

The Possible Origin of miRNAs Differentially Regulated in Leiomyoma Progenitors

Mariangela Di Vincenzo

Polytechnic University of Marche: Universita Politecnica delle Marche

Concetta De Quattro

University of Verona: Universita degli Studi di Verona

Marzia Rossato

University of Verona: Universita degli Studi di Verona

Raffaella Lazzarini

Polytechnic University of Marche: Universita Politecnica delle Marche

Giovanni Delli Carpini

Polytechnic University of Marche: Universita Politecnica delle Marche

Andrea Ciavattini

Polytechnic University of Marche: Universita Politecnica delle Marche

Monia Orciani (✉ m.orciani@staff.univpm.it)

Polytechnic University of Marche: Universita Politecnica delle Marche <https://orcid.org/0000-0003-4503-3693>

Research

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Abstract

Background: Leiomyoma are the most common indication for hysterectomy in the world and have a strong economic impact on health care systems; many different mechanisms have been considered for their aetiology, such as inflammation, dysregulated progenitor cells or different regulation of miRNAs. After performing a whole genome miRNA profiling in progenitor cells (PCs) derived from healthy myometrium (MPCs) and from leiomyoma (LPCs), only 15 miRNAs were identified as differentially expressed between MPCs and LPCs. Progenitor cells from Amniotic Fluid (AFPCs) are considered the most undifferentiated cells after the embryonic ones. Here we try to clarify if the miRNAs differently regulated between leiomyoma and myometrium cells arise as a conversion of MPCs along the differentiation process or if they may originate from a divergent cell commitment. To track the origin of the dysregulation, miRNA expression was analyzed in AFPCs (considered as surrogate for embryonic cells), MPCs and LPCs.

Methods: MPCs, LPCs and AFPCs were isolated and subjected to whole genome miRNA profiling; the expression of the 15 miRNAs previously identified as differentially regulated in MPCs and LPCs was compared to that detected in AFPCs.

Results: Clustering analysis sub-grouped the 15 miRNAs into 4 major clusters that converge to the KEGG pathways: Adherens junction, ECM-receptor interaction, TGF β signaling and cell cycle. miRNAs are differentially regulated in MPCs and LPCs compared to AFPCs and 10/15 of them show statistically significant variations between MPCs and LPCs.

Conclusion(s): Our results seem to point that a linear physiological differentiation axis exists from AFPCS to MPCs that, under particular insults, pathologically continues toward LPCs. Clustering analysis sub-grouped the 15 miRNAs into 4 major clusters that converge to the KEGG pathways: Adherens junction, ECM-receptor interaction, TGF β signaling and cell cycle. miRNAs are differentially regulated in MPCs and LPCs compared to AFPCs and 10/15 of them show statistically significant variations between MPCs and LPCs.

Our results seem to point that a linear physiological differentiation axis exists from AFPCS to MPCs that, under particular insults, pathologically continues toward LPCs.

Background

Uterine fibroids, also called leiomyomas, are the most common benign gynaecologic tumors in the reproductive years, with an incidence directly related to age [1, 2]. Their aetiology is not fully clear and epigenetic mechanisms, gene mutations, chronic inflammation, disrupted controls in progenitor cells, dysregulation of miRNAs have been all evaluated as potential causes.

Previously [3–5], we demonstrated that progenitor cells (PCs) isolated from fibroids and normal myometrium: i) can differently sustain acute and chronic inflammation promoting a microenvironment

suitable for leiomyoma onset; ii) out of 2646 miRNAs, only 15 miRNAs were differentially expressed in a robust manner between leiomyoma and normal myometrium, supporting the hypothesis that leiomyoma derives from alterations affecting progenitor cells.

Today it is noted that a population of progenitor cells occurs in almost all human tissues [6]. They are referred to as mesenchymal stem cells and, according to the criteria by Dominici [7], must be plastic adherent, positive for CD73, CD90, CD105 and negative for HLA-DR, CD14, CD19, CD34, CD45 and able to differentiate towards oste-, chondro- and adipogenic lineages. While satisfying these criteria, mesenchymal cells deriving from adult tissues show tissue-specific features that during differentiation become even more characterizing [8]. Among mesenchymal stem cells, amniotic fluid progenitor cells (AFPSCs) are of particular interest, since they express both adult and embryonic cell markers, indicating them as cells within an intermediate stage between embryonic and adult phenotype [9]. In a theoretical line of increasing differentiation, AFPSCs should be before PCs of myometrium and fibroids, having a lower degree of differentiation than the latter.

miRNAs are switchers able to modulate the cell fate by turning on/off specific gene targets and their aberrant expression can proportionally affect these critical processes leading to various pathological outcomes [10, 11]. We recently demonstrated that 15 miRNAs are differentially expressed between PCs from leiomyoma and healthy myometrium⁴, but we still don't know the mechanisms underlying these differences and its origin. In particular, when do these alterations occur? Do they arise during differentiation from embryonic cells to PCs (ie, differential miRNA expression leads to different PCs in normal myometrium and fibroids, hypothesis A, Fig. 1a) or later during the differentiation process (ie, miRNA dysregulation affects PCs from myometrium initially and subsequently exacerbates thus determining the acquisition of PCs with fibroids' features, hypothesis B, Fig. 1b)? Since the use of embryonic cells to answer these research questions is forbidden, AFPSCs that maintain a lower degree of differentiation than PCs were used as surrogate of the embryonic ones, with the aim to follow the onset of these alterations during the differentiation process.

Methods

Tissue collection

Amniotic fluid (AF) samples (n° 9), 3 ml, were obtained from amniocentesis after the 16th week of pregnancy for routine prenatal diagnosis. The gestation age (GA) was determined by ultrasonic biparietal diameter and femur length measurements of the foetus. AF was collected by ultrasound-guided transabdominal puncture. The indications were advanced maternal age (34–36) and the cytogenetic analyses revealed normal karyotypes.

Healthy and fibrotic myometrium samples (n°12), 3mm², were obtained from women of childbearing age (range, 30–35 years) undergoing myomectomy for symptomatic leiomyomas.

All patients provided their written informed consent to participate in the study, which was approved by the institutional ethics committees (2016-03600R) and was conducted in accordance with the Declaration of Helsinki.

Cells were isolated, cultured and characterized as previously described [3, 12, 13]. After samples collection, cells were characterized by testing the minimal criteria identified by Dominici [7] for mesenchymal definition.

Briefly, for immunophenotyping 2.5×10^5 cells at 3rd passage were stained for 45 min with fluorescein isothiocyanate (FITC)-conjugated antibodies (Becton-Dickinson) against: HLA-DR, CD14, CD19, CD34, CD45, CD73, CD90 and CD105. Differentiation into osteocytes, chondrocytes and adipocytes was assessed using STEMPRO® Osteogenesis, Chondrogenesis and Adipogenesis Kits (GIBCO, Invitrogen,) respectively. Cells cultured in DMEM/F-12 with 10%FBS were used as negative controls.

Osteogenic differentiation was assessed by Von Kossa and Alkaline phosphatase (ALP) stainings; adipogenic differentiation was tested by Oil Red staining.

For chondrogenesis, cells were cultured in pellet culture system and sections were exposed to a solution of Safranin-O [14].

miRNA profiling

Total RNA was extracted in triplicate using Norgen Total RNA Kit (Norgen, Biotek Corporation, Thorold, ON, Canada) from a pool of mixed cells obtained from the twelve cultures of MPCs (Myometrium Progenitor Cells) or of LPCs (Leiomyoma Progenitor Cells) and from the nine cultures of AFPSCs in triplicate. RNA purity and amount were measured using a NanoDrop Spectrophotometer (NanoDrop Technologies, INK, Wilmington, DE, USA), while RNA integrity (RNA integrity number ≥ 8.0) was assessed using RNA 6000 Nano Kit (Agilent Technologies). miRNA-sequencing libraries were generated with the QiAseq miRNA kit (QIAGEN), assessed by capillary electrophoretic analysis with the Agilent 4200 Tape station and sequenced using 1X75bp-reads on an Illumina NextSeq500 generating about 8 million fragments per sample.

Bioinformatics analysis

Starting from raw FASTQ files, quality of reads obtained from each sample was assessed using FastQC software (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), adapters were trimmed and reads with length < 18 bp or > 27 bp were filtered out with cutadapt (1.16). Filtered reads were aligned to the miRBase 22 using SHRiMP2 (2.2.3). Differential miRNA expression analysis between LPCs, MPCs and AF-MSCs was performed with DESeq2 (1.16.1). The expression of microRNAs was normalized as CPM (counts per million reads mapped) and subsequently the 15 miRNAs previously identified as differentially expressed between MPCs and LPCs were clustered by an unsupervised hierarchical clustering using spearman rank correlation and the average linkage method.

miRNAs targets analysis

To identify molecular pathways potentially altered by the expression of single or multiple miRNAs, Diana mir- Path Software was used [15]. This web-based application performs enrichment analysis of numerous miRNA target genes comparing each set of miRNA targets to all known KEGG (Kyoto Encyclopedia of Genes and Genomes, Kyoto, Japan) pathways.

The input dataset enrichment in each KEGG pathway is represented by the negative natural logarithm of the *P* value ($-\ln P$).

Results

MPCs, LPCs and AFPCs respectively isolated from healthy myometrial, fibroid tissue and amniotic fluids met the three criteria established by the International Society for Cellular Therapy (ISCT) for the identification of human mesenchymal stem cells [7]. Cells were plastic-adherent in standard culture conditions, strongly positive for CD105, CD73, and CD90 and negative for CD45, CD34, CD14, CD19, and HLA-DR surface molecules and able to differentiate towards mesenchymal lineages in vitro (data not shown) [16–18].

RNA obtained from the three cellular pools was used for miRNA profiling by RNA-sequencing, which was performed in triplicate [5]. Principal Component Analysis reported in Fig. 2 is based on more than 2600 miRNAs expressed in the samples analysed and shows that MPCs and LPCs present with a more similar miRNA expression profile as compared to AFPCs. Subsequently, we retrieved the expression of the 15 miRNAs previously found with a differential expression in MPCs versus LPCs, namely hsa-miR-146b-5p; hsa-miR-335-3p; hsa-miR-335-5p; hsa-miR-135b-5p; hsa-miR-10a-3p; hsa-miR-10a-5p; hsa-miR-200a-3p; hsa-miR-146a-5p; hsa-miR-576-3p; hsa-miR-122-5p; hsa-miR-1973; hsa-miR-595; hsa-miR-658; hsa-miR-4284; hsa-miR-924. Clustering analysis sub-grouped the 15 miRNAs into 4 major clusters according to their expression level across the cell type analyzed (Fig. 3a). The clusters were composed by miRNAs that converged to the following KEGG pathways: Adherens junction (has-miR-10a-5p, has-miR-10a-3p, has-miR-135b-5p, has-miR-200a-3p, 27 targeted genes), ECM-receptor interaction (has-miR-146b-5p, has-miR-335-3p, has-miR-335-5p, 32 targeted genes), TGF β signaling (has-miR-122-5p, has-miR-576-3p, has-miR-595, has-miR-1973, 21 targeted genes) and cell cycle (has-miR-924, has-miR-146a-5p, has-miR-4284, has-miR-658, 13 targeted genes).

miRNAs converging to the four pathways were differentially expressed in MPCs and LPCs compared to AFPCs and 10/15 of them showed statistically significant variations between MPCs and LPCs (Fig. 3b). In detail, the four miRNAs that converge to the KEGG pathway “adherens junction” are significantly downregulated in MPCs and mostly in LPCs compared to AFPCs; the three miRNAs targeting genes related to ECM-receptor interaction pathway show a significant increase in MPCs compared to AFPCs, while their expression is reduced in LPCs versus AFPCs. The expression of the four miRNAs involved in TGF β signaling pathway is notably higher in LPCs than in AFPCs whereas it does not change in 2 of 3 miRNAs in MPCs compared to AF-MSCs; finally, all the four miRNAs related to cell cycle are significantly downregulated in MPCs while only two were significantly reduced in LPCs compared to AFPCs

Discussion

The expression of 15 miRNAs previously identified as differentially regulated between myometrial PCs (MPCs) and leiomyoma PCs (PCs) [5] was compared to that observed in Amniotic Fluid PCs (AFPCs) to evaluate if the dysregulation of the 15 miRNAs occurs: i) as the result of an early commitment of undifferentiated cells (Fig. 1A) or ii) as an exacerbation of the physiological differentiation from AFPCs to MPCs (Fig. 1B). The regulation of the expression level of the miRNAs across the cell type analysed does not allow giving unique suggestions.

Firstly, cells isolated from healthy and fibrotic uterine tissues as well as from amniotic fluids were characterized according to the minimal criteria established by the International Society for Cellular Therapy (ISCT) for the identification of human mesenchymal stem cells⁷ and were able to satisfy them.

After characterization, the first issue to address was the validation of the choice of AFPCs as the most undifferentiated cells to be used as a surrogate of the embryonic ones [9, 19]. Consistent with the fact that AFPCs are the most undifferentiated cells among the three cell types analyzed, they cluster separately from MPCs and LPCs that are instead closer to each other.

The heat map and the histogram show that the expression of the majority of 15 miRNAs is very different between AFPCs and LPCs/MPCs (mean of absolute FC 2.6 ± 2.8). Clustering analysis followed by DIANA mir-Path investigation allowed identifying four KEGG pathways related to the miRNA-clusters defined based on expression: Adherens junction, ECM-receptor interaction, TGF β signaling and cell cycle.

To address the question of which hypothesis was more consistent (A, early commitment; B, exacerbation), the trend of expression across the cell types of each identified pathway was evaluated. Only adherens junction and TGF β signaling pathways show a step-wise manner along the differentiation process.

To investigate what is the most probable hypothesis explaining the origin of LPCs, (A, early commitment; B, exacerbation), we evaluated in details the pattern of miRNA expression across the three cell-types. Only the 4 miRNA linked to regulation of adherens junction and one linked to TGF β signaling pathways (miR-122-5p) show a progressive decreased or increased expression (respectively) from AFPCs to LPCs, ie along the hypothetical differentiation process.

Interestingly, LPCs expressed the lowest values of miRNAs related to adherens junction pathway and, most-likely as consequence, also the highest level of targeted proteins. As expected, the expression of adhesion molecules is higher in connective tissues where fibroblasts/myofibroblasts develop strong bounds to ECM than in AFPCs [20]. While the increase in the expression of proteins related to adherens junction from AFPCs to MPCs has to be considered physiological, its further weighting towards LPCs may be connected to the leiomyoma onset [21, 22].

Notably MED12, implicated as an oncogene in about 70% of uterine leiomyoma [23, 24], is a target of hsa miR-10a-5p, whose expression is gradually reduced from AFPCs up to LPCs, possibly explaining MED12

upregulation in leiomyoma.

While the expression of the three miRNAs converging in TGF β signalling pathway (has-miR-122-5p; has-miR-576-3p; has-miR-595) is weakly increased in MPCs compared to AFPCs, they are strongly upregulated in LPCs. The TGF β pathway has been reported as deeply dysregulated in leiomyoma [25–27]; the genes targeted by these miRNAs belong to a cascade that involves SMAD 2 and SMAD4 and leads to apoptosis and G1 cell cycle arrest; the high expression of the selected miRNAs in LPCs may intervene by suppressing these proteins. This is in line with previous researches [27] reporting that leiomyomas are refractory to the antiproliferative effects of TGF β 1 and TGF β 3 observed in normal myometrium.

For the other two pathways, the expression of miRNAs across the cell type does not show a linear trend that could reinforce the hypothesis of exacerbate differentiation from MPCs to LPCs. Previous studies about miRNA profiling from tissues samples of fibroid and myometrium identified a list of involved pathways that encloses the four here considered [28].

Interestingly, even if our pathways are enclosed in the list, the identified miRNAs are different.

It is not surprising that more miRNAs target the same genes but that the same pathways have been found differentially regulated in progenitor as well as in differentiated cells confirms the involvement of the selected pathways in the onset of leiomyoma. Indirectly, it also strengthens the hypothesis that the modulation of the expression of miRNAs during differentiation is a key mechanism in the pathogenesis of leiomyoma.

Conclusion

In conclusion, the regulation of miRNA expression across the cell type analysed does not lead to a unique conclusion: for adherens junction and TGF β signalling pathways, it seems that the differences previously detected between leiomyoma and myometrium cells could arise in step-wise manner along the differentiation process from AFPCs, whereas results from cell cycle and ECM-receptor interactions pathways suggest that the different expression of miRNAs may originate from a divergent cell commitment. Nevertheless, by combining our results with others previously reported, data seem to point toward hypothesis B, namely that a linear physiological differentiation axis exists from AFPCS to MPCs that, under particular insults, pathologically continues toward LPCs.

These results may support and integrate previous hypotheses about leiomyoma development: miRNAs play a pivotal role acting directly at the first steps of PCs differentiation and, as last hypothesis, creating the observed (epigenetic) predisposition.

Abbreviations

PCs: Progenitor Cells

MPCs: Myometrium Progenitor Cells

LPCs: Leiomyoma Progenitor Cells

AFPCs: Amniotic Fluid Progenitor Cells

miRNA: microRNA

KEGG: Kyoto Encyclopedia of Genes and Genomes

ECM: Extra Cellular Matrix

TGF β : Transforming Growth Factor β

GA: Gestation Age

CD: Cluster of Differentiation

FITC: Fluorescein Isothiocyanate

DMEM/F12: Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12

FBS: Fetal Bovine Serum

ALP: Alkaline phosphatase

CPM: counts per million

$-\ln P$: negative natural logarithm of the P value

PCA: Principal Component Analysis

ISCT: International Society for Cellular Therapy

MED12 - mediator complex subunit 12

SMAD: Small Mother Against Decapentaplegic

Declarations

Authors' contributions:

MDV: analyses and interpretation of cellular data, writing original draft; **CDQ** and **MR**: miRNA analysis production and elaboration; draft the manuscript; **RL**: contribution to data interpretation and draft discussion; **GDC**: sample collection and draft discussion; **AC**: sample collection and the related data interpretation, participation to study design and coordination; **MO**: conceptualization, supervision, writing -review & editing

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Availability of data and material:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

Ethics approval and consent to participate:

institutional ethics committees (2016-03600R); all patients provided their written informed consent to participate

Consent for publication:

not applicable

Competing interest:

The authors declare that they have no competing interests

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none

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Figures

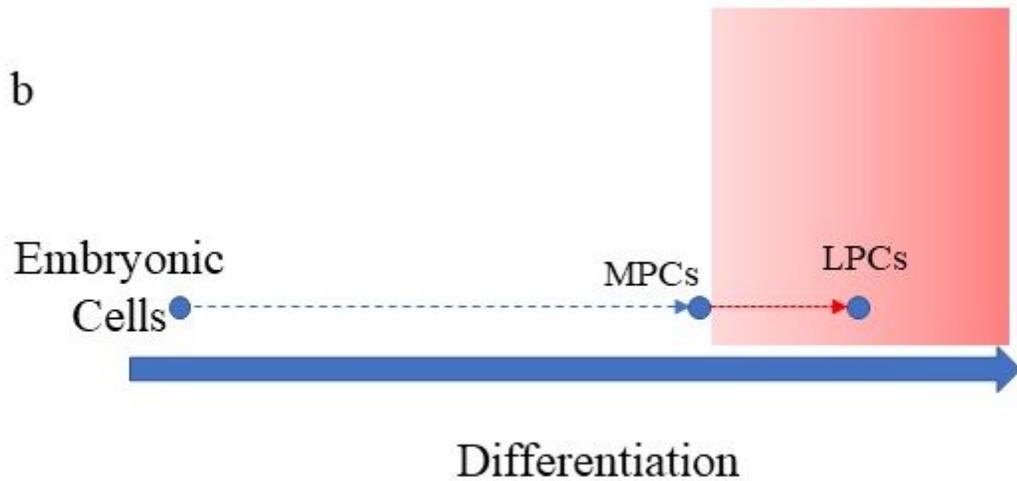
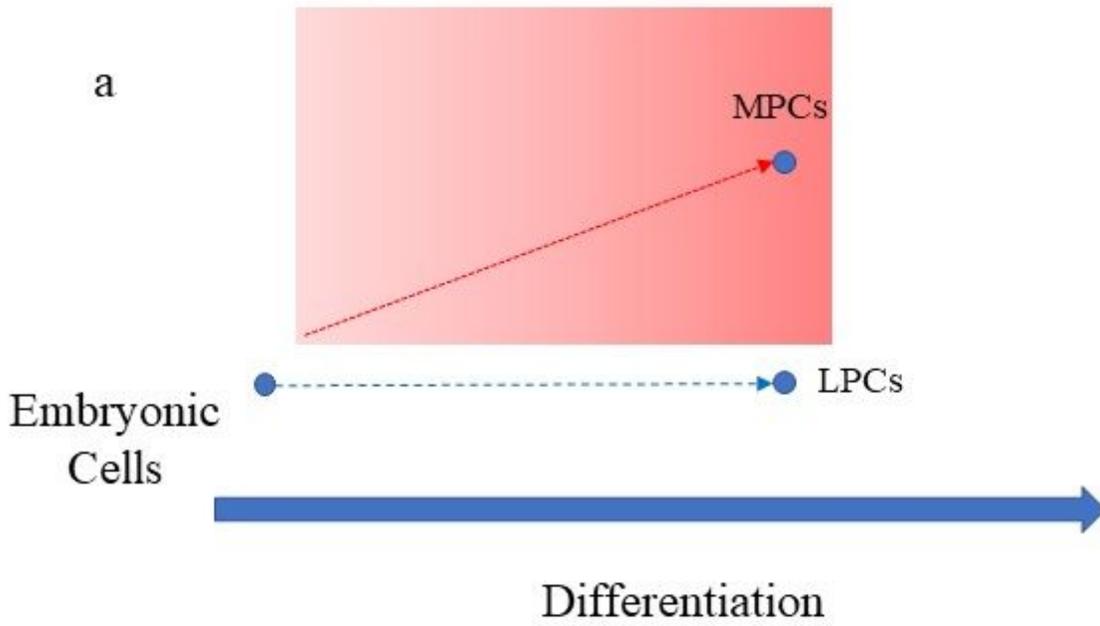


Figure 1

Two different hypotheses for the origin of miRNAs differentially regulated between MPCs and LPCs. (A) MPCs and LPCs are the results of divergent differentiation from embryonic stem cells; (B) embryonic stem cells physiologically differentiate in MPCs and a further pathological differentiation produces LPCs. Red areas indicate the spectrum of pathology.

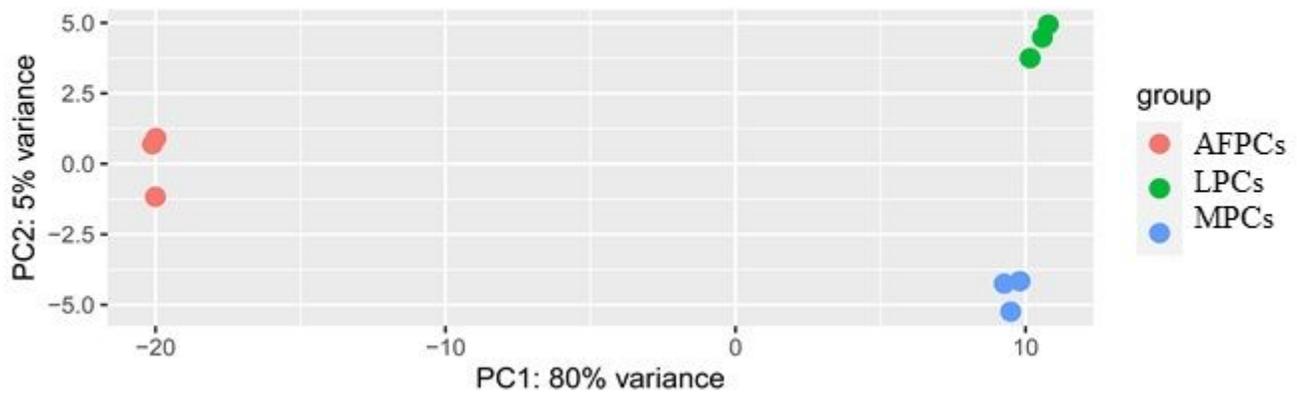


Figure 2

Principal Component Analysis (PCA) showing the variance of samples analysed in the study, based on the full miRNA profile (2646 expressed miRNAs). The analysis was conducted using the DESeq2 package and the values on each axis represent the percentages of variation explained by the principal components. PC1, principal component 1; PC2, principal component 2.

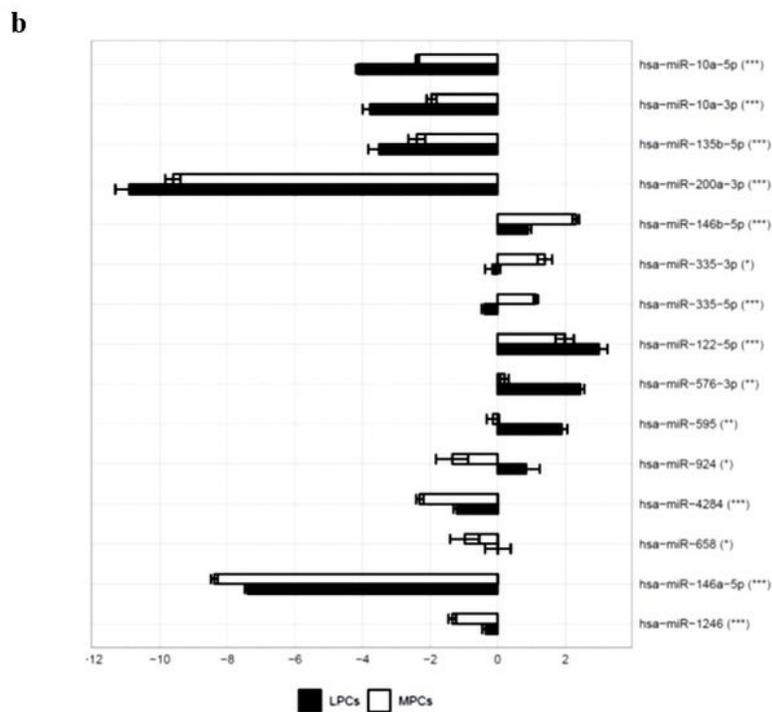
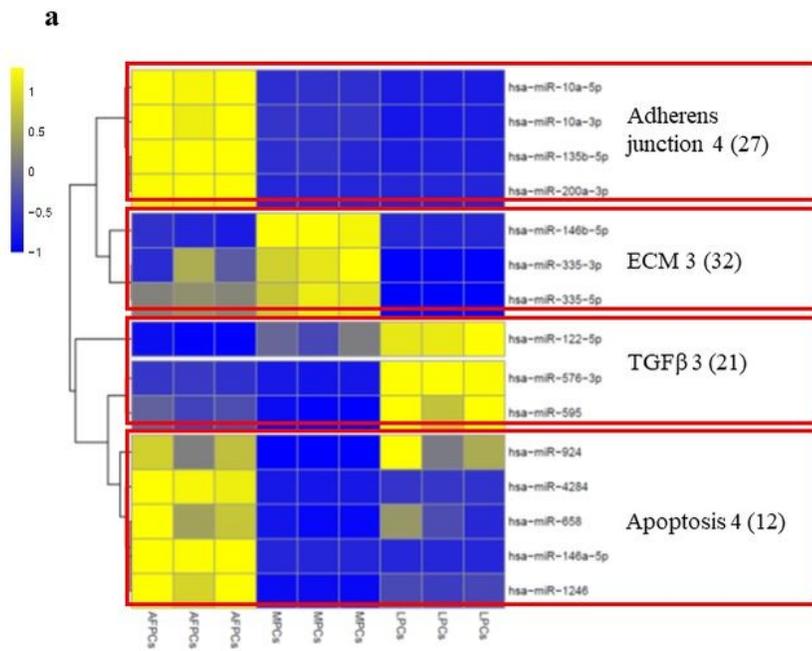


Figure 3

Heat map and expression fold change of 15 miRNAs previously identified as differentially expressed between MPCs and LPCs based on next generation sequencing analysis. (A) Each row represents a different miRNA, and each column represents one sample from the AFPC, MPC or the LPC RNA pool of 12 specimens. Clusters were obtained by unsupervised hierarchical clustering using spearman rank correlation and the average linkage method. Colour key illustrates normalized expression levels (z-scores

of normalized counts - cpm) of miRNAs across all samples. Gradient from yellow to blue indicates higher to lower expression. In the right column, the KEGG pathways identified by DIANA-miRPath software as targets of the analysed miRNAs. In brackets the number of targeted genes per pathways. (B) Expression fold changes (log₂-transformed) of the 15 selected miRNAs in MPCs and LPCs as compared to AFPCs. adjusted P-value <0.05: *** both comparisons, ** only LPCs, * only MSCs.