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Age-Associated *miR-26a* Elevation Correlated with Loss of Stemness in Senescent Breast Adipocytes from Obese Women

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Abstract

Obesity and aging are closely interconnected biological states that collectively accelerate the decline in tissue homeostasis and regenerative capacity. However, the molecular mechanisms linking obesity to accelerated aging is still unclear. In this study, we have shown that obesity decreased the level of miR-26a in mature breast adipocytes derived from obese women, as compared to mature breast adipocytes from lean women, and its level significantly increased upon their senescence. Interestingly, inhibition of miR-26a in senescent adipocytes restores their proliferative/migration capabilities. Additionally, we have shown that miR-26a binds to specific sequences on the 3'UTR of the stemness markers KLF4, MYC, SOX2 and LIN28A and decreases their expression upon its inhibition in senescent adipocytes. These results show that miR-26a is implicated in the activation of breast adipocytes in obese women through targeting the stemness markers and could be utilized to restoring cellular vitality and delaying age-associated tissue degeneration.

Keywords: Obesity; Aging; MicroRNA; Stem markers.

Introduction

Obesity and aging are closely interconnected biological states that collectively accelerate the decline in tissue homeostasis and regenerative capacity [1]. Both conditions are characterized by chronic low-grade inflammation, mitochondrial dysfunction, oxidative stress, and impaired stem cell function, all of which contribute to progressive deterioration in cellular health and tissue integrity [2,3]. With advancing age, adipose tissue undergoes significant structural and functional changes, including reduced adipogenic potential, altered adipokine secretion, and accumulation of senescent cells. Similarly, obesity induces premature cellular aging by promoting metabolic stress and inflammatory signaling within adipose depots [4]. This convergence between obesity-associated metabolic dysregulation and aging-related cellular senescence suggests that obesity may act as a driver of accelerated biological aging, particularly in metabolically active tissues such as adipose tissue [3,5].

At the cellular level, adipocyte progenitors and mesenchymal stem cells exhibit diminished proliferative and differentiation capacities with both chronological aging and obesity [4]. These alterations are often mediated by dysregulation of key transcriptional and epigenetic pathways that control stemness, self-renewal, and metabolic homeostasis [4]. Consequently, adipose tissue from obese and aged individuals displays reduced regenerative potential and impaired capacity to maintain tissue health [6]. Understanding the molecular mechanisms linking obesity to accelerated aging is therefore essential for identifying biomarkers of adipose tissue dysfunction and for developing therapeutic strategies aimed at restoring cellular vitality and delaying age-associated tissue degeneration. In this

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study, we aimed at investigating the potential molecular link between aging and obesity by studying the role of miR-26a in breast adipocytes derived from normal lean and obese women underwent mammoplasty.

Methods

Tissue collection

Breast adipose tissues were collected from reduction mammoplasty according to the guidelines of the Ethical committee of the KFSHRC (RAC# 2140017). Informed consent was obtained from all subjects.

Generation of primary adipocytes and luminal cells from breast tissues

The obtained tissues were immediately digested with collagenase to separate preadipocytes from the stroma vascular fraction, as previously described [7]. Preadipocytes were first cultured in special medium (PAGM) containing fetal calf serum (0.05 ml/ml), Endothelial Cell Growth Supplement (0.004 ml/ml), Epidermal Cell Growth Factor (10 ng/ml), Hydrocortisone (1 µg/ml) and Heparine (90 µg/ml), and used until confluency. Preadipocytes were then differentiated into mature adipocytes by replacement of the preadipocyte medium with serum-free preadipocytes differentiation medium (SFPADM), supplemented with D-Biotin (8 µg/ml), Insulin (0.5µg/ml), Dexamethasone (400 ng/ml), IBMX (44 µg/ml), L-Thyroxine (9 ng/ml) and Ciglitazone (3 µg/ml). Mature adipocytes were then cultured using adipocyte nutrition medium (ANM) containing fetal calf serum (0.03 ml/ml), D-Biotin (8 µg/ml), Insulin (0.5µg/ml), Dexamethasone (400 ng/ml). All supplements were purchased from PromoCell GmbH, Germany.

RNA purification and quantitative RT-PCR

Total RNA, containing miRNAs, was purified using the miRNeasy mini kit (Qiagen, UK) according to the manufacturer's instructions and was treated with RNase-free DNase. 1 µg RNA was used to synthesize cDNA utilizing either Advantage RT-PCR kit (Clontech Laboratories, Mountain View, CA, US) or miScript II RT kit (Qiagen, UK) for mature miRNAs. Quantitative RT-PCR was performed in triplicate using 4 µl cDNA mixed with 2x FastStart Essential DNA Green qPCR mastermix (Roche, New York, NY, US) and 0.3 µM forward and reverse primers. Amplifications were performed utilizing the

LightCycler 96 Real-time PCR detection system (Roche) using the following cycle conditions: 95°C for 10 min (1 cycle); 95°C for 10 sec, 59°C for 20 sec, 72°C for 30 sec (45 cycles). GAPDH expression levels were used for normalization, and gene expression differences were calculated using the threshold cycle (Ct). The obtained values were plotted as mean ± SD. Three independent experiments were performed for each reaction. (See table 1 in the expanded view for the sequence of the primers).

Transfection/Viral Infection

pLKO.1-miRZipmiR-26a (Inhibitor of miR-26a) and the control plasmid were used at 1 µg/ml each for transfection of 293FT cells. Lentiviral supernatants were collected 48 h post-transfection. Culture media were removed from the target cells and replaced with the lentiviral supernatant and incubated for 24 h in the presence of 1 µg/ml Polybrene (Sigma-Aldrich). Transduced cells were selected after 48 h with puromycin.

miRNA Target Prediction

miRNA targets were predicted using algorithms, including miRanda Human miRNA targets, miRDB, RNA22, and miROrg. To identify the genes commonly predicted by these different algorithms, the results of predicted targets were intersected using miRWalk.

Cell migration and proliferation

These assays were performed in a label-free real-time settings using the xCELLigence RTCA technology (Roche, Germany) that measures impedance changes in a meshwork of interdigitated gold microelectrodes located at the well bottom (E-plate) or at the bottom side of a micro-porous membrane (CIM plate 16) [8,9]. Cell migration was assessed as per the manufacturer's instructions. In brief, $2x10^4$ cells in serum-free medium were added to the upper wells of the CIM-plate non-coated with a thin layer of Matrigel (BD

Biosciences) basement membrane matrix. Complete medium was used as a chemo-attractant in the lower chambers. Subsequently, the plates were incubated in the RTCA for 24 h and the impedance value of each well was automatically

Table 1: Primer Sequences utilized for qRT-PCR.

GAPDH	5'-GAGTCCACTGGCGTCTTC-3'	5'-GGGGTGCTAAGCAGTTGGT-3'
KLF4	5'-CATCTCAAGGCACACCTGCGAA-3'	5'-TCGGTCGCATTTTTGGCACTGG-3'
MYC	5'-CCTGGTGCTCCATGAGGAGAC-3'	5'-CAGACTCTGACCTTTTGCCAGG-3'
SOX2	5'-GCTACAGCATGATGCAGGACCA-3'	5'-TCTGCGAGCTGGTCATGGAGTT-3'
LIN28A	5'- CCAGTGGATGTCTTTGTGCACC-3'	5'- GTGACACGGATGGATTCCAGAC-3'
mature miR-26a	UAACACUGUCUGGUAAAGAUGG	

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monitored by the xCELLigence system and expressed as Cell Index (CI) value, which represents cell status based on the measured electrical impedance change divided by a background value. Each assay was performed in triplicate.

For the proliferation assay, exponentially growing cells ($2x10^4$) were seeded in E-plate with complete medium as per the manufacturer's instruction. Cell proliferation was assessed for 48h. All data were recorded and analyzed by the RTCA software. Cell Index was used to measure the change in the electrical impedance divided by background value, which represents cell status. Each assay was biologically performed in triplicate [10].

Ki-67 immunostaining

The proportion of S phase cells was determined by assessing the levels of Ki-67 (Abcam) by immunostaining. Briefly, cells were fixed in 1:1 acetone/methanol, and a standard indirect immunoperoxidase procedure was applied using Ki-67 antibody, followed by peroxidase-conjugated anti-rabbit antibody. Sites of antibody binding were visualized by the deposition of brown polymer of DAB chromogen. The percentage of Ki-67-labeled cells (LI) was determined for at least 500 cells per data point and expressed as a mean and standard error of triplicate determinations.

Assessment of SA–β-Galactosidase activity

Endogenous senescence-associated β-galactosidase (SA-β-gal) activity was assessed histochemically. Cells were fixed in 0.5% glutaraldehyde in PBS for 15 minutes and then permeabilized with detergent wash solution (0.02% NP-40, 0.1% sodium deoxycholate, 2mM magnesium chloride in PBS) for 15 minutes, followed by incubation (4 hrs at 37°C) in a 1 mg/ml of X-gal substrate (5-bromo-4-chloro-3-indolyl-BD-galactopyranoside) in X-gal solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% sodium deoxycholate and 0.02% NP40 at an acidic pH (6.0). The proportion of β-gal–positive cells was assessed in a total count of 500 cells and expressed as a mean and standard error of triplicate determinations.

Statistical analysis

Statistical analysis was performed using GraphPad software. The results are presented as mean ±SD or SEM of at least three independent experiments. Statistical significance was determined using two-tailed Student's t-test and *p* values of 0.05 and less were considered as statistically significant.

Results

miR-26a is down-regulated in breast stromal adipocytes derived from obese cancer-free females

The expression of miR-26a was first assessed in mature adipocytes derived from obese women (Obadi) and lean women (Leadi), which were used simultaneously at similar passages. To this end, total RNA was prepared and specific primers for *miR-26a* was utilized for amplification by quantitative RT-PCR (qRT-PCR) using U6 for normalization. Figure 1A shows low level of *miR-26a* in Obadi cells as compared with its level in Leadi cells.

miR-26a is up-regulated in old breast stromal adipocytes derived from obese cancer-free females

To study the effect of cellular aging on the level of miR-26a, Obadi cells were simultaneously cultured and serially passaged, and at population doubling (PD) PD2 and PD10, cells were analyzed. Figure 1B shows clear change in the morphology of Obadi-8 with passaging. Indeed, Obadi-8 cells at PD10 became flat, larger and highly vacuolated (Figure 1B). In addition, these cells stopped proliferating and showed lower levels of the replication marker Ki-67 as compared to their younger counterparts at PD2 (Figure 1B). To confirm the senescent status of Obadi-8 at PD10, we assessed the level of the senescence-associated (SA) beta-galactosidase (SA-β-Gal). Figure 1B shows that old Obadi-8 cells at PD10 express very high level of the senescence marker, which was almost undetectable in their younger counterparts at PD2.

Additionally, total RNA was prepared and specific primers for *miR-26a* was utilized for amplification by qRT–PCR using U6 for normalization. Figure 1C shows strong increase in the miR-26a level in Obadi cells at PD10, as compared with their younger counterparts at PD2. This indicates that the level of miR-26a is low in Obadi cells, as compared to Leadi cells, and its level increases with aging.

To investigate the possible implication of miR-26a in the Obadi aging, Obadi-1 cells at PD10 were transfected with either miR-26a inhibitor or mock as a control. Total RNA was prepared and specific primers for miR-26a was utilized for amplification by qRT–PCR using U6 for normalization. Leadi-2 cell (PD2), Obadi-1 (PD10), Obadi-1 (PD10)-ctrl were utilized as controls. Figure 2A shows that inhibition of miR-26a in old Obadi-1 cells decreased the expression of the mature forms of miR-26a.

Next, the proliferation and migration capabilities of these cells expressing miR-26a inhibitor were assessed using the xCelligence system. Leadi-2 cell (PD2), Obadi-1 (PD10), Obadi-1 (PD10)-ctrl were utilized as controls. Figure 2B shows that, while the proliferation and migration of young obadi-1 cells were higher than those of young Leadi-1 cells, their proliferation and migration capacities decreased upon aging. Interestingly, inhibition of miR-26a in the old Obadi cells resumes their capabilities and become similar to the young Obadi-1 cells.

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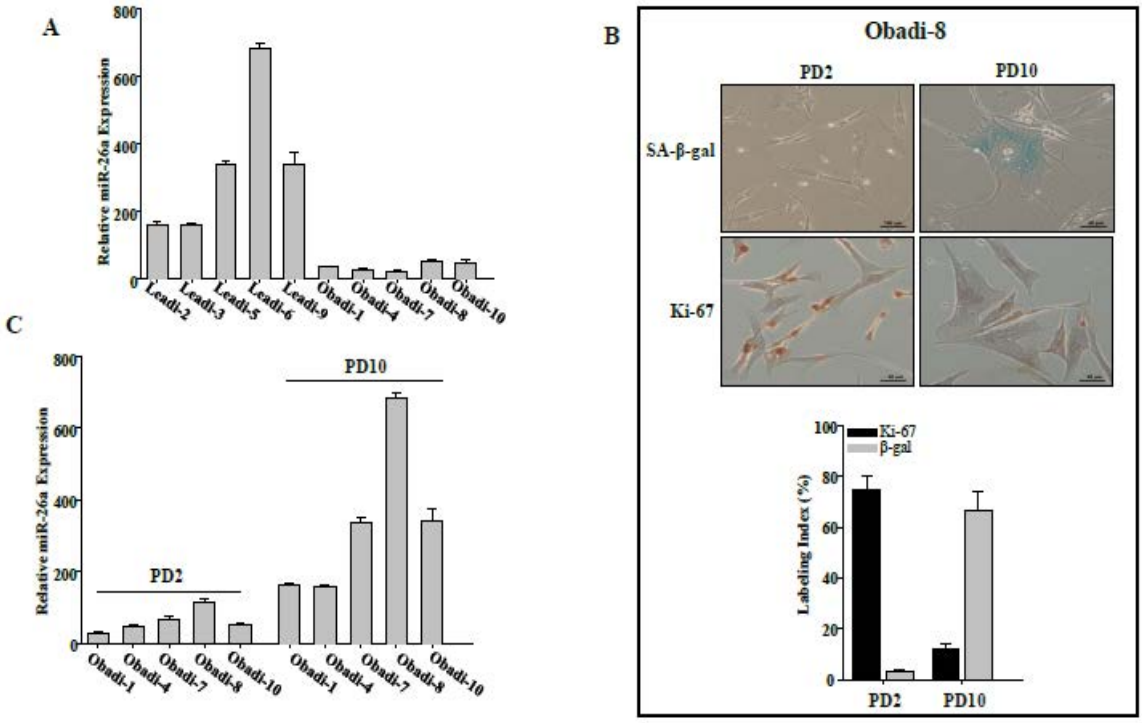


Figure 1: Breast adipocytes from obese females express low level of miR-26a. (A) Total RNA was prepared from the indicated adipocytes and utilized to assess the level of the miR-26a by qRT-PCR. Error bars represent means ± SD, values of 3 independent experiments. (B) Obadi-8 cells were sub-cultured, and then used at PD2 and PD10. SA-β-gal and Ki-67 staining for the indicated cells, scale bars represent 50 µm (Upper panel), Labeling index of SA-β-gal and Ki-67. (Lower panel). Error bars represent means ± SD, values of 3 independent experiments. (C) The indicated Obadi cells were sub-cultured and total RNA was then prepared from cells at PD2 and PD10 and utilized to assess the level of the miR-26a by qRT-PCR. Error bars represent means ± SD, values of 3 independent experiments.

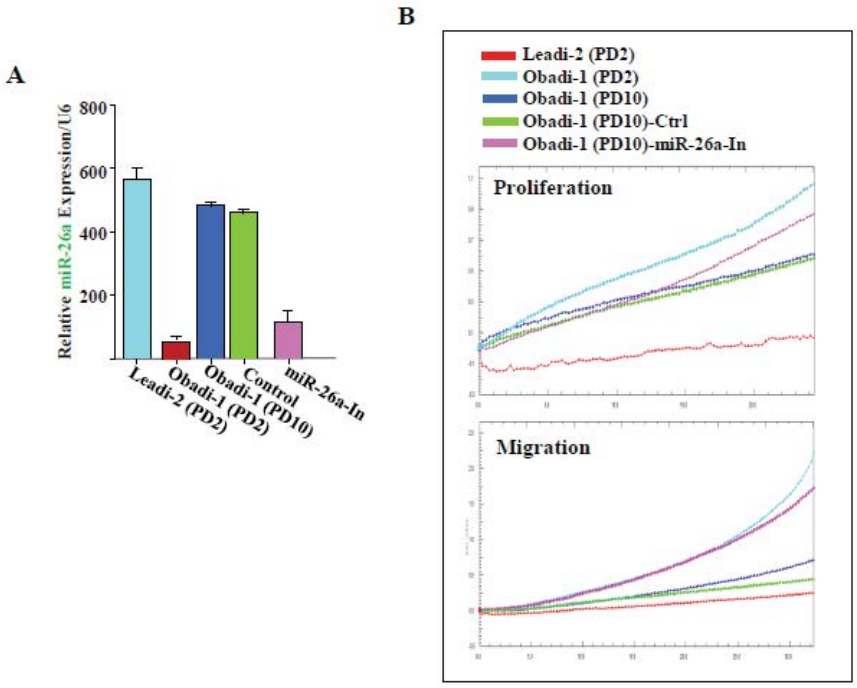


Figure 2: Inhibition of miR-26a in old breast adipocytes from obese females restore their proliferation and migration capabilities. (A) Obadi-1 cells at PD10 were transduced with miR-26a inhibitor or control plasmid. Total RNA was then prepared from PD2 and PD10 and utilized to assess the level of the miR-26a by qRT-PCR using U6 for normalization. Leadi-2 cell (PD2) and Obadi-1 (PD10) were utilized as controls. Error bars represent means ± SD, values of 3 independent experiments. (B) The proliferation and migration capabilities of the indicated cells were assessed using the RTCA-xCELLigence system (The graphs are representative of different experiments).

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miR-26a negatively regulates the expression of stemness markers via their 3'UTR

To further elucidate the molecular and functional link between miR-26a and aging of obadi cells, we first assessed the mRNA level of the stem markers including *KLF4, MYC, LIN28A* and *SOX2* by qRT–PCR. Figure 3A shows that the level of these transcripts is higher in Obadi cells as compared to the Leadi ones at similar passages. This indicates an obesity-related increment in the level of stem cell markers. Interestingly, their levels significantly decreased at PD10 as compared to PD2 (Figure 3B), indicating an age-related reduction in their levels.

Next, we sought to investigate whether miR-26a has a role in the regulation of these transcripts with aging in Obadi. To this end, we analyzed the miRNA databases, which showed that the *KLF4* 3'UTR contains one potential binding site for miR-26a located at bases 222 (mirSVR score= -0.1735). The *MYC* 3'UTR contains one potential binding site located at bases 206 for both miR-26a-1* and miR-26a-2* (mirSVR score= -0.1391). The *SOX2* 3'UTR contains one potential binding site for miR-26a located at bases 1001 (mirSVR score= -0.2000). The *LIN28A* 3'UTR contains 2 potential binding sites for miR-26a located at bases 240 (mirSVR score: -0.1115) and bases 1003 (mirSVR score: -0.0019) (Figure 3C).

Next, we evaluated the potential contribution of the miR-26a binding sites in the *KLF4, MYC, SOX2 and Lin28A* mRNA 3'UTR on the control of their expression. To this end, total RNA was prepared from Obadi-1-miR-26-In cells and their control counterparts and the level of *KLF4*, *MYC*, *SOX2* and *Lin28A* mRNAs was assessed by qRT-PCR. Figure 4A shows a clear increase in the levels of these transcripts upon miR-26a inhibition as compared to the control cells. This indicates a miR-26a-related reduction in the level of *KLF4*, *MYC*, *SOX2* and *LIN28A* in old Obadi cells.

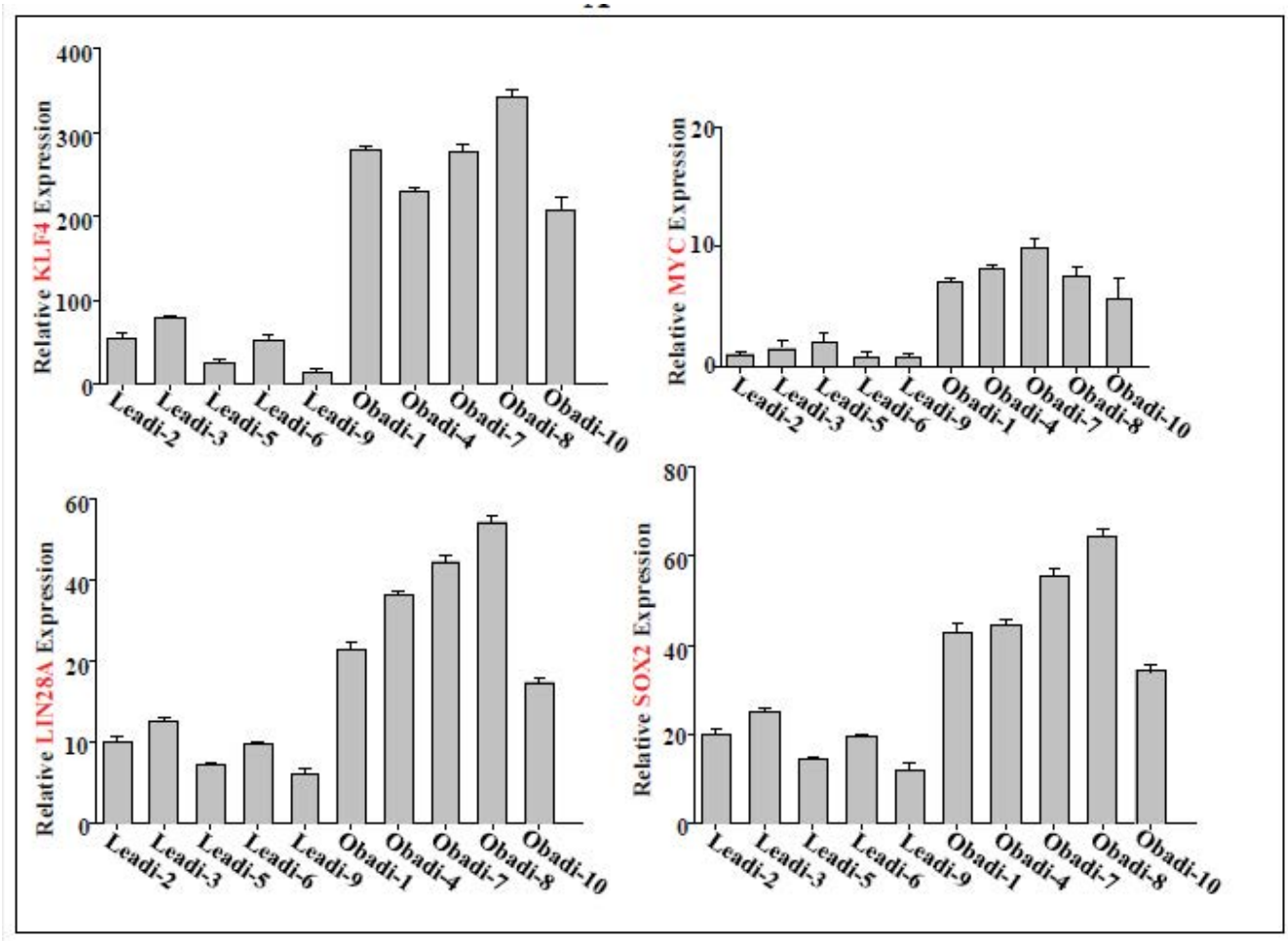
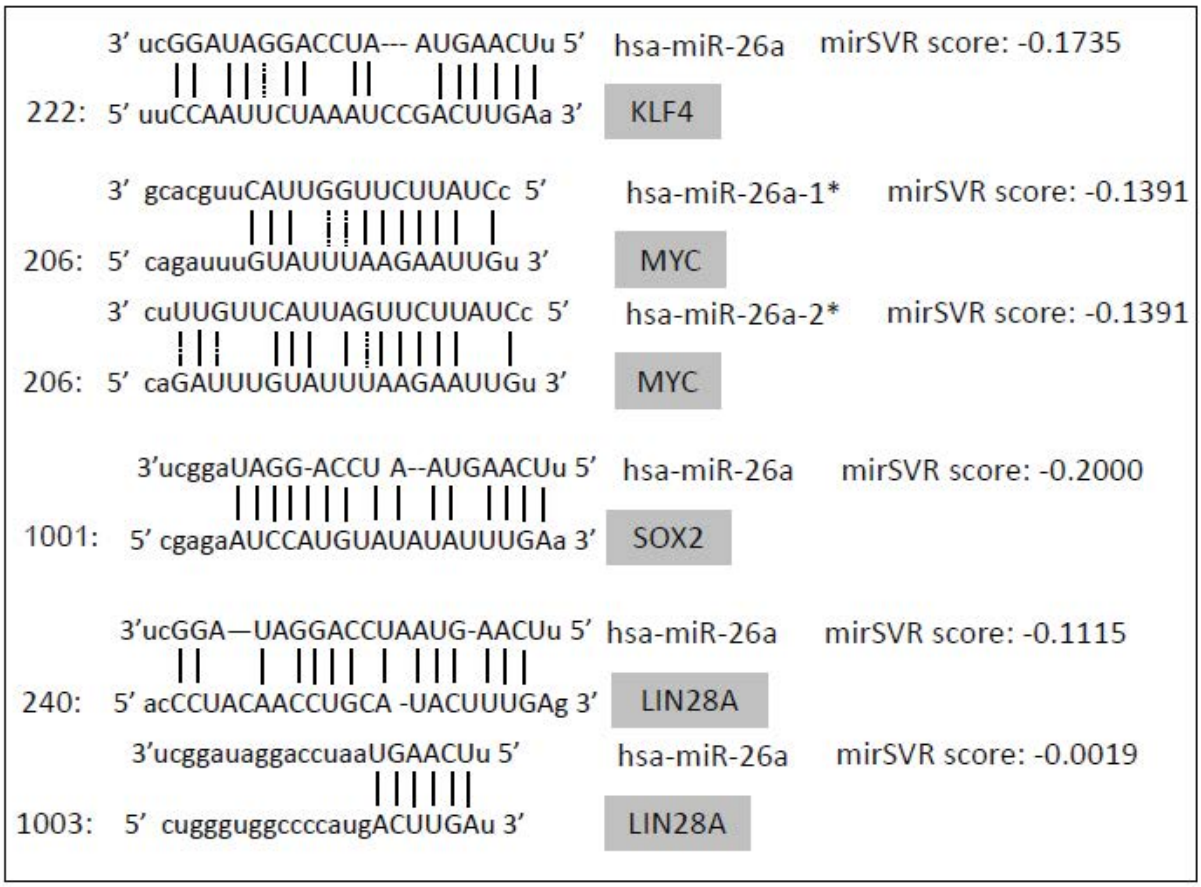


Figure 3: The expression level of stemness markers increased in obadi cells and decreased in an age-dependent manner. (A, B) Total RNA was prepared from the indicated cells and used to assess the level of the indicated transcripts by qRT-PCR. Error bars represent means ± SD, values of 3 different experiments.

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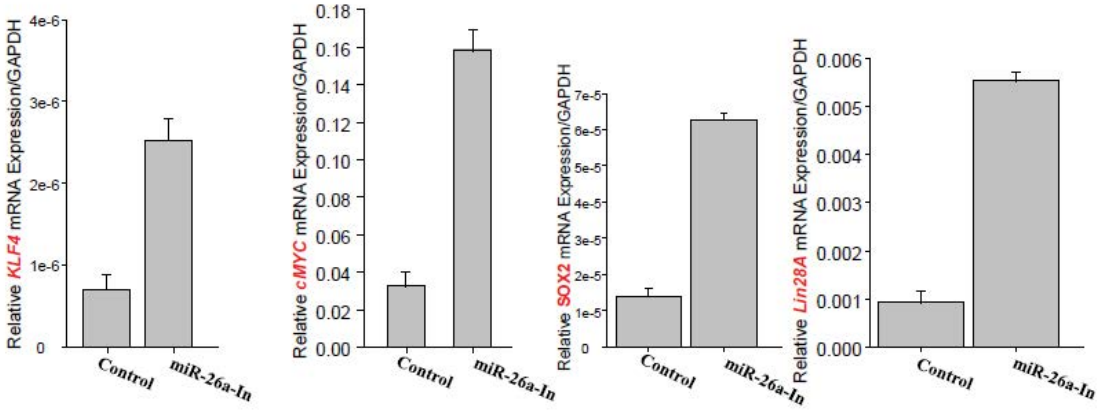


Figure 4: *miR-26a* control the Stem cell markers mRNA expression via their 3'UTR. (A) Sequence alignment of human miR-26a binding sites in the indicated 3'UTRs. (B) Total RNA was prepared from the indicated cells and used to assess the level of the indicated transcripts by qRT-PCR. Error bars represent means ± SD, values of 3 different experiments.

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Discussion

Cellular senescence within adipose tissue is increasingly recognized as a key driver and a central feature of obesity-associated dysfunction, contributing to impaired adipogenesis, chronic inflammation, and systemic metabolic disturbances [11,12]. The present study provides new insight into the regulatory role of miR-26a in human breast adipocytes and highlights its context-dependent modulation by both obesity and aging. Our data demonstrate that miR-26a expression is significantly reduced in breast adipocytes derived from obese women compared with those from lean individuals. This decrease may reflect an adaptive or compensatory response to the metabolically stressed microenvironment associated with obesity, which is characterized by chronic low-grade inflammation, altered adipokine secretion, and extracellular matrix remodeling [12]. In this setting, decreased miR-26a expression may function to preserve adipocyte viability and plasticity, potentially acting as a protective molecular mechanism aimed at maintaining tissue homeostasis despite adverse metabolic conditions. Given the central role of breast adipocytes in maintaining structural and paracrine support within the mammary niche [13], the down-regulation of miR-26a in obesity may represent an intrinsic attempt to sustain regenerative capacity and prevent premature cellular dysfunction. In contrast, aging was associated with a pronounced increase in miR-26a levels, accompanied by a significant reduction in adipocyte proliferative capacity and overall cellular fitness. This observation suggests that miR-26a plays a critical role in maintaining adipocyte health and regenerative potential over time. Aging is known to induce cellular senescence, mitochondrial dysfunction, and diminished responsiveness to regenerative cues in adipose tissue [14,15]. The observed upregulation of miR-26a may therefore contribute to these age-related alterations by impairing key pathways involved in self-renewal and proliferation. Importantly, the increase in the miR-26a expression correlated with reduced proliferation markers, supporting the hypothesis that miR-26a acts as a negative

regulator of adipocyte growth and turnover. These findings align with emerging evidence that microRNAs serve as central modulators of adipose tissue aging and stem cell exhaustion.

Mechanistically, our results indicate that the functional effects of miR-26a are mediated, at least in part, through regulation of core stemness-associated transcription factors, including *KLF4*, *MYC*, *SOX2*, and *LIN28A*. These factors constitute a well-established network governing cellular plasticity, self-renewal, and reprogramming capacity [18,19]. The negative association between miR-26a expression and the upregulation of these stem markers suggests that miR-26a may act upstream to sustain a progenitor-like state within breast adipocytes, thereby preserving their proliferative and regenerative competence. Increased miR-26a levels with aging may lead to suppression of this stemness network, resulting in diminished cellular renewal, increased susceptibility to senescence, and impaired tissue maintenance. Conversely, depletion of miR-26a expression appears to reactivate this transcriptional program, enhancing proliferative activity and potentially reversing aspects of age-related adipocyte decline. Such a mechanism is consistent with emerging paradigms in which reversal of senescence requires engagement of developmental or stemness pathways, rather than simple inhibition of cell cycle arrest [20]. The observed modulation of stemness markers supports a model in which miR-26a acts upstream of transcriptional networks governing cellular identity and regenerative potential.

These findings have important implications for adipose tissue biology in obesity. Senescent adipocytes and progenitors accumulate with increasing adiposity and age, leading to exhaustion of the regenerative pool and disruption of tissue homeostasis [21,22]. By restoring stemness features, miR-26a may contribute to replenishing functional adipocyte populations and improving tissue remodeling capacity. This raises the possibility that targeting miR-26a networks could complement or surpass existing strategies aimed at reducing inflammation or promoting adipogenesis alone [16,17].

Notably, the dual impact of miR-26a on senescence and stemness distinguishes it from interventions that target either process in isolation [23,24]. This integrative effect may be particularly relevant in the context of obesity, where impaired regeneration and persistent senescence coexist. Moreover, given the pleiotropic nature of microRNAs, miR-26a is likely to regulate a network of targets rather than a single pathway, potentially enabling coordinated remodeling of the adipose microenvironment.

In addition, these findings collectively support a model in which miR-26a functions as a central regulator of breast adipocyte health, linking metabolic status, aging, and stemness pathways. While obesity is associated with reduced miR-26a expression, possibly as a compensatory mechanism to counteract metabolic stress, aging leads to its progressive elevation, contributing to reduced adipocyte vitality and regenerative potential. This dual effect is particularly important, as it not only suppresses the detrimental aspects of senescence but also promotes regenerative capacity within adipose tissue. These findings position miR-26a at the intersection of senescence control and adipose tissue plasticity and contribute to a growing body of evidence positioning microRNAs as critical regulators of cellular aging and metabolic dysfunction [25,26]. This is particularly relevant in the context of obesity-related complications where dysfunctional adipose tissue contributes to insulin resistance, cardiovascular disease, and chronic inflammation [27-29]. The ability of miR-26a inhibition to enhance proliferation

and reactivate stemness markers represents a novel strategy for regenerative metabolic therapeutic potential. Modulation of the miR-26a–stemness axis may therefore represent a promising strategy for rejuvenating aging adipose tissue, improving tissue quality, and restoring regenerative function within the breast microenvironment.

However, several limitations should be acknowledged. First, the study is based on *in vitro* models derived from obese individuals, which, although physiologically relevant, do not fully recapitulate the complexity of *in vivo* adipose tissue architecture or systemic metabolic regulation. Second, while the modulation of stemness markers is evident, the precise downstream signaling pathways and direct gene targets of miR-26a require further validation. Future work integrating transcriptomic and proteomic profiling will be essential to resolve these mechanisms. Additionally, long-term effects and safety considerations of miR-26a-dependent senescence reversal strategies require careful evaluation, particularly in light of potential risks associated with uncontrolled cellular proliferation or dedifferentiation particularly in clinical settings. Therefore, future studies are warranted to further delineate the upstream regulators of miR-26a in adipocytes and to clarify its downstream molecular targets within the stemness network. In addition, investigating the paracrine effects of miR-26a-modulated adipocytes on surrounding epithelial and stromal cells will be essential to understanding its broader impact on breast tissue homeostasis and regeneration. Ultimately, therapeutic strategies aimed at abolishing miR-26a levels in aging adipose tissue may offer novel opportunities for enhancing tissue repair, combating age-related degeneration, and improving outcomes in regenerative and reconstructive applications, in addition to assessing the therapeutic potential of miR-26a delivery in models of obesity and metabolic disease. The development of targeted delivery platforms will be critical to enable tissue-specific modulation while minimizing off-target effects. It will also be important to determine whether similar mechanisms operate across different adipose depots and patient populations, including comparisons between lean and obese individuals.

In summary, our findings identify miR-26a as a regulator of adipocyte senescence and cellular plasticity, capable of promoting a shift toward a more regenerative state through modulation of stemness pathways. These results provide a conceptual framework for targeting microRNA-mediated networks to restore adipose tissue function in obesity and related metabolic disorders.

Conflicts of interest : None declared

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Author contributions

Huda H Al-Khalaf and Alaa Alhussain contributed equally to this work. Alaa AlHussain and Ibrahim O Alanazi (Methodology, data analysis and investigation), Huda H Al-Khalaf, Rafa Almeer, and Abdelilah Aboussekhra (Review and editing), and Huda H. Alkhalaf (Funding acquisition).

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