Retinoid-Regulated FGF8f Secretion by Osteoblasts Bypasses Retinoid Stimuli to Mediate Granulocytic Differentiation of Myeloid Leukemia Cells

Parvesh Chaudhry1, Xiaochun Yang1, Michael Wagner4, Ambrose Jong2,3, and Lingtao Wu1,3

Abstract
Signaling from the human hematopoietic stem cell (HSC) niche formed by osteoblastic cells regulates hematopoiesis. We previously found that retinoic acid receptor alpha (RARα), a transcription factor activated by retinoic acid (RA), mediates both granulocytic and osteoblastic differentiation. This effect depends on decreased phosphorylation of serine 77 of RARα (RARαS77) by the cyclin-dependent kinase-activating kinase (CAK) complex, a key cell-cycle regulator. In this article, we report that, by suppressing CAK phosphorylation of RARα, RA induces FGF8f to mediate osteosarcoma U2OS cell differentiation in an autocrine manner. By contrast, paracrine FGF8f secreted into osteoblast-conditioned medium by U2OS cells transduced with FGF8f or a phosphorylation-defective RARαS77 mutant, RARαS77A, bypasses RA stimuli to cross-mediate granulocytic differentiation of different types of human leukemic myeloblasts and normal primitive hematopoietic CD34+ cells, possibly through modulating mitogen-activated protein kinase (MAPK) pathways. Further experiments using recombinant human FGF8f (rFGF8f) stimuli, antibody neutralization, and peptide blocking showed that paracrine FGF8f is required for mediating terminal leukemic myeloblast differentiation. These studies indicate a novel regulatory mechanism of granulocytic differentiation instigated by RA from the HSC niche, which links loss of CAK phosphorylation of RARα with paracrine FGF8f-mediated MAPK signaling to mediate leukemic myeloblast differentiation in the absence of RA. Therefore, these findings provide a compelling molecular rationale for further investigation of paracrine FGF8f regulation, with the intent of devising HSC niche-based FGF8f therapeutics for myeloid leukemia, with or without RA-resistance.

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Introduction
Retinoic acid (RA) therapy for acute promyelocytic leukemia (APL) represents the most successful example of differentiation–induction therapy in current clinical oncology (1–3); however, this success has not extended to the remaining 85% of myeloid leukemia subtypes, largely because the mechanisms of RA-induced myeloid differentiation remain unclear. RA signaling is elicited through both the classical genomic pathway and rapid non-genomic signal transduction. The role of the classical RA-induced genomic pathway (4) in mediating cancer cell differentiation (5, 6) is well recognized (7). On the other hand, RA can exert rapid non-genomic effects independently of retinoic acid receptor (RAR)-mediated gene transcription (8) to induce cell differentiation or apoptosis (9, 10). Such non-genomic effects are mediated through cytoplasmic signaling kinases, for example, mitogen-activated protein kinase (MAPK) pathways related to phosphatidylinositol-3 kinase (PI3K) or extracellular signal–related kinase (ERK) signal transduction (10, 11). Moreover, recent insights into the role of osteoblast-formed hematopoietic stem cell (HSC) niche cues in modulating HSC development (12, 13) and leukemogenesis (13, 14) have gained attention on RA-mediated epigenetic regulation of terminal differentiation of myeloid leukemia cells.

It is known that RA-activated RARα regulates myeloid differentiation by transcriptional regulation of differentiation target gene (4, 6, 7). RARα, a phosphoprotein and transcription factor, is a substrate of CAK complex (6, 15). Human CAK is an enzyme consisting of cyclin-dependent kinase 7 (CDK7; ref. 16), cyclin H (17), and MAT1 (18).
Serine 77 (Ser-77) in the ligand-independent AF-1 domain of RARα (RARαS77) is the main residue phosphorylated by CAK (6, 15). CAK exists in cells either as free CAK or as part of the general transcription factor IIIH (TFIIH) complex. Both free CAK and TFIIH-containing CAK phosphorylate Ser-77 of RARα (6, 15). Free CAK controls G1 exit, a stage in which cells commit to proliferation or differentiation (19–21). We have found that either RA-decreased CAK phosphorylation of RARα or expression of the phosphorylation-defective RARαS77 mutant, RARαS77A, not only mediates granulocytic differentiation of both malignant and normal hematopoietic precursors (20–22) but also regulates osteoblastic differentiation and inhibits osteosarcoma formation (23). These studies show that RA-induced CAK-RARα signaling is involved in regulating differentiation of both osteoblastic and hematopoietic precursors.

Osteoblastic cells represent a regulatory component of the bone marrow microenvironment that mediate myelopoiesis, B-lymphocyte commitment, and HSC plasticity (12, 13, 24, 25), whereas disruption of this niche signal induces leukemogenesis (13, 26). Osteoblasts derived from human mesenchymal stem cells constitute the HSC niche (13, 24), and RA induces osteoblastic differentiation of both mesenchymal stem cells and osteosarcoma cells (23, 27). Striking success in epigenetic reversion of the genetic malignant phenotypes, as exemplified by RA treatment of APL harboring a PML-RARα fusion gene (2), provides proof of concept that RA-mediated HSC niche signaling can effect changes in the differentiation state of myeloid leukemia cells, even as the genome remains malignant and unstable (28). Previous studies strongly indicated the existence of a reciprocal relationship between osteoblasts and hematopoietic cells (12, 29), but the dimension of these interactions is yet to be defined. Because RA-induced loss of CAK phosphorylation of RARα or phosphorylation-defective RARαS77A mediates osteoblastic differentiation pathway through induction of FGF8f (23), we sought to investigate whether this osteoblast-derived FGF8f mediates granulocytic differentiation in a paracrine manner. Our studies show that osteoblast-secretion of FGF8f induced by either RA or RARαS77A regulates terminal granulocytic differentiation of myeloid leukemic cells, revealing a novel CAK-RARα signaling induced by RA to coordinate granulocytic differentiation at the paracrine level.

Materials and Methods

Cell culture

Human myeloid leukemic HL60, HL60R (RA-resistant), NB4 (APL), and human osteosarcoma U2OS cells were cultured as described previously (20, 21, 23). Cells within 5 to 15 passages of HL60 and U2OS cell lines, expanded immediately after receiving the cells from the American Type Culture Collection, were used for less than 5 months. HL60R (20) and NB4 cells (21) were tested to be Mycoplasma free by PCR methods after receiving cells from our collaborators, and each of those 5 to 15 passages of HL60R and NB4 cells was used for less than 5 months. The cancer cells were authenticated by their ability to form cancers in non-obese diabetic/severe combined immunodeficient, and/or nude mice. Normal human primitive hematopoietic CD34+ cells were obtained from AllCells and maintained with myeloid medium as described (22). The myeloid medium adapted for inducing granulopoiesis (MM-G) is supplemented with hydrocortisone for blocking the growth of lymphoid cells, which eliminates erythropoietin to inhibit the growth of erythroid cells (22). CD34 cells, certified to be HIV- and Mycoplasma-free by AllCells, were cultured for maxi mum 12 days without passaging after their initial expansion by following the manufacturer’s instructions. ATRA (RA) was from Sigma. RA concentration of 1 μM was used in the experiments. Recombinant human FGF8f was from R&D Systems.

Characterization of nuclear segmentation

Granulocytic differentiation, as judged by morphologic nuclear segmentation, has been described previously (20). Briefly, cells were cytocentrifuged for 5 minutes at 400 rpm in a Cytospin, fixed by using methanol, and stained with Wright–Giemsa stain (Sigma). The morphologic indicators of differentiation (nuclear/cytoplasmic ratio, nuclear shape, and degree of nuclear segmentation) were evaluated under a Zeiss Axioplan microscope. Images were color balanced in Adobe Photoshop.

Osteogenic differentiation

U2OS cells treated with RA or transduced with lentiviral pCCL-FGF8f or vector (Supplementary Fig. S1) were grown in 24-well plates. After reaching 70% to 80% confluence, the cells were washed and cultured for 21 days with bone differentiation medium (culture medium supplemented with 10 nmol/L dexamethasone (Sigma, #D2915), 20 mmol/L β-glycerolphosphate (Sigma, #G9891), 50 μmol/L L-Ascorbic acid 2-phosphate (Sigma, #A8960)). Cells were then fixed with 10% buffered formalin, and bone differentiation was judged by matrix mineralization as described previously (30) using Alizarin Red S (ARS; Sigma) staining.

Cell proliferation analysis

Cell duplication was determined by cell count as described previously (31).

Lentiviral transduction

Transduction of U2OS cells with lentiviral human RARαS77A or FGF8f (23) has been described previously (22).

Western blotting

Western blotting was done as described previously (20). Antibodies for FGF8, P-p38-MAPK (Thr 180/Tyr 182), P-p42/44-ERK (Thr-202/Tyr-204), p38-MAPK, p42/p44-ERK, and β-actin were from Santa Cruz Biotechnology.
Measurement of RA levels in the medium
The retained RA in the RA-osteoblast-conditioned medium (OCM) was monitored by using F9 reporter cells containing a LacZ reporter gene as described previously (32). X-gal was from Promega.

Quantitative real-time PCR
cDNAs were generated from DNase I–treated RNA (iScript; Bio-Rad), and quantitative real-time PCR (qRT-PCR) was carried out with QuantiTect SYBR Green PCR kits (Qiagen) by following the manufacturer’s instructions, using 384-well optical plates on the 7900HT Fast qRT-PCR System (Applied Biosystems). The housekeeping gene GAPDH was used as an internal control. Two negative controls were carried in parallel through all steps of the experiments. Standard curves for cDNA were composed of three 10-fold dilutions of control cDNA. The primers sequences and amplification conditions are given as Supplementary Table S1.

Analysis of FGF8f protein in the OCM
To assay secreted FGF8f, media conditioned by RA treatment of U2OS cells and FGF8f (or RARoS77A) overexpression in U2OS cells were harvested. These different OCM were concentrated using Pierce microconcentrators (Pierce Biotechnology, Inc.), and the protein quantity was measured by the improved Lowry assay using Bio-Rad DC Protein Assay (33). The presence of FGF8f in the OCM was confirmed by Western blot analysis.

Neutralization of FGF8f
Antibody neutralization of FGF8f has been described previously (23). Blocking peptide against FGF8 antibody (sc-27144P; Santa Cruz Biotechnology) was used as described previously (23).

Flow cytometric analysis
Flow cytometry using a direct immunofluorescence staining was carried out as described (20) for analysis of myeloid differentiation marker CD11b. CD11b-APC and CD34-PE antibodies were from BD Biosciences. Corresponding isotypes conjugated to irrelevant antibodies, isotype APC and isotype PE (BD Biosciences), were used as controls.

Statistical analysis
Student unpaired 2-tailed t test was used when appropriate.

Results
Osteoblast–secreted FGF8f in the presence of RA regulates osteoblastic differentiation in an autocrine manner
Because the HSC niche formed by osteoblastic cells mediates HSC development (13, 24, 25) through secreting its regulatory signaling molecules (24, 25, 34), while RA stimuli or expression of a phosphorylation-defective RARoS777 mutant, RARoS777A, in osteosarcoma U2OS cells upregulates FGF8f expression (23), we investigated whether such upregulated FGF8f can be sufficiently secreted by U2OS cells and in turn directly mediates osteoblastic differentiation of U2OS cells. In a series of experiments using U2OS cells treated with RA or transduced with lentiviral RARoS777A or FGF8f (Supplementary Fig. S1), we found that overexpression of RARoS777A or FGF8f mimicked the effect of RA or RARoS777A on inducing cellular morphology change (Fig. 1A), upregulating FGF8f secretion (Fig. 1B), inhibiting proliferation, and enhancing expression of osteoblastic differentiation regulator osteopontin (Fig. 1C). Moreover, bone differentiation analysis showed that, similar to RA stimuli, overexpression of FGF8f induces bone differentiation (Fig. 1D, top). Furthermore, to verify the effect of FGF8f on inducing bone differentiation, we decided to carry out antibody neutralization of FGF8f. Because of the special genetic structure of FGF8f shared with its alternatively spliced products FGF8e, FGF8b, and FGF8a (23, 35), an antibody specifically against FGF8f cannot be produced. However, in U2OS cells, FGF8f is the main isoform induced by RA or RARoS777A, and FGF8 antibodies only recognize a major 30-kDa protein, corresponding to the molecular mass of FGF8f (23). We, therefore, used FGF8 antibodies to neutralize the effect of FGF8f, as described previously (23), on inducing osteoblastic differentiation. We found that removal of FGF8f activities by FGF8 antibody neutralization significantly reduced osteoblastic differentiation (Fig. 1D, bottom). These data collectively show that RA-induced FGF8f is sufficiently secreted by U2OS cells, which exhibits a direct regulatory effect on mediating osteoblastic differentiation at the autocrine level.

RA-mediated FGF8f secretion by differentiating U2OS cells inhibits proliferation and induces differentiation of different subtypes of leukemic myeloblasts at the paracrine level
Osteoblast-formed HSC niche secretes signaling molecules to cross-mediate myelopoiesis (24, 25, 34), whereas FGF8f secreted by U2OS cells treated with RA or transduced with lentiviral FGF8f or RARoS777A (Fig. 1; ref. 23) is functionally active, as reflected by mediating osteoblastic differentiation in an autocrine manner (Fig. 1D). We, therefore, tested whether such osteoblast-secreted FGF8f can function in the absence of RA at the paracrine level to inhibit proliferation of myeloid leukemia cells, an indispensable biologic process required for induction of granulocytic differentiation. By coculturing HL60 cells with U2OS cells overexpressing FGF8f or RARoS777A, we found that the proliferation of both RA-sensitive HL60 and RA-resistant HL60R cells was inhibited (Fig. 2A). Because HL60R cells harbor a truncated ligand-dependent AF-2 domain of RARx (36, 37), the results show that osteoblast-derived paracrine FGF8f can bypass the upstream defect occurring at the levels of RA:RARx interaction to inhibit proliferation of RA-resistant leukemic myeloblasts, similar to the effect of RA on inhibiting proliferation of
RA-sensitive cells. Moreover, by culturing NB4 cells, which harbor a PML-RARα fusion gene (2), as well as HL60 cells in the presence of either RA or OCM collected from U2OS cells transduced with RARαS77A (S77A-OCM) or FGF8f (FGF8f-OCM), we found that cell proliferation in both HL60 and NB4 cells was inhibited (Fig. 2B). These results

Figure 1. Osteoblast-secreted FGF8f, in the presence of RA, regulates osteoblastic differentiation in an autocrine manner. A, morphology of U2OS cells treated with RA or transduced with lentiviral RARαS77A or FGF8f. B, Western blot (WB) analysis of FGF8f in the conditioned medium collected from U2OS cells treated with RA (left) or transduced by RARαS77A (middle) or FGF8f (right). C, proliferation analysis by cell count (left) and qRT-PCR depiction of osteopontin (OPN; right). * P < 0.05. D, bone differentiation induced by RA stimuli or FGF8f overexpression, as judged by ARS staining (top), and FGF8 antibody neutralization of FGF8-induced bone differentiation (bottom). Ab, antibodies.

Figure 2. RA-mediated FGF8f secretion by differentiating U2OS cells inhibits proliferation and induces differentiation of different subtypes of leukemic myeloblasts at the paracrine level. A, proliferation analysis of HL60 (left) and HL60R (right) cells cocultured with U2OS cells expressing RARαS77A or FGF8f. B, proliferation analysis of HL60 (left) and NB4 (right) cells cultured with different OCM. C, granulocytic morphologic differentiation of HL60 cells. D, flow cytometric analysis of the expression of CD11b. The corresponding isotypes as well as CD34 antibodies were used as controls. PE, phycoerythrin.
Paracrine FGF8f Signaling

Paracrine FGF8f derived from osteoblastic niche cells is required for mediating granulocytic differentiation of myeloid leukemic cells

To verify the inhibitory effect of paracrine FGF8f on leukemia cell proliferation, we chose to use FGF8 antibody neutralization of paracrine FGF8f effect, as described above (Fig. 1D). We cultured both HL60 and HL60R cells with FGF8f-OCM in the presence or absence of FGF8 antibodies as described previously (23). The results showed that whereas FGF8f-OCM inhibited cell proliferation in either HL60 or HL60R cells, this inhibitory effect of paracrine FGF8f was neutralized by FGF8 antibodies (Fig. 3A). Moreover, because FGF8f is a downstream target of RA signaling, we next verified whether the inhibitory effect of paracrine FGF8f is mediated by RA signaling. We found that, in the presence of negative (blank, vector) and positive (RA-OCM) controls, FGF8 antibodies neutralized the inhibitory effect of either RA-recombinant FGF8f-OCM or FGF8f-OCM-containing FGF8f on cell proliferation, while the blocking peptides of FGF8 antibodies overrode such counteraction of FGF8 antibodies (Fig. 3B). In parallel, the RA levels in RA-OCM were monitored to not exceed physiologic levels (10−9 mol/L) by using F9 RA-reporter cells (Supplementary Fig. S2). Therefore, the above-described data show that OCM-containing paracrine FGF8f is required for inhibiting proliferation of both RA-sensitive and RA-resistant leukemia myeloblasts.

To show that the observed effects of paracrine FGF8f are not due to unknown cytokines cosecreted and present in the conditioned medium, we first cultured HL60 cells in the presence of human recombinant FGF8f (rFGF8f). The result showed that rFGF8f significantly inhibited HL60 cell proliferation in the absence of RA (Fig. 3C). To examine that paracrine FGF8f specifically mimicked RA induction of granulocytic differentiation, we cultured HL60 and NB4 cells in the presence of RA or rFGF8f. The results showed that similar to RA stimuli, rFGF8f actually induces granulocytic morphology differentiation of HL60 and NB4 cells, an essential phenotype of terminal granulocytic differentiation (Fig. 3D, top; sections 3 vs. 2, respectively). Furthermore, using the antibody neutralization approach to define that paracrine FGF8f in OCM is a key factor in inducing granulocytic differentiation, HL60 cells were cultured with RA-OCM in parallel to different controls, including vector-OCM, RA stimuli, and blank. Distinctively, while FGF8 antibodies reversed the effect of RA-OCM containing FGF8f on inducing granulocytic morphologic differentiation of HL60 cells (Fig. 3D, bottom; sections 5 vs. 6), vector-OCM with FGF8 antibodies showed no effect on cellular status, similar to blank and vector-OCM groups (Fig. 3D, bottom; sections 4 vs. 1 and 3). Taken together, these data showed that paracrine FGF8f secreted by osteoelastic niche cells specifically cross-regulates granulocytic differentiation of different subtypes of leukemia myeloblasts.

Paracrine FGF8f induces terminal granulocytic differentiation of CD34+ cells

Hematopoietic development and granulopoiesis have been well characterized (38, 39). Although other groups and we have determined that RA mediates granulocytic differentiation of both normal and malignant hematopoietic precursors (1, 6, 20–22, 40), it remains unknown whether signaling instigated by RA from the HSC niche cross-regulates granulopoiesis. Given that paracrine FGF8f bypasses RA stimuli to induce terminal granulocytic differentiation of leukemia myeloblasts (Figs. 2 and 3), and that many pathways associated with cancer normally regulate stem cell development (41), we investigated whether paracrine FGF8f has similar effect on mediating terminal granulocytic differentiation of normal primitive hematopoietic precursors. CD34+ cells were cultured with RA or cultured with RA-OCM or S77A-OCM or FGF8f-OCM, in parallel to different controls. The results showed that both RA- and OCM-containing FGF8f inhibited proliferation of CD34+ cells (Fig. 4A and B). Because change in morphology is an essential phenotype for assessment of terminal granulocytic differentiation, we further analyzed granulocytic morphologic differentiation of CD34+ cells treated with RA or cultured with RA-OCM or S77A-OCM or FGF8f-OCM, in parallel to different controls. The results showed that RA-OCM-containing FGF8f mimics the effect of RA on inducing granulocytic differentiation (Fig. 4C). To verify that the observed response in CD34+ cells is not due to unknown cytokines cosecreted in the RA-OCM, we treated CD34+ cells with RA-OCM or S77A-OCM or FGF8f-OCM, in parallel to different controls. The results showed that RA-OCM-containing FGF8f mimics the effect of RA on inducing granulocytic differentiation (Fig. 4D). To verify that the observed response in CD34+ cells is not due to unknown cytokines cosecreted in the RA-OCM, we treated CD34+ cells with RA-OCM in the presence and absence of FGF8 antibodies. The results showed that, while RA-OCM-containing FGF8f mimics the effect of RA on inducing terminal granulocytic morphology differentiation of CD34+ cells (Fig. 4D; sections 5 and 11 vs. 2 and 8), FGF8 antibodies spatiotemporally neutralized such effect of RA-OCM.
containing FGF8f (Fig. 4D; sections 5 and 11 vs. 6 and 12). These data provide evidence that RA-mediated secretion of FGF8f by osteoblasts can bypass RA stimuli to inhibit proliferation and induce granulocytic differentiation of normal hematopoietic precursors.

**RA-mediated induction of FGF8f by osteoblasts modulates MAPK activities in both osteoblastic and hematopoietic precursors**

The above-described data show that osteoblast-secretion of FGF8f mediated by RA signaling coordinates osteoblastic differentiation (Fig. 1) with granulocytic differentiation of hematopoietic precursors (Figs. 2–4). These findings prompted us to ask a question: Which pathway is possibly involved in coupling FGF8f effect at both the autocrine and paracrine levels? Because FGF signaling is largely transduced through MAPK pathway (42) and because, in parallel to FGF8f induction, RA upregulates expression of MAPK signaling molecules in osteoblasts (23), we investigated whether MAPK signaling lies downstream of RA-originated FGF8f regulation in those osteoblastic and hematopoietic precursors. Western blot

Figure 3. Paracrine FGF8f derived from osteoblastic niche cells is required for mediating granulocytic differentiation of myeloid leukemic cells. A, FGF8 antibodies (100 ng/mL) neutralized the inhibitory effect of FGF8f-OCM on HL60 (left) and HL60R (right) cell proliferation. B, the inhibitory effect of paracrine FGF8f in S77A-OCM or FGF8f-OCM or RA-OCM on HL60 cell proliferation (blue bar) was neutralized in the presence of FGF8f antibodies (yellow bar), whereas such neutralizing effect of FGF8f antibodies was reversed in the presence of blocking peptides (red bar). FGF8 Ab versus FGF8 Ab + peptide: **, P < 0.002; *, P < 0.007; #, P < 0.02. P-FGF8f, paracrine FGF8f that presents in S77A- or FGF8f- or RA-OCM; B-peptide, blocking peptide for FGF8 antibodies. C, HL60 cells treated with rFGF8f. #, P < 0.0005; *, P < 0.002. D, rFGF8f mimicked the effect of RA on inducing terminal granulocytic differentiation of HL60 and NB4 cells (top). The effect of RA-OCM containing FGF8f on inducing terminal granulocytic morphology differentiation was reversed by FGF8 antibodies (bottom).
analysis using specific antibodies against different phosphorylated MAP kinases showed that, in FGF8f-mediated osteoblastic differentiation of U2OS cells (Fig. 1), p38-MAPK phosphorylation was inhibited (Fig. 5A, top) but p44-ERK phosphorylation was upregulated in association with decreased p42-ERK phosphorylation (Fig. 5A, bottom), in contrast to their even levels of total proteins. Interestingly, a similar dynamic pattern was also detected in HL60 cells cultured with RA-OCM, as shown by upregulated p44-ERK phosphorylation (Fig. 5B, bottom), accompanied by downregulated phosphorylation of both p38-MAPK (Fig. 5B, top) and p42-ERK (Fig. 5B, bottom).
Furthermore, in CD34+ cells cultured with RA-OCM, the phosphorylation of p42/44-ERK was periodically sustained (Fig. 5C, bottom) whereas the p38-MAPK phosphorylation was inhibited in general (Fig. 5C, top) during granulocytic differentiation induced by RA-OCM (Fig. 4). Altogether, these results support the notion that possibly through modulation of MAPK signaling at both the autocrine and paracrine levels, RA-induced FGF8f coordinates differentiation of osteoblastic and hematopoietic precursors.

Discussion

Osteoblast-derived paracrine FGF8f mediates granulocytic differentiation of both malignant and normal hematopoietic precursors in an RA-independent manner

The difficulty of integrating signals instigated by the genetic programs of hematopoietic precursors with the developmental cues originating from osteoblast-constituted HSC niche, where myeloid leukemia cells appear to arise, is a major barrier to effectively manipulate RA therapy for diverse types of myeloid leukemia. Our studies show that RA-mediated FGF8f induction by osteoblasts coordinates osteoblastic maturation (Fig. 1) with granulocytic differentiation of both leukemic myeloblasts and normal hematopoietic precursors (Figs. 2–4). Moreover, such paracrine FGF8f is capable of inhibiting proliferation of myeloid leukemic cells by overcoming RA-resistance (Figs. 2A and 3A), which results from mutation deletion of ligand-dependent AF-2 domain of RARα (36, 37). This effect of paracrine FGF8f on cross-regulating granulocytic differentiation of hematopoietic precursors is possibly through modulation of MAPK signaling (Fig. 5) via FGF8f-mediated interaction with the FGFR receptor (FGF-R; Fig. 6). Therefore, these findings provide evidence that, in the absence of RA, the downstream regulatory target of RA signaling, HSC niche-derived FGF8f, is able to execute the RA-induced signaling effect on inducng granulocytic differentiation of both malignant and normal hematopoietic precursors (Figs. 2–4). Success in identifying which FGFR on hematopoietic precursors interacts with paracrine FGF8f is crucial to determine the mechanisms of HSC niche-mediated granulopoiesis through modulation of MAPK signaling.

RAα hypophosphorylation induces FGF8f to coordinate osteoblastic maturation with granulocytic differentiation

How does RA mediate osteoblast induction of FGF8f to coordinate the regulation of osteoblastic differentiation with granulocytic differentiation? The important role of RARα is identified with myeloid development (3) as well as its mutation-resistance to myeloid differentiation (36, 37). It has been shown that RA-activated RARα regulates myeloid differentiation by transcription of differentiation target genes (1, 3, 4, 7), and that RA-suppressed CAK phosphorylation of RARα on RARαS77 of the AF-1 domain (6, 15) induces differentiation of both osteosarcoma cells (23) and leukemic myeloblasts (20, 21). Previous studies have shown that the human FGF8 promoter contains RARα-binding sites, and that RA-mediated interaction of RARα with these sites induces CAT activity (43). In addition, we showed that RA-suppressed CAK phosphorylation of RARα remodels RARα-chromatin interaction to induce transcription of differentiation target genes (6), including FGF8f that is induced by RA stimuli or overexpression of RARαS77A (23). Moreover, whereas FGFRs are expressed on nearly every cell of hematopoietic origin, FGFs are basically produced by bone marrow stromal cells, and cells from some mature peripheral blood lineages (13, 44). These findings indicate that RA-mediated FGF8f induction by osteoblasts (Fig. 1B; ref. 23) cross-regulates granulocytic differentiation (Figs. 2–4), possibly through its interaction with specific FGF-R expressed ubiquitously on hematopoietic cells (13, 44) in the bone marrow (Fig. 6). Therefore, determining the mechanism of RA-mediated transactivation of FGF8f in the HSC niche as well as the interaction of FGF8f/FGF-R on hematopoietic precursors are crucial to establish the role of RA-instigated epigenetic regulation of granulocytic differentiation.

Figure 6. RA-induced paracrine FGF8f from the osteoblastic niche cells bypasses RA stimuli to cross-regulate granulocytic differentiation of hematopoietic precursors. FGFR, FGFR receptor.
Paracrine FGF8f Signaling

Our studies show that RA-decreased CAK phosphorylation of RA-Ro induces FGF8f (23). The induced FGF8f (Fig. 1B), in turn, mediates osteoblastic differentiation in an autocrine manner (Fig. 1) and couples granulocytic differentiation of hematopoietic precursors at the paracrine level (Figs. 2–4). Such bidirectional regulation of FGF8f is associated with a dynamic change in ERK phosphorylation in both osteoblastic and hematopoietic precursors (Fig. 5). These findings indicate that RA coordinates CAK-RARo regulation with FGF8f-ERK signaling to couple osteoblastic maturation with granulocytic differentiation (Fig. 6). It is therefore important, in future studies, to delineate the involvement of ERK signaling mediated by FGF8f-dependent epigenetic regulation, using combined loss-of-function strategies including antibody neutralization, short-hairpin RNA silencing, and inhibition of ERK pathways. However, because FGF8f is also engaged in cross-talk with Wnt8B or WISP3 (23) through GSK3 or catenin signals (49) to activate the FGF–PI3K–AKT pathway in mediating granulocytic differentiation, the above-mentioned loss-of-function approaches might encounter difficulty in modulating granulocytic differentiation through blocking FGF8f-ERK pathway. If so, it is necessary to alternatively test whether paracrine FGF8f mediates granulocytic differentiation through cross-talk with Wnt signaling to activate the PI3K–AKT pathway, which has been implicated in the granulocytic differentiation of myeloid leukemia cells (50). Success in determining the mechanisms of this orchestration mediated by paracrine FGF8f should provide new insights into the niche-derived myeloid leukemogenesis.

In conclusion, our studies discover that FGF8f, a downstream targeting molecule induced by RA signaling in osteoblastic nicle cells, is capable of cross-mediating granulocytic differentiation of both malignant and normal hematopoietic precursors in the absence of RA. These findings show that, without RA stimuli, modulation of the downstream targeting molecule of RA signaling not only can efficiently implement the effect of RA on inducing granulocytic differentiation of myeloid leukemic cells but also can bypass the upstream defect occurring at the levels of RA:RARo interaction in RA-resistant cells. These findings thereby present a compelling molecular rationale for developing effective differentiation therapies against different types of myeloid leukemia by targeting paracrine FGF8f and its potential cofactors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Parvesh Chaudhry, Xiaochun Yang, Michael Wagner, et al.


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