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A role for miR-142-3p in colony-stimulating factor 1-induced monocyte differentiation into macrophages

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ABSTRACT

The differentiation of human peripheral blood monocytes into macrophages can be reproduced ex vivo by culturing the cells in the presence of colony-stimulating factor 1 (CSF1). Using microarray profiling to explore the role of microRNAs (miRNAs), we identified a dramatic decrease in the expression of the hematopoietic specific miR-142-3p. Up- and down-regulation of this miRNA in primary human monocytes altered CSF1-induced differentiation of monocytes, as demonstrated by changes in the expression of the cell surface markers CD16 and CD163. One of the genes whose expression is repressed by miR-142-3p encodes the transcription factor Early Growth Response 2 (Egr2). In turn, Egr2 associated with its co-repressor NGFI-A (Nerve Growth Factor-Induced gene-A) binding protein 2 (NAB2) binds to the pre-miR-142-3p promoter to negatively regulate its expression. Interestingly, the expression of miR-142-3p is abnormally low in monocytes from patients with the most proliferative forms of chronic myelomonocytic leukemia (CMML), and miR-142-3p re-expression in CMML dysplastic monocytes can improve their differentiation potential. Altogether, miR-142-3p which functions in a molecular circuitry with Egr2 is an actor of CSF1-induced differentiation of human monocytes whose expression could be altered in CMML.

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1. Introduction

Human peripheral blood monocytes are seminal effectors and regulators of the host inflammatory and innate immune responses. They are produced in the bone marrow, circulate in the blood, and enter into tissues under inflammatory conditions to give rise to a variety of macrophages and inflammatory dendritic cells, depending on their environment [1]. *Ex vivo* exposure of monocytes to colony-stimulating factor 1 (CSF1), also known as macrophage-colony stimulating factor (M-CSF), induces their differentiation into macrophages.

CSF1 signals through the CSF1 receptor tyrosine kinase (CSF1R), encoded by the *C-FMS* proto-oncogene [2], to trigger a series of phosphorylation cascades that mediate this cellular response [3,4]. The interaction of CSF1 with its receptor generates waves of phosphatidyl inositol 3-kinase (PI3K)/Akt activation, leading after 2–3 days to the activation of caspases that actively contribute to the formation of resting macrophages [3,5,6]. These signaling pathways may also connect CSF1R to the transcription machinery [7]. For example, MafB and c-Maf could prevent the proliferation of differentiated monocytes and macrophages [8] whereas Egr2 (early growth response 2, also known as Krox20) activates macrophage-specific genes such as *C-FMS*, in cooperation with Spi-1/PU.1 [9–11].

A role for microRNAs (miRNAs) in the complex regulatory network that governs monocyte differentiation into macrophages has been identified. When inducing the differentiation of human monocytes into macrophages with a so-called M1, pro-inflammatory phenotype, GM–CSF provokes a decrease in the expression of miR-223, miR-15a, and miR-16, which may contribute to this specific phenotype [12]. The miRNAs involved in the CSF1-induced differentiation of monocytes, leading to macrophages with a M2 anti-inflammatory phenotype, are less known, even though *C-FMS* expression has been shown to be

Abbreviations: cAMP, cyclic 3′5′-adenosine monophosphate; CCNT2, cyclin T2; CMML, chronic myelomonocytic leukemia; CSF1, colony-stimulating factor 1; CUX1, cut-like homeobox 1; EGR2, early growth response 2; GFI-1, growth factor independent-1; KLF4, Krüppel-like factor 4; MAFB, musculoaponeurotic fibrosarcoma oncogene homolog B; M-CSF, macrophage-colony stimulating factor; miRNA, microRNA; NAB2, nerve growth factor-induced gene-A binding protein 2; NR3C1, nuclear receptor subfamily 3 group C member 1 glucocorticoid receptor; PI3K, phosphatidyl inositol 3-kinase; RTK, CSF1 receptor tyrosine kinase; RAC1, ras-related C3 botulinum toxin substrate 1; Spi-1, spleen focus forming virus (SFFV) proviral integration oncogene; TAB2, TGF-β activated kinase 1/MAP3K7 binding protein 2

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indirectly regulated by miRNAs 17-5p-20a-106a that repress the transcription factor RUNX1 and by miR-155 that targets Spi-1/PU.1 and C/EBPβ [13,14].

We report here that miR-142-3p is a regulator of CSF1-induced macrophage differentiation and forms with Egr2 a positive feedback loop. More precisely, exposure of human monocytes to CSF1 induces a decrease in the expression of miR-142-3p, which enhances the expression of the transcription factor Egr2. In turn, Egr2 interacts with the miR-142-3p promoter to down-regulate its expression. The expression of this miRNA can be down-regulated in monocytes from patients with chronic myelomonocytic leukemia (CMML), a myeloproliferative/myelodysplastic syndrome which main characteristic is the accumulation of monocytes in peripheral blood, bone marrow and spleen [15].

2. Material and methods

2.1. Cell culture, differentiation and treatment

The human erythroleukemic K562 cell line (American Type Culture Collection, Manassas, VA, USA) and human purified monocytes were grown in RPMI 1640 Glutamax medium (BioWhittaker, Cologne, Germany) supplemented with 10% fetal calf serum (BioWhittaker), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) (BioWhittaker). Mononucleated cells were selected by Ficoll Hypaque (Eurobio, Les Ulis, France). The AutoMacs system (Miltenyi Biotec, Cologne, Germany) was then used to perform cell enrichment. Positive selection with CD14+ magnetic isolation kit (Miltenyi Biotec) was performed for monocyte enrichment for the purpose of miR-142-3p and Egr2 expression analyses. Concerning monocyte transfection, negative selection was used (Miltenyi Biotec). Monocytes were plated at 0.5×10^6 cells/mL and incubated for different times in the presence or absence of CSF1 (100 ng/ml) (Miltenyi Biotec), at 37 °C in a 5% CO_2 humidified atmosphere. Cell morphology was analyzed on cytospin slide preparations stained with May–Grünwald–Giemsa.

2.2. Constructs

A fragment of 1210 bp, encompassing the entire human Egr2 3′UTR, was PCR-amplified with the following sense (5′-GATGAGACTCAGGCTGATACACCAGCTCCC-3′) and antisense (5′-ATTGTTGAAAAGTATTTATTTACACTATAGTCACAAACCATCC-3′) primers using standard procedures and a proofreading polymerase (Platinum Pfu) (Invitrogen, Carlsbad, CA, USA). The cDNA clone, which contains the full length of Egr2 3′ UTR was used as template. The PCR product was sub-cloned into the pCR 2.1 vector (Invitrogen). The Egr2 3′UTR inserts were removed from the pCR 2.1 plasmid by XbaI digestion and ligated into a XbaI site located downstream of the firefly luciferase (f-luc) reporter gene (pGL3-Promoter vector) (Promega, Madison, WI, USA) driven by a 202 bp 5′ flanking region from human SV40 gene. The authenticity and orientation of the inserts relative to the luciferase gene were confirmed by sequencing. The resulting plasmids were designated pEgr2 3′UTR. Point mutations in miR-142-3p seed region were introduced with the QuikChange site-directed mutagenesis kit II (Stratagene, Santa Clara, CA, USA) by using the pEGR2 3′UTR vector as template, and by following the protocol provided in the kit. Mutagenesis was performed with the following sense (5′-GCACAACGACCCCGAGCACCCTTTCCTGTCC-3′) and antisense (5′-GGACAGGAAAGGGTGCTCGGGGTCGTTGTGC-3′) primers. Mutations were confirmed by sequencing.

2.3. MicroRNA microarray analysis

In order to prepare RNA samples for miRNA microarray or miRNA quantification, total RNA enriched with small RNAs was isolated using mirVana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA preparations from monocytes and CSF1-treated monocytes were sent to LC Sciences™ (Houston, TX, USA) for miRNA microarray analysis using the mParaflo microfluidic chips and detailed process can be found at http://www.lcsciences.com. The microarray data reported in this study have been deposited in the Gene Expression Omnibus (GEO), http://www.ncbi.nlm.nih.gov/geo/ (accession number GSE31306).

2.4. Flow cytometry analysis

Fluorescence activated cell sorting (FACS) analysis was performed on untreated or CSF1-treated monocytes. In brief, single-cell suspension was prepared and stained with primary antibodies. The cells were washed twice in PBS and re-suspended in PBS/0.1% bovine serum albumin (BSA 1%). Antibodies were added and incubated on ice. Antibodies CD16-PE, CD14-PE, CD163-PE, and CD71-APC were purchased from BD Biosciences. Flow cytometry was performed on LSRII (Becton Dickinson, Franklin Lakes, NJ, USA) using DIVA software (Becton Dickinson), and automatically compensations were applied. The results were analyzed using FlowJO software.

2.5. RNA isolation, reverse transcription, quantitative real-time PCR, 5′-RACE

Total RNA was extracted with TRI Reagent® (Ambion, Austin, TX, USA) and reverse transcribed with random primer (Promega). Quantitative real-time PCR was performed in triplicates in an Applied Biosystems 7500 Fast thermocycler (Foster City, CA, USA). For Pri-miRNA expression, commercial ready-to-use primers/Taqman probe mixes were used (Applied Biosystems) (pri-miR-142: Hs03303162_pri). Expression of mature miR-142-3p was determined using miRNA-specific quantitative real-time PCR assay (Applied Biosystems, 000464). Human *cyclophilin A* (Hs99999904_m1*) and *RNU6B* (Applied Biosystems, 0001093) were used as endogenous controls. For *EGR2* expression, quantitative real-time PCR was performed using SyBr Green detection protocol as outlined by the manufacturer. Briefly, 15 ng of total complementary DNA, 50 nM of each primer and 1× SyBr Green mix were used in a total volume of 20 μl. The human specific forward and reverse primers were: *L32*, 5′-TGTCCTGAATGTGGTCACCTGA-3′ and 5′-CTGCAGTCTCCTTGCACACCT-3′ used as a standardizing control, *EGR2*, 5′-GCCCTTCGCCTGTGACTACT-3′ and 5′-GTGGCGCTTCCTCTCATCA-3′. *L32* was used as internal control. 5′ Rapid amplification of cDNA ends (5′-RACE) was carried out using the TaKaRa 5-Full RACE Kit (TaKaRa Bio. Inc., Shiga, Japan) according to the manufacturer's instructions. Two rounds of 5′ RACE-PCR were performed. The 5′ RACE product was gel purified, cloned into the pMD19-T vector (TaKaRa Bio. Inc.), then sequenced. The human 5′ end-phosphorylated RT primer was 5′-CCACATGTCCAG-3′. The specific primers used for RACE were the following: A1, 5′-AGACAGGCAGCCGCACATGAGAA-3′; S1, 5′-TTCGGAGATCACGCCACTGCT-3′; A2, 5′-CCGACGGGTCCGGAGGACTGA-3′; S2, 5′-CCACCATCTTCCTCGGCGCTC-3′.

2.6. Western-blot analysis

Untreated or CSF1-treated monocytes were re-suspended in cooled lysis buffer (1% SDS, 1 mM sodium vanadate, 10 mM Tris pH 7.4, protease inhibitor cocktail). Samples were incubated on ice for 30 min. Total protein extracts were boiled in Laemmli buffer then separated by SDS-PAGE and electro-blotted to a nitrocellulose membrane. Equivalent loading of lanes was controlled with Ponceau Red Stain (data not shown). Membranes were blocked in 1× PBS-T (0.1%) and fat-free dry milk (5%) during 1 h at room temperature. Membranes were incubated with anti-Egr2 (Aviva Systems Biology LLC, San Diego, CA, USA), anti-Egr1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HSC70 (Santa Cruz Biotechnology) or anti-Actin

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