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Desregulação de miRNAs em células-tronco mesenquimais derivadas de fluxo menstrual de mulheres com e sem endometriose

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RESUMO

ZUCCO OLIVEIRA, R. Desregulação de miRNAs em células-tronco mesenquimais derivadas de fluxo menstrual de mulheres com e sem endometriose. 2021. 62 p. Dissertação (Mestrado) – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 2021.

A relação fundamental entre a teoria de Sampson e a presença de células-tronco mesenquimais no fluxo menstrual (MenSCs), bem como, as mudanças nos processos regulatórios pós-transcricionais como atores na etiopatogenia da endometriose, são pouco compreendidas. Nenhum estudo até o momento investigou o desequilíbrio de miRNAs em MenSCs relacionados à doença. Assim, por meio da literatura e de análises *in silico*, selecionamos quatro miRNAs previstos como reguladores de *EGR1, SNAI1, NR4A1, NR4A2, ID1, LAMC3* e *FOSB* envolvidos nas vias de apoptose, angiogênese, resposta a hormônios esteróides, migração, diferenciação e proliferação celular. Deste modo, um estudo caso-controle foi realizado com MenSCs de mulheres com e sem endometriose (dez amostras por grupo). Cruzando as informações obtidas nos bancos de dados STRING, PubMed, miRPathDB, miRWalk e DIANA TOOLS, optamos por explorar a expressão dos miRNAs miR-21-5p, miR-100-5p, miR-143-3p e miR-200b-3p por RT-qPCR. Encontramos uma regulação positiva do miR-200b-3p em MenSCs de endometriose (P = 0,0207), com uma alteração de 7,93 vezes (razão entre as médias geométricas) em comparação com o controle. A superexpressão do miR-200b tem sido associada ao aumento da proliferação celular, *stemness* e ao processo de transição mesenquimal-epitelial acentuado no endométrio eutópico de mulheres com endometriose. Acreditamos que o miR-200b-3p desregulado pode estabelecer alterações primárias nas MenSCs, e assim, favorecendo a implantação de tecido no local ectópico.

Palavras-chave: miR-200b-3p, endometriose, MenSCs, sangue menstrual, RT-qPCR.

ABSTRACT

ZUCCO OLIVEIRA, R. Dysregulation of miRNAs in menstrual blood-derived mesenchymal stem cells from healthy and endometriosis women. 2021. 62 p. Masters dissertation - Ribeirao Preto Medical School, University of São Paulo, 2021.

The key relationship between Sampson's theory and the presence of mesenchymal stem cells in the menstrual flow (MenSCs), as well as the changes in post-transcriptional regulatory processes as actors in the etiopathogenesis of endometriosis, are poorly understood. No study to date has investigated the imbalance of miRNAs in MenSCs related to the disease. Thus, through literature and *in silico* analyses, we selected four predicted miRNAs as regulators of *EGR1, SNAI1, NR4A1, NR4A2, ID1, LAMC3,* and *FOSB* involved in pathways of apoptosis, angiogenesis, response to steroid hormones, migration, differentiation, and cell proliferation. Therefore, a case-control study was conducted with MenSCs of women with and without endometriosis (ten samples per group). Crossing information obtained from the STRING, PubMed, miRPathDB, miRWalk, and DIANA TOOLS databases, we chose to explore the expression of miR-21-5p, miR-100-5p, miR-143-3p, and miR-200b-3p by RT-qPCR. We found an upregulation of the miR-200b-3p in endometriosis MenSCs (P=0.0207), with a 7.93-fold change (ratio of geometric means) compared to control. Overexpression of miR-200b has been associated with increased cell proliferation, stemness and accentuated mesenchymal-epithelial transition process in eutopic endometrium of endometriosis. We believe that dysregulated miR-200b-3p may establish primary changes in the MenSCs, thus favoring tissue implantation at the ectopic site.

Keywords: miR-200b-3p, endometriosis, MenSCs, menstrual blood, RT-qPCR.

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LISTA DE ABREVIATURAS MAIS UTILIZADAS

DEGs: genes diferencialmente expressos

EGR1: early growth response 1

eMSCs – células-tronco mesenquimais endometriais

EMT: transição epitelial-mesenquimal

FDR: taxas de falsas descobertas

FOSB: FosB proto-oncogene, AP-1transcription factor subunit

ID1: inhibitor of DNA binding 1, HLH protein

LAMC3: laminin subunit gamma 3

RT-qPCR: transcrição reversa – reação em cadeia da polimerase quantitativa

RQ: quantificação relativa

MenSCs: células-tronco mesenquimais derivadas do fluxo menstrual

MET: transição mesenquimal-epitelial

NR4A1: nuclear receptor subfamily 4 group A member 1

NR4A2: nuclear receptor subfamily 4 group A member 2

RIN: número de integridade de RNA

SFs: células fibroblásticas do estroma

SNAI1: snail family transcriptional repressor 1

SUMÁRIO

INTRODUÇÃO GERAL

Endometriose é uma doença ginecológica benigna, estrogênio-dependente, resistente a progesterona, inflamatória crônica, que afeta mundialmente de 5 a 10% das mulheres em idade reprodutiva (ZONDERVAN et al., 2020). O quadro clínico é heterogêneo, sendo as lesões identificadas em 7% de mulheres assintomáticas submetidas à ligadura tubária, 50% de adolescentes com dismenorreia descontrolada, 5% a 24% de mulheres com dor acíclica persistente e 10% a 40% de mulheres inférteis (CRAMER; MISSMER, 2002; EISENBERG et al., 2018). A doença é caracterizada histologicamente pela presença de implantes de tecido endometrial (glândulas e/ou estroma) fora da cavidade uterina (tecido ectópico), mais frequentemente localizados na pélvis (AGARWAL; SUBRAMANIAN, 2010). É considerada um problema de saúde pública devido ao impacto na saúde física e psicológica, além do impacto socioeconômico para tratamento, diagnóstico e monitoramento (SIMOENS et al., 2012; CULLEY et al., 2013).

A presença de tecido endometrial ectópico e as manifestações clínicas dessa doença são resultado da combinação de vários processos biológicos aberrantes, como: 1) menstruação retrógrada, disseminação linfovascular e/ou metaplasia em mulheres com resposta imunológica imprópria, 2) predisposição genética e/ou eventos epigenéticos que favorecem o estabelecimento de lesões endometrióticas, 3) endométrio (eutópico e ectópico) composto por células com mecanismos moleculares alterados, e 4) microambiente hormonal, inflamatório e pró-oxidativo alterado (SOURIAL et al., 2014).

A origem do endométrio ectópico tem sido objeto de muita investigação. Uma das teorias mais consensuais para a etiologia da endometriose é a de que haveria aderência de tecido endometrial decorrente de fluxo menstrual retrógrado, que carrega células viáveis capazes de permitir sua implantação e desenvolvimento ao atingir a cavidade peritoneal e órgãos adjacentes (SAMPSON, 1927). Embora seja uma teoria fundamentada, apresenta controvérsias, uma vez que 90% das mulheres apresentam menstruação retrógrada, mas apenas

5% a 10% delas desenvolvem a doença (HALME et al., 1984). Com isso, diversas hipóteses e teorias complementares foram elaboradas (SOURIAL et a., 2014). Nesse sentido, nas últimas duas décadas a hipótese de que há participação de células progenitoras ou células-tronco oriundas do endométrio (eMSCs - *endometrial mesenchymal stem cells*), e consequentemente presentes no fluxo menstrual (chamadas MenSC), no desenvolvimento e manutenção das lesões ectópicas (SASSON; TAYLOR, 2008; GARGETT et.al., 2016; LIU et al., 2020a)

A primeira menção na comunidade científica sobre a possibilidade da existência de células-tronco no endométrio foi por Prianishnikov (1979). Essa ideia surgiu devido à estrutura em duas camadas do endométrio, a funcional e a basal, que são sujeitas a mais de 400 ciclos de crescimento, diferenciação e descamação durante o período reprodutivo da mulher (MAYBIN; CRITCHLEY, 2015). Devido a propriedade de renovação cíclica do endométrio e as propriedades já conhecidas de células-tronco (autorrenovação, clonogenicidade e capacidade de diferenciação), essa hipótese tem sido reforçada (PRIANISHNIKOV et al., 1979 (PADYKULA et al., 1989; TANAKA et al., 2003). Entretanto, apenas em 2004, Chan e colaboradores comprovaram experimentalmente a existência de células com capacidade clonogênica no endométrio humano, demonstrando a existência de populações de células progenitoras epiteliais e estromais. Outras evidências da presença dessas células no tecido endometrial surgiram de estudos com um subtipo celular, chamado *side population*, que apresenta características semelhantes a células-tronco (MASUDA et al., 2015). É estimado que a *side population* compreende menos de 5% das células endometriais, e são mais abundantes nas fases proliferativa e menstrual do ciclo menstrual (KATO et al., 2007; MASUDA et al., 2010). Além disso, em experimentos de diferenciação celular, *side population* epitelial e estromal apresentam capacidade adipogênica e osteogênica (CERVELLÓ et al., 2010).

Logo após a comprovação da existência no endométrio de células-tronco mesenquimais (eMSCs) e epiteliais, alguns estudos demonstraram também que em lesões de endometriose

(FORTE et al., 2009; CHAN et al., 2011; KAO et al., 2011; SAVILOVA et al., 2017; LIU et al., 2020b) e no fluxo menstrual possuíam células-tronco mesenquimais/estromais (MENG et al., 2007; HIDA et al., 2008; MUSINA et al., 2008; PATEL et al., 2008), denominadas respectivamente de eMSCs e MenSCs dependendo de onde são isoladas. O primeiro estudo que extraiu MenSCs de pacientes com endometriose e relacionou seus achados à possível participação destas células na etiopatogenia da endometriose foi em 2014. Neste estudo, é relatada maior capacidade proliferativa e invasiva, aumento da expressão gênica e proteica dos imunorreguladores indoleamina 2,3-dioxigenase 1 (IDO1) e ciclooxigenase 2 (COX-2), além de maiores secreções das moléculas inflamatórias interferon-γ, interleucina (IL)-10 e proteína quimioatraente de monócitos (MCP)-1 (NIKOO et al., 2014).

As MenSCs têm aparência fibroblastóide, com alto potencial proliferativo e clonogênico, mantêm um cariótipo estável por até 68 duplicações celulares e são capazes de se diferenciar em 9 linhas celulares distintas (cardiomiocíticas, epiteliais respiratórias, neurocíticas, miocíticas, endoteliais, pancreáticas, hepáticas, adipocíticas e osteogênica) (MENG et al., 2007; MUSINA et al., 2008; PATEL et al., 2008). Semelhante às eMSCs, MenSCs preenchem os critérios definidos pela *International Society for Cellular Therapy* em relação ao perfil imunofenotípico (DOMINICI et al., 2006; PATEL et al., 2008). As diferenças descritas até agora entre esses dois tipos de células referem-se ao crescimento *in vitro*, onde eMSCs, mas não MenSCs, requerem fatores de crescimento e matriz extracelular para crescer (ALLICKSON; XIANG, 2012), e a forma de isolamento em sistema de cultura celular. As eMSCs geralmente são isoladas pela coexpressão dos marcadores perivasculares $CD146^{+}/CD140b^{+}$ (MCAM ou *melanoma cell adhesion molecule*/ PDGFRB ou *platelet derived growth factor receptor beta*) ou pelo marcador único $SUSD2^{+}$ (*sush domain containing 2*) (SCHWAB; GARGETT, 2007; MASUDA et al., 2012), e as MenSCs comumente são isoladas por capacidade de adesão a plástico (CHEN et al., 2019). Contudo, estes dados

comparativos ainda são restritos e a concordância na literatura de um marcador específico para separar as eMSCs de células fibroblásticas do estroma (SFs) ainda é algo discutível (TEMPEST et al., 2018).

Concomitante aos achados destas células progenitoras, avanços nas técnicas de alto rendimento têm permitido o estudo amplo de diversas doenças complexas, como a endometriose (SHENDURE; JI, 2008; MOROZOVA et al., 2009; POLI-NETO et al., 2020). Neste sentido, alguns estudos de expressão gênica diferencial direcionados a entender melhor o comportamento do transcriptoma em SFs e progenitoras mesenquimais (eMSCs e MenSCs) foram realizados utilizando diferentes propostas experimentais em endométrio, lesão endometriótica (SPITEZER et. al., 2012; BARRAGAN et al., 2016; REKKER et al., 2017; LIU et al., 2020b), e em células do fluxo menstrual (WARREN et al., 2018; MEOLA et al., 2021).

Os estudos focados nos componentes celulares presentes no fluxo menstrual são até o momento muito escassos, embora sejam uma interessante fonte de investigação para a origem e desenvolvimento da doença. Warren e colaboradores (2018) encontraram 31 genes diferencialmente expressos (DEGs) ao comparar uma população de células SFs (caracterizadas como $CD45^-/CD326^-/CD31^-/CD90^+/CD105^+/CD73^+/CD140b^+$) obtidas de fluxo menstrual de mulheres com e sem endometriose (n=3 por grupo). Neste trabalho os autores apresentaram uma menor capacidade de decidualização (induzida em cultivo) destas células no grupo endometriose e destacaram os genes *IGFBP1*, *SST*, *PRL*, *BCL2L11*, *WNT5A* e *FOXO1* como importantes nesse processo. Em condições de cultivo diferentes, nosso grupo analisou o transcriptoma de MenSCs de mulheres com endometriose avançada (n=10) e sem endometriose (n=10), e destacamos 42 transcritos diferencialmente expressos (FDR < 0,1) que enriquecem vias relacionadas à transição mesenquimal-epitelial, diferenciação celular, neuroangiogênese, migração celular e sinalização Notch (MEOLA et al., 2021). Ademais, comparando nossos

achados com duas meta-análises recentes de nosso grupo, os genes *EGR1*, *SNAI1*, *NR4A1*, *NR4A2*, *ID1*, *LAMC3* e *FOSB* são frequentemente superexpressos na condição de endometriose e, portanto, são alvos interessantes para futuros estudos (POLI-NETO et al., 2020; 2021; MEOLA et al., 2021). Contudo a participação do endométrio como carreador de alterações primárias não é totalmente esclarecida.

Tendo em vista que a expressão gênica é modulada a nível transcricional, pós-transcricional, translacional e por modificações pós-traducionais, é de extrema importância avaliar os diferentes estágios de regulação para a compreensão da endometriose (POPE; MEDZHITOV, 2018). Além disso, estudos revelam uma discordância entre os níveis de transcritos e suas proteínas correspondentes, que pode estar associada ao processo fisiopatológico da endometriose e a participação de mecanismos regulatórios pós-transcricionais (WREN et al., 2007; FASSBENDER et al., 2010; 2012).

MicroRNAs ou miRNAs são uma classe de pequenos RNAs não codificantes de aproximadamente 22 nucleotídeos, são reguladores da expressão gênica pós-transcricional e atuam por dois mecanismos principais: induzindo a maquinaria celular a degradar o RNA mensageiro alvo ou impedindo o processo de tradução dele (HA; KIM, 2014). Os miRNAs desempenham papéis essenciais em quase todos os processos biológicos do corpo humano, mantendo a homeostase celular e, devido a esse vasto papel, expressões aberrantes dessas moléculas já foram relacionadas a diversos mecanismos patológicos (VISHNOI; RANI, 2017; MATSUYAMA; SUZUKI, 2019), incluindo a endometriose (PAN et al., 2007; OHLSSON-TEAGUE et al., 2009; CHEGINI, 2010; FILIGHEDDU et al., 2010; HAWKINS et al., 2011; SANTAMARIA; TAYLOR, 2014; BRAZA-BOILS et al., 2015; SAARE et al., 2017; AGRAWAL et al., 2018). Nos últimos anos, o papel central dos miRNAs no controle da proliferação, sobrevivência, autorrenovação e diferenciação de várias células-tronco tem sido discutido, principalmente em processos de reprogramação celular (MATHIEU; RUOHOLA-

BAKER, 2013). Em um estudo recentemente concluído por nosso grupo, avaliamos a expressão dos genes centrais responsáveis pela biogênese de miRNAs (*DROSHA, DGCR8, XPO5, DICER* e *AGO1-4*) em MenSCs. Uma regulação negativa de *DROSHA* foi encontrada em mulheres com endometriose (MD 0,48 ± 0,14), sendo aproximadamente 2 vezes menos expressa que no grupo controle (MD 0,97 ± 0,35) (CRESSONI et al., 2021), achado esse que sugere comprometimento da síntese de miRNAs e um possível impacto na expressão global dessas moléculas regulatórias.

Assim, acreditamos que as MenSCs de mulheres com endometriose, que caem na cavidade peritoneal, devido ao fluxo menstrual retrógrado, possuem expressão de miRNAs alterada, o que contribui para o estabelecimento de tecido endometrial ectópico.

OBJETIVOS

1) Predizer por associações funcionais dos genes *EGR1, SNAI1, NR4A1, NR4A2, ID1, LAMC3* e *FOSB*, as vias enriquecidas que possivelmente têm envolvimento com a etiopatogenia da endometriose;

2) Buscar e selecionar quatro miRNAs preditos como reguladores dos genes *EGR1, SNAI1, NR4A1, NR4A2, ID1, LAMC3* e *FOSB* através da literatura e por análises *in sílico*;

3) Explorar a expressão diferencial dos miRNAs selecionados por RT-qPCR em MenSCs de mulheres com e sem endometriose.

MANUSCRITO

Periódico alvo Reproductive Sciences

Title: Overexpression of miR-200b-3p in menstrual blood-derived mesenchymal stem cells from endometriosis women.

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Abstract

The key relationship between Sampson's theory and the presence of mesenchymal stem cells in the menstrual flow (MenSCs), as well as the changes in post-transcriptional regulatory processes as actors in the etiopathogenesis of endometriosis, are poorly understood. No study to date has investigated the imbalance of miRNAs in MenSCs related to the disease. Thus, through literature and *in silico* analyses, we selected four predicted miRNAs as regulators of *EGR1, SNAI1, NR4A1, NR4A2, ID1, LAMC3,* and *FOSB* involved in pathways of apoptosis, angiogenesis, response to steroid hormones, migration, differentiation, and cell proliferation. Therefore, a case-control study was conducted with MenSCs of women with and without endometriosis (ten samples per group). Crossing information obtained from the STRING, PubMed, miRPathDB, miRWalk, and DIANA TOOLS databases, we chose to explore the expression of miR-21-5p, miR-100-5p, miR-143-3p, and miR-200b-3p by RT-qPCR. We found an upregulation of the miR-200b-3p in endometriosis MenSCs (P=0.0207), with a 7.93-fold change (ratio of geometric means) compared to control. Overexpression of miR-200b has been associated with increased cell proliferation, stemness and accentuated mesenchymal-epithelial transition process in eutopic endometrium of endometriosis. We believe that dysregulated miR-200b-3p may establish primary changes in the MenSCs, thus favoring tissue implantation at the ectopic site.

Keywords: miR-200b-3p, endometriosis, MenSCs, menstrual blood, RT-qPCR.

Introduction

Endometriosis is likely the result of a combination of several aberrant biological processes, which include: 1) retrograde menstruation, lymphovascular dissemination, and/or metaplasia in women with impaired immune function; 2) cells with anomalous molecular mechanisms composing the eutopic and ectopic endometrium; 3) genetic predisposition and/or epigenetic for ectopic implants development; 4) the presence of altered progenitor cells originating from the endometrium and desquamated in the menstrual flow acting in the development and maintenance of ectopic lesions; and 5) a receptive, hormonal, inflammatory and pro-oxidant peritoneal microenvironment [1–3].

In fact, the exact origin of the ectopic tissue is still uncertain. In this scenario, Sampson's theory [4] is one of the most consensual ideas for the appearance of ectopic tissue, and that brings us the insight that the cellular components present in menstruation are important players in the development of endometriosis [5]. Among these cellular elements, mesenchymal stem cells originating from the endometrium (eMSCs) and consequently present in the menstrual flow (MenSCs) have gained prominence in the development and maintenance of ectopic lesions [6–8].

However, the participation of the eutopic endometrium as a carrier of primary alterations is yet to be fully understood. In two recent meta-analyses, our group has described important particularities that are evidenced in the endometrium of women with endometriosis, highlighting gene pathways directly related to immune surveillance, stem cell self-renewal and mesenchymal-epithelial transition [9, 10]. In parallel with these pursuits, we have analyzed the transcriptome by RNA-seq of MenSCs obtained from women with and without endometriosis (unpublished data). The *EGR1, SNAI1, NR4A1, NR4A2, ID1, LAMC3,* and *FOSB* are frequently overexpressed in the endometriosis condition in these three studies, and therefore, they are interesting targets for investigations.

Nevertheless, some studies have revealed inconsistencies between the expression levels of transcripts and their protein counterparts as connected with the pathophysiological process of endometriosis and with post-transcriptional regulatory mechanisms [11–13]. Thus, a strong role of miRNAs with several endometriosis outcomes has been investigated [14–22].

MicroRNAs or miRNAs are a class of small RNAs of approximately 22 non-coding nucleotides (ncRNA - non coding RNAs) that are regulators of post-transcriptional gene expression by two main mechanisms: inducing the cellular machinery to degrade the target mRNA or by preventing the process of mRNA translation by 30% of the genes [19]. In recent years, the central role of miRNAs in controlling the proliferation, survival, self-renewal and differentiation of several stem cells has been discussed, especially in cell reprogramming processes [23]. In endometriosis, several triggering mechanisms of ectopic lesions can be regulated, such as cell migration and proliferation, evasion of apoptosis and the immune system, adhesion and invasion to the peritoneum and adjacent organs [17].

No study to date has investigated the imbalance of miRNAs in menstrual blood mesenchymal stem/stromal cells related to the endometriosis process. Here, we describe through literature and *in silico* analyses the search and selection of four predicted miRNAs as regulators of *EGR1, SNAI1, NR4A1, NR4A2, ID1, LAMC3,* and *FOSB* involved in pathways of apoptosis, angiogenesis, response to steroid hormones, migration, differentiation, and cell proliferation. In addition, we explore the expressions of these target miRNAs using reverse transcription-quantitative PCR (RT-qPCR). We believe that deregulated microRNAs that have functional associations with the above genes may establish primary changes in the MenSCs, favoring tissue implantation at the ectopic site.

Materials and Methods

Ethical approval

This is a case-control study approved by The Research Ethics Committee of the University Hospital of the Ribeirao Preto Medical School (HCRP 3644/2019). The MenSC used in this work were collected from November 2014 to December 2016 following the ethics guidelines established by the Declaration of Helsinki (HCRP 15227/2012). They have been transferred to a biorepository of the Human Reproduction Section at the Department of Gynecology and Obstetrics of the Ribeirao Preto Medical School (HCRP 3644/2019). All participants have provided written informed consent.

Patients and Eligibility criteria

Twenty women's clinical data and cell samples were obtained from a biorepository (HCRP 3644/2019, approved 04/06/2019) (**Table 1**). We included women between 18 and 40 years old with eumenorrheic menstrual cycles (intervals from 24 to 32 days ± three days; 2 to 7 days of duration) who had not used hormone therapy at least three months before sample collection, nor had smoking and drinking habits. Additionally, they did not have any systemic disease, tumor, endocrinopathy, cardiovascular or rheumatological diseases. The case group included ten women at the mean age of 36.0 (SD±2.9), with a histological and laparoscopic diagnosis of endometriosis classified as III or IV according to the ASRM [24]. We selected patients who had undergone surgical treatment an average of 4 years (SD ± 1.3) before collection and presenting diagnostic imaging suggestive of endometrioma as evidence of active endometriosis in the pelvic cavity at the time of collection. For the healthy control group, we selected ten fertile women with a mean age of 35 years (SD ± 3.3), at least with two children,

no history of recurrent abortion nor clinical and laparoscopic diagnosis of endometriosis verified during laparoscopy for tubal ligation.

Samples collection, MenSCs isolation and characterization

The protocols described in this section were conducted from November 2014 to December 2016 before storing the MenSCs in the biorepository. These experiments were performed with cells in the third cell culture passage (P3). Briefly, the menstrual blood was collected through a silicone menstrual cup (Inciclo, Brazil) previously sterilized with gamma radiation and inserted into the vagina for 3 hours during the 2nd, third or fourth day of the menstrual cycle. The method used for MenSCs isolation was earlier reported by Meng et al. [25] with modifications [26]. Twenty-three markers immunophenotypically characterized the cells following the manufacturer's instructions on the FACSCalibur flow cytometer (BD Biosciences, USA) using CellQuest™ version 4.0 software (BD Biosciences, USA). The composition of the panel markers is available in **Table 2**. Still meeting the minimal criteria for multipotent mesenchymal stem cells (MSCs) as defined by the International Society for Cellular Therapy [27], the cells were differentiated into adipocytes and osteocytes using the conditions of the differentiation medium described by Musina et al. [28] with modifications [26]. Part of the MenSCs in P3 were fixed in RNAlater Stabilization Solution (#AM7020, ThermoFisher, USA) for 24 hours before freezing at -80 °C for the expression experiments (carried out in January 2021).

In silico selection of target miRNAs

For the prediction of functional associations and enriched pathways of *EGR1*, *SNAI1*, *NR4A1*, *NR4A2*, *ID1*, *LAMC3* and *FOSB*, we used the STRING database, v.11, mean confidence score 0.400 (Gene Ontology Enrichment Analysis) (https://string-db.org/) [29], and

then we correlated these signaling pathways with the etiopathogenesis of endometriosis. In addition, a search in the PubMed database (https://pubmed.ncbi.nlm.nih.gov/) was performed using the terms "*endometriosis" AND "miRNA*", "*endometrium" AND "miRNA*", "*endometrial mesenchymal stem cells" AND "miRNAs expression*" to select miRNAs with altered functions and expressions in this disease. Based on the cross information obtained from the STRING and PubMed databases, we used miRPathDB v.2.0 (Gene Ontology Enrichment Analysis) (https://mpd.bioinf.uni-sb.de/) [30] to select miRNAs that act in pathways common with target genes. Furthermore, we searched for miRNAs predicted as regulators of at least one of the genes through the databases miRWalk v.2.0 (http://mirwalk.umm.uni-heidelberg.de/) [31] and DIANA TOOLS (TarBase v.8) (http://diana.imis.athena-innovation.gr/) [32].

Total RNA isolation and miRNA cDNA synthesis

According to the manufacturer's instructions, the total RNA was extracted using the AllPrep DNA/RNA/miRNA Universal Kit (#80224, Qiagen, USA). To remove any contamination with genomic DNA, we treated total RNA with DNA-free Kit DNase Treatment and Removal Reagents (#AM1906, Invitrogen, USA). The RNA integrity number (RIN) was measured with the Agilent RNA 6000 Nano Kit (# 5067-1511, Agilent, USA) using the Agilent 2100 Bioanalyzer (Agilent, USA). We included in the study only samples with RIN $\geq$ 8 (see Supplementary Information **Figure S1**). Total RNA was quantified with the Qubit RNA BR Assay Kit (#Q10210, Invitrogen, USA) using the Qubit 2.0 Fluorometer (Invitrogen, USA). Fifty nanograms of total RNA were applied for miRNA cDNA synthesis using the TaqMan Advanced miRNA cDNA Synthesis Kit (#A28007, Applied Biosystems, USA) based on the manufacturer's workflow. The final template was diluted 1:10 with nuclease-free water and stored at -20°C until use.

RT-qPCR

Relative quantification (RQ) for target miRNAs was assessed using the TaqMan Advanced miRNA assays (≠A25576, Applied Biosystems, USA): hsa-miR-143-3p (477912_mir), has-miR-100-5p (478224_mir), has-miR-21-5p (477975_mir), and has-miR-200b-3p (477963_mir). It was chosen hsa-miR-191-5p (477952_mir), hsa-miR-361-5p (478056_mir), and hsa-186-5p (477940_mir) as endogenous control miRNAs. The manufacturer states that the amplification efficiency of the assays is very close to 100%, so it is not necessary to measure the efficiency [33]. We used the ViiA 7 Real-time PCR System (Applied Biosystems, USA) to perform the RT-qPCR amplification. The reaction was prepared with 5.0 µL of TaqMan Fast Advanced Master Mix (2X) (# 4444557, Applied Biosystems, USA), 0.5 µL of hydrolysis probe (20X) (≠A25576, Applied Biosystems, USA), 2.5 µL of cDNA (dilution 1: 10), and nuclease-free water to complete the final volume to 10 µl. The samples were run in three technical replicates with the maximum difference between Ct values up to 0.3 cycles. The cycles were as follows: one cycle at 95 °C for 20 seconds, 40 cycles of 1 second at 95 °C, and 20 seconds at 60 °C. No amplification was detected in the template control containing RNAse-free water instead of cDNA or mRNA. The RQ was determined for each analyzed target according to the $2^{-\Delta\Delta Ct}$ method [34] available in the free comprehensive Thermo Fisher Connect Platform (https://www.thermofisher.com/br/en/home/digital-science/thermo-fisher-connect.html). We used a cDNA pool of MenSCs controls as a reference sample and the hsa-miR-191-5p, hsa-miR-361-5p, and hsa-186-5p as endogenous controls to normalize the Ct raw data.

Statistics

Exploratory data analysis was performed using descriptive statistics. Clinical variables and immunophenotypic markers were compared between groups using the Mann-Whitney test

(independent samples). Gene expression variables (RQ) were transformed to log 10 and were compared using the independent samples t-Test. Analyzes were performed in SAS software, version 9.4. Data were presented as the median, minimum and maximum values, geometric mean, and the boxplot was used for graphically depicting groups. For interpretation, a p-value < 0.05 was considered significant.

Results

Clinical Variables and Characterization of MenSCs

No significant differences were observed regarding the patients' clinical data **(Table 1)** or cell characterization. The MenSCs agree with the minimal criteria for multipotent MSC [27] as they can differentiate into adipocytes and osteocytes (data not shown) and express the previously described immunophenotypic profile for MenSCs [28, 35, 36] **(Table 2).**

In silico selection of hsa-miR-21-5p, hsa-miR-100-5p, hsa-miR-143-3p e hsa-miR-200b-3p

The predicted association networks for the *EGR1, SNAI1, NR4A1, NR4A2, ID1, LAMC3* and *FOSB* targets using the STRING database can be explored at https://version-11-5.string-db.org/cgi/network?networkId=b8Gr6hLc5Dqo. However, we only selected pathways that are strongly involved in the pathophysiology of endometriosis, such as apoptosis, angiogenesis, steroid hormone response, cell migration, differentiation and proliferation [2, 37]. After extensive research in the literature, we chose hsa-miR-143-3p, hsa-miR-21-5p, hsa-miR-200b-3p and hsa-miR-100-5p, which had dysregulated expression in endometriosis **(Table 3)** and that act in pathways common to target genes according to the miRPathDB database **(Table 4)**. We certify that hsa-miR-21-5p (*EGR1, NR4A2*), hsa-miR-100-5p (*SNAI1, ID1*), hsa-miR-143-

3p (*EGR1, FOBS, LAMC3, NR4A1, NR4A2*) and hsa-miR-200b-3p (*SNAI1, FOSB, LAMC3*) are predicted miRNA-target interactions.

Overexpression of miR-200b-3p

As we can see in **Figure 1**, there is no significant difference between the three miRNAs (miR-143-3p, miR-21-5p, and miR-100-5p). Nevertheless, we found an upregulation of the miR-200b-3p in endometriosis MenSCs (P=0.0207), with a 7.93-fold change (ratio of geometric means) compared to control.

Discussion

To the best of our knowledge, the imbalance of miRNAs in menstrual blood mesenchymal stem/stromal cells as a player in the development of endometriosis has not yet been evidenced. In this work, we describe through the literature and *in silico* analyzes that miRNAs miR-143-3p, miR-100-5p, miR-21-5p and miR-200b-3p, regulators of *EGR1*, *SNAI1*, *NR4A1*, *NR4A2*, *ID1*, *LAMC3*, and *FOSB*, are involved in apoptosis, angiogenesis, steroid hormone response, migration, differentiation and cell proliferation pathways. We also explored the expressions of these miRNAs and found that miR-200b-3p is up-regulated in MenSCs from patients with endometriosis compared to the control group, and this imbalance may play an important role in the etiopathogenesis of endometriosis.

It is widely accepted that miRNAs play important roles in gynecological diseases, including endometriosis [19, 50]. The miRNA-200 family is composed of five members, cluster I (miR-200b, -200a and -429) located on chromosome 1, and cluster II (miR-200c and -141) located on chromosome 12 [51]. Its members are expressed in epithelial cells and are considered important regulators of the epithelial-mesenchymal transition (EMT) [52]. Specifically, miR-200b-3p is one of the most frequently reported miRNAs to be differentially

expressed in endometriosis [21] and, also, alterations in its expression are commonly related to the development of carcinomas [53]. It regulates several cellular processes such as cell motility, proliferation, migration, and differentiation [53] that may participate in the etiopathogenesis of endometriosis. It is also involved in angiogenesis through the regulation of VEGF (vascular endothelial growth factor) and EGFR2 (epidermal growth factor receptor), which could contribute to the establishment and maintenance of ectopic lesions [39].

In the context of endometriosis, miR-200b expression is reported to be decreased in endometriotic lesions [15, 17, 18, 39, 45–47] and increased in stromal [48] and mesenchymal stem cells from the endometrium of women with endometriosis [49], which strengthens our findings. Therefore, we believe that this dysregulation in MenSCs in the endometriosis condition confers a genetic programming that favors differentiation into epithelial cells, accentuating the mesenchymal-epithelial transition (MET). Since "EMT and MET are not binary processes" [54], these appear to be important for the formation of ectopic lesions [55].

The balance between cell proliferation and differentiation is necessary for the proper functioning of stem cells and that can be regulated by miRNAs. It is believed that this balance is unregulated in several diseases, including endometriosis, which results in alterations in both cell function and fate [49]. As previously reported, the overexpression of miR-200b can favor the colonization of ectopic sites by increasing the proliferation of stem cells and their stemness properties [48, 49, 56], which is largely dependent on the MET [57]. In addition, it has been reported that high levels of miR200b-3p result in the blocking of *ZEB1* (E-box-binding transcription factors 1) and, thus, possibly reverse EMT and induce MET [58]. The transcriptional repressors of E-cadherin, *ZEB1* and *ZEB2*, if inhibited by miR-200b-3p, will lead to an increased expression of this epithelial marker. [59]. Furthermore, the higher rates of cell proliferation and an enhanced side population phenotype are associated with upregulation of the transcription factor *KLF4* upon increased levels of miR-200b. These appear to be related

to a mechanism by which endometriosis acquires tumorigenic characteristics although maintaining a benign state [56, 60].

The greatest strength of our data is that we used strict inclusion criteria in search of biological groups as homogeneous as possible and, thus, more suitable to highlight biological effects. Despite this advantage, this study did possess some limitations. Our small sample size cannot evidence the presence or absence of alterations in the expression of miRNAs hsa-miR-21-5p, hsa-miR-100-5p, hsa-miR-143-3p. Furthermore, expression analysis after culture models must be carefully interpreted, as they can mask the real cellular environment, making it difficult to extrapolate the same results to *in vivo* systems [61]. Thus, future functional studies may clarify the role of miR-200b-3p in MenSCs and the importance of this primary change for endometriosis.

In summary, we describe for the first time that MenSCs from women with endometriosis have upregulated miR-200b-3p. This overexpression, which has been associated with increased cell proliferation, stemness, and accentuated mesenchymal-epithelial transition process in eutopic endometrium of endometriosis, may favor ectopic colonization sites when these cells are carried by menstrual reflux.

Authors' contributions

RZO applied the experiments, analyzed and interpreted the data, and drafted the article. FOB, ACLC, and LBCP performed a literature review, *in silico* analysis, and interpreted the data. The CCP assisted in the RT-qPCR experiments and the preparation of the laboratory experiments. PAT and MDO helped in cell culture, immunophenotyping, and cell differentiation. OBPN, JCRS, and RAF helped design the study, select the samples and review the manuscript. JM designed and coordinated the study, supervised the experiments, and edited the manuscript. All authors read and approved the final manuscript.

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Table captions

Table 1. Clinical data of women with and without endometriosis.

Table 2. Immunophenotypic profile of MenSCs using the FASCalibur flow cytometer.

Table 3: Dysregulation of target miRNAs in the endometriosis.

Table 4: Regulated signaling pathways in common by genes and miRNAs.

Table 1: Clinical data of women with and without endometriosis.

Control	Age (years)	BMI (kg/m2)	Menstrual cycle day	Endometriosis	Age (years)	BMI (kg/m2)	Menstrual cycle day	ASRM Classification	USG/ endometrioma	Symptoms
C10	33	23.5	4	E2	39	26.7	3	IV	RO	Inf/pain
C17	39	26.4	3	E3	31	28.1	3	III	LO	Inf/pain
C22	31	29	2	E4	34	25.7	3	IV	RO/LO	Inf/pain
C29	36	28.1	2	E7	33	29.4	4	III	RO	Inf/pain
C31	37	23.2	2	E8	39	24.6	4	IV	LO	Inf/pain
C32	35	25.2	3	E9	34	20.0	2	III	LO	Inf/pain
C34	28	29.2	3	E11	39	23.19	2	III	RO	Inf/pain
C35	37	22.4	3	E12	37	25.51	2	III	LO	Inf/pain
C38	35	26.4	2	E13	39	21.6	2	IV	RO/LO	Inf/pain
C39	39	26.9	3	E27	35	27	2	III	RO	Inf/pain
Median	35.0	26.4	3.0	**Median**	36.0	25.6	2.5			
p-value	0.590	0.570	0.901							

Note. BMI: Body Mass Index; USG: Ultrasonography; LO: Left Ovary; RO: Right Ovary; Inf: Infertility. Statistics: Mann-Whitney test (independent samples) and significance p-value < 0.05.

Table 2: Immunophenotypic profile of MenSCs using the FACSCalibur flow cytometer.

Marker	Catalogue Number	Supplier	Control (%) N=10	Endometriosis (%) N=10	Expression Classification	p-value
CD45	340040	BD Simultest	0.07 (0.0; 1.3)	0.04 (0; 0.7)	negative (<2%)	0.208
CD14	555397	BD Pharmingen	0.10 (0.0; 1.6)	0.12 (0.0; 1.0)	negative (<2%)	0.969
CD34	340430	BD Biosciences	0.01 (0.0; 0.5)	0.28 (0.0; 1.3)	negative (<2%)	0.104
CD31	555445	BD Biosciences	0.15 (0.0; 1.0)	0.06 (0.0; 0.7)	negative (<2%)	0.567
EpCAM	324204	BioLegend	0.00 (0.0; 1.0)	0.03 (0.0; 1.9)	negative (<2%)	0.155
CD106	551146	BD Pharmingen	0.31 (0.0; 1.3)	0.03 (0.0; 0.9)	negative (<2%)	0.127
HLA-DR	555813	BD Pharmingen	0.05 (0.0; 0.3)	0.00 (0.0; 0.2)	negative (<2%)	0.148
STRO-1	MAB1038	R&D Systems	0.31 (0.0; 5.0)	0.19 (0.0; 0.2)	negative (<2%)	0.969
CD51/CD61	555505	BD Pharmingen	6.36 (0.8; 46.0)	7.85 (1.4; 31.3)	low (2 - 20%)	0.578
CD146	560846	BD Pharmingen	27.70 (10.5; 58.8)	20.9 (12.0; 63.2)	medium (20 - 60%)	0.435
SUSD2	130-106-326	MACS	39.10 (12.0; 70.2)	29.0 (13.2; 68.5)	medium (20 - 60%)	0.684
CD140b	558821	BD Pharmingen	56.00 (15; 80.7)	40.40 (19.0; 67.6)	medium (20 - 60%)	0.279
CD166	559263	BD Pharmingen	61.50 (42.1; 87.2)	54.70 (22.8; 77.3)	medium (20 - 60%)	0.344

HLA-ABC	555552	BD Pharmingen	72.00 (45.5; 92.7)	64.30 (47.5; 83.0)	high (>60%)	0.630
CD44	555478	BD Pharmingen	74.59 (51.4; 87.4)	75.48 (60.5; 87.0)	high (>60%)	0.970
CD49e	555617	BD Pharmingen	82.41 (62.1; 88.9)	76.04 (53.2; 87.6)	high (>60%)	0.190
CD54	555511	BD Pharmingen	87.57 (73.6; 91.9)	82.22 (65.0; 89.4)	high (>60%)	0.123
CD13	557454	BD Pharmingen	88.75 (69.6; 94.5)	88.84 (68.8; 93.5)	high (>60%)	0.850
CD105	560819	BD Pharmingen	71.65 (29.5; 91.49)	67.79 (44.8; 90.8)	high (>60%)	0.909
CD73	550257	BD Pharmingen	86.56 (74.6; 92.5)	84.45 (71.9; 90.4)	high (>60%)	0.472
CD90	555596	BD Pharmingen	93.95 (91.2; 97.9)	93.44 (87.89; 95.8)	high (>60%)	0.190
CD29	559883	BD Pharmingen	95.44 (89.9; 96.4)	94.46 (89.2; 95.5)	high (>60%)	0.140
Control γ1/2a	340041	BD Simultest	--------	--------	negative isotype control	

Note: Data presented as median (minimum; maximum). Statistics: Mann-Whitney test (independent samples) and significance p-value < 0.05.

Table 3: Dysregulation of target miRNAs in endometriosis.

miRNA	Comparison (tissue)	Expression level	Reference
hsa-miR-21-5p	Endometrioma x Eutopic endometrium	↑	Ramón et al. [38]
	Lesion x Control endometrium	↑	Yang et al. [39]
	eMSC treated with endometriosis serum	↑	Abdel-Rasheed et al. [40]
	Lesion (peritoneal and deep) x Eutopic endometrium	↓	Haikalis et al. [41]
	Endometrioma x Peritoneal lesion	↓	Haikalis et al. [41]
hsa-miR-100-5p	Lesion x Eutopic endometrium	↑	Teague et al. [15]
	Endometrioma x Eutopic endometrium	↑	Filigheddu et al. [17]
	Endometrioma x Control endometrium	↑	Hawkins et al. [18]
	Stromal cells of endometrioma x Stromal cells of control endometrium (leiomyoma)	↑	Abe et al. [42]
	Lesion x Control endometrium	↑	Wright et al. [43]
hsa-miR-143-3p	Lesion x Eutopic endometrium	↑	Teague et al. [15]
	Endometrioma x Eutopic endometrium	↑	Filigheddu et al. [17]
	Lesion x Eutopic endometrium	↑	Zheng et al. [44]
	eMSC treated with endometriosis serum	↑	Abdel-Rasheed et al. [40]
hsa-miR-200b-3p	Lesion x Control endometrium	↓	Teague et al. [15]
	Endometrioma x Eutopic endometrium	↓	Filigheddu et al. [17]
	Endometrioma x Control endometrium	↓	Hawkins et al. [18]

Lesion x Control endometrium	↓	Braza-Boils et al. [45]
Peritoneal lesion x Healthy peritoneum	↑	Saare et al. [46]
Stromal cells x Epithelial cells (both in control endometrium)	↑	Saare et al. [46]
Lesion x Eutopic endometrium	↓	Shi et al. [47]
Lesion x Control endometrium	↓	Yang et al. [39]
Eutopic endometrium x Control endometrium (sorting for stromal cells)	↑	Logan et al. [48]
eMSC (eutopic endometrium) x eMSC (control endometrium)	↑	Mashayekhi et al. [49]

Note: Expression levels refer to the first tissue in the comparisons. Eutopic endometrium: women with endometriosis; Control endometrium: healthy women. eMSC: endometrial mesenchymal stem cells. ↓: downregulated. ↑: upregulated.

Table 4: Signaling pathways regulated in common by the genes and miRNAs.

Genes	**Signaling pathways**	**miRNAS**
EGR1, SNAI1, NR4A2, ID1	Apoptosis	hsa-miR- 21-5p hsa-miR-100-5p hsa-miR143-3p hsa-miR-200b-3p
EGR1, ID1, NR4A1	Angiogenesis	hsa-miR- 21-5p hsa-miR143-3p hsa-miR-200b-3p
SNAI1, NR4A2, ID1, NR4A1	Cell migration	hsa-miR- 21-5p hsa-miR-100-5p hsa-miR143-3p hsa-miR-200b-3p
FOSB, NR4A2, NR4A1	Steroid hormone response	hsa-miR- 21-5p hsa-miR-100-5p hsa-miR143-3p hsa-miR-200b-3p
EGR1, SNAI1, NR4A2, LAMC3, ID1, NR4A1	Cell differentiation	hsa-miR- 21-5p hsa-miR143-3p hsa-miR-200b-3p
ID1, NR4A1	Cell proliferation	hsa-miR- 21-5p hsa-miR-200b-3p

Note: Data obtained by cross information between STRING and miRPathDB databases.

Figure captions

Figure 1: Gene expression profiles of miRNAs in MenSCs of healthy women (C) and with endometriosis (E) using RT-qPCR methodology**.** The horizontal line inside the box represents the median. Whiskers represents minimum [Q1-(1.5*IQR)] and maximum [Q3 + (1.5*IQR)] values, excluding outlier values (displayed as separate points). IQR: interquartile range. The box-plot was created in MedCalc statistical software v.20 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020). ▪ geometric mean. * p-value < 0.05.

Supplementary information accompanies this paper

Figure S1: Total RNA quality control. RNA Integrity Number (RIN) was carried out using Agilent 2100 Expert B.02.07.SI532.

Figure 1

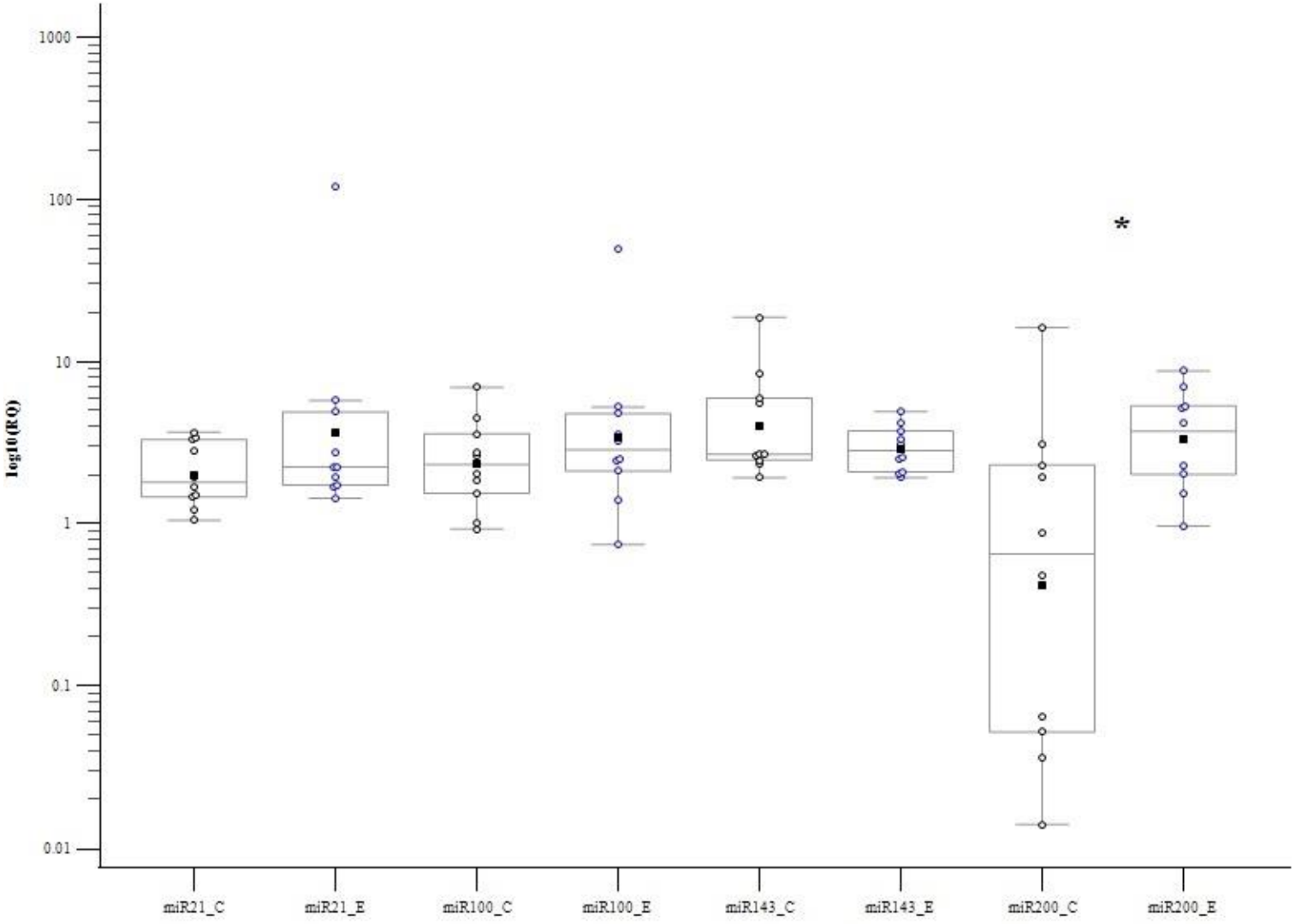
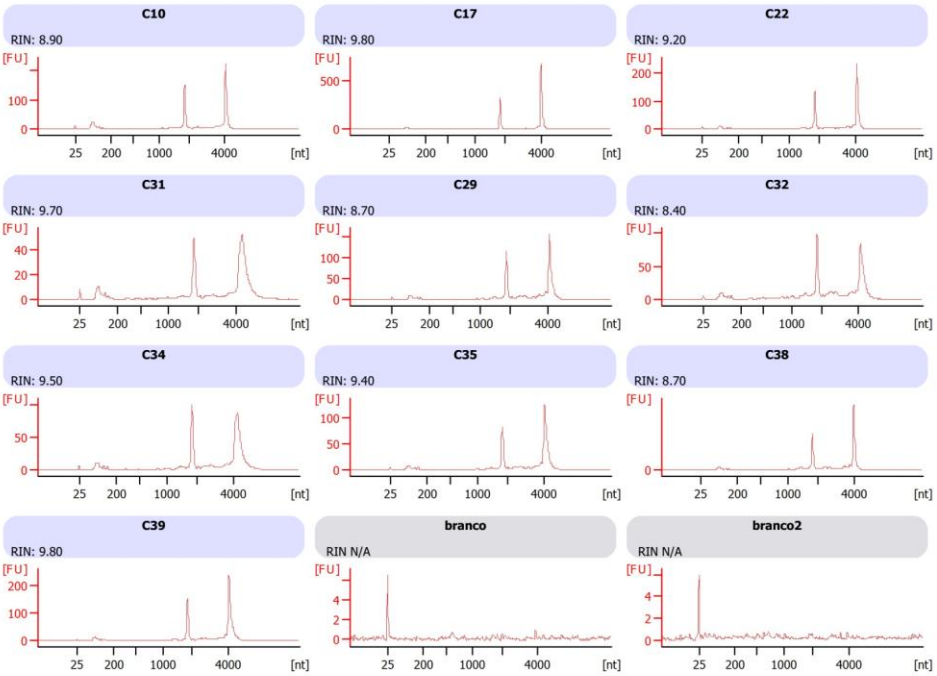
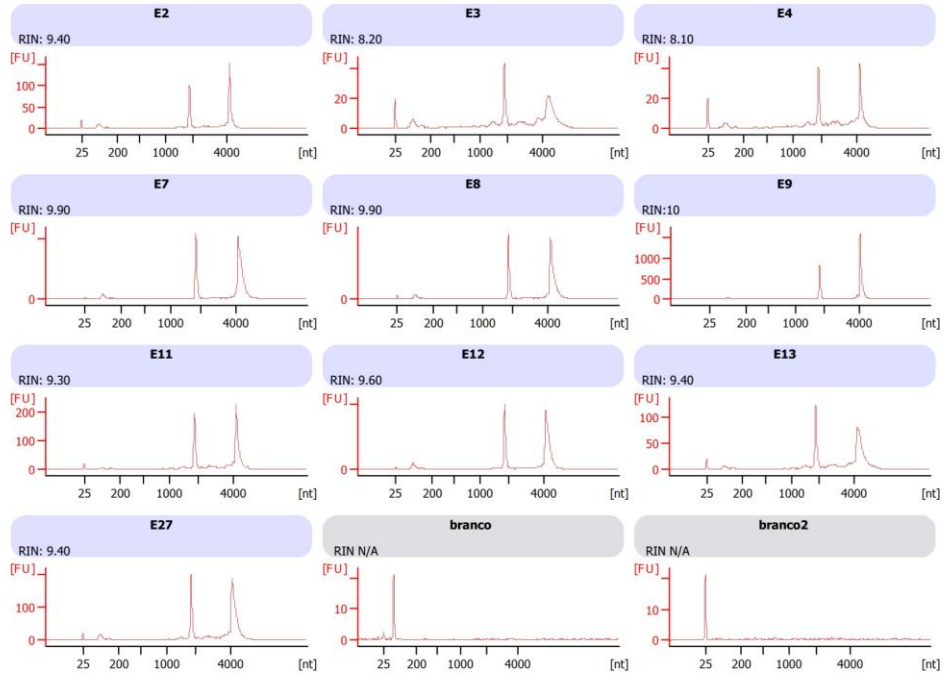

1000
100
10
1
0.1
0.01
log10(RQ)
*
miR21_C
miR21_E
miR100_C
miR100_E
miR143_C
miR143_E
miR200_C
miR200_E

Figure S1

Control



Endometriosis



CONCLUSÕES

1) As vias de sinalização de apoptose, angiogênese, resposta a hormônios esteróides, migração, diferenciação e proliferação celular foram enriquecidas para os genes *EGR1, SNAI1, NR4A1, NR4A2, ID1, LAMC3* e *FOSB* e são reguladas em comuns pelos quatro miRNAs alvos selecionados;

2) Os miRNAs miR-21-5p, miR-100-5p, miR-143-3p e miR-200b-3p foram selecionados por participarem de vias em comum com os genes alvos descritos acima e por estarem relacionados na literatura com a endometriose;

3) As predições de interações entre miRNAs-genes alvos foram: miR-21-5p (*EGR1, NR4A2*), miR-100-5p (*SNAI1, ID1*), miR-143-3p (*EGR1, FOBS, LAMC3, NR4A1, NR4A2*) e miR-200b-3p (*SNAI1, FOSB, LAMC3*);

4) Nossa casuística permitiu evidenciar variações significativas na expressão do miR-200b-3p.

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ANEXOS

ANEXO 1 – PROTOCOLOS DETALHADOS DO CULTIVO CELULAR

COLETA E ISOLAMENTO DAS CÉLULAS

O fluxo menstrual foi coletado entre o segundo e quarto dia após o início do ciclo menstrual, em um coletor menstrual de silicone (InCiclo, Brasil) esterilizado previamente com radiação gamma. Após a higienização das pacientes com sabonete líquido antisséptico a base de 2% de digluconato de clorexidina, o coletor foi introduzido na vagina por 2hs. O procedimento para isolamento das células mononucleares (CMN) baseia-se na separação por centrifugação em gradiente de *Ficoll-Hypaque* de densidade 1077 g/mL.

Brevemente, 1 a 5 mL fluxo menstrual foi transferido para um tubo falcon contendo Antibiotic Antimycotic Solution 10x (cat 15240-062, GIBCO, Life Technologies, EUA), ACD 10% (Citrato, Ácido cítrico e Dextrose) (JP Indústria Farmacêutica, SA) e completou-se o volume para 30 mL com PBS (1x) (cat P-3813, Sigma-Aldrich, St Louis, MO, EUA). Entre o momento da coleta e os passos seguintes, a amostra foi armazenada à 4°C por no máximo 4hs. Em seguida, foi transferida para uma sala estéril e então gentilmente e de forma contínua foi acrescentado 13 mL de *Ficoll-Paque*^{TM} *Plus* (cat 71-7167-00AG, Histopaque-1077, GE HealthCare BioSciences, Uppsala, Suécia). Esta solução foi então centrifugada a 800g por 30 min à temperatura de 22°C sem adicionar sistema de frenagem.

Após este processo, o anel esbranquiçado característico das CMN presentes na interface do sistema plasma/*Ficoll-paque* foi coletado com o auxílio de uma pipeta por meio de movimentos circulares e transferido para um novo tubo. Novamente foi acrescentado PBS 1x contendo ACD 10% e Antibiotic antimycotic Solution 1x (acrescido de penicilina e streptomicina de forma que suas concentrações finais atingissem 3x na solução final) completando o volume para 40 mL. As células então foram centrifugadas a 450g por 10 minutos a 22°C e sem freios. Esse processo foi realizado por duas vezes. Após as lavagens, o sobrenadante foi descartado e conforme a dimensão do botão celular foi adicionado 1-5 mL de meio de cultura *α-Minimum Essential Medium* (α-MEM) (cat 11900-016, GIBCO, Life Technologies, EUA) suplementado com 15% de SBF (cat SH30071.03, Fetal Bovine Serum Standard - HyClone™, Logan, UT, EUA), 100 U/mL penicilina e 100µg/mL streptomicina (cat 15140-122, GIBCO BRL, Gaithersburg, MD, EUA), 10mM Hepes (cat H4034, Sigma-Aldrich, St Louis, MO, EUA) e 20mM bicarbonato de sódio (cat 56297, Sigma-Aldrich, St Louis, MO, EUA) para contagem da suspensão celular no equipamento BC-2800 Auto Hematology

Analyzer (Mindray Medical International Limited, EUA). Após a contagem, células mononucleares foram semeadas em garrafas para expansão.

EXPANSÃO DAS CÉLULAS

A concentração de CMN plaqueadas variou de 1,6 x 10^5 a 2,0 x 10^7. As células foram expandidas em frascos T-25 ou T-75 (Corning, Cambridge, MA, USA) contendo 10 a 15 mL de meio α-MEM suplementado conforme descrição acima e cultivadas na incubadora *steril-cult 200* (Forma scientific-Thermopharma) com 85% de umidade a 37°C com 5% de CO_2. Após 5 a 7 dias, dependendo do número de células aderidas à garrafa, foi trocado todo o meio e consequentemente houve a remoção das células não aderentes. Posteriormente, o meio foi trocado mais uma vez após 3 dias. O tempo de cultura variou de 14 a 16 dias até que a confluência de 80 a 90% fosse atingida e a primeira tripsinização ocorresse para que as células fossem colhidas. O cultivo celular foi monitorado por microscopia de contraste de fase (Microscópio, Olympus, IX71) semanalmente.

No protocolo de tripsinização foram realizadas duas lavagens das células aderentes com 10 mL de PBS 1x, seguido da adição de solução de Tripsina-EDTA na concentração de 5mg/mL (0,05% tripsina e 0,53 mM de EDTA) (GIBCO™, Life Technologies, EUA) por 5 minutos a 37°C. Após a centrifugação à 450g por 10 minutos a 4°C, o pellet celular foi ressuspendido em 3 mL de α-MEM + 15% de SBF para contagem celular e as células replaqueadas em frascos de cultura de 75 cm^2 (Corning, Cambridge, MA, USA), o cultivo celular durou em média 6 dias (primeira passagem – P1).

Na segunda passagem (P2), foi feito o repique de aproximadamente 1x10^5 de células e o restante foi congelado em SBF com 10% Dimetilsulfóxido (DMSO, Sigma-Aldrich, St Louis, MO, EUA) na concentração de 1 x 10^6 células/mL em crio-tubos de congelamento (Greiner Bio-one, Frickenhausen, Alemanha) e armazenadas em nitrogênio líquido.

Quando necessário, as células foram descongeladas em banho Maria a 37ºC, ressuspensas em meio RPMI (cat 23400-013, GIBCO™, Life Technologies, EUA) com 5% de SBF e centrifugadas por 10 minutos a 328*g*. A seguir foram lavadas em PBS 1x, ressuspensas em α-MEM com 15% SBF e destinadas ao experimento previsto após terem sido contadas no equipamento BC-2800 Auto Hematology Analyzer.

CITOMETRIA DE FLUXO

As análises de imunofenotipagem foram realizadas para as amostras na P3 utilizando-se o citômetro de fluxo FASCalibur (BD, San Jose, CA, USA) e o software *CELLQuest*^{TM} (BD, San Jose, CA, USA). Foram analisados 10.000 eventos celulares para cada marcação imunofenotípica e os resultados foram gerados em gráficos de histograma para imunomarcação e em "Dot Plot" para definição do tamanho/granularidade (FSC/SSC) interna.

O protocolo de imunomarcação descrito a seguir será realizado para todos os marcadores citados na **Tabela 2** pois são todos marcadores de membrana. As suspensões celulares de 1 x 10^5/100µL de células diluídas em PBS 1x foram colocadas em tubos de poliestireno 12 x 75mm (Falcon, EUA) com 5 µL dos diferentes anticorpos monoclonais marcados com fluorocrômos específicos e incubados no escuro à temperatura ambiente por 15 minutos. A seguir, após terem sido acrescentados 2 mL de PBS 1x em cada tubo para lavagem das amostras, estas foram centrifugadas à 300g por 3 minutos e o sobrenadante descartado. Neste momento apenas para o marcador STRO-1 de imunomarcação indireta, acrescentou-se 3 µL do anticorpo secundário IgM de cabra anti-camundongo marcado com FITC e incubou-se por 15min no escuro à temperatura ambiente. Em seguida, novamente todos os marcadores foram lavados com PBS 1x e centrifugados. Cada botão celular foi ressuspenso em 200 µL de PBS 1x e analisado no citômetro.

DIFERENCIAÇÃO EM ADIPÓCITOS E OSTEÓCITOS

Após os protocolos de diferenciação descritos abaixo, as células foram avaliadas sob microscopia de luz, utilizando o miscroscópio Axioskop 2 *plus* (Carl Zeis, Alemanha). As imagens foram capturadas com a câmera digital Axiocam (Carl Zeis, Alemanha) e analisadas como auxílio do software AxioVision (Carl Zeis, Alemanha).

As células foram diferenciadas em adipócitos e osteócitos seguindo as condições do meio de diferenciação descrito por Musina et al. 2008. Usamos 4 x 10^4 células / mL para induzir a diferenciação e 5 x 103 células/mL para controle. As células foram cultivadas em placas GREINER CELLSTAR de 24 poços (# M8812, Merck, EUA) com lamínulas Deckgläser (# 100013, Knittel, Alemanha). As diferenciações celulares foram induzidas 24 a 48 horas após o início do cultivo e foram realizadas por 15 a 20 dias para diferenciação adipogênica e 31 dias para diferenciação osteogênica.

Após 24 – 48hs de cultivo, todo o meio de cultura foi removido e, a seguir, foi adicionado 1 mL de meio de indução:

Adipócitos - [α-MEM 10% SBF, suplementado com 1 µM de dexametasona (Decadron injetável, Prodome, Campinas, SP, Brasil), 2,5 mg/mL de insulina (Sigma-Aldrich, St. Louis, MO, EUA) e 100 µM de indometacina (Sigma-Aldrich, St. Louis, MO, EUA)] ou somente o meio α-MEM 7,5% SFB nos poços controles. As placas foram reincubadas em estufa úmida a 37ºC com 5% de CO2 e metade do meio foi trocado a cada 3-4 dias durante 15-20 dias.

Após a indução da diferenciação adipogênica, os vacúolos de gordura foram corados usando o corante Oil red O (cat O0625, Sigma). Após a retirada do meio de indução, as células foram lavadas com PBS 1x e foram fixadas com 1 mL de formalina 10% por 1h à temperatura ambiente (TA). Em seguida, após a retirada da formalina e lavagem com água Milli-Q, foi adicionado 1 mL de isopropanol 60% por 5min à TA. Após a retirada do isopropanol, adicionou-se 500µL de oilred (0,3g/100mL) por 1h à TA. Depois da retirada do corante, 3 lavagens com água Milli-Q e secagem dos poços por pelo menos 24hs, 300 µL de solução de Hematoxilina de Harris (cat.HHS32, Sigma) foi adicionado por 1min à TA. O material foi montado em lâmina com glicerina.

Osteócitos - [α-MEM 7,5% SBF suplementado com 0,1 mM de dexametasona (Decadron injetável, Prodome, Campinas, SP, Brasil), 20 mM de ácido ascórbico e 1M de β glicerolfosfato (Reagem, Rio de Janeiro, RJ, Brasil)] ou somente o meio α-MEM 7,5% SFB nos poços controles. As placas foram reincubadas em estufa úmida a 37ºC com 5% de CO2 e metade do meio foi trocado a cada 3-4 dias durante 31 dias.

Após a indução da diferenciação osteogênica, o acúmulo de cálcio nas células mineralizadas foi corado com Alizarin red (cat O5600, FluKa Analytica). Após a retirada do meio de indução e lavagem das células com PBS 1x, estas foram fixadas com paraformaldeído 4% por 1h à TA. Em seguida a retirada do paraformaldeído e lavagem com PBS 1x, adiciona-se 500µL de Alizarin red (2g/mL) por 45 min à TA protegido da luz. Após 4 lavagens, as lamínulas foram secas e embebidas por 1 min no xilol para montagem das lâminas com Permout SP 15 (Fisher, Hampton, NH, Inglaterra).

ANEXO 2 – APROVAÇÃO DO COMITÊ DE ÉTICA

HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA
DE RIBEIRÃO PRETO DA UNIVERSIDADE DE SÃO PAULO

FMRP-USP
RIBEIRÃO PRETO

Ribeirão Preto, 24 de junho de 2019.

Ofício nº 1906/2019
CEP/MGV

PROCESSO HCRP nº 3644/2019

Prezada Pesquisadora,

O trabalho intitulado **"CÉLULAS TRONCO MESENQUIMAIS ENDOMETRIAIS (eMSC) E PERSPECTIVAS NA ETIOPATOGENIA DA ENDOMETRIOSE: PERFIL DE TRANSCRITOS E IMPACTO DO FLUIDO PERITONEAL"**, foi analisado pelo Comitê de Ética em Pesquisa, em sua 492ª Reunião Ordinária realizada em 17/06/2019, e enquadrado na categoria: **APROVADO,** bem como o Termo de Consentimento Livre e Esclarecido e para Guarda de Material Biológico Versão 2 – 04/06/2019.

De acordo com Carta Circular nº 003/2011/CONEP/CNS, datada de 21/03/2011, o sujeito de pesquisa ou seu representante, quando for o caso, deverá rubricar todas as folhas do Termo de Consentimento Livre e Esclarecido – TCLE – apondo sua assinatura na última do referido Termo; o pesquisador responsável deverá da mesma forma, rubricar todas as folhas do Termo de Consentimento Livre e Esclarecido – TCLE – apondo sua assinatura na última página do referido Termo.

Este Comitê segue integralmente a Resolução nº 466/2012 CNS/MS.

Lembramos que devem ser apresentados a este CEP, o Relatório Parcial e o Relatório Final da pesquisa.

Atenciosamente.

DRA. MARCIA GUIMARÃES VILLANOVA
COORDENADORA DO COMITÊ DE ÉTICA EM
PESQUISA DO HCRP E DA FMRP-USP

Ilustríssima Senhora
JULIANA MEOLA LOVATO
Depto. de Ginecologia e Obstetrícia