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Vitamin D_3-driven signals for myeloid cell differentiation - Implications for differentiation therapy

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Abstract

Primitive myeloid leukemic cell lines can be driven to differentiate to monocyte-like cells by 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), and, therefore, $1,25(OH)_2D_3$ may be useful in differentiation therapy of myeloid leukemia and myelodysplastic syndromes (MDS). Recent studies have provided important insights into the mechanism of $1,25(OH)_2D_3$-stimulated differentiation. For myeloid progenitors to complete monocytic differentiation a complex network of intracellular signals has to be activated and/or inactivated in a precise temporal and spatial pattern. $1,25(OH)_2D_3$ achieves this change to the 'signaling landscape' by: *i)* direct genomic modulation of the level of expression of key regulators of cell signaling and differentiation pathways, and *ii)* activation of intracellular signaling pathways. An improved understanding of the mode of action of $1,25(OH)_2D_3$ is facilitating the development of new therapeutic regimens.

Keywords

Vitamin D_3; Deltanoids; Leukemia; Differentiation therapy; Cell signaling

1. Introduction

Treatment of human myeloid leukemia cells, including HL60 myeloblastic cells [1-4], U937 monoblastic cells [5], and THP-1 cells [6], with physiological concentrations of $1,25(OH)_2D_3$ induces their differentiation into functional monocytes. For complete functional differentiation to occur the leukemic cells have to be exposed to $1,25(OH)_2D_3$ for between

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36-48 hours as during this period 'differentiating' adherent CD14-expressing HL60 cells revert to an undifferentiated phenotype if $1,25(OH)_2D_3$ is removed [7-9]. Examination of the behavior of single HL60 myeloid cells exposed to $1,25(OH)_2D_3$ revealed a complex response as to cell behavior - there is an initial burst of proliferation which gives way to growth arrest and terminal differentiation [10,11].

Direct regulation of the transcription of genes encoding proteins that control the cell cycle, prevention of apoptosis and differentiation is important to $1,25(OH)_2D_3$-driven monocytic differentiation. Increased expression and/or activation of several intracellular signaling pathways is also crucial. These include several protein kinase C (PKC) isoforms [12,13], the phosphatidylinositol 3-kinase (PI3K)-AKT pathway [6,14-16], the p42 extracellular regulated kinase (p42-ERK), p38-ERK and the c-Jun N-terminal kinases (JNK) families of mitogen activated protein kinases (MAPKs) [17-22]. Pharmacological or genetic blockade of these pathways abrogates $1,25(OH)_2D_3$-driven monocytic differentiation. Control of these signaling pathways is necessarily complex since they have to be both temporally and spatially integrated so that the correct sequence of regulatory signals are generated in response to $1,25(OH)_2D_3$. Importantly, it is increasingly apparent that pathways are interconnected into networks, with nodal points at which several pathways intersect. While these networks have not been well delineated at this time, some suggested interactive pathways are presented in the Figures 1-3, and an example of a nodal point may be c-Raf 1 [23]. In this review we examine the signaling interplay that is provoked by $1,25(OH)_2D_3$.

2. Translocation of vitamin D receptor (VDR) to the nucleus plays a central role in $1,25(OH)_2D_3$-induced monocytic differentiation

Vitamin D receptor (VDR) is a member of the nuclear hormone receptor super family, and $1,25(OH)_2D_3$ acts similarly to the other steroid hormones, such as the thyroid hormone. VDR functions as a ligand-activated transcription factor. Ligated VDR forms a heterodimer with the retinoid X receptor (RXR) which regulates target genes by binding to vitamin D response elements (VDREs) in the promoter regions of genes resulting in either gene activation or repression [24-26]. Similarly, the VDR can directly interact with a number of other proteins which can regulate its activity. For instance, VDR and β-catenin can physically interact so that β-catenin functions are suppressed and VDR transcriptional activities are enhanced [27]. Conversely, the promyelocytic leukemia zinc finger protein (PLZF), which is often over-expressed in acute promyelocytic leukemia (APL), physically interacts with VDR in U937 myeloid cells, neutralizes VDR function, and blocks $1,25(OH)_2D_3$-stimulated monocytic differentiation [28].

$CD34^{+ve}$ progenitor cells from VDR knockout mice failed to differentiate into monocytes when challenged with $1,25(OH)_2D_3$ *in vitro* [29]. However, the production of monocytes (and other blood cells) appears to be normal in VDR knockout mice suggesting that VDR may not be absolutely essential for monocyte differentiation *in vivo* [29]. Whether the latter is true in humans is not known. However, myeloid progenitors isolated from patients with type II vitamin D resistant rickets (which contain non-functional mutated VDRs) are refractory to $1,25(OH)_2D_3$-induced differentiation [30]. Similarly, reducing VDR protein levels by antisense oligonucleotides [31] reduces the sensitivity of U937 cells to $1,25(OH)_2D_3$-driven differentiation [Hughes, unpublished observations]. Treatment of THP-1 cells with lipopolysaccharide reduces VDR expression and interferes with $1,25(OH)_2D_3$-driven monocytic differentiation [32]. Conversely, topoisomerase II inhibitors potentiate $1,25(OH)_2D_3$-induced monocytic differentiation of HL60 and U937 cells, and this relates to increased VDR expression [33].

A role for the formation of VDR-RXR heterodimers during differentiation was revealed by the enhancement of $1,25(OH)_2D_3$-induced HL60 monocyte differentiation by RXR agonists, and abrogation by RXR antagonists, but not by RAR antagonists [34]. RXRα is the principal partner for VDR binding and formation of this heterodimer is an absolute requirement for translocation to the nucleus and the activation of gene transcription [35]. Prevention of VDR-RXR hetero-dimerization, and subsequent recruitment of transcriptional co-activators, has been observed to reduce $1,25(OH)_2D_3$-stimulated monocytic differentiation of myeloid cell lines [34,36-38]. Similarly, preventing the association of the VDR/RXR heterodimer with VDREs, by co-expression of DR3-VDRE oligonucleotide decoys, reduced $1,25(OH)_2D_3$-mediated monocytic differentiation of HL60 cells [39].

In unstimulated cells most of the VDR is found in the cytoplasm, and upon $1,25(OH)_2D_3$ stimulation rapidly translocates to the nucleus [40-44]. This requires activation of the mitogen activated kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways [43]. Additionally, VDR expression increases within a few hours of exposure of myeloid leukemic cells to $1,25(OH)_2D_3$ [32,40-44]. This is due to increased transcription (which is indirect as the VDR gene does not have a VDRE) and, perhaps, a reduction in proteosome-mediated degradation of VDR [43]. It has recently been shown that the cardiotonic steroid bufalin enhances VDR-mediated gene trans-activation, and hence monocytic differentiation, in HL60 cells by prolonging the period that the VDR is retained in the nucleus after $1,25(OH)_2D_3$ stimulation, probably by preventing degradation of the VDR [45,46]. That translocation of the VDR to the nucleus is required for monocytic differentiation is evidenced by the following observations: *i)* VDR failed to accumulate in the nucleus in $1,25(OH)_2D_3$-resistant HL60 cells [41] and THP-1 sub-lines [40], and *ii)* in HL60 cells there is a correlation between the potency of side chain-modified vitamin D analogs in inducing differentiation and their ability to drive nuclear localization of VDR [44]. Interestingly, over-expression of the AML-associated gene translocation products PLZF-RARα, PML-RARα and AML-ETO-1 in U937 cells blocked $1,25(OH)_2D_3$-stimulated translocation of the VDR to the nucleus and reduced the responsiveness of cells to $1,25(OH)_2D_3$-stimulated monocytic differentiation [47,48].

3. Activities of lipid signaling pathways are increased during $1,25(OH)_2D_3$-driven monocytic differentiation

3.1 Increased expression and activation of protein kinase C isoforms is important

The PKC family is made up of a number of highly homologous serine/threonine kinases, that differ in their activation requirements and substrate specificities [49]. Members of the family play important regulatory roles in many aspects of hematopoietic cell function including differentiation [50]. An early indication that activation of PKC is important to monocytic differentiation can be taken from the observation that a single dose of the potent but relatively non-specific PKC activator 12-O-tetradecanoylphorbol 13-acetate (TPA), which cannot be metabolized, differentiates myeloid progenitor cell lines towards monocytes, whereas multiple doses of 1,2-dioctanylglycerol, a metabolized PKC activator ($t^{½}$ ~ 8 hours), are needed to achieve differentiation. This relatively crude experiment suggests that a long lasting PKC signal is required for cells to complete the monocytic differentiation program, but does not identify which of the PKC isoforms are involved [51].

Myeloid cells express all three subclasses of PKC isoforms [52], including the classical diacylglycerol (DAG)- and calcium-activated PKC isozymes (α, βI, βII and γ). PKCα and PKCβI/βII are important in different facets of $1,25(OH)_2D_3$-induced monocytic differentiation and in particular, PKCα is important in the maintenance of terminal differentiation [52]. The failure of KG-1a cells to differentiate may relate to a low basal level of PKCβ and a failure to up-regulate expression in response to TPA [53]. Resistance of a HL60 sub-line to TPA-

mediated monocytic differentiation appears to be associated with failure of a cytosol-to-membrane translocation of PKC isoforms [54].

PKC activity is increased following $1,25(OH)_2D_3$ treatment of myeloid cell lines. In particular, PKCα and PKCβI/βII activity starts to increase ~ 6-8 hours after exposure to $1,25(OH)_2D_3$ and remains elevated for several days (see figure 1) [51,53,55-58]. The importance of PKC to $1,25(OH)_2D_3$-mediated monocytic differentiation has been revealed by a number of approaches. Pre-treatment of cells with small molecule inhibitors of specific PKC isoforms and antisense oligonucleotides against PKCβI or PKCβII block $1,25(OH)_2D_3$-driven monocytic differentiation [58]. Treatment of myeloid cell lines with sub-differentiating concentrations of $1,25(OH)_2D_3$ for at least 12-24 hours, but for no more than 36-48 hours, 'primes' the cells so that they become 'supersensitive' to the differentiating actions of TPA. This involves the activation of classical PKC isoforms and tyrosine kinases [59,60]. Similarly, treatment of a HL60 sub-line that is resistant to TPA-mediated monocytic differentiation with a low concentration of $1,25(OH)_2D_3$ for 24 hours restores sensitivity to TPA. This appears to be mediated by an increase in the level of expression of PKCβ (figure 1) [61]. There are many examples of synergy between $1,25(OH)_2D_3$ and PKC-activating phytochemicals in inducing monocytic differentiation. For example, both silibinin or artemisinin synergise with $1,25(OH)_2D_3$ and increase the expression and activities of PKCβ and PKCα. Accordingly, HL-60 cell differentiation induced by silibinin or artemisinin in combination with $1,25(OH)_2D_3$ is blocked by PKC inhibitors [62,63].

3.2. Nuclear translocation and activation of phospholipase C by $1,25(OH)_2D_3$

Increased cellular levels of both DAG and calcium are required for the activation of the classical PKC isoforms [49,50]. Phospholipase C (PLC) hydrolyses phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) to generate DAG and inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3), a second messenger involved in the release of calcium [49,50]. $1,25(OH)_2D_3$ does not produce a rapid (within minutes of stimulation) Ins(1,4,5)P_3-dependent increase in $[Ca^{2+}]_i$ in HL60 cells [21,64]. Therefore, any changes in $[Ca^{2+}]_i$ seen in myeloid leukemic cell lines must rely on direct activation of store-operated Ca^{2+} entry (SOCE). Indeed, in HL60 cells $[Ca^{2+}]_i$ rises slowly to 20-30% above basal after 72-96 hours exposure to $1,25(OH)_2D_3$ [21,64]. A similar response is seen following stimulation of freshly isolated human peripheral blood mononuclear (PBM) cells with $1,25(OH)_2D_3$. In PBM the $1,25(OH)_2D_3$-stimulated increase in $[Ca^{2+}]_i$ was produced by classical SOCE mechanisms :- an initial depletion of intracellular calcium stores followed by a prolonged period of calcium entry due to activation of a Ca^{2+} release activated Ca^{2+} channel (CRAC) [65]. However, neither activation nor pharmacological inhibition of internal calcium stores or calcium influx have a significant effect on $1,25(OH)_2D_3$ provoked monocytic differentiation of HL60 cells [21].

Treatment of HL60 or THP-1 myeloid leukemic cells with exogenous PtdIns-specific PLC is sufficient to induce monocytic differentiation, which is associated with persistent activation of several classical PKC isoforms [66,67]. However, $1,25(OH)_2D_3$ failed to stimulate PLC activity in HL60 cells. Also, inhibitors and activators of PLC activity failed to have any effect on $1,25(OH)_2D_3$-mediated differentiation [21]. Even so, the $1,25(OH)_2D_3$-mediated monocytic differentiation of HL60 cells is associated with an increased nuclear expression of several PLC isoforms. Detection of intranuclear $PLC\beta_2$ and $PLC\gamma_2$ increases progressively from around 48 hours post exposure to $1,25(OH)_2D_3$, and peaks at ~96 hours, while $PLC\beta_3$ increases between 48-72 hours, and then decreases until 96 hours post $1,25(OH)_2D_3$ [68,69]. As yet, the importance of these increases is unclear, though it is possible that increased intra-nuclear levels of PtdIns(4,5)P_2 can have effects on chromatin structure and RNA processing [70].

3.3. Phospholipase D is activated by 1,25(OH)$_2$D$_3$

DAG can be obtained from the breakdown of membrane phospholipids, such as phosphatidylcholine, by the sequential actions of phospholipase D (PLD) and phosphatidate phosphohydrolase (Figure 1) [71]. PLD activity is increased during monocytic differentiation of U937 cells, induced by dibutyryl cyclic AMP [72], and during GM-CSF/IL-4-stimulated differentiation of monocytes into macrophages [73]. TPA-induced monocytic differentiation of U937 cells is augmented by the PLD activator Se-methylselenocysteine or by over-expression of PLD-1 [74]. 1,25(OH)$_2$D$_3$-stimulated PLD activity has been observed in HL60, U937, THP-1 and NB4 cells [21], and inhibitors of PLD and phosphatidate phosphohydrolase blocked the 1,25(OH)$_2$D$_3$-stimulated differentiation of HL60, U937 and THP-1 cells [21,75, 76]. Hence, PLD-mediated generation of DAG, which in turn activates PKC isoforms, appears to be important to 1,25(OH)$_2$D$_3$-driven monocytic differentiation.

3.4. 1,25(OH)$_2$D$_3$-induced stimulation of phospholipase A$_2$ generates a differentiation enhancing signal

The PLA$_2$ super family of enzymes hydrolyses a variety of phospholipids generating a free fatty acid (e.g. arachidonic acid), and lysophospholipid. Myeloid cells contain each of the five main types of PLA$_2$:- the secreted (sPLA$_2$'s), the cytosolic (cPLA$_2$'s), the Ca^{2+}-independent (iPLA$_2$'s), the PAF acetylhydrolases, and the lysosomal PLA$_2$'s. 1,25(OH)$_2$D$_3$ caused PLA$_2$-mediated release of arachidonic acid from HL60 and U937 cells, starting within a few hours and lasting at least 48 hours (figure 1) [77-79]. Addition of exogenous arachidonic acid potentiated 1,25(OH)$_2$D$_3$-mediated monocytic differentiation [79]. In keeping with all of this, inhibition of PLA$_2$ (with dexamethasone) blocked TPA-induced monocytic differentiation of HL60 cells and 1,25(OH)$_2$D$_3$-stimulated monocytic differentiation of U937 cells. Arachidonic acid can be further metabolized in myeloid cells, by either the cycloxygenases (to produce prostaglandins) or lipoxygenase (to produce leukotrienes) pathways. However, to date no prostaglandins or leukotrienes have been identified that either inhibit or potentiate 1,25 (OH)$_2$D$_3$-mediated monocytic differentiation.

3.5. Sphingomyelinase is activated by 1,25(OH)$_2$D$_3$

Sphingolipid breakdown products (ceramide, sphingosine and sphingosine-1-phosphate) are a new class of lipids that regulate proliferation, apoptosis and differentiation [80]. A transient rise in ceramide has been observed during TPA- and 1,25(OH)$_2$D$_3$-stimulated monocytic differentiation of HL60 cells [81-83]. Post-1,25(OH)$_2$D$_3$-treatment there is also increased expression and activation of a Mg^{2+}-independent neutral sphingomyelinase in HL60 cells [84] and an acidic sphingomyelinase in THP-1 cells [85]. Furthermore, treatment of HL60 cells with exogenous bacterial sphingomyelinase enhanced the ability of low doses of 1,25 (OH)$_2$D$_3$ to induce monocytic differentiation [81]. Synthetic ceramides when added with sub-threshold concentrations of 1,25(OH)$_2$D$_3$ triggered HL60 cells to differentiate to monocytes without further conversion to sphingosine [81], suggesting that ceramide is a mediator of myeloid cell differentiation. Recently, it has been shown that 1,25(OH)$_2$D$_3$-mediated monocytic differentiation is potentiated by several ceramide derivatives, via modulation of the activity of a signaling pathway involving PI3K, PKC, JNK and ERK [86].

Ceramide can be further metabolised to sphingosine (by ceramidase) and sphingosine-1-phosphate (S1P, by sphingosine kinase), and activation of sphingosine kinase generates an anti-apoptotic signal during 1,25(OH)$_2$D$_3$-mediated monocytic differentiation. The mechanism by which 1,25(OH)$_2$D$_3$-stimulated S1P production prevents apoptosis in myeloid cells is not understood.

4. The phosphatidylinositol 3-kinase-Akt-1 signaling pathway plays an important role in 1,25(OH)$_2$D$_3$-stimulated monocytic differentiation

Phosphatidylinositol 3-kinases (PI3Ks) generate lipid second messengers that control many aspects of cell function, including growth, differentiation survival, metabolism and motility [87,88]. In mammalian cells eight distinct PI3K isoforms have been described. These are divided into three sub-classes depending on subunit composition and mode of activation [89]. The class I PI3Ks are heterodimers composed of a catalytic subunit (p110α, p110β, p110γ) which physically associates with a regulatory subunit (p85 in the case of p110α, β, and δ or p87/p101 for p110γ). Both p110α and p110β are found in most cells whilst p110δ and p110γ are usually only found in cells of hematopoietic origin [90]. Upon receptor activation, class I PI3Ks synthesize the messenger lipids PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 in the plasma membrane where they coordinate the recruitment and activation of pH-domain containing protein effectors (e.g, the serine kinase Akt, sometimes called protein kinase B) [91]. PtdIns (3,4,5)P_3 is required for translocation of Akt to the plasma membrane where it is activated by sequential phosphorylation by phosphoinositide-dependent kinase-1 (PDK-1) and mammalian target of rapamycin complex 2 (mTORC2) or DNA-dependent protein kinase (DNA-PK) [92,93]. Pharmacological or genetic inhibition of several components of the PI3K signaling pathway point to an important role in both the survival and proliferation of hematopoietic progenitors and in myeloid differentiation [94-98]. For example, Akt plays important roles during lineage specification of hematopoietic progenitor cells whereby increasing Akt activity promoted neutrophil and monocyte development, whilst reducing its activity resulted in eosinophil differentiation [98]. Transplantation of CD34^{+ve} cells ectopically expressing constitutively active Akt into NOD/SCID mice resulted in enhanced neutrophil and monocyte development [98].

Inhibitor studies suggest that activation of both PI3K and Akt are crucial to 1,25(OH)$_2$D$_3$-mediated protection against apoptosis and induction of monocytic differentiation [6,14-16, 99]. For example, the 1,25(OH)$_2$D$_3$-mediated increase in the expression and activity of steroid sulphatase (a marker of myeloid differentiation) was blocked by pharmacological and genetic inhibition of either PI3K or Akt and involved activation of the transcription factor NF-κB by a PI3K/Akt-dependent mechanism [15]. CD11b and CD14 are two cell surface markers that are commonly used to assess the progress of 1,25(OH)$_2$D$_3$-stimulated monocytic differentiation. However, no recognizable VDREs can be found in the promoter regions of either gene. Binding of several universal transcription factors to their cognate response elements in the promoter region of the CD11b and CD14 genes has been associated with their up-regulation during monocytic differentiation. For example, PU.1, Sp1 and perhaps c-jun have been reported to regulate expression from the CD11b promoter [100,101]. Similarly, Sp1 can activate the CD14 promoter in myeloid cells [102-105]. Although not formally demonstrated at the CD14 promoter in myeloid cells, it has been shown that 1,25(OH)$_2$D$_3$ affects the transcription of several genes following the binding of a VDR-Sp1 complex to an Sp1 response element [106-108] Transcription of the CD14 gene is regulated also by a C/EBPβ transcription factor [103], whose expression is regulated by 1,25(OH)$_2$D$_3$ [19,37]. A 1,25(OH)$_2$D$_3$-stimulated increase in the activity and binding of the myeloid zinc finger-1 (MZF-1) transcription factor to the proximal promoter of CD11b and of CD14 may be essential for expression of CD11b and CD14. Both the DNA binding functions and the transcriptional activity of MZF-1 are dependent on a 1,25(OH)$_2$D$_3$-driven activation of PI3K [109].

5. 1,25(OH)$_2$D$_3$ modulates mitogen activated protein kinase signaling pathways

The mitogen activated kinases (MAPKs) are a family of serine threonine kinases that play important roles in coupling cell surface receptors to changes in transcriptional programs. The MAPKs are grouped into 3 principal families: the extracellular signal-regulated protein kinases (ERKs): the p38 MAPKs and the c-Jun N-terminal kinases (JNKs) [110] (see Figures 2 and 3). MAPK signaling involves the creation of a multi-protein signaling complex (a signalosome) and cellular targets include transcription factors that drive differentiation [111,112]. Recent evidence suggests that the MAPK family plays an important role in regulating many aspects of hematopoiesis [113]. As discussed below, 1,25(OH)$_2$D$_3$-mediated monocytic differentiation is associated with increased ERK and JNK activity and is augmented by inhibiting p38 MAPK, but most likely only its α and β isoforms (Zhang J and Studzinski GP, unpublished data). In contrast, all-*trans*-retinoic acid (ATRA)-mediated granulocytic differentiation of HL60 cells is thought to be associated with selective utilisation of ERK MAPKs, but not JNK or p38 MAPKs [114].

5.1 Activation of the Ras-Raf-ERKs signaling pathway has multiple roles in 1,25(OH)$_2$D$_3$-stimulated growth arrest and monocytic differentiation

The ERK MAPKs are activated in response to both tyrosine kinase- and G-protein-coupled receptors. Following activation of the small G-protein Ras [110-112], the serine/threonine kinase Raf-1 is recruited to the plasma membrane and activated by multi-site phosphorylation by PKC, protein kinase A and the Src family of tyrosine kinases. In the classical MAPK- ERK pathway, activated Raf-1 phosphorylates mitogen-activated protein kinase (MAP) kinase (MEK-1) which in turn phosphorylates and activates the p42 ERK1 and the closely related p44 ERK2 MAPKs. Active ERK is then released from MEK to dimerize and translocate into the nucleus [110-112,115]. The 1,25(OH)$_2$D$_3$-stimulated p42 ERK MAPK pathway activates the C/EBP family of transcription factors, which play an important role in driving monocytic differentiation [19].

In serum-starved HL60 and NB4 cells, 1,25(OH)$_2$D$_3$-stimulates an increase in p42 ERK activity (as assessed by its phosphorylation status) which starts within minutes and lasts less than an hour (Figure 2) [21,116-118]. This rapid time frame remains to be demonstrated in non-starved cells. Under both conditions, there is a more persistent delayed increase, lasting between 24-48 hours, and then ERK activity gradually fades [21,23]. A kinetically similar increase in Raf-1 activity is observed [23,116]. The initial rise in p42 ERK activation following 1,25(OH)$_2$D$_3$-stimulation of HL60 cells was blocked by pharmacological antagonists of VDR, but not by RXR antagonists [15]. Inhibitors of PKCα, Src tyrosine kinase and Ras-Raf-1 interactions blocked 1,25(OH)$_2$D$_3$-induced activation of the p42 ERK MAPK [21,117,118]. Thus, components of the canonical pathway appear to mediate 1,25(OH)$_2$D$_3$ activation of p42 ERKs. Inhibitor studies suggest that activation of p42 ERK MAPK signaling cascade is essential to 1,25(OH)$_2$D$_3$-stimulated monocytic differentiation of myeloid cells (Figures 2 and 3). However, the specificity of many of the small molecule inhibitors used in the above studies has been questioned, especially when compounds are used at high concentrations [119-122]. Therefore, it seems desirable that the conclusions based on pharmacological inhibition alone be reinforced by the use of more specific genetic or molecular biological approaches.

Raf-1 and its binding partners play roles in 1,25(OH)$_2$D$_3$-stimulated monocytic differentiation. Compounds that prevent the recruitment of Ras and Raf-1 to the plasma membrane, or block the physical association of Ras with Raf-1, block 1,25(OH)$_2$D$_3$-mediated monocytic differentiation [21]. Similarly, transfection of myeloid leukemic cell lines with antisense Raf-1 or short interfering mRNAs (siRNA) against Raf-1 reduced 1,25(OH)$_2$D$_3$-stimulated

monocytic differentiation [23,123]. In contrast, sensitivity of myeloid cell lines to 1,25 $(OH)_2D_3$-induced monocytic differentiation was enhanced by over-expression of Raf-1 [23], by direct small molecule Raf-1 activators [21] or indirect Raf-1 activation [124].

The transcription factor C/EBPβ, and its association with the retinoblastoma protein (Rb), are essential for 1,25$(OH)_2D_3$ stimulated monocytic differentiation. Up-regulation of C/EBPβ and retinoblastoma protein (Rb) expression in response to 1,25$(OH)_2D_3$ stimulation appears to be mediated by activation of the Raf-1/MEK/ERK MAPK signaling cascade [123]. Genetic knockdown of the Raf-1 prevents the 1,25$(OH)_2D_3$-induced up-regulation in C/EBPβ of Rb expression, and abolished C/EBPβ binding to Rb [123].

Raf-1 appears to have an additional signaling role during terminal monocytic differentiation of HL60 cells. This is mediated via Raf-1 activation of p90 ribosomal S6 kinase (p90 RSK), and independent of p42 ERK activation. During the later stages of 1,25$(OH)_2D_3$-stimulated HL60 differentiation, p90 RSK is still active when MEK and ERK activation has returned to basal levels [23]. Late p90 RSK activity was not reduced by inhibition of MEK or ERK, but was abrogated by Raf-1 inhibition. Interestingly, p90 RSK plays a role in activating C/EBPβ in many cell systems [125,126], and this could be important to monocytic differentiation.

There also seems to be a novel regulatory link between the PI3K-Akt signaling pathway and the p42 ERK pathway. As described above, 1,25$(OH)_2D_3$ stimulates an initial increase in Akt activity lasting for ~48-72 hours which then fall away as the cells enter growth arrest and terminal differentiation. Activated Akt can bind to and inactivate Raf-1 signaling [Figure 3]. Wang et al [123] have reported that over-expression of Akt inhibited p42 MAPK signaling, down-regulated $p21^{CIP\text{-}1/waf\text{-}1}$ and $p27^{KIP\text{-}1}$ and blunted differentiation in response to 1,25 $(OH)_2D_3$, while knockdown of Akt (by RNA interference) gave reverse effects. Therefore, the loss of Akt activity seen prior to 1,25$(OH)_2D_3$-induced growth arrest of myeloid cells seems to remove a functional brake on the p42 ERK signaling pathway. Wang et al [123] propose that as Akt activity wanes Raf-1 is released from the inhibitory Akt-Raf-1 complex, leaving it free to activate MEK and p42 ERK. It was also suggested that an indirect activation of p42 ERK is an absolute requirement for the 1,25$(OH)_2D_3$-mediated expression of $p21^{CIP\text{-}1/waf\text{-}1}$ and $p27^{KIP\text{-}1}$ in myeloid cells, and hence for growth arrest and terminal differentiation [123].

Several scaffolding proteins that modulate Raf-1 function are direct 1,25$(OH)_2D_3$ targets in myeloid cells. For example, transcription of the genes encoding the scaffolding protein kinase suppressor of ras-1 (KSR-1) and KSR-2 are directly increased by ligated VDR [127,128]. Both KSR-1 and KSR-2 can associate with and phosphorylate Raf-1 in a stimulus-dependent manner in several model systems, and can act as scaffolds to facilitate the assembly at the cell membrane of Raf-1 protein and its downstream targets [129,130]. However, the scaffold function of KSR-2, though likely on structural grounds, has not been formally demonstrated. Scaffolding protein, such as KSR1 can play an important part in regulating the intensity, duration and specificity of signaling pathways. Importantly, the relative stoichiometry of a scaffold protein and its binding partners are important to signaling, since as the level of expression of the scaffolding protein and Raf-1 approach parity, an optimal differentiation inducing signal is generated [112]. Conversely, an excess of a scaffold protein actually inhibits downstream signaling by titrating the client proteins, binding them individually rather than to the same scaffold molecule at the same time [130-132].

Manipulating KSR-1 and KSR-2 expression can influence the sensitivity of myeloid leukemic cell lines to differentiating stimuli (Figures 2, 3). Anti-sense knockdown of KSR-1 reduced monocytic differentiation induced by low concentrations of 1,25$(OH)_2D_3$. At low 1,25 $(OH)_2D_3$ concentrations, p42 ERK MAPK and p90 RSK activation was also diminished following KSR-1 knockdown [133]. Ectopic expression of a KSR-1 construct amplified the

monocytic differentiation-inducing signals at low $1,25(OH)_2D_3$ concentrations [133], while siRNA knockdown of KSR-2 reduced the proportion of highly differentiated monocyte-like cells in HL60 cultures treated with $1,25(OH)_2D_3$ [128]. Knockdown of KSR-2 has also revealed a role in increased cell survival indicating that optimal differentiation to monocytes requires enhanced anti-apoptotic (including Bcl-2/Bax- and Bcl-2/Bad-mediated) events [128].

5.2. Inhibiting p38 MAP kinase signaling potentiates $1,25(OH)_2D_3$-mediated monocytic differentiation

The p38 MAPK family is made up of four members: p38α, p38β, p38γ and p38δ [134,135]. These proteins are encoded by separate genes and are approximately 60% identical at the amino acid level. All four members of the p38 family are thought to be expressed in myeloid cells or their precursors, with p38α being the most abundant and p38β, p38γ and p38δ expressed to a lesser extent [136,137, Studzinski, unpublished observations]. It is thought that each of the p38 isoforms may play important roles in regulating several aspects of myeloid cell proliferation and differentiation although, the exact function of each of the p38 isoforms is still unclear. Multiple stimuli activate the members of the p38 MAPK family by phosphorylation mediated by the following kinase cascade:- the MAPK kinases MKK 3 and MKK6 are the primary upstream activators of p38 MAPK, although MKK4 has also been shown to activate p38 MAPK in some cell types [134,135]. A variety of upstream MAPK Kinase Kinases (MAP3Ks), including Tpl2/cot-1, are known to phosphorylate and activate specific MKKs in different cell types [134,135,138]. p38 MAPK can also be activated by autophosphorylation [134,135].

One study has suggested that lower than normal levels of phosphorylated p38 (an indirect measurement of its activation status) can be observed in hematopoietic progenitors found in bone marrow core biopsy samples from patients with myeloproliferative disorders and this has been suggested to be involved the increased proliferation seen in these cells [139]. In contrast, p38 MAP kinase activity appears to be constitutively activated in myeloid cells from patients with myelodysplastic syndromes (MDS) [139,140]. This has been correlated with incomplete differentiation and enhanced apoptosis of MDS hematopoietic progenitors [139,140]. In keeping, pharmacologic inhibitors of p38α/β decrease apoptosis in MDS $CD34^{+ve}$ progenitors and which leads to dose-dependent increases in myeloid colony formation. Similarly, siRNA knockdown of p38 leads to enhancement of hematopoiesis in MDS progenitors grown *ex vivo* [139-142].

The effect of $1,25(OH)_2D_3$ on p38 MAPK activity in myeloid cells is quite complex. In HL60 cells $1,25(OH)_2D_3$ caused a fairly rapid and long lasting (~ 24 hours) activation of p38 [18], followed by a fairly rapid return to basal level [18]. However, the degree of monocytic differentiation induced by low doses of $1,25(OH)_2D_3$ in myeloid leukemic cells is enhanced by specific inhibition of p38α and β [143-145]. Similarly, treatment of freshly isolated human monocytes with ouabain, which increases p38 activity, was associated with loss of expression of CD14 [146]. Inhibition of p38 MAPK activity was associated with an increase in the activity of the p42 ERK and particularly the JNK MAPK signaling pathways in myeloid cells [143-145] and hepatocytes [147]. Hence, there appears to be either negative cross-talk or a negative feedback loop between the MAPK signaling cascades.

5.3 The role of the c-Jun N-terminal kinases (JNKs) family of MAP kinases in $1,25(OH)_2D_3$-driven monocytic differentiation of myeloid cells

Three classes of the c-jun N-terminal kinase family (JNK) of MAP kinases are encoded by *jnk1, jnk2, jnk3* genes, and 10 separate JNK isoforms result from alternative splicing of these gene transcripts [148-150]. HL60-40AF cells express both JNK1 and JNK2 [151], whilst in THP-1 myeloid leukemic cells JNK1β1, JNK2α1, and JNK2α2 are found [152]. Recent

evidence suggests that JNK1 and JNK2 may have mutually antagonistic roles in the regulation of monocytic differentiation [151].

$1,25(OH)_2D_3$ stimulation of myeloid cells is associated with a fairly rapid but persistent increase in JNK phosphorylation and activity [18,153]. Pharmacological inhibition of JNK abrogated $1,25(OH)_2D_3$-mediated monocytic differentiation [153]. Translocation of JNK to the nucleus is essential to $1,25(OH)_2D_3$-mediated monocytic differentiation [151]. Further insight to the importance of JNK to monocytic differentiation came from studies using the HL60-40AF cell line, which is resistant to the differentiating effect of $1,25(OH)_2D_3$ [42,154]. In this cell line, $1,25(OH)_2D_3$ failed to stimulate JNK activity. Resistance of HL60-40AF cells to $1,25(OH)_2D_3$ was reversed by co-treatment with $1,25(OH)_2D_3$, carnosic acid (a plant derived antioxidant), and the p38 MAPK inhibitor SB203,580 (DCS cocktail) [42,145,151] which increased total JNK activity [155]. The central role of JNKs was reinforced by the observation that the degree of $1,25(OH)_2D_3$-mediated differentiation and JNK activity in HL60 myeloblastic cells were augmented in parallel following co-stimulation with ceramide derivatives [86].

It is now clear that the interplay between JNK1 and JNK2 is important to resistance of HL60-40AF cells to $1,25(OH)_2D_3$ [151]. In unstimulated HL60-40AF cells the basal level of JNK2 activity was found to be much higher than the basal activity of JNK1. In control HL60 cells the reverse was true and phosphorylated JNK1 (*p*-JNK) translocated to and accumulated in the nucleus within a few hours of stimulation with $1,25(OH)_2D_3$, while in HL60-40AF cells, expression of *p*-JNK1 was restricted to the cytosol. Hence, exclusion of *p*-JNK1 from the nucleus may be restraining differentiation in HL60-40AF cells. Consistent with this notion, in HL60-40AF cells the DCS cocktail partially restored the appearance of phosphorylated *p*-JNK1 in the nucleus, and of phosphorylated c-Jun (a marker of JNK1 activation). This indicated that an imbalance in nuclear JNK2 and JNK1 signaling restrains monocytic differentiation. When siRNA was used to knock-down JNK1 in HL60-40AF cells, the ability of the DCS cocktail to induce differentiation was reduced and this was associated with reduced activation of the c-Jun/AP-1 transcription factor complex. On the other hand, knock-down of JNK2 amplified the effectiveness of the DCS cocktail as revealed by up-regulation of activated JNK1 and increased activities of the JNK-regulated transcription factors which are essential for monocytic differentiation (e.g c-Jun, ATF2 and Jun B as well as C/EBPβ)[151]. These results show that JNK2 signaling is restraining JNK1's activity in driving $1,25(OH)_2D_3$-stimulated monocytic differentiation.

6. Role of microRNA in $1,25(OH)_2D_3$-stimulated monocytic differentiation

MicroRNAs are small, noncoding and highly conserved RNA molecules that regulate expression of genes post-transcriptionally by binding to the 3′-UTR regions of the mRNAs [156,157]. Many studies have demonstrated the importance of individual microRNAs to diverse physiological processes, including hematopoietic cell development [158-161]. Several microRNAs are widely expressed in hematopoietic cells, and their altered expression (e.g. by chromosomal translocations) has been correlated with leukemia [162,163].

MicroRNAs are down-regulated, in a dose- and time-dependent manner, during $1,25(OH)_2D_3$-stimulated monocytic differentiation of HL60 and U937 cells (Figure 3) [164]. The microRNAs down-regulated are members of the *miR-181* family; the one most markedly down-regulated was *miR-181a. In silico* studies have revealed *miR-181a* binding sites in human and mouse $p27^{Kip1}$ 3′-UTRs. In myeloid leukemia cells treated with $1,25(OH)_2D_3$, *miR-181a* contributes to the control of G1 to S phase transition by modulating expression of the cell cycle regulator $p27^{Kip1}$. *MiR-181a* also inhibits $1,25(OH)_2D_3$-induced expression of CD14 and markedly reduces G1 arrest of the cells. In proliferating HL60 cells, there is a high level of

expression of *miR-181a* and the levels of $p27^{Kip1}$ mRNA and protein are low and insufficient to inhibit Cdk4/6 activity and trigger cell cycle arrest. $1,25(OH)_2D_3$ down-regulates the level of *miR-181a*, resulting in an increase first in the level of $p27^{Kip1}$ mRNA, and then protein, leading to G1 block [164]. Similarly, down regulation of *miR-181a* and a concomitant rise in the level of expression of $p27^{kip1}$ mRNA prior to G1 arrest has been associated with TPA-induced monocytic differentiation of HL60 cells [165]. It is tempting to speculate that down-regulation of *miR-181b* during ATRA-induced granulocytic differentiation of APL cells might also relate to cell cycle control [166]. These studies are consistent with the report that levels of expression of *miRNA-181a* are higher in poorly differentiated AML blasts (M1 and M2 subtypes) than in subtypes M4 and M5, which show partial monocytic differentiation [167]. Together, the above studies [166-167] support the hypothesis that a high constitutive level of expression of *miR-181* family members may contribute to the malignant transformation of myeloid cells.

7. Strategies for improving the clinical utility of $1,25(OH)_2D_3$

Animal studies have shown that $1,25(OH)_2D_3$ significantly prolongs the survival of mice transplanted with leukemic cells by promoting cell differentiation [168,169]. However, oral administration of supra-physiological doses of $1,25(OH)_2D_3$ to MDS patients has only produced modest increases in neutrophil and platelet counts in a small minority of patients treated [170-174]. There were no significant increases in patient survival. Moreover, a significant proportion of AMLs are either refractory, or rapidly acquire resistance, to $1,25(OH)_2D_3$-mediated differentiation. The clinical utility of $1,25\text{-}(OH)_2D_3$ in these patients has also been compromised by the severe toxicity of therapeutic doses of $1,25(OH)_2D_3$, primarily by potentially fatal drug-induced hypercalcemia [175].

Attempts to resolve the hypercalcemia problem have focused on the generation $1,25(OH)_2D_3$ analogs ('deltanoids'), with reduced calcemic activity whilst retaining the ability to induce growth arrest and differentiation. Hundreds of such compounds have been developed [176] ; some of them are used in the treatment of psoriasis [177], but their usefulness in treating MDS and AML has yet to be demonstrated. For example, the non-calcemic deltanoid 1α-hydroxyvitamin D_3 ($1\alpha(OH)D_3$) was more effective than $1,25(OH)_2D_3$ in *in vitro* studies [168], but no clear beneficial effect was seen in MDS patients treated with the compound [170,172]. Similarly, 19-Nor-$1,25(OH)_2D_2$ (Paricalcitol, Zemplar) is a potent inducer of monocytic differentiation in myeloid leukemic cell lines *in vitro* [178], but real clinical benefit in MDS patients has not been observed [179]. Other low calcemic analogs which deserve further attention include 1,25-dihydroxy-16-ene-5,6-trans-cholecalciferol (Ro25-4020), which significantly prolonged the survival time of mice inoculated with the myeloid leukemic cell line WEHI $3BD^+$ at concentrations that did not affect calcium levels [180]. To date, the anti-leukemic effects of this compound have not been evaluated in humans [180]. The Gemini family of non-calcemic vitamin D analogs [181] are also worthy of examination in patients. These compounds are considerable more potent than $1,25(OH)_2D_3$ at driving growth arrest and monocytic differentiation of a variety of myeloid leukemic cell lines *in vitro* [182]. One of the family members, 1,25-dihydroxy-21(3-hydroxy-3-methyl-butyl)-19-nor-cholecalciferol (19-nor-Gemini; Ro27-5646), has shown some promise in a mouse model of myeloid leukemia at non-toxic doses [183]. However, the effects of the Gemini family of compounds have not been evaluated in human subjects.

An important issue as to the failure of early clinical trials of $1,25(OH)_2D_3$ for treatment of MDS and AML is the heterogeneous nature of these diseases. It has recently become appreciated that a detailed understanding of a patient's cytogenetic and 'genomic' background has contributed to the introduction of more effective 'patient-specific' chemotherapeutic regimes [184], therefore it is likely that similar considerations will help identify subgroups of

patients who will respond favourably to differentiation therapies from those that will not [184]. A new WHO classification of AML identifies four main groups: AML with recurrent genetic abnormalities, AML with MDS-related changes, therapy-related myeloid neoplasms, and AML not otherwise specified [185]. The first group contains diseases that are different as to genetic background, prognosis and treatment. For example, APL patients with specific chromosomal translocation t(15;17)(q22;q12), which generates the PML-RARα fusion protein, are unresponsive to the differentiating effect of 'physiological' doses ATRA but the blockage in differentiation can be overcome by supra-physiological amounts of ATRA, especially if combined with arsenic trioxide. ATRA treatment of APL patients significantly improved clinical outcomes [186,187]. Similarly, 5-10% of paediatric patients with leukaemia have chromosomal translocations involving 11q23 breakage. This is prevalent in patients with acute lymphoblastic leukaemia (ALL), acute myelogenous leukaemia (AML) of the M4 and M5 types according to the French-American-British (FAB) classification and mixed lineage leukaemia (MLL), and is usually associated with a poor clinical outcome. A panel of cell lines with translocations involving 11q23 has been established, each of which exhibit a differing sensitivity to ATRA- or $1,25(OH)_2D_3$-induced differentiation [188]. Those cell lines in which expression of the cyclin-dependent kinase 4 and 6 (CDK4 and CDK6) inhibitor *p*16 is compromised by the presence of 11q23 translocations failed to respond to either ATRA or $1,25(OH)_2D_3$, whereas those cell lines that express *p*16 responded to both ATRA and $1,25(OH)_2D_3$ [188]. It is, therefore, possible that differentiation therapy using $1,25(OH)_2D_3$ or other deltanoids might be limited to a specific sub-type(s) of AML. *In vitro* studies are underway to identify whether AML subtypes can be further classified by their sensitivity or resistance to $1,25(OH)_2D_3$-driven differentiation. Therefore, perhaps only those patients who carry favourable mutations or cytogenetic abnormalities should be included in clinical trials of deltanoids.

Differentiation therapy strategies can be devised from our understanding of $1,25(OH)_2D_3$-driven signaling pathways. Toxicity can be avoided by combining relatively low doses of deltanoids which have low calcemia-inducing activity with signal transduction pathway enhancers. For example, $1,25(OH)_2D_3$-induced monocytic differentiation of HL60 cells *in vitro* may be enhanced by co-treatment with ascorbic acid and vitamin E [189] by a mechanism that is believed to involve pertubations in arachidonic acid metabolism and cyclic AMP generation [79]. Other antioxidants such as carnosic acid, curcumin, ebselen and silibinin are also effective in potentiating monocytic differentiation of cells treated with low concentrations of $1,25(OH)_2D_3$ *in vitro* [145]. Recently, it was reported that in a mouse model of AML, Balb/c mice inoculated with murine WEHI-3B D leukemia cells, treatment of the mice with a low calcemic deltanoid (19-nor-Gemini) and carnosic acid markedly extended the life span of leukemia-bearing mice [183]. The myelodysplastic disease was reverted to normal and there was no significant liver toxicity or hypercalcemia. Hence, a combination of an antioxidant and a deltanoid may be useful in the treatment of AML. As described above, inhibition of p38 MAPKα/β markedly enhances monocytic differentiation of HL60 cells treated with a low dose of $1,25(OH)_2D_3$. A combination of carnosic acid and an inhibitor of p38MAPKα/β is extremely effective at increasing the sensitivity of HL60 cells to $1,25(OH)_2D_3$-stimulated differentiation *in vitro* and *ex vivo* [42,145]. It would be interesting to examine whether a further increase in survival can be obtained in the Balb/c model by adding a p38 inhibitor to the cocktail. Inhibitors of p38 kinase are very promising agents for combination therapy, since several of them are now in pre-clinical and clinical trials to treat inflammatory diseases [190,191]. Such studies will establish doses that are safe to use in the clinic. However, it seems likely that the role of different isoforms of p38MAPK will have to be separately delineated. Thus, signaling of differentiation by $1,25(OH)_2D_3$ remains a fertile field for further pre-clinical investigations.

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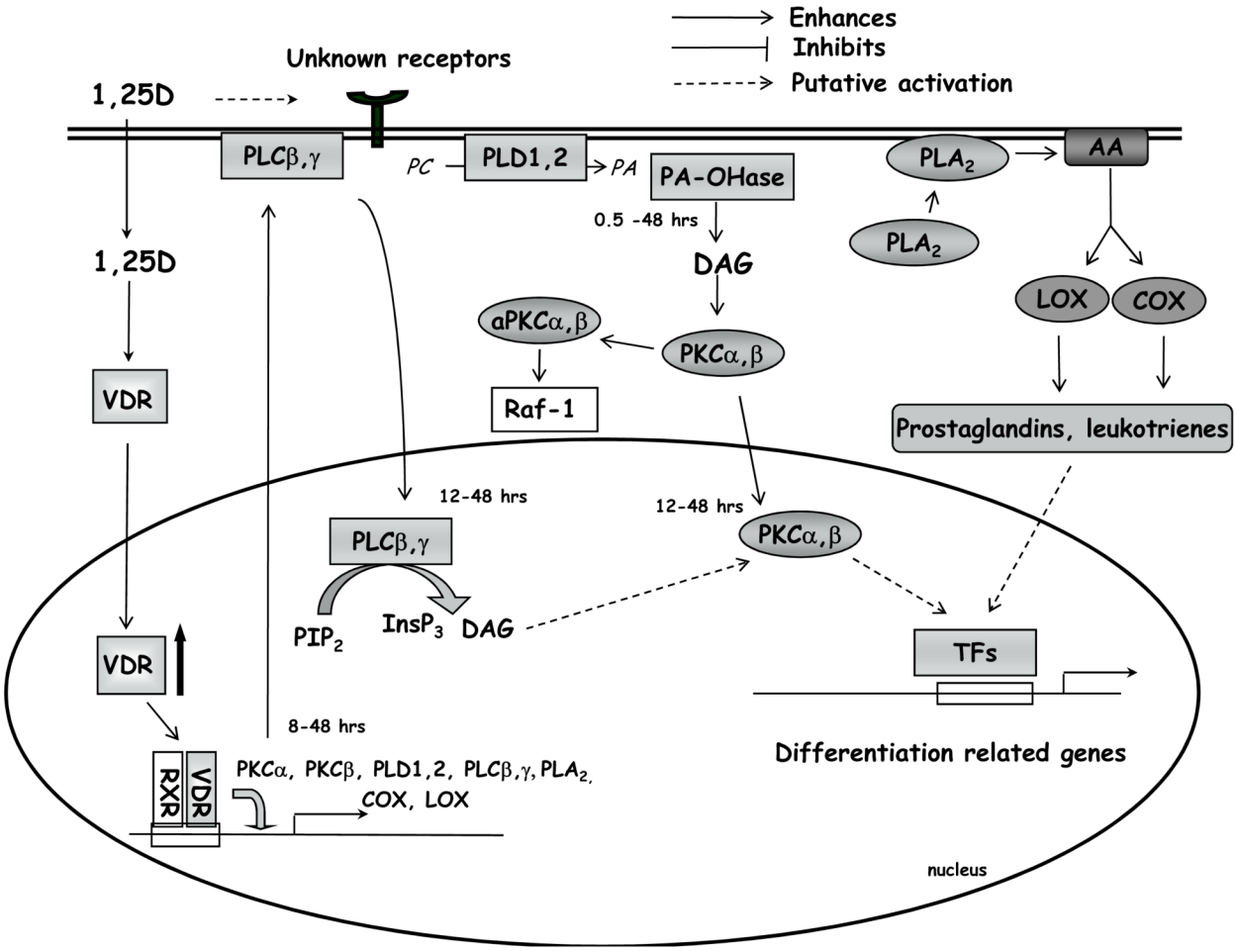

Figure 1. Activities of lipid signalling pathways during $1,25(OH)_2D_3$-driven monocytic differentiation

$1,25(OH)_2D_3$ crosses the cell membrane and binds to VDR in the cytosol. Ligated VDR translocates to the cell nucleus and, as a heterodimer with RXR, activates transcription of 1,25 $(OH)_2D_3$-regulated genes. In addition, $1,25(OH)_2D_3$, through an unknown mechanism, slowly activates and induces nuclear translocation of PLC isoforms. This leads to production of DAG and $InsP_3$ and to an increase in intracellular Ca^{2+}. Another source of DAG is provided by activated PLD, followed by the action of phosphatidate phosphohydrolase (PA-OH-ase). Increased levels of DAG and Ca^{2+} cause activation of PKCα and β, which is indispensable for cell differentiation. Activation of PLA_2 causes production of prostaglandins and leukotrienes, which, through unknown mechanisms, influence monocytic cell differentiation. For references see text. Signal transduction downstream to Raf-1 will be discussed in next figures.

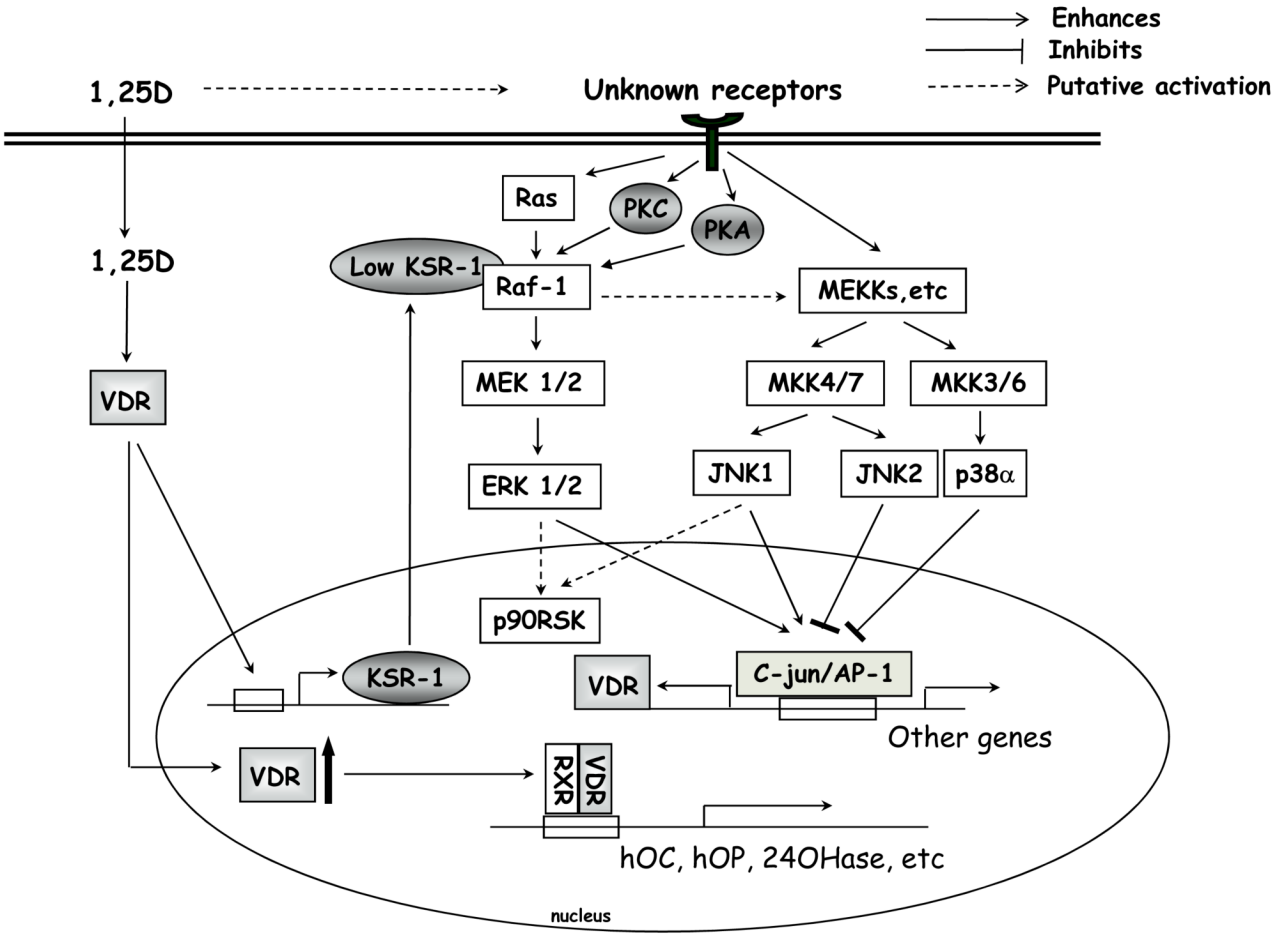

Figure 2. Reported signal transducers at early (0-24h) stages of 1,25(OH)$_2$D$_3$-induced monocytic differentiation

1,25(OH)$_2$D$_3$, through an unknown mechanism, induces rapid activation of various MAPKs which leads to an increase in AP-1 activity. Ras-Raf-ERK activation is additionally modulated by KSR-1, which is up-regulated by 1,25(OH)$_2$D$_3$. Activation of ERK1/2 and JNK1 positively regulates cell differentiation, while p38 MAPK α/β and JNK2 have a negative influence. There is also a potential negative feedback mechanism between p38 MAPK and ERK MAPK signal transduction pathways, as ERK activities increase when p38 α/β is inhibited.

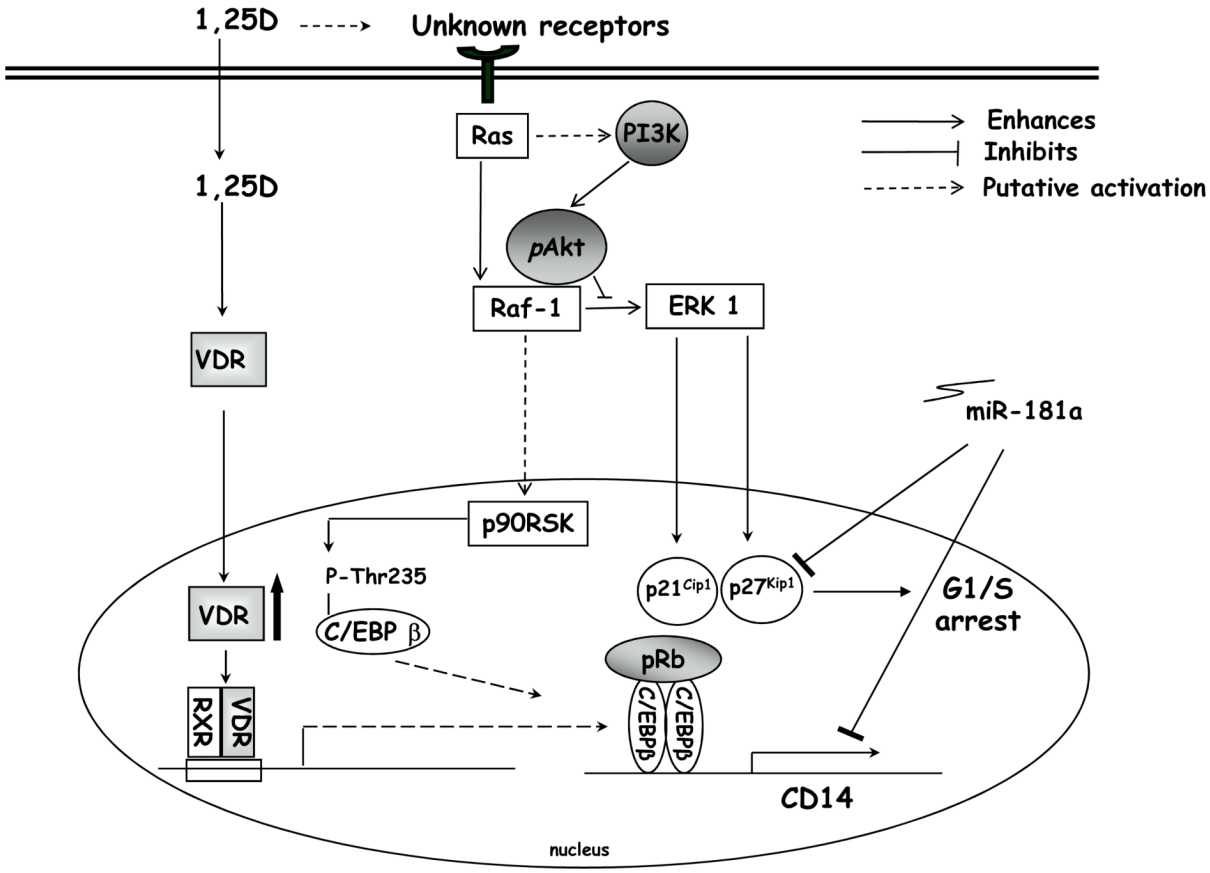

Figure 3. Examples of known signal transducers at late (24-48h) stages of 1,25(OH)$_2$D$_3$-induced monocytic differentiation

At later stages of 1,25(OH)$_2$D$_3$-induced monocytic differentiation there is increased expression of the transcription factor C/EBPβ. C/EBPβ is then phosphorylated and translocates to the cell nucleus, where it regulates many differentiation-related genes. Activation of the Ras-Raf-ERK1 signal transduction pathway may contribute to increases in the cyclic dependent kinase inhibitors p21$^{CIP\text{-}1/waf\text{-}1}$ and p27$^{KIP\text{-}1}$, which causes cell cycle arrest. Activated Akt (*p*Akt) may inhibit the activation of ERK by binding to Raf. Increases in the cyclic dependent kinase inhibitors appear to be reversed by high levels of either *miR-181a*.