Influence of rs2228570 on Transcriptional Activation by the Vitamin D Receptor in Human Gingival Fibroblasts and Periodontal Ligament Cells

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Background: rs2228570 is the only known single nucleotide polymorphism of the vitamin D receptor (*VDR*) that alters the protein structure. VDRs can be distinguished using the restriction endonuclease FokI and accordingly divided into three genotypes: FF, Ff, and ff. Influence of rs2228570 on transcriptional activation by VDRs in human gingival fibroblasts (hGFs) and periodontal ligament cells (hPDLCs) is investigated in this study.

Methods: From 15 donors, hGFs and hPDLCs were cultured, genomic DNA was extracted, and genotypes were determined using the polymerase chain reaction (PCR)-restriction fragment length polymorphism method. Cells were stimulated with calcitriol with or without VDR antagonist ZK159222 or osteogenic induction. Alkaline phosphatase, osteocalcin, and *VDR* messenger RNA (mRNA) expression were detected using real-time PCR. Alkaline phosphatase and osteocalcin protein expression were detected by enzyme activity assays with p-nitrophenyl phosphate substrate and enzyme-linked immunosorbent assay, respectively.

Results: Among the 15 donor cell cultures, the number of FF, ff, and Ff genotypes were 5, 3, and 7, respectively. There were no significant differences in expression of alkaline phosphatase or osteocalcin among the three genotypes in hGFs. However, after stimulation with calcitriol, alkaline phosphatase and osteocalcin mRNA levels in FF-hPDLCs were significantly higher than in other hPDLCs genotypes, as was osteocalcin protein expression. Furthermore, when ZK159222 was included, this difference disappeared, and when osteogenic induction was performed, alkaline phosphatase and osteocalcin mRNA and protein levels were higher in FF-hPDLCs than in the other hPDLCs genotypes.

Conclusion: The FF-VDR genotype is associated with the most remarkable upregulation of alkaline phosphatase and osteocalcin in hPDLCs. *J Periodontol 2017;88:915-925.*

KEY WORDS

Periodontium; polymorphism, single nucleotide; receptors, calcitriol; transcriptional activation.

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Calcitriol, the active hormonal metabolite of vitamin D_3, plays an important role in regulation of bone metabolism and immune function.[1,2] Calcitriol exerts its biologic effects through the vitamin D receptor (VDR), a member of the nuclear receptor superfamily.[3] Functional domains of the VDR consist of an N-terminal DNA binding domain and a C-terminal ligand binding domain, with two zinc atoms forming zinc finger DNA binding motifs within the DNA binding domain.[4] Heterodimerization with retinoid X receptors and recruitment of the basal transcription factor IIB (TFIIB) to the promoter via the VDR-retinoid X receptors heterocomplex are both essential for the transcriptional activity of VDR.[5-7] There are docking sites for TFIIB at both the N terminus and C terminus of VDR, and TFIIB delivered by the VDR-retinoid X receptors heterocomplex stabilizes the RNA polymerase II preinitiation complex, facilitating repeated transcription of target genes.[7]

Multiple single nucleotide polymorphisms of the human *VDR* gene have been identified and investigated,[8,9] of which rs2228570 in exon 2 involves a C to T transition to generate a new initiation codon (ATG) three codons upstream that can be identified using the *FokI* restriction endonuclease[10,11] (rs2228570 was sometimes called the *VDR-FokI* gene polymorphism). Thus, there are either two ATG start codons separated by six nucleotides or only the 3′ ATG codon due to a T to C substitution in the 5′ ATG. If the 5′ ATG codon is lost, so is the recognition sequence for *FokI*, and the resultant VDR protein (424 aa) is three amino acids shorter at the N terminus and is designated the F genotype. In contrast, when the 5′ ATG codon is intact, a VDR protein of 427 amino acids is produced that is designated the f genotype.[11] The rs2228570 single nucleotide polymorphism has not been linked to any other VDR polymorphisms[8,9] and is the only single nucleotide polymorphism that causes an actual difference in the structure of the VDR protein.[12] Therefore, influence of rs2228570 on biologic activity of the VDR is worth studying. It was reported that the calcitriol-dependent transcriptional activity of a reporter construct under the control of a vitamin D response element was significantly greater (≈1.7-fold) for the shorter F-VDR form than for the longer f-VDR form.[13] Similarly, expression of F-VDR resulted in a higher transcriptional activity than did expression of f-VDR.[7] Similar results have been reported by other groups,[14,15] but others found no significant differences between transcriptional activities of f-VDR and F-VDR.[16,17] Thus, influence of rs2228570 on transcriptional activation by VDR remains controversial.

To date, only a few studies[18-22] have focused on the relationship between rs2228570 and periodontitis. In 2003, 74 patients with chronic periodontitis (CP) and 94 healthy controls were studied, but no association between rs2228570 and CP was observed.[18] In a South Korean study, 93 patients with generalized aggressive periodontitis (AgP) and 143 healthy controls were assessed, and the shorter VDR (FF-VDR) was correlated with an increased risk of generalized AgP (odds ratio [OR] = 1.83).[19] Similarly, a Chinese study of 51 patients with generalized AgP and 53 healthy controls concluded that the FF-VDR (OR = 2.90) and the F allele (OR = 2.02) might increase susceptibility of AgP.[20] In another study, 99 patients with CP and 97 healthy controls in Libya were investigated, and no association between rs2228570 and CP was detected.[21] Recently, FF and Ff genotypes were associated with increased susceptibility to severe CP in 1,460 Thai patients.[22] Thus, there is some evidence of an association between rs2228570 and periodontitis, but the underlying mechanism remains unknown, and the influence of rs2228570 on cells originating from the periodontium might provide insight.

In the present study, the influence of rs2228570 on transcriptional activation by VDR in human gingival fibroblasts (hGFs) and human periodontal ligament cells (hPDLCs), both of which originate from the periodontium, is investigated.

MATERIALS AND METHODS

Ethics Statement

The study protocol was approved by the institutional review board of Peking University School and Hospital of Stomatology, Beijing, PR China (PKUSSIRB-2011007), and written informed consent was obtained from each participant in accordance with the Helsinki Declaration of 1975, as revised in 2013.

Study Population

A total of 178 patients (70 males and 108 females, aged 16 to 64 years; mean age: 29.5 years) participated in the study. Sixty-six patients with generalized AgP, 52 patients with CP, and 60 healthy controls were recruited from July 2001 to October 2007. The study population was the same as those in a previous study by the authors.[23] A 2-mL EDTA anticoagulated peripheral blood sample was obtained from each patient by venipuncture for the following analysis.

Cell Culture

Primary cultures of hGFs and hPDLCs were grown and maintained according to previously described methods.[24,25] Briefly, 15 systemically and periodontally healthy volunteers (six males, nine females, aged 20 to 31 years; mean age: 24 years) were enrolled from October 2011 to August 2012, and gingiva tissue was collected, minced, and inoculated into 24-well plates to obtain hGFs. Meanwhile, hPDLCs were obtained from extracted third molars from the same 15 donors. PDL tissue attached to the middle third of the roots was curetted using a surgical scalpel, minced,

and inoculated into 24-well plates. Cultures were maintained in Dulbecco modified Eagle medium (DMEM)‖ supplemented with 10% (v/v) fetal bovine serum (FBS),¶ 100 U/mL penicillin G, and 100 mg/mL streptomycin. All plates were maintained in a humidified atmosphere of 5% (v/v) CO_2 at 37°C. After reaching 80% confluence, hGFs and hPDLCs were digested with 0.25% (wt/vol) trypsin and 0.02% (wt/vol) EDTA and subcultured at a ratio of 1:2.

DMEM without phenol red bioreagent,# 10% dextran-coated charcoal-stripped FBS (DCC-FBS),** and cells from passages 4 to 6 were used in all subsequent experiments, all of which were performed in biologic triplicate.

In subsequent experiments, two different types of media were used: 1) regular medium containing DMEM supplemented with 10% DCC-FBS and 2) osteogenic induction medium containing DMEM supplemented with 10% DCC-FBS, 10 mM β-glycerophosphate, 50 μg/mL ascorbic acid, and 100 nM dexamethasone.†† Ethanol served as the vehicle for osteogenic induction medium, at a concentration of 4 μL ethanol per 100 mL medium. Ethanol was also used as the vehicle for calcitriol at 100 μL ethanol per 100 mL medium after addition of calcitriol. Ethanol was also used for VDR antagonist ZK159222[26,27]‡‡ at 100 μL per 100 mL medium after addition of ZK159222. As is shown in Figure 1, donor cells were divided into four groups: 1) group 1, the control group; 2) group 2, the calcitriol group; 3) group 3, the ZK159222 group; and 4) group 4, the osteogenic induction group. Cells from all four groups were seeded into six-well plates at a density of 2,000/cm^2. Osteogenic induction medium was used for group 4 throughout the entire experiment, whereas other groups were retained in regular medium containing 0.004% (v/v) ethanol. After culturing for 48 hours, media were renewed, and after another 47 hours, 1,000 nM ZK159222 was added to the medium of group 3, and 0.1% (v/v) ethanol was added to the media of the other three groups. After 1 hour, the media were renewed, and 10 nM calcitriol was added to the media of groups 2 and 3, osteogenic induction medium was added to the medium of group 4, and 0.1% (v/v) ethanol was added to the medium of group 1. After 48 hours, supernatants were collected, and cells were scraped from wells and placed in phosphate-buffered saline containing 0.2% nonionic surfactant.§§ Cells in identical wells were digested, and the number of cells was counted prior to RNA extraction using a reagent.‖‖ All samples were chilled immediately and stored at –80°C until needed.

Analysis of VDR FokI*-Discernable Genotypes*

Genomic DNA was extracted from blood samples or collected hGFs and hPDLCs using a genomic DNA extraction kit¶¶ following instructions provided by the manufacturer. DNA integrity was checked using agarose gel electrophoresis.

Cleaved amplification polymorphism sequence-tagged sites (CAPs) were used for *FokI* genotyping. Briefly, 100 to 500 ng genomic DNA was mixed with a polymerase chain reaction (PCR) cocktail containing 10× reaction buffer (containing 20 mM $MgCl_2$), 0.25 mM dNTP, 1.0 U Taq polymerase,## and 1.0 μM each of the forward (5′-AGCTGGCCCTGGCACTGACTCTGGCTCT-3′) and reverse (5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′) primers in a final volume of 20 μL. All reactions were performed in a thermal cycler.*** Amplification was performed with an initial denaturation at 95°C for 10 minutes, followed by 35 cycles at 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds, with a final elongation at 72°C for 7 minutes. PCR products were checked by 1% (wt/vol) agarose gel electrophoresis, and 8 μL of the target fragment was digested with 1 U of *FokI*††† in a volume of 20 μL and incubated overnight according to instructions provided by the manufacturer. Digested products were detected by 2.5% (wt/vol) agarose gel electrophoresis and ethidium bromide staining.

*Detection of Alkaline Phosphatase (*ALP*), Osteocalcin (*OCN*), and* VDR *messenger RNA (mRNA) Expression*

RNA was extracted using a reagent and reverse-transcribed to complementary DNA using a reverse transcription kit.‡‡‡ Real-time PCR was performed using a kit§§§ and a real-time thermocycler.‖‖‖‖‖‖‖‖ Data were analyzed using software¶¶¶ according to instructions provided by the manufacturer. GAPDH was used as an internal control. Sequences of primers were as follows: *ALP* forward primer, 5′-CACCCACGTCGATTGCATCT-3′; *ALP* reverse primer, 5′-TAGCCACGTTGGTGTTGAGC-3′; *OCN* forward primer, 5′-CACTCCTCGCCCTATTGGC-3′; *OCN* reverse primer, 5′-CCCTCCTGCTTGGACACAAAG-3′; *VDR* forward primer, 5′-GGTGGAGGGAGCCATCCTT-3′; *VDR* reverse primer, 5′-TGGGACAGCTCTAGGGTCACA-3′; *GAPDH* forward primer, 5′-GAAGGTGAAGGTCGGAGTC-3′; *GAPDH* reverse primer, 5′-GAAGATGGTGATGGGATTTC-3′. Data are presented as relative mRNA levels calculated

‖ Gibco, Thermo Fisher Scientific, Waltham, MA.
¶ HyClone, GE Healthcare, Logan, UT.
# Sigma-Aldrich, St. Louis, MO.
** TBD, Tianjin, PR China.
†† Sigma-Aldrich.
‡‡ VDR antagonist ZK159222, Bayer Pharma AG, Leverkusen, Germany.
§§ Triton X-100, Sigma-Aldrich.
‖‖ Trizol, Invitrogen, Thermo Fisher Scientific.
¶¶ Watson Biotechnologies, Shanghai, PR China.
DingGuo, Beijing, PR China.
*** HBPX220, PCR Express Thermal Cycler, Thermo Hybaid, Ashford, U.K.
††† TaKaRa Biotechnology, Dalian, PR China.
‡‡‡ Fermentas, Vilnius, Lithuania.
§§§ Roche, Basel, Switzerland.
‖‖‖‖‖‖‖‖ ABI 7500, Applied Biosystems, Thermo Fisher Scientific.
¶¶¶ SDS software, Applied Biosystems, Thermo Fisher Scientific.

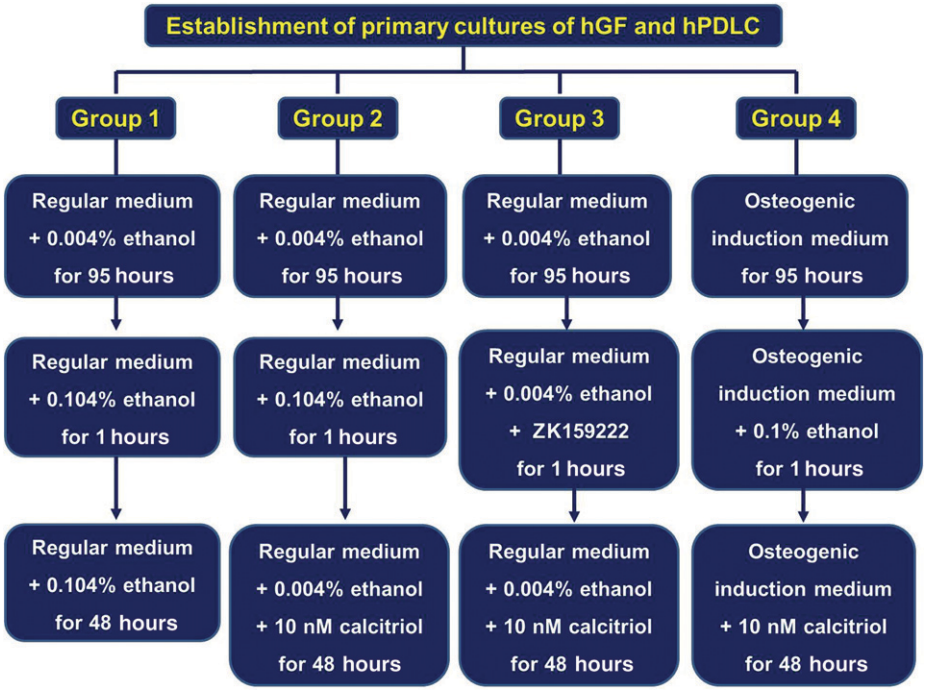

Figure 1.
Explanatory diagram of the four groups. Group 1 = control group; group 2 = calcitriol group; group 3 = ZK159222 group; group 4 = osteogenic induction group.

using the $2^{-\Delta Ct}$ method (ΔCt = Ct of the target gene – Ct of GAPDH).[28]

Determination of ALP Activity

ALP activity in cell lysates was measured by enzyme assay with p-nitrophenyl phosphate (p-NPP) substrate as reported previously.[29] In brief, 50 μL/well cell lysates were added to 96-well plates and incubated with 100 μL substrate containing 10 mmol/L p-NPP and 1 mmol/L $MgCl_2$ for 30 minutes at 37°C. Reactions were stopped by adding 100 μL of 1 mol/L NaOH. Optical density (OD) was measured at 405 nm using a spectrophotometer.### OD values were divided by number of cells prior to statistical analysis.

Measurement of OCN Protein Production

OCN concentration in cell supernatants was measured by radioimmunoassay as reported in previous studies.[23,30] Briefly, a commercially available radioimmunity assay kit**** with a lower limit of 0.35 ng/mL was used, and assays were performed according to the manufacturer protocols. To avoid bias, origins of samples were not known to scientists performing the analysis. Amount of OCN was divided by number of cells prior to statistical analysis.

Detection of VDR Protein Expression

VDR protein expression by hGFs and hPDLCs in group 2 were detected using Western blots. In brief, hGFs and hPDLCs in group 2 were harvested using lysis buffer, and protein concentration was determined using a protein assay kit.†††† Twenty micrograms of total protein from each sample was loaded onto a gel comprising a 5% (wt/vol) stacking gel and a 10% (wt/vol) running gel. At the end of the electrophoresis, samples were transferred onto nitrocellulose blotting membranes.‡‡‡‡ Blots were probed with a rabbit polyclonal antibody to VDR (diluted 1:1,000)§§§§ or a mouse monoclonal antibody to β-actin (diluted 1:1,000).|||||| Secondary antibodies¶¶¶¶ against rabbit and mouse IgG were both diluted 1:2,500. Antigen–antibody complexes were detected using an appropriate reagent.#### Band volume of VDR was analyzed and normalized to that of the corresponding β-actin using software.*****

Statistical Analyses

Association between periodontitis and rs2228570 was analyzed using logistic regression analysis. The Shapiro–Wilk test was used to determine distribution of the variants, and one-way analysis of variance (ANOVA) was used to compare differences among expression levels of ALP, OCN, and VDR in different genotype groups. Post hoc comparison was performed using the least significant difference method. Comparison of ALP, OCN, and VDR expression in hGFs and hPDLCs was carried out using the paired-samples *t* test. Statistical analyses were carried out using a statistical software package,††††† and $P < 0.05$ was considered statistically significant.

RESULTS

Among the 66 patients with AgP, the number of FF, Ff, and ff genotypes were 27, 28, and 11, respectively. Among the 52 patients with CP, the number of FF, Ff, and ff genotypes were 16, 21, and 15 and among the 60 healthy controls were 12, 37, 11, respectively. As presented in Table 1, the frequency of FF genotype was significantly higher than those of FF and Ff

Model 550, Bio-Rad, Hercules, CA.
**** HTA, Beijing, PR China.
†††† Applygen Technologies, Beijing, PR China.
‡‡‡‡ Amersham Pharmacia Biotech, Little Chalfont, U.K.
§§§§ Santa Cruz Biotechnology, Santa Cruz, CA.
|||||| Santa Cruz Biotechnology.
¶¶¶¶ Beijing Zhongshan Golden Bridge Biotechnology, Beijing, PR China.
Enhanced chemiluminescence reagent, Applygen Technologies.
***** Quantity One software, v4.31, Bio-Rad.
††††† SPSS Statistics for Windows, v11.5, SPSS, Chicago, IL.

Table 1.

Association Between Periodontitis and rs2228570

Genotypes	Patients With AgP (n = 66)	Patients With CP (n = 52)	Healthy Controls (n = 60)	OR of AgP (95% CI)	OR of CP (95% CI)
FF	27	16	12	2.45 (1.08 to 5.57)*	2.01 (0.72 to 5.63)
Ff and ff	39	36	48	1.00	1.00

CI = confidence interval.
* Significantly different from "Ff and ff."

genotypes in patients with AgP (OR = 2.45, 95% confidence interval [CI] = 1.08 to 5.57, $P<0.05$). No significant association between CP and FF genotype was detected (OR = 2.01, 95% CI = 0.72 to 5.63, $P>0.05$).

From the 15 donors of hGFs and hPDLCs, the number of FF, Ff, and ff genotypes were 5, 7, and 3, respectively, and the male:female ratio in FF, Ff, and ff groups was 2:3, 3:4, and 1:2, respectively. Results of agarose gel electrophoresis are shown in Figure 2.

As presented in Figure 3A, no significant differences in mRNA expression levels for ALP in hGFs were detected among the four groups. However, in hPDLCs in groups 2 and 4 (Fig. 3B), mRNA expression levels of ALP of FF genotypes were significantly higher than those in the other two genotypes. No significant differences were detected for OD in hGFs among the four groups (Fig. 3C). Significant differences in OD values in hPDLCs were only detected in group 4, and OD values were higher in the FF genotype than the Ff and ff genotypes (Fig. 3D; 15.82 ± 2.36 versus 10.04 ± 1.77 and 10.43 ± 1.85, respectively, $P<0.05$). As shown in Figure 3E, there were no significant differences in OCN mRNA levels among the four groups in hGFs. However, in hPDLCs in groups 2 and 4, OCN mRNA expression was higher in the FF genotype than the other two genotypes (Fig. 3F). Similarly, there was no significant difference in OCN protein levels among the four groups in hGFs (Fig. 3G); however, in group 2 (1.30 ± 0.36 versus 0.53 ± 0.24 and 0.47 ± 0.30 ng/10^5 cells, respectively, $P<0.05$) and group 4 (1.77 ± 0.18 versus 0.95 ± 0.35 and 0.83 ± 0.39 ng/10^5 cells, respectively, $P<0.05$), the FF genotype displayed greater OCN expression in hPDLCs than the other two genotypes (Fig. 3H).

Expression of VDR mRNA was not significantly different among different genotypes in hGFs (Fig. 4A) or hPDLCs (Fig. 4B). Similarly, expression of VDR protein in group 2 was not different among different genotypes in hGFs or hPDLCs (Figs. 4C through 4F).

Results of paired-sample *t* tests (Fig. 5) showed that mRNA expression of ALP and OCN in hPDLCs was significantly higher than in hGFs. Statistically significant differences in ALP activity between hGFs and hPDLCs were only observed in group 2 (5.13 ± 0.96 versus 5.88 ± 1.06, $P<0.05$) and group 4 (7.64 ± 1.38 versus 12.05 ± 3.33, $P<0.05$. Similarly, hPDLCs secreted more OCN than hGFs in group 2 (0.43 ± 0.08 versus 0.77 ± 0.47 ng/10^5 cells, $P<0.05$) and group 4 (0.56 ± 0.18 versus 1.20 ± 0.51 ng/10^5 cells, $P<0.05$. No significant difference was detected in the relative expression of VDR in hGFs and hPDLCs.

DISCUSSION

The association between AgP and FF-VDR genotype detected in the present study is in line with results of previous studies.[7,13-15] Racial differences in *VDR-FokI* gene nucleotide polymorphisms exist. It was reported that the frequency for FF, Ff, and ff genotypes was about 40%, 45%, and 15% in whites,[31,32] respectively. In African Americans,[33] the frequency for FF, Ff, and ff genotypes was about 61%, 35%, and 4%, respectively. There is also diversity among East Asian populations for *VDR-FokI*. In southern Koreans,[19] the frequency for FF, Ff, and ff genotypes was about 35%, 48%, and 17%, respectively. In

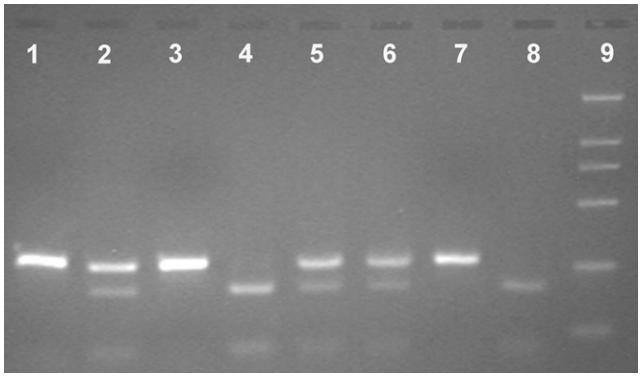

Figure 2.
Agarose gel electrophoresis of different genotypes of VDR. Lane 1 = PCR products of 267 bp before restriction endonuclease digestion; lanes 3 and 7 = FF-VDR; lanes 2, 5, and 6 = Ff-VDR; lanes 4 and 8 = ff-VDR; lane 9 = DNA marker.

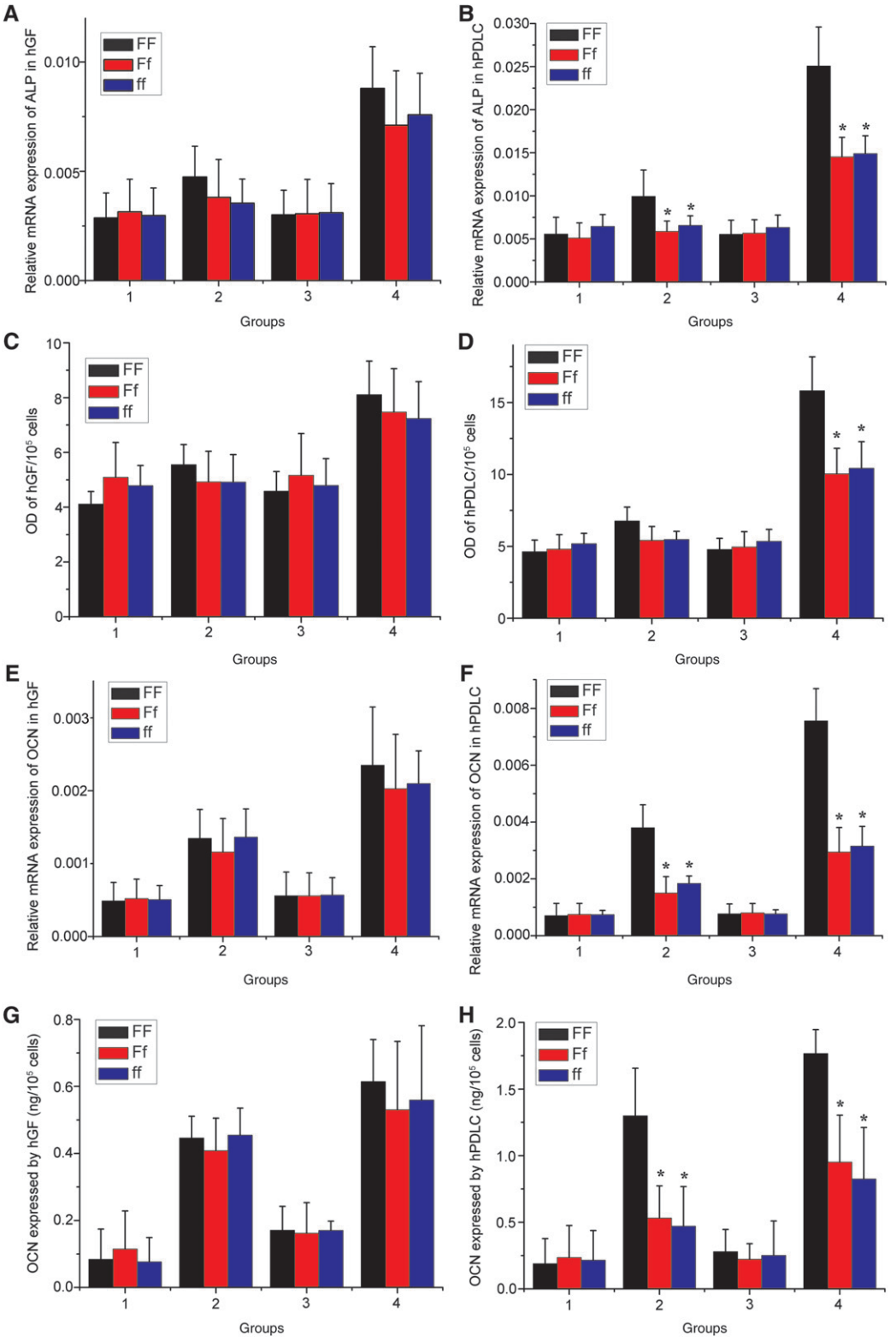

Figure 3.

*Influence of rs2228570 on expression of ALP mRNA in hGFs **(A)** and hPDLCs **(B)**. Influence of rs2228570 on ALP activity in hGFs **(C)** and hPDLCs **(D)**. Influence of rs2228570 on expression of OCN mRNA in hGFs **(E)** and hPDLCs **(F)**. Influence of rs2228570 on expression of OCN protein in hGFs **(G)** and hPDLCs **(H)**. Experiments were performed in biologic triplicate. ALP activity, OCN protein production, and mRNA expression levels of ALP and OCN were compared using one-way ANOVA, and data are presented as mean ± SE. Group 1 = control group; group 2 = calcitriol group; group 3 = ZK159222 group; group 4 = osteogenic induction group. *Significantly different from FF genotype in the same group.*

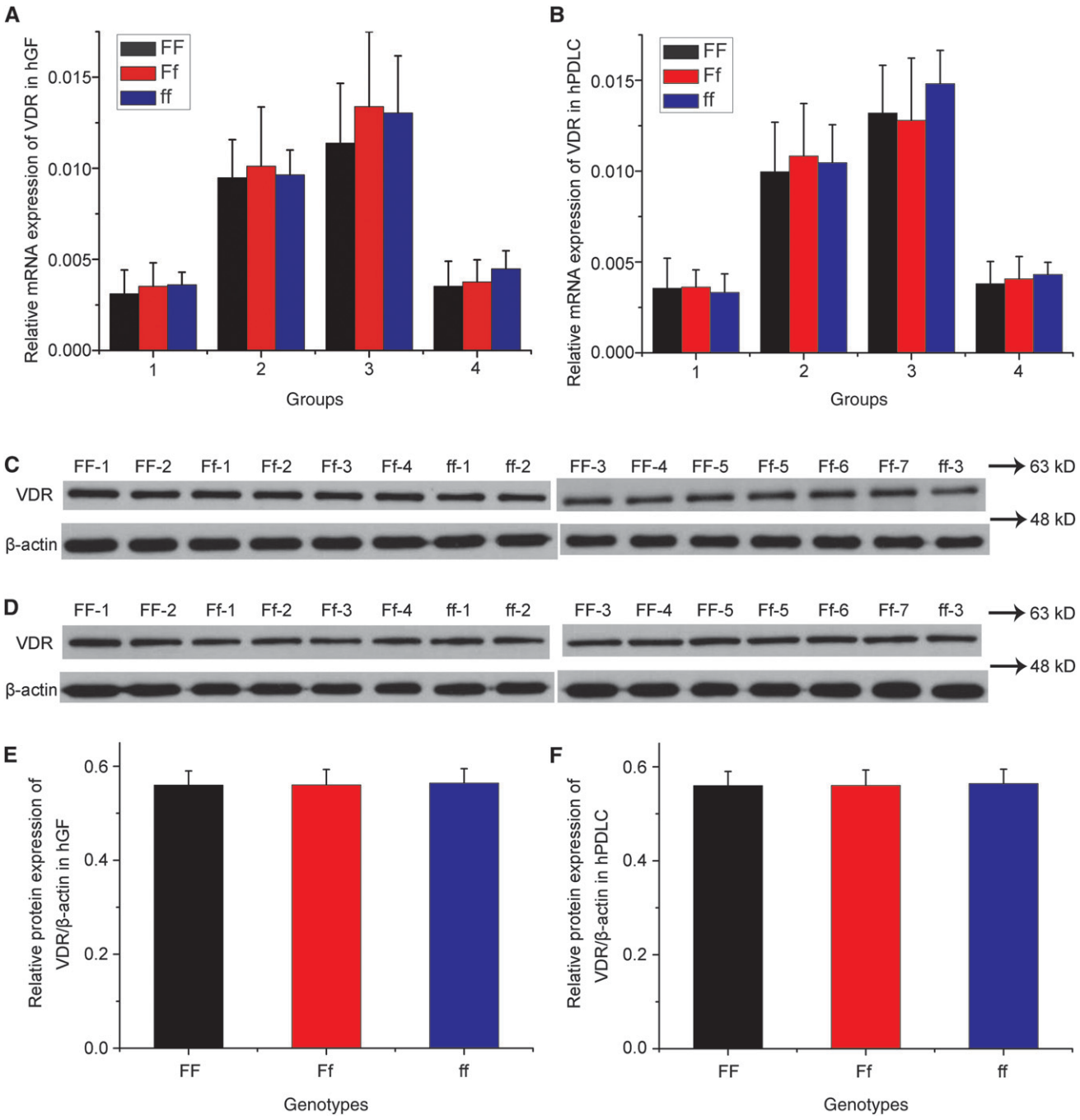

Figure 4.

Expression of VDR mRNA in hGFs ***(A)*** *and hPDLCs* ***(B)*** *with different genotypes of rs2228570.* ***C through F)*** *Expression of VDR protein in hGFs (C and E) and hPDLCs (D and F) with different genotypes of rs2228570 after stimulation with 10 nM calcitriol for 48 hours. Experiments were performed in biologic triplicate. mRNA and protein expression levels of VDR were compared using one-way ANOVA, and data are presented as mean ± SE. Group 1 = control group; group 2 = calcitriol group; group 3 = ZK159222 group; group 4 = osteogenic induction group.*

Japanese populations,[18,32] the frequency for FF, Ff, and ff genotypes was about 42% to 43%, 43% to 47%, and 10% to 15%, respectively. In Chinese populations,[20,34,35] it was reported that the frequency for FF, Ff, and ff genotypes was about 27% to 33%, 40% to 53%, and 16% to 28%, respectively. In the present study, all frequencies are similar to those reported in previous studies.

After calcitriol stimulation, hPDLCs with the FF-VDR genotype synthesized more ALP and OCN than did hPDLCs with either the Ff-VDR or ff-VDR genotype, indicating that FF-VDR supports a higher

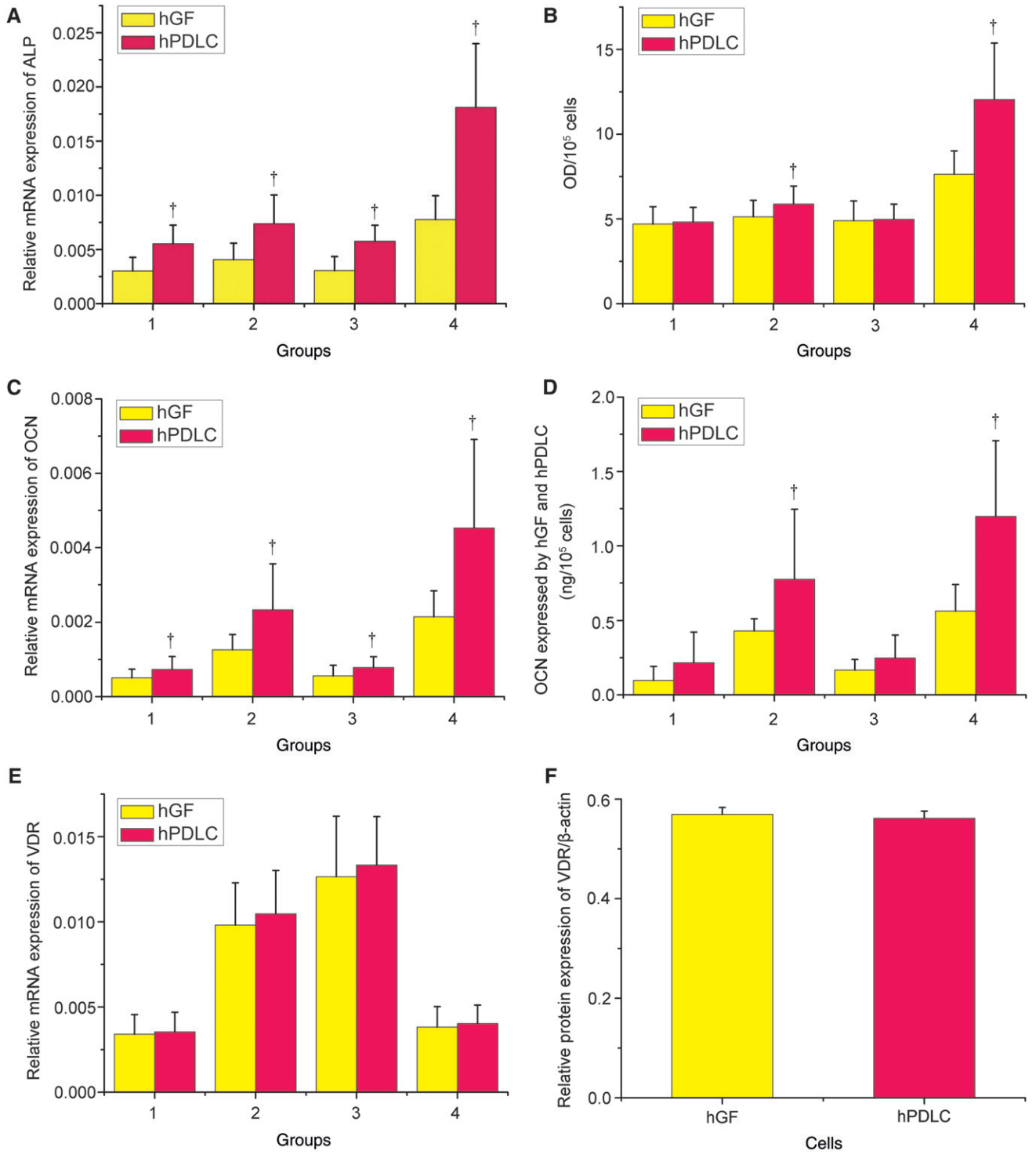

Figure 5.
Paired comparison of hGFs and hPDLCs. ***A)*** *mRNA expression of ALP.* ***B)*** *ALP activity.* ***C)*** *mRNA expression of OCN.* ***D)*** *Protein expression of OCN.* ***E)*** *mRNA expression of VDR.* ***F)*** *Protein expression of VDR after stimulation with 10 nM calcitriol for 48 hours. Experiments were performed in biologic triplicate. Comparison was carried out using the paired-samples t test, and data are presented as mean ± SE. Group 1 = control group; group 2 = calcitriol group; group 3 = ZK159222 group; group 4 = osteogenic induction group.* †*Significantly different from hGF in same group.*

transcriptional activity than Ff-VDR or ff-VDR in hPDLCs. Two positively charged arginine residues in the TFIIB docking site at the N terminus of VDR are believed to be crucial for the interaction between these proteins.[7] There are an additional three amino acids in ff-VDR compared with FF-VDR, and the negatively charged glutamic acid residue in the middle of these three amino acids might preclude this interaction between the N terminus of VDR and TFIIB.[7] If so, ff-VDR may be less efficient at supplying TFIIB to the RNA polymerase II preinitiation complex than FF-VDR, resulting in a decrease in transcriptional initiation and mRNA synthesis of vitamin D–responsive genes. This putative molecular mechanism could explain the higher transcriptional activity of FF-VDR in hPDLCs. Differences in VDR transcriptional activity must be reconciled with the observation that expression of FF-VDR was not significantly higher than either Ff-VDR or ff-VDR in hPDLCs.

Because ALP and OCN are both encoded by vitamin D–responsive genes,[4,36,37] particularly in hGFs and hPDLCs,[38] ALP and OCN were chosen to investigate transcriptional activation by VDR in the present study. In addition, to further validate the results, the VDR antagonist ZK159222 was added, and osteogenic induction was also performed. ZK159222 was used to suppress VDR function and to test transcriptional activity of different VDR genotypes. Expression of ALP and OCN were not significantly different between groups 1 and 3, indicating that the transcriptional activity of different VDR genotypes accounted for differences in expression of ALP and OCN. Since ALP and OCN are both associated with mineralization, the effect of osteogenic induction was investigated, and this further amplified observed differences in expression of ALP and OCN.

In previous studies,[7,13-15,17] exogenous VDR expression was investigated using expression vectors. Transcriptional activation by both endogenous VDR and exogenous VDR expressed from engineered constructs with different *FokI* genotypes have been compared in only two previous studies,[16,39] which generated inconsistent results. Significant differences in transcriptional activity were not observed among different endogenous or exogenous VDR genotypes in one study,[16] but transcriptional activity of exogenous FF-VDR was significantly higher in the other.[39] However, no significant differences were observed when endogenous *FokI* genotypes were tested in fibroblast cell lines, suggesting different results can be obtained from endogenous and exogenous VDR.[39] Therefore, to better understand this process ex vivo, an investigation was made in the present study of different endogenous VDR genotypes. In a study by Gross et al.,[16] fibroblasts were primarily cultured from skin biopsies, whereas in the other study,[39] fibroblast cell lines were derived from patients or the American Type Culture Collection, and exact sources were not mentioned, which could explain why the apparent influence of rs2228570 on transcriptional activation by VDR might differ in different cell types.

The rs2228570 single nucleotide polymorphism can generate three genotypes: FF, ff, and Ff. Heterozygotes (Ff) are able to synthesize both long and short VDR proteins.[7,16] In most previous studies, only exogenous VDR was analyzed,[7,13-15,17] and the heterozygote Ff has only been investigated once[13] in a study in which both FF-VDR and ff-VDR expression vectors were transfected into cells to simulate the heterozygote Ff. It was revealed that the transcriptional activity of Ff-VDR was significantly higher than that of ff-VDR and significantly lower than that of FF-VDR.[13] In the present study, transcriptional activity of Ff-VDR in hPDLCs was significantly lower than that of FF-VDR but not significantly different from that of ff-VDR. Because a natural heterozygote was investigated in the present study, results might represent more closely the actual situation in vivo.

Both hGFs and hPDLCs are used in experiments in the present study, and differences in the transcriptional activation by VDR are observed between them. It was previously demonstrated that both hGFs and hPDLCs display similarity to osteoblasts, and cells exhibiting phenotypic markers of osteoblasts could be isolated from both gingiva and periodontal ligaments.[38,40] However, hPDLCs are more similar to osteoblasts than hGFs and are superior for facilitating hard tissue formation.[41,42] *ALP* and *OCN* genes chosen for evaluating the transcriptional activity by VDR are both associated with mineralization; hence, a higher propensity for hPDLCs for mineralization could account for the more pronounced differences among VDR genotypes in hPDLCs. It should be noted that when hGFs and hPDLCs were compared in previous studies,[38,42] heterogeneity of cells from different donors was reported to exert some influence. In the present study, comparison of hGFs and hPDLCs from the same donor avoids this potential source of heterogeneity, potentially making the real differences between hGFs and hPDLCs more observable. Furthermore, only hGFs and hPDLCs from the same donor that were successfully cultured were used in subsequent experiments; hence, only 15 volunteers were enrolled. This meant that only three ff genotype samples were included, because this genotype is of a relatively low frequency in the Chinese population,[20,34,35] and individual differences among these cells may affect results, which might be a limitation of this study.

As well as an involvement in periodontitis, the FF-VDR genotype has been linked to a variety of other conditions, such as a lower susceptibility to type 1

diabetes mellitus[43] and severe respiratory syncytial virus bronchiolitis,[44] higher bone mineral density,[13] and a higher risk of alcohol addiction.[45] However, complete functional explanations for these associations remain elusive. The elevated transcriptional activity of FF-VDR in hPDLCs in the present study provides insight on the association between rs2228570 and periodontitis, but the exact relevance of the observed differences requires future research.

CONCLUSION

According to results of the present study, FF-VDR is associated with more remarkable upregulation of ALP and OCN than other VDR genotypes in hPDLCs.

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