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ORIGINAL ARTICLE

Effects of DDR1 on migration and adhesion of periodontal ligament cells and the underlying mechanism

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Abstract

Background and objective: As one of the widely expressed cell surface receptors binding to collagen, the most abundant component of the extracellular matrix (ECM), knowledge of the expression, functions, and mechanisms underlying the role of discoidin domain receptor 1 (DDR1) in human periodontal ligament cells (hPDLCs) is incomplete. This study determined the expression of DDR1 in hPDLCs and the effect of DDR1 upon migration and adhesion to hPDLCs, as well as the related regulatory mechanisms.

Materials and Methods: The expression of DDR1 and the DDR1 isoforms in hPDLCs from six donors were tested. The migratory ability (horizontal and vertical) and adhesive capacity of hPDLCs with or without specific knockdown of DDR1 were evaluated. After treatment with MEK-ERK1/2 inhibitors (PD98059 and U0126) with or without RNAi, the migratory and adhesive capacity of hPDLCs were re-tested. Western blotting was performed to verify p-MEK1/2 and p-ERK1/2, the key factors of the MEK-ERK1/2 signaling pathways.

Results: DDR1 was detected in hPDLCs in the mRNA and protein level; DDR1b was the dominant isoform. Knockdown of DDR1 almost halved the migratory capacity and significantly downregulated the adhesive capacity of hPDLCs. The use of MEK-ERK1/2 inhibitors caused declined migratory and adhesive capacity of hPDLCs as well. After DDR1 was knocked down, the expression of p-MEK and p-ERK protein declined significantly while total MEK and ERK showed no obvious change, which means the ratio of p-MEK/MEK and p-ERK/ERK was markedly reduced.

Conclusions: DDR1 plays an important role in the migration and adhesion of hPDLCs and might be regulated via the MEK-ERK1/2 signaling pathway.

Yuhan Wang and Bing Han contributed equally to this work.

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KEYWORDS
adhesion, discoidin domain receptor 1, MAPK, migration, periodontal ligament cell

1 | INTRODUCTION

The extracellular matrix (ECM) is a three-dimensional meshwork composed of non-cellular components that form the physical environment surrounding cells.[1] Cell surface receptors attach directly to ligands of the ECM, thus forming cell-ECM adhesion complexes. Specifically, ECM serves as the structural support for cell adhesion and tissue organization.[2] As a force-bearing structure responding to forces from the surrounding environment, cell-ECM adhesion plays an essential role in regulating cellular migratory behavior in coordination with cell–cell adhesion.[3,4] It is well-known that collagens are the most abundant ECM constituents.[5] Among all the cell surface receptors interacting directly with collagen, the most widely expressed receptors are integrins and discoidin domain receptors (DDRs).[6] Indeed, the functional significance of collagen interactions with integrins has been well-studied,[7–9] while knowledge of DDRs is incomplete.[10]

Discoidin domain receptors are a separate family of collagen-specific receptors, including DDR1 and DDR2, that exhibit tyrosine kinase activity.[10,11] DDR1 and DDR2 regulate cell-collagen interactions in normal and pathologic conditions.[12] A couple of studies have reported that DDR1 not only associates with more intracellular signaling cascades, but also has an indispensable role in the regulation of cell adhesion,[13] migration,[12,14] wound healing,[15,16] and other functions in various organs (kidneys and skin) compared with DDR2. DDR1 has five isoforms (a–e); DDR1a and DDR1b are the most abundant and functional isoforms.[14,17]

Discoidin domain receptor 1 (DDR1) is a member of the receptor tyrosine kinase (RTK) family. RTK is one of the classic signaling cascades of the mitogen-activated protein kinase (MAPK) pathway, especially the MEK-ERK1/2 pathway.[18,19] Therefore, it is highly possible for the MEK-ERK1/2 pathway to act as the downstream signaling pathway of DDR1.[20,21] DDR1 has been confirmed to activate cascades via the MEK-ERK1/2 pathway in several other cells, such as the mesangial cells, J774A.1 murine macrophages, and several types of tumor cells.[22–24] Additionally, DDR1 affects cell migration via the MEK-ERK1/2 pathway in smooth muscle cells, A431 and HEK 293 cells.[25,26]

Periodontal ligaments (PDLs) are collagen-rich connective tissues between the cementum and alveolar bone[27] that transmit forces from teeth to the alveolar bone and provide nutrition to teeth.[28] Human periodontal ligament cells (hPDLCs) are a heterogeneous group of cells located in PDLs that are essential components of PDLs and a promising candidate for periodontal tissue regeneration.[29,30] Previous studies have confirmed that hPDLCs and the ECM have an intimate connection to maintain periodontium health.[31] It has been reported that decreased ECM stiffness caused by periodontal diseases inhibits hPDLCs from forming periodontal tissues.[32,33] In addition, several studies have elucidated the role of DDR1 in periodontal tissue development,[34,35] which is a process requiring cell migration and adhesion to lay a foundation of establishing the appropriate organization and integrity of the tissue.[36,37] During this process, loss of DDR1 leads to a periodontal breakdown in mice.[35] DDR1 also supports collagen mechanical reorganization in periodontal tissues.[34,38] In a DDR1-knockout mouse model, collagen reorganization of periodontal tissues declined over 30%.[34] Considering the involvement of DDR1 in cell-ECM interactions and the impacts on periodontal development, DDR1 might be of importance in the cellular behavior of PDLs; however, the expression of DDR1 and the role of DDR1 in regulating the migration and adhesion of hPDLCs have not been established. Thus, we hypothesized that DDR1 promotes the migration and adhesion of hPDLCs through the MEK-ERK1/2 pathway.

2 | MATERIALS AND METHODS

2.1 | Culture of hPDLCs

Extracted third molars were obtained from six volunteers with healthy periodontal tissues. Cells from different donors were not mixed. It was approved by the institutional review board of Peking University School and Hospital of Stomatology (PKUSSIRB-2011007). The primary cells were cultured per our previous protocols.[39–41] Briefly, PDLs were collected from the central section of the extracted molar root by gently curetting and mincing. T25 flasks (Corning, Inc., Corning, NY, USA) were used for inoculation of hPDLCs isolated from the periodontal tissue blocks. Then, hPDLCs were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Waltham, MA, USA), with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS; Kang Yuan Biology, Tianjin, China). hPDLCs were placed in an incubator containing 5% CO_2 at a temperature of 37°C.

When hPDLCs reached 80% confluence, cells were digested with 0.25% trypsin and 0.02% EDTA. Cells from passage 4 were used in all subsequent experiments. All experiments were performed in triplicate.

2.2 | Detection of DDR1 expression in hPDLCs

Human periodontal ligament cells used in this part were obtained from donor A, B, and C. Six-well plates (Corning) were used to inoculate cells (5×10^4 cells/well). RNA was obtained with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription was performed using a PrimeScript RT Master Mix Kit (Takara Bio, Shiga, Japan) and 500 ng of RNA to obtain cDNA. PCR

amplification was performed using the conventional method.[42] The total volume of the PCR amplification reaction was 20 μl. Each reaction system contained 2×Taq PCR StarMix with loading dye (GeneStar, Beijing, China), the specific forward primers, the specific reverse primers, and cDNA. The primer sequences for all DDR1 isoforms are shown in Table 1. The reference gene was β-actin. The PCR products were visualized by electrophoresis on a 1.5% agarose gel.

Immunofluorescence was used to locate the expression of DDR1 protein in hPDLCs using previously described methods.[43] In brief, hPDLCs were seeded on coverslips, then fixed with ice-cold 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 for 30 min at room temperature. Fixed cells were then incubated for 1 h in 10% goat serum (ZSGB-BIO, Beijing, China) at room temperature, stained with the primary antibody (anti-DDR1 antibody: #5583; Cell Signaling Technology, Inc., CST, Danvers, MA, USA; 1:1600) overnight at 4°C, then exposed to the secondary antibody (FITC-labeled goat anti-mouse IgG; ZSGB-BIO; 1:50) for 1 h, and mounted using antifade mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Beyotime Biotechnology, Shanghai, China). Then, three fields from each well were photographed through a fluorescence microscope (Leica, Wetzlar, Germany) and the number of cells in each field were counted.

2.3 | Knockdown of DDR1 RNA expression

After reaching 80% confluence, three hPDLCs (from donors A, B, and C) were transfected by small interfering RNA (siRNA; GenePharma, Shanghai, China) to knockdown DDR1 expression. There were two groups. One group had knocked down DDR1 expression (KD), and the other group was a negative control without DDR1 RNA knockdown (NC). The siRNA used in the negative control group was non-homologous to any known gene sequence. The siRNA sequences for DDR1 were 5′-CCACCAACUUCAGCAGCUUTT-3′ (sense) and 5′-AAGCUGCUGAAGUUGGUGGTT-3′ (antisense). Ten nanomolar siRNA was pre-complexed with jetPRIME transfection reagent (Polyplus-Transfection, Strasbourg, France) at room temperature for 10 min before addition to hPDLCs. Then, cells were cultured in 5% CO_2 at 37°C until collection for RT-qPCR. RT-qPCR was then performed with SYBR Green (Solarbio, Beijing, China) with a real-time thermocycler (Applied Biosystems, Carlsbad, CA, USA). The thermal profile started with an initialization step of 10 min at 95°C, followed by 40 cycles of denaturation (15 s at 95°C), primer annealing (1 min at 55°C), and extension (45 s at 72°C). The primer sequences are displayed in Table 1.

2.4 | Migratory and adhesive capacity of hPDLCs with and without DDR1 knockdown

Wound-healing migration and Transwell migration assays were performed to determine the horizontal and vertical migration ability of hPDLCs, respectively. A cell adhesion assay was used to determine the adhesive capacity of hPDLCs. In this part, three hPDLCs from donors B, C, and D were used.

The wound-healing migration assay was performed according to the following protocol. hPDLCs were plated in 6-well culture plates (5×10^4 cells/well) and incubated in serum-free DMEM at 37°C before reaching a confluence of 80%. The experiments involved three groups, as follows: blank control group (Blank) without any treatment; negative control group (NC), in which the cells were treated with siRNA non-homologous to any known gene sequence; and knockdown group (KD), in which RNA interference was performed with siRNA to knockdown DDR1 expression. The cells in all groups were scratched across the surface of the well using a 200-μl pipette. Following incubation at 37°C of 24 h, the scratches were visualized and images were obtained with an optical microscope (magnification ×100; Olympus, Tokyo, Japan) at 0, 6, 12, and 24 h.

The Transwell migration assay was performed according to the following protocol. The groups were the same as the groups in the wound-healing migration assay. The Transwell chamber with an 8.0-μm pore polycarbonate membrane (Corning) was placed into a 6-well culture plate; the chamber was designated as the upper chamber and the culture plate was designated as the lower chamber. The hPDLCs (5×10^4 cells/well) were resuspended in a serum-free medium and seeded in the upper chamber. DMEM containing 10% FBS was added to the lower chamber. Then, the cells were incubated for 24 h at 37°C. After a portion of the cells invaded the lower chamber, the cells were treated with 4% paraformaldehyde

TABLE 1 Primers for PCR

Gene	Forward Primers	Reverse Primers	Size of amplified products
DDR1a	5′-CCCCAATGGCTCTGCCTA-3′	5′-AACAATGTCAGCCTCGGCATA-3′	115 bp
DDR1b	5′-GGCCAAACCCACCAACAC-3′	5′-AACAATGTCAGCCTCGGCATA-3′	124 bp
DDR1c	5′-CCCTTTGCTGGTAGCTGTCAA-3′	5′-ACCAAGAATGCCAGCTTCTCC-3′	151 bp
DDR1d	5′-TCATCTCTGATGTGGTGAACAA-3′	5′-AGGTGCACCAGAGCCATT-3′	349 bp
DDR1e	5′-TCATCTCTGATGTGGTGAACAA-3′	5′-TGGCTCTCTAGGACCTTGCT-3′	271 bp
GAPDH	5′-GAAGGTGAAGGTCGGAGTC-3′	5′-GAAGATGGTGATGGGATTTC-3′	226 bp
β-actin	5′-CAAGATCAACCGGGAAAAGATGA-3′	5′-TGGATGGCGACATACATGGC-3′	75 bp

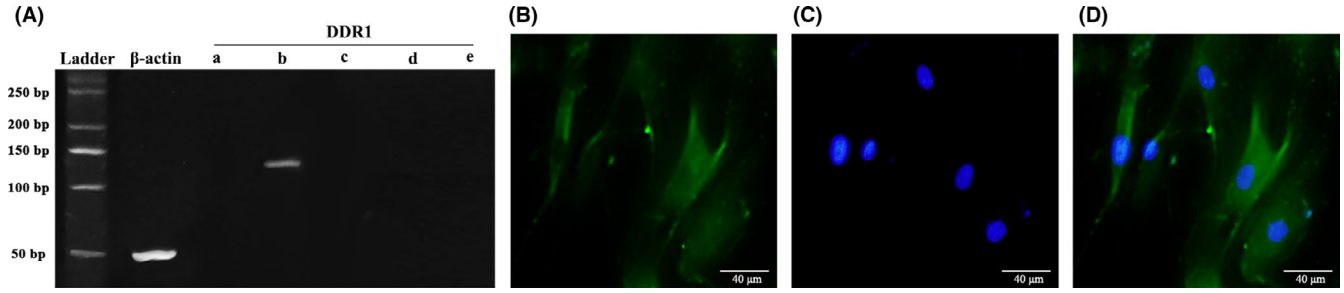

FIGURE 1 DDR1 expression in hPDLCs. (A) DDR1b was the only isoform that tested positive in hPDLCs at the mRNA expression level. (B-D) DDR1 was detected in hPDLCs via immunofluorescence staining (the blue-labeled area) at the protein expression level

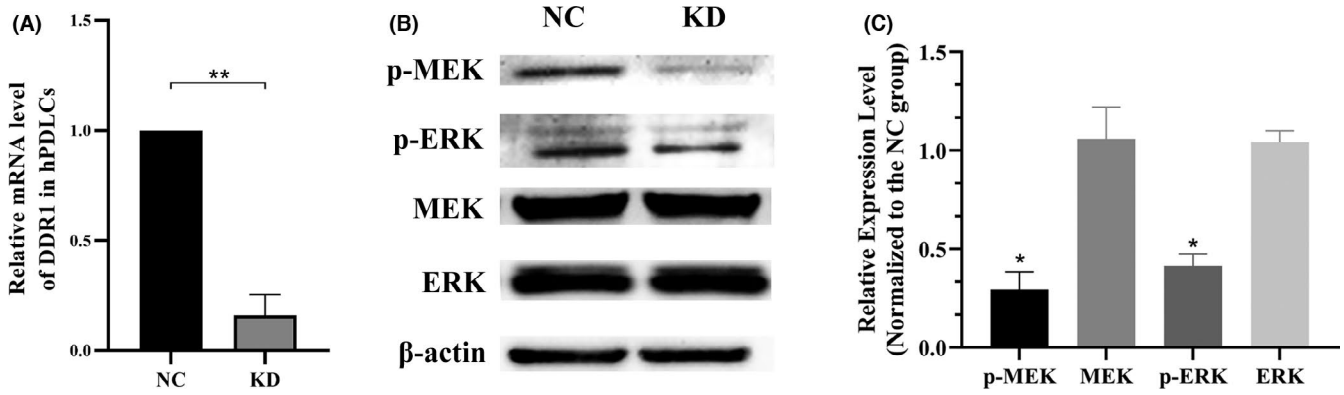

FIGURE 2 Effects of DDR1 siRNA on the expression of DDR1 and the MEK-ERK1/2 signaling pathway. (A) After hPDLCs were transfected by DDR1 siRNA, DDR1 expression at the mRNA level was knocked down over 80% ($p < .05$). (B,C) The expression of p-MEK1/2 and p-ERK1/2 was significantly reduced while MEK1/2 and ERK1/2 showed no marked difference after DDR1 knockdown ($p < .05$). NC is the abbreviation for the negative control group. KD is the abbreviation for the DDR1 knockdown group. The data are shown as the mean ± SE. "*" marked a statistical difference between the NC group and the KD group (one-way ANOVA test, $n = 3$)

and 0.1% crystal violet (Solarbio) at room temperature. The images of the membrane were captured using an optical microscope and three fields (magnification ×100; Olympus) from each well were randomly selected.

The following is the detailed protocol for the cell adhesion assay. A 96-well plate was coated with 10 μl of type I collagen derived from rat tail (Sigma, St. Louis, MO, USA). Then, the plates were incubated at 37°C for 1 day and washed twice with PBS (Gibco). The groups were identical to those in the wound-healing migration and Transwell migration assays. The hPDLCs were pretreated as above and seeded into the collagen-coated 96-well plate at a density of 5×10^5 cells/ml in 100 μl. After 2 h, the plates were washed twice with PBS and a Cell Counting Kit-8 Reagent (Beyotime Biotechnology) was added. A microplate reader (BioTek, Instruments Inc., Winooski, VT, USA) was used to measure the absorbance at 450 nm to confirm the relative number of remaining cells.

2.5 | MEK-ERK1/2 inhibition by PD98059 and U0126 with or without DDR1 knockdown

MEK-ERK1/2 inhibitors, including PD98059 and U0126 (Selleck Chemicals, Houston, TX, USA), were used with or without DDR1 siRNA. There were seven groups, as follows: (1) Blank, without any treatment; (2) NC, treated with siRNA non-homologous to any known gene sequence; (3) KD, treated with DDR1 siRNA; (4) PD98059, treated with ERK1/2 inhibitor (PD98059) at a concentration of 20 μM for 24 h; (5) U0126, treated with MEK1/2 inhibitor (U0126) at the concentration of 20 μM for 24 h; (6) PD98059+KD, treated with PD98059 and DDR1 siRNA simultaneously; and (7) U0126+KD, treated with U0126 and DDR1 siRNA simultaneously. Then, the seven groups (hPDLCs from donor D, E, and F) were assessed using the wound-healing migration, Transwell migration, and cell adhesion assays according to the above protocols.

2.6 | Detection of p-MEK1/2 and p-ERK1/2 expression in hPDLCs after DDR1 knockdown

DDR1 protein expression was detected using western blotting, as described in our previous study.[44] The hPDLCs used for protein extraction were from donor D, E, and F. The protein concentration was measured using a BCA protein assay kit (Solarbio). Equal amounts of protein were separated by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gels, then the gels were electroblotted onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 30 min in 5% non-fat milk. After blocking, the membranes were

incubated overnight with primary antibodies at 4°C. The primary antibodies used were anti-phospho-MEK1/2 (Bioss, Beijing, China; 1:1000), anti-phospho-ERK1/2, anti-MEK1/2, and anti-ERK1/2 (ABclonal, Wuhan, China; 1:1000). After washing with TBST, the membranes were incubated with second antibodies (#7074; CST; 1:2000) and detected by peroxidase-conjugated secondary antibodies using an enhanced chemiluminescence (ECL) system (NCM Biotech, Suzhou, China). To assure equal loading of protein in each lane, the blots were stripped and incubated again with an antibody against β-actin (Bioss). The blots were scanned using a western blot detection system. The band intensity was analyzed using ImageJ (https://imagej.net/Fiji). Protein expression was normalized to β-actin.

2.7 | Statistical analysis

Data analysis was performed using SPSS25.0 software (SPSS, Inc., Chicago, IL, USA). The Shapiro-Wilk test was used to demonstrate the distribution of variants. If the data were normally distributed, one-way ANOVA was used, and the data are presented as the mean ± SE. If the data were not normally distributed, the Kruskal-Wallis test was used, and the data are presented as the median and interquartile range. A p-value <.05 was considered statistically significant.

3 | RESULTS

3.1 | Expression of DDR1 in hPDLCs

DDR1b was shown to be expressed in hPDLCs, while DDR1a, DDR1c, DDR1d, and DDR1e had minimal expression (Figure 1A). The expression of DDR1 protein was also detected using immunofluorescence staining (Figure 1B-D). DDR1 was immunostained with antibodies labeled with FITC (green) and nuclei were counterstained with DAPI (blue).

3.2 | Efficiency of DDR1 RNA interference and Impact of DDR1 on p-MEK1/2 and p-ERK1/2 levels

DDR1 was successfully knocked down over 80% ($p < .05$) in comparison with cells treated with negative control siRNA (Figure 2A). Since activation of MEK and ERK occur only upon phosphorylation of their conserved activation loop, p-MEK and p-ERK serve as active forms while total MEK and ERK contain both active and inactive MEK and ERK. Knockdown of DDR1 induced a distinctive decline in p-MEK1/2 ($p < .05$) and p-ERK1/2 ($p < .05$) expression while MEK1/2 and ERK1/2 showed no significant difference between the two groups (Figure 2B,C). Namely, compared with the NC group (cells treated with siRNA non-homologous to any known gene sequence), the ratio of p-MEK1/2 to MEK1/2 and p-ERK1/2 to ERK1/2 were decreased in the KD group (cells treated with DDR1 siRNA).

3.3 | Influence of DDR1 on the migratory and adhesive capacity of hPDLCs

The wound-healing assay showed that the wound gap was larger in the KD group (cells treated with DDR1 siRNA) compared to the Blank group (cells without any treatment) and the NC group (cells treated with siRNA non-homologous to any known gene sequence) after 24 h. In contrast, the gap in the NC group was filled with cells like the blank control group (Figure 3A), indicating that downregulated DDR1 significantly slowed down cell migration by almost 50% ($p < .05$, Figure 3B). The Transwell migration assay also showed that the decreased expression of DDR1 was correlated with a significant reduction (over 50%) in the number of cells invading the lower compartment ($p < .05$, Figure 3C,D). The hPDLCs attached to the plates in the KD group were significantly fewer than the NC group, in the cell adhesion assay ($p < .05$, Figure 3E), indicating the role of DDR1 in promoting hPDLC adhesion to collagen.

3.4 | Influence of MEK-ERK1/2 on the migration and adhesion of hPDLCs

Cells were divided into seven groups as follows: (1) Blank, without any treatment; (2) NC, treated with siRNA non-homologous to any known gene sequence; (3) KD, treated with DDR1 siRNA; (4) PD98059, treated with ERK1/2 inhibitor (PD98059) at a concentration of 20 μM for 24 h; (5) U0126, treated with MEK1/2 inhibitor (U0126) at the concentration of 20 μM for 24 h; (6) PD98059+KD, treated with PD98059 and DDR1 siRNA simultaneously; and (7) U0126+KD, treated with U0126 and DDR1 siRNA simultaneously. Based on the wound-healing migration assay, cells treated with MEK inhibitors (PD98059 [$p < .05$] or U0126 [$p < .05$]) alone migrated relatively slower. Cells treated with PD98059+KD ($p < .05$) or U0126+KD ($p < .05$) moved at a slower pace compared to cells in the NC group (Figure 4A,B). Compared to cells treated with DDR1 siRNA only, the groups with the addition of PD98059 and U0126 did not reflect a statistically significant difference ($p > .05$; Figure 4A,B). Moreover, the Transwell migration assay results were consistent with the wound-healing migration assay. Compared to knocking down DDR1 only, the combination of MEK-ERK1/2 inhibitors and DDR1 siRNA did not exhibit a greater effect on inhibiting migration of hPDLCs ($p > .05$; Figure 5A,B). Similarly, application of PD98059 ($p < .05$) or U0126 ($p < .05$) resulted in a significantly reduced ability of adhesion to collagen, while DDR1 siRNA did not significantly influence this tendency ($p > .05$; Figure 5C).

4 | DISCUSSION

The positive effects of DDR1 on cellular migration and adhesion in hPDLCs were confirmed in the present study, which is in agreement with other cell types, such as epithelial and smooth muscle cells.[14,45] In addition, such effects might be regulated through the

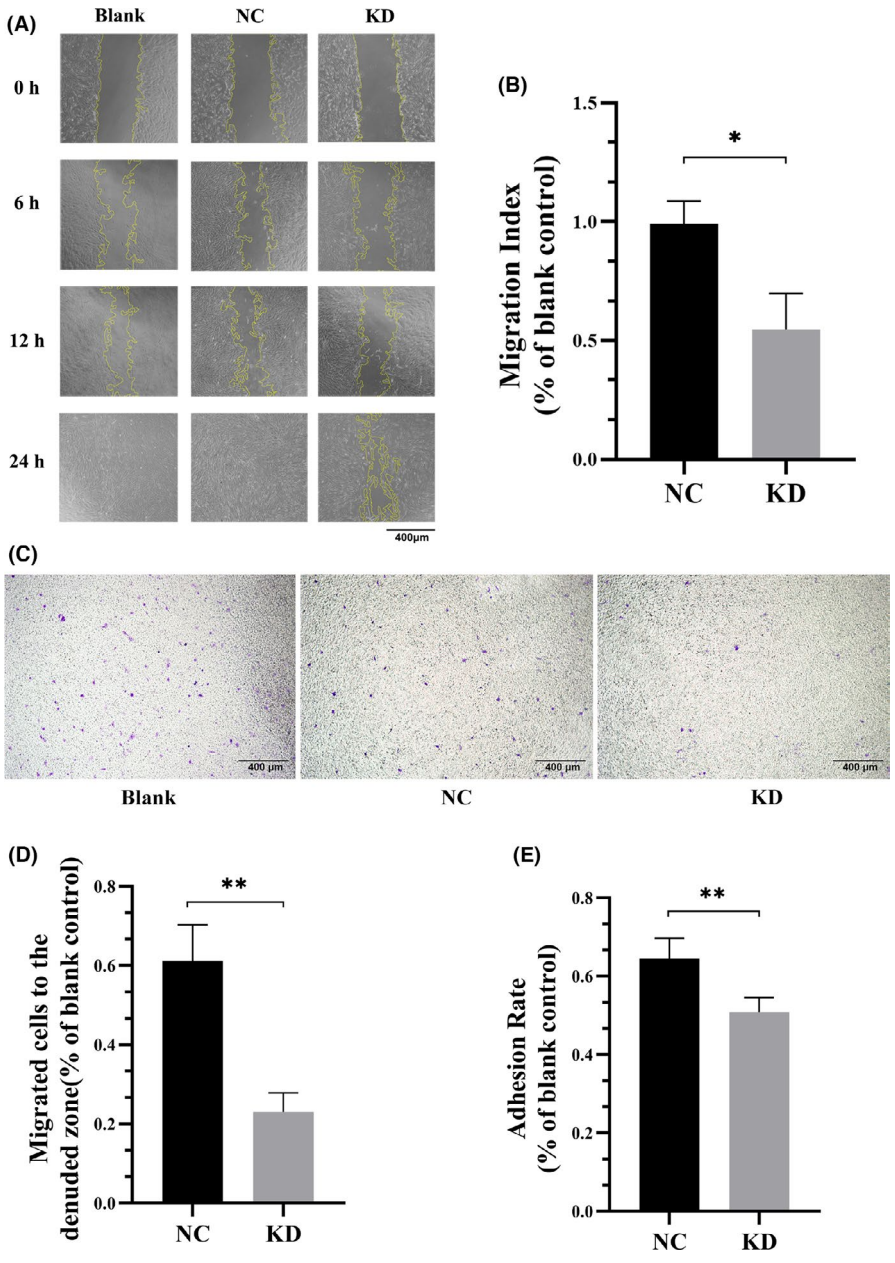

FIGURE 3 Influence of DDR1 on migration and adhesion of hPDLCs. (A) hPDLCs in the KD group moved distinctly slower in the horizontal direction than the control groups in the 24-h observation period, while cells in the Blank and NC groups migrated at an essentially identical rate. (B) Taking the Blank group as the baseline, the migration rate showed significant variation between the KD and NC groups ($p < .05$). (C) In the vertical direction, cells stained with crystal violet were fewer in the KD group than the Blank and NC groups. (D) Knockdown of DDR1 significantly decreased the number of cells passing through the membrane compared to the NC group ($p < .05$). (E) The lower expression of DDR1 significantly downregulated the adhesive capacity of hPDLCs compared to the NC group. NC is the abbreviation for the negative control group. KD is the abbreviation for the DDR1 knockdown group. The data are shown as the mean ± SE. Any two groups marked by the same "*" showed a statistical difference (one-way ANOVA test, $n = 3$)

MEK-ERK1/2pathway. Thus far, our hypothesis that DDR1 plays a positive regulatory role in migration and adhesion of hPDLCs and the MEK-ERK1/2 pathway might be involved in the regulation was affirmed.

The DDR1 gene spans ~24 kb and contains 17 exons. The extracellular domain is encoded by exons 1–8, the transmembrane domain by exon 9, the intracellular juxtamembrane (IJXM) domain by exons 10–12, and the catalytic domain by the remaining exons. All DDR1 isoforms are similar in the extracellular and transmembrane domains, but differ in the intracellular domain: Of the five isoforms, only DDR1a, DDR1b, and DDR1c are functional receptors; DDR1d and DDR1e lack a functional kinase domain.[46] The difference among five isoforms has been confirmed to make effects on the extent of glycosylation, phosphorylation, protein interactions, expression patterns, and functions.[11] DDR1a and DDR1b have been most studied because DDR1a and DDR1b are the most widely expressed isoforms. In the IJXM domain, a 37-residue segment, which contains important phosphorylation sites is unique to DDR1b.[11] While other isoforms of DDR1 are inclined to bind to collagen fibrils, DDR1b shares similarities with DDR2 to bind to soluble, monomeric collagen instead of filamentous collagen. Unlike DDR2, DDR1b forms globular clusters in response to monomeric collagen, which are more stable than other kinds of clusters.[47] In the present study, DDR1b was the dominant DDR1 isoform in hPDLCs, which is similar to the observation involving myelinating oligodendrocytes of the central nervous system.[48] Studies[49–51] have shown that hPDLCs display a potential of neurogenesis, which might partially explain the similarity between the two cell types. The dominance of DDR1b was verified using RT-qPCR, and only generic antibody for all DDR1 isoforms was used because of the unavailability of commercial specific antibody for DDR1b. Detection using specific antibody for DDR1b is needed in a future study.

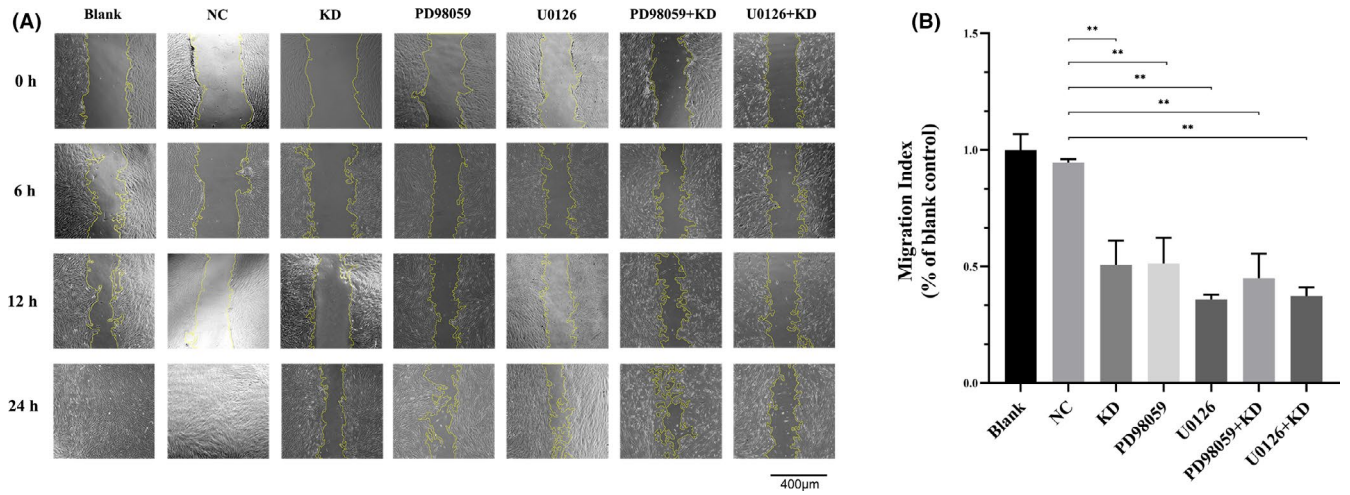

FIGURE 4 Influence of MEK-ERK1/2 on the horizontal migration of hPDLCs. (A) Cells in the PD98059, U0126, PD98059+KD, and U0126+KD groups did not occupy the vacant area, while cells in the NC group filled the entire field of vision after 24 h. (B) The Blank group was used as the baseline. The migration rate showed that cellular migration in the PD98059, U0126, PD98059+KD, and U0126+KD groups was markedly inhibited compared to the NC group ($p < .05$), while there were no significant differences among the PD98059, PD98059+KD, U0126, and U0126+KD groups ($p > .05$). The abbreviations are listed as follows: (1) Blank, without any treatment; (2) NC, treated with siRNA non-homologous to any known gene sequence; (3) KD, treated with DDR1 siRNA; (4) PD98059, treated with ERK1/2 inhibitor (PD98059) at a concentration of 20 μM for 24 h; (5) U0126, treated with MEK1/2 inhibitor (U0126) at the concentration of 20 μM for 24 h; (6) PD98059+KD, treated with PD98059 and DDR1 siRNA simultaneously; and (7) U0126+KD, treated with U0126 and DDR1 siRNA simultaneously. The data are shown as the mean ± SE. Any two groups marked by the same "*" showed a statistical difference (one-way ANOVA test, $n = 3$)

The results from the wound-healing migration and Transwell migration assays showed that the horizontal and vertical migration abilities exhibited significant reductions after DDR1 was knocked down, which illustrates the vital function of DDR1 in the migration of hPDLCs. The wound-healing migration assay is concise and explicit to reflect the migration rate, but there is a possibility that the proliferation of cells during the observation period might cause bias. In addition, because hPDLCs were cultured in serum-free DMEM in the wound-healing migration assay, the adverse effects produced by the cultivation environment without FBS cannot be dismissed. Thus, the Transwell migration assay was designed to overcome those disadvantages, and our results indicate that DDR1 impacts migration in hPDLCs. In addition, not only did the migratory capacity of hPDLCs decline due to DDR1 knockdown, but DDR1 exerts apparent effects upon hPDLCs adhesion.

hPDLCs have been shown to have the strongest regenerative properties when compared to other cells derived from the periodontium.[52,53] It is essential to ensure hPDLCs preferentially occupy the root surface to facilitate periodontal regeneration. Cells must interact with their environment through adhesion receptors to migrate. Thus, cell-matrix adhesion is a prerequisite for cell migration.[54] Therefore, migration and adhesion were the focus of the present study. Both enhanced migratory and adhesive capacity regulated by DDR1 indicated the potential importance of DDR1 in periodontal regeneration.

Confirmation of DDR1 effects on the migration and adhesion of hPDLCs is not sufficient. Although there were some studies focusing on the effect and mechanism of DDR1 upon cellular migration and adhesion in other cells,[12,13,22–26] more research is needed to demonstrate this effect and its related signal transduction pathway in hPDLCs. DDR1 is a member of the RTK family. MAPK signaling pathway could be activated as a downstream signaling pathway of the RTK family.[55] Thus, DDR1 might have a close connection with the MAPK signaling pathway. The generic signaling pathway consists of the following four distinct cascades[56]: extracellular signal-related kinases (ERK1/2); Jun amino-terminal kinases (JNK1/2/3); p38-MAPK; and ERK5. Specifically, the MEK-ERK1/2 signaling pathway has been reported to be associated with cell differentiation, migration, adhesion, and other cellular processes.[56] In several cell types, such as mammary epithelial and smooth muscle cells, DDR1 is positively correlated with the MEK-ERK1/2 signaling pathway.[25,57] In fact, this is the reason we chose to explore the relationship between DDR1 and the MEK-ERK1/2 signaling pathway in this study.

Western blotting showed that DDR1 knockdown caused a significant reduction in the expression of p-MEK1/2 and p-ERK1/2, which is the first evidence in support of the hypothesis that the MEK-ERK1/2 signaling pathway functions as the downstream pathway of DDR1. Additionally, we used MEK inhibitors (PD98059 and U0126) to further verify this hypothesis. Knockdown of DDR1 and blocking the MEK-ERK1/2 signaling pathway had a similar influence on the migration and adhesion of hPDLCs provided the second evidence for the hypothesis. Third, the greater decrease in migration and adhesion of hPDLCs in the U0126 group than the PD98059 group could be attributed to the stronger inhibitory effects of U0126 than PD98059, which further indicated the involvement of the MEK-ERK1/2 signaling pathway in the migration and adhesion of hPDLCs.

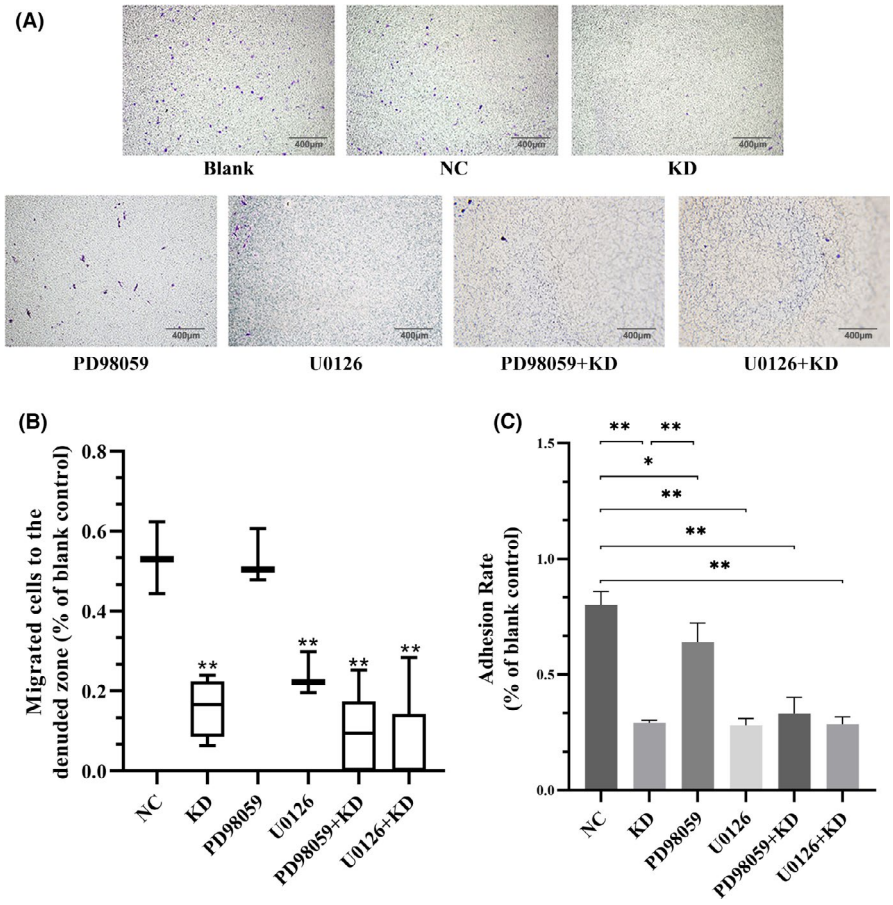

FIGURE 5 Influence of MEK-ERK1/2 on vertical migration and adhesion of hPDLCs. (A) The number of cells stained with crystal violet was less in the PD98059, U0126, PD98059+KD, and U0126+KD groups than the NC group. (B) Taking the Blank group as the baseline, cells migrating to the denuded zone were significantly fewer in the PD98059, U0126, PD98059+KD, and U0126+KD groups than the NC group ($p < .05$). There were no statistical differences among the PD98059, PD98059+KD, U0126, and U0126+KD groups ($p > .05$). (C) Cells in the PD98059, U0126, PD98059+KD, and U0126+KD groups had decreased adhesion to collagen compared with the NC group ($p < .05$). The difference between the PD98059 and PD98059+KD groups or between the U0126 and U0126+KD groups was less pronounced ($p > .05$). The abbreviations are listed as follows: (1) Blank, without any treatment; (2) NC, treated with siRNA non-homologous to any known gene sequence; (3) KD, treated with DDR1 siRNA; (4) PD98059, treated with ERK1/2 inhibitor (PD98059) at a concentration of 20 μM for 24 h; (5) U0126, treated with MEK1/2 inhibitor (U0126) at the concentration of 20 μM for 24 h; (6) PD98059+KD, treated with PD98059 and DDR1 siRNA simultaneously; and (7) U0126+KD, treated with U0126 and DDR1 siRNA simultaneously. The data are shown as the mean ± SE. Any two groups marked by the same "*" showed a statistical difference (the Kruskal-Wallis test, $n = 3$)

There were no synergistic effects between DDR1 knockdown and blocking of the MEK-ERK1/2 signaling pathway. Thus, we cannot exclude the possibility that other pathways might also be involved in the regulation of migration and adhesion, except for the MEK-ERK1/2 signaling pathway.

The results of this study verified the influence of DDR1 on the migration and adhesion of hPDLCs, which was in accordance with the previous studies in other cells.[14,34] The expression of DDR1 would promote cell migration in NIH3T3 cells by associating with non-muscle myosin IIA (NMIIA) during cell migration on collagen; if non-muscle myosin II activity is blocked, the cell migration over collagen will be inhibited.[14] DDR1 activation induced by collagen promoted adhesion to collagen and increased the association of DDR1 and NMIIA in fibroblasts.[34] Although it was indicated that DDR1 might regulate cellular adhesion and migration through the MEK-ERK1/2 signaling pathway in the present study, the mechanism for the influence of DDR1 on the adhesion and migration of hPDLCs still needs further exploration, and the association between DDR1 and NMIIA in hPDLCs will be investigated in our future study.

In the study of Chavez et al., it is found that periodontal breakdown would happen in mice with genetic ablation of DDR1.[35] It was speculated that lack of DDR1 might cause junctional epithelium attachment impairment or cellular repair deferral, which resulted in periodontal breakdown and indicated the importance of DDR1 in periodontal health.[35] Similarly, knockdown of DDR1 reduced the attachment and migration of hPDLCs in the present study, which also indicated the potential role DDR1 played in periodontal repair and regeneration. Furthermore, according to the speculation of Chavez et al., DDR1 expressed in a wide range of immune cells, and its knockout might influence the adhesion, migration, and immune defense function of different types of immune cells, which might be another reason for the DDR1 knockout-related periodontal

breakdown. hPDLCs could participate in periodontal immune defense, and to some extent could also be deemed as a kind of immune cell.[39,40] According to our present results, the immune defense function of PDLCs might be compromised as well in DDR1-knockout mice, which might help to explain the DDR1 knockout-related periodontal breakdown. Additionally, some other *in vivo* studies also confirmed that migration/adhesion-impaired hPDLCs might aggravate periodontal defects.[58–60] Although the results reported in this study agreed with Chavez's findings that DDR1-knockout mice developed periodontitis, further research is needed to examine the above speculations.

In conclusion, DDR1 might be indispensable in the migration and adhesion of hPDLCs, and the function of DDR1 could be achieved via the MEK-ERK1/2 signaling pathway. Nevertheless, it remains difficult to interpret the underlying mechanism in detail, which merits future study.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

AUTHOR CONTRIBUTIONS

Xiaoyan Wang and Kaining Liu have contributed to conception and design. Bing Han and Yuhan Wang took responsibility for data acquisition, data analysis, and manuscript writing. Xiaoyan Wang and Kaining Liu have critically revised the manuscript critically for important intellectual content. All of the authors approved the final version of the manuscript to be published. All authors agreed to be accountable for all aspects of the work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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