Human blastocysts exhibit unique microRNA profiles in relation to maternal age and chromosome constitution

Blair R. McCallie · Jason C. Parks · Alyssa L. Strieby · William B. Schoolcraft · Mandy G. Katz-Jaffe

Received: 10 December 2013 / Accepted: 7 April 2014 / Published online: 24 April 2014
© Springer Science+Business Media New York 2014

Abstract

Purpose To determine microRNA (miRNA) expression in human blastocysts relative to advanced maternal age and chromosome constitution.

Methods Cryopreserved human blastocysts were warmed and underwent a trophectoderm biopsy for comprehensive chromosomal screening. Select blastocysts were then lysed, reverse transcribed, and pre-amplified prior to running real-time PCR. Statistical analysis was performed using an internal constant housekeeping miRNA. Significant microRNA's of interest were then analyzed for their predicted genes and biological pathways. Additional cryopreserved blastocysts were warmed and stained for the SIRT1 protein for validation. **Results** Human blastocysts exhibit unique miRNA expression profiles in relation to maternal age and chromosome constitution. miR-93 was exclusively expressed in blastocysts from women in their forties and further up-regulated with an abnormal chromosome complement. Up-regulated miR-93 resulted in an inverse down-regulation of targets like SIRT1, resulting in reduced oxidative defense.

Conclusions MiRNAs play an important role in aging as well as chromosome constitution and have downstream effects that regulate proteins which can compromise embryonic development.

Capsule MiRNAs play an important role in aging as well as chromosome constitution and have downstream effects that regulate proteins which can compromise embryonic development.

B. R. McCallie · J. C. Parks · A. L. Strieby · M. G. Katz-Jaffe (✉)
National Foundation for Fertility Research, 10290 RidgeGate Circle,
Lone Tree, CO 80124, USA
e-mail: mkatz-jaffe@fertilityresearch.org

W. B. Schoolcraft
Colorado Center for Reproductive Medicine, Lone Tree, CO 80124,
USA

Keywords Microna · Human · Blastocyst · Advanced maternal age · Aneuploidy

Introduction

MicroRNAs are endogenous, small (21–22 nucleotides), non-coding RNA molecules that post-transcriptionally regulate the expression of mRNA targets in a sequence specific manner [1]. To date, more than 1,500 human miRNAs have been identified and catalogued (<http://mirbase.org/>). Studies have shown that a significant proportion of miRNAs are conserved across vertebrates with many miRNAs predicted to regulate the expression of hundreds of different genes [2]. MicroRNAs have also been associated with a variety of biological functions including cell proliferation, apoptosis, metabolism, aging, and development [3,4].

In the complex world of aging, environmental stimuli and genetic factors play important contributory roles. A number of aging associated pathways have been characterized including the insulin/insulin-like growth factor signaling pathway, which was identified through loss of function alleles of the genes *age-1* and *daf-2* to extend longevity in *C. elegans* [5]. MicroRNAs have also been shown to play a role in regulating gene and protein signatures which affect the aging process at both the tissue and organism level, and can act in both pro- and anti-longevity regulatory pathways [6]. In an aging human population, nine miRNAs displayed decreased expression when compared to young individuals, with five of these decreased miRNAs characterized in cancer pathogenesis [4]. Decreased miRNA expression is also common during cellular senescence and an increasing number of senescent cells are accumulated with the aging process [7].

Women in the developed world are increasingly postponing childbearing until well into their fourth and fifth decades. This trend is leading to an aging reproductive society and the

fertility complications that are well documented with advanced maternal age defined as >35 years (ACOG Committee Opinion 2010; www.acog.org). One of the main contributors to maternal age-related infertility is the declining number of oocytes as a woman approaches menopause, in addition to the compromised quality of these remaining oocytes [8]. Oocytes from women with advanced maternal age have been shown to have increased mitochondria number and size which could be contributing to a decline in developmental potential [9]. Chromosome abnormalities are also more prevalent in the aged oocyte, resulting in compromised oocyte quality and clinical pregnancies that contribute to >70 % of spontaneous miscarriages [8,10]. Chromosome abnormalities have similarly been identified in malignant cells from numerous cancer types which, along with other genetic alterations, including distorted miRNA expression, are associated with disease prognosis and cancer progression [11].

Given that maternal aging is the most significant risk factor associated with human infertility, the aim of this study was to investigate the association between advanced maternal age, embryo chromosome constitution and miRNA expression. In the present study, we evaluate whether advanced maternal age and embryo chromosome constitution impact miRNA function and contribute to the decline of oxidative defense mechanisms in aged and aneuploid blastocysts.

Results

In total, 60 out of 377 miRNAs (16 %) were expressed across morphologically equivalent human blastocysts, with a unique microRNA expression signature being observed relative to maternal age and independent of blastocyst chromosome constitution. Positive expression was defined as a Ct value ≤ 36 in at least 5 out of 6 samples in each group; negative expression was defined as a Ct value ≥ 37 in at least 5 out of 6 samples in each group. Statistical analysis comparing miRNA expression profiles between young oocyte donor derived blastocysts (n=5) and blastocysts produced from women in their forties (n=5) revealed 42 differentially expressed miRNAs ($P<0.05$; Table 1). The vast majority of these 42 miRNAs displayed increased expression in the blastocysts derived from older women, with only 2 miRNAs showing decreased expression (miR-186 and miR-628-5p; $P<0.05$). In addition, 11 miRNAs were exclusively expressed in blastocysts from women in their forties (miR-15b, miR-18a, miR-184, miR-195, miR-20b, miR-212, miR-222, miR-367, miR-515-5p, and miR-518a-3p, miR-93; $P<0.05$) (Table 1). Negative expression was only observed for these 11 miRNAs in young oocyte donor derived blastocysts.

Statistical analysis comparing miRNA expression profiles between chromosomally normal (n=5) and abnormal blastocysts (n=5) revealed a panel of 38 differentially expressed

miRNAs ($P<0.05$), with 29 (74 %) showing increased expression in chromosomally abnormal blastocysts (Table 2). Three of these miRNAs were exclusively expressed in chromosomally abnormal blastocysts including; miR-218, miR-381 and miR-508-3p ($P<0.05$) (Table 2). Pathway analysis using Pathway Studio software generated several pathways and biological processes for the exclusively expressed miRNAs including apoptosis, cell proliferation, and cell differentiation.

MiR-93 was exclusively expressed in blastocysts from women in their forties and further increased in expression with an abnormal chromosome constitution ($P<0.05$). In a recent study, increased expression of miR-93 was shown to have been associated with aging in rat liver [12]. Further investigation suggested SIRT1, an oxidative stress defense protein, as a target gene of miR-93, and studies using Western blotting validated the repression of SIRT1 with the increased expression of miR-93 [12]. We performed immunofluorescence staining for the SIRT1 protein in human blastocysts from women in their forties (n=29) and blastocysts from young, donor oocytes (n=31). SIRT1 was observed in the nucleus of all blastocyst cells and this was confirmed by overlay with a nuclear DAPI stain. Nuclear localization of SIRT1 has been previously reported [13]. A significant decrease in expression of the SIRT1 protein was found in the aged blastocysts compared to blastocysts from young oocyte donors (Fig. 1). Therefore, increased expression of miR-93 reflects a repression of its target gene, SIRT1, a vital oxidative defense gene, in human blastocysts from women of advanced maternal age.

Discussion

It is well documented that maternal aging is the most significant risk factor associated with human infertility, and that embryo chromosome constitution is associated with the decline in reproductive potential as women enter their 40's. The aim of this study was to investigate an association between advanced maternal age, embryo chromosome constitution and miRNA expression. Our results indicate that advanced maternal age and embryo chromosome constitution impact miRNA expression and target gene function, and may specifically contribute to the decline of oxidative defense mechanisms in aged blastocysts.

MicroRNA profiling of blastocysts from women in their forties compared to blastocysts from young fertile donor oocytes revealed 42 differentially expressed miRNAs including 11 miRNAs that were exclusively expressed in blastocysts from women of advanced maternal age. One of these exclusively expressed microRNAs was miR-15b. This microRNA has been shown to be involved in the cell cycle and its over expression results in cell cycle arrest [14]. Another exclusively expressed miRNA in our study was miR-93 which is

Table 1 Analysis of miRNA expression in euploid blastocysts derived from young oocyte donors in comparison to euploid blastocysts from women in their forties. In total, 60 miRNAs were expressed with 42 showing differential expression ($P<0.05$). Fold changes with an * were statistically significant ($P<0.05$). miRNAs with Ct values=40 were considered not present

miRNA	Young, Eup Avg Ct	Aged, Eup Avg Ct	Fold Change
hsa-miR-106a-4395280	28.9	27.32	3.7*
hsa-miR-106b-4373155	31.48	30.84	1.9
hsa-miR-125a-5p-4395309	32.75	31.95	2.2
hsa-miR-146b-5p-4373178	32.88	29.45	>10*
hsa-miR-15b-4373122	40	32.07	>10*
hsa-miR-17-4395419	27.94	24.46	>10*
hsa-miR-184-4373113	40	32.32	>10*
hsa-miR-186-4395396	31.08	32.1	0.6*
hsa-miR-18a-4395533	40	31.94	>10*
hsa-miR-191-4395410	25.5	25.67	1.1
hsa-miR-192-4373108	31.48	28.27	>10*
hsa-miR-193b-4395478	31.27	29.01	5.9*
hsa-miR-195-4373105	40	33.6	>10*
hsa-miR-197-4373102	31.87	29.63	5.9*
hsa-miR-19b-4373098	29.05	25.43	>10*
hsa-miR-200c-4395411	30.24	27.48	8.4*
hsa-miR-203-4373095	30.91	29.93	2.4*
hsa-miR-20b-4373263	40	33.43	>10*
hsa-miR-212-4373087	40	30.26	>10*
hsa-miR-218-4373081	40	40	N/A
hsa-miR-222-4395387	40	29.79	>10*
hsa-miR-24-4373072	28.05	27.18	2.3*
hsa-miR-28-3p-4395557	32	29.67	6.2*
hsa-miR-302a-4378070	31.18	28.91	6.0*
hsa-miR-302b-4378071	29.22	27.64	3.7*
hsa-miR-302c-4378072	29.82	29.38	1.7
hsa-miR-30c-4373060	32.07	29.14	9.4*
hsa-miR-31-4395390	31.62	29.32	6.1*
hsa-miR-320-4395388	28.89	28.22	2.0*
hsa-miR-323-3p-4395338	30.62	29.63	2.5*
hsa-miR-342-3p-4395371	31.19	30.04	2.8
hsa-miR-345-4395297	31.35	29.27	5.2*
hsa-miR-367-4373034	40	29.66	>10*
hsa-miR-371-3p-4395235	28.13	26.83	3.1*
hsa-miR-372-4373029	21.65	20.59	2.6*
hsa-miR-373-4378073	30.19	29.26	2.4
hsa-miR-374a-4373028	32.45	30.94	3.5*
hsa-miR-374b-4381045	32.29	29.53	8.4*
hsa-miR-381-4373020	40	40	N/A
hsa-miR-454-4395434	32.94	31.3	3.9*
hsa-miR-484-4381032	28.16	27.26	2.3*
hsa-miR-508-3p-4373233	40	40	N/A
hsa-miR-512-3p-4381034	22.95	22.78	1.4
hsa-miR-515-3p-4395480	30.6	31.9	0.5

Table 1 (continued)

miRNA	Young, Eup Avg Ct	Aged, Eup Avg Ct	Fold Change
hsa-miR-515-5p-4373242	40	32.99	>10*
hsa-miR-517a-4395513	28.48	26.55	4.7*
hsa-miR-517c-4373264	29.06	26.89	5.6*
hsa-miR-518a-3p-4395508	40	32.35	>10*
hsa-miR-518b-4373246	30.13	29.68	1.7
hsa-miR-518e-4395506	29.36	29.02	1.6
hsa-miR-518f-4395499	32.2	30.84	3.2*
hsa-miR-519a-4395526	30.38	28.74	3.9*
hsa-miR-519d-4395514	31.12	28.61	7.1*
hsa-miR-520b-4373252	32.88	31.63	2.9
hsa-miR-520 g-4373257	29.87	29.58	1.5
hsa-miR-521-4373259	34.09	33.97	1.3
hsa-miR-522-4395524	31.89	31.47	1.7
hsa-miR-525-3p-4395496	30.39	29.15	2.9
hsa-miR-548a-3p-4380948	33.02	32.23	2.1
hsa-miR-628-5p-4395544	32.5	33.39	0.7*
hsa-miR-886-5p-4395304	29.97	29.24	2.1
hsa-miR-93-4373302	40	31.73	>10*
MammU6-4395470	22.2	22.51	1.0

associated with oxidative stress and was also identified to be further up-regulated with an abnormal chromosome constitution of an aged blastocyst.

Oxidative stress negatively alters cell-signaling communication required for normal cell growth and proliferation. Oxidative stress is considered one of the causes of normal aging [15] and may contribute to several disease states affecting female reproduction, including poor oocyte quality [16]. SIRT1, a NAD-dependent histone deacetylase, has been identified in anti-oxidative stress regulation during aging, possibly by depleting ROS to maintain cell survival [17]. miR-93 has been shown to target oxidative stress defense proteins, including SIRT1 [18]. During the aging process, miR-93 potentially reduces the production of SIRT1 proteins and their transcription factors, which would result in the loss of oxidative defense [12]. Immunofluorescence staining for the SIRT1 protein in blastocysts from women in their forties, compared to blastocysts from young fertile donor oocytes, showed decreased expression of SIRT1 in aged blastocysts. Therefore, it appears that there is an association between the increased expression of miR-93 in aged blastocysts that could reflect a repression and down-regulation of its target gene, SIRT1.

In summary, this novel study showed that human blastocysts exhibit unique miRNA expression profiles in relation to maternal age and chromosome constitution. A set of 11 miRNAs exclusively characterized blastocysts from women in their forties and a unique panel of 38 miRNAs were differentially expressed in relation to blastocyst chromosome

Table 2 Analysis of miRNA expression comparing euploid blastocysts to aneuploid blastocysts from women in their forties revealed 38 differentially expressed miRNAs ($P<0.05$). Fold changes with an * were statistically significant ($P<0.05$). miRNAs with Ct values=40 were considered not present

miRNA	Aged, Eup Avg Ct	Aged, Aneup Avg Ct	Fold Change
hsa-miR-106a-4395280	27.32	27.64	0.8
hsa-miR-106b-4373155	30.84	29.78	2.1*
hsa-miR-125a-5p-4395309	31.95	33.46	<0.5*
hsa-miR-146b-5p-4373178	29.45	28.08	2.6*
hsa-miR-15b-4373122	32.07	31.32	1.7
hsa-miR-17-4395419	24.46	25.38	0.5
hsa-miR-184-4373113	32.32	31.2	2.2
hsa-miR-186-4395396	32.1	32.04	1.0
hsa-miR-18a-4395533	31.94	31.29	1.6
hsa-miR-191-4395410	25.67	25.96	0.8
hsa-miR-192-4373108	28.27	28.22	1.0
hsa-miR-193b-4395478	29.01	28.12	1.9*
hsa-miR-195-4373105	33.6	31.92	3.2*
hsa-miR-197-4373102	29.63	29.77	0.9
hsa-miR-19b-4373098	25.43	26.5	0.5*
hsa-miR-200c-4395411	27.48	26.75	1.7*
hsa-miR-203-4373095	29.93	30.28	0.8
hsa-miR-20b-4373263	33.43	32.31	2.2*
hsa-miR-212-4373087	30.26	31.01	0.6
hsa-miR-218-4373081	40	16.21	>10*
hsa-miR-222-4395387	29.79	30.8	0.5
hsa-miR-24-4373072	27.18	27.04	1.1
hsa-miR-28-3p-4395557	29.67	29.1	1.5*
hsa-miR-302a-4378070	28.91	26.99	3.8*
hsa-miR-302b-4378071	27.64	26.2	2.7*
hsa-miR-302c-4378072	29.38	30.42	0.5*
hsa-miR-30c-4373060	29.14	27.88	2.4*
hsa-miR-31-4395390	29.32	29.55	0.9
hsa-miR-320-4395388	28.22	27.78	1.4*
hsa-miR-323-3p-4395338	29.63	29.61	1.0
hsa-miR-342-3p-4395371	30.04	29.44	1.5*
hsa-miR-345-4395297	29.27	29.85	0.7
hsa-miR-367-4373034	29.66	28.96	1.6*
hsa-miR-371-3p-4395235	26.83	26.29	1.5*
hsa-miR-372-4373029	20.59	20.23	1.3*
hsa-miR-373-4378073	29.26	28.08	2.3*
hsa-miR-374a-4373028	30.94	30.44	1.4*
hsa-miR-374b-4381045	29.53	28.57	1.9*
hsa-miR-381-4373020	40	27.91	>10*
hsa-miR-454-4395434	31.3	29.92	2.6*
hsa-miR-484-4381032	27.26	26.69	1.5*
hsa-miR-508-3p-4373233	40	32.71	>10*
hsa-miR-512-3p-4381034	22.78	23.01	0.9
hsa-miR-515-3p-4395480	31.9	30.88	2.0*
hsa-miR-515-5p-4373242	32.99	32.13	1.8

Table 2 (continued)

miRNA	Aged, Eup Avg Ct	Aged, Aneup Avg Ct	Fold Change
hsa-miR-517a-4395513	26.55	27.16	0.7
hsa-miR-517c-4373264	26.89	27.8	0.5*
hsa-miR-518a-3p-4395508	32.35	32.35	1.0
hsa-miR-518b-4373246	29.68	31.01	<0.5*
hsa-miR-518e-4395506	29.02	30.07	0.5*
hsa-miR-518f-4395499	30.84	19.66	>10*
hsa-miR-519a-4395526	28.74	28.63	1.1
hsa-miR-519d-4395514	28.61	29.75	0.5
hsa-miR-520b-4373252	31.63	30.51	2.2*
hsa-miR-520 g-4373257	29.58	30.93	0.4*
hsa-miR-521-4373259	33.97	32.22	3.4*
hsa-miR-522-4395524	31.47	31.97	0.7*
hsa-miR-525-3p-4395496	29.15	29.2	1.0
hsa-miR-548a-3p-4380948	32.23	32.28	1.0
hsa-miR-628-5p-4395544	33.39	15.1	>10*
hsa-miR-886-5p-4395304	29.24	31.66	0.2*
hsa-miR-93-4373302	31.73	31.29	1.4*
MammU6-4395470	22.51	22.51	1.0

constitution. MiR-93 was exclusively expressed in blastocysts from women in their forties and further up-regulated with an abnormal chromosome complement. Up-regulated miR-93 appears to be associated with an inverse down-regulation of targets like SIRT1, resulting in reduced oxidative defense. Further investigations of these miRNAs will reveal critical roles in gene modulation that could reflect compromised embryonic development.

Experimental procedures

Surplus, cryopreserved, in vitro produced, day 5 blastocysts ($n=75$) from the Colorado Center for Reproductive Medicine were donated to research with patient consent and Institutional Review Board approval. All blastocysts analyzed were of equivalent morphology and graded as expanded blastocysts on day 5 of development: A) Chromosomally normal blastocysts from young, oocyte donors (mean 26.4 years), B) Chromosomally normal blastocysts from women in their forties (range 40–44 years), and C) Chromosomally aneuploid blastocysts, all of which were incompatible with life, from women in their forties (range 40–44 years). The infertile female patients presented for infertility treatment with normal ovarian reserve for their respective maternal age, and had an expected ovarian response to stimulation. Their indication for IVF was only advanced maternal age with no other infertility diagnosis including no male factor.

Fig. 1 Immunofluorescence staining for the SIRT1 protein in human blastocysts from women in their forties ($n=29$) and blastocysts from young, donor oocytes ($n=31$) to investigate this known miR-93 target gene. Immunofluorescence staining revealed a significant decrease in expression of SIRT1 protein (FITC, green) in the blastocysts from women of advanced maternal age. DAPI (blue) was used as a counter stain for the nucleus

Blastocyst culture

All gamete and embryo manipulations were performed in a pediatric isolette to control for humidity, temperature, and pH fluctuations. Semen preparation was performed using a 50–70–95 discontinuous gradient of Pure Sperm (Nidacon, Gothenburg, Sweden) and the resulting pellet was washed in fertilization medium. Intracytoplasmic sperm injection (ICSI) was performed on mature oocytes that had been denuded in hyaluronidase using a Nikon inverted microscope (Nikon Instruments, Melville, NY) with Narashige micromanipulators (Narashige International, East Meadow, NY). Assessment of fertilization took place 15–18 h after insemination. Embryos with two pronuclei were group cultured for 96 h in microdrops of sequential media (Cooper Surgical, Trumbull, CT) at 5 % O_2 , 6 % CO_2 at 37 °C. On the morning of day 5, blastocyst grading was performed using the Gardner and Schoolcraft system [19].

Blastocyst biopsy of trophectoderm cells for comprehensive chromosome screening

Expanded blastocysts underwent biopsy of 3–5 trophectoderm cells to determine chromosome constitution using previously published standard laboratory techniques [20]. Briefly, under an inverted Nikon microscope (Nikon) with Narishige manipulators and injectors (Narishige), trophectoderm cells were aspirated into a biopsy pipette and detached from the blastocyst by firing several pulses of a laser (Hamilton-Thorne Research, Beverly MA) at the area of constriction to separate them from the rest of the blastocyst. The aggregate of TE cells was placed intact into a PCR tube after several washes through hypotonic solution.

Comprehensive chromosome screening for all 23 pairs of human chromosomes was performed using a quantitative real-time PCR based method developed by Reproductive Medicine Associates of New Jersey [21]. Briefly, trophectoderm cells were lysed in KOH and incubated at 65 °C for 10 min. The lysed DNA was then used as a template for multiplex pre-amplification of 96 loci with the use of TaqMan Copy Number Assays and TaqMan Pre-amplification Master Mix as recommended by the supplier (Life Technologies, Carlsbad CA) in a 50 μ l reaction for 18 cycles. Real-time PCR was then performed in quadruplicate for each individual 96 loci on a 384-well plate using TaqMan Gene Expression Master Mix as recommended by the supplier (Life Technologies) in a 5 μ l reaction. A novel method of the standard delta delta threshold cycle method of relative quantitation was applied [21].

Blastocyst vitrification and warming

Following TE biopsy, blastocysts were vitrified using the Cryotop with a DMSO/ethylene glycol protocol developed by Kuwayama et al. [22] and used routinely in the IVF laboratory. Blastocysts were then stored in liquid nitrogen. Upon study recruitment blastocysts were warmed in a thawing solution of 1 mol/L sucrose for 45–60 s at 37°C. Warmed embryos were then transferred to a dilution solution of 0.5 M sucrose for 3 min, followed by washing with medium containing no sucrose for 5 min before placing in embryo culture media (Vitrolife, Englewood, CO) for re-expansion.

Reverse transcription, Pre-amplification, and quantitative real-time PCR

After warming, blastocysts were lysed using a previously validated protocol from the TaqMan® MicroRNA Cells-to-

CtTM Kit (Life Technologies) with minor modifications. Briefly, blastocysts were washed through phosphate-buffered saline (PBS) before being transferred into 10ul of a lysis/DNase solution and incubated at room temperature for 8 min. 1ul of Stop Solution was then added and incubated for an additional 2 min at room temperature. The lysed blastocyst was snap-frozen in liquid nitrogen and stored at -80°C . A negative control was also utilized throughout the procedure to ensure the absence of false amplification.

Samples were reverse transcribed using the MegaplexTM RT Primers Human Primer Pool A and the TaqMan[®] MicroRNA Reverse Transcription Kit (Life Technologies). 4.3ul of a master mix containing 20 mM dNTPs, 75U reverse transcriptase, 2U ribonuclease inhibitor, 22.5 mM MgCl₂, and 1× RT Primers was combined with 3.2ul of each sample and incubated for 40 cycles at 16 °C for 2 min, 42 °C for 1 min, and 50 °C for 1 s, followed by a 5 min hold at 85 °C. The entire volume of cDNA was then pre-amplified by combining it with 17.5ul of MegaplexTM PreAmp Primers Human Pool A and TaqMan[®] PreAmp Master Mix and incubated using the following thermal cycling profile: 95 °C for 10 min, 55 °C for 2 min, 72 °C for 2 min, 14 cycles at 95 °C for 15 s and 60 °C for 4 min, with a final hold at 99.9 °C for 10 min. The pre-amplified product was then diluted by adding 75ul of 0.1× TE for a final volume of 100ul and stored at -20°C until real-time PCR was performed.

Quantitative real-time PCR was performed by combining 9ul of the diluted pre-amplified product to 450ul of TaqMan[®] Universal PCR Master Mix, No AmpErase[®] UNG and 441ul of nuclease-free water. 100ul of this mixture was added to each of the 8 ports on the TaqMan[®] Array Human MicroRNA A Card, which was then centrifuged, sealed, and run on the ABI7900HT Fast Real-Time PCR System at 50 °C for 2 min, 94.5 °C for 10 min, and 40 cycles at 97 °C for 30 s and 59.7 °C for 1 min.

Cycle thresholds were analyzed using RQ Manager 1.2.1 (Life Technologies) and statistical analysis for PCR was performed using the internal constant housekeeping miRNA, MammU6, and REST 2009 software (Qiagen, Valencia, CA). REST software uses randomization and bootstrapping techniques to determine statistical significance. The mathematical model uses the correction for exact PCR efficiencies and the mean crossing point deviation between sample and control groups. The resulting expression ratio is tested for significance by a Pair Wise Fixed Reallocation Randomization Test. Significance was defined at $P < 0.05$. A list of exclusively expressed miRNAs was uploaded into Pathway Studio (Elsevier, Waltham MA) to identify relationships between these miRNAs, their predicted target genes, and biological processes and pathways.

Immunofluorescence

Cryopreserved blastocysts were warmed and transferred into a dish containing 400ul 2 % formaldehyde microtubule

stabilization buffer (MTSB-XF) [23] and then incubated at 37 °C for 30 min. Blastocysts were then washed in MOPS containing no protein, transferred into a dish containing the primary antibody, SIRT1 (Abcam, Cambridge MA) (1:100 dilution), and incubated at 4 °C overnight in a humidity chamber. The next morning, blastocysts were put through a series of three washes consisting of PBS supplemented with 1 % BSA, 0.2 % powdered milk, 2 % normal goat serum, 0.1 M glycine, 0.1 % Triton-X, and 2 % sodium azide before an overnight incubation at 4 °C with the secondary antibody, polyclonal goat anti-rabbit IgG conjugated to DyLight[®] 488 (Abcam) (1:500 dilution). Following this final incubation, blastocysts were washed another three times before mounting onto glass slides using VECTASHIELD[®] Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) to visualize nuclei. Slides were viewed under an Olympus BX52 fluorescent microscope at 40× using the DAPI and FITC exposures and then photographed. All analysis was performed blinded using Metamorph Image Analysis Software and average FITC intensities were recorded (Molecular Devices, Sunnyvale, CA). Images were standardized for exposure time and image scaling. The SIRT1 antibody was tested for specificity and a negative control was performed by staining with only the secondary antibody, which resulted in no fluorescence.

References

1. Bushati N, Cohen SM. microRNA functions. Annual review of cell and developmental biology. 2007;23:175–205. doi:[10.1146/annurev.cellbio.23.090506.123406](https://doi.org/10.1146/annurev.cellbio.23.090506.123406).
2. Thomas M, Lieberman J, Lal A. Desperately seeking microRNA targets. Nat Struct Mol Biol. 2010;17(10):1169–74. doi:[10.1038/nsmb.1921](https://doi.org/10.1038/nsmb.1921).
3. Hackl M, Brunner S, Fortschegger K, Schreiner C, Micutkova L, Muck C et al. miR-17, miR-19b, miR-20a, and miR-106a are down-regulated in human aging. Aging cell. 2010;9(2):291–6. doi:[10.1111/j.1474-9726.2010.00549.x](https://doi.org/10.1111/j.1474-9726.2010.00549.x).
4. Hooten N, Abdelmohsen K, Gorospe M, Ejiogu N, Zonderman AB, Evans MK. microRNA expression patterns reveal differential expression of target genes with age. PloS one. 2010;5 (5):e10724. doi:[10.1371/journal.pone.0010724](https://doi.org/10.1371/journal.pone.0010724).
5. Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R. A C elegans mutant that lives twice as long as wild type Nature. 1993;366(6454):461–4. doi:[10.1038/366461a0](https://doi.org/10.1038/366461a0).
6. Smith-Vikos T, Slack FJ. MicroRNAs and their roles in aging. J Cell Sci. 2012;125(Pt 1):7–17. doi:[10.1242/jcs.099200](https://doi.org/10.1242/jcs.099200).
7. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol. 2007;8(9):729–40.
8. Selesniemi K, Lee HJ, Muhlhauser A, Tilly JL. Prevention of maternal aging-associated oocyte aneuploidy and meiotic spindle defects in mice by dietary and genetic strategies. Proc Natl Acad Sci U S A. 2011;108(30):12319–24. doi:[10.1073/pnas.1018793108](https://doi.org/10.1073/pnas.1018793108).

9. Lodish H, Berk A, Zipursky SL, et al. Molecular cell biology. New York: W. H. Freeman; 2000.
10. Reproductive E, Infertility C. Family physicians advisory C, maternal-fetal medicine C, executive, council of the society of O et al. Advanced reproductive age and fertility. Journal of obstetrics and gynaecology Canada : JOGC. Journal d'obstetrique et gynecologie du Canada : JOGC. 2011;33(11):1165–75.
11. Calin GA, Croce CM. MicroRNAs and chromosomal abnormalities in cancer cells. *Oncogene*. 2006;25(46):6202–10. doi:[10.1038/sj.onc.1209910](https://doi.org/10.1038/sj.onc.1209910).
12. Li N, Muthusamy S, Liang R, Sarojini H, Wang E. Increased expression of miR-34a and miR-93 in rat liver during aging, and their impact on the expression of Mgst1 and Sirt1. *Mech Ageing Dev*. 2011;132(3):75–85. doi:[10.1016/j.mad.2010.12.004](https://doi.org/10.1016/j.mad.2010.12.004).
13. Campagna M, Herranz D, Garcia MA, Marcos-Villar L, Gonzalez-Santamaria J, Gallego P, et al. SIRT1 stabilizes PML promoting its sumoylation. *Cell Death Differ*. 2011;18(1):72–9. doi:[10.1038/cdd.2010.77](https://doi.org/10.1038/cdd.2010.77).
14. Xia H, Qi Y, Ng SS, Chen X, Chen S, Fang M, et al. MicroRNA-15b regulates cell cycle progression by targeting cyclins in glioma cells. *Biochem Biophys Res Commun*. 2009;380(2):205–10. doi:[10.1016/j.bbrc.2008.12.169](https://doi.org/10.1016/j.bbrc.2008.12.169).
15. Kregel KC, Zhang HJ. An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. *American journal of physiology Regulatory, integrative and comparative physiology*. 2007;292(1):R18–36. doi:[10.1152/ajpregu.00327.2006](https://doi.org/10.1152/ajpregu.00327.2006).
16. Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: a review. *Reproductive biology and endocrinology : RB & E*. 2012;10:49. doi:[10.1186/1477-7827-10-49](https://doi.org/10.1186/1477-7827-10-49).
17. Hasegawa K, Wakino S, Yoshioka K, Tatematsu S, Hara Y, Minakuchi H, et al. Sirt1 protects against oxidative stress-induced renal tubular cell apoptosis by the bidirectional regulation of catalase expression. *Biochem Biophys Res Commun*. 2008;372(1):51–6. doi:[10.1016/j.bbrc.2008.04.176](https://doi.org/10.1016/j.bbrc.2008.04.176).
18. Maes OC, An J, Sarojini H, Wang E. Murine microRNAs implicated in liver functions and aging process. *Mech Ageing Dev*. 2008;129(9):534–41. doi:[10.1016/j.mad.2008.05.004](https://doi.org/10.1016/j.mad.2008.05.004).
19. Gardner DK, Schoolcraft WB. In-vitro culture of human blastocysts. In: Jansen R, Mortimer D, editors. Towards reproductive certainty: fertility and genetics beyond 1999. Carnforth: Parthenon Press; 1999. p. 378–88.
20. Schoolcraft WB, Treff NR, Stevens JM, Ferry K, Katz-Jaffe M, Scott Jr RT. Live birth outcome with trophectoderm biopsy, blastocyst vitrification, and single-nucleotide polymorphism microarray-based comprehensive chromosome screening in infertile patients. *Fertil Steril*. 2011;96(3):638–40. doi:[10.1016/j.fertnstert.2011.06.049](https://doi.org/10.1016/j.fertnstert.2011.06.049).
21. Treff NR, Scott Jr RT. Four-hour quantitative real-time polymerase chain reaction-based comprehensive chromosome screening and accumulating evidence of accuracy, safety, predictive value, and clinical efficacy. *Fertil Steril*. 2012. doi:[10.1016/j.fertnstert.2012.11.007](https://doi.org/10.1016/j.fertnstert.2012.11.007).
22. Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. *Theriogenology*. 2007;67(1):73–80. doi:[10.1016/j.theriogenology.2006.09.014](https://doi.org/10.1016/j.theriogenology.2006.09.014).
23. Albertini MR, Nicklas JA, Chastenay BF, Hunter TC, Albertini RJ, Clark SS, et al. Analysis of T cell receptor beta and gamma genes from peripheral blood, regional lymph node and tumor-infiltrating lymphocyte clones from melanoma patients. *Cancer immunology, immunotherapy : CII*. 1991;32(5):325–30.