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Effect of luteal-phase support on endometrial microRNA expression following controlled ovarian stimulation

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Abstract

Background: Studies suggested that microRNAs influence cellular activities in the uterus including cell differentiation and embryo implantation. In assisted reproduction cycles, luteal phase support, given to improve endometrial characteristics and to facilitate the implantation process, has been a standard practice. The effect of different types of luteal phase support using steroid hormones in relation to endometrial miRNA profiles during the peri-implantation period has not seen described. This study was designed to evaluate the expression of miRNAs during the luteal phase following controlled ovarian stimulation for IVF and the influence of different luteal phase support protocols on miRNA profiles.

Methods: The study was approved by the Johns Hopkins Hospital Institutional Review Board. Endometrial biopsies were obtained on the day of oocyte retrieval from 9 oocyte donors (group I). An additional endometrial biopsy was obtained 3–5 days later (Group II) after the donors were randomized into three groups. Group IIa had no luteal-phase support, group IIb had luteal support with micronized progesterone (P), and Group IIc had luteal support with progesterone plus 17-beta-estradiol (P + E). Total RNA was isolated and microarray analysis was performed using an Illumina miRNA expression panel.

Results: A total of 526 miRNAs were identified. Out of those, 216 miRNAs were differentially regulated ($p < 0.05$) between the comparison groups. As compared to the day of retrieval, 19, 11 and 6 miRNAs were differentially regulated more than 2 fold in the groups of no support, in the P support only, and in the P + E support respectively, 3–5 days after retrieval. During the peri-implantation period (3–5 days after retrieval) the expression of 33 and 6 miRNAs increased, while the expression of 3 and 0 miRNAs decreased, in the P alone and in the P + E group respectively as compared to the no steroid supplementation group.

Conclusion: Luteal support following COS has a profound influence on miRNA profiles. Up or down regulation of miRNAs after P or P + E support suggest a role(s) of luteal support in the peri-implantation uterus in IVF cycles through the regulation of associated target genes.

Keywords: MicroRNA, Ovarian stimulation, Luteal phase support, Microarray

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Background

MicroRNAs (miRNAs) are a class of single-stranded, non-coding small RNAs that regulate gene expression at the translational level and play fundamental roles in several biological processes, including cell differentiation, proliferation, development and apoptosis [1-3]. It is believed that mammalian miRNAs are responsible for the regulation of over 60% of all human genes [4]. Either by controlling mRNA degradation or by translational repression, miRNAs have emerged as key regulators of gene expression [5,6]. Each miRNA is predicated to have a broad range of target mRNAs and each mRNA may be regulated by multiple miRNAs [7,8].

The role of miRNAs in the female reproductive system and particularly in the endometrium has been the focus of several studies in recent years [9,10]. So far it has been established that miRNAs are indeed expressed in the human endometrium and they are also subjected to hormonal regulation [10,11]. Hawkins et al. were able to identify a number of miRNAs that were differentially regulated in endometriotic tissues as compared to normal endometrium [12]. The overall regulatory role of miRNAs in the pathophysiology of endometriosis has been reviewed extensively by Ohlsson Teaque *et al.* [13].

Ovarian stimulation protocols with gonadotropins have been invariably associated with luteal phase deficiency and poor implantation rates [14,15]. While the exact reasons for this phenomenon are still unclear, luteal phase support, given to improve endometrial characteristics and to facilitate the implantation process, has been a standard practice. Progesterone is a universally accepted agent for luteal phase support and can be administered orally, intramuscularly, or vaginally [16,17]. Estrogens in the form of 17β- estradiol or estradiol valerate have also been used for luteal phase support [18], although studies aimed to evaluate the concept of estrogen addition during the luteal phase have lead to inconclusive results [14,19] . It has been suggested that during ovarian stimulation for IVF, the endometrial receptivity starts to occur in mid luteal phase after oocyte retrieval [20]. Prior to, and during the implantation process, the expression of multiple endometrial genes and gene products is highly regulated [21-23]. The role of miRNAs in regulating cellular processes during the endometrial transition has recently attracted a great deal of attention [10,24-28]. For example, Kuokkanen *et al.* reported distinct miRNA gene expression signatures in the late proliferative and mid-secretory phase endometrial epithelium [24]. However, the effect of different types of luteal support in relation to endometrial miRNA profiles during the period of peri-implantation has not been described. In this study, we have investigated the impact of two commonly used luteal phase support protocols, progesterone alone and progesterone plus estrogen, on the expression profiles of 526 miRNAs in the human endometrium following ovarian stimulation with a gonadotropin/ GnRH antagonist protocol.

Methods

Oocyte donors and ovarian stimulation

The study was approved by the Johns Hopkins Hospital Institutional Review Board. Nine oocyte donors who enrolled in the Johns Hopkins oocyte donation program participated in the study. All donors were 21 to 31 years of age and underwent a standard screening protocol for oocyte donation, in accordance with the recommendations of the American Society for Reproductive Medicine [29]. The risks of the procedure were discussed in detail, with particular emphasis on the risks associated with the endometrial biopsy and the use of steroids during luteal phase, and written informed consents were obtained.

Study subjects underwent ovarian stimulation according to a gonadotropin / GnRH antagonist protocol as described previously [30]. Briefly, ovarian stimulation was initiated with gonadotropins on the second day of vaginal bleeding following discontinuation of oral contraceptive pills. On the 6th day of stimulation, a daily subcutaneous evening dose of 0.25 mg ganirelix acetate (Schering-Plough Corp, West Orange, NJ, USA) was added. When at least three follicles reached a mean diameter of 18 mm, ovulation was triggered with a single dose of hCG (Profasi, 10,000 IU; Serono Inc. Rockland, MA, USA). Sonographically guided transvaginal oocyte retrieval was performed 34–36 hours after the hCG administration. The retrieved oocytes were used for IVF procedures and the resulting embryos were either transferred to matched recipients or cryopreserved for future use.

Luteal-phase support and tissue collection

Endometrial biopsies on oocyte donors were performed using a Pipelle catheter (Unimar, Wilton, CT) on the day of oocyte retrieval and served as baseline (group I). At that time, the donors were randomized into three groups, with three subjects in each group. Group IIa received no luteal phase support after retrieval. Group IIb had luteal phase support with micronized progesterone (P) in the form of vaginal suppositories (200 mg every 6 h starting from the day after retrieval). Group IIc received a daily oral dose of 2 mg 17β-estradiol in addition to the micronized progesterone (P + E). Endometrial biopsies were obtained again 3–5 days (each of treatment groups contains 2 samples from day 3 and 1 sample from day 5) after retrieval. All specimens were stored in liquid nitrogen at –196°C immediately after the biopsy.

RNA preparation and miRNA analysis

Total RNA was isolated and extracted from individual endometrial samples using the Trizol Reagent method (Invitrogen, Carlsbad, California 92008, cat. no. 15596–026). The quality of the RNA samples was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The integrity of miRNA was assessed by a miRNA specific

RT-PCR using an ABI (Applied Biosystems; Foster City, CA) Taqman assay for U6 snRNA (AB Assay ID 001973). The results indicated an average Ct of 20.1 (SD 0.84) for all samples with a minimum Ct of 18.3 and maximum Ct of 22.

Illumina miRNA expression profiling (Catalog # MI-501-1001) was carried out according to manufacturer's recommended protocols. Briefly 200ngs of total RNA for each sample was polyadenylated and converted to cDNA using a biotinylated oligo-dT primer with a universal PCR sequence at its 5'-end. Biotinylated cDNA was annealed to query oligos. Each query oligo consisted of a universal PCR priming site at the 5'end, an address sequence that complements a corresponding capture sequence on the array, and a microRNA-specific sequence at the 3'end. This mixture was bound to streptavidin-conjugated paramagnetic particles to select the cDNA/oligo complexes; second strand cDNA synthesis was completed by primer extension. All cDNA templates were amplified with a pair of common PCR primers. The primer on the strand complementary to the array was fluorescently labeled for subsequent hybridization to the arrays.

Validation of the selected miRNAs, shown to be regulated by Illumina miRNA microarray, was performed by RT-PCR. QRT-PCR was performed using the RT^2 Profiler^{TM} Human miFinder miRNA PCR Array (MAH-001A) from SuperArray (SABiosciences, Gaithersburg, MD). RT^2 Profiler^{TM} PCR Arrays are designed for relative quantitative QRT-PCR based on SYBR Green detection and performed on a one sample/one plate 96-well format, using primers for a preset list of 88 most abundantly expressed and best characterized micro RNA sequences. In brief, miRNA was converted to cDNA via a universal tailing and reverse transcription reaction. CDNA volumes were adjusted to ~2.5 ml with SuperArray RT^2 Real-Time SYBR Green/ROX PCR 2X Master Mix (PA-012) and 25 μl of cDNA mix was added to all wells. The PCR plate was sealed and spun at 1500 rpm X 4 min. Real time PCR was performed on an Applied Biosystem (Foster City, CA) 7300 Real Time PCR System. ABI instrument settings included setting reporter dye as "SYBR", passive reference is "ROX"; Delete UNG Activation, and add Dissociation Stage.

To correlate differentially expressed miRNAs and their regulated genes, we used differentially regulated and selected miRNAs against an established miRNA database for predicted target genes (Sanger miRBase, v9.1, February 2007 release). MicroRNA data was also analyzed through the use of Ingenuity Pathway Analysis (IPA, Ingenuity$^{®}$ Systems, www.ingenuity.com). Pathway enrichments were calculated using the NIAID DAVID functional enrichment tool [31,32].

Statistical analysis

Preliminary analysis of the scanned data was performed using Illumina BeadStudio software which returns single intensity data values/miRNA following the computation of a trimmed mean average for each probe type represented by a variable number of bead probes/gene on the array. Data was globally normalized by scaling each array to a common median value, and significant changes in gene expression between class pairs were calculated using the Student t-test. Significant gene lists were calculated by selecting genes which satisfied a significance threshold criteria of t-test *p*-values less than or equal to 0.05 and a fold change ± 2 or greater.

Relative miRNA expression derived from QRT-PCR was calculated by using the 2^{-Ct} method, in which Ct indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches detection threshold [33]. The normalized ΔCt value of each sample is calculated using an endogenous control small molecular weight RNA (U6 snRNA). Fold change values are presented as average fold change = $2^{-(average\ Ct)}$ for genes in treated relative to control samples. The criteria of significance used for the RT-PCR results were the same as used for the Illumina miRNA arrays.

Results

Demographic characteristics

Demographic characteristics for all study participants were similar in all treatment groups. The mean age of the study participants was 24 years and mean body mass index was 21.3 ± 1.2 kg/m2. Overall, the baseline serum FSH, LH and E2 levels, the length of the stimulation , total amount of gonadotropins used, peak estradiol levels, and number of oocytes retrieved were comparable (P > 0.05) between the groups (Table 1).

MiRNA profiles and comparisons between groups

To establish endometrial miRNA profiles, we used a microarray platform consisting of 526 miRNA probes. Triplicates of each group samples were used, which proved that genes from same condition of samples are reproducible. Levels of miRNA expression are similar in the same sample groups including the samples from either day 3 or day5. The fluorescent intensity of each expressed transcript in each sample group was compared to the median fluorescence intensity of each transcript in the paired comparison group. Individual transcripts with increased (red) and decreased (green) miRNA abundance in the given comparisons were identified, as shown in the hierarchical clustering map in Figure 1. It is demonstrated that there is a high degree of overall concordance between and within treatments for later versus early luteal phase and, in particular a striking concordance, for hormone treated versus non-treated groups at days 3–5 after oocyte retrieval. Following global normalization, the mean expression value for each group was subjected to statistical analysis. A 2 fold change in the expression was arbitrarily selected as a cut-off level. Individual miRNAs that have shown a significant change in their expression (greater than 2fold and/or p < 0.05 between the comparison groups)

Table 1 Group characteristics

Characteristics	Group IIa (no support)	Group IIb (P support)	Group IIc (P + E support)	P
N	3	3	3	
Age (yr)	25.7 ± 3.2	23.6 ± 0.8	22.8 ± 1.2	0.494
BMI (kg/m^2)	23.3 ± 1.4	21.6 ± 1.8	20.2 ± 1.2	0.096
Day 2 FSH (mIU/ml)	4.5 ± 0.9	5.6 ± 1.1	4.0 ± 0.3	0.178
Day 2 LH (mIU/ml)	2.4 ± 0.8	4.0 ± 1.3	5.2 ± 0.2	0.507
Day 2 E2 (pg/ml)	36.7 ± 11.6	34.5 ± 12.5	20.5 ± 3.5	0.646
Gonadotropins used (IU)	2850 ± 525	2400 ± 645	2625 ± 675	0.387
Peak E2 level (pg/ml)	1928 ± 100.0	2514 ± 400	2625 ± 480	0.563
Days of stimulation	10.3 ± 1.1	9.3 ± 1.2	10.1 ± 0.7	0.588
No. of oocytes	14.5 ± 5	18.4 ± 3	16.0 ± 4	0.398

are shown in an Additional file 1: Table S1 with a total of 248 miRNAs listed.
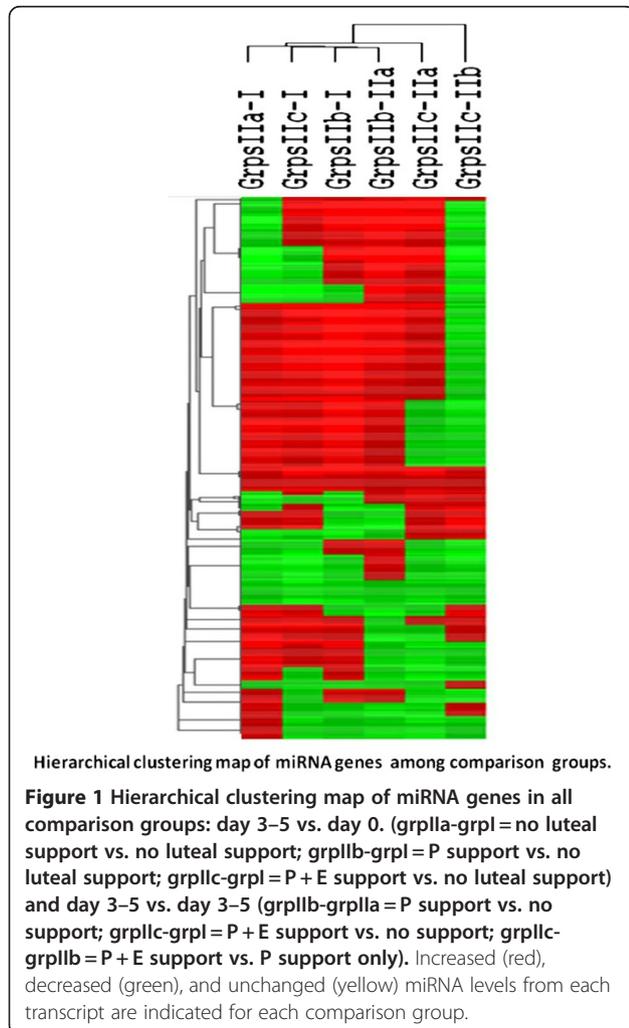
Initially we compared miRNA expression in the endometrial samples obtained on the day of retrieval to those obtained 3–5 days later (Figure 2, the 3 comparison columns on the left). In the group with no luteal phase support, 14
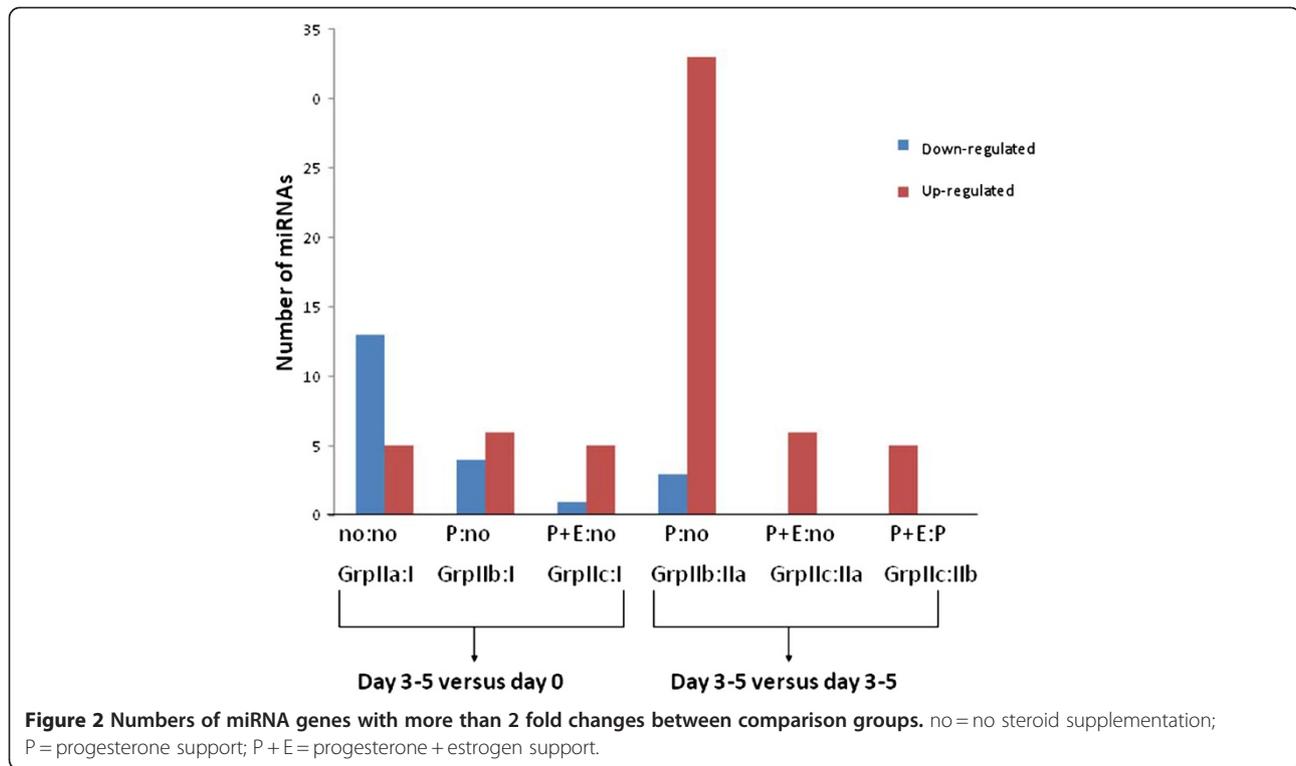

Hierarchical clustering map of miRNA genes among comparison groups.

Figure 1 Hierarchical clustering map of miRNA genes in all comparison groups: day 3–5 vs. day 0. (grpIIa-grpI = no luteal support vs. no luteal support; grpIIb-grpI = P support vs. no luteal support; grpIIc-grpI = P + E support vs. no luteal support) and day 3–5 vs. day 3–5 (grpIIb-grpIIa = P support vs. no support; grpIIc-grpI = P + E support vs. no support; grpIIc-grpIIb = P + E support vs. P support only). Increased (red), decreased (green), and unchanged (yellow) miRNA levels from each transcript are indicated for each comparison group.

miRNAs (HS_202.1, HS_209.1, HS_284.1, hsa-miR-202*:9.1, hsa-miR-346, hsa-miR-363*, hsa-miR-504, hsa-miR-569, hsa-miR-302d, hsa-miR-632, HS_17, HS_145.1, hsa-miR-133b, hsa-miR-144:9.1) were down-regulated and 5 miRNAs were up-regulated (HS_130, hsa-miR-876-5p, hsa-miR-876-3p, hsa-miR-122, hsa-miR-9) at greater than 2 fold changes. In the P alone group, 4 miRNAs (hsa-miR-144:9.1, hsa-miR-486-5p, HS_97, HS_203) were down regulated and 7 (HS_163, hsa-miR-614, hsa-miR-610, hsa-miR-559, hsa-miR-876-5p, HS_18, hsa-miR-876-3p) were up regulated, while in the P + E support group, 1 miRNA (hsa-miR-449a) was underexpressed and 5 (HS_276.1, hsa-miR-876-5p, HS_18, HS_111, hsa-miR-876-3p) were overexpressed .

Subsequently, we compared miRNA gene expression between the different treatment groups during mid-luteal phase at 3–5 days after retrieval, as shown in Figure 2, the 3 comparison columns on the right. In the progesterone support group an overexpression (more than 2 fold increase) was observed for 33 miRNAs (HS_149, HS_166.1, HS_175, HS_202.1, HS_209.1, HS_284.1, HS_41, hsa-miR-1468, hsa-miR-202*:9.1,hsa-miR-346, hsa-miR-504, hsa-miR-512-5p, hsa-miR-560:9.1, hsa-miR-563, hsa-miR-638, hsa-miR-663, hsa-miR-302d, hsa-miR-302b*, hsa-miR-632, hsa-miR-622, HS_17, HS_163, hsa-miR-518b, HS_108.1, hsa-miR-614, hsa-miR-610, HS_263.1, HS_30, hsa-miR-512-3p, HS_32, HS_282, HS_169, HS_145.1) and in the P + E support group for 6 miRNAs (HS_149, HS_276.1, HS_41, hsa-miR-563, HS_17, hsa-miR-144:9.1) as compared to the no steroid supplementation group. On the other hand, underexpression of 3 miRNAs (HS_176, HS_97, HS_203) were seen only in P support group. In the comparison between E + P and P supplementation groups, 5 miRNAs (hsa-miR-144:9.1, hsa-miR-486-5p, HS_176, HS_97, HS_203) were up-regulated and none were down regulated at greater than 2 fold levels.

Venn diagram analysis of differentially expressed miRNA genes

A total of 216 miRNAs were differentially regulated (p < 0.05) between the study groups. MiRNAs with significant

Figure 2 Numbers of miRNA genes with more than 2 fold changes between comparison groups. no = no steroid supplementation; P = progesterone support; P + E = progesterone + estrogen support.

changes in common (shared miRNAs) between groups are shown in Figure 3. Panel A shows changes in miRNA expressions between day 3–5 and day of retrieval. Among the 3 comparison groups, 3 miRNAs (hsa-miR-876-3p, hsa-miR-155, and hsa-miR-503) were shared by all 3 groups and 5, 10 and 13 miRNAs respectively were shared in each pair of groups. Panel B compares groups on day 3–5 at all possible combinations. Group IIb vs. IIa and Group IIc vs. IIa shared 4 miRNAs (HS_241.1, hsa-miR-346, hsa-miR-503, and hsa-miR-99a); Group IIc vs. IIa and Group IIc vs. IIb shared 1 miRNA (hsa-miR-766) and Group IIb vs. IIa and Group IIc vs. IIb shared 3 miRNAs (hsa-miR-501-5p, hsa-miR-512-5p and hsa-miR-146a).
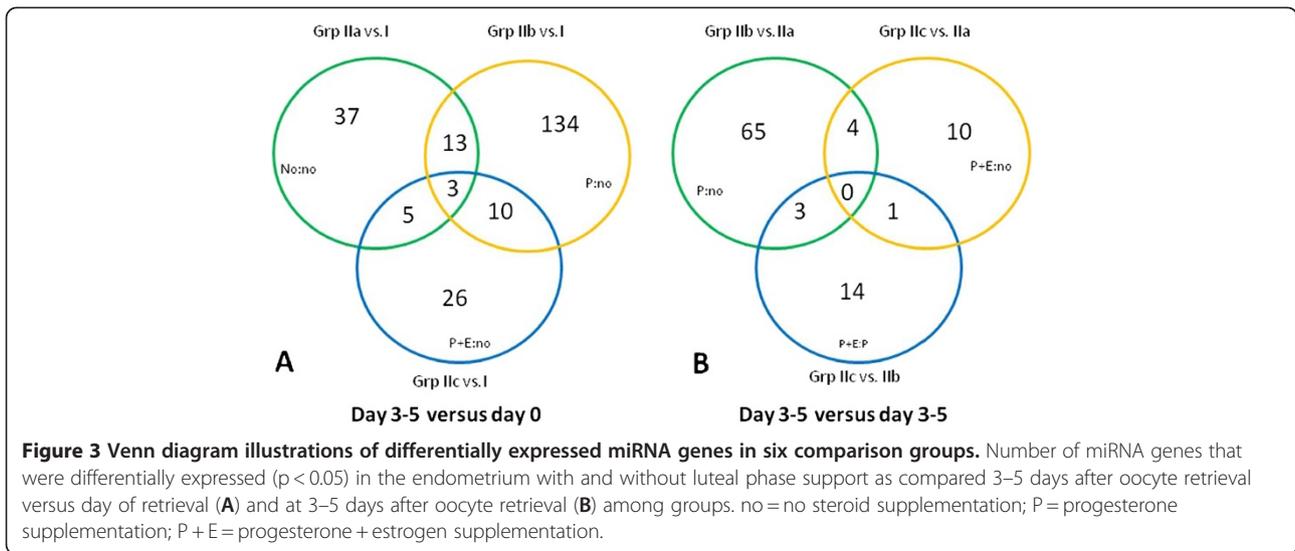
Validation analysis

Array based RT-PCR with 88 miRNAs was used to validate our Illumina array expression findings. We were able to map 19 miRNAs between the two platforms. Of these, 14/19 demonstrated concordance at the level of the direction of regulation (increased or decreased) at a hypergeometric probability of $p < 0.014$. Nine representative miRNAs were selected for groups IIa vs. I and IIc vs. IIa as indicated in Figure 4. The trends for up-regulation and down- regulation of these miRNAs were consistent between the two array measurements.

MiRNA and target genes

To explore the biological relationship between differentially expressed miRNAs and their regulated genes, we used differentially regulated ($p < 0.05$) miRNAs on day 3–5 after oocyte retrieval against an established miRNA database for predicted target genes (Sanger miRBase, v9.1, February 2007 release). Interestingly, there are large numbers of predicted target genes for a given miRNA per miRBase. We were able to identify nineteen miRNAs and their selected target genes in this defined study categories which are shown in Table 2.

In order to further investigate the possible biological implications for those miRNAs which were cross validated by both QRT-PCR and Illumina array data (Figure 4), the relationship of these microRNAs and their known gene targets was evaluated using the IPA miRNA Target Filter software. This group of miRNAs is regulated between day 3–5 and day 0 and also at day 3–5 between P + E (Grps IIc) and no support (Grps IIa) groups. IPA was able to identify 7 out of the 9 miRNAs from Figure 4 (excluding hsa-miR-144, and hsa-mir-181b). The gene targets were identified for these miRNAs based upon the selection of the most stringent criteria requiring experimental observation of a given miRNA and its target. Gene targets were further filtered for known involvement in endocrine system disorders. The results of this analysis (Table 3) that shows pathway enrichments were calculated for the entire gene set. The findings of the analysis demonstrated a significant involvement of genes of extracellular matrix, cell proliferation, and response to steroid hormone stimulus between days 3–5 versus day 0 at no steroid support groups (Table 3, Grps IIa-I). Interestingly, this effect was almost completely abrogated by progesterone and estrogen
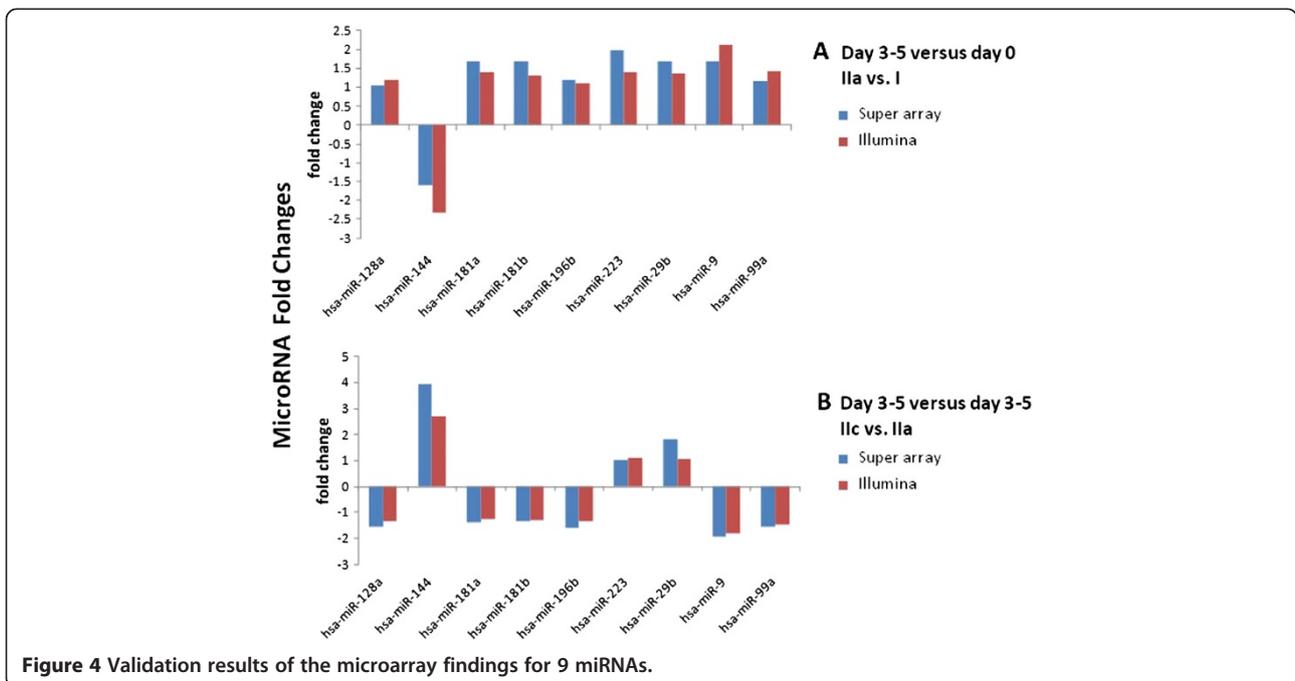
Figure 3 Venn diagram illustrations of differentially expressed miRNA genes in six comparison groups. Number of miRNA genes that were differentially expressed (p < 0.05) in the endometrium with and without luteal phase support as compared 3–5 days after oocyte retrieval versus day of retrieval (**A**) and at 3–5 days after oocyte retrieval (**B**) among groups. no = no steroid supplementation; P = progesterone supplementation; P + E = progesterone + estrogen supplementation.

treatment (Table 3, GrpsIIc-IIa) for genes of cellular proliferation and response to steroid hormones but not for extracellular matrix.

Discussion

In the past few years, the field of miRNA research has evolved rapidly. Various studies have provided strong evidence for the widespread expression and the regulatory functions of miRNAs on gene expression under either physiologic or pathologic conditions. MicroRNAs have now been recognized as key players in the process of cell proliferation and differentiation. Global analysis of miRNAs in human tissues have showed that, in addition to the brain, the uterus, the cervix, and the ovaries have the highest restricted enrichment in individual miRNAs [34]. The identification of miRNA as well as the functional analysis of individual expressed miRNA in the uterus has shed light onto the cycling changes that occur in response to steroids and during pregnancy. The impact of the ovarian steroids on miRNA expression and regulation in the uterus has been

Figure 4 Validation results of the microarray findings for 9 miRNAs.

Table 2 Selected miRNAs and gene targets (comparisons are at day3-5 after oocyte retrieval)

miRNA	P vs. no	P + E vs. no	P + E vs. P	Predicted target genes
hsa-miR-335	↑↑			IMP2,CD79B,WWP1,AP3S1,HOXD8,MAX,SP1,MAP2,MAK3,STARD7,CAP350,PANK2, SRPR,PPP6C,LASS5,ATP1B1
hsa-miR-346	↑	↑		IMP1,EIF3S1,BCL6,ABCC12,LIF,FSTL4,KGFLP2,KRAS,RAF7,FGF7,TMEM28,IGSF4B, PPP1R9B,COL2A1,HCG3, CALN1,HBP1,SF1
hsa-miR-448	↑			DOCK9,PPM2C,NTF3,CAP1,MAP2K6,ITM1,PRKAR2B,PAPPA,CDC2L6,CNTN4, IGF1R,SOCS5,CLK1,HOXA11,WWP1,FOXJ3,WDR22,MPPED2,ADD1,PRKA2
hsa-miR-504	↑			DCX,ATP1B4,IL1RAPL1,MNT,KLF13,PRKAR2A,IL16,LIF,FXR2,NRF1,CAMK2G,MMD, LOC284296,DND1,CNTFR, SORT1,NFIX
hsa-miR-512-5p	↑↑		↓	PIK3R1,CTNNB1,EMX2,SOX21,RIPK5,MBD6,SRPK, VNN3, ERP29,PHF15,FBXW11,LOC285074,MAP1A,CHD9
hsa-miR-520 g	↑↑			ETF1,CAMK2N1,NLK,TNFSF11,CNR1,EFTUD1,HMGB3,FBN2,ENC1,MARK4,TFEB, TNFSF12,PRKAR2A,TNKS1BP1,EIF4E,PPP3CA,IMP1,MAP3K14,TMTC2,TTN, GTF2IRD2,PTK2B,DNAJB5,TNRC6A,VEGF,EIF4G2,FOXO1A,MAP3K9
hsa-miR-204	↑			RUNX2,SOX4,NRBF2,MAP1LC3B,CDC42,ATP2B1,AKAP1,MAP3K3,CENTD1,IGF2R, NTRK2,TGFBR2,AP3M1,
				NEUROG1,P53CSV,TCF7L1,CDH2, CDC25B,TCF12,ELF2
hsa-miR-369-3p			↓	TCF8,PKIA,TLN1,CHD7,NCK2,CD2AP,CDC2L6,ELMOD1,CCNE2,FOXG1B,HOXB3,ADAMTS19, GIT2,ADAMTS3,TCF12, SRPK2,ADAMTS6,MAP2,ADAM10,FOXO1A,VEGFC
hsa-miR-328	↑			AK6,ITGA5,PRX,IGSF4C,MAX,SOX11,PTPN9,DPH2, HIST1H4D,USP37,VSIG4,RPP14,SF4, ULK2,FGD1,PLAG1
hsa-miR-186	↑			APLP2,ITGA6,RPS6KB1,CDC42,PRDM10,IGSF11,EFCBP1,TCF20,CAST,LMBR1,TMED2,TGFBR2,ICMT, IL2,CCNT2,HOXB8,PAK7,FOXD1,PTGES3,MAP3K2,VEGF,COL3A1, SRPK2,MAPKAP1,C16orf52,MAP2
hsa-miR-517a	↑			AMMECR1,ACACA,NPAS4,BSN,HNRPU,PTK2B,CDKN2A,CBLN2,RAPGEFL1,LOC201895,FOXJ3, PHF13,TMCC1
hsa-miR-365			↓↓	EIF4E3,MAP2K7,LAMP2,ENTPD7,PCNP,ADAMTS6, COL7A1,PPP5C,REV3L,PTGDR,KCNH2, RBM12,PKD2L2
hsa-miR-221	↓↓			CDC2L6,TIMP3,EIF4E3,NTF3,IMP2,HTLF,CDV3,NL,EIF5A2,NRK,PAK1,CDKN1C,FAT2,LIFR,TMCC1, MAP3K10,VGLL4,FAM13A1,TCF12,HOXC10,MAPK10,HMGCLL1,ADAM11,CD4,CTCF
hsa-miR-495	↓↓			MAPK6,CDK6,EML4,ILF3,PTK9,PRR7,HBEGF,HOMER1,MARK3,SP4,TGFB2,LHX2,HOXC6,PRKX, AP3M1,SOX9,GMFB,HMGCLL1,FOXO3A,EDG3,NKRF,HOXB9,TIMP2, IGSF4,CD164,TNFRSF21
hsa-miR-146a	↓		↑	FBXL10,IRAK1,TRAF6,CD79B,SP8,FLJ33814,SFRS6,NPAS4,CXorf22,EIF4G2,MMP16,USP3, KCTD15,SMAD4,LOC440944,SEC23IP,BCORL1,TM6SF2,DLGA1
hsa-miR-99a	↓	↓		EPDR1,FZD8,HS3ST2,EIF2C2,HS3ST3B1,FGFR3,BAZ2A,MBNL1,CYP26B1,KBTBD8,SMARCA5, FRAP1,ZZEF1,ICMT,C4orf16,ADCY1,MTMR3,CTDSPL,HOXA,RAVER2, INSM1,TRIB2,SLC44A1
hsa-miR-181c,d	↑↑			ETF1,COL16A1,NLK,TNFSF11,MAP3K3,MAP2K1,ITGA3,TCERG1,MAPT,MAPK1,MAP1B,CDH13, ITGB8,PCGF2,ADAMTS18,LMBRD2,MMP14,CD163,LIF,ADAMTS6, TNFRSF11B,CDC42BPA
hsa-miR-200b			↓	TCF8,NTF3,CYLN2,HMGB3,PRKAR2B,MPP5,GIT2,MAP4K3,FLJ21103,E2F3,CSNK1G3,MMD2, ZNF53, EIF5B,ERRFI1
hsa-miR-196b		↓		IMP1,CDYL,COL14A1,SSR1,IMP3,CDV3,CALM3,COL24A1,CDKN1B,ELF4,HOXC8,HMGA2, HOXA5,MAP4K3,PARP6,COL3A1,HOXA1,TNFSF12,COL1A2, HOXA7,HOXB6

↑ or ↓= up or down regulated, $p < 0.05$; ↑↑ or ↓↓= up or down regulated, $p < 0.01$.
See website http://www.mirbase.org (http://microrna.sanger.ac.uk) regarding additional predicted target genes.

evidenced by the fact that treatment with 17β estradiol or RU-486 resulted in differential regulation of miRNAs in the myometrium and leiomyomas [35].

In the present study, we have examined 526 different miRNAs in the human endometrium following COS and identified a rich number of miRNAs with at least 2 fold changes in the level of expression during the luteal phase (Figure 2, Additional file 1: Table S1). Statistical analysis identified that the changes were significant (p < 0.05) for 216 of miRNAs (Additional file 1: Table S1). These changes were observed not only in the within the group analysis at

different times during luteal phase (comparison between day 0 and day 3–5) but also in the analysis between groups at the same time frame (comparison between the treatment groups at day 3–5). As demonstrated in Figure 1and Figure 2, there was a substantial increase in miRNA expression in the groups treated with progesterone alone as compared to the no supplementation group. In genome-wide identification of endometrial miRNA in natural and stimulated cycles reported by Sha *et al.* [36], 22 miRNAs were significantly dysregulated on the day of hCG + 7 in stimulated cycles as compared with day of LH + 7 in natural

Table 3 Cross validated miRNAs and their selected target genes

Symbol	GrpsIIa-I [ILL-FC]	GrpsIIc-IIa [ILL-FC]	Source	Symbol	Entrez gene name	Pathway (enrichment)	P Value	FDR
miR-223-3p (GUCAGUU)	1.389	1.111	1	VIM	vimentin			
miR-223-3p (GUCAGUU)	1.389	1.111	1,2	RHOB	ras homolog family member B			
miR-223-3p (GUCAGUU)	1.389	1.111	1	IRS1	insulin receptor substrate 1			
miR-29b-3p (AGCACCA)	1.375	1.068	2,3	TUBB2A	tubulin, beta 2A class IIa			
miR-29b-3p (AGCACCA)	1.375	1.068	1,2,3,4	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	extracellular matrix	1.29E-07	4.44E-06
miR-29b-3p (AGCACCA)	1.375	1.068	1,2,3	PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)			
miR-29b-3p (AGCACCA)	1.375	1.068	2,3	MYBL2	v-myb myeloblastosis viral oncogene homolog (avian)-like 2			
miR-29b-3p (AGCACCA)	1.375	1.068	1,2	COL5A3	collagen, type V, alpha 3	extracellular matrix	1.29E-07	4.44E-06
miR-29b-3p (AGCACCA)	1.375	1.068	32,3	COL5A2	collagen, type V, alpha 2	extracellular matrix	1.29E-07	4.44E-06
miR-29b-3p (AGCACCA)	1.375	1.068	1,2,4	COL4A1	collagen, type IV, alpha 1	extracellular matrix	1.29E-07	4.44E-06
miR-29b-3p (AGCACCA)	1.375	1.068	1,2,3,4	COL1A2	collagen, type I, alpha 2	extracellular matrix	1.29E-07	4.44E-06
miR-29b-3p (AGCACCA)	1.375	1.068	1,2,4	COL15A1	collagen, type XV, alpha 1	extracellular matrix	1.29E-07	4.44E-06
miR-9-5p (CUUUGGU)	2.104	−1.802	1	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1			
miR-9-5p (CUUUGGU)	2.104	−1.802	1,2	FOXO1	forkhead box O1			
miR-9-5p (CUUUGGU)	2.104	−1.802	1,2	FOXG1	forkhead box G1	positive regulation of cell proliferation	1.21E-08	1.67E-06
miR-9-5p (CUUUGGU)	2.104	−1.802	1,2,3	CDH1	cadherin 1, type 1, E-cadherin (epithelial)			
miR-181a-5p (ACAUUCA)	1.376	−1.24	1,4	TRA@	T cell receptor alpha locus			
miR-181a-5p (ACAUUCA)	1.376	−1.24	1,2	TIMP3	TIMP metallopeptidase inhibitor 3			
miR-181a-5p (ACAUUCA)	1.376	−1.24	1,2	NOTCH4	notch 4	positive regulation of cell proliferation	1.21E-08	1.67E-06
miR-181a-5p (ACAUUCA)	1.376	−1.24	1,2	**KRAS**	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	response to steroid hormone stimulus	6.24E-07	4.65E-05
miR-181a-5p (ACAUUCA)	1.376	−1.24	1,2,4	HOXA11	homeobox A11			
miR-181a-5p (ACAUUCA)	1.376	−1.24	1,2	GATA6	GATA binding protein 6			
miR-181a-5p (ACAUUCA)	1.376	−1.24	1,2	ESR1	estrogen receptor 1	response to steroid hormone stimulus	6.24E-07	4.65E-05
miR-181a-5p (ACAUUCA)	1.376	−1.24	1,2	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	positive regulation of cell proliferation	1.21E-08	1.67E-06
miR-181a-5p (ACAUUCA)	1.376	−1.24	1,2,3,4	**BCL2**	B-cell CLL/lymphoma 2	response to steroid hormone stimulus	6.24E-07	4.65E-05

Table 3 Cross validated miRNAs and their selected target genes *(Continued)*

miR-196a-5p (AGGUAGU)	1.092	−1.342	1	IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta			
miR-196a-5p (AGGUAGU)	1.092	−1.342	1,2,4	HOXC8	homeobox C8			
miR-196a-5p (AGGUAGU)	1.092	−1.342	1,3	ANXA1	annexin A1			
miR-99a-5p (ACCCGUA)	1.427	−1.48	1,2,3	MTOR	mechanistic target of rapamycin (serine/threonine kinase)	positive regulation of cell proliferation	1.21E-08	1.67E-06
miR-99a-5p (ACCCGUA)	1.427	−1.48	1,2,3	IGF1R	insulin-like growth factor 1 receptor	positive regulation of cell proliferation	1.21E-08	1.67E-06
miR-99a-5p (ACCCGUA)	1.427	−1.48	1,2,3	FGFR3	fibroblast growth factor receptor 3	positive regulation of cell proliferation	1.21E-08	1.67E-06
miR-128 (CACAGUG)	1.184	−1.345	1,2	TXNIP	thioredoxin interacting protein	response to steroid hormone stimulus	6.24E-07	4.65E-05
miR-128 (CACAGUG)	1.184	−1.345	2,3	**TGFBR1**	transforming growth factor, beta receptor 1	response to steroid hormone stimulus	6.24E-07	4.65E-05
miR-128 (CACAGUG)	1.184	−1.345	1,2	LDLR	low density lipoprotein receptor	response to steroid hormone stimulus	6.24E-07	4.65E-05
miR-128 (CACAGUG)	1.184	−1.345	1,2	E2F3	E2F transcription factor 3	positive regulation of cell proliferation	1.21E-08	1.67E-06
miR-128 (CACAGUG)	1.184	−1.345	1,2	ADORA2B	adenosine A2b receptor			

Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). MiRNA Target Filter was applied using the strictest criteria (experimentally observed microRNA/gene targets only) filtered for genes previously identified for involvement in endocrine system disorders. Each of the seven miRNAs has multiple gene targets. Fold changes between groups as determined by Illumina miRNA array measurements are shown. Ingenuity target identifications are generated from multiple databases (Source). Pathway enrichments were calculated for the entire gene set using the NIAID DAVID functional enrichment tool [31,32]. Genes that feature in both the cellular proliferation and the steroid hormone pathways are in bold (KRAS, BCL2, TGFBR1).

[ILL-FC] = Illumina array-fold change; Source = 1.miRecords, 2.TargetScan Human, 3.Ingenuity Expert Findings, 4.TarBase; FDR = False Discover Rate.

cycles. Among those, 11 miRNAs exhibited putative estrogen response elements or progesterone response elements in the promoters. In a study of examining gene expression profile in natural cycle and stimulated cycles during luteal phase (LH + 2 or 7; hCG + 2 or 7), Haouzi *et al.* [37] demonstrated that COS regimens altered endometrial receptivity in comparison with natural cycle. These and our studies indicate that ovarian stimulation or altered steroid hormone levels may affect miRNA profiles, consequently, affect endometrial receptivity. Furthermore, we found that the addition of estradiol in the regimen resulted in a significant attenuation of effect of progestone (Figure 1, Figure 2) on the level of miRNA expression. These findings support the notion that the well known anti-proliferative effect of progesterone on the endometrium could be possibly exerted by a localized increase in miRNA expression. The addition of estradiol at the same time could reverse this effect partially by attenuating this increase. Whether this effect is directly or indirectly associated with ovarian stimulation or the type of drug delivery for luteal support (estradiol was administered orally whereas progesterone was administered vaginally in this study) requires further investigation.

By microarray, Northen blot and in situ hybridization, Hu *et al.* [38] was able to identify eight specific miRNAs that were significantly up-regulated at implantation sites. Chakrabarty et al. have showed in the mouse uterus, that two specific miRNAs, the mmu-miR-101a and the mmu-miR-199a*, were differentially expressed during implantation in coordination with the expression of cyclooxygenase-2(Cox-2), a gene critical for implantation [39]. Studies on temporal and spatial regulation of miRNAs in the rat uterus, during embryo implantation, have identified the let-7a and mir-320 specifically in the uterine endometrium with higher expression level on gestation day 6–7 [26,27]. These evidences and our findings of differential expression of miRNAs in the peri-implantation period with and without luteal phase support suggest role(s) of miRNAs during the remodeling process of endometrium in association with implantation.

Neo-angiogenesis is a pivotal process in reproductive function where it regulates endometrial regeneration, corpus luteum formation and finally placentation. The regulatory function of miRNAs in the process of neo-angiogenesis has been illustrated in several in vitro and in vivo models [9]. For example, the role of miRNAs in the neo-angiogenesis has been reported in experiments with Dicerex ½ mouse embryos (altered function of Dicer required for miRNA processing) which suffer from defective angiogenesis, due to disruption in the expression of vascular endothelial growth factor (VEGF) as well as to its receptor flt-1 [40]. We have noticed in our study that several miRNAs including miR-520 g, miR-369-3p, and miR-186 (Table 2), with VEGF as predicted target gene, were differentially regulated during the peri-implantation period. More specifically there was a significant increase in the expression of miR-520 g in the group that received only progesterone as compared to the other groups. In contrast, in the same group, there was a pronounced suppression of miR-221, which is known to regulate endothelial nitric oxide synthase, one of the key regulators of endothelial biology and angiogenesis [41]. Whereas our findings support the regulatory effect of miRNAs in the process of neo-angiogenesis, the precise impact of this action remains obscure. Individual targets of specific miRNAs responsible for the phenotypes have been proposed in experimental settings, although it is likely that many miRNAs function through cooperative regulation of multiple mRNAs [7]. Indeed, Revel et al. evaluated the expression of miRNAs in the secretory endometrium of repeated implantation failure patients and identified 13 miRNAs were differentially expressed (10 were overexpressed and 3 were underexpressed), which putatively regulated the expression of 3800 genes.

In addition, in this study, based on the most stringent criteria requiring experimental observations, IPA miRNA Target analysis for cross validated microRNAs identified 7 out of 9 miRNAs and their gene targets which were further subjected for pathway analysis. The results revealed significant involvement of genes of extracellular matrix, cell proliferation, and response to steroid hormone stimulus from day 0 to day 3–5 after oocyte retrieval in a group with no steroid support (Table 3). Conversely, this effect was almost completely abolished by supplementation of progesterone and estrogen (Table 3, GrpsIIc-IIa) for genes of cellular proliferation and response to steroid hormones bur not for genes of extracellular matrix.

Under the influence of the ovarian steroids the human endometrium undergoes cyclic changes. Estradiol promotes epithelial cell proliferation, while progesterone inhibits this estrogen-induced effect, promotes differentiation, and has decidualizing effects on endometrial stroma later in the secretary phase. We hypothesize that ovarian steroids may regulate multiple genes related to the uterine tissue remodeling and endometrial receptivity, at least in part, through modulating miRNA expression profiles.

We realize that there are several limitations in this study. The relatively small sample size due to limited number of donors that have agreed to participate could represent one of those. Unfortunately due to the design of our experiment it was extremely difficult to obtain more specimens. Furthermore, due to the fact that the same women were biopsied twice during the same COS cycle the first biopsy may induce gene expression differences that are likely to be reflected in the miRNA expression profile of the second biopsy. Additional group(s) with only one biopsy for each subject for a given group and given day of biopsy would provide another layer of control to strengthen the findings in this study. On the other hand, the limited sample size also reflects the difficulty in obtaining these samples. In addition, although group II contains 3 samples in each sub-group, there are 2 samples from day 3 and 1 sample from day 5 which may potentially

affect miRNA profiles. However, after normalization and careful comparison, samples from day 3 and day 5 showed similar expression level on miRNAs profile in the same treatment group. Since day 3–5 are all in mid-secretory period of the cycle, we combined day 3 and day5 samples as one stage of the luteal phase for analysis.

Despite these limitations nevertheless, our array-based global miRNA profiling describes, for the first time, the miRNA expression profile in the human endometrium during the luteal phase following COS for IVF and luteal support with steroid supplementation. We have shown that this profile is under considerable influence by ovarian steroids, even though the molecular mechanism of this interaction still remains unclear. Importantly, several miRNAs found to have enriched or depleted transcript load during the luteal phase may have specific roles in the control of endometrial receptivity. Further studies are necessary to create a detailed expression profile for these miRNAs in relation to their target genes in the endometrium throughout the natural cycle as well as the stimulated cycle for IVF. We plan to further investigate several significantly regulated miRNAs and associated target gene pathways in relation to endometrial receptivity and implantation. Functional study will also be designed to link the imperative miRNAs in potential clinical applications.

Conclusions

The array-based study presented here has revealed several findings: 1) there is an expression of a unique set of miRNAs in the endometrium following controlled ovarian stimulation; 2) the level of expression for these miRNAs undergoes significant changes during the peri-implantation period; 3) the expression is influenced by ovarian steroids; 4) expression of miRNAs may be associated with target genes and gene pathways. The miRNAs found to have enriched or depleted transcript load during the luteal phase may have specific roles in the control of endometrial receptivity during the peri- implantation period through regulation of their target genes. Further studies are necessary to create a detailed expression profile for these miRNAs as well as their associated target genes throughout the natural cycle and the stimulated cycle for IVF in the endometrium. Studies for specifically regulated miRNAs and their target genes as well specific gene pathways in relation to endometrial receptivity and implantation are also proposed.

Additional file

Additional file 1: Table S1. MiRNA with greater than 2 fold changes and/or significantly regulated between comparison groups.

Abbreviations

miRNA: MicroRNAs; COS: Controlled ovarian stimulation; GnRH: Gonadotropic releasing hormones; IVF: In vitro fertilization; hCG: Human chorionic gonadotropin; LH: Luteinizing hormone; FSH: Follicle-stimulating hormone; P: Progesterone; P + E: Progesterone plus 17-beta-estradiol; IPA: Ingenuity pathway analysis.

Authors' contributions
YZ, NV designed the protocol, developed the study, managed specimens, and drafted the manuscript. HZ provided valuable suggestions for the study and actively involved in manuscript preparation. NN participated in data analysis and manuscript preparation. CC, JF carried out microarray analysis, performed the statistical analysis, and contributed in manuscript preparation. All authors read and approved the final manuscript.

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