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Article

The influence of overexpressed fibroblast growth factor receptors towards vitamin D receptor expression and activity

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Abstract: (1) Background: Many malignancies are driven by mutations which affect the gene for fibroblast growth factor receptor (FGFR) 1. Previously we have documented that signal transduction from FOP2–FGFR1 fusion protein in KG1 cells downregulated the expression of vitamin D receptor (*VDR*) gene. In this paper we investigated if also other FGFRs were responsible for the regulation of the *VDR* expression. (2) Methods: We used human myeloid leukemia cells U937, and bone cancer cell line U2OS, and cell transfection methods in order to address the above questions. (3) Results: In myeloid leukemia cells overexpression of FGFR 1-4 caused shift to granulocytic differentiation, upregulated expression of *VDR*, and sensitized these cells to 1,25-dihydroxyvitamin D (1,25D)-induced monocytic differentiation, while in bone cells, signal transduction activated by FGF1 was not responsible for regulation of VDR expression and activity. (4) Conclusions: Since the overexpression of FGFRs occurs in many neoplasms, it may be reasonable to use 1,25D analogs in these cancers, in which overexpression of FGFRs leads to *VDR* upregulation.

Keywords: 1,25-dihydroxyvitamin D; vitamin D receptor; vitamin D receptor; fibroblast growth factor receptor; signal transduction, differentiation.

1. Introduction

The major and the best known role of 1,25-dihydroxyvitamin D (1,25D) is to maintain the calcium-phosphate homeostasis of the organism [1]. 1,25D regulates also other vital processes, such as detoxification of the organism, differentiation of keratinocytes, and development of immune cells [2-4]. The high level of vitamin D receptor (VDR) in monocytes, and their ability to produce 1,25D from its precursor, 25-hydroxyvitamin D, have been known [5]. VDR plays roles in proper functioning of T cells [6] and of B cells [7] as well. The findings from the studies in acute myeloid leukemia (AML) cells support the use of 1,25D as an anticancer agent [8]. Preclinical studies using mouse models of systemic AML confirmed the anti-leukemic action of 1,25D [9,10]. In AML cells 1,25D triggers a complicated set of events involving the coordinated actions of transcription factors and intracellular signaling molecules which result in transcription of target genes [11]. The gene encoding 24-hydroxylase of 1,25D (CYP24A1) is the most strongly upregulated by VDR [12]. Hydroxylation of 1,25D at carbon atom C-24 catalyzed by CYP24A1 provides a negative feedback to the activity of 1,25D [13].

Our previous research has shown that 1,25D and its synthetic analogs are effective only against certain subtypes of AML, and that some mutations might confer resistance, while others confer susceptibility to 1,25D-based differentiation therapy [14-16]. AML cell line named KG1 [17] is resistant to 1,25D-induced differentiation, and our earlier experiments revealed that this was caused by a very low expression level of *VDR* gene and low protein level [18]. Later, we found that there are two mechanisms that keep *VDR* expression low in these cells. One is maintained by a retinoic acid receptor α (RARα) [19], and the other, more potent, is maintained by a constitutively activated fusion protein containing part of fibroblast growth factor receptor 1 (FGFR1) and a part of FGFR1 oncogene partner 2 (FOP2) [16].

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The fibroblast growth factor (FGF) superfamily consists of structurally-related polypeptides with a high affinity to heparin [20]. The biological actions of FGFs are mediated through binding to the high-affinity cell surface receptors, FGFRs. The FGFR family contains five genes, out of which four encode transmembrane tyrosine kinase receptors, which exist in multiple splicing variants. Binding of FGFs to FGFRs results in a dimerization of these receptors and transphosphorylation of their tyrosine kinase domains [21]. As a result, FGFRs activate different signaling cascades including MAPK, PI3K and phospholipase Cγ (PLCγ) [22]. In addition, constitutively active FGFRs cause downstream activation of signal transducer and activator of transcription (STAT) pathways [23].

Now, we wanted to investigate if overexpression of normal FGFRs in AML cells has any effect on *VDR* gene expression and activity of VDR protein. We also wanted to test if the influence of fusion proteins containing kinase domain of FGFR1 towards *VDR* expression, is limited to KG1 cells or not.

2. Results

2.1. Influence of FGFRs on the VDR expression and activity in U937 cells

At first, we wanted to test if activation of pathways from FGF receptors may contribute to regulation of *VDR* expression in leukemic cells of myeloid origin. To test this, we transfected U937 cells with pcDNA3 plasmid encoding each of four FGF receptors, namely, FGFR1, FGFR2, FGFR3 and FGFR4 [24,25]. The expression of *FGFR1*, *FGFR2*, *FGFR3* and *FGFR4* genes in transfected cells has been tested in Real-time PCR, and the results are presented in figure 1.

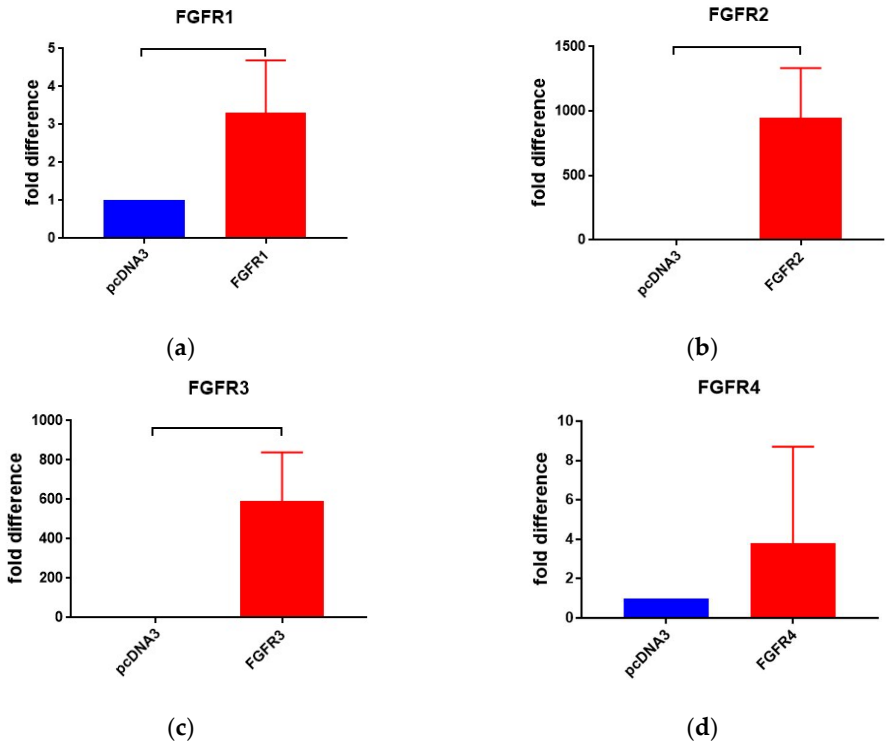

Figure 1. Expressions of *FGFRs* in transfected U937 cells. mRNA from mock-transfected U937 cells (pcDNA3) and the cells transfected with vectors encoding FGFR 1-4 was isolated and translated into cDNA. Then the expressions of *FGFR1* (**a**), *FGFR2* (**b**), *FGFR3* (**c**) and *FGFR4* (**d**) relative to *GAPDH* mRNA levels was measured in Real-time PCR. The bar charts show mean values (±SEM) of fold differences in gene expression obtained in three experiments. Values that differ significantly from those obtained for mock-transfected cells are marked with whiskers.

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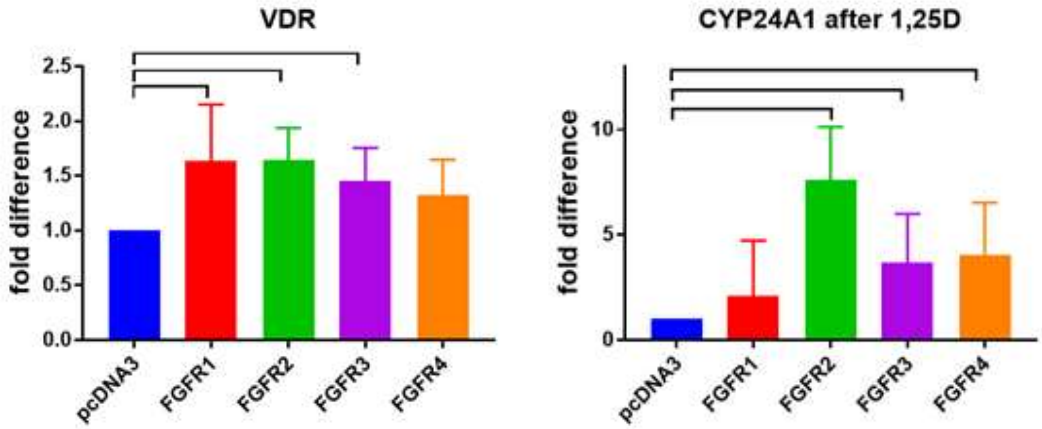
Then, the expressions of *VDR* and *CYP24A1* were tested in the cells overexpressing *FGFRs*, and compared to the mock-transfected cells. Since expression of *CYP24A1* is regulated by a complex of 1,25D-VDR, in order to study this gene, the cells were exposed to 10 nM 1,25D for 96 hours [19]. On the other hand, expression of *VDR* does not depend in human blood cells on 1,25D [26], thus to study *VDR*, the cells were left untreated. As presented in figure 2a, the expression of *VDR* was slightly, but significantly higher in the cells overexpressing *FGFRs*, than in mock-transfected cells. Figure 2b shows that in all cells overexpressing *FGFRs*, *CYP24A1* was higher than in mock-transfected cells, but only in the case of *FGFR2* the difference was significant.



(**a**) (**b**)

Figure 2. Expressions of *VDR* and *CYP24A1* in transfected U937 cells. Mock-transfected U937 cells (pcDNA3) and the cells transfected with vectors encoding FGFR 1-4 were left untreated (**a**), or exposed to 10 nM 1,25D for 96 hours (**b**). Then mRNA from was isolated and translated into cDNA. Expressions of *VDR* (**a**) and *CYP24A1* (**b**) were measured relative to *GAPDH* mRNA levels was measured in Real-time PCR. The bar charts show mean values (±SEM) of fold differences in gene expression obtained in five experiments. Values that differ significantly from those obtained for mock-transfected cells are marked with whiskers.

2.2. Effects of FGFRs on differentiation of U937 cells

In the following experiments we examined whether overexpression of *FGFRs*, and subsequent higher expression of *VDR* had any consequence towards the sensitivity of the cells to 1,25D-induced cell differentiation. In figure 3 the results of flow cytometry experiments are presented. These experiments revealed that overexpression of *FGFR 1-3* induces shift in differentiation of U937 cells into granulocytic pathway, documented by an increase of CD11b-positive cells (figure 3a). Addition of 10 nM 1,25D to these cells changes their differentiation into monocytic pathway, documented by an increase of CD11b, CD14-double positive cells (figure 3b).

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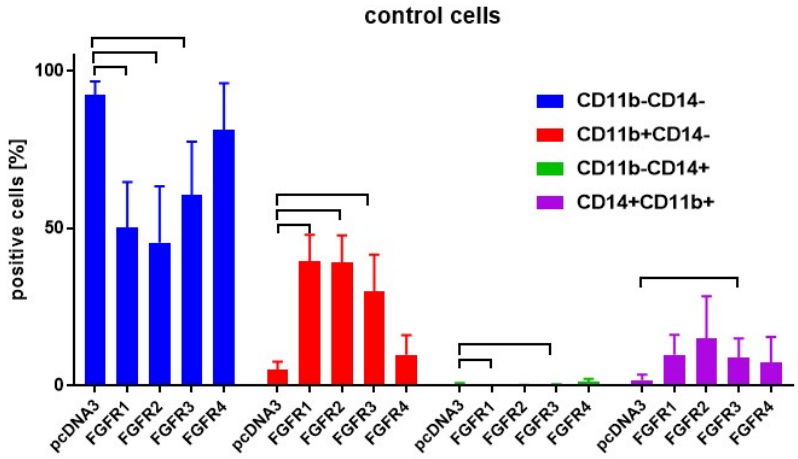
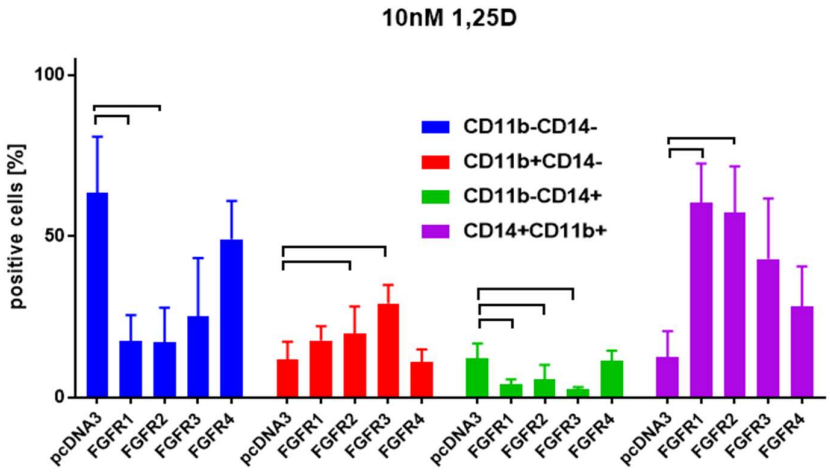


(**a**)



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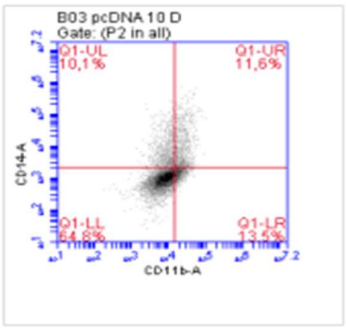
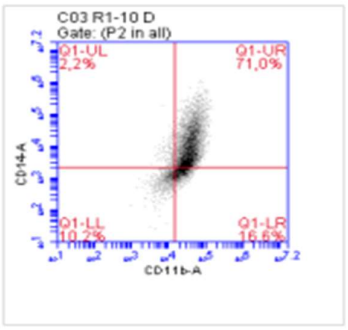


(**c**)



(**d**)

Figure 3. Levels of CD11b and CD14 cell differentiation markers in transfected U937 cells. Mock-transfected U937 cells (pcDNA3) and the cells transfected with vectors encoding FGFR 1-4 were left untreated (**a, c**), or exposed to 10 nM 1,25D for 96 hours (**b, d**). Then the levels of CD11b and CD14 cell surface markers were tested in flow cytometry. The bar charts show mean values (±SEM) of percentages of positive cells obtained in four experiments. Values that differ significantly from those obtained for mock-transfected cells are marked with whiskers. Representative dot-plots for mock-transfected (**c**) and *FGFR1* expressing (**d**) cells exposed to 10 nM 1,25D are presented.

2.4. Lack of expression of FGFR1 fusion genes in U937 cells

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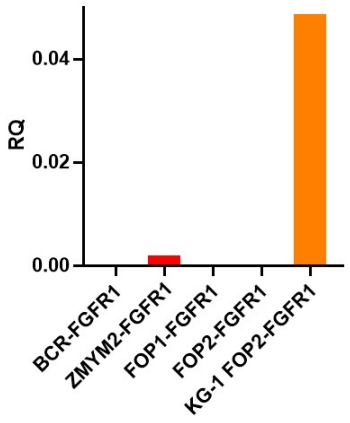
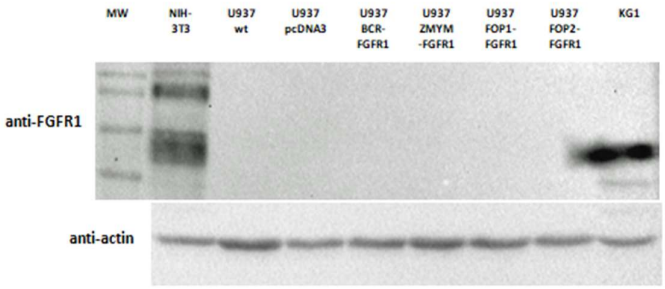
Our next task was to study influence of various fusion proteins containing kinase domain of FGFR1 on phenotype and function of U937 cells. For this purpose we used fusion genes obtained from Prof. John Cowell from the Cancer Center at Georgia Regents University. These fusion genes, *BCR-FGFR1* [29], *ZNF-FGFR1* [28] and *OP1-FGFR1* [27] were drivers of leukemogenesis in blood cells. We have also cloned *FOP2-FGFR1* fusion gene from KG1 cells. All the above fusion genes were cloned into pcDNA3 vector, and used for transfection of U937 cells. Since all transfections were succesful, as U937 cells were able to survive in a selection medium, the expressions of fusion genes were tested, relative to *GAPDH* expression, using Real-time PCR. Expression of *FOP2-FGFR1* fusion gene in KG1 cells was tested as a positive control. Surprisigly, neither of fusion genes in transfected U937 cells was expressed, as presented in figure 5a. Also the presence of fusion proteins was tested in Western-blots. In this case, FGFR1 in NIH-3T3 cells was used as an additional positive control. As presented in figure 5b, fusion proteins were not detectable in transfected cells.



(**a**)



(**b**)

Figure 5. Expressions of fusion genes and proteins in transfected U937 cells. (**a**) mRNA from mock-transfected U937 cells (pcDNA3), U937 cells transfected with vectors encoding fusion genes, and KG1 cells was isolated and translated into cDNA. Then the expressions of BCR-FGFR1, ZNF-FGFR1, OP1-FGFR1 and FOP2-FGFR1 relative to *GAPDH* mRNA levels were measured in Real-time PCR. The bar charts show mean expressions obtained from triplicates. (**b**) Protein lysates from the above cells, and from NIH-3T3 cells were tested in Western-blots using anti-FGFR1, and subsequently anti-actin antibodies. The presence of proteins containing FGFR1 kinase domain was visualized using chemiluminescence.

2.5 Influence of FGF1 signaling on the vitamin D receptor expression and activity in U2OS cells

Then we wanted to study if high levels of FGFRs may contribute to regulation of *VDR* expression also in the cells other than blood. For this purpose we used U2OS cells, which were obtained from the bone tissue of a fifteen-year-old human girl suffering from osteosarcoma [27]. These cells are easy to transfect, and we have known from our previous

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studies that they have high expression of *VDR* and are responsive to 1,25D [28]. Thus, we transfected U2OS cells with same plasmids encoding four FGF receptors. The overexpression of *FGFR1*, *FGFR2*, *FGFR3* and *FGFR4* genes in transfected cells has been tested in Real-time PCR. The expression of all receptors except *FGFR1* has been significantly upregulated in U2OS cells transfected with vectors encoding *FGFRs* in comparison to mock-transfected ones (figure 6).

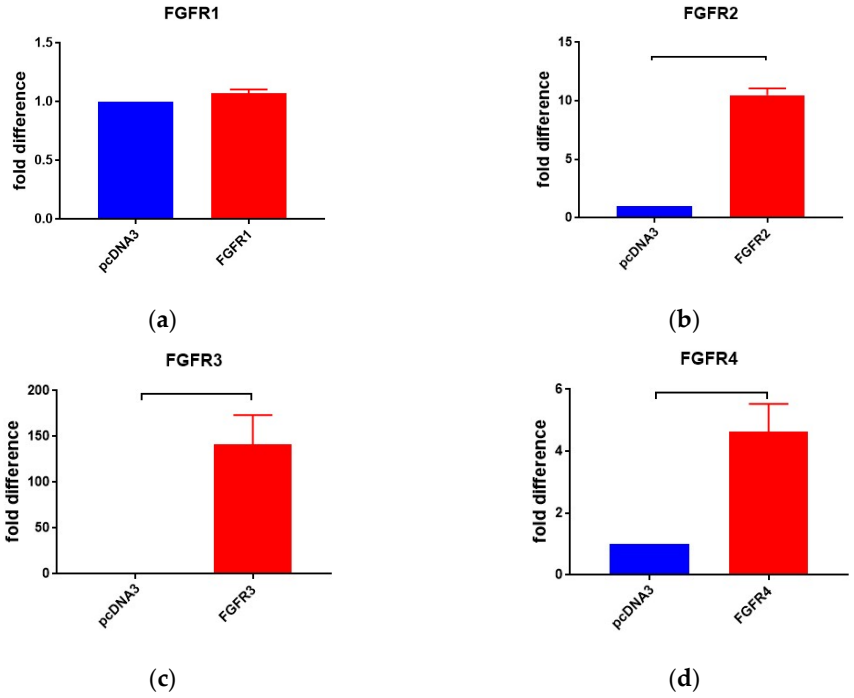

Figure 6. Expressions of *FGFRs* in transfected U2OS cells. mRNA from mock-transfected U2OS cells (pcDNA3) and the cells transfected with vectors encoding FGFR 1-4 was isolated and translated into cDNA. Then the expressions of *FGFR1* (**a**), *FGFR2* (**b**), *FGFR3* (**c**) and *FGFR4* (**d**) relative to *GAPDH* mRNA levels was measured in Real-time PCR. The bar charts show mean values (±SEM) of fold differences in gene expression obtained in four experiments. Values that differ significantly from those obtained for mock-transfected cells are marked with whiskers.

Since three of the four *FGFRs* were significantly overexpressed in U2OS cells, in the next step we tested the expression of *VDR* in untreated cells and the expression of *CYP24A1* in the cells exposed to 10 nM 1,25D for 24 hours, which is the time-frame at which CYP24A1 achieves its maximum in these cells [29]. The results indicate that overexpression of *FGFR 2-4* does neither induced increase in *VDR's* expression (figure 7a) nor in VDR's activity (figure 7b).

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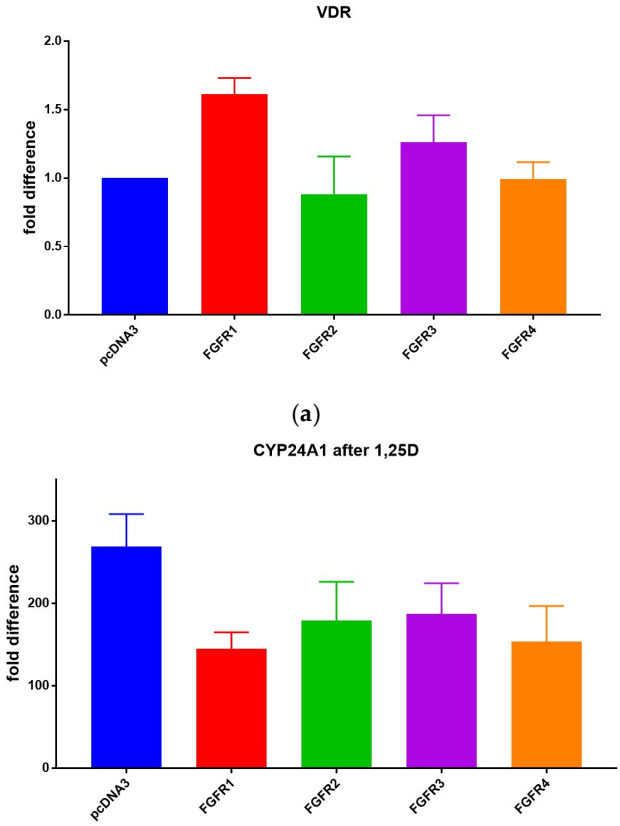

Figure 7. Expressions of VDR and CYP24A1 in transfected U2OS cells. Mock-transfected U2OS cells (pcDNA3) and the cells transfected with vectors encoding FGFR 1-4 were left untreated (**a**), or exposed to 10 nM 1,25D for 24 hours (**b**). Then mRNA from was isolated and translated into cDNA. Expressions of *VDR* (**a**) and *CYP24A1* (**b**) were measured relative to *GAPDH* mRNA levels was measured in Real-time PCR. The bar charts show mean values (±SEM) of fold differences in gene expression obtained in four experiments. Values that differ significantly from those obtained for mock-transfected cells are marked with whiskers.

3. Discussion

Many malignancies are driven by chromosomal translocations which involve the gene for FGFR1 and fuse it to the distant amino-terminal partners. In blood cells, these translocations are associated with the disease called 8p11 myeloproliferative syndrome, which typically presents with bilineage characteristics (myeloid and lymphoid), and rapidly transforms to AML [30]. In our previous experiments we discovered that constitutively active fusion protein FOP2-FGFR1 is responsible for activation of STAT1 signal transduction pathway, a high level of interferon (IFN) stimulated genes (ISGs), and in consequence for a very low expression level of *VDR* gene and low level of VDR protein [16].

In addition to chromosomal translocations, *FGFR* genes may be affected by other mutations. Gene amplification of *FGFR1* was discovered in squamous cell lung cancers and ER-positive breast cancers, whereas *FGFR2* in some gastric cancers and in some triple-negative breast cancers [31,32]. Recent data indicate that also in some cases of AML *FGFR1* gene is amplified [33].

Therefore, in our recent study we addressed the question whether overexpression of *FGFR 1-4* in myeloid leukemia cells may affect their phenotype and function. For that purpose U937 cells were transfected either with empty pcDNA3 plasmid, or with the same plasmid encoding each of four FGF receptors, namely, FGFR1, FGFR2, FGFR3 and FGFR4.

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Our flow cytometry experiments have revealed that overexpression of receptors 1, 2 and 3 upregulates the level of CD11b protein on the surface of U937 cells. Another name for CD11b is integrin αM (ITGAM), which is a subunit in heterodimeric integrin α-M β-2 molecule. This integrin is expressed by macrophages, neutrophils, NK cells and activated lymphocytes. Integrin α-M β-2 is responsible for leukocyte adhesion and migration to mediate the inflammatory response [34]. CD11b can also bind complement element C3bi, thus facilitating complement-mediated phagocytosis [35]. Our data suggest that signaling induced by FGFR 1-3 allows AML cells to differentiate along the myeloid pathway.

Then we examined the expression of *VDR* gene in the above cells. Overexpression of *FGFRs 1-3* caused slight but significant increase in the levels of *VDR* transcripts. Consequently, in the next step the cells were exposed to 10 nM 1,25D for 96 hours, and expression of VDR-target gene *CYP24A1* was tested in Real-time PCR, while the levels of differentiation markers were tested in flow cytometry. Overexpression of each of *FGFRs 2-4* caused increase in the levels of *CYP24A1* transcripts, thus indicating higher activity of VDR protein. Flow cytometry data revealed that the cells with overexpression of *FGFR 1-3* were more sensitive to 1,25D than mock-transfected cells. The levels of CD14 antigen, which were increased after exposure of the cells to 1,25D, were higher in cells overexpressing each of FGFR1-3, than in mock-transfected cells. CD14 is a protein present on macrophages responsible for binding bacterial lipopolysaccharide [36].

The next question to address was whether the FGFR-induced upregulation in VDR expression and activity is cell type-dependent or not. For this purpose we needed the cells responsive to 1,25D that originate from the tissue different than blood. We decided to use U2OS cells in our investigation. These cells were obtained from the bone tissue of a fifteen-year-old human girl suffering from osteosarcoma [27], they have high expression of *VDR* and are responsive to 1,25D, and they are easy to transfect [28]. So, also U2OS cells were transfected either with empty pcDNA3 plasmid, or the same plasmid encoding each of four FGF receptors. In these cells overexpression of FGFRs affected nether VDR gene expression nor transcriptional activity.

One more issue to discuss is the lack of expression of FGFR1 fusion genes in U937 cells. These cells were properly transfected, since after transfection they became resistant to geneticin. Moreover, when the same vector carried genetic information for normal FGF receptors, their expression was high. Therefore, there is a mechanism in U937 cells that either leads to inhibition of expression of fusion genes, or to degradation of mRNA encoding fusion genes. We suppose that this mechanism might be connected with the response to non-self nucleic acids in the cells of the immune system.

4. Materials and Methods

4.1. Cell Lines

U937, KG1 and U2OS cells were purchased from the German Resource Center for Biological Material (DSMZ GmbH, Braunschweig, Germany). NIH-3T3 were kind gift from Prof. Antoni Wiedłocha from the Institute for Cancer Research at The Norwegian Radium Hospital (Oslo, Norway). U937 and KG1 cells were cultured in RPMI-1640, and U2OS and NIH-3T3 adherent cultures in DMEM medium (both Biowest, Nuaillé, France), both supplemented with 10% FBS, 2mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin (all from Sigma-Aldrich, St. Louis, MO). The cells were kept at standard cell culture conditions of humidified atmosphere of 95% air and 5% CO_2 at 37°C. For all experiments the cells were suspended in a fresh medium containing 1,25D, FGF1 or an appropriate solvent.

4.2. Chemicals and Antibodies

1,25D was purchased from Cayman Europe (Tallinn, Estonia) and dissolved in an absolute ethanol to 10 μM, and subsequently diluted in the culture medium to 10 nM concentration. FGF1 was a kind gift from Dr. Daniel Krowarsch from our Department.

The protein was dissolved to the concentration of 100ng/ml in PBS with BSA (0.1mg/ml) containing heparin (concentration 10U/ml) and kept at -20°C. Antibody CD11b-FITC and matching isotype control were from BioLegend CNS, Inc. (San Diego, CA), while CD14-PE, and matching isotype control were from ImmunoTools (Friesoythe, Germany). Anti-FGFR1 antibody was from Cell Signalling (Rabbit, 9740, 1:1000) and horseradish-conjugated anti-rabbit antibody was from Jackson Immunoresearch, 1:5000).

4.3. cDNA Synthesis and Real-Time PCR

Total RNA was isolated using RNA Extrazol (Blirt, Gdańsk, Poland) as per manufacturer`s recommendations. RNA quantity was determined using Nanodrop (Thermo Fisher Scientific Inc. Worcester, MA) and the quality of RNA was verified by gel electrophoresis. RNA was transcribed into cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Real-time RT-PCR reactions were performed using SensiFAST SYBR Hi-ROX 2000 (Bioline, London, UK) and CFX real-time PCR System (Bio-Rad Laboratories Inc., CA). The sequences of *GAPDH*, *CYP24A1* and *VDR* and *FOP1-FGFR1* primers and reaction conditions were as described previously [26,37]. Remaining primers were as follows:

BCR-FGFR1 forward AGACCATCTTCTTCAAAGTGCCT and reverse GGGTCTTCGGGAAGCTCATAC

ZMYM2-FGFR1 forward ATGTTCCAGTGCCTATCCCTG and reverse AGGGTCTTCGGGAAGCTCATA

FOP2-FGFR1 forward AGAAGAACCCCAGAGTTCATG and reverse ATCAGTCGGCCTTGGAACTTA

Relative quantification of gene expression was calculated with the ΔΔCt method [38] using *GAPDH* as the endogenous control.

4.4. Flow Cytometry

U937 cells were collected by vigorous washing and washed with ice-cold PBS supplemented with 0.1% BSA (PBS/BSA). The cells were incubated for 1 h on ice with antibodies in the amounts suggested by the producers. Then the cells were washed and suspended in 350 μl of PBS/BSA prior to analysis on the Becton Dickinson Accuri C6 (San Jose, CA, USA). Data analysis was performed using Becton Dickinson Accuri C6 software.

4.5. Cloning of FOP2–FGFR1 fusion gene from KG1 cells

The 5′ and 3′ ends of *FOP2–FGFR1* fusion gene in KG1 cells were determined using SMARTer® RACE 5′/3′ Kit from Takara Bio (Japan). Then whole ORF of fusion gene encoding the tyrosine kinase was amplified from cDNA obtained from KG1 cells, and cloned into pcDNA vector (ThermoFisher) using an In Fusion cloning kit (Takara Bio). The full sequence of the fusion gene ORF is presented in supplementary Table S1.

4.6. Preparation of Constructs and Cell Transformation

The vectors pcDNA3-FGFR1, pcDNA3-FGFR2, pcDNA3-FGFR3 and pcDNA3-FGFR4 were kind gifts from prof. Antoni Wiedłocha from the Institute for Cancer Research at The Norwegian Radium Hospital (Oslo, Norway). After obtaining the vectors were amplified in *Escherichia coli* StellarTM Competent Cells (Takara Bio; Kusatsu, Japan), and the coding inserts were sequenced by Microsynth (Göttingen, Germany). The insert encoding FGFR2 had a point mutation, which was corrected using site-directed mutagenesis.

The vectors pMIEG3-BCR-FGFR1 [39], pMIEG3-ZNF-FGFR1 [40] and pMIEG3-FOP1-FGFR1 [37] were kind gifts from prof. John Cowell from the Cancer Center at Georgia Regents University in Augusta (GA, USA). All fusion genes have been cloned into pcDNA3 vector (ThermoFisher) using an In Fusion cloning kit from Takara Bio.

U937 and U2OS cells were transfected using Viromer® RED from Lipocalyx GmbH (Halle, Germany) according to user manual, and then stably transfected cells were selected using geneticin at a concentration of 1μg/ml.

4.7. Western Blotting

In order to prepare cell lysates, the cells were washed with PBS and lysed using SDS -page sample buffer (Tris-HCL, SDS, glycerol, β-mercaptoethanol, Bromophenol BLUE). Then the lysates were briefly sonicated and boiled for 10 min. For western blotting 20 µl of lysates were separated on 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were then dried, and incubated sequentially with primary (overnight) and a horseradish peroxidase-conjugated secondary antibody (1 h) at room temperature. The protein bands were visualized with a chemiluminescence. Then, the membranes were stripped, dried again, and probed with subsequent antibodies.

4.8. Statistical Analysis

One-way ANOVA and Student's t-test were used to analyze the statistical significance of the results obtained (GraphPad Prism 7; GraphPad Software Inc. and Excel; Microsoft).

5. Conclusions

The data presented in this paper indicate that FGF receptor signaling cooperates with VDR signaling in blood cell's differentiation towards macrophages.

Supplementary Materials: The full sequence of the *FOP2-FGFR1* fusion gene ORF from KG1 cells is presented in supplementary Table S1.

Author Contributions: Conceptualization, E.M.; methodology, E.M., A.M., A.J., P.K.; investigation, E.M., A.M., A.G., A.J., P.K..; resources, E.M.; data curation, E.M., A.M., A.G., A.J.; writing—original draft preparation, E.M.; writing—review and editing, E.M., A.M., A.J.; supervision, E.M.; project administration, E.M.; funding acquisition, E.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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