miR-34c works downstream of p53 leading to dairy goat male germline stem-cell (mGSCs) apoptosis

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Abstract

Objectives: Recent lines of evidence have indicated that miR-34c can play important roles in regulation of the cell cycle, cell senescence and apoptosis of mouse and human tumour cells, spermatogenesis, and male germ-cell apoptosis. However, there is little information on the effects of miR-34c on proliferation and apoptosis of livestock male germ cells. The dairy goat is a convenient domestic species for biological investigation and application. The purpose of this study was to investigate the effects of miR-34c on apoptosis and proliferation of dairy goat male germline stem cells (mGSCs), as well as to determine the relationship between p53 and miR-34c in this species.

Materials and methods: Morphological observation, miRNA *in situ* hybridisation (ISH), bromodeoxyuridine staining, flow cytometry, quantitative-RT-PCR (Q-RT-PCR) and western blotting were utilized to ascertain apoptosis and proliferation of mGSCs, through transfection of miR-34c mimics (miR-34c), miR-34c inhibitor (anti-miR-34c), miR-34c mimics and inhibitors co-transfected (mixture) compared to control groups.

Results: Results manifested that miR-34c over-expression promoted mGSCs apoptosis and suppressed their proliferation. Simultaneously, a variety of apoptosis-related gene expression was increased while some proliferation-related genes were downregulated. Accordingly, miR-34c promoted apoptosis in mGSCs and reduced their pro-

liferation; moreover, expression of miR-34c was p53-dependent.

Conclusions: This study is the first to provide a model for study of miRNAs and mechanisms of proliferation and apoptosis in male dairy goat germ cells.

Introduction

miRNAs are endogenous, non-coding, small RNAs – length 20–25 nt, functioning as negative regulators post-transcription (1). MiRNAs play multiple important roles in biological processes such as cell proliferation, apoptosis and organism development (2–4). Non-coding RNAs downregulate specific gene expression by binding to the 3' UTR region, which would lead to target mRNA degradation or inhibition of translation. MiR-34c, miR-34a and miR-34b belong to the miR-34 family, which is evolutionarily highly conserved (5,6). The MiR-34 family is found to have the following functions:

- (1) Cell cycle arrest. Tarasov *et al.* (7) transfected miR-34c into osteosarcoma cell line U2OS and found increase in cell number with cells arrested in G1 phase, and reduced number in S phase compared to control groups;
- (2) Promotion of cell aging. It has been reported that transfection of miR-34c into human fibroblasts leads to cell aging (8,9);
- (3) Induction of apoptosis (10). Bommer *et al.* (11) found that miR-34c targets and inhibits activity of Bcl-2, which plays an essential role in prevention of apoptosis by the caspase pathway, in colon carcinoma cell line SW-480, resulting in increased apoptosis. p53 and caspase3 are closely related to apoptosis, and their upregulation promotes cell death (12–14).

Cyclin D1 is active in G1-S phase transition in the cell cycle and increased cyclin D1 expression can reduce

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G1 phase cells (15); down-regulation of c-Myc and PCNA result in attenuated proliferation (16). Such are key markers that determine whether a cell population will succumb to apoptosis. The multiple functions of miR-34c result not only from its various targets, but also due to the many genes that directly regulate miR-34c expression. It has been reported that miR-34c is accommodated by p53 expression and participates in the p53 pathway (17). By comparing 688 miRNAs in p53, active and non-active, in lung cancer cells, Raver-Shapira *et al.* (18) found elevated miR-34c expression in the p53 active group. Tarasov *et al.* (7) analysed miRNA expression patterns in p53 knock-out ovary endothelial cells, which revealed significant reduction in miR-34c. As an important factor in the p53 network, miR-34c regulates genes, which control p53 expression in a feedback regulation manner. miR-34c is largely p53-independent and is involved in late spermatogenesis in mouse testis and in regulation of male germ-cell apoptosis (19,20).

Male germline stem cells (mGSCs) can be isolated, originating from spermatogonial stem cells (SSCs). Additionally, these cells share characteristics of embryonic stem cells (ESCs) and SSCs. Thus, these cells can be regarded as a new and promising source for cell therapy in infertility, and also can be used as a model in cell and developmental biology. The dairy goat is a convenient domestic species for biological investigation and application, due to its natural diversity, its valuable products, relatively short gestation period and strong adaptive ability (21,22). In our laboratory we have obtained dairy goat mGSCs and shown that these cells have typical characteristics of mGSCs (21,22); however, mechanisms of self-renewal and differentiation of dairy goat mGSC remained to be investigated. As miR-34c is found to be critical in apoptosis of mouse and human tumour cells, spermatogenesis and male germ cell apoptosis (5,16,19,20), we decided to explore miR-34c's affects on dairy goat mGSCs, and to further discuss the relationship between miR-34c and p53.

Materials and methods

Cell transfection

The dairy goat mGSCs were cultured in our laboratory (21,22) and medium used was DMEM/F12 (Invitrogen, Carlsbad, CA, USA) containing 20% knock-out serum replacement (KSR; Invitrogen), supplemented with 0.1 mM of 2-mercaptoethanol (Invitrogen), 2 mM L-glutamine (Invitrogen), 1% non-essential amino acids (Invitrogen), 10 ng/ml recombinant human basic fibroblast growth factor (bFGF; Millipore, Billerica, CA, USA)

and BIO (2.5 µM, Merck, Whitehouse Station, NJ, USA).

miR-34c mimics and miR-34c inhibitor were obtained from Applied Biosystems (Van Allen Way PO Box 6482, Carlsbad, CA, USA). mGSCs were transfected with miR-34c mimics, miR-34c inhibitor, mixture and scrambled oligonucleotides as control. RNA was diluted to 200 ng in 50 µl Opti-MEM (Invitrogen) reduced serum medium; mixed gently, then 0.5 µl PLUSTM Reagent (Invitrogen) was added directly to the diluted RNA and the mixed medium was incubated for 5 min at room temperature. LipofectamineTM LTX Reagent (Invitrogen) was mixed gently before use, then 1 µl was added to the diluted RNA. All were then gently rocked and incubated for 30 min at room temperature. A volume of 50 µl RNA-LipofectamineTM LTX complexes was added, and transfection medium was replaced 5 h later by fresh medium; cells were observed 48 h later using an inverted phase contrast microscope.

5-bromo-2-deoxyuridine (BrdU) incorporation assay

Proliferation of the mGSCs was assayed using the BrdU incorporation method, performed similarly to previous reports (23,24), but with modification. First, cells were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature then were washed three times for 10 min each, in PBS (pH 7.4) containing 0.1% Triton X-100. Cells were then washed three times in PBS (pH 7.4) alone. Anti-BrdU (1:100; Santa Cruz, CA, USA) dissolved in 0.1 M PBS (pH 7.4) containing 5% normal goat serum was added and cells were incubated overnight at 4 °C. Cells were washed in PBS (pH 7.4) three more times, then incubated in corresponding secondary antibody (1:500, FITC; Millipore), for 1 h at room temperature. Three more washes were carried out, cells were visualized using a Leica fluorescence microscope, and were analysed for BrdU uptake to determine BrdU positive percentage, by manual counting (22,25,26).

Flow cytometric analysis for apoptosis

After treatment with miR-34c for 48 h, cells were washed in PBS and digested with 0.25% trypsin. They were transferred to a centrifuge tube for centrifugation at 200 g for 5 min after digestion, then supernatant was removed and cells were collected. They were resuspended gently in PBS, and $1-5 \times 10^5$ resuspended cells were centrifuged at 200 g for 5 min; once more the supernatant was removed and cells were resuspended gently in 500 µl mixture. 5 µl annexin V-FITC and 5 µl propidium iodide were added to the medium and mixed gently. Cells were incubated

(covered by aluminium foil) at room temperature for 10 min in the dark, then underwent FACS analysis.

Doxorubicin-treated cells

Doxorubicin (ShangHai in China), diluted to 2.5, 5, 10, 20, 40 µg/ml was added in DMEM/F12 (Invitrogen), supplemented with 10% FBS. Then cells were treated in different concentrations and 20 µg/ml for 6, 12 and 24 h, respectively. Expression levels of doxorubicin in the treated cells were determined by Quantitative RT-PCR (Q-RT-PCR). Finally, expression of p53, c-Myc and miR-34c treated with doxorubicin was analysed by Q-RT-PCR and western blotting. Nuclei were stained with DAPI (Sigma St. Louis, MO, USA) to help evaluate apoptosis.

Quantitative RT-PCR

Q-RT-PCR reactions were set up in 25 µl reaction mixture containing 12.5 µl 1 × SYBR® PremixExTaq™ (TaKaRa, Biotech. Co. Ltd., Dalian, China), 0.5 µl sense primer, 0.5 µl antisense primer, 11 µl distilled water and 0.5 µl template. Reaction conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 58 °C for 20 s. All expression levels were normalized to β-actin in each well. Expression was quantified as ratio of mRNA levels obtained from mGSCs in absence or presence of miR-34c mimics (48 h). Q-RT-PCR primers are listed in Table 1.

miRNA in situ hybridisation

miRNA-ISH detection system kit was purchased from Focobio (Guangzhou, China). Concrete steps are as follows: testicular tissues were washed twice in PBS for 10 min each, then slices were incubated in solution A for 20 min at room temperature. Slices were then treated

with solution B for 15 min at room temperature, washed in PBS for 10 min, fixed in 4% paraformaldehyde for 15 min and washed in PBS for 10 min. Tissue was removed from the hybridization chamber solution C was discarded. 10–200 µl of hybridization solution containing 1.5–2 µM oligonucleotide probe was added and hybridized overnight at 40–42 °C in an incubator. Next day the probe was removed, washed in washing buffer I for 15 min at room temperature, rinsed in washing buffer II twice (15 min again), then washed in 75% EtOH 2 min, 100% EtOH, 2 min and then air dried for 10 min. 20 µl DAPI was added 10 min in the dark. Finally, tissue was washed and analysed using an immunofluorescence microscope.

Immunofluorescence staining

All dairy goat testicular tissues used in this study were derived from 30 days postnatal, 90 days postnatal and adult testes and were dissected then fixed in 4% paraformaldehyde. Tissues were taken to paraffin wax, sectioned, deparaffinized and rehydrated following standard methods. To dehydrate sections were dipped in three changes of xylene for 6 min each, two changes of 100%, 95%, 75% alcohol for 3 min, and afterwards rinsed twice in water for 5 min. Slides were soaked in boiling citrate buffer for 15–25 min, followed by three washes in cold PBS, each for 5 min. Washed sections were blocked with blocking solution (PBST + 1% BSA) for a minimum of 30 min then incubated in primary antibody to P53 (1:100; Beyotime, Haimen, Jiangsu, China) overnight at 4 °C. Tissues were washed in PBS three times, then incubated in secondary antibody M488 (1:500; Chemicon International, Inc., Temecula, CA, USA) following manufacturer's manual. Cell nuclei were stained with DAPI. Negative controls were carried out for each run to test secondary antibody reaction (same protocol, but for one section or cell sample, pri-

Table 1. The primer sequences

Gene	Forward primer	Reverse primer	Annealing temp (°C)	Product size (bp)
β-actin	GCGGCATCCACGAACTAC	TGATCTCTCTGCATCCTGTC	104	58
Oct4	AGGAGTCCCAGGACATCAA	AGGGTGATCCTCTTCTGCTT	166	58
CD90	GATCCAGGACTGAGCTCTCGG	TCACGGGTCAGACTGAACTCATAC	195	58
Dazl	CAAGTTCACAGTTCAGGTATCAC	GACAACGGAGTTTCTCAGTCTATTC	299	58
Vasa	GCTGGCGTAATAGCGAAGAGG	GCACAGATGCGTAAGGAGAAAA	107	58
CyclinD1	TGAACCTACCTGGACCGCT	CAGGTTCACCTTGAGYTTGT	212	50
C-myc	CTGGTGGGCGAGATCATCA	CACTGCCATGAATGATGTTC	304	54
P53	GGAATCTTCTGGGACGG	CTTCTGGTCTTCGGGTAGC	336	49
Caspase3	GAAGATCATAGCAAAAGGAG	TGTCTCAATGCCACAGTCCA	207	58
5S	CTGGTTAGTACTTGGACGGGAGAC	GTGCAGGGTCCGAGGT	50	58
miR-34c	GCAGCCAGGCAGTGTAGTTAGC	GTGCAGGGTCCGAGGT	50	58

mary antibody being replaced with 1% BSA), or non-specific background staining.

Western blotting

Total cell extracts were prepared from mGSCs in transfected miR-34c or miR-34c inhibitor, and proteins were extracted in $1 \times$ SDS-PAGE sample loading buffer. Total cell protein was resolved by SDS-PAGE, transferred to PVDF membranes, and probed with antibodies for β -actin (1:1000, Beyotime, Haimen, Jiangsu, China), c-Myc (1:1000, Chemicon), PCNA (1:1000, Millipore), P53 (1:1000, Beyotime, Haimen, Jiangsu, China), Cyclin D1 (1:1000, Bioss Biotechnology, Beijing, China), caspase 3 (1:1000, Bioss Biotechnology). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as a secondary antibody (1:1000, Beyotime). Detection was performed using Thermo Scientific Pierce ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA, USA).

Statistical analysis

Effect of miR-34c on proliferation and apoptosis of mGSCs were evaluated based on Q-RT-PCR, Brdu staining. Data are presented as mean $\pm$ SEM and standard errors of the mean (SEM). In this study they were calculated for at least 3 replicates in each of 3 independent experiments. Statistical comparisons were assessed using Student's test; $P < 0.05$ was considered to be sta-

tistically significant, and $P < 0.01$ was considered to be highly significant.

Results

Characterization of dairy goat mGSCs

The dairy goat mGSCs were characterized using RT-PCR and immunofluorescence staining. Results illustrated that the cultured cells expressed Oct4 (also known as Pou5f1), Vasa, Dazl, Nanog and CD90 (Fig. 1a and 1b). This indicated that cells obtained were mGSC characteristic. Moreover, the cells had the potential to differentiate into embryoid bodies (EBs), in which various cell types were found of the three embryonic germ layers, as determined by immunofluorescence and/or histological staining (22).

Expression patterns of p53 and miR-34c

Q-RT-PCR analysis showed that p53 and miR-34c were highly expressed in the adult mGSCs and were generally expression in the 90 days postpartum (dpp) tissue; there was extremely low expression level in 30 dpp cells (Fig. 2a and 2c). Results of immunofluorescence were in agreement with the results of Q-RT-PCR (Fig. 2b). This showed that expression of p53 was significantly enhanced in adult dairy goat testis cells; conversely, expression of p53 was weak in 30 dpp samples. Percentage of p53 positive spermatogonia cells in adult testis

Figure 1. Cultured mGSCs expressed characteristic stem cell markers including Oct4, c-Myc, Vasa, Dazl and CD90, analysed by RT-PCR (a) and immunofluorescence (b).

Figure 2. Expression pattern of p53 and miR-34c in 30 dpp, 90 dpp and adult dairy goat testis mGSCs. (a, c) Q-RT-PCR analysis showed that p53 and miR-34c were more highly expressed in adult cells than in 30 dpp and 90 dpp samples. $*P < 0.05$, $**P < 0.01$; (b) Immunofluorescence showed p53 expression was adult than 30 dpp and 90 dpp samples; (d) miRNA ISH showed miR-34c expression was higher in adult than 30 dpp and 90 dpp samples.

was significantly higher than that in 30 dpp and 90 dpp testis. Results of miR-34c *in situ* hybridisation also supported miR-34c detection at Q-RT-PCR (Fig. 2d). This indicated that expression of miR-34c was significantly higher in adult dairy goat testis than in 30 dpp and 90 dpp tissues (Fig. 2c and 2d).

MiR-34c over-expression suppressed proliferation and enhanced apoptosis in the dairy goat mGSCs

mGSCs over-expressing miR-34c became rounded in shape while suspended in medium. Simultaneously, numbers of mGSCs in the over-expressed miR-34c group were significantly less than in control and miR-34c inhibitor groups (Fig. 3a). Some fragmentation appeared in nuclei of cells of over-expressed miR-34c mGSCs, with no similar cell phenotype in control, Mixture or miR-34 inhibitor groups (Supplementary Fig. S1). Results of FACS analysis showed that percentage of apoptotic mGSCs was significantly higher after miR-34c over-expression; however, percentage of apoptosis mGSCs in miR-34c inhibitor was specifically lower

compared to controls (Fig. 3b). Brdu incorporation assay (Fig. 4) indicated that percentage of Brdu positive cells was significantly lower in the miR-34c over-expression group compared to controls and Mixture groups; on the contrary, transfection of miR-34c inhibitor increased percentages of Brdu positive cells. These results further demonstrate that miR-34c over-expression reduced proliferation of the mGSCs, and miR-34c inhibitor specifically increased their proliferation. These results suggested that miR-34c appeared to reduce proliferation of the mGSCs, but this might have resulted from widespread apoptosis stimulated by miR-34c.

p53 overexpression activated miR-34c and inhibited c-Myc

Over-expression miR-34c in the mGSCs specifically down-regulated expression level of c-Myc and cyclin D1, and up-regulated expression of caspase 3. However, there were no significant differences in expression levels of either p53 or PCNA in over-expression miR-34c and its inhibitor groups, as analysed by Q-RT-PCR and wes-

Figure 3. mGSCs over-expressing miR-34c became round in suspension in the medium (a). FACS analysis showed that percentage of apoptotic mGSCs was significantly higher after miR-34c over-expression (b).

Figure 4. BrdU incorporation assay illustrated that percentage of BrdU positive cells was significantly lower in the miR-34c over-expression group compared to control, inhibitor and Mixture groups (a, b).

tern blotting (Fig. 5). In turn, over-expression of p53 gave rise to significant up-regulation of miR-34c, and down-regulated expression of c-Myc (Fig. 6). Results showed that p53 had stimulated transcriptional expression of miR-34c and inhibited the protein level of c-Myc (27). These effects were induced by miR-34c through specific binding sites to 3'UTR of c-Myc in miR-34c; evolutionarily, these specific binding sites are highly conserved.

Doxorubicin up-regulated p53 protein level and further up-regulated expression of miR-34c

The chemotherapeutic agent doxorubicin (Dox), a DNA-damaging agent, activates a p53-survivin signalling pathway inducing cell cycle arrest and apoptosis. Here, expression levels of miR-34c were up-regulated by doxorubicin in a dose- and time-dependent manner (Fig. 7a and 7b). Expression level of miR-34c reached its peak when doxorubicin was 20 µg/ml. Additionally, expression level of miR-34c showed a trend to increase over time. Thus, we treated the cells with 20 µg/ml doxorubicin to detect any effects on expression of p53, c-Myc and miR-34c; 6-fold increase in miR-34c expression was observed by 24 h of doxorubicin exposure. Meanwhile, protein level of p53 clearly increased; however, mRNA level of p53 was not significantly changed, which implied that miR-34c was p53-dependent. (Fig. 7c and 7d). Effects of doxorubicin gave rise to stimulation of miR-34c, p53 and c-Myc, then induced mGSCs apoptosis. Meanwhile, the nucleus was larger and dispersed from the whole to the small granular by doxorubicin exposure (Supplemented Fig. S2).

Discussion

Study of apoptosis, proliferation and differentiation of germ cells is one of the most important fields in the life sciences; miRNAs play important roles in almost every

biological process. Up to date, approximately 30% of human genes have been found to be regulated by miRNAs and it is becoming more and more important to uncover mechanisms of miRNA regulation underlying complex biology processes (28,29). Recent studies have shown that miR-34c is closely related to apoptosis and to cell proliferation (5,16), however up to now, study on miR-34c in maintaining germline stem-cell (mGSC) self-renewal has been minor (19).

miRNAs exist ubiquitously in mammals and induce target gene degradation and translational inhibition by binding complementary mRNA sequences, playing a critical role in the gene expression network (30–32). miR-34c and p53 feedback regulation has been discovered previously in mice. p53 accommodated miR-34c in altering expression of *Bcl-2*, *c-Myc* oncogenes and genes for G1-associated cyclins (cyclin D1, cyclin E2), in suppression of cancer (33); yet, miR-34c inhibits SIRT1 (silent mating type information regulation) and enhances p53 activity (34). However, relationships between p53 and miR-34c in male germ cells of dairy goat up to now have not been clear. Understanding exact mechanisms between these two molecules is a valuable resource.

By using morphological observation, BrdU labelling, flow cytometry, Q-RT-PCR and western blotting analysis, we found that miR-34c over-expression in our dairy goat mGSCs resulted in floating, rounded cells, indicating signs of apoptosis. Flow cytometry results showed that miR-34c over-expression lead to apoptosis. Up-regulation of caspase3 in mRNA level also suggested that miR-34c promoted apoptosis. However, p53 expression at either mRNA or protein level did not change clearly; these results suggest that mGSC apoptosis by miR-34c over-expression would not influence p53 expression. Higher doses of miR-34c reduced BrdU signal as well as cell density, together with lower c-Myc mRNA and protein levels; it is thus, suggested that miR-34c reduced proliferative ability of the mGSCs. Meanwhile, over-

Figure 5. Over-expression of miR-34c specifically down-regulated expression level of c-Myc, cyclin D1, and up-regulated expression of caspase 3 in mGSCs. However, there were no significant differences in expression of p53 and PCNA in over-expression of miR-34c and inhibitor group analysed by Q-RT-PCR (a) and western blotting (b). * $P < 0.05$.

Figure 6. Over-expression of p53 in the mGSCs gave rise to significant induction of miR-34c level, and down-regulated expression of c-Myc, as analysed by Q-RT-PCR (a) and western blotting (b). * $P < 0.05$; ** $P < 0.01$.

expression of miR-34c lead to down-regulation of cyclin D1, indicating that miR-34c inhibited G1-S transfer during cell cycle.

microRNA-34, a potent mediator of p53, has been previously been reported to function as a tumour suppressor, and miR-34a has been found to be down-regu-

Figure 7. Doxorubicin up-regulated expression of p53 and miR-34c. Expression levels of miR-34c were up-regulated by doxorubicin in a dose- and time- dependent manner (a, b); (c and d), p53 was increased by Dox; however, c-Myc was reduced by doxorubicin. * $P < 0.05$; ** $P < 0.01$.

lated in malignant prostate cancer cells (20). Here we discovered that miR-34c promoted our mGSC apoptosis and reduced their proliferative ability. Over-expressed p53 mGSCs up-regulated expression of miR-34c and moreover, activation of p53 protein by doxorubicin increased miR-34c. Thus, we found miR-34c is p53-dependent in dairy goat mGSCs, which provides us with a better basis for discovering underlying mechanisms regulating miR-34c. The role of miR-34c in later steps of spermatogenesis is indispensable (27); remarkably, its main function is to suppress cell proliferative ability through down-regulation of multiple targets (35) and/or to promote apoptosis (36). In our study, Q-RT-PCR, immunofluorescence and miRNA ISH analysis first showed that p53 and miR-34c were significantly enhanced in adult tissues. The studies have suggested that miR-34c was essential for apoptosis of dairy goat mGSCs *via* p53, and regulated development and apoptosis of mGSCs.

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Supporting Information

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

Fig. S1 Fragmentation appeared in nuclei of miR-34c over-expression mGSCs (arrow indicated); no similar cell phenotypes were seen in control and miR-34 inhibitor groups.

Fig. S2 Nuclei of mGSCs treated with doxorubicin were larger and dispersed from whole fragments to small granular types.