dramatically decreases throughout the differentiation process. Studies by Sun et al. [18] on the association of *Klf4* with NANOG have 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.

The results obtained from the histochemical analysis of the comparison of the expression pattern of *Klf4* and SOX9 show that these two genes do not colocalize in adult mouse testis. Since SOX9 has been introduced as a marker of testicular Sertoli cells [19], our results indicate that *Klf4* lacks protein expression in Sertoli cells. In 2002, Behr et al. [20] stated that *Klf4* is expressed in somatic Sertoli cells in mouse testis, but in the case of the human testicle, Sertoli cells do not show any sign of *Klf4* protein expression. Also, Sze et al. [21] reported in 2008 that follicle-stimulating hormone strongly induces *Klf4* expression in mouse Sertoli cells and that *Klf4* is involved in the activation of some proteins involved in tight junctions, so it appears to regulate the movement and translocation of germ cells to cross the blood-testis barrier Deletion of *Klf4* in Sertoli cells disrupts the germinal epithelium and Sertoli cell morphology during puberty, despite the fact that the mice are fertile and have normal testicular morphology.

The results obtained from Fluidigm analysis also, in a way, confirm the results obtained from immunostaining. The graphs obtained show a significant increase in the expression of *Klf4*

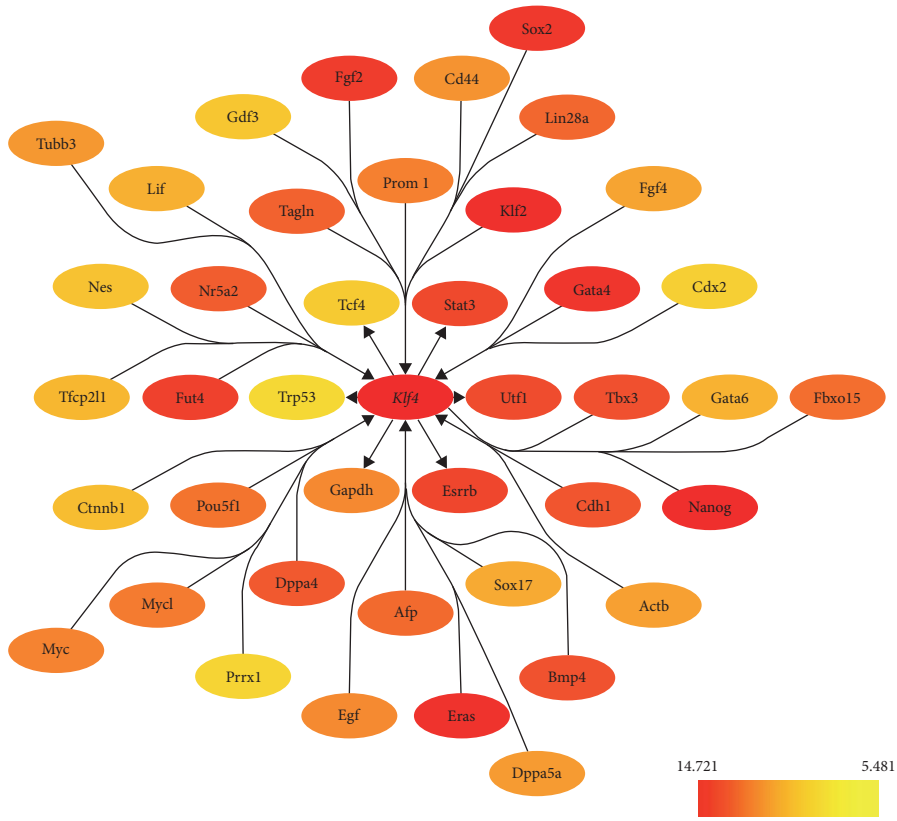


(a)

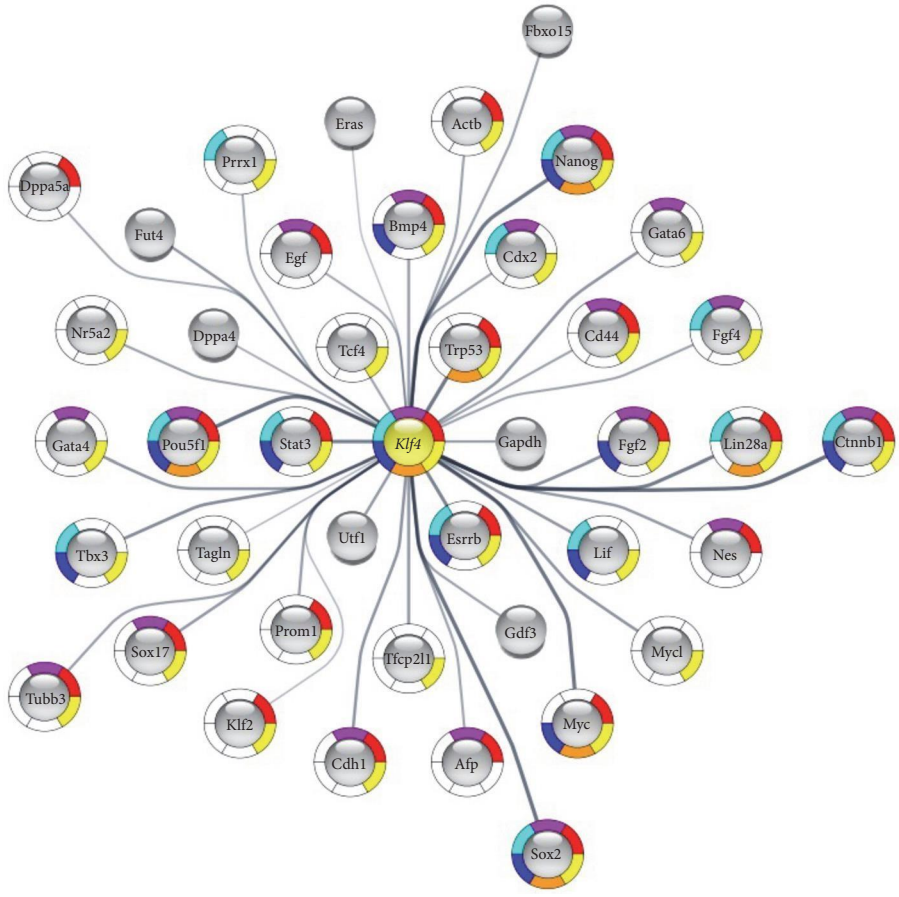


(b)

Figure 7: STRING functional gene analysis using Cytoscape. (a) PPI network of *Klf4* as predicted by STRING and the first 50 genes identified. Network nodes are labeled with gene symbols. Each node represents a protein, and edges indicate protein–protein interactions, with line thickness being indicative of evidence strength for a predicted interaction. Yellow node represents *Klf4* (confidence score cutoff >0.40). (b) Using the MCODE Cytoscape plugin, the native network is broken down into subnetworks up to the motif level. Each module subnetwork from the native network represents with MCODE score.



(a)



(b)

Figure 8: Network using cytoHubba plugin and enrichment analysis. (a) Hub gene networks identified from the protein–protein interaction network using the EPC method of the Cytoscape app cytoHubba (https://apps.cytoscape.org/apps/cytohubba) and comparing the

importance of each node in the network. Red represents the most important node, and the fainter the color, the less important that protein is in the network. (b) The functional enrichment analysis of selected genes. Different colored parts of the circles refer to the related biological processes, with line thickness being indicative of evidence strength for a predicted interaction.

Table 2: Name, color, and details of each function specified in Figure 6(b).

Category	Chart color	Term name	Description	FDR value
TISSUES		BTO:0000556	Germ layer	3.37E − 27
TISSUES		BTO:0006078	Pluripotent stem cell	3.71E − 33
TISSUES		BTO:0001268	Somatic cell	9.62E − 14
GO biological process		GO:0019827	Stem cell population maintenance	9.72E − 16
GO biological process		GO:0030154	Cell differentiation	2.53E − 13
KEGG pathways		mmu04550	Signaling pathways regulating pluripotency of stem cell	1.74E − 14

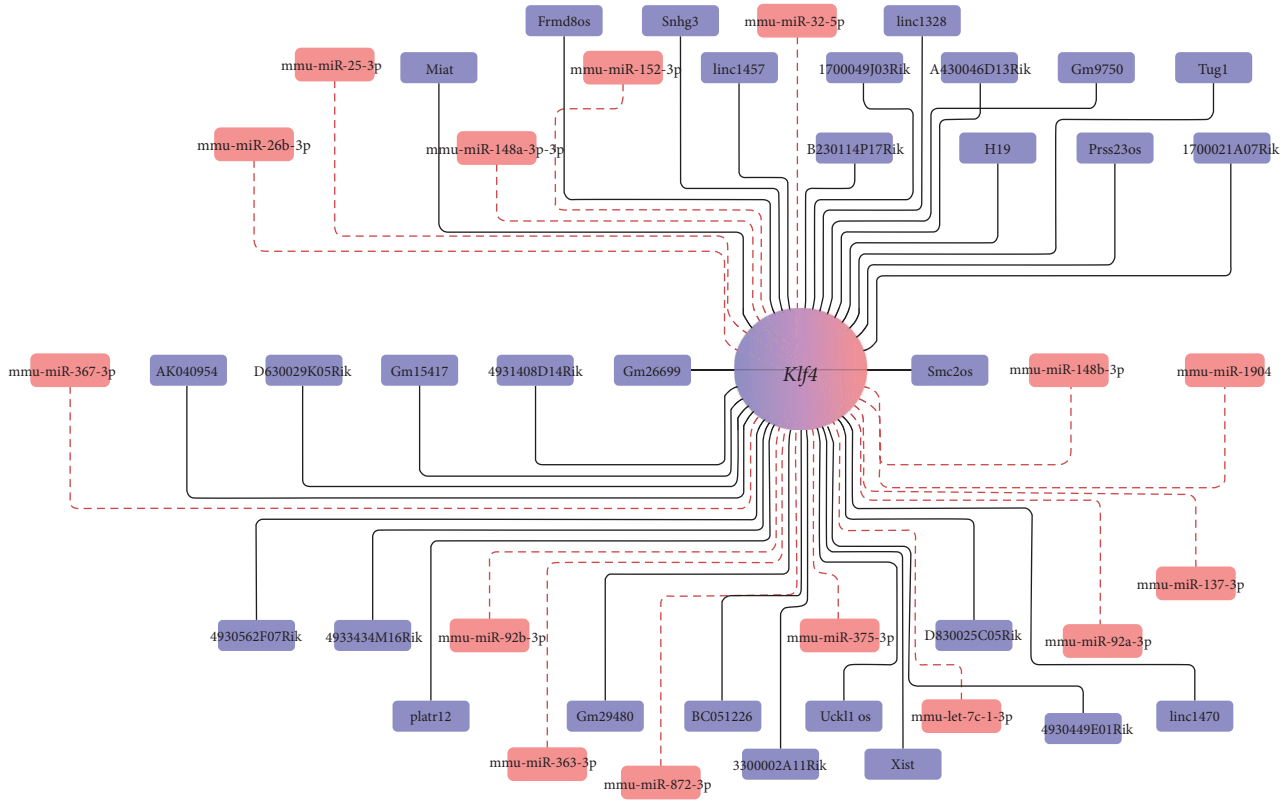


Figure 9: Visualization of miRNA–protein and lncRNA–protein interaction network. Identification of hub miRNAs and lncRNAs whose target gene is *Klf4*, using four separate databases, including TargetScan, NPInter, miRDB, and RNAInter for miRNA and RNAInter database for lncRNA in mouse microorganisms. Fifteen miRNAs were common among all databases, and the first 30 lncRNAs were identified. Pink rectangular nodes indicate miRNA and the red dash indicates their edges. Blue rectangular nodes indicate lncRNAs, and black lines show their edges.

during the differentiation of adult mouse SSCs. However, this difference is not significant in neonate mice. The expression of *Klf4* changes in differentiated and undifferentiated adult SSC stages. In newborn mice, the expression of *Klf4* is almost the same in all populations of SSCs, and *Klf4* may not stimulate the differentiation of SSCs. Our data indicate that the expression of *Klf4* is significantly different in the differentiated SSC population between adult mice and neonate mice, and adult mice have

a higher expression than neonate mice. Also, the results obtained 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. All these results could indicate that in addition to *Klf4* being a differentiating factor in adult mice, its expression level can also be age-dependent, and even the dual function of *Klf4* may be age-dependent. Similar to the results of our experiments, Yang et al. [22] have 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. Also, in 2015, Azizi et al. [14] concluded that the dedifferentiation and spontaneous transformation of SSCs into pluripotent ESCs is age-dependent, and this could be due to the change in the expression pattern of SSCs genes, including *Klf4*. From another perspective, we 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 [23]. 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 noncancerous tissues, and its expression is inversely related to tumorigenesis, which supporting the role of *Klf4* as a tumor suppressor. These studies might imply that *Klf4* can play a dual role depending on different cellular contexts and types of cancer. This behavior is similar to other factors, such as TGF-β [8, 23]. In addition, Zhang et al. [17] have 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 [22].

Our bioinformatics analyses show that *Klf4* is also found in somatic cells, as it has many roles in maintaining pluripotency. This itself can indicate the dual function of this factor, although the main reason for this type of function is still unknown, and many factors, such as signaling pathways, can be one of the reasons. On the other hand, in network enrichment analysis, it has been shown that *Klf4* also plays a role in cell differentiation; its value is 2.53E-13, which is generally lower than its value in stem cell population maintenance. Godmann et al. [24] have reported that *Klf4* is involved in epithelial cell terminal differentiation. In the absence of *Klf4*, stomach keratinocytes, mucosal epithelial cells, and corneal outer epithelial cells fail to develop. Additionally, they used microarray expression analysis to look into the transcriptional network that *Klf4* is a part of during the late stages of male germ cell development. Surprisingly, they found that several zinc finger proteins from the same family of Krüppel C2H2-type zinc finger proteins as *Klf4* had increased expression. The PPI network of *Klf4* shows its extensive connections with many genes. Furthermore, it has been shown that in addition to gene interactions that affect *Klf4* function, interactions between miRNAs and lncRNAs with *Klf4* should not be ignored. The regulatory role of these RNAs on *Klf4* is very impressive and proven by several studies.

Induced *Klf4* expression has been linked to upregulation of several genes involved in cell cycle arrest and downregulation of genes that promote cell proliferation, according to research by Chen et al. [25]. Moreover, we consider that perhaps the conditions of the culture media may affect the expression of *Klf4* [26]. For example, the LIF can activate the JAK-STAT signaling pathway and subsequently activate STAT3, which directly binds to the *Klf4* gene promoter and affects its expression. The connection of LIF and Stat3 with *Klf4* in the obtained bioinformatics data is also significant [27]. The specific effects of estradiol, progesterone, FGF, and EGF on *Klf4* expression in SSCs in culture media conditions are not mentioned in the given information. Therefore, it is unclear how these factors may affect *Klf4* expression in this context. Further research would be needed to determine the influence of estradiol, progesterone, FGF, and EGF on *Klf4* expression in SSCs. Also, the expression of *Klf4* is regulated at both transcriptional and posttranscriptional levels [8]. These concepts can indicate that *Klf4* is a versatile transcription factor that is involved in the regulation of several cellular processes, but its function can be dependent on several factors, such as age, which we have observed in our obtained data. However, it cannot be stated precisely that *Klf4* is definitely a differentiation factor, but it can be stated that it plays its role as a differentiation factor in adult mice.

5. Conclusion and Future Perspective

In summary, our study shows the expression pattern of *Klf4* during the differentiation of SSCs in the mouse testis and states that *in vitro Klf4* expression is significantly higher in differentiated SSCs than in undifferentiated cultured cells. Since *Klf4* plays significant roles in many cellular and developmental processes and also plays a major role in regulating the characteristics of stem cells, it can be used as a therapeutic or diagnostic marker. Various complex interactions are involved in the process of differentiation, proper spermatogenesis, and thus reproduction, and one of the most interesting and controversial factors is *Klf4*, since *Klf4* can show opposite functions in different conditions. The results from our experiments become more controversial since the biological literature mentions *Klf4* as a factor that maintains pluripotency and stem cells. Our findings could provide a better understanding of the involvement of *Klf4* in the molecular mechanisms during testicular development and adult SSC stages, leading to discovering or optimizing existing methods for infertility treatment and cancer.

Data Availability

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

Ethical Approval

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

Disclosure

The present manuscript has been published as a preprint in the Research Square platform (https://www.researchsquare.com/article/rs-1426870/v1). However, due to insufficient data to prove the authors' hypotheses, part of its data has been removed, and new analyses have been added to it.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Kiana Sojoudi carried out and designed the experiment, designed the bioinformatics data, assembly of data, data analysis, and wrote and edited the final document; Hossein Azizi carried out and designed the experiment, project administration; Thomas Skutella provided critical feedback and data analysis. The authors read and approved the final manuscript.

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