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RESEARCH ARTICLE

Effects of MiR-335 on the Proliferation in Yak Preadipocytes

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Abstract

To explore the effect of miR-335 on the proliferation of yak precursor adipocytes, the expression of miR-335 was detected by real-time fluorescence quantitative PCR (qRT-PCR) during the proliferation phase of yak precursor adipocytes in this experiment, when cells were transfected with miR-335 mimic and mimic NC, miR-335inhibitor and inhibitor NC at 70% cell growth density, EdU staining was used to detect cell proliferation, and the expression of cell proliferation marker genes (*CCNB1, CCND2, PCN4,* and *CDK4*) was detected by qRT-PCR; wild-type and mutant vectors of the miR-335 target gene SP1 were constructed and transfected into 293T cells, and miR-335 was assayed with SP1 using a dual luciferase assay reporter. The results showed that the expression of miR-335 increased and then decreased during the proliferation of yak precursor adipocytes. overexpression of miR-335 inhibited the expression of *CCNB1, CCND2, PCN4,* and *CDK4* in the cells, and EdU-positive cells decreased. The results of the dual luciferase reporter suggest a targeted regulatory relationship between miR-335 and *SP1*.

Keywords: Mir-335; Yak; Preadipocytes; Proliferation

Introduction

The yak is a unique livestock resource in the alpine and subalpine regions bordering the Tibetan Plateau [1], it obtains nutrients mainly by feeding on natural pasture, which makes the fat content change. Animal fat content depends to a large extent on the number of adipocytes and their size [2]. Usually, before birth and in the early stages of growth and development the fat content is determined by the number of adipocytes and the capacity of lipid synthesis; while in the fattening period of the animal fat content is determined by the volume of adipocytes [3]. Changes in the number and volume of yak adipocytes affect their fat deposition and thus their production performance. Exploring the mechanism of yak adipocyte proliferation is of great significance for the improvement of yak production performance and the increase of farmers' income.

As early as 1993, a class of non-coding single-stranded RNAs of 19 to 22 nt was discovered in nematodes, which researchers called microRNAs (miRNAs) [4], it is widely distributed in animals, plants and microorganisms. Numerous studies have shown that miRNAs are one of the key factors in the regulation of cell proliferation, and their overexpression or interference affects cell proliferation. It was confirmed that miR-92a-3p inhibited the proliferation of 3T3-L1 precursor adipocytes [5], miR-125b-5p inhibits 3T3-L1 precursor adipocyte proliferation [6], miR-136 promotes the proliferation of precursor adipocytes in small-tailed han sheep [7]. miRNAs can play a regulatory role by binding to the 3' UTR region of target genes, mediating the degradation of target gene mRNAs or inhibiting their translation process. The expression of miR-17-92 reaches its highest level during the proliferative phase of preadipocytes and promotes cell proliferation by targeting and regulating the tumor suppressor protein Rb2/p130 [8]. It was found that miR-143a-5p targets MAPK7 during the proliferative phase of 3T3-L1 precursor adipocytes thereby inhibiting cell proliferation [9]. miR-21 targets signal transduction and activators of transcription (S-TATs) 3 and inhibits the proliferation of human adipose-derived MSC cells [10].

miR-335 was expressed in several tissues, with particularly significant expression in brain, liver, skeletal muscle, heart, white adipose, and brown adipose tissues [11]. Lipid metabolism, changes in lipid content, and 3T3-L1 cell differentiation were closely associated with miR-335 expression. Studies have shown that miR-335 target genes are mainly enriched in the Wnt signaling pathway, MAPK signaling pathway, calcium signaling pathway, cell cycle and other signaling pathways, and through the regulation of factors in these pathways, thereby affecting the exocrine pancreatic secretion, lipid kinase activity, as well as tumor development and progression, and other biological processes [12]. Researchers found that the expression level of miR-335 was up--regulated in liver and fat of obese mice [13]. However, few studies have been reported on the effects of miR-335 on yak precursor adipocytes. Inhibitory effect of miR-335 on cancer cell proliferation [14], it is hypothesized that miR-335 may be relevant to the proliferation of adipocytes. SP1 (specificity protein 1), a common transcription factor, is associated with a variety of biological processes, including autophagy, apoptosis, differentiation, growth and other processes, and is widely expressed in a variety of cells in the body [15,16]. It has been found that *SP1* promotes adipogenesis by up-regulating the expression of sterol regulatory element-binding protein-1c (*SREBP-1c*) and fatty acid synthase (*FASN*) genes in cancer cells [17]. Roy [18] et al. found that SP1 has a role in promoting lipid deposition in mouse 3T3-L1 cells. Liu [19] et al. reported that SP1 could promote breast cancer cell proliferation by up-regulating the expression of tissue differentiation-inducing non-protein coding RNA (TINCR). Li [20] et al. showed that microRNA-376a could inhibit the proliferation of glioma cells by down-regulating the protein expression of *SP1* mRNA through targeted degradation. In this study, we chose *SP1*, the target gene of miR-335, to explore how miR-335 affects yak cell proliferation by regulating *SP1*. Yak precursor adipocytes were used to analyse the effect of miR-335 on their proliferation. The mechanism of miR-335 in yak precursor adipocytes was determined by cell transfection, EdU staining and dual luciferase activity test, which provided a reference for exploring the molecular mechanism of yak growth and development.

Materials and Methods

Materials

Experimental Cell

Yak precursor adipocytes were preserved from laboratory pre-culture.293T cells were purchased from Wuhan Proximity Biologicals, Hubei, China.

Main Reagents and Instruments

DMEM/F12, Fetal Bovine Serum (FBS), 100x Penicillin (Dual Antibody), miR-335 mimic and mimic NC, miR-335 inhibitor and inhibitor NC, Total RNA Extraction Kit (Tiangen, China), SPARKscript II All-in-one RT SuperMix for qPCR (With gDNA Eraser) (Ciscojet), 2xSYBR Green qPCR Mix (With ROX) (Ciscojet), miRcute Enhanced miRNA Fluorescence Quantitative Detection Kit (SYBR Green) (Tiangen, China), Lipofectamine® 3000 Liposome Transfection Kit, EdU-555 Cell Proliferation Detection Kit (Dalian Meilun, China), Dual-Luciferase Activity Test Kit, 6-well plate, micropipette, pasteurized burette, CO_2 incubator, ultra-clean bench, water bath, centrifuge, PCR instrument.

Methodologies

MiR-335 Target Gene Prediction

The miRNA target gene online prediction software TargetScan 8.0 (https://www.targetscan.org/vert_80/) and miRDB (https://mirdb.org/mirdb/index.html) were utilized for target gene prediction of miR-335 to select adipose cell proliferation-related target genes. The primer design was also performed through NCBI (National Center for Biotechnology Information (nih.gov)).

MiR-335 Temporal Expression Profile

Cells were collected from yak precursor adipocytes at 0 d, 3 d, 6 d and 9 d of the proliferative phase, and total RNA was extracted from the cells at different periods according to the total RNA extraction instructions, and the expression of miR-335 was detected, respectively.

Cell Transfection and RNA Extraction

After the cell growth fusion reached about 70%, miR-335 mimic, mimic NC, miR-335 inhibitor, and inhibitor NC were transfected into yak cells co-cultured for 48 h using Lipofectamine® 3000 liposomes, and then total RNA was extracted according to the instructions of the total RNA extraction kit.

EDU Staining

10^6 cells were inoculated in 6-well plates and cultured for 48 h to return to normal. The test was performed according to the EdU kit instructions.

Real-Time Fluorescence Quantitative PCR Assay

The expression of proliferation-related genes and miR-335 were detected according to the instructions of 2xSYBR Green qPCR Mix (With ROX) and miRcute Enhanced miRNA Fluorescence Quantitative Detection Kit (SYBR Green), respectively.

Dual Luciferase

The dual luciferase reporter gene vector was GP-mirGLO, in which sea kidney luciferase was used as the detection reporter gene and firefly luciferase as the internal reference reporter gene. SP1-3'UTR-WT (wild type), SP1-3'UTR-MUT (mutant), and SP1-3'UTR-WT and miR-335 mimic, SP1-3'UTR-WT and miR- 335 mimic NC, SP1-3'UTR-MUT and miR-335 mimic, SP1-3'UTR-MUT and miR-335 mimic NC, respectively, were co-transfected into 293T cells and co-cultivated for 48 h. After co-cultivation, according to the instructions of the dual-luciferase activity assay kit Perform the assay.

Statistical Analysis of Data

SPSS 26.0 (IBM) was used to statistically analyze the qRT-PCR data obtained in this experiment. One-way ANOVA test was used for analysis of variance (ANOVA), with P<0.05 as the significance of difference and P<0.01 as the criterion for determining highly significant differences. Graphs were plotted using GraphPad.Prism.9.5 graphing.

Table 1: Primer information

Primer name	Primer sequence（5’-3’）	Length
SP1-3' UTR-WT	F:CAT AGA CGC GTC CAT TCT GTG ACC AAA ATA	129
	R:TAA CTG TTT AAA CCA AGA CAA CAT CTA TTC GAA	
SP1-3' UTR-MUT	F:CAT AGA CGC GTC CAT TCT GTG ACC AAA ATA	129
	R:TAA CTG TTT AAA CAC CTC CAA CAT CTA TTC GAA	
miR-335	F: GCC CGT CAA GAG CAA TAA CGA AAA AT	
miR-335	R: AGT GCA GGG TCC GAG GTA TT	
U6	F:CGC TTC ACG AAT TTG CGT GTC AT	
U6	R:GCT TCG GCA GCA CAT ATA CTA AAA T	
CCNB1	F:GGC TCC AGT GCT CTC CTC CTC	84
CCNB1	R:AAT CTT CGT GTT CCT GGT GAT CCG	
CCND2	F:ATG GAA CCT GGC GGC TGT C	103
CCND2	R:CTG GGC GTG CTT GCG AAT C	
PCNA	F:CCT TCT ACC TGT AGC CGT GTC ATT G	81
PCNA	R:CTT TCT TCA AGA TGG AGC CCT GGA C	
CDK4	F:ACA CAA GCG AAT CTC TGC CTT CC	77
CDK4	R:TTG TTC ACT CTG CGT CAC CTT CTG	
SP1	F:ACG GCA ACA ATG GCA GTG AGT C	91
SP1	R:TGC TGG TTC TGA AGG TTG GAA GTG	
GAPDH	F:CAA GTT CAA CGG CAC AGT CAA GG	124
GAPDH	R:CTC GCT CCT GGA AGA TGG TGA TG	

Result

Mir-335 Target Genes

There were 243 target genes of miR-335 obtained by predictive target gene software analysis. After a literature review, *SP1* was screened to be associated with adipocyte proliferation, and the binding site information of miR-335 and *SP1* was found (Figure 1). Therefore, *SP1* was screened as a candidate target gene for miR-335 to regulate yak precursor adipocytes.

Figure 1: Binding site information of *SP1*

Time Course Expression Profile

Throughout the proliferation of yak precursor adipocytes, miR-335 expression changed. As shown in Figure 2, the expression of miR-335 showed a trend of increasing and then decreasing in the 0th, 3rd, 6th and 9th d of precursor adipocyte proliferation, in which the lowest expression was found in the 0th d of cell proliferation and the highest expression was found in the 3rd d of proliferation ($P<0.01$), and then, the expression of miR-335 decreased significantly in the 6th and 9th d of cell proliferation ($P<0.05$).

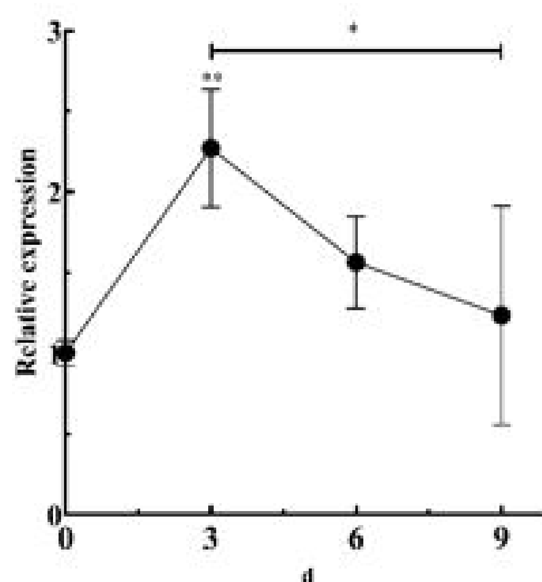


Figure 2: Temporal expression profile of miR-335

Mir-335 Overexpression and Interference Efficiency Assay

After transfecting miR-335 mimic and mimic NC and miR-335 inhibitor and inhibitor NC into Qinghai yak precursor adipocytes, respectively, we found that after overexpression of miR-335, the expression level of miR-335 in the precursor adipocytes increased 1.7-fold ($P<0.01$), and after interfering with miR-335 expression, the expression level of miR-335 in the precursor adipocytes decreased 0.59-fold ($P<0.01$) (Figure 3). After interfering with miR-335 expression, miR-335 expression level in precursor adipocytes was highly significantly decreased by 0.59-fold ($P<0.01$) (Figure 3)

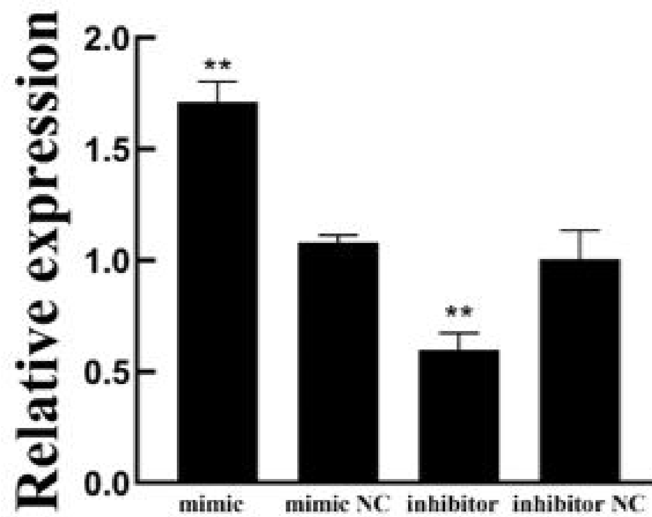


Figure 3: Transfection efficiency of miR-335

Effects of Overexpression and Interference with Mir-335 on Cell Proliferation

In order to investigate the effect of miR-335 on the proliferation of yak precursor adipocytes, the cells were treated with overexpression and interference of miR-335, respectively, and the relative mRNA expression of cell proliferation-related genes *CCNB1*, *CCND2*, *PCNA* and *CDK4* was detected by qRT-PCR. It was found that the expression of cell proliferation-related genes *CCNB1*, *CCND2*, *PCNA* and *CDK4* was highly significantly decreased after overexpression of miR-335 ($P<0.01$). In contrast to the results of overexpression of miR-335, the expression of *CCNB1*, *CCND2*, *PCNA* and *CDK4* was highly significantly increased after interfering with the expression of miR-335 ($P<0.01$) (Figure 4).

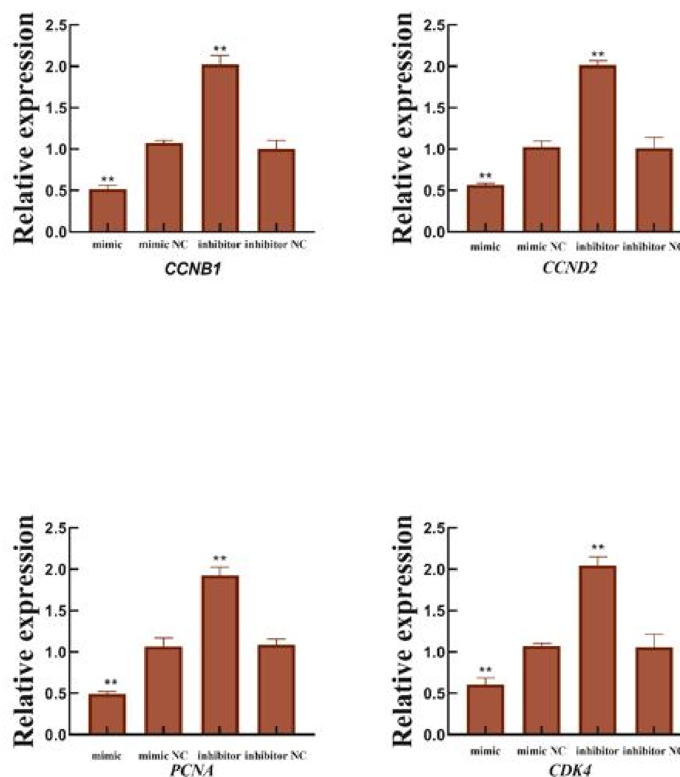
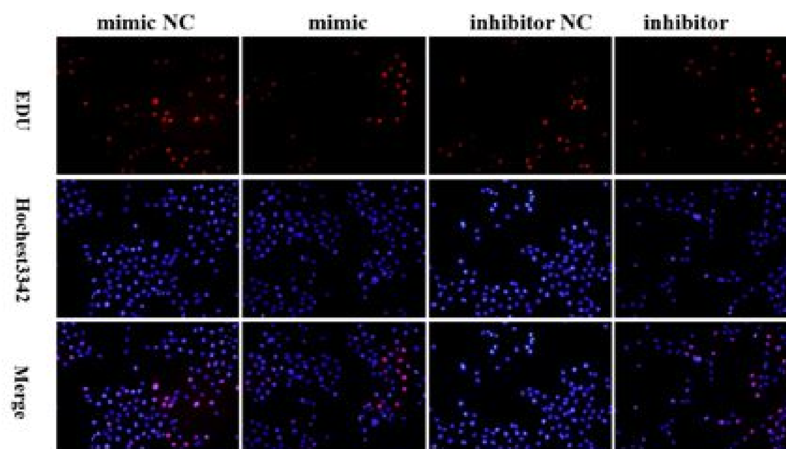


Figure 4: Effect of miR-335 on the expression of cell proliferation-related genes

The role of miR-335 in cell proliferation was further explored using EdU staining, as shown in Figure 5, which revealed that overexpression of miR-335 decreased the proportion of EdU-positive cells; interference with miR-335 expression increased the proportion of EdU-positive cells.



Dual Luciferase Reporter Assay

The relative luciferase activities of the co-transfected *SP1*-3'UTR-WT (wild type) and miR-335 mimic groups were highly significantly lower than those of the co-transfected *SP1*-3'UTR-WT (wild type) and miR-335 mimic NC groups ($P<0.01$, Figure 6). The relative luciferase activities of the co-transfected *SP1*-3'UTR-MUT (mutant) and miR-335mimic groups were highly significantly lower than those of the co-transfected *SP1*-3'UTR-MUT (mutant) and miR-335 mimic NC ($P<0.01$, Figure 6). This leads to the preliminary conclusion that miR-335 and *SP1* have a targeting relationship, suggesting that *SP1* is a direct target of miR-335.

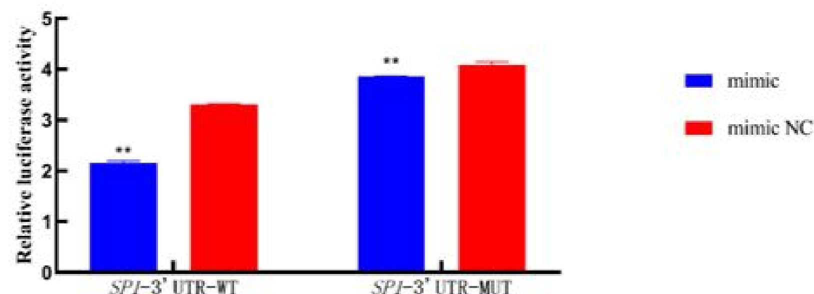


Figure 6: *SP1* relative dual luciferase activity

Discussion

The whole process of precursor adipocyte proliferation is rapid and short, occupying a very small proportion of the entire growth period of the cells. The primary yak precursor adipocytes generally take about 10 days from extraction to full growth, and the passaged cells take about 5 days from inoculation to full growth. Cell proliferation is mainly divided into synthetic phase (S phase) and mitotic phase (M phase), between which there are two intermediate interphases, G1 and G2 [21]. The expression of miR-335 changed during the proliferation of precursor adipocytes, suggesting that miR-335 may regulate the proliferation of precursor adipocytes.

It has been shown that cell proliferation is mainly regulated by cell cycle-related genes *CCNB1, CCND2, PCNA* and *CDK4*. Cell cycle-related genes include cyclins, a group of proteins that appear and disappear periodically during the cell cycle [22]. Cyclin B1 (CCNB1) is a member of the cyclin family, with a gene length of approximately 8800 bp, and acts at the G2/M detection site of mitosis in eukaryotic cells [23]. Cell cycle protein D2 (cyclin D2, CCND2) is a member of the cyclin family, which acts as an initiator of the cell cycle, is expressed in the G1 phase, and functions at the G1/S transition [24]. Proliferating cell nuclear antigen (PCNA) is an indispensable cofactor for DNA polymerase during replication, and its ability to bind DNA to polymerase and significantly increase its synthesis has led to the use of PCNA as an important marker of cell proliferation status [25]. Cyclin-dependent kinase 4 (CDK4) is thought to play a major role in early G1 cell cycle progression. CCND binds to CDK4 or CDK6 and acts on cells in the G1 to initiate the cell cycle and promote cellular DNA synthesis, thereby promoting cell proliferation [26]. However, some studies converge on the idea that miRNAs can be involved in the regulation of the cell proliferation process through regulatory genes or pathways. In recent years miR-335 studies on proliferation have focused on the regulation of cancer cells. Studies have shown that miR-335 inhibits human breast cancer cell proliferation by targeting and regulating the expression of EphA4 [27]. miR-335 inhibits proliferation of gastric cancer cells through MAPK10 (mitogen-activated protein kinase 10) [28]. miR-335 inhibits head and neck squamous cell carcinoma development by regulating MAP3K2 expression [29]. Upregulation of miR-335-5p may inhibit colon cancer cell proliferation by targeting G6PD [30]. Relevant studies on the effect of miR-335 on the proliferation of yak precursor adipocytes have not been found. In this experiment, we verified its effect on the proliferative effect of yak precursor adipocytes by overexpressing and interfering with miR-335. The results revealed that overexpression of miR-335 extremely significantly down-regulated the expression of cell proliferation marker genes *CCNB1, CCND2, PCN4, and CDK4*, and EDU-positive cells were reduced. The expression of cell proliferation-related genes was highly significantly elevated after interfering with miR-335 expression ($P<0.01$). This study identified that miR-335 inhibited the proliferation of yak precursor adipocytes in terms of both overexpression and interference, but the mechanism of inhibition still needs to be further investigated.

Among the available studies on miRNA-targeted genes to regulate cell proliferation and apoptosis, no studies on the targeting relationship between miR-335 and SP1 have been found. The method of choice for numerous targeting relationship validation assays is the dual luciferase activity assay [31]. Dong et al [32] confirmed that miR-18a-5p negatively regulated THBD expression by dual luciferase assay. Pass-through dual luciferase assay reveals negative regulation of miR-214 with TRMT61A [33]. Dual luciferase reporter gene analysis revealed that miR-210 was targeted and regulated by binding to the AjE2F3-3'UTR region in the range of 108 nt to 128 nt [34]. Target gene identification is the key to reveal the mechanism of miRNA action. Many studies have shown that miRNAs can affect adipogenesis by directly targeting adipose-related genes. Overexpression of miR-200b in 3T3-L1 inhibits the expression level of PPARγ [35], thereby affecting adipogenesis. NCOA3 acts as a target gene for miR-17-5p in adipocyte differentiation in pigs [36]. Similar to miR-335 targeting regulation of SP1 to inhibit cell proliferation [37]， in this study, the targeting relationship between miR-335 and SP1 was verified by dual luciferase detection assay, further refining the regulatory network of miR-335.

Conclusion

In this experiment, the expression pattern of miR-335 during cell proliferation was detected by qRT-PCR, and it was demonstrated that miR-335 inhibited the expression of cell proliferation-related genes. And it was found that miR-335 inhibited the proliferation of yak precursor adipocytes by targeting the regulation of SP1.

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Informed Consent Statement

Not applicable.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest

The authors declared that they had no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

The Animal Welfare statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.

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