Telocytes regulate macrophages in periodontal disease

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Abstract

Telocytes (TCs) or interstitial cells are characterised in vivo by their long projections that contact other cell types. Although telocytes can be found in many different tissues including the heart\textsuperscript{1}, lung\textsuperscript{2} and intestine\textsuperscript{3}, their tissue-specific roles are poorly understood. Here we identify a specific cell signalling role for telocytes in the periodontium whereby telocytes regulate macrophage activity. We performed scRNA-seq and lineage tracing to identify telocytes and macrophages in mouse periodontium in homeostasis and periodontitis and carried out HGF signalling inhibition experiments using Tivantinib. We show that telocytes are quiescent in homeostasis, however, they proliferate and serve as a major source of HGF in periodontitis. Macrophages receive telocyte-derived HGF signals and shift from an M1 to a M1/M2 state. Our results reveal the source of HGF signals in periodontal tissue and provide new insights into the function of telocytes in regulating macrophage behaviour in periodontitis through HGF/Met cell signalling, that may provide a novel approach in periodontitis treatment.
Introduction

Periodontitis is an inflammatory disease of the periodontal ligament, the tissue that connects teeth to alveolar bone. It is a prevalent, incurable and continuous degenerative disease that results in bone loss and tooth loss. Individuals with periodontitis often exhibit gingiva recession, bleeding and tooth mobility. All periodontitis (with attachment loss) develops from gingivitis (no attachment loss) with poor prognosis but not all gingivitis develops into periodontitis.

Numerous studies have focused on the causes of periodontitis. Briefly, pathogens accumulate on the tooth surface (forming plaque), invade the periodontium tissue and release LPS (lipopolysaccharide), that results in inflammation and immunologic events. LPS causes the polarization of pro-inflammatory macrophages (M1). M1 macrophages release cytokines such as TNF-α, IFN-γ, IL-6 and IL-12, that contribute to the development and progression of inflammation-induced tissue destruction.

Hence, understanding the regulation of the inflammatory response is critical to understanding and treating periodontitis. The periodontal ligament (PDL), albeit only present as a thin layer, contains several different cell types. However, the understanding of cell populations in the PDL, their interactions, signalling pathways and how these are impacted upon by disease and inflammation are poorly understood.

In this study, we describe a cell type not previously identified in the PDL, namely telocytes (interstitial cells). Telocytes are an enigmatic interstitial cell type that are best characterised by their unusual morphology having very long projections that make direct contacts with other cells. They are believed to a play a role in direct cell-cell communication establishing three-dimensional networks guiding tissue
organization, mechanical sensing, regulating immune responses and phagocytic-like properties. Transmission electron microscopy (TEM) of telocytes show extracellular vesicles bulging out from their membranes suggesting active physical communication with other cells. To date possible cell signalling roles played by telocytes have not been fully described. It is only known that in the intestine, sub-epithelial telocytes are identified as an important source of Wnt signals to maintain the proliferation of intestinal stem cells.

Telocytes are identified in tissues by dual immunolabelling, most commonly CD34+/CD31-, CD34+/c-Kit+, CD34+/Vim+, CD34+/PDGFRα+. In this study, we use CD34+/CD31- to identify PDL-resident telocytes combined with genetic lineage tracing and Sc-RNA sequencing to determine the role of telocytes in homeostasis and periodontitis. We show that quiescent telocytes located near blood vessels are activated in periodontitis and regulate macrophages via the HGF/Met signalling pathway. The resulting transition of macrophages from an M1 to M2 state provides a possible therapeutic strategy for treating periodontitis.
Results

ScRNA-seq analysis reveals a telocyte population in PDL

The PDL is made up of both neural crest-derived and mesodermal-derived cell types and we have previously described the constituent cell populations using single cell transcriptomics\textsuperscript{14}. This analysis compared adult PDL in homeostasis with PDL tissue from a ligature-induced periodontitis mouse model\textsuperscript{16}. The two datasets were integrated by performing a canonical correlation analysis (CCA)\textsuperscript{17} identifying 2,270 cells for analysis after filtering. These cells were further divided to 18 unsupervised clusters for annotation (Figure 1-a). Differential expression (Figure 1—figure supplement 1) revealed cell clusters, including endothelial cells (\textit{Pecam1}), epithelial cells (\textit{Krt14}, \textit{Krt5}), B cells (\textit{Cd79a}, \textit{Cd79b}), T cells (\textit{Skap1}, \textit{Trac}), dendritic cells (\textit{Cd209a}, \textit{Mg12}), macrophages (macrophage 1 (\textit{Plek}, \textit{Cd80}), macrophage 2 (\textit{Wfdc17}, \textit{Mpeg1}), macrophage 3 (\textit{Apoe}, \textit{Cxcr1}), macrophage 4 (\textit{Arg1}, \textit{Cd36})), mast cells (\textit{Cmal}, \textit{Tpsb2}), neutrophils (neutrophil 1 (\textit{Retnlg}, \textit{Mmp8}, \textit{Mmp9}), neutrophil 2 (\textit{Ngp}, \textit{Cd177}, \textit{Chil3}), neutrophil 3 (\textit{Fcnb}, \textit{Chil3})), a proliferative cell population (\textit{Stmn1}, \textit{Mki67}, \textit{Pclaf}), cemento/osteolineage cells (\textit{Ibsp}, \textit{Bglap}, \textit{Spp1}), fibroblasts (\textit{Postn}, \textit{Aspn}) and an intermediate cluster between cluster 2, 15 and 14. A small population of cells (cluster 14) were found in the mesenchymal cell population that expresses \textit{Cd34}, however, they did not express the endothelial cell marker \textit{Cd31} and we thus identified these cells as telocytes (TCs) (Figure 1-b).

According to the literature, telocytes can be identified by dual immunolabelling, most commonly \textit{CD34+/CD31-}, \textit{CD34+/c-Kit+}, \textit{CD34+/Vim+}, \textit{CD34+/PDGFRα+}\textsuperscript{10,12–15}. However, in our ScRNA sequencing data, we found that the expressions of \textit{Kit}, \textit{Vim}
and *Pdgfra* are either low with not much overlap with *Cd34* or ubiquitous (Figure 1—
figure supplement 2). To confirm these cells as telocytes we first cultured PDL cells
*in vitro* and searched for cells with a typical telocyte morphology. Typical telocyte cell
morphology was observed with small cell bodies and a long cellular process called
telopodes (Figure 1-c). Telopodes consist of dilated portions (podoms) and thin
segments in between (podomers). To determine if the telocyte-like cells are derived
from neural crest, we collected PDL cells from *Wnt1*\textsuperscript{Cre/\,+;Rosa26}\textsuperscript{mTmG/+} mice, which
labels neural crest derived cells\textsuperscript{18}, and stained for GFP and CD34. GFP positive cells
showed telocytes structures with podoms and podomers, which were also positive
for CD34 (Figure 1-d). To identify telocyte locations *in situ*, we co-immunostained
sections with CD34 and CD31. As shown in Figure 1-e, telocytes (CD34+/CD31-)
were found in close association with blood vessels (CD34+/CD31+), had small cell
nuclei and long cell protrusions. However, pericytes are also in close proximity to
blood vessels. Therefore, to discriminate between telocytes and pericytes, we
compared genes that are expressed in pericytes, *Cd146* and *Ng2*. We found that
these genes were not expressed in telocytes (Figure 1-f). These data collectively
suggest that CD34+/CD31- telocytes are present in the PDL located in the vicinity of
blood vessels.

**Quiescent telocytes are activated in periodontitis**

Since telocytes only account for a small subset of cells in the RNAseq datasets in
PDL during homeostasis together with the big overall increase in immune cells in the
disease datasets thereby masking any changes in telocyte numbers, we addressed
whether these cells are quiescent or actively proliferating during homeostasis directly
on tissue sections. Lineage tracing using *Cd34*\textsuperscript{Cre\,ERT2/+;Rosa26}\textsuperscript{tdTomato/+} mice\textsuperscript{19}
followed by a 1d-1yr post-tamoxifen chase period revealed that CD34+/CD31- cell numbers did not increase to any significant extent (Figure 2), suggesting that telocytes are a small, quiescent cell population in homeostasis. To investigate if telocytes responded to disease, we used our established ligature-induced periodontitis mouse model where sutures are placed around the second molars\textsuperscript{14}. The ligature leads to plaque accumulation and thus facilitates the invasion of bacteria\textsuperscript{20}. By measuring the distance between alveolar bone crest and cemento-enamel junction (ABC-CEJ distance) (Figure 3-a), we found that bone loss reached a maximum between day 4-7 for all three molars (Figure 3-b). Notably, even though only the second molar was subjected to a ligature, the first and third molars also showed some bone loss at early time points, suggesting they are affected by the ligature-induced periodontitis to some extent. However, the first and third molar bone loss was recovered at longer time points (Figure 3-b), indicating the self-recovery ability from milder periodontitis.

We visualised telocytes with CD34 and CD31 antibodies in ligature PDL at different time points. After 2 hours, there was an obvious increase in the number of CD34+/CD31- telocytes close to blood vessels (Figure 3-c, Figure 3—figure supplement 1). $\text{Cd34}^{\text{creERT2+/}};\text{Rosa26}^{\text{tdTomato/+}}$ mice\textsuperscript{19} tracing for 7 days also showed an increase in tdTomato+/CD31- cells in periodontitis (Figure 3-d,e, Figure 3—figure supplement 2). These telocytes also expressed the proliferation marker Ki67 (Figure 3-f). These data indicate that telocytes proliferate in the PDL following ligature-induced periodontitis.
It has been demonstrated that telocytes can secrete extracellular vesicles\textsuperscript{11}, suggesting that they may have a role in cell signalling. Gene enrichment analysis of the single cell RNA-seq datasets identified angiogenesis, leukocyte migration and inflammatory responses as the three top pathways in PDL telocytes (Figure 3-g).

**Telocytes regulate macrophages via HGF/Met signalling pathway**

To functionally understand the differences between homeostasis and periodontitis, we interrogated the RNA-seq datasets to compare the two conditions with respect to cell-cell communication pathways. We identified the CHEMERIN, HGF, IFN-I, IL16, LIFR and APELIN pathways as not being active during homeostasis but rather, to be active in periodontitis (Figure 4-a). Telocytes were found to express $Hgf$ and $Flt3$ (Figure 4-b), highlighting a potential role for the HGF signalling pathway.

HGF (hepatocyte growth factor) was originally found in liver as a potential hepatocyte mitogen\textsuperscript{21}. It is involved in repair and regeneration as a healing factor\textsuperscript{22–24}. In mouse ligature-induced periodontitis, our RNA-seq cell-cell communication analysis identified macrophages as a target of HGF signalling from telocytes (Figure 4-c). Of the 4 macrophage clusters identified, telocytes are identified as potentially interacting with macrophage clusters 1 and 4. To confirm that telocytes express HGF, triple immunofluorescence staining was performed with CD34, CD31 and HGF. Endothelial cells (CD34+/CD31+) did not express HGF, whereas telocytes (CD34+/CD31-) with telopodes expressed HGF (Figure 4-d). This is consistent with Sc-RNA seq analysis (Figure 4-e,f). In conclusion, telocytes express HGF and based on the ScRNAseq analysis, are the only cells that produce this signal in periodontitis.
The recipient cells are macrophages which express the HGF-receptor HGFR (Hepatocyte growth factor receptor), encoded by Met (Figure 4-c).

Macrophages receiving HGF signals show M1-M2 transition

Four clusters of macrophages were identified from the single cell transcriptomics analysis (Figure 5). DEG (differentially expressed genes) analysis suggested macrophage cluster 1 express Acod1, Trem1; macrophage cluster 2 express Mc4a4c, Ccr2; macrophage cluster 3 express Aif1, Mrc1; macrophage cluster 4 express Cd36, Arg1 (Figure 5-a). To gain a better understanding of these cells, we performed unsupervised clustering on the 4 clusters. In each re-clustered plot, clusters on the left are cells from periodontitis and right from homeostasis – they are clearly separated and the periodontitis cells occur in three linked clusters (0, 3 and 6) (Figure 5-b,e,f).

The HGF signalling pathway has a sole ligand-receptor pair, HGF and HGFR (Met). Interestingly, in the datasets, macrophages expressing Met also expressed Arg1 (encodes Arginase1, Arg1) and Nos2 (encodes iNOS, inducible nitric oxide synthase) (Figure 5-b).

In periodontitis, iNOS mediates the pathological effect of LPS and it is a marker of inflammatory M1 macrophages, which are an indicator of disease progression. Arg1, a gene expressed in M2 macrophages, however, is believed to decrease LPS-induced pro-inflammatory cytokine production. Therefore, macrophages activated by HGF signals in the PDL can be considered to be in a M1/M2 state. Previous studies indicate that monocytes in an inflammatory environment first polarize to M1, then M2 subject to micro-environmental signals. It is well known that Arg1
competes with iNOS for their common substrate L-arginine. Moreover HGF/Met is reported to induce Met+/Nos2+ macrophages to an M2-like phenotype via overexpression of Arg1.

During homeostasis, macrophages in PDL do not express Met protein, however, macrophages start to express Met in periodontitis (Figure 5-c, Figure 5—figure supplement 1). Met expressing cells are located in close proximity to the telopodes of telocytes (Figure 5-d), indicating that telocytes likely make physical contact with Met+ macrophages. Thus, it is conceivable that telocytes promote the overexpression of Arg1 in periodontitis which further leads to the M1/M2 state of macrophages. Additionally, RNA velocity analysis suggests that these Arg1+/Nos2+ macrophages present in cluster 0 (Figure 5b) are linked with Ccr2\textsuperscript{hi}Aif1\textsuperscript{lo} (cluster 3) cells and Ccr2\textsuperscript{lo}Aif1\textsuperscript{hi} cells (cluster 6) (Figure 5-e, f).

To experimentally determine if Arg1 expression in macrophages is increased by HGF signals from telocytes, tivantinib (ARQ197), a small molecule Met inhibitor was used to inhibit HGF/Met signalling. Tivantinib is a highly selective, non-ATP competitive, orally available inhibitor of Met. We observed that when tivantinib was administered to mice with ligature-induced periodontitis, Arg1+ cell numbers significantly decreased (Figure 6-a, b), indicating that inhibition of the HGF-Met signalling interaction in macrophages blocks their polarisation.

To demonstrate that telocytes are a source of HGF signals for macrophage transition from M1 to M2, we FAC-sorted CD34+/CD31- telocytes and co-cultured these with M0/M1 macrophages. It is important to point out that the CD34+/CD31- expression
profile is currently the best identity marker for telocytes described in the literature but other undetermined cells also have this expression profile. The FAC-sorted cells are a heterogenous population as not all adopted a characteristic telocyte morphology following 24 hours in culture. Nonetheless, we obtained sufficient cells with telocyte morphology. For better characterisation of macrophage polarisation markers, we noted that the literature describes a number of markers including Nos2 (Inos) and Cd80 as M1 markers, and, Arg1 and Cd163 as M2 markers. However, only Inos and Arg1 are consistently reliable in our qPCR experiments as highly expressed in macrophages compared to telocytes (Figure 6—figure supplement 1). Therefore, we used Inos/Arg1 as M1/M2 polarisation markers for subsequent qPCR analyses. Upon analysing the expression levels of Inos and Arg1 by qPCR, in comparison to control macrophage-only cultures, the presence of telocytes led to a significant increase in Arg1 expression with simultaneous reduction in Inos expression (Figure 6-c). However, this did not occur when tivantinib was added to the culture (Figure 6-c). Together, these data suggest that M1 macrophages respond to HGF signals secreted by telocytes via Met to adopt an M1/M2 phenotype.
Based on *in vitro, in vivo* and *in silico* studies, we identified a neural crest-derived cell population by morphology and expression of CD34+/CD31- in mouse periodontium. These cells, telocytes, are in a quiescent state in normal periodontal tissue unless challenged by periodontitis whereupon they increase in number and secrete HGF. Although we did not detect an obvious increase in telocyte numbers in our periodontitis sequencing dataset, this was likely a result of the overall relative decrease in stromal cell numbers caused by the increase in immune cells. In periodontitis, our sequencing data showed a larger proportion of inflammatory cells and relatively fewer stromal cells, indicating the successful induction of the inflammatory disease. In periodontitis, the emergence of a new macrophage population not present in homeostasis together with signalling changes in telocytes raised the possibility of a possible interactions between telocytes and macrophages.

We observed telocytes, as a major source of HGF, making physical contact with macrophages. Cleaved HGF can activate Met and downstream signalling pathways\(^3\). It is believed that pro-HGF, the inactive precursor is secreted and processed by HGF activator (HGFAC), a zymogen in the circulation to achieve its function\(^3\). Macrophages receiving HGF signals express *Met, iNos* and *Arg1*, representing a M1/M2 state. The *Arg1+/Nos2+* macrophages have been recently proposed to exist in other tissues\(^3\). These cells do not exist in homeostasis but are detected in periodontitis identifying HGF/Met as a key macrophage regulatory pathway. Our findings are consistent with reports that LPS causes M1 polarisation and a shift towards M2 polarisation mediated by HGF signals\(^3\). Thus, expression of
two competing enzymes, iNOS and Arg1 in Met expressing cells results from regulation by telocytes, which shifts M1 macrophages to an M2 phenotype, resulting in a M1/M2 state. The transition can be effectively inhibited by an HGF/Met inhibitor, tivantinib. It is reported that the transition from LPS-induced M1 macrophages to M2 macrophages is controlled by PI3K or CaMKKβ-AMPK signalling pathway in Met expressing cells via induction of Arg1 expression\textsuperscript{28,36}. We propose the underlying mechanism is possibly the activation of HGF/Met signalling pathway triggered the activation of PI3K or CaMKKβ-AMPK signalling pathway in macrophages.

The presence of M1 macrophages can cause bone loss whereas M2 macrophages can help prevent bone loss in the PDL. telocytes showed the ability to shift M1 to M2, indicating that HGF secreted by telocytes should be beneficial in reducing bone loss. By comparing the bone loss of the second molars with the first molars and third molars from 1 day to 1 month post ligature treatment, we found that only the molars without ligature treatment showed an ability to recover bone loss caused by periodontitis. Second molars, which have the ligature throughout, showed consistent bone loss indicating any ability of telocytes to reduce bone loss is limited by constant physical stimuli, supporting the importance of maintaining oral hygiene in future clinical applications.

Ligature-induced periodontitis is considered as an appropriate model to mimic human periodontitis\textsuperscript{37}. However, whether telocytes are able to control the progression of human periodontitis requires further investigation. From the aspect of this study, an optimistic outcome is expected given the activation of telocytes or HGF/Met pathway under careful maintenance of oral hygiene. Coincidentally,
exogenous application with HGF was found to improve periodontal bone regeneration in swine\textsuperscript{38}.

Additionally, telocytes may have a role in angiogenesis as shown in the gene enrichment terms (Figure 3-e). More CD31+ cells were noticed in periodontitis samples (Figure 3-d). Vascularisation is considered important for periodontal regeneration\textsuperscript{39}. Therefore, the role of telocytes in periodontitis may not only be the regulation of macrophages through HGF/Met signalling pathway but also through angiogenesis. Telocytes are the cells that provide niche signals in the intestine\textsuperscript{3}. Future work may focus on the signals that telocytes send to adjacent niche cells including stem cells\textsuperscript{40}, endothelial cells\textsuperscript{41} and nerve cells\textsuperscript{40}.

Collectively, our study demonstrates for the first time that telocytes increase in number in periodontitis and communicate with immune cells to positively regulate periodontitis via HGF. The activation of HGF/Met signalling pathway or the exogenous use of activated telocytes may be a promising therapeutic measure against periodontitis. This function of telocytes may also present in other inflammatory disease where telocytes exist such as arthritis\textsuperscript{42}. Furthermore, our study also has implications for cancer research, where telocytes were found present and HGF/Met signalling were found essential for cancer metastasis\textsuperscript{43,44}. 
Methods and Materials

Mice

All mice were maintained in the Biological Service Unit, New Hunts House, King's College London. Mice were exposed to a 12 hour light-dark cycle and with food and water available ad libitum. Wild type CD1 mice were obtained from CRL (Charles River Laboratory, UK), Wnt1<sup>Cre/+ ; Rosa26<sup>mTmG/+ </sup>mice<sup>18</sup> were from JAX 003829 and 007576 respectively. The Cd34<sup>creERT2/+ ; Rosa26<sup>tdTomato/+ </sup>mouse<sup>19</sup> was a kind gift from Prof. Qingbo Xu (King’s College London)<sup>16</sup>. Three intraperitoneal injections of tamoxifen were given at a dose of 2 mg/30 gbw (Sigma, T5648) for 3 consecutive days. Mice were sacrificed by exposure to a rising concentration of carbon dioxide or cervical dislocation followed by tissue dissection and tissue processing. All mouse work was approved by UK Home Office under the project license 70/7866 and P5F0A1579, approved by the KCL animal ethics committee.

Animal disease model

Animals older than 8 weeks were used to induce periodontitis. Mice were anaesthetized with Ketavat and Domitor, injected 10 mL/kg i.p. The ligature procedure was performed as described<sup>45</sup>. Briefly, 5-0 wax coated braided silk suture (COVIDIEN, S-182) was tied around the upper second molar in order to induce periodontitis. Samples were collected at desired time points.
**HGF/Met pathway inhibition**

CD1 mice were used to induce periodontitis. Tivantinib (130mg/kg in corn oil with 2.5% DMSO) was orally applied at day 5 post procedure. A control group was given corn oil with 2.5% DMSO. Samples were collected 12 hours later (n=3).

**Immunofluorescence**

Maxillae were dissected and fixed in 4% PFA overnight. Samples were decalcified in 19% EDTA until soft enough to cut (~7 days). Processed samples were then dehydrated with 30% sucrose followed by embedding in OCT on dry ice with ethanol. Cryosections were fixed by 4% PFA. Sections were then subject to permeabilization by 0.2% Triton X-100 (Sigma, X100), heat-induced antigen retrieval, and blocking with 3% BSA. Sections were stained by the following antibodies: anti-RFP (Abcam, Ab62341), anti-CD34 (Abcam, Ab81289 and Ab8158), anti-CD31 (Abcam, Ab7388 and Ab24590), anti-GFP (Abcam, Ab13970), anti-Arg1 (Abcam, Ab92274), anti-Met (Abcam, Ab51067), anti-HGF (Abcam, Ab83760) and anti-Ki67 (Abcam, Ab16667). Secondary antibodies included Alexa Fluor 488 (Invitrogen, A11039), Alexa Fluor 568 (Invitrogen, A11077), Alexa Fluor 633 (Invitrogen, A21052) and Alexa Fluor 488 (Invitrogen, A11008). Tyramide signal amplification (NEL744001KT, PerkinElmer) was performed for weak signals. Hoechst 33342 (Invitrogen 62249, 1:500) was used for DNA staining. Slides were mounted using Citifluor AF1 (EMS, 171024-AF1) and cover-slipped for microscopy. Zeiss Apotome or Leica TCS SP5 systems was used for acquiring images. Image J and Adobe Photoshop were used for image processing.
Single-cell RNA sequencing and analysis

For ScRNA-seq, adult CD1 mice were used. CD1 mice were sacrificed and dissected under a stereomicroscope with the gingiva carefully removed. Teeth were extracted and only the intact molars were kept. For periodontitis, only the second molars were used for subsequent use. The harvested molars were pooled and dissociated with 3 U/mL Collagenase P (COLLA-RO, ROCHE) followed by incubation for 45 minutes in a 37°C shaking water bath. The dissociation process was aided by dispersion with a 1 mL pipette every 15 minutes. Cells were then passed through a 40 μm strainer (Falcon 352340) followed by FACS sorting for alive cells. Single cells in PBS with 0.04% ultrapure BSA were processed following a standard 10x genomic protocol (Chromium Single Cell 3’ v3). Count matrices were generated from the fastq files via CellRanger pipeline using Ensembl 97 genome annotation. Ambient and background RNA from the count matrices were first removed using CellBender remove-background tool\cite{46}. Cells express >1,000 features and with less than 20% mitochondria gene content were kept. 2270 cells were used for analysis. Batch effect was removed by the Seurat (v3.2.0) CCA approach\cite{47}. Integrated data were subsequently scaled and PCA was performed. 30 dimensions were calculated based on variable features followed by UMAP\cite{48} for embedding and Louvain\cite{49} clustering (resolution 1) on knn graph. Macrophages (613 cells) were selected and re-clustered. RNA velocity data were generated using the velocyto tool\cite{50}. For gene enrichment analysis, metascape (https://metascape.org/gp/index.html#/main/step1)\cite{51} was used: genes highly expressed in telocytes cluster with avg_logFC >0 were selected, 863 input gene was used. Finally, cell-cell communication was estimated based on cell groups by using CellChat (v1.0.0)\cite{52}, a method which provides a database that takes into account
multi-subunit structures of ligand-receptor pairs, soluble agonists and antagonists, as well as membrane-bound co-receptors. The communication between cell types was analysed based on the secreted signalling database.

In vitro studies

PDL cells from CD1 mice (n=3) or Wnt1\textsuperscript{Cre/+};Rosa26\textsuperscript{mTmG/+} mice (n=3) were collected for cell culture. Tissues were treated as above to harvest single cell suspension. DMEM/F12 media (3:1) supplemented with 20% FBS, L-glutamine and P/S was used for cell culture. Cells at passage 1 were used for analysis.

Isolation of telocytes by FAC-sorting

Molar teeth and tongues were dissected in L-15 medium (Thermofisher, 21083027) from 8 adult female mice for isolation and sorting of telocytes as CD34+/CD31- cells. Dissected pulp, PDL and tongues were transferred into 1.5ml tubes, excess L-15 was removed and 100µl of 20U/ml Papain (27mg/ml, Sigma, P3125) in L-15 medium was added to each tube. Cells were dissociated at 37°C in a heated shaker, triturating using a filtered low-binding tip every 5 minutes for a total of 40 minutes. The dissociation reaction was stopped by adding 1:1 volume of prewarmed sample buffer (1% fetal bovine serum in L-15). Cells were strained using a 40µm nylon sterile cell strainer (Falcon, 352340) into a 50ml tube and transferred to a 5ml FACS tube (Falcon, 352235). Cells were stained with fluorescent conjugated anti-CD34-APC (Invitorgen 50-0341-80) and anti-CD31-PE (Invitorgen12-4321-80) at 1:200 dilution. DAPI (1mg/ml) was added (1:1000) immediately prior to FAC-sorting using the BD FACSAria sorters into 1.5ml low-binding tubes with 100µl of full alpha MEM culture media.
Telocyte-Macrophage co-culture

Murine bone marrow derived macrophages were generated from bone marrow cells harvested from 6 wildtype animals and cultured in full alpha MEM media containing 50ng/ml recombinant mouse M-CSF (macrophage colony-stimulating factor) protein (Biotechne-416-ML-050/CF). The cells were cultured and expanded for 3 days prior co-culture with telocytes. FAC-sorted telocytes were seeded into a 48 well plate with full alpha MEM media. After 24 hours, in the control group, macrophages with 50ng/ml M-CSF and 1µg/ml LPS diluted in alpha MEM were added to the telocytes. In the experimental group, macrophages with 50ng/ml M-CSF, 1µg/ml LPS and 100nM Tivantinib were added to the telocytes. Cells were cultured for a further 6 days in vitro.

Quantitative (q) RT-PCR

cDNA from RNA extracted from telocyte-macrophage co-culture were subjected to qPCR analysis with the AriaMx Real-Time PCR System (Agilent Technologies) using SYBR green and gene specific primers. Reactions were repeated in triplicates. Relative expression levels were calculated using $2^{-\Delta\Delta CT}$ method using $Rsp19$ as an endogenous housekeeping gene. Differences between experimental groups were compared using an unpaired two-tailed Student's t-test and P-value ≤0.05 was considered statistically significant.

Microcomputed tomography

Maxilla samples were fixed in 4% PFA overnight followed by three washes in PBS. Samples were scanned on a SCANCO μCT50 scanner with 70kVp voltage and a
tube current of 114 μA at 6μm isotropic voxel size. Scans were analysed by MicroView software.

Statistical analysis

Statistical analysis was performed using an unpaired Student’s t-test using GraphPad Prism software. P < 0.05 were considered statistically significant.
**Data Availability**

Sequencing data have been deposited in the GEO database under accession code GSE160358 and GSE167917. Scripts used to perform analysis are available on GitHub: [https://github.com/JingZhaoK/telocytes.git](https://github.com/JingZhaoK/telocytes.git)

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**Declaration of interests**

The authors declare no competing interests.


18. Graves DT, Fine D, Teng Y-TA, Van Dyke TE, Hajishengallis G. The use of
rodent models to investigate host-bacteria interactions related to periodontal
051X.2007.01172.x
Are Crucial for Endothelial Repair of Injured Artery. *Circulation Research*.
2021;129:e146–e165. DOI: 10.1161/CIRCRESAHA.121.319494
20. Nakamura T, Nawa K, Ichihara A. Partial purification and characterization of
hepatocyte growth factor from serum of hepatectomized rats. *Biochem
Biophys Res Commun*. 1984;122(3):1450-1459. doi:10.1016/0006-
291X(84)91253-1
Myocardial protection from ischemia/reperfusion injury by endogenous and
doi:10.1172/JCI10226
22. Matsumoto K, Funakoshi H, Takahashi H, Sakai K. HGF–Met Pathway in
doi:10.3390/biomedicines2040275
Promotes Peripheral Nerve Regeneration by Activating Repair Schwann Cells.
24. Yang Z, Ming X-F. Functions of Arginase Isoforms in Macrophage
Inflammatory Responses: Impact on Cardiovascular Diseases and Metabolic
25. Italiani P, Boraschi D. From Monocytes to M1/M2 Macrophages: Phenotypical
doi:10.3389/fimmu.2014.00514

doi:10.1016/j.archoralbio.2017.03.006


40. Popescu LM, Gherghiceanu M, Suciu LC, Manole CG, Hinescu ME. Telocytes


Figure Legends

Figure 1 Telocytes in the PDL

a. PDL single cells from control mice and ligature treated mice were combined and clustered into 18 clusters. b. Identification of each cluster. Telocyte cluster were identified by CD34+ and CD31-. Macrophages are in four clusters. c. In vitro cell culture with CD1 PDL cells at passage 1 show characteristic telocyte structure, including podoms (red arrows, the dilated portion) and podomers (between two arrows, the thin segments between podoms). d. Wnt1 lineage traced cells (GFP in green) were cultured and stained with CD34 in red, they show piriform cell body and moniliform podoms (red arrows) and podomers. e. Telocytes (CD34+CD31-) was detected near blood vessel (CD34+CD31+) in vivo, CD34 in green, CD31 in red. White arrow indicate the small nuclei and white triangle show the elongation of telocyte respectively. f. CD34 expression was compared with pericyte markers, CD146 (Mcam) and NG2 (Cspg4) expression.

Figure 2 Cd34creERT2 lineage tracing showing limited contribution of telocytes to PDL homeostasis in adulthood

In the adult stage, Cd34creERT2/+; Rosa26tdTomato/+ mice were used to trace from 7 weeks for 1 day (a), 1 month (b), 2 months (c) and 1 year (d). CD34 lineage traced cells in red, CD31 were co-stained in green. CD34+ CD31- cells are telocytes which were rarely found in periodontal tissue and randomly dispersed during homeostasis. Increase of CD34+CD31- cell number was not detected as the extending of tracing time. Scale bars = 100 μm. B: bone, T: tooth. Periodontal tissue in dot line.
Figure 3 Telocytes are activated in response to periodontitis by increasing their number

a. Illustration of bone loss measurement. For the first and second molars, the ABC-CEJ distance of mesial and distal roots in parallel to the root long axis and the ABC-CEJ distance in the trifurcation area were measured, for the third molar, the ABC-CEJ distance in the middle of the tooth was measured. b. Quantification from micro-CT results indicates the change of bone loss in periodontitis plotted by time course (n=3). Hard tissue around the second molar is severely affected, the time for all molars reaching the bone loss plateau is between day 5 to day 7. 1 unit = 0.4mm (n=3). c. An accumulation of telocytes was found in periodontitis as early as 2 hours after the ligation procedure. These telocytes (CD34, green) were mostly found around blood vessels (CD31, red), especially tissue toward the crown. Scale bars = 10μm. d. Cd34creERT2/+; Rosa26tdTomato/+ mice were given three tamoxifen injections started from the procedure day and harvested at day 7. Significantly increased CD34+ cells (red) were observed in the periodontitis group. There were more endothelial cells (green) observed at day 7 of periodontitis. The telocyte (red) derived cells were not overlapping with endothelial cells (green). Scale bars = 20μm. e. Statistical analysis of numbers of telocytes comparing homeostasis (n=3) and periodontitis (n=4). f. Telocytes (CD34+, CD31-) express a proliferation marker, Ki67 in periodontitis. CD34: yellow, CD31: cyan, Ki67: magenta, nuclei: grey. g. 863 input genes highly expressed in telocytes cluster with avg_logFC >0 were selected for gene enrichment analysis. The 20-best p-value terms was plotted. The bar plot is colored by p-values. B: bone, P: periodontal tissue.
**Figure 4 Telocytes regulate macrophages via HGF/Met signalling pathway**

a. Comparison analysis show HGF pathway is upregulated in periodontitis b. Cell-cell communication analysis was performed on disease dataset based on secreted signals database. The outcoming patterns were plotted. Bubble plot suggest that telocytes send signal molecules in FLT3 and HGF signalling pathways exclusively in periodontitis (dark purple dots). c. Circle plot and heatmap suggest telocytes send HGF signals to macrophage clusters 1, 3, 4 in periodontitis. d. Immunostaining on CD1 mice periodontitis tissue for CD34+/CD31- cells indicate telocytes (white arrows) and CD34+/CD31+ cells for endothelial cells (orange arrow) from CD1 mice. The typical morphology of telocytes, podoms are denoted with arrow heads. The telocytes were expressing HGF (magenta). Scale bars = 10μm. e. Expression of CD34 (marker of telocyte) and Hgf were both found in the telocyte cluster and some cells in the intermediat cell cluster. f. CD34 is expressed in endothelial cells and telocytes, Hgf is expressed in telocytes but not endothelial cells and some intermediate cells that close to telocytes.

**Figure 5 HGF/Met signalling drives M1 to M2 transition**

a. Heatmap presenting the DEG of 4 macrophage subpopulations (top 20 DEG genes). b. Macrophages was extracted from the complete dataset and re-clustered. In a feature plot, all the macrophages in disease dataset are in the left cell cluster, separated from macrophages in homeostasis (cells on the right). Feature plots show Met expressing macrophages express M1 marker Nos2 and M2 marker Arg1. c. Met protein was not detected in PDL homeostasis but in periodontitis. Met: magenta, nuclei: blue. Scale bars = 50μm. d. Telocytes (yellow, indicated by arrows) are
making contact with Met expressing cells (magenta) by using their protrusions (telopodes indicated by arrow heads). CD34: yellow, CD31: cyan, Met: magenta, nuclei: grey. Scale bar = 10μm.  

**e.** RNA velocity shows, *Met*+ macrophages (cluster 0) are related to those in clusters 3 and 6. **f.** Macrophages expressing *Met* (cluster 0) are related to those in cluster 6 (*Ccr2+Aif1$^{hi}$*) and cluster 3 (*Ccr2+ Aif1$^{lo}$*) macrophages.

*Figure 6 Telocytes promote M1 to M2 transition via HGF/Met signalling*

**a.** CD1 mice were used to induce periodontitis. Corn oil or tivantinib (Tiv) were given once at day 5 post procedure. Samples were collected 12 hours after drug delivery. Mice given corn oil (left) show more cells expressing Arg1+ cells (yellow) in periodontium than the mice given Tivantinib (right). Scale bars = 10μm.  

**b.** Statistical analysis shows significant difference in Arg1 expression between control group (corn oil) and tivantinib treated group (*p*<0.05).  

**c.** qPCR data showing FAC-sorted telocytes (TC) cultured with M0/1 macrophages (M) in the presence of LPS leads to an increase in the M2 marker *Arg1*, with simultaneous reduction in the M1 marker *Inos*. Transition of M1 to M2 is significantly reduced upon addition of the HGF/Met inhibitor tivantinib. Error bars represent the standard error of mean (*p*<0.05). B: bone; T: tooth; P: periodontal tissue.

*Supplementary Figures*

**Figure 1—figure supplement 1.** Heatmap of cell clusters show telocytes distinct from other mesenchymal cells. Panel b shows a zoomed region of boxed region in a.  

**Figure 1—figure supplement 2.** Telocyte identification makers
Figure 3—figure supplement 1. An increased number of telocytes was observed at day 2 and day 4 post procedure.

Figure 5—figure supplement 1. Met expression in homeostasis and periodontitis

Figure 6—figure supplement 1. Macrophage polarisation marker gene expression in cultured M1 macrophages and FAC-sorted telocytes.

Figure 3—figure supplement 2. Telocytes show lower Cd34 expression comparing to endothelial cells. Figure 3-d with separate channels is shown. Endothelial cells (CD34+CD31+) are indicated with blue arrows and telocytes (CD34+CD31-) are indicated with yellow arrows. magenta: RFP, green: CD31. The expression of Cd34 was significantly lower in telocytes than in endothelial cells. Gene expression level from scRNA-seq indicate Cd34 expression level in telocytes as distinct from endothelial cells.
Ligature

Ctrl

Met