Long noncoding RNA related to periodontitis interacts with miR-182 to upregulate osteogenic differentiation in periodontal mesenchymal stem cells of periodontitis patients

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Periodontitis impairs the osteogenic differentiation of human periodontal mesenchymal stem cells (hPDLSCs), but the underlying molecular mechanisms are still poorly understood. Long noncoding RNAs (lncRNAs) have been demonstrated to have significant roles under both physiologic and pathological conditions. In this study, we performed comprehensive IncRNA profiling by IncRNA microarray analysis and identified a novel IncRNA, osteogenesis impairment-related IncRNA of PDLSCs from periodontitis patients (lncRNA-POIR), the expression of which was significantly decreased in PDLSCs from periodontitis patients (pPDLSCs) and was upregulated by osteogenic induction. To study the functions of lncRNA-POIR, we prepared cells with overexpression and knockdown of lncRNA-POIR and found that lncRNA-POIR positively regulated osteogenic differentiation of hPDLSCs and pPDLSCs both in vitro and in vivo. Using quantitative real-time PCRs (qPCRs) and luciferase reporter assays, we demonstrated that lncRNA-POIR may act as a competing endogenous RNA (ceRNA) for miR-182, leading to derepression of its target gene, FoxO1. In this process, lncRNA-POIR and miR-182 suppress each other and form a network to regulate FoxO1. FoxO1 increased bone formation of pPDLSCs by competing with TCF-4 for β-catenin and inhibiting the canonical Wnt pathway. Finally, inflammation increased miR-182 expression through the nuclear factor-κB pathway, and the miR-182 overexpression in the inflammatory microenvironment resulted in an imbalance in the lncRNA-POIR-miR-182 regulatory network. In conclusion, our results provide novel evidence that this lncRNA-miRNA (microRNA) regulatory network has a significant role in osteogenic differentiation of pPDLSCs and that it has potential as a therapeutic target in mesenchymal stem cells during inflammation. Cell Death and Disease (2016) 7, e2327; doi:10.1038/cddis.2016.125; published online 11 August 2016

Human periodontal mesenchymal stem cells (hPDLSCs) are a type of mesenchymal stem cells (MSCs) that can be isolated from periodontal ligament tissue and that have the potential to differentiate into multiple cell types, including osteogenic, adipogenic and chondrogenic lineages. Because of their osteogenic capacity and tissue origin, hPDLSCs are considered to be excellent cellular source for alveolar bone regeneration and repair. However, changes in the microenvironment because of age, hypoxia and inflammation can impair the multiplex differentiation potential of hPDLSCs. Periodontitis is a highly prevalent chronic inflammatory bone disease, and it is the main cause of tooth loss. Growing evidence suggests that inflammation suppresses the regenerative capacity of hPDLSCs. Although some studies have suggested that the Wnt/β-catenin and nuclear factor-κB (NF-κB) pathways may have roles in the impairment of osteogenesis of PDLSCs from periodontitis patients (pPDLSCs), the exact underlying mechanisms were still unknown. Noncoding RNAs (ncRNAs), which have been recently recognized, have no protein-coding capabilities but may act as key regulators to control biological and pathological processes. The main types of small ncRNAs are miRNAs, which regulate gene expression by directly binding to the 3'-UTR region of mRNAs directly. A subset of miRNAs, including miR-29b, miR-204/211 (ref. 23), and miR-182, participate in the regulation of osteogenesis of MSCs. Long noncoding RNAs (lncRNAs) are another important type of ncRNAs that are longer than 200 nt in length. Multiple studies have indicated that lncRNAs are involved in and may be

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Abbreviations: ALP, alkaline phosphatase; ChIP, chromatin immunoprecipitation; Col1, collagen type1; DKK1, dickkopf-1; GO, gene ontology; HA-TCP, hydroxyapatite-tricalcium phosphate; H&E, hematoxylin and eosin; hPDLSCs, human periodontal mesenchymal stem cells; IncRNAs, long noncoding RNAs; IncRNA-POIR, osteogenesis impairment-related IncRNA of periodontal mesenchymal stem cells from periodontitis patients; miRNAs, microRNAs; MSCs, mesenchymal stem cells; NF-κB, nuclear factor-κB; P65, RelA; pPDLSCs, periodontal mesenchymal stem cells from periodontitis patients; qPCR, quantitative real-time PCR; RISC, RNA-induced silencing complex; RIP, RNA-binding protein immunoprecipitation

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vital to a variety of diseases associated with aberrant cellular control, including neurological, autoimmune, and cardiovascular conditions, as well as cancer.\textsuperscript{25} Moreover, substantial evidence indicates that some lncRNAs, such as MEG3, H19, and IncRNA-ANCR, could regulate stem cell osteogenic differentiation under normal and aberrant conditions.\textsuperscript{26–28}

Unlike miRNAs, lncRNAs can either positively or negatively regulate the expression of protein-coding genes through a variety of mechanisms. Recently recognized, a large number of lncRNAs and miRNAs suppress each other as ceRNAs and form an accurate regulatory network to regulate target genes of miRNAs.\textsuperscript{29} On the one hand, lncRNAs that regulate transcription involves base pairing with miRNAs, which effectively depletes miRNAs by acting as a sponge or a decoy.\textsuperscript{30–32} On the other hand, miRNAs are able to bind to lncRNAs and trigger lncRNAs decay.\textsuperscript{33} There are growing evidences that the regulatory network composed of lncRNAs and miRNAs have significant roles in cellular differentiation. For instance, expression of the lncRNA LINCM1D1 is induced during myoblast differentiation, and it acts as a sponge of miR-133 and miR-135 to control expression of the transcription factors MAML1 and MEF2C, which activate muscle-specific gene expression.\textsuperscript{34} Although roles of lncRNA-miRNA networks in various biological processes have been reported, their functions in the osteogenesis of MSCs under normal or inflammatory conditions remain poorly understood.

In this study, we have performed comprehensive lncRNA profiling and have found that a crucial lncRNA, pPDLC osteogenesis impairment-related lncRNA (IncRNA-POIR), positively regulates the osteogenic differentiation of hPDLCs in inflammatory microenvironments. During this process, IncRNA-POIR and miR-182 suppress each other and form a network to regulate the target gene of miR-182, FoxO1. FoxO1 increases bone formation by inhibiting canonical Wnt signaling. Furthermore, aberrant activation of the NF-\(\kappa\)B pathway during inflammation may increase miR-182 expression and result in an imbalance in the IncRNA-POIR-miR-182 regulatory network. This report is the first to demonstrate the control of osteogenic differentiation of MSCs by a lncRNA-miRNA regulatory network in an inflammatory microenvironment.

Results

LncRNA-POIR expression, which is significantly altered in hPDLCs and pPDLCs, is correlated with osteogenic differentiation of pPDLCs. To identify lncRNAs that affect the osteogenic potential of pPDLCs, we assessed the lncRNA expression profiles of two pairs of hPDLCs and pPDLCs using microarrays. Our analysis resulted in the identification of 89 lncRNAs and 387 mRNAs that were differentially expressed (fold change >2.0, \(P\)-value <0.05) between the two groups of cells (Figure 1a and Supplementary Figure S3A).

The results of Gene Ontology (GO) and pathway analyses of the differentially expressed mRNAs are shown in Supplementary Figures S3B–D.

Next, we confirmed the microarray results using qPCR. The expression changes of the lncRNAs detected using qPCR were consistent with the microarray results (Figure 1b). Then, we chose five lncRNAs with high fold changes (fold change >5.0, \(P\)-value <0.05) between the hPDLCs and pPDLCs and assessed whether they affect pPDLC osteogenesis. Among the studied samples, expression of the lncRNA ENST00000446358 (IncRNA-POIR) was the most significantly altered between the non-induced group and osteogenic-induced group (Figure 1c), exhibiting a gradual increase until 14 days after osteogenic induction (Figure 1d). Moreover, the IncRNA-POIR and Runx2/Col1 (collagen type 1) expression levels were highly correlated at 0, 1, 7 and 14 days after osteogenic induction (Figure 1e).

LncRNA-POIR increases osteogenic differentiation of pPDLCs. To determine the biological effects of lncRNA-POIR on the osteogenic differentiation of pPDLCs, we constructed shlncRNA-POIR plasmids for IncRNA-POIR knockdown (shlncRNA-POIR) and IncRNA-POIR-overexpressing lentiviruses for IncRNA-POIR overexpression. To control for potential off-target shRNA effects, three different shRNAs were designed against IncRNA-POIR, and the most efficient construct was selected for transfection (Figure 2a). We also selected cells that stably overexpressed IncRNA-POIR (Figure 2b).

We found that IncRNA-POIR overexpression significantly increased the mRNA levels of osteogenic genes including Runx2, ALP and Col1 in pPDLCs (Figure 2c). Conversely, shlncRNA-POIR decreased the expression of these genes in pPDLCs (Figure 2d). Alizarin red staining, alkaline phosphatase (ALP) staining, and ALP activity assay also revealed that IncRNA-POIR overexpression increased ALP activity and mineralized bone matrix formation in pPDLCs, whereas they were decreased by shlncRNA-POIR (Figures 2e–g).

To further evaluate the osteogenic function of IncRNA-POIR in pPDLCs in vivo, pPDLCs from the control groups, IncRNA-POIR-overexpressing groups, and shlncRNA-POIR groups were loaded onto hydroxyapatite-tricalcium phosphate (HA-TCP) and implanted into NOD/SCID mice for 4 weeks. The new bone tissue stained red with hematoxylin and eosin (H&E) stain and stained blue with Masson’s Trichrome stain; in addition, osteoid formation was increased in the IncRNA-POIR-overexpressing group compared with the negative control groups. Conversely, osteoid formation by pPDLCs was decreased in the shlncRNA-POIR group (Figures 2h and i). These results strongly suggest that IncRNA-POIR promotes the osteogenesis of pPDLCs both in vitro and in vivo.

LncRNA-POIR knockdown decreases osteogenic differentiation of hPDLCs. To further demonstrate that the downregulation of IncRNA-POIR expression causes decreased osteogenesis in pPDLCs compared with hPDLCs, we transfected shlncRNA-POIR plasmids into hPDLCs and observed the effects on osteogenesis. The results demonstrated that shlncRNA-POIR decreased the mRNA levels of Runx2, ALP and Col1, ALP activity and mineralized bone matrix formation in hPDLCs (Figures 3a–d).

Next, hPDLCs from negative control groups and shlncRNA-POIR groups were loaded onto HA-TCP and implanted into NOD/SCID mice for 4 weeks as described above. The results showed that the hPDLCs in the
shlncRNA-POIR group formed fewer osteoids than those in the negative control groups (Figures 3e and f).

**LncRNA-POIR acts as a sponge of miR-182.** Besides, shlncRNA-POIR and miR-182 could negatively regulate each other. To determine whether shlncRNA-POIR acts as a miRNA sponge that competes with mRNA for binding to miRNAs, we synthesized a miR-182 inhibitor (anti-miR-182) and assessed its efficiency by qPCR. The results revealed that anti-miR-182 significantly inhibited miR-182 expression compared with a blank control and miR-182 inhibitor NC (anti-miR-NC) (Figure 4a). Next, we performed miRNA target site prediction using MicroInspector online software (http://bioinfo.uni-plovdiv.bg/microinspector). We found that shlncRNA-POIR contains a single element complementary to miR-182 (Figure 4b) and that miR-182 expression was promoted by shlncRNA-POIR (Figure 4c). We also found that the shlncRNA-POIR level was increased following miR-182 inhibition (Figure 4d). Moreover, shlncRNA-POIR and miR-182 expression in pPDLSCs was highly negatively correlated at 0, 1, 7 and 14 days after osteogenic induction (Figure 4e).

To determine whether shlncRNA-POIR directly regulates miR-182, we generated luciferase reporter constructs. The results showed that the shlncRNA-POIR-wild-type reporter was strongly suppressed by miR-182 (Figure 4f). However, the mutant shlncRNA-POIR reporter was not affected by this miRNA. These results indicate that shlncRNA-POIR and miR-182 directly regulate each other.

We also found that shlncRNA-POIR is associated with the RNA-induced silencing complex (RISC) by performing an RNA-binding protein immunoprecipitation (RIP) assay. The shlncRNA-POIR and miR-182 RNA levels were higher in the anti-Ago2 group compared with the anti-normal IgG group (Figures 4g and h).

**LncRNA-POIR modulates FoxO1 through regulation of miR-182.** Reports have shown that miR-182 decreases bone formation of MSCs by directly targeting FoxO1.\(^{24}\) Consistent with these findings, the miR-182 inhibitor significantly reduced the FoxO1 mRNA and protein levels in pPDLSCs (Figures 5a and b). Further, luciferase reporter assay demonstrated that miR-182 inhibited the luciferase activity of the 3′-UTR of the FoxO1 luciferase reporter (Figures 5c and d). To determine whether shlncRNA-POIR affects FoxO1 expression, we monitored FoxO1 expression at both the mRNA and protein levels in shlncRNA-POIR-overexpressing and shlncRNA-POIR
A significant increase in FoxO1 expression was observed in response to lncRNA-POIR overexpression. Moreover, shlncRNA-POIR negatively regulated the expression of FoxO1 (Figures 5e–h).

The miR-182 and FoxO1 have opposite effects on osteogenic differentiation of pPDLSCs. To control for potential off-target siFoxO1 effects, three different siRNAs were designed against FoxO1. Then, we assessed the efficiency of siFoxO1 by qPCR and western blotting, and the most efficient construct was selected for transfection (Figures 6a and b). Moreover, we assessed the osteogenic function of FoxO1 using siFoxO1. siFoxO1 was found to inhibit osteogenic processes in pPDLSCs (Figures 6c–f). We also found that the effects of lncRNA-POIR overexpression on osteogenic markers could be offset by siFoxO1.
which strongly demonstrated that lncRNA-POIR regulated bone formation through FoxO1. Next, we also observed the osteogenic function of FoxO1 in vivo and found that the pPDLSCs in the siFoxO1 group formed fewer osteoids than those from the negative control groups (Figures 6i and j).

Furthermore, we synthesized a miR-182 inhibitor to identify the osteogenic effects of miR-182. The results of qPCR, western blot analyses, and Alizarin red staining showed that the miR-182 inhibitor promoted the osteogenesis of pPDLSCs (Supplementary Figures S4A–D).

Foxo1 regulates osteogenic differentiation of pPDLSCs through negative regulation of canonical Wnt pathway.

To identify the effects of the canonical Wnt pathway on the osteogenesis of pPDLSCs, we treated the pPDLSCs with 50 ng/ml dickkopf-1 (DKK1; a specific inhibitor of the canonical Wnt pathway). After 7 days of osteogenic induction, the DKK1-treated pPDLSCs exhibited an increased osteogenic capacity compared with the control cells (Figures 7a and b).

Next, we assessed whether FoxO1 inhibits the canonical Wnt pathway and rescues the osteogenesis of pPDLSCs. We found that in the presence of siFoxO1, expression of the canonical Wnt pathway targets cyclin D1, Axin and c-myc was significantly upregulated in pPDLSCs (Figures 7c and d). Immunoprecipitation assay revealed that FoxO1 decreased the amount of β-catenin bound to TCF-4 (Figure 7e). These results indicate that FoxO1 can impair the binding of β-catenin to TCF-4 without affecting β-catenin expression in the total nuclear extracts.

Inhibition of the canonical Wnt pathway via DKK1 completely offsets the negative osteogenic effects of siFoxO1 (Figures 7f–h), which indicates that FoxO1 may regulate osteogenic differentiation of pPDLSCs through this pathway.

Overactivation of the NF-κB pathway in inflammation is the main cause of dysregulation of the lncRNA-POIR and miR-182 regulatory network. In this part, we found that the strong upregulation of miR-182 had an important role in suppressing the expression of lncRNA-POIR in pPDLSCs. qPCR revealed that the inhibition of miR-182 reversed the downregulation of lncRNA-POIR expression in the inflammatory environment (Figure 8a). However, the effects of the inflammatory environment on miR-182 upregulation were consistent regardless of whether lncRNA-POIR was inhibited or not (Figure 8b). Western blotting revealed that the NF-κB pathway was activated in pPDLSCs (Figure 8c), and chromatin immunoprecipitation (ChIP) assay showed that RelA (P65) and c-Rel directly bound to the promoter region of pri-miR-182 (Figure 8d). The binding sites on the promoter region of pri-miR-182 were predicted by JASPAR (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl). These findings indicate that overactivation of the NF-κB pathway is the main cause of dysregulation of the lncRNA-POIR and miR-182 network. To verify this finding, we inhibited the NF-κB pathway.
pathway using siIKKα (the efficiency of siIKKα was determined by qPCR) (Figure 8e) and found that the increase in miR-182 expression was blocked and that lncRNA-POIR expression was increased in the inflammatory microenvironment (Figures 8f and g).

**Discussion**

Recently, many studies have focused on the functional deficiency of stem cells in several disease conditions. Interestingly, this stem cell dysfunction is always continuous...
and contributes to the disease pathology.\textsuperscript{35,36} Reports have shown that hPDLSCs lose their osteogenic potential in inflammatory microenvironments.\textsuperscript{18} Moreover, the function deficiency of pPDLSCs could be long-lasting and irreversible in ex vivo expansion without inflammatory supplements. The multidifferentiation potential of pPDLSCs is impaired, even in cells that have been passaged nine times.\textsuperscript{37} This impairment might be due to the fact that pPDLSCs secrete TNF-\(\alpha\) and IL-1\(\beta\), which make cells still subjected to inflammatory microenvironment, after in vitro culturing without inflammatory supplements for several weeks.\textsuperscript{18} Our results also showed that pPDLSCs that have been passaged seven times still lose their osteogenic ability and secrete cytokines. In fact, these characteristics of pPDLSCs are consistent with hPDLSCs which were treated with TNF-\(\alpha\) and IL-1\(\beta\)\textsuperscript{18} (Supplementary Results and Supplementary Figure S2).

Figure 6  The opposite effects of miR-182 and FoxO1 on osteogenic differentiation. (a and b) Transfection effects of siFoxO1 was determined by qPCR and western blot. (c-f) The functions of siFoxO1 on the expression of osteogenic markers of pPDLSCs were measured by Alizarin red staining, qPCR and western blot 7 or 14 days after osteogenic induction. (g and h) Runx2 and Col1 mRNA levels and Runx2 protein level in pPDLSCs were measured after being transfected with siFoxO1 under the treatment of overexpression of IncRNA-POIR or controls at 7 or 14 days after osteogenic induction. (i and j) siFoxO1 inhibits osteogenesis of hPDLSCs in vivo. pPDLSCs were mixed with HA-TCP and transplanted into the dorsal region of nude mice for 4 weeks. Then, the results were measured by H&E staining and Masson's trichrome staining. Quantitative analysis of the new bone area determined by Image-Pro Plus 6.0 software. At least three fields were randomly selected from each transplant. Six implants were engrafted into three mice per treatment. All experiments were repeated three times. Relative expressions of mRNAs were normalized by \(\beta\)-actin in qPCR. Data represent mean \(\pm\) S.D. *\(P<0.05\), **\(P<0.01\) and NS, not significant. The scale bar in the micrographs represents 200 nm. Con, control; IncRNA-POIR, lentivirus for upregulating IncRNA-POIR; NB, new bone; NC, lentivirus negative control; OD, optical density; siFoxO1, FoxO1 oligo; siNC, negative control.

LncRNAs have important roles in numerous developmental pathways, including the maintenance of stem cell pluripotency and the regulation of apoptosis, erythropoiesis and keratinocyte differentiation.\textsuperscript{38} As knowledge of lncRNA functions grows, interest in their role in the commitment of MSCs is increasing. Important studies have revealed that lncRNAs...
regulate osteogenic capacity under both normal and abnormal conditions.\textsuperscript{27,28}

To investigate the role of lncRNAs in the regulation of hPDLSCs osteogenesis in the context of periodontitis, we performed microarray expression profiling of lncRNAs in hPDLSCs and pPDLSCs and confirmed the results by qPCR (Figures 1a and b). The qPCR results showed a similar trend as the microarray results, but there were differences. These differences might be due to differences in either the sample size or the sensitivity of the methods. Therefore, the data from the microarray analysis must be confirmed by qPCR, which is more accurate. (The comparison between the array data and qPCR data could be found in Supplementary Table S2.)

Among the differentially expressed lncRNAs, we chose lncRNA-POIR as a target because its expression was the most significantly altered in pPDLSCs before and after osteogenic induction (Figure 1c) and the relative expression levels of lncRNA-POIR and Runx2 (Col1) were strongly correlated ($R>0.8$, $P<0.01$) (Figure 1e). LncRNA-POIR was only recently discovered by lncRNA sequencing technology, and its biological function has not yet been determined. Therefore, to identify the function of IncRNA-POIR, pPDLSCs were constructed with IncRNA-POIR overexpression and knockdown, revealing that lncRNA-POIR promoted bone formation both in vitro and in vivo (Figure 2).

We also observed the effects of lncRNA-POIR in hPDLSCs and found that it affected the osteogenic differentiation of these cells. These results demonstrate that reduced lncRNA-POIR expression in pPDLSCs decreases osteogenic differentiation and that lncRNA-POIR can serve as a target to regulate bone formation in hPDLSCs and pPDLSCs (Figure 3).

LncRNAs, a type of ncRNAs, could regulate their targets in ncRNA networks by interacting with other ncRNAs, similar to miRNAs. In this study, we found that IncRNA-POIR, miR-182 and FoxO1 were strongly correlated because both IncRNA-POIR and FoxO1 3'-UTR region were found to be targets of miR-182, as determined by bioinformatics analysis and luciferase assay. Interestingly, IncRNA-POIR and miR-182 form an autoregulatory loop by negatively regulating each

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**Figure 7** FoxO1 regulate osteogenic differentiation of pPDLSCs through negative regulation of canonical Wnt pathway. (a and b) ALP staining and ALP activity was detected following 7 days of osteogenic differentiation medium and DKK1 induction. (c and d) Some targets of canonical Wnt pathway were measured by qPCR and western blot in pPDLSCs after being transfected with siFoxO1. (e) pPDLSCs were transfected with siFoxO1, immunoprecipitated with an anti-TCF-4 or anti-IgG antibody and probed with an anti-$\beta$-catenin antibody. The TCF-4 or $\beta$-catenin expressions in nuclear extracts were also measured. (f–h) Alizarin Red S and osteogenic gene mRNA levels in pPDLSCs were measured after being transfected with siFoxO1 under the treatment of DKK1 or not at 7 or 14 days after osteogenic induction. All experiments were repeated three times. Relative expressions of mRNAs were normalized by $\beta$-actin in qPCR. Data represent mean ± S.D. *$P<0.05$, **$P<0.01$ and NS, not significant. Con, control; DKK1, 50 ng/ml recombinant human DKK1; OD, optical density; siFoxO1, FoxO1 oligo; siNC, negative control
other. This mechanism may have an important role in regulating the biological functions of stem cells. Moreover, the RISC, by which miRNAs recognize their complementary mRNA targets and exert their functions, has an important role in this reciprocal repression. 

In our study, both lncRNA-POIR and miR-182 were found to associate with the RISC by binding to Ago2, a core component of the RISC (Figures 4g and h). FoxO1, as a regulator of the antioxidant process and redox balance, can regulate bone formation in a controversial manner. FoxO1 has been reported to promote osteogenesis, and its deletion, specifically from osteoblasts and osteoblast...
progenitors, has been reported to decrease the osteoblast number, bone formation rate, and bone volume.\textsuperscript{42,43} Kim et al.\textsuperscript{24} have reported that miR-182 negatively regulates osteogenesis by targeting FoxO1. Our study revealed similar results in pPDLSCs (Figure 5). Moreover, the positive osteogenic effects of IncRNA-POIR were attenuated by siFoxO1, which strongly supports the association of the osteogenic functions of IncRNA-POIR with FoxO1 (Figure 6).

To further examine the mechanism of FoxO1 in regulating bone formation in pPDLSCs, we focused on the canonical Wnt pathway, which has a very important role in osteogenesis. The majority of studies have suggested that the canonical Wnt pathway inhibits pPDLSC osteogenic differentiation.\textsuperscript{17,18,44} Under inflammatory conditions, the expression of β-catenin and its target, cyclin D1, are increased, and DKK1, a specific inhibitor of the canonical Wnt pathway, restores the osteogenic capacity of pPDLSCs. Our results showed that DKK1 obviously increased the osteogenic capacity of pPDLSCs, verifying the negative effect of the canonical Wnt pathway on bone formation (Figures 7a and b).

Interestingly, FoxO1 often acts as a canonical Wnt pathway inhibitor by competing with the TCF/LEF complex for β-catenin and the expression of cyclin D1, a direct downstream target of β-catenin/TCF, is inhibited by FoxO1.\textsuperscript{45–47} Thus, FoxO1 may regulate osteogenic differentiation by competing with the TCF/LEF complex for β-catenin and inhibiting the canonical Wnt pathway. Our results are in support of our hypothesis. We found that siFoxO1 inhibited the expression of the Wnt target genes cyclin D1, c-myc and Axin (Figures 7c and d), and immunoprecipitation analysis demonstrated that FoxO1 decreased the amount of β-catenin bound to TCF-4 (Figure 7e). These results verify that FoxO1 regulates the canonical Wnt pathway by competing with TCF-4 for β-catenin. Moreover, we found that the negative osteogenic effect of siFoxO1 was diminished by DKK1, suggesting that siFoxO1 inhibited bone formation through regulation of the canonical Wnt pathway (Figures 7f–h). Some studies have reported that FoxO1 attenuates the differentiation of osteoblasts by inhibiting the canonical Wnt pathway.\textsuperscript{45,48} However, the functions of this pathway vary with changes in the microenvironment. Under inflammatory conditions, the canonical Wnt pathway has a negative role in osteogenic differentiation, explaining the FoxO1-mediated promotion of bone formation of pPDLSCs, which occurs through regulation of this pathway.

To identify the mechanism that impairs the expression of IncRNA-POIR in pPDLSCs, we found that the strong upregulation of miR-182 had an important role. In the absence of miR-182, the effects of the inflammatory environment on downregulation of IncRNA-POIR expression disappeared (Figure 8a). In inflammation, increased miR-182 caused the low level of IncRNA-POIR expression. Considering IncRNA-POIR and miR-182 often negatively regulate each other, as described above, an abnormally low IncRNA-POIR level may lead to a further increase in miR-182 expression in a vicious circle. These results strongly demonstrate that inflammation may regulate cell functions through IncRNA-miRNA regulatory network and this mechanism has never been discussed before.

In addition, we observed that miR-182 expression was regulated by the NF-κB pathway. The NF-κB/Rel pathway, which is often activated during inflammation, induces the transcription of downstream genes.\textsuperscript{49,50} Chen et al.\textsuperscript{19} have demonstrated that this pathway is strongly activated in pPDLCs and that its activation interferes with the bone formation of pPDLCs. Similarly, our results showed that the NF-κB pathway was upregulated in pPDLCs (Figure 8c). Further, we found that aberrant activation of this pathway was the key factor leading to miR-182 overexpression during inflammation. This is mainly because P65 and P50, important transcription factors of the NF-κB pathway, directly bind to the promoter regions of pri-miR-182 (Figure 8d). Inhibition of the NF-κB pathway using siIKKα resulted in a decrease in the miR-182 level and an increase in the IncRNA-POIR expression in the inflammatory microenvironment (Figures 8f and g). All of these results suggested that the activated NF-κB pathway during inflammation disrupted the balance of the IncRNA-POIR and miR-182 regulatory network.

According to the ceRNA hypothesis, IncRNAs may act as a ceRNA for miRNAs and lead to derepression of target genes.\textsuperscript{25} In this process, as targets of miRNAs, IncRNAs are also under the regulation of miRNAs. This IncRNA-miRNA regulatory network has a significant role in mediating cell functions in normal and abnormal conditions.\textsuperscript{30–34,40,41} In our study, we indicated an innovative model to depict the core effects of IncRNA-POIR-miR-182 network in osteogenesis of pPDLCs. In inflammation, this network, which is regulated by the NF-κB pathway in inflammatory microenvironments, may influence the osteogenic differentiation of pPDLCs through the FoxO1/canonical Wnt pathway (Figure 8h). Our study has provided some new insights into the roles of this IncRNA-miRNA regulatory network in MSC differentiation and provided the new target points and strategies for the periodontitis.

Materials and Methods

Samples and cell cultures. Primary hPDLSC cultures were obtained from 10 individuals, aged 31–40 years, who were undergoing no premolar extractions for orthodontic reasons or third molar extractions. pPDLCs were obtained from 10 individuals, aged 27–43 years, who were diagnosed with stable periodontitis and had two-thirds alveolar bone loss and more than one pocket (depth > 5 mm). None of these selected subjects showed any clinical evidence of recent infection or systemic disease, and none had a history of smoking or of maxillofacial surgery, radiotherapy, or chemotherapy. All samples were collected at the Dental Clinic of the Fourth Military Medical University. Written informed consent was provided by all participants, and the study was approved by the hospital’s ethics committee (license number: IRB-REV-2015038).

The methods used for the culturing and identification of hPDLSCs and pPDLCs can be found in Supplementary Methods, Results and Supplementary Figure S1.

Microarray and data analysis. A total of six samples (three pPDLCs and three hPDLCs from six individuals), each which contained ~1 × 10\textsuperscript{6} cells, were prepared, and total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA quantity and quality were measured by NanoDrop ND-1000 (Thermo Fisher Scientific, Boston, MA, USA). RNA integrity was assessed by standard denaturing agarose gel electrophoresis. All labeled IncRNAs and miRNAs were hybridized onto an Arraystar Human LncRNA Microarray V3.0 (Arraystar, Kangchen, Shanghai, China), which included 30 586 IncRNAs and 26 109 coding transcripts.

Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technologies, Santa Clara, CA, USA) with minor modifications. Briefly, mRNA was purified from total RNA after removal of rRNA (mRNA-ONLY Eukaryotic mRNA Isolation Kit;
Epicentre, San Diego, CA, USA). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length without 3' bias using a random priming method (Arraystar Flash RNA Labeling Kit; Arraystar, Rockville, MD, USA). The labeled cRNAs were purified with an RNase Mini Kit (Qiagen, Hilden, Germany). The concentrations and specific activities of the labeled cRNAs (pmol Cy3 per μg cRNA) were measured using a NanoDrop ND-1000. One microgram of each labeled cRNA was fragmented by adding 5 μl of 10 x Blocking Agent and 1 μl of 25 x Fragmentation Buffer, and then the mixture was heated to 60 °C for 30 min. Finally, 25 μl of 2 x GE Hybridization buffer was added to dilute the labeled cRNA. Fifty microliters of hybridization solution were dispensed onto gasket slides, and cRNA expression microarrays were assembled. The slides were incubated for 17 h at 65 °C in an Agilent Hybridization Oven (Agilent, Santa Clara, CA, USA). The hybridized arrays were washed, fixed, and scanned using an Agilent DNA Microarray Scanner (part number G2505C).

Agilent Feature Extraction software (version 11.0.1.1) (Agilent, Santa Clara, CA, USA) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using GeneSpring GX v12.1 software package (Agilent Technologies). After quantile normalization of the raw data, lncRNAs and mRNAs that were flagged as Present or Marginal (‘All Targets Value’) in at least three out of six samples were chosen for further analysis using two-tailed unpaired Student’s t-test, with adjustments of the P-values for multiple testing via Bonferroni correction for post hoc analysis.

The data discussed in this publication have been deposited into NCBI’s Gene Expression Omnibus and are accessible through the GEO series accession number GSE78074 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE78074).

Mineralization assay. See Supplementary Methods for more details.

Immunoprecipitation. Nuclear proteins were extracted using a Nuclear Extraction Kit (Millipore, Billerica, MA, USA) according to the manufacturer’s protocol. To analyze β-catenin-TCF-4 interactions, cell lysates were immunoprecipitated with a TCF-4 antibody or normal IgG. The immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and co-immunoprecipitated β-catenin was analyzed by western blotting with a β-catenin antibody.

Western blot. See Supplementary Methods for details.

qPCR. See Supplementary Methods for details.

Oligonucleotide and cell transfection. Both the miRNA mimics, miRNA inhibitors and siRNAs were synthesized by Ribobio (Guangzhou, China). Oligonucleotide transfection was conducted using Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer’s recommendations. After 48 h of transfection, the cells were collected and used for further investigations.

Plasmids and lentiviruses. The following plasmids and lentiviruses were designed and constructed by GeneChem (Shanghai GeneChem, Shanghai, China): lncRNAs, pGC-FU-POIR-3FLAG-SV40-EGFP-IRE5-puromycin for lncRNA-POIR overexpression and pGC-FU-3FLAG-SV40-EGFP-IRE5-puromycin for lncRNA-POIR NC overexpression.

The lncRNA-POIR sequence was amplified using the indicated primers: F: 5′-CCA CCCATGCTAAGGAG-3′; R: 5′-GCTCGCCGTCACTGCAGCAGG-3′ and inserted into an SV40 plasmid vector. We used a blank SV40 plasmid vector as an NC. Sequencing was performed to verify the correctness of the sequence. For lentivirus packaging, 293T cells were transfected with an lncRNA-POIR plasmid vector and the packaging plasmids Helper 1.0 and Helper 2.0 (Shanghai GeneChem). The harvested lentiviruses were concentrated, purified and preserved at −80 °C. hPDLSCs and pPDLSCs were cultured at a concentration of 2 x 10^5 cells per well in six-well plates. After the cells had grown to 30–40% confluency, they were transfected with lentiviruses in the presence of polybrene at a multiplicity of transfection of 50.

The sequences of the lncRNA-POIR RNA interference plasmids are as follows: SD11-lncRNA-POIR (4043-1), 5′-TCGTCGACCTGACTGAACGACTGTA-3′; SD11-lncRNA-POIR (4042-2), 5′-CTCTCCCTACGAGGTTTAT-3′; SD11-lncRNA-POIR (4044-1), 5′-GACCAGACCCAGACCCATAGA-3′; and SD11-NC, 5′-TTTCCAGACAGTCTGA CGT-3′.

Plasmid transfection was conducted using Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer’s protocol. After 48 h of transfection, the cells were collected and used for further investigations.

In vivo transplantation. The 6-week-old NOD/SCID mice (Fourth Military Medical University, Xi’an, China) were randomly divided into 12 groups (grouping situation can be found in Supplementary Table S3). Each group included three mice and six transplant complexes (each mouse was transplanted with two complexes in the dorsal region). For a single transplant complex, ~5 x 10^6 cells were mixed with 20 mg HA-TCP (Sigma, St Louis, MO, USA) and subcutaneously implanted into pockets of NOD/SCID mice in the dorsal region. General anesthesia was administered by intramuscular injection of pentobarbital sodium (0.1 ml/100 g) for all surgical procedures. All animal procedures were approved by the Animal Care Committee of the Fourth Military Medical University (license number: 2015, kq-012). After 4 weeks, the mice were killed by cervical dislocation under general anesthesia. The implants were then removed, fixed with 4% paraformaldehyde and decalcified in 10% EDTA (pH 6.0) for 7 days. For histological analyses, the implants were then embedded in paraffin, sectioned and stained with H&E or Masson’s Trichrome stain (BaSO Diagnostic Inc., Guangdong, China) according to the manufacturer’s instructions.

RNA immunoprecipitation. RIP assay was performed using an RNA Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer’s instructions with an anti-Ago2 antibody (2 μg; Millipore) and normal mouse IgG as an NC. qPCR was performed using Taqman Universal PCR Mix as described above. All experiments were repeated three times.

Chromatin immunoprecipitation. ChiP assay was performed with a ChiP Assay Kit (Millipore) according to the manufacturer’s instructions with an anti-P65 antibody (2 μg; Millipore), anti-c-Rel antibody (2 μg; Millipore) and normal mouse IgG as an NC. qPCR was performed using Taqman Universal PCR Mix as described above, and the primers used for amplification of the immunoprecipitated DNA are listed in Supplementary Table S1. All experiments were repeated three times.

 Luciferase reporter assay. The putative miR-182 target binding sequence in wild-type lnRNA-POIR (lnRNA-POIR wt) and a mutant (lnRNA-POIR mut, with mutation of individual bases in the binding site) were synthesized and cloned downstream of the luciferase gene in pmirGLO luciferase vectors (Promega, Madison, WI, USA).

To determine whether miR-182 directly targets lnRNA-POIR and the FoxO1 3'-UTR, we constructed a wild-type FoxO1 3'-UTR (FoxO1 3'-UTR wt) reporter plasmid and mutated (FoxO1 3'-UTR mut, with mutation of individual bases in the binding site) FoxO1 3'-UTR reporter plasmid with pmirGLO luciferase vectors.

The plasmids, mRNA mimics (miR-NC and miR-182) and Renvila luciferase plasmid (Promega, Madison, WI, USA), used as a normalization control, were co-transfected into cells. Firefly and Renilla luciferase activities were measured consecutively by Dual Luciferase Assay (Promega) at 48 h after transfection. All experiments were repeated three times.

Dkk1 treatment. Cultures in complete medium were treated with 50 ng/ml of a Wnt inhibitor, Dkk1 (human recombinant Dkk1; PeproTech, Rocky Hill, NJ, USA), and the cultures containing Dkk1 were changed every other day. On culture day 7, the cells were harvested and subjected to assays of in vitro osteogenic differentiation. ALP activity assay and ALP staining have been described previously.

Statistical analyses. All results are presented as the mean ± S.D. from at least three independent experiments and were analyzed using two-tailed unpaired Student’s t-test. Pearson’s product-moment correlation coefficients (r) were used to establish the association of the two variables. For analysis of multiple groups, the P-values were adjusted using the Bonferroni correction for post hoc analysis. A P-value < 0.05 was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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