Downregulated expression of hsa-miR-181c in Fanconi anemia patients: implications in TNFα regulation and proliferation of hematopoietic progenitor cells

SHORT TITLE: The Role of hsa-miR-181c in Fanconi Anemia

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ABSTRACT

Fanconi anemia (FA) is an inherited genetic disorder associated with BM failure and cancer predisposition. In this study we aimed to elucidate the role of miRNAs in the hematopoietic defects observed in FA patients. Initial studies showed that three miRNAs, hsa-miR-133a, hsa-miR-135b and hsa-miR-181c were significantly down-regulated in lymphoblastoid cell lines and fresh peripheral blood cells from FA patients. In vitro studies with cells expressing the luciferase reporter fused to the TNFα 3'UTR confirmed in silico predictions suggesting the interaction of hsa-miR-181c with TNFα mRNA. These observations were consistent with the downregulated expression of TNFα mediated by hsa-miR-181c in cells from healthy donors and FA patients. Because of the relevance of TNFα in the hematopoietic defects of FA patients, BM cells from FA patients were transfected with hsa-miR-181c to evaluate the impact of this miRNA in their clonogenic potential. Strikingly we observed that hsa-miR-181c markedly increased the number and size of the myeloid and erythroid colonies generated by FA BM cells. Taken together our results offer new clues to understand the biological basis of the BM failure in FA patients, and open new perspectives for the treatment of the hematological dysfunction in FA patients based on miRNA regulation.
INTRODUCTION

MicroRNAs (miRNA) are 20-24 nucleotide (nt) RNAs that act regulating gene expression mainly by destabilizing target mRNAs through partial base pairing complementary sites. Since their original description, multiple miRNAs have been shown to regulate the development and differentiation of different tissues, including the hematopoietic system. Additionally, miRNAs have been shown to act as tumor suppressors or oncogenes in the development of hematological malignancies.

Because Fanconi anemia (FA) is a complex disease mainly associated with BM failure and cancer predisposition, we aimed to investigate whether specific miRNAs were deregulated and play a role in the hematopoietic dysfunction of FA patients. Among the hematological dysfunctions of FA patients, macrocytosis is often the first detected abnormality, followed by thrombocytopenia and neutropenia. A high incidence of cancer, principally acute myeloid leukemia (AML) and squamous cell carcinomas is also associated with FA. Data from the International FA Registry showed that in these patients the actuarial risk of developing BM failure, hematological and non-hematological malignancies by 40 years of age is 90%, 33% and 28%, respectively. Differences in the clinical symptoms of FA patients are difficult to interpret. Nevertheless, because FA is a chromosomal instability disorder, FA cells accumulate DNA damage at an increased rate. As recently reviewed, unrepaired DNA damage, may activate apoptotic pathways, thus potentially leading to BM failure, or induce additional mutations and translocations that may finally result in solid tumors or leukemias.

Although the exact function of FA proteins is not clearly understood, eight FA proteins (FANCA, B, C, G, F, E, M and L) interact to form a FA core complex, responsible for the monoubiquitination of FANCD2 and FANCI, known as the ID complex. After monoubiquitination, both proteins migrate to sites of DNA damage, forming DNA repair foci in association with other proteins, including FANCJ/BRIP1, FANCD1/BRCA2, FANCN/PALB2. An additional molecule, the nuclease FAN1, has been recently shown to be an essential partner in the FA pathway due to its interaction with the ID complex and its...
further recruitment to sites of DNA damage\textsuperscript{16-19}. Finally, the observation that FANCP (SLX4)\textsuperscript{20,21} had endonuclease activity, is allowing to unravel the role of the FA/BRCA pathway in the repair of DNA interstrand cross-links (ICLs) during replication\textsuperscript{22}.

In the present study, miRNA expression analyses in lymphoblastic cell lines (LCLs) and peripheral blood (PB) cells from FA patients and healthy donors showed that three different miRNAs were specifically down-regulated in FA hematopoietic cells. \textit{In silico} studies together with \textit{in vitro} experiments of gene interference evidenced that one of the miRNAs that was consistently down-regulated in FA samples was \textit{hsa-miR-181c}. Moreover, our results demonstrate that this miRNA interacts with TNF\textalpha{}, playing an important role in the functional properties of FA hematopoietic progenitors.

**MATERIALS AND METHODS**

**Cell lines and primary cells.**

FA patients were diagnosed based on clinical symptoms and chromosome breakage tests of PB cells using DNA cross-linker drugs\textsuperscript{23}. Patients and healthy donors (HD) were encoded to protect their confidentiality, and informed consents were obtained in all cases according to Institutional regulations. Epstein-Barr virus-transformed LCLs from FA patients were grown in RPMI medium (Gibco) supplemented with 15% FBS, Glutamine and antibiotics (0.5% of Penicillin and Streptomycin).

Peripheral blood mononuclear cells (MNC) were obtained by Ficoll fractionation. Bone Marrow samples were depleted from erythrocytes with Hydroxyethyl Starch (HES) as previously described\textsuperscript{24} and cultured in IMDM (Iscove’s modified medium) supplemented with 20% FBS and antibiotics (0.5% of Penicillin and Streptomycin) in the presence of thrombopoietin (TPO, 50 ng/ml; R&D Systems, Minneapolis, MN), stem cell factor (SCF, 150 ng/ml; Peprotech, London, UK) and Flt3ligand (Flt3-L, 50 ng/ml; Invitrogen, Carlsbad, CA).
The RKO cell line (colon carcinoma cell line) was grown in DMEM supplemented with 10% FBS, Glutamine and antibiotics (0.5% of Penicillin and Streptomycin).

RNA extraction, reverse transcription and Real-Time PCR quantification of miRNAs.

A number of 2-5.10^6 cells either from LCLs or PB from healthy and FA-A patients was used for RNA extraction using the Trizol total RNA isolation reagent (Molecular Probes). cDNA was synthesized using gene specific primers designed by Applied Biosystems (http://www5.appliedbiosystems.com/tools/mirna/) following the Taqman MicroRNA assay protocol (PE Applied Biosystems, Foster City, CA). Reaction was prepared as previously published. Real Time qPCR was performed using and Applied Biosystems 7300 Sequence Detection system and a Rotor Gene using 1.33 μl of RT product, 1xTaqman Universal PCR master mix and 1μl of primers and probe mix of the Taqman MicroRNA assay protocol (PE Applied Biosystems). The reaction was incubated at 95ºC for 10 min, followed by 40 cycles of 95ºC for 15s and 60ºC for 10 min. The Ct data was calculated using default threshold settings. Normalization of the data was done using RNU6B gene as endogenous control. Relative quantification of expression of analyzed miRNAs was calculated with the 2^ΔΔCt method (Applied Biosystems. User Bulletin N°2 (P/N 4303859)). Data are presented as log 2^ΔΔCt of the relative quantity of miRNAs, normalized and compared with the mean relative expression value of control cell lines or PB samples. A supervised analysis using the SAM algorithm (Significant Analysis of Microarrays) was performed in order to identify miRNAs with statistically significant changes in expression between both groups.

DNA methylation analysis

DNA was extracted using QIAmp DNA Mini Kit (Qiagen, Hilden, Germany). Methylation specific PCR (MSP) was used to analyze the methylation status of the CpG sites located 5’ upstream of hsa-miR-181c and was performed as
previously described. For hsa-miR-181c-MSP primers hsa-miR-181c-MD (5´-GTTTCGTAGATTAGGTTAGGCG-3´) and hsa-miR-181c-MR (5´-CAATAATCGCACAATTCGAC-3´) for the methylated reaction, which amplify a 151 bp product and primers hsa-miR-181c-UD (5´-GTTTGTAGATTAGGTTAGGGTG-3´) and hsa-miR-181c-UR (5´-CTCAATAATCTCAACAAATTCGAC-3´) for the unmethylated reaction, which amplify a 154 bp product were used. PCR conditions for hsa-miR-181c-MD/hsa-miR-181c-MR primers were 94ºC for 10 min, followed by 35 cycles at 94ºC for 1 min, 60ºC for 1 min and 72ºC for 1 min. PCR conditions for hsa-miR-181c-UD/hsa-miR-181c-UR primers were 94ºC for 10 min, followed by 35 cycles at 94ºC for 1 min, 58ºC for 1 min and 72ºC for 1 min. The final extension was at 72ºC for 10 min. The products were separated by electrophoresis on 1.8% agarose gel.

The 4 CpG dinucleotides that are directly located in the genomic sequence that results in mature miRNA hsa-miR-181c, were analyzed by the technique of pirosequencing as previously described. After bisulfite treatment of DNA, “hot start”PCR was performed using hsa-miR-181c-PD (5´-TTTATGAGGAAAAGGGGTTTTTAT-3´) and hsa-miR-181c-PR (biotine-5´-AAAAATAACAAATCTCAACAAATTCGAC-3´). PCR conditions were 94ºC for 10 min, followed by 40 cycles at 94ºC for 1 min, 58ºC for 1 min and 72ºC for 1 min. The final extension was at 72ºC for 10 min. The resulting biotinylated PCR products were immobilized to streptavidin Sepharose High Performance beads (GE healthcare, Uppsala, Sweden) and processed to yield high quality ssDNA using the PyroMark Vacuum Prep Workstation (Biotage, Uppsala, Sweden), according to the manufacturer’s instructions. The pyrosequencing reactions were performed using the Pyromark™ ID (Biotage, Uppsala, Sweden) and sequence analysis was performed using the hsa-miR-181c-SEQ1 (5´-ATGTTTTTTGTTTTTTGTTATTTTATTTA-3´) and hsa-miR-181c-SEQ2 (5´-TTTGTGTTATGTTTTTTGTTATTTTATTTA-3´) primers and the PyroQ-CpG analysis software (Biotage, Uppsala, Sweden).

Human male genomic DNA universally methylated for all genes (Intergen Company, Purchase, NY) was used as a positive control for methylated alleles (not shown). Water blanks were included with each assay. The results were
always confirmed by repeating MSP assays or pyrosequencing after an independently performed bisulfite treatment.

**Cells Transfection and transduction with miRNAs.**

Lymphoblast cell lines and primary BM samples from FA-A patients were transfected either with a negative-precursor miRNA control (Pre-miR control #1, Applied Biosystems) or a Pre-miR-181c precursor molecule using siNeoFX reagent (Ambion). Briefly, retronectin coated plates were used to transfect 2.5x10⁵ LCLs or BM cells with Pre-miR molecules. Transfection was repeated after 24h, and 48h later cells were harvested for functional and expression assays. To determine the transfection efficiency in these experiments, a carboxyfluorescein (FAM™) dye-labeled Pre-miR Negative Control (Applied Biosystems) was used. The transfection efficiency was determined as the percentage of FITC positive cells, measured by flow cytometry two days after transfection. Cell viabilities were determined by analyzing the proportion of propidium iodide negative cells by flow cytometry (EPICS XL; Coulter Electronics, Hialeah, FL).

To stably transduce BM cells from FA patients, infective supernatants containing lentiviral vectors carrying the Pre-miR-181c and the EGFP genes (Pre-miR-181c-EGFP LV; System Biosciences) or a control vector (EGFP LV) were generated as previously described ²⁷. Briefly, 5x10⁵ FA-BM cells were cultured in IMDM supplemented with 20% FBS and 300ng/ml hSCF (Peprotech, London, UK), 100ng/ml hTPO (R&DSystems, Minneapolis, MN) and 100ng/ml Flt3-L (Invitrogen, Carlsbad, CA) in retronectin-coated wells (Retronectin, Takara Shuzo, Otsu, Japan). Two rounds of infection were conducted with either LV: Pre-miR-181c-EGFP or LV:EGFP and the efficiency of transduction was analyzed by the percentage of EGFP⁺ cells measured by flow cytometry in a Becton Dickinson FACS LSRFortessa.

**mRNA expression analyses of target genes.**

BM samples transfected with Pre-miR control or Pre-miR-181c were harvested 48h after transfection and RNA was extracted with trizol as previously
described. Total RNA was obtained and the expression of *TNF*α, *IL-1*β, *RAD54B* and *β-ACTIN* was studied with Sybergreen (Applied Biosystems) using the following primers:

**TNF*α-F**: 5’-CAGCCTCTTCTCTTCTTGAT-3’ and **TNF*α-R**: 5’-GCCAGAGGGCTGATTAGAGA-3’.

**Il-1*β-F**: 5’-CTGTCTGCGTGGTTGAAAGA-3’ and **IL-1*β-R**: 5’-TTGGGTAATTTTGGGATCTACA-3’.

**RAD54B-F**: 5´-TCATGATCTGCTTGACTGTGAG-3’ and **RAD54B-R**: 5’-TTTTTCCAACGAATCACCTGT-3’.

**hDNA-RNA-βACTIN-F**: 5´-ATTGGCAATGAGCGGTTCC-3’ and **hRNA-βACTIN-R**: 5´-CACAGGACTCCATGCCC-3’.

**TNF*α analyses.**

Intracellular levels of TNFα in erythrocyte-depleted BM cells previously transduced or transfected with miRNA constructs were determined by flow cytometry 48 hours after miRNA transfection. Samples were incubated for five hours either with 50ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma), with lipopolysaccharide (LPS;1µg/ml; Sigma), or with resiquimod (R848;1 µg/ml; Alexis Biochemicals, Paris, France), together with 5 µg/ml brefeldin A (Sigma), which inhibits intracellular transport of cytokines into the Golgi complex, avoiding the extracellular release of the cytokines. After the O.N. incubation, cells were washed in PBA (PBS+0,1% BSA) and incubated for 30 min. at 4ºC with anti-CD45-FITC or anti-CD45-PC5 (Beckman Coulter,Inc., Brea, CA). Stained cells were resuspended for 20 min. at 4ºC in Cytofix/Cytoperm fixation/permeabilization solution (BD Biosciences, San Diego,CA) according to the manufacturer’s instructions. After permeabilization, cells were washed twice with washing buffer and stained for intracellular TNFα using an anti-TNFα-PE antibody (BD Biosciences). Flow cytometry analysis was conducted in a Becton Dickinson FACS LSRFortessa.
Luciferase reporter assays.

For reporter assays a region of WT 3’UTR from TNFα, the 3’UTR from TNFα mutated, 3’UTR from IL1β and the Target hsa-miR-181c (which is perfectly complementary to hsa-miR-181c sequence) were constructed annealing the following primers:

3’UTR-TNF-α.F: 5’-CTAGTATTATTTATTTACAGATGAATGTATTTATTTGGGAGACCGGGGTA-3’ and 3’UTR-TNF-α.R: 5’-AGCTTACCCCGGTCTCCCCAAATAAAATACATTCATCTGTAATAAATAAATAAAT TA -3’

3’UTR-TNF-α mutated. F: 5’-TCGAGATTATTTATTTACAGATCTGCGTATTTATTTGGGAGACCGGGG TGC-3’ and 3’UTR-TNF-α mutated. R: 5’-GGCCGCACCCCGGTCTCCCCAAATAAAATACGCAGATCTGTAAATTAATAAAT AATC-3’

3’UTR-IL1-β.F: 5’-TCGAGGTACCAGAGAGTGCTGTGCTGAATGTGGACTCAATCCCTAGGGC TGGGC-3’ and 3’UTR-IL1-β.R: 5’-GGCCGCCAGCCCTAGGGATTTTGACACATTCAGCACAGGACTCTCTGG GTACC-3’

3’UTR-Target miRNA181c.F: 5’-TCGAGATTATCTACCCAGAGGTTGTAATGTGGACTCAATCCCTAGGGC GTGC-3’ and 3’UTR-Target miRNA181c.R: 5’-GGCCGCACCCCGGTCTCCCCAAATAAAACATTCAACCTGTGAGTGTA TAATC-3’

Annealing was conducted incubating both primers 4 min. at 95°C and 10 min. at 70°C in annealing buffer (100mM potassium acetate, 30mM HEPES pH7.4 and 2mM magnesium acetate). Primers where then phosphorylated and cloned into the siCHECK2 vector from Promega digested with Xho I and Not I.
Fifty thousand RKO cells were plated in DMEM containing 10% FBS. 24h later cells were transfected with the siCHECK vectors either with the Pre-miR control or the Pre-miR-181c using lipofectamine (Invitrogen) following manufacturer’s instructions. For inhibition experiments an anti-miR control or an anti-miR-181c were transfected. In the case of LCLs, nucleofection was conducted using Cell line optimization 96-well nucleofector kit from Amaxa (Lonza, Germany), according to manufacturer’s recommendations. Nucleofection was done combining siCHECK-TNFα or siCHECK-mutTNFα with Pre-miR-181c. 24-72 h post-transfection luciferase reporter assay was done using Dual-Glo luciferase kit (Promega).

The ratio between the firefly and the Renilla luciferase allows the normalization of luciferase values. Ratios were normalized against the control plasmid. RKO experiments with miRNA transfection were performed five times in duplicates and anti-miR experiments were conducted three times also in duplicates. In LCLs, three independent experiments were conducted. In all cases the expression of endogenous miRNA after transfection was analyzed by RT-qPCR. Statistical differences were determined using Student’s t test.

In vitro culture of hematopoietic progenitors.

For the assessment of colony forming cells (CFCs), samples consisting of at least 150,000 mononuclear or erythrocyte-depleted BM cells were cultured at 37°C, in 5% CO₂, and 95% humidified air, in Methocult H4434 medium containing Stem Cell Factor, GM-CSF, IL-3 and erythropoietin as growth factors (Stem Cell Technologies, Vancouver, BC, Canada) according to standardized procedures. Colonies were scored after two weeks in culture in an inverted microscope (Olympus IX70 WH10x/22; objective 4x).

Statistical analysis.
Results are shown as the mean±s.e. Differences between groups were assessed using the two tailed Student’s t-test. Statistical analysis of the data was performed by using Graph Pad Prism 4.0 software (La Jolla, CA, USA).

RESULTS

Down-regulated expression of specific miRNAs in lymphoblast cell lines and primary blood cells from FA-A patients.

To study the involvement of miRNAs in FA we initially determined the expression levels of 157 miRNAs in lymphoblast cell lines (LCLs) obtained from 6 healthy donors and 8 FA-A patients using the TaqMan MicroRNA Assay kit. After normalizing the data using the RNU6B as control, we determined the mean expression level of each miRNA in the different FA cell lines and compared with the mean values determined in control LCLs. These analyses showed a down-regulated expression of 8 miRNAs in FA LCLs (hsa-miR-99a, hsa-miR-133a, hsa-miR-135b, hsa-miR-139, hsa-miR-181c, hsa-miR-182, hsa-miR-183, hsa-miR-199s (all of them with p<0.01 and Sam score>0.9; data not shown).

To confirm data obtained in LCLs, fresh PB MNCs from 9 FA-A patients and 6 healthy donors were used. In these experiments the expression levels of 7 out of the 8 miRNAs that were down-regulated in FA-LCLs were analyzed (hsa-miR-199s miRNA was not analyzed because it has been eliminated from the miRNA annotation list). As shown in Figure 1, no evident changes of expression were noted in four out of seven miRNAs, while a consistent down-regulated expression of three miRNAs, hsa-miR-133a, hsa-miR-135b and hsa-miR-181c, was observed in fresh FA PB cells.

Because in gastric carcinomas a reduced expression of hsa-miR-181c had been associated with the methylation of CpG sites located upstream of this miRNA, we conducted these analyses in LCLs from two healthy donors and two FA-A patients, where a downregulated expression of hsa-miR-181c was confirmed. Methylation specific PCR (MSP) analyses revealed, however, that the CpG sites upstream of the hsa-miR-181c were strongly methylated in LCLs.
not only from FA patients but also from HD (Supplementary Figure 1a). The analysis by pyrosequencing of the 4 CpG dinucleotides located in the genomic sequence that results in mature miRNA *hsa-miR-181c* showed a similar methylation of the 4 CpG dinucleotides in all samples (Supplementary Figure 1b).

**hsa-miR-181c regulates the expression of TNFα by interacting with its 3′UTR region**

Aiming to identify potential targets of the miRNAs that were down-regulated in FA samples, *in silico* analyses were performed with the MIRANDA software. These analyses showed that *hsa-miR-181c* had three potential target genes, *TNFα, IL-1β* and *RAD54B*, that could be relevant in the disease. Strikingly, two of these genes, *TNFα* and *IL-1β*, have been reported to be over-expressed in FA cells.

To test the effect of *hsa-miR-181c* upon mRNA expression of *TNFα, IL-1β* and *RAD54B*, LCLs from different FA-A patients were transfected with a control pre-miRNA and Pre-miR-181c. As shown in Figure 2, the ectopic expression of Pre-miR-181c in FA-A LCLs significantly decreased the expression of *TNFα* while no change in the mRNA expression of *IL-1β* and *RAD54B* was observed. Because several studies have shown *TNFα* overexpression in FA-C cells, the relevance of *hsa-miR-181c* in the downregulation of *TNFα* was also confirmed in FA-C LCLs (See Supplementary Figure 2). Importantly, because miRNAs can also interfere the expression of their target genes by translational repression, our studies of RNA expression do not exclude the possibility that miR-181c may also reduce the *IL-1β* and/or *RAD54B* protein levels.

To investigate whether *hsa-miR-181c* interacts with the *TNFα* mRNA, we cloned the 3′UTR region that is hypothetically recognized by the miRNA in a luciferase reporter vector (siCHECK2-TNFα). Additionally, a similar vector carrying the 3′UTR from the *IL-1β* was generated (siCHECK2-IL1β). As a negative control, a vector carrying a mutated 3′UTR of *TNFα* was constructed (siCHECK2-mutTNFα). As a positive control, a vector with the sequence that
exactly resembles the region recognized by the *hsa-miR-181c* (siCHECK2-TargetmiR181c) was also generated.

In preliminary experiments we confirmed that the endogenous expression of *hsa-miR-181c* in RKO cells was similar to that observed in LCLs from healthy donors (data not shown). To investigate whether the endogenous expression of *hsa-miR-181c* in RKO cells could be sufficient to inhibit the expression of the *Renilla luciferase* fused to TNFα 3’UTR, the luciferase activity was firstly determined in RKO cells transfected with siCHECK2-TNFα, and also with the negative and positive controls (siCHECK2-mutTNFα, and siCHECK2-TargetmiR181c, respectively). As shown in Figure 3a a significant decrease in the *Renilla* luciferase activity (normalized to firefly luciferase) was observed in RKO cells transfected with the positive control (siCHECK2-TargetmiR181c) and also with the siCHECK2-TNFα, compared to cells transfected with the negative control (siCHECK2-TNFα mutated).

Because other miRNAs potentially expressed in RKO cells - apart from *hsa-miR-181c* - could account for the down-regulated *Renilla* luciferase activity observed in siCHECK2-TNFα-transfected cells, in the next set of experiments RKO cells were co-transfected with different siCHECK2 vectors: siCHECK-TNFα, siCHECK-IL1β or siCHECK-mutTNFα and also with a Pre-miR control (white bars in Figure 3b) or with the Pre-miR-181c (black bars). Three days after co-transfection, the relative *Renilla* luciferase activity was determined. When cells were transfected with siCHECK-TNFα, a significant decrease of *Renilla* luciferase activity was induced by the co-transfection with Pre-miR-181c compared to the Pre-miR control (see first two bars in Figure 3b). In samples transfected with siCHECK-IL1β, no significant inhibition was observed between groups co-transfected with Pre-miR-181c and the control Pre-miR (Figure 3b), in good consistency with data observed in Figure 2. As expected, when siCHECK-mutTNFα was used, no inhibition was induced by Pre-miR-181c, compared with the control Pre-miR (Figure 3b).

To verify the interaction of miR-181c with the TNFα 3’UTR region in cells of hematopoietic nature, similar experiments to those shown in RKO cells (Figure 3b) were conducted with LCLs co-transfected with Pre-miR-181c together with
siCHECK-TNFα or siCHECK-mutTNFα. As it was observed in RKO cells, LCLs transfected with the siCHECK-TNFα showed a reduced luciferase activity compared to cells transfected with siCHECK-mutTNFα (Figure 3c). Taken together, these results demonstrate that hsa-miR-181c down-regulates the expression of TNFα by means of the interaction with its 3′ UTR.

Finally, to confirm that the TNFα 3′ UTR region is a target of hsa-miR-181c, in the next set of experiments RKO cells were co-transfected with siCHECK-TNFα, siCHECK2-IL1β or siCHECK-mutTNFα, and also with an anti-miR control (white bars in Figure 3d) or with a hsa-miR-181c anti-miR (anti-miR-181c; black bars). Cells transfected with the siCHECK-TNFα vector, showed an increased expression of Renilla luciferase activity after co-transfection with the hsa-miR-181c anti-miR compared to the control anti-miR (Figure 3d). In good consistency with data from Figure 2, no expression changes were observed when the siCHECK-IL1β or the siCHECK-TNFα mutated vectors were used (Figure 3d). These results confirm that hsa-miR-181c targets the 3′ UTR of TNFα, mediating a down-regulated expression of the gene.

**Regulation of TNFα by hsa-miR-181c in BM cells from healthy donors and FA patients.**

In a next set of experiments we investigated the production of TNFα in BM cells from healthy donors (HD) and FA patients 48h after transfection with the control Pre-miR or with Pre-miR-181c. When TNFα was analyzed in untransfected BM samples from either HD or FA patients, a very low number of cells expressed detectable levels of intracellular TNFα. Samples were, therefore, activated either with PMA, LPS or with R848. Activations with LPS or R848 markedly increased the proportion of TNFα− expressing cells, mainly within the CD14+ population, while the generation of TNFα− expressing CD14+ cells was much lower in PMA-stimulated cells (See representative analyses in Figure 4a). When hsa-miR-181c was transfected into LPS- and R848-activated BM cells from a healthy donor, moderate decreases in the intracellular levels of TNFα were observed (Figure 4b). When similar experiments were conducted with BM samples from FA-A patients, less consistent results were obtained. In this case,
while either LPS or R848 induced a significant TNFα overexpression in cells from two FA patients (FA-110 and FA-536), no TNFα overexpression was induced by these molecules on cells from a third FA patient (FA-13). Additionally, while *hsa-miR-181c* downregulated the expression of TNFα in LPS-activated cells - but not in R848-activated cells - from patient FA-110, only R848-activated cells from patient FA-536 showed a downregulated expression of TNFα (not shown). The use of lentiviral vectors carrying the *Pre-miR-181c* and the *EGFP* marker gene clarified, however, that FA cells with a downregulated expression of TNFα corresponded to *hsa-miR-181c* expressing cells (Supplementary Figure 3).

*hsa-miR-181c* transfection in BM cells from FA patients reproducibly improves the growth of FA hematopoietic progenitors *in vitro*.

Finally, to investigate the functional consequences mediated by *hsa-miR-181c* on the growth of FA hematopoietic progenitors, BM cells from five FA patients - without evidences of myelodisplasia or leukemia deduced from morphological and karyotypic assessment in BM aspirates - were transfected either with the control Pre-miR or with Pre-miR-181c, and then cultured in semisolid medium for 14 days. As shown in Figure 5a, *hsa-miR-181c* mediated a marked increase in the number of hematopoietic colonies in all tested FA BM samples. The effects of *Pre-miR-181c* were evident not only by the increased number of colonies, both myeloid and erythroid, but also by the larger size of the colonies (see representative analyses in Figure 5b). In all instances, the expression of *hsa-miR-181c* was confirmed by qPCR performed in pooled hematopoietic colonies from each patient (not shown).

**DISCUSSION**

In this study we aimed to dissect the potential role of miRNAs in the hematological manifestations of FA, a severe inherited disease associated with several cellular dysfunctions affecting the proliferation and differentiation of hematopoietic cells. Previous results from Gruber *et al.* demonstrated that mice deficient in *Ars2*, a protein involved in the proper miRNA processing, developed
BM failure\textsuperscript{33}, reinforcing our hypothesis that a number of miRNAs might be altered in FA. In consistency with this hypothesis, our results showed the downregulation of eight miRNAs in LCLs from FA patients, three of which (hsa-miR-133a, hsa-miR-135b and hsa-miR-181c) were also down-regulated in fresh FA PB MNCs. Interestingly one of the miRNAs that was systematically down-regulated in FA cells, hsa-miRNA-181c, had TNF\textgreek{a}, IL-1\textgreek{b} and RAD54B as possible target genes, although only TNF\textgreek{a} and IL-1\textgreek{b} have been previously described to be up-regulated in FA cells\textsuperscript{28,31,32}.

While the inhibitory studies with the siCHECK2 vector showed that hsa-miR-181c could target the TNF\textgreek{a} 3'UTR, this effect was not observed with the IL-1\textgreek{b} 3'UTR (Figure 3). Levels of TNF\textgreek{a} down-regulation obtained in our luciferase experiments were similar to those described with hsa-miR-125b, another miRNA also capable of down-regulating TNF\textgreek{a}\textsuperscript{34}. In that study the authors found that miR-125b also targets the 3'-untranslated region of TNF\textgreek{a} transcripts, and proposed that its down-regulation in response to LPS may be required for the proper TNF\textgreek{a} production.

Due to the relevance of TNF\textgreek{a} in the etiology of the BM failure and cancer progression in FA, we focused our next studies on the effects of hsa-miR-181c upon the expression of TNF\textgreek{a} in FA BM cells, and also upon the growth of hematopoietic progenitors from FA patients. In this respect, early studies showed that altered responses to this cytokine had a critical role in the pathogenesis of acquired aplastic anemia due to the apoptotic loss of hematopoietic stem and progenitor cells (see review in\textsuperscript{35}). Significantly, increased levels of this cytokine were found in the serum of FA patients\textsuperscript{36,37}, and also in supernatants from LCLs\textsuperscript{37,38} and BM cells from these patients\textsuperscript{28}. Additionally, the neutralization of the interaction of this cytokine with its receptor significantly increased the growth of BFU-E and CFU-GM progenitors from FA patients\textsuperscript{28,39}, in good consistency with mechanistic studies showing a deregulated apoptosis and hypersensitivity of FA BM progenitor cells exposed to TNF\textgreek{a}\textsuperscript{38,40-44}. In addition to studies showing the relevance of TNF\textgreek{a} on the etiology of FA BMF, studies in \textit{FancC}\textsuperscript{-/-} mice have shown that this cytokine
provides a selective pressure for apoptosis resistant FA cells, thus facilitating the malignant progression of FA clones with accumulated mutations\textsuperscript{45,46}.

Our data showing the interaction of \textit{hsa-miR-181c} with the TNF\textsubscript{\alpha} 3'UTR (Figure 3) are consistent with analyses indicating that this miRNA downregulates the expression of TNF\textsubscript{\alpha} in LCLs and primary cells from HDs and FA patients (see Figures 2, 4 and Supplementary Figure 3). These observations together with our data showing a reduced expression of \textit{hsa-miR-181c} in FA cells (Figure 1) indicate that this miRNA could be involved, in addition to other molecules such as MMP-7, in the altered levels of expression/secretion of TNF\textsubscript{\alpha} in FA cells\textsuperscript{31}.

The functional relevance of \textit{hsa-miR-181c} upon the hematopoiesis of FA patients is deduced from our \textit{in vitro} culture experiments conducted with BM cells from FA patients (Figure 5). As happened in cultures of FA BM cells treated with TNF\textsubscript{\alpha} fusion protein inhibitors\textsuperscript{28,39}, our data in Figure 5 clearly show that the expression of \textit{hsa-miR-181c} on FA patient’s BM significantly improved the growth of myeloid and erythroid progenitors, probably due to the downregulated expression of TNF\textsubscript{\alpha} in FA progenitor and/or mature cells; i.e. CD14\textsuperscript{+} cells present in transfected BM or generated during the colony growth.

The fact that BM samples from FA patients corresponding to three different complementation groups (FA-A, FA-G and FA-J) showed similar improvements in colony growth after the ectopic expression of \textit{hsa-miR-181c} suggests that the downregulated expression of this miRNA in FA patients should be involved - either directly or indirectly - in the impaired growth of their hematopoietic progenitors, independent of the FA complementation group. Additionally, the observation of an improved colony growth in cultures of BM cells from patient FA-13, whose TNF\textsubscript{\alpha} levels were low even after LPS- or R848- activation would suggest that this miRNA might also target other negative regulators involved in the growth of hematopoietic progenitors. Our results, together with the fact that miRNAs can be modified to improve their stability after \textit{in vivo} infusion\textsuperscript{47-49} open the possibility of using miRNAs, in particular \textit{hsa-miR-181c}, for the treatment of the BM failure in FA patients, as already conducted with TNF\textsubscript{\alpha} inhibitors\textsuperscript{50}.

Interestingly, when BM cells from a FA patient with acute myeloid leukemia were transfected with \textit{hsa-miR-181c}, no increase in the number of colonies was
observed (data not shown). Although further studies are necessary to confirm this observation, it is of significance that studies conducted in Fancc−/− mice showed that malignant clones were not sensitive to TNFα45,46, indicating that these malignant clones may have developed mechanisms of TNFα resistance and/or degradation.

The mechanisms accounting for the decreased expression of different miRNAs, including hsa-miR-181c, in FA cells still remains unclear. Epigenetic modifications of genes encoding for these miRNA, such as promoter hypermethylation or histone modifications51-53, may have a critical role in this effect. Moreover, previous results in gastric carcinomas have shown that hsa-miR-181c can be down-regulated by methylation30. However, methylation analyses conducted in LCLs from healthy donors and FA-A patients showed a similar heavy methylation of CpG sites located near the hsa-miR-181c and of the CpG dinucleotides located in the mature hsa-miR-181c. These results strongly suggest that the methylation of these CpG sites is not the mechanism accounting for the down-regulation of this miRNA in FA cells.

The observation from O`Conell et al.5 showing that hsa-miR-181c is enriched in mouse HSCs and human CB CD34+ cells is of particular interest, taking into account the reduced expression of this miRNA in FA hematopoietic cells, and its role in both TNFα downregulation and hematopoietic progenitors cell growth. In this context it would be of interest to determine hsa-miR-181c levels in the very rare population of FA CD34+ and HSCs. Similarly, understanding the involvement of this miRNA in the homing and the repopulating properties of FA-HSCs would be highly relevant, considering O’Conell observations showing that the ectopic expression of this miRNA impaired the competitive repopulating ability of BM cells from healthy mice.

Taken together, we have shown for the first time that FA hematopoietic cells are characterized by a down-regulated signature of several miRNAs, and demonstrated that one of these down-regulated miRNAs, hsa-miR-181c, interacts with 3’UTR of TNFα, inhibiting its expression and toxic effects in hematopoietic progenitors from FA patients. These observations offer new clues to understand the biological basis of the BMF in FA patients, and hopefully may
help in the development of new therapeutic strategies for the treatment of this severe disease.

ACKNOWLEDGMENTS:

The authors also wish to thank the technical assistance of Aurora de La Cal and Sergio Losada (CIEMAT/CIBERER). This work was supported by grants from the European Program “7FWP, Health” (PERSIST; Ref Grant Agreement no: 222878), the Ministry of Science and Innovation: Programa de Fomento de Cooperación Científica Internacional (110-90.1) and Plan Nacional de Salud y Farmacia (SAF 2009-07164), Fondo de Investigaciones Sanitarias, ISCIII (Programa RETICS-RD06/0010/0015). The authors also thank the Fundación Marcelino Botín for promoting translational research at the División de Hematopoyesis y Terapia Génica at the CIEMAT. CIBERER is an initiative of the Instituto de Salud Carlos III.

AUTHOR CONTRIBUTIONS:


CONFLICT OF INTEREST:

No conflict of interest.
REFERENCES:


FIGURES

Figure 1: Comparative analysis of miRNA expression levels in primary blood cells from FA-A patients and healthy donors. The figure shows the relative expression of hsa-miR-99a, -133a, -135b, -139, -181c, -182 and -183 determined by qPCR in fresh mononuclear cells obtained from healthy donors (squares) and FA-A patients (triangles). Upper graphics show microRNAs whose expression was not modified in samples from FA patients, compared to healthy donors (HD). The lower graphics show microRNAs significantly down-regulated in FA-A patients. Expression levels were related to the expression of the RNU6B gene and normalized to mean values corresponding to HD samples.

Figure 2: Relative expression of IL-1β, TNFα and RAD54B in LCLs from FA-A patients transfected with a Pre-miR control and Pre-miR-181c. In all instances, mRNA expression levels were analyzed by qPCR two days after transfection with a Pre-miR control (white bars) or Pre-miR-181c (black bars). Expression levels were related to the expression of the human β-actin gene, and normalized to mean values corresponding to LCLs transfected with the Pre-miR control. Bars corresponding to IL-1β and TNFα expression represent mean values ± s.e. of data deduced from the analysis of three FA patients. In the case of RAD54B, data from two patients is shown. For each determination, three independent experiments were conducted.

Figure 3: Pre-miR-181c regulates the expression of TNF-α by interacting with its 3'UTR sequence. The figure represents the normalized Renilla luciferase activity in RKO cells transfected with the different constructs. a) RKO cells were transfected with the different siCHECK2 plasmids. b) RKO cells were co-transfected with Pre-miR control (white bars) or Pre-miR-181c (black bars) together with the different siCHECK2 plasmids. c) LCLs from healthy donors were transfected with siCHECK2-mut TNFα or siCHECK2- TNFα and Pre-miR-181c. d) RKO cells were co-transfected with anti-miR control (white bars) or with an anti-miR-181c (black bars) together with the different siCHECK2 plasmids. In all instances Renilla luciferase activity was normalized to firefly...
luciferase activity. Bars show mean values ± SE corresponding to 3-5 independent experiments.

**Figure 4: Downregulated expression of TNFα in LPS-activated BM cells transfected with Pre-miR-181c.** a) Representative flow cytometry analysis showing intracellular expression of TNFα in CD14+ cells in fresh BM and in BM cells activated with PMA; LPS or R848. b) Representative analysis of intracellular TNFα in LPS-activated BM cells transfected with Pre-miR control or with Pre-miR-181c. Gray histogram corresponds to non activated cells; empty histograms correspond to LPS-activated cells transduced with the Pre-miR control (dotted line) and with Pre-miR-181c (continuous line). Data correspond to BM sample from a healthy donor.

**Figure 5: Pre-miR-181c improves the clonogenicity of hematopoietic progenitors from FA patients.** a) Analysis of the number of hematopoietic progenitors from FA patients cultured in methylcellulose 48h after transduction with a Pre-miR control (white bars) or with Pre-miR-181c (black bars). b) Representative analysis of CFU-GM and BFU-E colonies corresponding to patient FA-287. Pictures correspond to patient FA-13.
Figure 1

Relative expression

miR99a  p = 0.097  miR139  p = 0.054  miR182  p = 0.912  miR183  p = 0.358

miR133a  p = 0.0053  miR135b  p = 0.0135  miR181c  p = 0.0031
Figure 2

Relative expression

- **IL1β**
  - NS
  - p = 0.90

- **TNFα**
  - **
  - p = 0.0017

- **RAD54B**
  - NS
  - p = 0.32
Figure 3

(a) Normalized Luciferase activity

(b) Normalized Luciferase activity

(c) Normalized Luciferase activity

(d) Normalized Luciferase activity
Figure 4

a) 

Non-Act

PMA

LPS

R848

CD14-FITC

b) 

Count

anti TNFα-PE

Pre-miR control

Pre-miR-181c
Figure 5

(a) Number of CFCs per 5x10^6 cells

- FA-13 (FA-A)
- FA-287 (FA-J)
- FA-467 (FA-A)
- FA-110 (FA-A)

(b) CFU-GMs/10^6 cells and BFU-Es/10^6 cells

- Pre-miR control
- Pre-miR-181c

CFU-GM and BFU-E images for:
- Pre-miR control
- Pre-miR-181c
Downregulated expression of *hsa-miR-181c* in Fanconi anemia patients: implications in TNF α regulation and proliferation of hematopoietic progenitor cells

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