CD49f promotes proliferation of male dairy goat germline stem cells

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Abstract

Objectives: CD49f enhances multipotency and maintains stemness in embryonic stem cells (ESCs), however, whether it would be effective in mGSCs has remained unclear. Moreover, better standards for mGSC enrichment and purification are necessary. The present study was conducted to determine roles of CD49f in mGSC enrichment and regulation.

Materials and methods: CD49f expression patterns were investigated in dairy goats. CD49f positive cells were purified and enriched using magnetic-activated cell sorting (MACS), and characteristics of the cultured cells were assayed using alkaline phosphatase (AP) analysis, quantitative real-time PCR (QRT-PCR) and immunofluorescence analysis. Furthermore, the exogenous *CD49f* gene was transfected into mGSCs and its effects were analysed.

Results: CD49f was found to be conserved in both mRNA and amino acid sequences and that it was an efficient marker for dairy goat mGSC identification, enrichment and purification. CD49f positive cells expressed higher levels of mGSC-specific markers, and proliferated faster than CD49f negative cells. Overexpression CD49f promoted proliferation of dairy goat mGSCs, and Oct4 expression was upregulated; histone H3-lysine 9 dimethylation (H3K9me2) was reduced.

Conclusions: Taken together, our data suggest that CD49f plays novel and dynamic roles in regulating maintenance of pluripotency in mGSCs via Oct4 crosstalk and histone methylation dynamics, which may provide new solutions for mGSCs stability *in vitro*.

Introduction

The male germline stem cells (mGSCs) are adult stem cells located on basement membranes of testicular seminiferous tubules, and they undergo self-renewal or differentiation in male fertility throughout life. Spermatogenesis is a complex and productive process that originates from mGSCs and ultimately results in formation of mature spermatozoa. A number of studies have shown superiority of mGSC transplantation in exploration of biological properties and male infertility (1). Not only characterization of mGSCs *in vivo*, but also specific markers maintained mGSCs cultured *in vitro*, have also been found (2).

Considering mGSCs cultured *in vitro*, significant progress has been achieved in mouse (2), and Glial cell line-Derived Neurotrophic Factor (GDNF) has been believed to be an essential growth factor for mGSCs culture (3). It has been shown that mGSCs also have the potential to dedifferentiate into pluripotent stem cells in appropriate conditions (4), this imbues them with the unique ability to be employed as an efficient source for cell therapy, which could thus bypass moral and ethical obstacles faced by embryonic stem cells (ESCs), and potential lack of safety of transgenes of induced pluripotent stem cells (iPSCs).

Many mGSCs markers have been identified in mouse and man, such as Oct4, GFRA1 (glial cell-line derived neurotrophic factor), PLZF (promyelocytic leukaemia zinc finger), CD49f and CD90 (5). CD49f, also known

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as $\alpha 6$ integrin, was first found in 1999 in the context of CD49f positive cells having greater colony formation ability after transplantation into recipient mouse testis (6). Previous study has shown that CD49f is a valid marker of mouse mGSCs (2,6,7) and recently, its positive cells have also been believed to form sperm-like cells after co-culture with Sertoli cells (8). CD49f is a laminin receptor, a major component of seminiferous tubule basement membranes with CD29. As mGSC transplantation now is believed to be the gold standard to determine capacity of mGSCs, CD49f and CD29 could be the consistent markers of mGSCs. Recent study has shown that CD49f enhanced cell multipotency by regulation with pluripotency factors Oct4 and SOX2 (5). Also, CD49f has been associated with focal adhesion kinase (FAK) (9), which is necessary for integrin-stimulated cell migration (10). It can be cross regulated in multiple pathways such as those of MEK/ERK and p53 (11), and affect expression of pluripotency markers (12,13). Series of micro RNAs such as miR-221, miR-23a, miR-29b, miR-24, miR-29a, miR-199b, miR-199a, miR-27a, and miR-21 have been shown to have higher expression levels in CD49f positive cells involved in cell-cycle biological processes and the cell-cycle KEGG pathway (14).

However, there is little information concerning the effects of CD49f on the fate of livestock mGSCs. Here, we investigated expression patterns of CD49f in dairy goat testis and explored its effects on proliferation of dairy goat mGSCs. Our results demonstrate that CD49f is an efficient marker of mGSCs, and that it might be involved in crosstalk with Oct4 and chromosome modifier histone H3-lysine 9 (H3K9), to enhance dairy goat mGSC proliferation.

Materials and methods

Sample collection

Guanzhong dairy goat testes were obtained from the Yaoan abattoir, and collected from goats of different ages (neonates, prepubertals and adults) and treated as previously described (15). They were immediately transported to the laboratory (within 4 h) and prepared for cell isolation and culture. Goats were killed specifically for our experiments and all procedures were approved by Shaanxi Centre of Stem Cell Engineering & Technology, Northwest A&F University.

Cell culture and DNA transfection

After collection, goat testicular tissues were prepared as previously described (15). Briefly, tissues were washed 5–8 times in PBS containing penicillin (100 U/ml) and

streptomycin (100 mg/ml). Seminiferous tubules from each testis were cut into small pieces using forceps and scissors. Seminiferous epithelial cells were dissociated by modified enzymatic digestion, then two-step successive differential plating methods were used to plate cells and cultured them in petri dishes, pretreated with polylysine (16).

Male germline stem cells (mGSC) used in this study were from an immortalized dairy goat mGSC line mGSCs-I-SB (17). Cells were cultured in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (Hyclone, Logan, Utah, USA), 0.1 mM β -mercaptoethanol (Sigma, St. Louis, MO, USA) and 2 mM glutamine (Invitrogen) at 38.5 °C in a humidified atmosphere with 5% CO₂.

To transfect the *CD49f* gene into mGSCs, pCMV6-CD49f plasmid (5) was incubated with opti-MEM (Gibco, Gaithersburg, MD, USA). Tubefect as transfection reagent was added in ($V = m$ of plasmids/2) and co-incubated at room temperature for 15 min, then added to the cells. 12 h after transfection, medium was discarded and replaced with fresh DMEM/F12 with appropriate supplements.

Magnetic activated cell sorting (MACS)

The dairy goat mGSCs were isolated as previously reported (18). Cells were plated in 10-cm diameter culture dishes at 37 °C, 5% CO₂ and saturated humidity, overnight, to remove potential contamination of Sertoli and myoid cells. Then non-attached cells were collected for MACS by centrifuging at 1000 rpm for 5 min. After washing in PBS, cells were resuspended in MACS buffer (PBS containing 2 mM EDTA and 0.5% BSA). In the order of 1.3×10^7 cells were incubated with rabbit anti-CD49f polyclonal antibody resuspended in 80 μ L MACS buffer at 37 °C for 1 h. After centrifuging at 1000 rpm for 5 min, they were washed in MACS buffer and incubated in secondary antibody. Cell suspensions were passed through the MACS column in MACS separator (Milenyi Biotec GmbH, Teterow, Germany). Unlabeled cells (CD49f-negative) were collected, whereas CD49f-labeled cells still reacted to the magnetic field. Next, the magnetically labeled CD49f positive cells were flushed out with the aid of a plunger, into a collection tube. Recovery levels of CD49f-positive cells were calculated as a ratio between number of CD49f-positive cells to total cell number.

Quantitative real-time polymerase chain reaction

Total RNAs were extracted from testis, heart, liver, spleen, lung, kidney, skin, muscle, adipose tissue, intes-

tine and pancreas, of the dairy goats and were cracked using TRIzol (Tiangen Biotech Co. Ltd, Beijing, China); primers are listed in Table 1. Protocol details for RT-PCR and gel electrophoresis were performed as described in previous studies (19). Further, expression pattern of CD49f in different tissues, and Oct4, Gfra1, PLZF, PCNA, Scp3 and CCND1 expression in cell samples were analysed by QRT-PCR as described previously (20).

Immunofluorescence staining

For immunofluorescence staining, tissues were fixed in 4% paraformaldehyde (PFA) overnight, then passed through 50%, 70%, 80%, 90%, 100% alcohols and xylene for gradient dehydration; they were then embedded in paraffin wax followed by, sections being cut. Rehydration was performed through xylene, 100%, 90%, 80%, 70%, 50% alcohols, followed by washing twice in phosphate buffer saline for 10 min each. Antigens were retrieved in citric acid for heated to boiling in a microwave oven. Samples were blocked with 1% bovine serum albumin (BSA) for 30 min at room temperature (RT) before incubation in primary antibodies against CD49f (1:50, Bioss, Beijing, China), CD90 (1:200, Santa Cruz, Inc., CA, USA), PLZF (1:100; Santa Cruz), PGP9.5 (1:50, Bioss), ETV5 (1:50, Bioss), H3K9me2 (1:200, Bioworld, Nanjing, China) at 4 °C overnight. After washing 3 times in PBS, tissues were incubated in secondary antibodies (goat anti-mouse or goat anti-rabbit) conjugated to fluorescein at RT for 1 h. They were

then washed 3 times in PBS and stained in 5 µg/ml DAPI for 5 min. Sections were then assessed using fluorescence microscopy.

For cell immunofluorescence, cells were cultured under normal conditions then fixed in 4% PFA at RT for 15 min. They were then permeated with 0.1% triton X-100 for 10 min at RT and washed 3 times in PBS followed by blocking with 1% BSA for 30 min at RT. Primary antibodies used were against CD49f (1:50, Bioss), PLZF (1:100; Santa Cruz), Stra8 (1:100; Santa Cruz), REC8 (1:100; Santa Cruz), GFRa1 (1:100; Santa Cruz), Oct4 (1:200; Millipore) at 4 °C overnight. Remaining steps were identical as those used on the tissues samples.

Alkaline phosphatase staining

Alkaline phosphatase (AP) activity was determined essentially as described previously (21). Briefly, cells were washed three times in PBS and fixed in 4% PFA for 10–15 min at RT. After further washing three times in PBS samples were stained with naphthol AS-MX phosphate (200 µg/ml, Sigma) and Fast Red TR salt (1 mg/ml, Sigma) in 100 mM Tris-buffer, pH 8.2–8.4, for 10–30 min at room temperature. They were washed twice again in PBS to terminate the staining.

Western blot analysis

Proteins were extracted from cell samples transfected with pCMV6-CD49f plasmid and pDesRed plasmid (control), and protein concentration was detected using the BCA Protein Quantification Kit (Vazyme, Piscataway, NJ, USA). After heat denaturation in 5% SDS–PAGE sample loading buffer, protein samples were resolved in SDS–PAGE and transferred to PVDF membranes. They were probed with actin and GAPDH (1:1000, Beyotime, Bioss), and antibodies against CD49f (1:500, Bioss), H3K9me2 (1:200; Bioworld), Oct4 (1:500; Millipore) were used as previously described (22). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin was the secondary antibody (1:1,000, Bioworld). Detection was performed using Thermo Scientific Pierce ECL Western blotting substrate, and results were analyzed with the Tanon-410 automatically gel imaging system.

Statistical analysis

mRNA and protein level gene expressions were statistically analyzed using a two-tailed t-test analysis (Excel, Microsoft 2007). $P < 0.05$ were considered statistically significant. All data were collected from 3 independent experiments.

Table 1. The quantitative Real-time PCR primers

Gene	Strand	Sequence	Length of PCR fragment (bp)
Gapdh	F	CCACGCCATCACTGCCACCC	116
	R	CAGCCTTGGCAGCGCCAGTA	
Gfra1	F	GGACAGGCAGCAGGAAATA	200
	R	GTCTCCTGTCCCAGTCAAA	
PLZF	F	CACCGCAACAGCCAGCACTAT	127
	R	CAGCGTACAGCAGGTCATCCAG	
PCNA	F	AGTGGAGAACTTGGAAATGGAA	167
	R	GAGACAGTGGAGTGGCTTTTGT	
CCND1	F	TGAACTACCTGGACCGCT	212
	R	CAGGTTCCACTTGAGYTTGT	
Scp3	F	GTATGGAGGACTTGGAGA	138
	R	GAGACTTTCGGACACTTGC	
Oct4	F	AAGCAGTGACTACTCCCAACG	156
	R	GGGAATGGGACCGAAGAGTA	
CD49f	F	CGAAGCACGAATCCCGAGAC	235
	R	TGCTCTACACGAACAATCGCTTT	

Results

CD49f was an efficient marker to identify dairy goat mGSCs

To identify any similarity of CD49f between species, the gene sequence of dairy goat CD49f (mRNA and protein levels) were obtained by electronic cloning; this is listed in Table S1. Homology of CD49f mRNA and amino acid sequences of mouse, rat, man, cattle and goat were compared, and these results proved to be of great similarity to CD49f mRNA and amino acid sequences, of the five species tested (Fig. 1a,b). To identify CD49f expression pattern in the dairy goat, QRT-PCR was performed on testis, heart, liver, spleen, lung, kidney, skin, muscle, adipose

tissue, intestine and pancreas. This assay indicated that the highest mRNA expression level was in the spleen, with relatively high levels in muscle, intestine and heart, greater than in lung, kidney, adipose tissue or skin. CD49f expression level in testis was moderate compared to the other tissues (Fig. 1c). To identify location of CD49f in testis, straining of testis sections of different age animals (neonate, prepubertal and adult) were performed and results showed that CD49f positive cells were located on the basement membranes of the seminiferous tubules (Fig. 1d). This is in accord with location of mGSCs and co-location of CD90, PLZF, ETV5, H3K9me2 and PGP9.5, previously demonstrated mGSC markers (Fig. 2).

Figure 1. Homology of CD49f between species and CD49f expression pattern assay of the goat. (a) Homology comparison of CD49f mRNA sequences between mouse, rat, man, cattle and goat. (b) Homology comparison of CD49f amino acid sequences between mouse, rat, man, cattle and goat. (c) CD49f expression pattern on mRNA level of goat organ tissues analyzed by QRT-PCR. * $P < 0.05$, ** $P < 0.01$. (d) Immunofluorescence staining of CD49f in semeniferous tubules of dairy goat in different developmental stages, and mouse, Bar = 200 μ m.

Figure 2. CD49f was an efficient marker to identify dairy goat mGSCs *in vivo*. Immunofluorescence staining of germ cell markers (CD49f, CD90, PLZF, ETV5 and PGP9.5) and epigenetic modification marker (H3K9me2) in dairy goat testis, Bar = 200 μ m.

CD49f was an efficient marker for identification and enrichment of dairy goat mGSCs in vitro

To identify biological characteristic of CD49f in mGSCs cultured *in vitro*, enrichment of CD49f positive cells was performed with MACS (18). Efficiency of their enrichment by MACS selection, using anti-CD49f rabbit polyclonal antibody, was nearly 0.867%. CD49f positive

cells had higher percentages of CD49f, GFRa1, Oct4 and PLZF positivity compared to CD49f negative cells (Fig. 3a,b). After being cultured *in vitro* for 14 days on MEF feeder, CD49f positive cells had greater growth potential than CD49f negative cells (Fig. 3c) and formed typical mGSCs colonies with AP positive staining (Fig. 3d). Positive immunofluorescence staining of Oct4

Figure 3. CD49f was an efficient marker to identify and enrich dairy goat mGSCs *in vitro*. (a) Immunofluorescence staining of SSC markers (CD49f, GFRa1, Oct4 and PLZF) in enriched CD49f positive (CD49f+) and CD49f negative (CD49f-) cells, Bar = 200 μm. (b) Relative fluorescence intensity of GFP in immunofluorescence staining of A, CD49f, GFRa1, Oct4 and PLZF expression levels in CD49f+ cells were higher than CD49f- cells. (c) Cell proliferation assay of enriched CD49f+ and CD49f- cells cultured *in vitro*, Cell number assay of CD49f+ and CD49f- cells cultured for 14 days from 0.3×10^4 cells, respectively, number of cells at day 14 in CD49f+ was about 2.6×10^4 cells and CD49f- was 0.8×10^4 cells, number of cells in CD49f+ significantly increased compared to CD49f- cells, three biological repeats were performed. * $P < 0.05$. (d) AP staining of enriched CD49f+ and CD49f- cells. (Above, CD49f+ cells and below, CD49f- cells, Bar of the left column = 100 μm, Bar of the right column = 50 μm).

and PLZF revealed that CD49f positive cells cultured for 14 days *in vitro* maintained better characteristics of mGSCs (Fig. 4A,B).

CD49f positive cells had greater colony formation ability, after retinoic acid (RA) induction for 7 days, immunofluorescence staining showing cells induced from CD49f positive cells expressing markers of meiosis STRA8 and REC8 (Fig. 4C,D), and CD49f positive cells showing greater ability in formation of sperm-like cells (Fig. 4E).

CD49f promoted proliferation of dairy goat mGSCs by Oct4 upregulation and epigenetic modification

Integrin interactions initiate cascades of signaling pathways involved in cell survival and proliferation. To

further assess whether CD49f plays roles in dairy goat mGSC proliferation, pCMV6-CD49f plasmid was transfected to the immortalized dairy goat mGSC line mGSCs-I-SB (17), termed mGSC-CD49f, and pDesRed plasmid was simultaneously transfected as control termed mGSC-DesRed. QRT-PCR analysis revealed higher expression levels of Oct4 in mGSC-CD49f compared to control mGSC-DesRed, suggesting CD49f overexpression resulted in strengthening of germline cell characteristics. Significant higher expression of PCNA and CCND1 showed efficient promotion of proliferation in mGSC-CD49f. However, Scp3 was up-regulated in mGSC-CD49f, thus we conceived the hypothesis that this proliferation may affect meiotic progress (Fig. 5a). Immunofluorescence staining and western blotting of CD49f both showed higher expression levels of CD49f

Figure 4. CD49f functioned in proliferation and differentiation of mGSCs cultured *in vitro*. (A–B) Immunofluorescence staining of germ cell markers (Oct4 and PLZF) in CD49f+ and CD49f– mGSCs cultured for 14 days *in vitro*, Bar = 200 μ m. (C–D) Immunofluorescence staining of markers of meiosis (Stra8 and REC8) in CD49f+ and CD49f– mGSCs induced by RA, Bar = 200 μ m. (E) a. Morphology of CD49f– mGSCs induced by retinoic acid for 7 days. b. Morphology of CD49f+ mGSCs induced by RA for 7 days, Bar = 50 μ m.

in mGSC-CD49f compared to mGSC-DesRed (Fig. 5b, c). Oct4 also showed the same tendency with CD49f increase (Fig. 5d,e), while H3K9me2 had lower levels in mGSC-CD49f compared to mGSC-DesRed (Fig. 5d,e), suggesting that epigenetic modification dynamics by CD49f may regulate proliferation of goat mGSCs.

Discussion

Male GSCs retain the self-renewal potentiality of stem cells and male germline-oriented differentiation. There are many kinds of cells in the niche which balance the microenvironment, thus mGSCs occupy only a small proportion of the testis, so it is exceptionally important to establish a specific standard protocol for mGSC isolation and identification (23). Identifying germ cell-specific markers for mGSC enrichment and purification is urgent. CD49f has been believed, since 1999, to be an efficient

marker of mouse mGSCs (24); this was demonstrated by transplantation of CD49f positive and CD49f negative cells isolated from mouse testis, into a spermatogenesis-deficient model. Considering further species, preliminary studies have found dairy goat mGSCs to be similar to those of man and mouse (4,25). In our investigation, we found the *CD49f* gene to be highly homologous between mouse, rat, man, cattle and the dairy goat, and it had moderate expression levels in dairy goat testis. Furthermore, we determined by immunofluorescence staining of testicular tissues and analysis of the cells biological function *in vitro*, whether CD49f would be an efficient marker in goat testis. We found it to be located specifically on basement membranes of seminiferous tubules. Co-localisation of CD49f with male spermatogonial stem cell (SSC) markers such as PLZF, CD90 and PGP9.5, also supports our view that it is an efficient marker of dairy goat mGSCs, in common with other domestic animals (24). We also elucidated the role of CD49f in

Figure 5. CD49f promoted proliferation of dairy goat mGSCs. (a) Gene expression (Oct4, Gfra1, PLZF, PCNA, Scp3 and CCND1) of mGSC-DsRed and mGSC-CD49f analyzed by QRT-PCR, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (b) Immunofluorescence staining of CD49f in mGSC-DsRed and mGSC-CD49f. (c) Western blot analysis of CD49f expression in mGSC-DsRed and mGSC-CD49f, Bar = 200 μ m. (d) Western blot analysis of Oct4 and dimethyl histone H3 lysine 9 (H3K9me2) in mGSC-DsRed and mGSC-CD49f. (e) Statistical analysis of Oct4 and dimethyl histone H3 lysine 9 (H3K9me2), average gray intensity was measured using Image-ProPlus 6.0 Software.

maintenance of self-renewal and differentiation potential of dairy goat mGSCs.

In our study, CD49f was found co-localized with germ cell-specific genes *CD90*, *PLZF*, *ETV5*, *H3K9me2* and *PGP9.5*, proving it to be an efficient marker for verification and enrichment of dairy goat mGSCs. CD49f positive cells were efficiently enriched by MACS; they expressed higher levels of CD49f, GFRa1, Oct4 and PLZF and formed better mGSC colonies, suggesting that CD49f might work with other germ cell-specific genes to co-regulate self-renewal and differentiation of mGSCs.

CD49f positive cells exhibited better stability when cultured *in vitro* for longer periods of time compared to CD49f negative cells, and self-renewal gene expression was expressed stably in CD49f positive cells. In addition, mGSCs could be induced to differentiate into spermatogenic cells (16). In our study, CD49f positive cells expressed meiosis markers STRA8 and REC8, and

showed greater ability to form spermatid-like cells compared to CD49 negative cells after induction by RA, suggesting that CD49f plays roles in advancement of mGSC differentiation fate.

According to recent studies, Oct4 plays important roles in regulating proliferative capacity, colony formation, and lineage differentiation potencies of mesenchymal stem cells (26). In our investigation, we found that mGSCs generated from CD49f-positive cells had higher expression of Oct4, suggesting that Oct4 binds to the CD49f promoter region and regulates CD49f transcription in mGSCs, overexpressed CD49f up-regulated germ cell-specific genes and promoted proliferation of goat mGSCs. Lysine methylation interaction with germ cell-specific genes in mGSCs (which exhibits both chromatin structure repression and activation in a context-specific manner), and histone methylation dynamics of H3K9, make pluripotency of mGSCs *in vitro* more stable.

H3K9me2 is associated with pluripotency of stem cells (27). Our results also revealed that H3K9me2 expression was regulated by CD49f.

Taken together, these findings suggest that CD49f contributed to maintenance of pluripotency in mGSCs via Oct4 crosstalk and histone methylation dynamics.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 The sequence of dairy goat CD49f.