Biochemical and Biophysical Research Communications 422 (2012) 751–757

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Decalpenic acid induces early osteoblastic markers in pluripotent mesenchymal cells via activation of retinoic acid receptor γ

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ARTICLE INFO

Article history:
Received 10 May 2012
Available online 19 May 2012

Keywords:
Decalpenic acid
Retinoid
Retinoic acid receptor γ
Pluripotent mesenchymal cell
Osteoblast differentiation

ABSTRACT

Decalpenic acid is a natural small molecule previously isolated from the fermentation broth of fungi that induces early osteoblastic markers in pluripotent mesenchymal cells. Treatment of mouse pluripotent mesenchymal C3H10T1/2 cells with decalpenic acid gave rise to a morphological change similar to that induced by the treatment with retinoic acid, i.e. the cells adopted a more elongated spindle shape. Using a retinoic acid response element reporter and receptor activity assays, we show that decalpenic acid is a new retinoid with selectivity towards retinoic acid receptors γ and α. The induction of early osteoblastic markers by decalpenic acid was significantly inhibited by treatment with the retinoid antagonist, LE540, or with small interfering RNA-mediated knockdown of retinoic acid receptor γ. These results demonstrated that decalpenic acid induces early osteoblastic markers in pluripotent mesenchymal cells through activation of retinoic acid receptor γ.

1. Introduction

Osteoblasts produce most of the proteins present in the bone extracellular matrix and are responsible for bone formation during both embryonic development and adult life [1,2]. In adult, the completed skeleton undergoes continuous remodeling that is characterized by the sequential tethering of bone formation by osteoblasts and bone resorption by osteoclasts. Dysregulation of this coupled remodeling can lead to bone diseases such as osteoporosis [3]. Therefore, understanding the molecular mechanisms that regulate osteoblast differentiation is of great interest for bone disease therapies and bone regenerative medicine. In this context, small molecules that induce osteoblastogenesis may be useful tools for investigating the mechanism of osteoblast differentiation.

Osteoblasts arise from mesenchymal stem cells (MSC) that have the potential to differentiate into various cell lineages including osteoblasts, chondrocytes, myoblasts and adipocytes [4]. Osteoblast differentiation from MSC is controlled by a hierarchy of essential transcription factors including Runx2 and Osterix/Sp7. During osteoblast differentiation, osteoblastic genes including *alkaline phosphatase* (*Alpl*), *osteopontin* (*Opn*), *type I collagen* (*Coll*) and *osteocalcin* (*Ocn*) are strongly induced [5]. Several signaling pathways involved in regulating a diverse range of developmental process, such as bone morphogenetic proteins (BMPs) and Hedgehog, play an important role in the regulation of osteoblast differentiation [2,5,6]. In addition, it has been shown that retinoic acid, a naturally active vitamin A metabolite, could stimulate osteoblast differentiation via the induction of the osteoblastic genes such as *Alpl* and *Opn* [7–10]. Retinoic acid exerts a wide range of effects on vertebrate development and adult tissue homeostasis by regulating cell proliferation, differentiation and apoptosis. Retinoic acid activates three members of the nuclear receptor superfamily, RARα, RARβ and RARγ, which function as ligand-dependent transcriptional regulators by binding, as heterodimers with rexinoid receptors (RXRs) to retinoic acid response elements (RAREs) located in target genes [11,12].

The mouse mesenchymal cell line, C3H10T1/2, exhibits MSC-like pluripotency and therefore provides a useful model system for the study of osteoblast differentiation [6,13,14]. Previously, we screened libraries of microbial fermentation broths using this model system and isolated a novel natural compound named decalpenic acid [15] (Fig. 1A). In the present study, we aimed to investigate the molecular mechanism by which decalenic acid mediates its osteogenic effect.

Abbreviations: MSC, mesenchymal stem cell; BMP, bone morphogenetic protein; RARE, retinoic acid response element; DPA, decalpenic acid; ATRA, all-*trans* retinoic acid; RA, retinoic acid; PM, purmorphamine; RAR, retinoic acid receptor; RXR, rexinoid receptor; LBD, ligand binding domain.

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http://dx.doi.org/10.1016/j.bbrc.2012.05.075

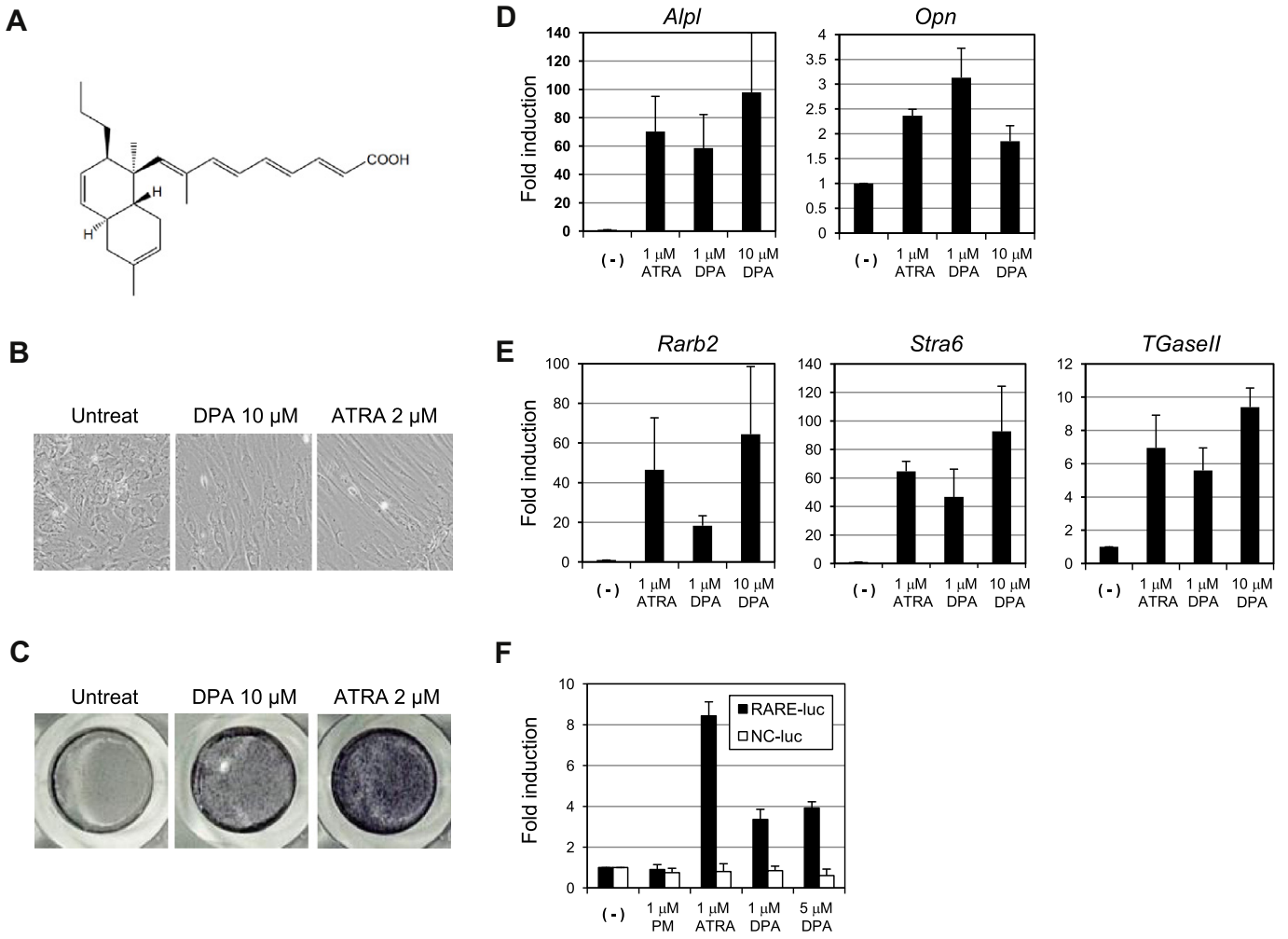


Fig. 1. Biological activities of DPA and ATRA in the mouse C3H10T1/2 mesenchymal cell line. (A) Relative stereochemistry of DPA. (B) Effect on cellular morphology. Cells were treated with 10 μM DPA or 2 μM ATRA for 72 h. (C) ALP staining. Cells were treated with 2 μM DPA or 2 μM ATRA for 4 days and then stained for alkaline phosphatase activity. (D) Real-time RT-PCR analysis of the osteoblastic marker genes, *Alpl* and *Opn*. Cells were treated with 1 or 10 μM DPA, or 1 μM ATRA, for 72 h, and then total RNA was extracted and subjected to real-time RT-PCR. (E) Real-time RT-PCR analysis of the retinoid target genes, *Rarb2*, *Stra6,* and *TGaseII*. Cells were treated with 1 or 10 μM DPA or 1 μM ATRA for 72 h, and then total RNA was extracted and subjected to real-time RT-PCR. (F) Retinoic acid response element (RARE) reporter assay. Cells were transiently transfected with the Cignal™ RARE reporter kit. After treatment for 24 h with ATRA, DPA, or PM (Purmorphamine; a hedgehog signal agonist), the dual luciferase assay was performed. RARE-luc is a reporter plasmid in which the firefly luciferase gene is under the control of a tandem of RAREs. NC-luc is a negative control reporter plasmid lacking the RARE element. Each error bars represents the mean ± S.D. obtained from three independent samples.

2. Materials and methods

2.1. Materials and cell culture

ATRA, 9-*cis* RA and Purmorphamine were purchased from Calbiochem (La Jolla, CA, USA). LE540 was purchased from Wako Pure Chemical Industry (Tokyo, Japan). siRNA SmartPool for mouse *Rarγ* and non-targeting control siRNA were purchased from Dharmacon/Thermo (Lafayette, CO, USA). Mouse C3H10T1/2 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). HEK293T cell line was obtained from GenHunter Corporation (Nashville, TN, USA). These cells were cultured in DMEM (Wako Pure Chemical Industry) containing 10% fetal bovine serum. ALP staining was performed as previously described [15].

2.2. RNA isolation and real-time RT-PCR

Total RNA was isolated from cells using an RNeasy Plus Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized using the SuperScriptVILO First-strand Synthesis System (Invitrogen). Real-time RT-PCR was performed on a Thermal Cycler Dice Real Time System (Takara, Shiga, Japan) using SYBR® Premix Ex Taq™II (Takara). All the primers were purchased from Takara. All reactions were run in triplicate, and relative expression levels were calculated using the ΔΔCT method. Values were normalized to the level of *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*).

2.3. DNA microarray analysis

Total RNA was isolated from the cells using the RNeasy Plus Kit (Qiagen). An oligonucleotide microarray (Mouse Whole Genome 4 × 44 K, Agilent) was used to monitor the relative abundance of transcripts. Double strand cDNA was synthesized from 1 μg of total RNA with oligo (dT) primer, amplified with T7 RNA polymerase up to ~100 μg of cRNA, and hybridized to the oligonucleotide microarray according to the manufacturer's instructions. The microarray experiments were repeated three times with different RNA preparations, and the data were analyzed using the GeneSpring GX ver.11 software package (Agilent).

2.4. Generation of reporter constructs

Gal4-hRARα and Gal4-hRXRα constructs were gifts from Dr. H. Srinivas (University of Pittsburgh). Human RARβ and RARγ cDNAs

were amplified from a cDNA pool of human Hs578T cells by PCR using Platinum *Pfx* DNA polymerase (Invitrogen). PCR primer pairs were as follows: for RARβ4 (sense: 5′-TAT GGA TCC ATT TAC ACT TGT CAC CGA GAT AAG; antisense: 5′-TAT AAG CTT TTA TTG CAC GAG TGG TGA CTG ACT G), for RARγ1 (sense: 5′-TAT GGA TCC GCC ACC AAT AAG GAG CGA CTC TTT G; antisense: 5′-TAT AAG CTT TCA GGC TGG GGA CTT CAG GCC C). The resulting PCR products were cloned into the pFA-CMV vector (Stratagene) at the *Bam*HI and *Hind*III sites.

2.5. Reporter assays

For RARE reporter assays, C3H10T1/2 cells were plated at an initial density of 1×10^4/well in 96-well plates (SUMILON, Tokyo, Japan). After culture for 24 h, cells were transfected with the Cignal™ RARE Reporter Kit (SABiosciences, Frederick, MD, USA) using MultiFectam (Promega, Madison, WI, USA) according to the manufacturer's instructions. 23 h after transfection, cells were treated with compounds in OPTI-MEM medium (Invitrogen) containing 0.5% FBS. After 24 h treatment, cells were solubilized using Reporter Lysis Buffer (Promega) and assayed for firefly and *Renilla* luciferase activities using the Dual-Glo™ Luciferase Assay System (Promega) according to the manufacturer's instructions. Firefly luciferase activity was normalized to the transfection efficiency using *Renilla* luciferase activity.

For the Gal4-RAR reporter assay, HEK293T cells were plated at an initial density of 1×10^5/well in collagen I-coated 6-well plates (SUMILON). After culture for 24 h, the cells were transfected with 700 ng of GAL4-RAR expression plasmid, 1 μg of pGL4.31 reporter (Promega) and 100 ng of pcDNA3.1LacZ (Invitrogen) using FuGeneHD transfection reagent (Promega) according to the manufacturer's instructions. One day after transfection, the cells were replated at a density of 8×10^3/well into collagen-coated 96-well plates. Cells were treated for 24 h with either vehicle or the indicated concentrations of the various compounds in OPTI-MEM medium containing 2.5% FBS. After treatment for 24 h, cells were solubilized using Passive Lysis Buffer (Promega) and assayed for firefly luciferase and LacZ activities using Luciferase Assay Reagent (Promega) and Lumi-Gal 530 (Lumigen, Southfield, MI, USA), respectively. Firefly luciferase activity was normalized to the transfection efficiency using LacZ activity. The relative transcriptional activity for the receptors in cells treated with 2 μM 9-*cis* RA was set as 100%.

2.6. Silencing of mouse Rarγ

C3H10T1/2 cells were seeded at an initial density of 2×10^5/well in 6-well plates 24 h prior to transfection. siRNA for *Rarγ* or control siRNA were transfected at a final concentration of 5 nM using LipofectAMINE RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. The siRNA/Lipofectamine complex was replaced 24 h later with DMEM containing 10% FBS and 1 μM DPA.

2.7. Western blot analysis

The cells were washed two times with ice-cold PBS and solubilized in lysis buffer (20 mM Hepes (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 50 mM NaF, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 25 μg/ml each of antipain, leupeptin and pepstatin). The lysates were centrifuged at 13,000*g* for 20 min at 4 °C. The supernatants were boiled in SDS buffer containing 0.5 M β-mercaptoethanol. These samples were separated by SDS–PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Protein levels were detected using the following antibodies: rabbit polyclonal antibodies specific for RARα (#2554, Cell signaling technology) and RARγ (ab12012, Abcam), mouse monoclonal antibodies specific for RXRα (K8508, PerseusProteomics, Tokyo, Japan) and α-Tubulin (T-5168, Sigma). The blots were visualized using ECL Plus Western Blotting Detection Kit (GE Healthcare Japan, Tokyo, Japan).

3. Results

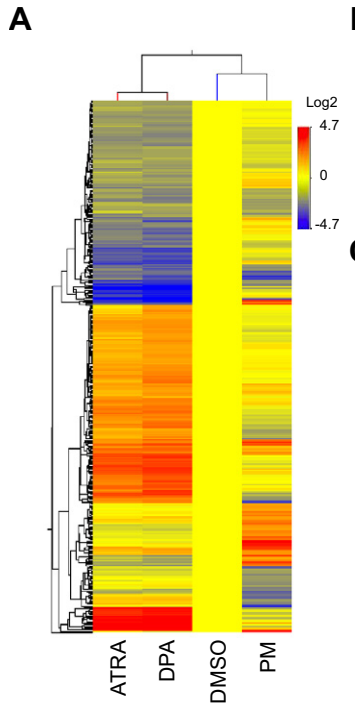
Previously, we demonstrated that treatment with decalpenic acid (DPA) alone induces early osteoblastic markers such as alkaline phosphatase (ALP) and osteopontin (OPN) in C3H10T1/2 cells, and that the effect of DPA was distinct from those of the hedgehog signaling agonist and BMP-2 [15]. These results suggest that DPA induces early osteoblastic markers through activation of signaling pathway(s) other than hedgehog and BMP.

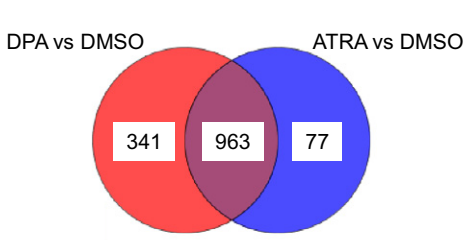
Because it has been shown that retinoids can stimulate osteoblast differentiation [7–10], we compared the effect of DPA in C3H10T1/2 cells with that of all-*trans* retinoic acid (ATRA), which is the most important endogenous retinoid (Fig. 1). First, we noticed that the morphology of cells treated for 72 h with micromolar concentrations of DPA changed to a more elongated spindle shape, which is similar to that induced by treatment with micromolar concentrations of ATRA (Fig. 1B). An ALP staining assay showed that, similar to treatment with DPA, treatment with micromolar concentrations of ATRA induced the activity of the early osteoblastic marker, ALP (Fig. 1C). Real-time RT-PCR analysis revealed that the mRNAs for the early osteoblastic markers *Alpl*, the gene encoding ALP, and *Opn* were significantly induced by these treatments (Fig. 1D). These data led us to hypothesize that DPA may act as a retinoid in C3H10T1/2 cells.

To examine whether DPA acts as a retinoid, we performed real-time RT-PCR analysis of known retinoic acid (RA)-responsive genes, such as *Rarb2*, *Stra6* and *TGaseII* [12,16,17] (Fig. 1E). In C3H10T1/2 cells, the mRNAs of these genes were strongly induced by 72 h treatment with micromolar concentrations of both ATRA and DPA. Moreover, a reporter assay using a luciferase reporter driven by a multimerized RARE demonstrated that treatment with micromolar concentrations of DPA induced an approx. 4-fold increase in luciferase activity over control (Fig. 1F). These data strongly suggest that DPA acts as a retinoid and activates the retinoic acid receptor signaling pathway in C3H10T1/2 cells.

Next, we performed DNA microarray analysis to compare the effects of DPA and ATRA on global gene transcription in C3H10T1/2 cells (Fig. 2). Cells were treated independently with 10 μM DPA, 1 μM ATRA, 2 μM Purmorphamine (PM, a hedgehog signal agonist [14]) and 1% DMSO for 72 h, and then mRNAs were isolated and subjected to an Agilent Mouse Whole Genome 4×44 K array, which represent over 41,000 genes and transcripts with public domain annotations. Among these 41,000-plus genes and transcripts, 1304 (3.2%), 1040 (2.5%) and 565 (1.4%) were differentially expressed ($\geqslant$3.0-fold change; $P \leqslant 0.05$) in response to DPA, ATRA or PM, respectively. The hierarchical clustering analysis for a total 1683 genes and transcripts demonstrated that the global gene expression signature of DPA correlates highly with that of ATRA (Fig. 2A). 73.8% (963/1304) of the DPA-responsive genes and transcripts overlap with those of ATRA, and 92.6% (963/1040) of the ATRA-responsive genes and transcripts overlap with those of DPA (Fig. 2B). Furthermore, all of the top 20 genes (including known RAR target genes such as *Cyp26b1* and *Rarb* [11]) up-regulated by DPA treatment were also strongly up-regulated by ATRA treatment (Fig. 2C). Thus, our data demonstrated that DPA mimics the effect of ATRA on global gene transcription, and that the main biological activity of DPA in C3H10T1/2 cells is the retinoid activity.

There are two families of retinoic acid receptors known as RARs (retinoic acid receptors) and RXRs (rexinoid receptors), which





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ProbeName	GeneSymbol	Description	Fold change (Log2) DPA	ATRA
A_52_P226664	Thsd4	Mus musculus thrombospondin, type I, domain containing 4 (Thsd4), transcript variant 1	9.42	7.48
A_51_P501844	Cyp26b1	Mus musculus cytochrome P450, family 26, subfamily b, polypeptide 1 (Cyp26b1), transcript variant 1	8.01	7.08
A_52_P600417	Rgs8	Mus musculus regulator of G-protein signaling 8 (Rgs8)	7.87	6.94
A_51_P255699	Mmp3	Mus musculus matrix metallopeptidase 3 (Mmp3)	7.75	8.04
A_51_P208603	Dio2	Mus musculus deiodinase, iodothyronine, type II (Dio2)	7.65	6.54
A_51_P162162	Inmt	Mus musculus indolethylamine N-methyltransferase (Inmt)	7.58	6.99
A_51_P237565	Kank4	Mus musculus KN motif and ankyrin repeat domains 4 (Kank4)	7.47	6.69
A_51_P394210	Fap	Mus musculus fibroblast activation protein (Fap)	7.45	7.28
A_51_P380750	Cbfa2t3	Mus musculus core-binding factor, runt domain, alpha subunit 2, translocated to, 3 (human) (Cbfa2t3), transcript variant 1	7.40	6.39
A_52_P281145	Kank4	Mus musculus KN motif and ankyrin repeat domains 4 (Kank4)	7.00	6.15
A_52_P21459	Tmem176b	Mus musculus transmembrane protein 176B (Tmem176b), transcript variant 1	6.95	6.88
A_52_P264550	Gabra3	Mus musculus gamma-aminobutyric acid (GABA) A receptor, subunit alpha 3 (Gabra3)	6.83	6.41
A_52_P36847	Cpz	Mus musculus carboxypeptidase Z (Cpz)	6.76	6.07
A_52_P515094	Tmem176a	Mus musculus transmembrane protein 176A (Tmem176a), transcript variant 1	6.75	6.76
A_52_P432124	Gsta3	Mus musculus glutathione S-transferase, alpha 3 (Gsta3), transcript variant 1	6.72	5.73
A_51_P231820	C130026I21Rik	Mus musculus RIKEN cDNA C130026I21 gene (C130026I21Rik), transcript variant 1	6.50	6.56
A_51_P258098	Tmem176a	Mus musculus transmembrane protein 176A (Tmem176a), transcript variant 1	6.43	6.45
A_51_P255675	Cpz	Mus musculus carboxypeptidase Z (Cpz)	6.35	5.69
A_51_P202440	Rarb	Mus musculus retinoic acid receptor, beta (Rarb)	6.19	5.49
A_52_P13802	Cbfa2t3	Mus musculus core-binding factor, runt domain, alpha subunit 2, translocated to, 3 (human) (Cbfa2t3), transcript variant 1	6.18	5.10

Fig. 2. Global transcriptional analysis of DPA treated cells. Cells were treated independently with 10 μM DPA, 1 μM ATRA, 2 μM PM or 1% DMSO for 72 h, and then mRNAs were isolated and subjected to the Agilent Mouse Whole Genome 4 × 44 K array. The experiments were carried out as three completely biological replicates, for a total 12 reads, and the data were pooled for analysis. For each gene and transcript, the differences in expression levels between DPA, ATRA, PM-treated cells and DMSO-treated cells were calculated. (A) Hierarchical clustering analysis for a total of 1683 genes and transcripts that were differentially expressed ($\geqslant$3.0-fold change; $P \leqslant 0.05$) compared to DMSO in at least one treatment-group. Increased expression levels are shown in red and decreased expression levels are shown in blue. (B) Venn diagram of 1304 genes and transcripts (DPA vs. DMSO) and 1040 genes and transcripts (ATRA vs. DMSO). (C) List of the top 20 up-regulated genes following treatment with 10 μM DPA. The fold changes induced by the treatment with 10 μM DPA or 1 μM ATRA are shown.

activate transcription by transducing retinoic acid signals [11,12]. Since our results demonstrated that DPA mainly acts as a retinoid, we investigated whether DPA could activate the retinoic acid receptors, RARα, RARβ and RARγ, and the rexinoid receptor RXRα. Human HEK293T cells were transiently transfected with GAL4-fusion constructs for each of the human receptors, and the effects of DPA, ATRA and 9-*cis* RA on the transcriptional activation at the GAL4 upstream activating sequence were examined (Fig. 3). As previously reported, 9-*cis* RA showed high efficiency in activating the RARs and RXRα [11]. ATRA also showed high activity to RARs and a lesser activity to RXRα in our experimental conditions. In this system, we found that DPA induced a significant activation of RARγ and a weak activation of RARα. In contrast, DPA caused no significant activation of RARβ and RXRα at the concentrations tested. These results indicate that DPA is a new retinoid that exhibits selective activity towards RARγ and RARα.

Finally, we examined whether DPA induces early osteoblastic markers via the activation of retinoic acid receptors. To address this issue, we first used the synthetic RAR pan-antagonist, LE540, which inhibits RARα, RARβ and RARγ [18,19]. The induction of known RA-responsive genes (*Rarb2* and *Stra6*) by treatment with 1 μM DPA in C3H10T1/2 cells was almost completely inhibited by co-treatment with 5 μM LE540 (Fig. 4A). In parallel, the induction of early osteoblastic markers (*Alpl* and *Opn*) with DPA was also strongly inhibited by co-treatment with LE540 (Fig. 4A). These results strongly suggested that DPA induces early osteoblastic markers through the activation of RARs. Because DPA mainly activates RARγ (Fig. 3), we next performed RNA interference-mediated knockdown of mouse *Rarγ* in C3H10T1/2 cells. Western blot analysis demonstrated that transient transfection of specific siRNAs for mouse *Rarγ* strongly decreased the level of mouse RARγ protein without affecting the levels of RARα and RXRα proteins (Fig. 4B). Under these conditions, the induction of known RA-responsive genes (*Rarb2* and *Stra6*) and early osteoblastic markers (*Alpl* and *Opn*) with 1 μM DPA was strongly inhibited in *Rarγ*-targeting siRNA transfected cells (Fig. 4C). These results demonstrated that DPA induces early osteoblastic markers via the activation of RARγ in C3H10T1/2 cells at a concentration of 1 μM.

4. Discussion

The chemical structure of DPA consists of a carboxyl group, a central polyene linker and a hydrophobic bicyclic end group, which is similar to the general chemical structure of retinoic acid (Fig. 1A). Moreover, there are many synthetic retinoids with a hydrophobic bicyclic end group [20]. These facts support our conclusion that DPA is a new retinoid and activates retinoic acid receptor signaling pathway.

The RAR family is composed of three genes, which express the α, β and γ isotypes that correspond to distinct pharmacological targets [11,12]. We found that at micromolar concentrations, DPA exhibited significant activity towards RARγ and to a lesser extent towards RARα, but did not activate of RARβ and RXRα to any extent at the concentrations tested (Fig. 3). The crystal structures of both RAR and RXR ligand binding domains (LBDs) bound to various ligands have revealed that RXR LBD forms a shorter, more restrictive L-shaped pocket in contrast to the linear I shape of the RAR LBD [12,20]. This restrictive shape of the RXR LBD requires a sharp bend of the polyene linker in the retinoid skeleton to allow binding.

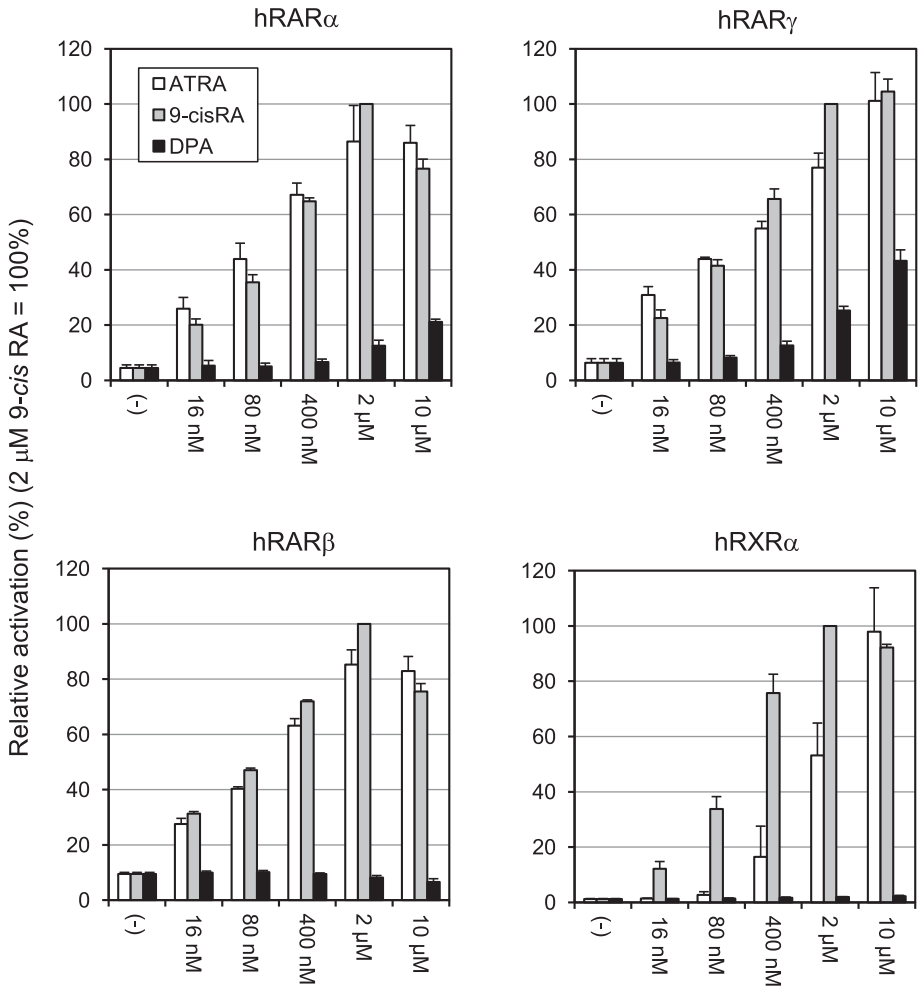

Fig. 3. Receptor activation profiles of DPA. The transcriptional activation activities of DPA on RARα, RARβ, RARγ or RXRα receptors were determined. HEK293T cells were transiently transfected with each of the GAL4-fused human receptors (RARα, RARβ, RARγ, or RXRα), pcDNA3.1LacZ, and pGL4.31 containing a GAL4 upstream activating sequence and a minimal adenoviral promoter upstream of the firefly luciferase gene. The effects of DPA, ATRA and 9-*cis* RA on the transcriptional activation at the GAL4 upstream activating sequence were examined by luciferase activity after treatment with the compounds for 24 h. Firefly luciferase activity was normalized to the transfection efficiency using LacZ activity. The relative transcriptional activity for the receptors in cells treated with 2 μM 9-*cis* RA was set as 100%. Each error bars represents the mean ± S.D. obtained from three independent samples.

Thus, 9-*cis* RA, which has a bent structure in the polyene linker, can bind and activate RXR, but ATRA, which has an unbent (all-*trans*) polyene linker, does not bind to RXR effectively. This is consistent with our finding that DPA, which possessed an all-*trans* polyene linker, similar to ATRA, does not activate RXRα (Fig. 3).

It has been reported that only three residues are divergent in the LBD among the RAR isotypes, and these are important for RAR isotype specification [20,21]. RARγ and RARβ differ at two residues in helices 5 and 11, whereas all three residues (in helices 3, 5 and 11) diverge in the LBDs of RARγ and RARα. Thus, the RARγ LBD is more similar to RARβ than RARα. We cannot account for the reason why DPA, which has significant activity towards RARγ, has a weak activity towards RARα but does not activate RARβ. Determination of the crystal structure of RARγ complexed with DPA is needed to address this question, and would provide useful information for the design of RAR subtype specific retinoids.

Despite of the RAR isotype selectivity of DPA, our DNA microarray analysis in C3H10T1/2 cells demonstrated that the global gene expression signature induced by 10 μM DPA correlates highly with that of 1 μM ATRA, which activates all of the RAR isotypes (Fig. 2). Our GAL4-RAR receptor assay showed that the treatment with 10 μM DPA activates RARγ and RARα significantly (Fig. 3). In addition, a previous study on ATRA-induced osteoblast differentiation in C3H10T1/2 cells revealed that the effects of ATRA are mediated by RARα and RARγ [8]. The RAR isotype selectivity of DPA and the profile of RAR isotype dependency in C3H10T1/2 cells may collectively account for the similarities of the global gene expression signatures induced by 10 μM DPA and 1 μM ATRA.

Several studies using *Rar* knockout cells have revealed isotype-specific effects on the induction of RA-responsive genes by ATRA. For example, the induction of *Stra6* is dependent on *Rarγ* and the induction of *Rarβ* is mediated by multiple Rar isotypes [17,22]. In contrast, siRNA-mediated knockdown of mouse *Rarγ* demonstrated that the induction of RA-responsive and osteoblastic marker genes by 1 μM DPA was dependent on Rarγ in C3H10T1/2 cells (Fig. 4). This observation is consistent with our receptor assay data, which suggest that 1 μM DPA primarily activates RARγ and activates RARα only slightly (Fig. 3). Therefore, DPA may be used to activate RARγ restrictively in a cellular context at a concentration of 1 μM. Because it has been reported that RARγ plays an important role in the self-renewal and differentiation of hematopoietic stem cells [23], DPA may be useful as a chemical tool for biological studies on hematopoietic stem cells.

In the present study, we have demonstrated that the natural small compound, DPA, is a new retinoid with selectivity towards RARγ and RARα. We also revealed that DPA induces early

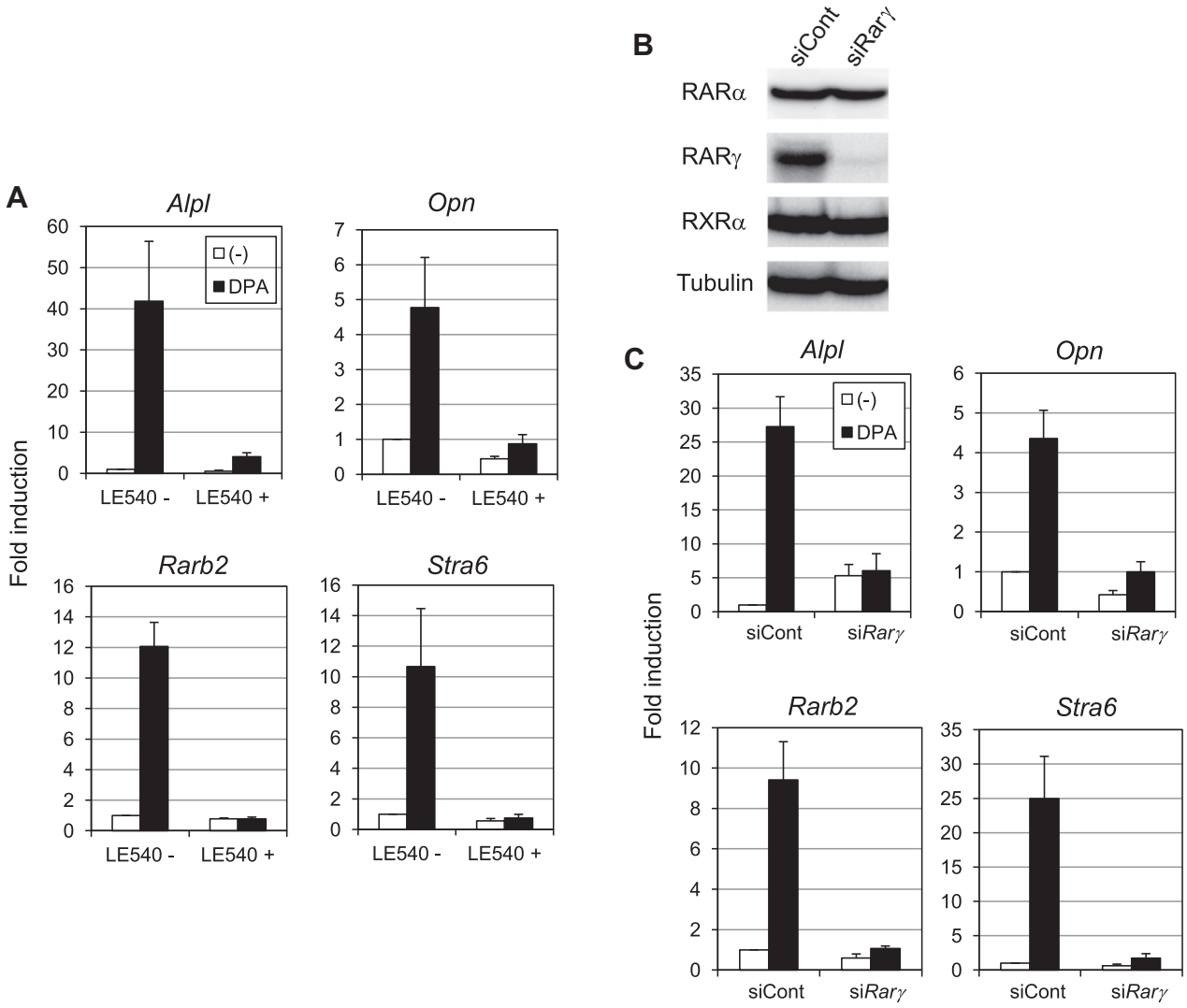

Fig. 4. Effect of retinoic acid receptor inhibition on the biological activity of DPA in C3H10T1/2 cells. (A) Cells were treated with 1 μM DPA in the absence or presence of 5 μM LE540 (pan-RAR antagonist) for 48 h, and the mRNA levels of the indicated genes were analyzed by real-time RT-PCR. (B) Western blot analysis of mouse RARα, RARβ and RXRα in siRNA transfected cells. Cells were transfected with 5 nM of control siRNA (siCont) or siRNA for mouse *Rarγ* (siRarγ). 24 h after siRNA transfection, cells were cultured in fresh medium for 48 h. Subsequently, cells were lysed, and 40 μg of whole cell lysates were separated on a 10% SDS-PAGE. α-Tubulin served as a loading control. (C) 24 h after siRNA transfection, cells were treated with 1 μM DPA for 48 h, and the mRNA levels of the indicated genes were analyzed by real-time RT-PCR. Each error bars represents the mean ± S.D. obtained from three independent samples.

osteoblastic markers in mouse pluripotent mesenchymal cells via activation of retinoic acid receptor γ. DPA may be used to activate RARγ restrictively in a cellular context and as a chemical tool for modulating biological events mediated by RARγ.

Acknowledgments

The authors thank Dr. H. Srinivas for providing the GAL-RARα and GAL-RXRα expression plasmids. This work was supported in part by a Grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to S.S.).

References

[1] P. Ducy, T. Schinke, G. Karsenty, The osteoblast: a sophisticated fibroblast under central surveillance, Science 289 (2000) 1501–1507.
[2] V. Krishnan, H.U. Bryant, O.A. MacDougald, Regulation of bone mass by Wnt signaling, J. Clin. Invest. 116 (2006) 1202–1209.
[3] G.A. Rodan, T.J. Martin, Therapeutic approaches to bone diseases, Science 289 (2000) (2000) 1508–1514.
[4] T. Komori, Regulation of osteoblast differentiation by transcription factors, J. Cell. Biochem. 99 (2006) 1233–1239.
[5] H. Hu, M.J. Hilton, X. Tu, et al., Sequential roles of Hedgehog and Wnt signaling in osteoblast development, Development 132 (2005) 49–60.
[6] T. Katagiri, A. Yamaguchi, T. Ikeda, et al., The nonosteogenic mouse pluripotent cell line C3H10t1/2 is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2, Biochem. Biophys. Res. Commun. 172 (1990) 295–299.
[7] N.S. Makhijani, D.S. Bischoff, D.T. Yamaguchi, Regulation of proliferation and migration in retinoic acid treated C3H10T1/2 cells by TGF-beta isoforms, J. Cell. Physiol. 202 (2005) 304–313.
[8] N. Wiper-Bergeron, C. St-Louis, J.M. Lee, CCAAT/enhancer binding protein β abrogates retinoic acid-induced osteoblast differentiation via repression of Runx2 transcription, Mol. Endocrinol. 21 (2007) 2124–2135.
[9] A. Wang, X. Ding, S. Sheng, et al., Retinoic acid inhibits osteogenic differentiation of rat bone marrow stromal cells, Biochem. Biophys. Res. Commun. 375 (2008) 435–439.
[10] W. Zhang, Z.L. Deng, L. Chen, et al., Retinoic acids potentiate BMP9-induced osteogenic differentiation of mesenchymal progenitor cells, PLoS ONE 5 (2010) e11917.
[11] J. Bastien, C. Rochette-Egly, Nuclear retinoid receptors and the transcription of retinoid-target genes, Gene 328 (2004) 1–16.
[12] P. Germain, P. Chambon, G. Eichele, et al., International union of pharmacology. LX. retinoic acid receptors, Pharmacol. Rev. 58 (2006) 712–715.
[13] S.M. Taylor, P.A. Jones, Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine, Cell 17 (1979) 771–779.

[14] X. Wu, J. Walker, J. Zhang, et al., Purmorphamine induces osteogenesis by activation of the hedgehog signaling pathway, Chem. Biol. 11 (2004) 1229–1238.
[15] S. Sakamoto, F. Kojima, M. Igarashi, et al., Akamatsu, decalpenic acid, a novel small molecule from *Penicillium verruculosum* CR37010, induces early osteoblastic markers in pluripotent mesenchymal cells, J. Antibiot. 63 (2010) 703–708.
[16] L.X. Zhang, K.J. Mills, M.I. Dawson, et al., Evidence for the involvement of retinoic acid receptor RARα-dependent signaling pathway in the induction of tissue transglutaminase and apoptosis by retinoids, J. Biol. Chem. 270 (1995) 6022–6029.
[17] H. Chiba, J. Clifford, D. Metzger, et al., Distinct retinoid X receptor-retinoic acid receptor heterodimers are differentially involved in the control of expression of retinoid target genes in F9 embryonal carcinoma cells, Mol. Cell. Biol. 17 (1997) 3013–3020.
[18] H. Umemiya, H. Fukasawa, M. Ebisawa, et al., Regulation of retinoidal actions by diazepinylbenzoic acids. Retinoid synergists which activate the RXR-RAR heterodimers, J. Med. Chem. 40 (1997) 4222–4234.
[19] Y. Li, Y. Hashimoto, A. Agardir, et al., Identification of a novel class of retinoic acid receptor β-selective retinoid antagonists and their inhibitory effects on AP-1 activity and retinoic acid-induced apoptosis in human breast cancer cells, J. Biol. Chem. 274 (1999) 15360–15366.
[20] A.R. De Lera, W. Bourguet, L. Altucci, et al., Design of selective nuclear receptor modulators: RAR and RXR as a case study, Nat. Rev. Drug Discovery 6 (2007) 811–819.
[21] M. Gehin, V. Vivat, J.M. Wurtz, et al., Structural basis for engineering of retinoic acid receptor isotype-selective agonists and antagonist, Chem. Biol. 6 (1999) 519–529.
[22] S. Dan, L.J. Gudas, Gene expression profiling elucidates a specific role for RARγ in the retinoic acid-induced differentiation of F9 teratocarcinoma stem cells, Biochem. Pharmacol. 75 (2008) 1129–1160.
[23] L.E. Purton, S. Dworkin, G.H. Olsen, et al., RARγ is critical for maintaining a balance between hematopoietic stem cell self-renewal and differentiation, J. Exp. Med. 203 (2006) 1283–1293.