Silencing Itch in human PBMCs promotes differentiation of monocytes into osteoclasts

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Research Article

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Silencing *Itch* in human PBMCs promotes differentiation of monocytes into osteoclasts

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Abstract

**Introduction:** Two clinical case reports of humans with mutations in *Itch* share distinct morphological defects such as stunted growth, macrocephaly, and dysmorphic features indicating a role for *Itch* in bone remodelling. Studies in mice have found that the encoded E3 ubiquitin ligase acts as a negative regulator of osteoclastogenesis, however no studies thus far have investigated whether this is translatable to a human model.

**Experimental Procedures:** Human whole blood samples were separated to isolate and culture peripheral blood monocytes (PBMCs) in M-CSF containing media. Media was later supplemented with RANKL to promote osteoclast differentiation. Transient siRNA-mediated *Itch* knockdown (si-*Itch*) in monocytes was verified by qPCR and western blot to confirm reduction in both *Itch* mRNA and protein respectively. PBMCs were aliquoted onto 96-well plates where confluence and osteoclast formation were analysed using automated cytometry analysis before and after staining for tartrate resistant acid phosphatase activity (TRAP). Cells were also stained with Hoechst33342 to look for multinucleate cells.

**Results:** Cells treated with si-*Itch* showed an 80% knockdown in *Itch* mRNA and >75% reduction in protein. Following the 7-day differentiation period, si-*Itch* caused a 17% increase in numbers of large cellular bodies as well as a 47% increase in multinucleate bodies, indicating an overall increase in mature osteoclast formation. Itch knockdown had little effect cellular toxicity compared to scrambled conditions.

**Conclusions:** Our data shows silencing *Itch* expression increases the potential of primary human PBMCs to differentiate into mature osteoclasts in-vitro and confirms that *Itch* has clinical significance in human bone remodelling.

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**Keywords**

*Itch*, PBMC, osteoclast, E3 ubiquitin ligase, knockdown
**Introduction**

The *Itch* gene encodes a Hect domain E3 ubiquitin ligase that was originally identified by Perry’s group in their study of non-agouti lethal mice [1]. These *Itch*-knockout (*Itch*-/-) mice were dubbed “Itchy” mice due to the excessive scratching phenotype they displayed along with other more severe autoimmune defects [1]–[3]. Since its discovery *Itch* has been shown to have several roles in regulating autoimmunity including the T-cell antigen receptor response [4], [5] T-cell anergy [6], [7], and differentiation of T-helper type 2 cells (T\(\text{H}2\)), T-follicular helper cells (T\(\text{FH}\)), and regulatory T-cells (T-reg) [3], [5], [8], [9].

Clinical significance of *Itch* in humans was not apparent until a case study by Lohr’s group in 2010. They followed a small closed group of children of old Amish heritage who presented with numerous symptoms including organomegaly, failure to thrive, stunted growth, macrocephaly, dysmorphic features, and inflammatory cell infiltration of lungs liver and gut [10]. Genome-wide autozygosity mapping and subsequent sequence analysis identified a single nucleotide insertion in exon 6 of the *Itch* gene, resulting in a frameshift mutation that causes the translated protein to lack three of the four WW binding motifs in addition to the catalytically active HECT domain [10]. A second report of an individual, unrelated to the Lohr cohort, had biallelic mutations in *Itch* resulting in a truncated version of the E3 ligase. The patient also displayed the same phenotypical defects with the addition of campodactyly of the fingers [11]. Whilst *Itch*’s role in regulating immunity is well documented and characterised from studies in *Itch*-/- mice, the other associated symptoms i.e. stunted growth, macrocephaly, dysmorphic features, and campodactyly indicates an additional role for *Itch* in healthy bone growth and remodelling. Furthermore, It has also been observed that patients being treated with clomipramine (CMI), a FDA approved tricyclic antidepressant and recently identified small molecule inhibitor of *Itch* [12], [13], have an associated dose-dependent increase in fracture risk [14].

Mice are a particularly useful model organism for the study of human skeletal disease as there are many similarities between the mouse and human skeletal system including the mechanisms and genes involved in bone development and metabolism [15], [16]. Like humans, mice develop bone through endochondral ossification and also show age-related declines in cancellous and cortical bone. The main advantage of using mouse tissue rather than human, besides ethical considerations, is that bone growth and remodelling processes are accelerated in mice due to their considerably shorter lifespan, which greatly increases convenience. Studies comparing *Itch*-/- mice to wild-type (wt) have observed that mice lacking *Itch* have greater average numbers of both osteoblasts and osteoclasts [17]–[19], cells that govern bone formation and bone resorption respectively. Dysregulation of the balance between these opposing processes can result in osteopetrosis or osteolysis which are associated with debilitating diseases humans. Whether or not the *Itch*-/- mice were osteopetrotic or osteoporotic however seemed age-dependent: young mice appear to have greater bone density whilst older mice have lower bone density [17], [19]. Another study also noted that mice treated with clomipramine/norclomipramine, had higher numbers of osteoclasts in bone tissue resulting in decreased bone density and volume [18].

The discovery of receptor activator of nuclear factor-κB (RANK) and osteoprotegrin (OPG) and their role in osteoclastogenesis was a breakthrough in understanding the bone remodelling process [20]–[24]. Interaction of RANK with RANKL (RANK ligand), secreted by osteoblasts and stromal cells in vivo [25]–[27], promotes osteoclast differentiation by inducing autoubiquitination of TNF receptor-
associated factor 6 (TRAF6) which upregulates nuclear factor-κB (NF-κB) transcriptional activity[17], [28], [29]. TRAF6-/ mice have impaired osteoclast function that results in osteopetrosis [30], causing defects in bone remodelling and tooth eruption. Studies in mice show that Itch negatively regulates osteoclast differentiation by associating with the deubiquitinating (DUB) enzyme cylindromatosis (CYLD). The Itch/CYLD complex then removes Ub from TRAF6 to prevent further NF-κB signalling. CYLD/- mice are hypersensitive to RANKL, have prolonged NF-κB signalling, and have severe osteoporosis [31].

Whilst mice are a powerful for modelling the human skeletal system, as with all models it is not perfect with several noted differences for example the lack of a Haversian system in mice [15], [16]. In this short study we attempt to replicate one aspect of what is seen mouse studies but apply it to a human ex-vivo model. We hypothesise that genetic knockdown of Itch in human peripheral blood monocytes (PBMCs) facilitates differentiation into mature osteoclasts. PBMCs isolated from whole blood were cultured and subject to transient Itch knockdown using siRNA before being exposed to culture conditions to promote osteoclast differentiation. Different staining methods and automated cytometry analysis were then used to assess mature osteoclast formation.

**Materials and Methods**

**PBMC isolation from whole blood:**

Whole blood was collected from healthy volunteers using a protocol approved by School of Medicine’s research ethics committee (MD9202). 10% EDTA was added to whole blood (10 µl per ml of blood) to prevent coagulation. Blood was then diluted 1:1 with cold PBS and layered on top of 10ml Histopaque (Sigma Aldrich). The tube was then transferred to a bench-top centrifuge and spun at 800 ×g for 30min with no brake. Using a sterile plastic pipette, PBMCs from the buffy coat layer were aspirated and moved to a separate falcon tube. Cells were washed 2-3 times with cold PBS, centrifuging at 300 ×g for 10mins (brake applied) and discarding the supernatant each time. PBMCs were resuspended in culture media so as to achieve a cell density of 10⁶ cells/ml. PBMCs were dispensed onto either 6-well plates (Corning) at 10⁶ cells/well, plain-surface or Osteo Assay Surface 96-well plates (Corning) at 1000 cells/well.

**Cell culture:**

PBMCs were cultured in α-MEM (ThermoFisher) culture media supplemented with 10% FBS, 1% Penicillin/Streptomycin and 20 ng/ml macrophage colony-stimulating factor (M-CSF - R&D Biosciences). Cells were incubated with 5% CO₂ at 37°C and media was changed every 3 days. To promote osteoclast differentiation the media was further supplemented with 50 ng/ml RANKL (R&D Biosciences) after which media was changed every 2 days.

**siRNA transfection:**

Itch-targeting siRNA duplex data was kindly supplied by Dr Simon Newman, Nanogenics Ltd. And synthesised by Eurogentec. Sense sequence: GCU-GUU-GUU-UGC-CAU-AGA-A (5’-3’), antisense
sequence: UUC-UAU-GGC-AAA-CAA-CAG-C (5’-3’). For a scrambled control we used a commercial negative control (Eurogentec). siRNA was combined with Lipofectamine RNAiMAX (ThermoFisher) in OPTImem to get a solution with a final siRNA duplex concentration of 125nM. After 6 days of PBMC culture siRNA solution was added to 6/96-well plates containing cells to a final concentration of 12.5nM/well. After 24 hours media was aspirated and replaced with fresh, RANKL-containing media. siRNA was re-applied after 72 hours RANKL exposure, once again only for 24 hours before being replaced with fresh media.

**Western blot:**
Lysates from 6-well plates were taken 48 hours after siRNA transfection using RIPA buffer to lyse cells. A BCA assay was used to determine protein concentration for each sample to make aliquots for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Primary antibodies used: rabbit anti-Itch D8Q6D (CST), mouse anti-β-actin 8H10D10 (CST). Secondary antibodies used: donkey anti-rabbit CW800 (Licor) and donkey anti-mouse RD680 (Licor). Membranes were imaged and quantified using a Licor Odyssey Scanner (Licor).

**RNA extraction and analysis by qPCR:**
Lysates from 6-well plates were taken 48 hours after siRNA transfection. RNA extraction and isolation, reverse transcription and qPCR master mix preparation was performed using Qiagen’s RNEasy Mini Kit, QuantiTect Reverse Transcription Kit and Rotor-Gene SYBR Green PCR Kit respectively (Qiagen) as per the manufacturer’s instructions. The primers used for relative qPCR were QuantiTECT Primer Assays (Qiagen) for GAPDH (housekeeping gene) and Itch. qPCR was performed using a Rotor-Gene Q (Qiagen) and associated software. Relative Itch expression was determined using the ΔΔCt method.

**Tartrate resistant acid phosphatase (TRAP) staining:**
Osteoclasts were stained for TRAP activity using the Acid Phosphatase Leukocyte Kit (Sigma Aldrich). General preparation and staining were done as per the manufacturer’s instructions however the protocol was adapted for 96-well plates rather than microscope slides. Cells were viewed under a light-microscope and images taken using Leica Application Suite V4 (Leica). Automated cytometry analysis (Celigo) was performed on the plates to quantify TRAP activity. Analysis algorithms were designed to screen for: A. all objects with TRAP⁺ activity and B. large multicellular objects with TRAP⁺ activity (positive findings with this algorithm were checked manually).

**Nuclear staining using Hoechst 33342:**
After TRAP staining, wells were air-dried and incubated with 100 µl of Hoechst staining solution (1:10,000 Hoechst 33342 - Life Technologies – in PBS) in the dark for 15 minutes at room temperature. Wells were imaged using automated cytometry analysis software and multinucleate cells were manually counted and recorded in a spreadsheet for further analysis.
Statistical analysis:
Graphs were generated in Graph Pad (version 9.3.1). To test for significance between the means of the test and control conditions Students’ t-test was used.

Results
Successful transient knockdown of *Itch* in human PBMCs:
Osteoclasts can be differentiated from monocytes derived from whole-blood samples, provided there are suitable growth conditions to encourage monocyte isolation [25], [32]. PBMCs were isolated and cultured in 6-well plates with media containing M-CSF to promote monocyte survival.

On day 6 cells were exposed to siRNA for 24 hours. siRNA-containing media was then replaced with osteoclast differentiation media. Western blot and qPCR were used to compare expression of *Itch* at both the protein level and mRNA level in monocytes. In siRNA-treated samples (si-Itch) there was >75% reduction in *Itch* protein expression compared to the untreated control and even more so compared to the scrambled control (Fig. 1A). These results were mirrored at the transcription level with an almost 80% reduction in mRNA expression compared to untreated cells (Fig. 1B). This data demonstrates that we can robustly knockdown Itch in primary human monocytes. Furthermore little observable difference in β-Actin protein indicated little or no change in cell viability during knockdown.

Figure 1: Successful siRNA-mediated knockdown of *Itch* in primary human monocytes: A. siRNA-mediated knockdown of Itch (si-Itch) confirmed by western blot. Itch (green) to β-Actin (red) signal ratio was determined and quantified relative to an untreated control. B. Itch mRNA quantified by qPCR in untreated, scrambled, and si-Itch treated cells. GAPDH was used as a housekeeping gene. C. Percentage confluence of wells in a 96-well plate that were
either untreated or subject to scrambled or Itch-targeted siRNA. Confluence determined by automated cytometry analysis (n=20 per condition).

After confirmation that we were able to transiently knockdown Itch in primary cells, PBMCs were aliquoted and cultured on one of two types of 96-well plates: either plain-surface or Osteo-Surface Assay plates[33] and were cultured for a total of 14 days; 7 days with M-CSF for promoting monocyte attachment and survival and 7 days with RANKL added (differentiation media). Automated cytometry analysis was used to assess well confluence for any differences in cell viability at the end of the culture period (Fig 1C) to determine if knockdown conditions had any effect. No significant difference was seen for si-Itch-treated samples compared to untreated controls however there was an 8% decrease in confluence for scrambled wells compared to si-Itch (mean = 23% & 31% for scrambled and si-Itch respectively, p=0.0012). Interestingly in the osteo-surface plate whilst there was no difference in confluence between conditions, overall confluence was on-average 10% lower than that observed in plain-surface 96 well plate (Fig S1), indicating a reduction in either attachment or viability of cells to the differing plate type.

**siRNA-mediated Itch knockdown facilitates mature osteoclast formation:**

After 7 days culture in osteoclast differentiation media cells were fixed and stained for TRAP activity (Fig. 3A.), TRAP staining being a classic histological method for identification of osteoclasts [34], [35]. TRAP-positive (TRAP+) cells were counted using automated cytometry analysis using a single-cell count algorithm adjusted to identify TRAP+ cells in 96-well plates (Fig. S2-A.). Untreated and si-Itch wells had comparable numbers of TRAP+ cells (mean = 2294 & 2162 respectively) whilst scrambled wells had less (mean = 1308) (Fig. 3B.), concurrent with the confluence data prior to TRAP staining.

Mature, fully differentiated osteoclasts are large multinucleate cells formed from the fusion of several individual premature, mononuclear osteoclasts. The same plates were scanned with a second cell-count algorithm that was designed to select for TRAP+ objects above a particular surface-area threshold (Fig. S2-B.). Wells treated with si-Itch had significantly higher numbers of hits compared to untreated cells (Fig 3C. mean = 63 & 54 for si-Itch and untreated respectively, p = 0.0376) indicating an increase in the number of mature osteoclasts. Scrambled-treated wells displayed far lower numbers (mean = 18). Data was also mirrored in the osteo-surface assay plate with si-Itch-treated wells having higher numbers of hits for both algorithms (Fig S2-C. & D.) despite little to no difference in confluence between each condition prior TRAP staining.

Whilst an increase in large cellular bodies is indicative of immature osteoclast fusion, a weakness of using automated cytometry analysis is that data could be an overestimate of the actual value as the output takes into account anything that fulfils the criteria of the algorithm applied, potentially leading to false positives. To further verify that mature osteoclasts have formed, cells on the plain-surface plate were stained with Hoechst33342 to look for multinucleate bodies. The plate was scanned again blue fluorescence (461 nm emission) and a brightfield filter and images merged (Fig 3D.). Due to technical limitations it was impossible to design an algorithm which could accurately discriminate cellular bodies with multiple nuclei from mononuclear cells so multinucleate bodies were counted manually for each well (Fig 3E.). *Itch* knockdown significantly resulted in an increase
in multinucleate bodies (mean = 25) compared to both untreated (mean = 17) and scrambled-treated (mean = 9) wells (t-test comparing si-Itch vs untreated, p < 0.0001). These results combined confirm that genetic knockdown of \textit{Itch} promotes differentiation of PBMCs into mature osteoclasts, replicating the work that has been shown in mice but in a human ex-vivo context. Furthermore, \textit{Itch} knockdown using siRNA seems to have little impact on overall cell survival as indicated from both the confluence data and the number of TRAP$^+$ cells.
Figure 3: Knockdown of Itch in primary human monocytes increases osteoclast differentiation ex-vivo:

A. TRAP stained cells in wells after siRNA treatment and differentiation period were imaged under a light microscope (10x magnification). Automated cytometry analysis for TRAP+ (B.) and large, TRAP+, cells/bodies (C.) in wells. D. Cell nuclei stained with Hoechst 33342 and imaged. Multinucleate bodies (red arrows) were manually counted and results plotted. Box plots show mean and standard deviation within each test condition, statistical significance was determined by Student’s t-test.
Discussion

Studies in *Itch*/- mice have revealed and outlined a mechanism by which *Itch* exerts influence upon bone remodelling by inhibiting osteoclast and osteoblast differentiation [18], [36], [37]. Although the morphological phenotypes shown in both the Lohr and Brittain *Itch*-mutant cohorts are not seen in mouse models, these common features are still indicative of a defect in bone growth and remodelling in humans. In this study we looked to see if inhibiting *Itch* function through genetic knockdown reproduces results seen in the aforementioned mouse studies but in a human ex-vivo setting. The study found that *Itch* knockdown increases formation of large, multinucleate TRAP$^+$ cellular bodies from healthy human PBMC indicative of increased formation.

In an adult organism, upregulation of proteins associated with increased osteoclastogenesis or osteoblastogenesis is usually due to localised inflammation causing persistent NF-κB signalling [17], [36]. In the case of osteoclasts, TRAF6 self-polyubiquitination is required to transduce RANKL/RANK signalling to promote NF-κB transcriptional activation. Mouse studies looking at osteoclast differentiation have identified that the DUB CYLD associates with Itch to deubiquitinate TRAF6 and inhibit NF-κB. Although *Itch* does act to regulate the immune response and inflammatory signalling pathways, the data shown in this study provides evidence that there is an intrinsic cellular mechanism by which *Itch* regulates osteoclast differentiation as the conditions were designed to study *Itch* in osteoclasts in a vacuum. During cell culture of PBMCs and osteoclasts there was a lack of external inflammatory agents, with the only additional cytokines being M-CSF and RANKL to promote differentiation. Furthermore, siRNA acts intracellularly to knockdown gene function through exploitation of endogenous RNA interference (RNAi) machinery.

Although we have shown that siRNA-mediated knockdown in monocytes is robust, repeatable, and efficient, further optimisation could be performed to broaden the range of gene-editing techniques for primary monocytes. Stable *Itch* knockout in PBMCs was attempted with CRISPR-Cas9 using a lentiviral vector for transduction (Fig S3). The advantage of HIV-1-derived lentiviral transduction methods is the ability to integrate and edit quiescent cells, expanding the pool of potentially editable cell lines [38]. However, it was found that PBMCs were refractory to lentiviral transduction, causing significant cell death and making it difficult to determine silencing efficiency due to lack of protein. previous work on PBMCs and lentiviral constructs have shown that they are resistant to HIV-1 lentiviral transduction vectors which led to an increased cellular toxicity [39]–[41]. Whilst this does weaken the case of using viral vectors for primary culture it does not rule out CRISPR as a method of stable knockout in PBMCs and that alternative vectors are required. As PBMCs do not divide in culture conditions a “pseudo-stable” knockout can in theory be generated from gene-editing machinery encoded in a plasmid that is transfected via traditional methods such as lipofection and nucleofection.

An interesting observation during our short study was the differences in well confluences between each of the treatment regimens and plate formats. Previous studies have reported that mouse osteoclast culture on dentine discs promoted cell survival [17] although the mechanism is unclear. The Osteo-Assay Surface Plates used in our experiments have wells that are coated in a synthetic bone-like matrix. In this study we observed that there was an overall 10% decrease in well confluence between the different plate format however the discrepancy in confluence between the
scrambled and other conditions is absent in the osteo-surface assay plate. Lipofection as a transfection technique has been reported to be toxic to cells[42] however confluence between si-Itch and untreated wells was similar despite the addition of transfection reagent. One possibility is that cells are simply more adherent to a plain cell culture surface over the osteo-surface.

A small molecule screen performed by Rossi et. al. found that the tricyclic antidepressant clomipramine (CMI) can inhibit Itch activity [12]. A later study then looked at the effect of CMI treatment on osteoclast formation and bone resorption in wt and Itch-/- mice 18. WT mice treated with CMI showed increased osteoclast formation resulting in an increase in bone resorption. Meanwhile Itch-/- were unaffected by the CMI regimen indicating that CMI-dependent bone loss is dependent on CMIs interaction with Itch. It would therefore be interesting to see if a treatment regimen of CMI in PBMCs produces osteoclast numbers that are comparable to that seen for the siRNA-mediated Itch knockdown. There is reported evidence of dose-dependent increase in fracture risk in patients receiving therapeutic CMI[14], however no link was made to Itch regulation and osteoclastogenesis in this cohort.

We have demonstrated that siRNA-mediated Itch knockdown is sufficient for promoting osteoclast differentiation from primary human PBMCs ex-vivo. Combined with the data from mouse studies and the clinical implications of mutant Itch in humans in the two cohort studies, targeting Itch signalling may be a potential therapeutic strategy in the treatment of bone diseases. However further research is needed to explore and clarify Itch/CYLD/TRAF6 expression and activity in broad range of diseases first.

Abbreviations:
PBMC; peripheral blood mononucleocyte, TH2; T-helper type 2 cell, TFH; T-follicular helper cell, T-reg; regulatory T cell, CMI; clomipramine, RANK; receptor activator of nuclear factor-κB, OPG; osteoprotegrin, RANKL; RANK ligand, TRAF6; TNF receptor-associated factor 6, NF-κB; nuclear factor-κB, Ub; ubiquitin, wt; wild-type, DUB; deubquitinating enzyme, CYLD; cylindromatosis, TRAP; tartrate resistant acid phosphatase, RNAi; RNA interference,

Authors Contributions
O.J.R, and D.J.H conception and design of research. O.J.R performed experiments and analysed data. O.J.R, and D.J.H interpreted results. O.J.R. drew figures and drafted manuscript. O.J.R and D.J.H edited and revised the manuscript.
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**Competing Interests**
The authors declare have no financial or non-financial competing interests to disclose

**Authors Contributions**
O.J.R, and D.J.H conception and design of research. O.J.R performed experiments and analysed data. O.J.R, and D.J.H interpreted results. O.J.R. drew figures and drafted manuscript. O.J.R and D.J.H edited and revised the manuscript.

**Ethics**
Patient-derived PBMCs were obtained using a method approved by the University of St Andrews School of Medicine’s research ethics committee (MD9202). Informed consent was obtained prior to taking blood.
**Supplementary Figures:**

**Figure S1:**

A. Percentage confluence of cells in 96-well osteo-surface assay plate after 14 days of culture (n = 20 for each condition).

B. Comparison of confluence between plain (blue) and osteo-surface (purple) plates for each condition. Student’s t-test confirmed statistically significant difference in mean values of untreated and si-Itch wells (p = 2.33x10^{-5} & 2.21x10^{-5} respectively).
**Figure S2:** A. Representative images of a section of a well before (left) and after (right) the TRAP⁺ cell counting algorithm was applied. B. Representative images of a section of a well before (left) and after (right) the large TRAP⁺ cell/body counting algorithm was applied. Positive hits that fulfill algorithm criteria are highlighted with a green outline. C. Quantification of automated cytometry analysis for TRAP⁺ cells in osteo-surface assay plate. Means for untreated, scrambled, and si-Itch wells are 449, 330, and 532 respectively. Asterix indicates statistically significant difference between the means of si-Itch and untreated wells (p = 0.0048). D. Quantification of large, TRAP⁺, cells/bodies algorithm in wells of osteo-surface assay plate. Means for untreated, scrambled, and si-Itch wells are 4, 2, and 9 respectively. Asterix indicates statistically significant difference between the means of si-Itch and untreated wells (p = 0.009).
Figure S3: A. Western blot image for Itch and β-Actin used in Figure 1 which includes the lysates for CRISPR-Cas9 treated cells. Cas9 with scrambled gRNA in lane 2 and Cas9 with Itch-targeted gRNA in lane 3. B. Whole western blot image.