Journal of Cellular Biochemistry 96:996–1002 (2005)

Optimized Transfection of Diced siRNA into Mature Primary Human Osteoclasts: Inhibition of Cathepsin K Mediated Bone Resorption by siRNA

Christina I. Selinger, Christopher J. Day, and Nigel A. Morrison*

School of Medical Sciences, Griffith University, Gold Coast campus, Southport, Qld, 4215, Australia

Abstract Osteoclasts are large multinucleated cells responsible for bone resorption. Bone resorption is dependent on the liberation of calcium by acid and protease destruction of the bone matrix by proteinases. The key proteinase produced by the osteoclast is cathepsin K. Targeted knock-down of cathepsin K was performed using small inhibitory RNA (siRNA). siRNA is a method that introduces short double-stranded RNA molecules that instruct the RNA-induced silencing complex (RISC) to degrade mRNA species complementary to the siRNA. Transfection of siRNA by lipid cations allows for short-term inhibition of expression of the targeted gene. We show that transfection of primary human osteoclasts with siRNA to cathepsin K reduces expression by $\geq$60% and significantly inhibits bone resorption with a reduction of both resorption pit numbers ($P = 0.018$) and resorbed area ($P = 0.013$). We also show that FuGENE 6 is an effective lipid transfection reagent with which to transfect primary human osteoclasts, that does not produce off-target effects. J. Cell. Biochem. 96: 996–1002, 2005.

Key words: osteoclast; siRNA; cathepsin K; bone resorption

The challenge facing those discovering gene targets is to define the role these genes play in specific cellular systems. A number of strategies have been employed to determine the function and impact of genes in certain cellular systems, such as mouse knockouts, chemical inhibitors, neutralizing antibodies, and antisense oligonucleotides. All of these strategies are deficient, mouse knockouts are expensive, chemical inhibitors are not very specific, while neutralizing antibodies must be used at high concentration and can only be used to target cell surface or secreted molecules. siRNA has emerged as a powerful tool that can overcome limitations presented by other methods such as lack of specificity and availability. siRNA involves the introduction of short double-stranded RNA molecules that direct the RNA-induced silencing complex (RISC) to degrade mRNA species complementary to the siRNA [Hannon, 2002]. siRNA is highly specific and its effects are more prolonged and less prone to degradation than antisense oligonucleotides [Bertrand et al., 2002]. Transfection of siRNA by lipid cations allows for short-term inhibition of expression of the targeted gene.

Cathepsin K (CTSK) is the key proteinase produced by osteoclasts [Drake et al., 1996] and is a widely accepted marker of osteoclast formation along with tartrate resistant acid phosphatase (TRAP) [Hayman et al., 1996] and calcitonin receptor (CTR) [Nicholson et al., 1986]. Bone resorption by osteoclasts occurs with demineralisation followed by degradation, a process involving cysteine proteases [Hill et al., 1994; Gowen et al., 1999]. The principal protease involved in bone resorption is CTSK [Saftig et al., 1998; Gowen et al., 1999]. CTSK is abundantly and selectively expressed in osteoclasts [Drake et al., 1996], and is released from lysosomes at the ruffled border into the resorption lacunae where it degrades type I [Garnero et al., 1998] and type II collagen [Kafienah et al., 1998].

*Correspondence to: Nigel A. Morrison, School of Medical Sciences, Griffith University, Gold Coast campus, Parklands drive, Southport, Qld, 4215, Australia.
E-mail: N.Morrison@griffith.edu.au

Received 1 June 2005; Accepted 6 June 2005
DOI 10.1002/jcb.20575

We have undertaken a study, which outlines the effectiveness of diced siRNA in primary human osteoclasts using a number of transfection reagents and diced siRNA concentrations. In order to determine the effectiveness of siRNA in primary human osteoclasts, we targeted a prominent gene involved in osteoclast bone resorption, CTSK and tested for changes in CTSK gene expression and the ability of osteoclasts treated with siRNA to resorb bone.

MATERIALS AND METHODS

Production of Diced siRNA

Primers were designed to amplify a 540 bp region of CTSK mRNA: Forward Primer 5′–3′ GCG TAA TAC GAC TCA CTA TAG GGA GAA ACA ACG CCT GTG GCA TTG CC, Reverse Primer 5′–3′ GCG TAA TAC GAC TCA CTA TAG GGA GAG TAA ACT TGT ACC AAA GAC TT. These primers included the T7 RNA polymerase promoter, which allows RNA synthesis to be performed from the generated PCR product. Nucleospin Extraction kit (BD Biosciences) was used to purify PCR product. *In vitro* transcription was performed using T7 RNA Polymerase (New England Biolabs) and incubated at 37°C for 3 h. RNA was treated with RQ1 DNase (Promega) and purified by ethanol precipitation and quantitated by spectrophotometry. RNase III Dicer Digestion was performed with ShortCut RNase III (New England Biolabs) and incubated for 20 min at 37°C. Ten microliter 10X EDTA was used to stop the digestion. siRNA was purified by ethanol precipitation, separated on a 12% polyacryamide gel to estimate size, and quantitated using spectrophotometry.

Cell Culture

Osteoclasts were cultured from PBMCs isolated from whole blood. PBMCs were isolated and cultured as previously described [Day et al., 2004]. Adherent PBMCs were grown on 24-well plates supplemented with 25 ng/ml of M-CSF and 40 ng/ml of RANKL (Peprotech, Rocky Hill, NJ) in modified Eagle's medium (MEM, Invitrogen, Carlsbad, CA) with weekly media changes. Studies of the effects of siRNA on gene expression were carried out using 14-day cultures grown on 24 well plates (BD Biosciences). Cultures of mature human osteoclasts were prepared for bone resorption assays by differentiating osteoclasts for 14 days on 6-well plates coated with type 1 collagen (BD Biosciences). Mature osteoclasts were then removed with dissociation buffer (Invitrogen, Carlsbad, CA), isolated by 100*g* centrifugation through 20% fetal calf serum (FCS) cushion in MEM, and plated out on chips of sperm whale dentine in 96-well plates with 5% FCS in MEM (supplemented with RANKL and M-CSF, as above) in a 5% CO_2 atmosphere. Cells were immediately exposed to the siRNA transfection complex for 6 h, washed with medium and then incubated for a further 96 h. After this time, dentine resorption pits were visualized by gold-sputter coating and scanning electron microscopy. Resorption pits were counted and total area of resorption measured. Tartrate resistant acid phosphatase (TRAP) enzyme staining was performed using a Leukocyte Acid Phosphatase Staining Kit (Sigma-Aldrich, St. Louis, MO).

siRNA Transfection

Complexes between siRNA and the transfection reagents were prepared according to the particular specifications of the individual manufacturers of each reagent. Generally, after siRNA-reagent complexes had formed, the mixture was exposed to cultures and incubated for 6 h before replacing the M-CSF/RANKL supplemented media. Both the siRNA transfection reagent comparison and the siRNA dose-response cultures were performed using siRNA concentrations of 25 nM and 0–25 nM, respectively. FuGENE 6 (Roche) was used as per guidelines for 24-well plates with a volume of 3 μl FuGENE 6 per well. One microliter of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 3 μl Oligofectamine (Invitrogen, Carlsbad, CA) was used per well following the manufacturer's procedures. Two microliters of JetPEI (Polyplus-transfection, Illkirch, France) was used per well following the appropriate protocol for 24-well plates. Transfection in 96-well plates for bone resorption assays were performed as per the 24-well plates scaled for smaller final volumes. All bone resorption assays were performed using FuGENE 6 as a transfection reagent.

Q-PCR Analysis of Gene Expression

Cultures were lysed using 4 M guanidium isothiocyanate, 1% lauryl sarcosine lysis and RNA was harvested using NucleoSpin RNA II kits (BD Biosciences). RNA was converted to cDNA using Improm-II reverse transcriptase

(Promega), essentially as described in Day et al. [2004]. Primers are as follows for OAS1 5′-3′ F-CCT GAG AAG GCA GCT CAC G, R-CAG CAG AAT CCA GGA GCT CAC and CTSB 5′-3′ F-CAA CTC CTG GAA CAC TGA CT, R-CCC ACG GCA GAT TAG ATC TT. Primers for CTSK and ribosomal 18s genes [Day et al., 2004] were used in Q-PCRs, which were performed and analyzed using SYBR Green I Supermix (Bio-Rad, Hercules, CA) as previously described. Data are derived from three repeated experiments performed on separate occasions, except for parallel comparisons of transfection reagents, which was done once.

Statistical Analysis

Results were analyzed using analysis of variance with Fisher's least significant difference post-hoc *t*-test using the SPSS version 11 computer program.

RESULTS

Optimization of siRNA Transfection into Osteoclasts

Four transfection reagents were initially tested with siRNA for their effectiveness to knock-down CTSK expression. The greatest knock-down was achieved with Lipofectamine 2000 (98%) followed by FuGENE 6 (87%), Oligofectamine (79%), and JetPEI (67%) (Fig. 1A). The two most effective transfection reagents (Lipofectamine 2000 and FuGENE 6) were then tested for non-specific knock-down of CTSK using siRNA for Green fluorescent protein (GFP). Lipofectamine 2000 significantly altered CTSK expression using non-CTSK specific siRNA ($P = 0.0002$), whereas FuGENE 6 did not ($P = 0.13$) (Fig. 1B). We, therefore, proceeded to use FuGENE 6 for siRNA experiments.

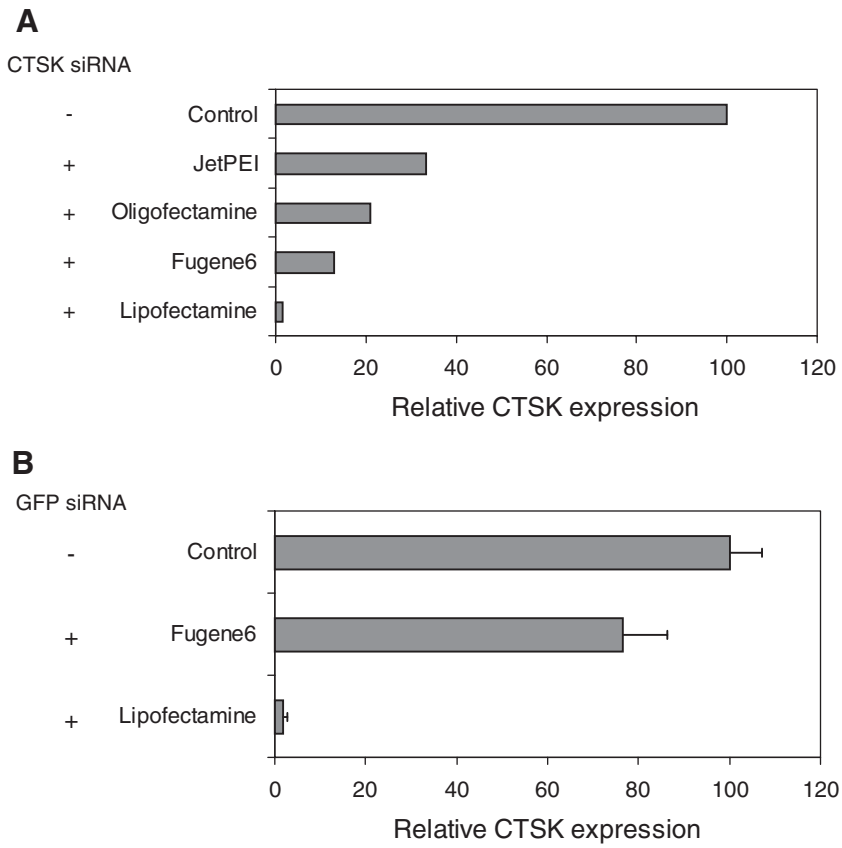

Fig. 1. Selection of transfection reagents for CTSK gene expression knock-down. **A**: Four transfection reagents were tested with siRNA to CTSK. Maximum knock-down was achieved with Lipofectamine 2000 (98%) followed by FuGENE 6 (87%), Oligofectamine (79%), and JetPEI (67%). **B**: Lipofectamine 2000 and FuGENE 6 were tested for non-specific knock-down of CTSK using siRNA for GFP. Lipofectamine 2000 significantly altered CTSK expression using GFP siRNA ($P = 0.0002$), whereas FuGENE 6 did not ($P = 0.13$). Error bars are the standard errors derived from three replicate experiments performed independently.

Specificity of Target Knockdown

FuGENE 6 was used to test concentrations of siRNA from 2.5–25 nM for downregulation of CTSK mRNA expression. Q-PCR was used to determine maximum downregulation of CTSK expression, which was achieved with 25 nM siRNA. Twenty-five nanomolar reduced CTSK expression by 91% compared with the control osteoclast culture (Fig. 2A). Twenty nanomolar and 12.5 nM reduced expression by 70% and 68%, respectively (Fig. 2A).

The interferon response gene 2′,5′-oligoadenylate synthetase 1 (OAS1) is an enzyme induced as part of the cellular response to viral infection [Nilsen et al., 1980], and was used to gauge the level of interferon response in osteoclasts treated with siRNA. Cultures treated with concentrations of siRNA between 12.5 nM and 25 nM were analyzed for OAS1 expression. Twenty nanomolar and 25 nM concentrations of siRNA treatment significantly increased OAS1 expression compared with no treatment ($P = 0.02$, $P = 0.01$), whereas 12.5 nM siRNA did not ($P = 0.39$) (Fig. 2B). These data suggest that 12.5 nM siRNA is sufficient for CTSK knock-down and within the concentration that fails to excite the interferon response.

Cathepsin B (CTSB) is a member of the cysteine protease family, related to cathepsin K and is expressed in osteoclasts [Inaoka et al., 1995]. If the CTSK siRNA effect resulted from non-specific repression of cathepsins, we may expect CTSB to be repressed. We analyzed the expression of CTSB in CTSK siRNA treated

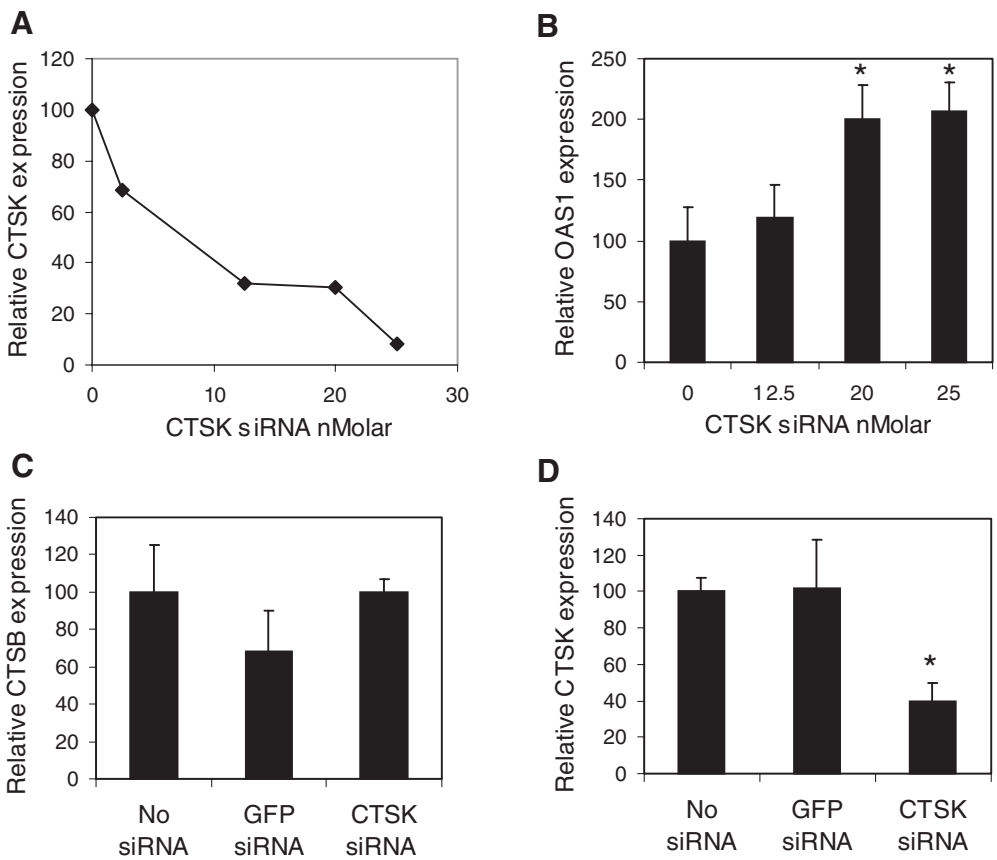

Fig. 2. Optimization of siRNA transfection. **A**: Using FuGENE 6, maximum downregulation of CTSK expression was achieved with 25 nM siRNA (91%). Twenty nanomolar and 12.5 nM reduced CTSK expression by 70% and 69%. respectively. **B**: Twenty nanomolar and 25 nM concentrations of siRNA treatment significantly increased OAS1 expression compared with no treatment ($P = 0.02$, $P = 0.01$), whereas 12.5 nM siRNA did not ($P = 0.39$). **C**: No significant difference in CTSB expression occurred between control cells and siRNA treated cells ($P = 0.50$), or between control cells and control siRNA treated cells ($P = 0.18$). **D**: siRNA treatment for CTSK significantly inhibited cathepsin K expression ($P = 0.02$) compared with untreated osteoclasts, and reduced expression by 60%. Control siRNA for GFP had no significant effect on CTSK expression ($P = 0.95$). Error bars are the standard errors derived from three replicate experiments performed independently.

osteoclasts to examine off-target knock-down and to indicate the specificity of the anti CTSK siRNA. We found no significant difference in CTSB expression between control cells and siRNA treated cells ($P = 0.50$) (Fig. 2C). In addition, there was no significant difference in CTSB expression between control cells and GFP siRNA treated cells ($P = 0.18$) (Fig. 2C).

Treatment of mature osteoclasts with siRNA for CTSK significantly inhibited cathepsin K expression ($P = 0.02$) compared with untreated osteoclasts, and reduced expression by 60% (Fig. 2D). Control siRNA for GFP had no significant effect on CTSK expression ($P = 0.95$) (Fig. 2D). Taken together these data indicate that CTSK siRNA transfection results in specific knock-down of cathepsin K mRNA.

Effect of Cathepsin K siRNA on Gene Expression on Bone Resorption

Mature osteoclasts were harvested from collagen-coated plates and seeded onto dentine chips to test the effects of siRNA on osteoclast function. Cultures were exposed to siRNAs and osteoclasts were allowed to resorb bone for 96 h. Controls with no siRNA exposure showed strongly TRAP positive multinucleate giant cells (Fig. 3A) that produced abundant resorption pits on dentine in the relatively short time of exposure (Fig. 3B). No significant differences in the appearance of cells or resorption pit formation (Fig. 3C and D, respectively) were observed in cells exposed to siRNA directed against GFP. Mature osteoclasts transfected with siRNA directed against CTSK had the usual visual appearance as TRAP positive multinucleate giant cells (Fig. 3E) but the formation of resorption pits on dentine was markedly curtailed (Fig. 3F). CTSK siRNA treatment of mature osteoclasts resulted in inhibition of bone resorption with significant reduction in resorption pit numbers ($P = 0.018$, Fig. 3G) and total area of resorption ($P = 0.013$, Fig. 3H) when compared to control untreated osteoclasts and GFP siRNA treated cells. GFP siRNA did not significantly alter resorption pit number ($P = 0.93$) or resorption area ($P = 0.91$) compared to untreated osteoclasts. CTSK siRNA treatment significantly reduced resorption pit number ($P = 0.036$) and resorption area ($P = 0.032$) when compared to GFP siRNA treatment. Reduced bone resorption observed in CTSK siRNA treated cells was not due to decreased cell number, with total cell count not significantly different between CTSK siRNA and controls ($P = 0.85$). Average cell count per photographed field of view was 95 cells for control osteoclasts and 94 cells for siRNA to CTSK treated osteoclasts.

DISCUSSION

Despite the popularity of siRNA technology, the successful use of siRNA in highly differentiated primary human cells is limited. We provide evidence that siRNA can be successfully used to specifically inhibit gene expression in mature primary human osteoclasts. CTSK is one of the most abundantly expressed genes in the osteoclast and plays a major role in osteoclast driven bone resorption. We have shown that 'DICER' synthesized siRNA gives knockdown of a highly expressed gene such as CTSK by $\geq$60%, with as little as 12.5 nM siRNA using the lipid transfection reagent FuGENE 6. In contrast to Lipofectamine 2000, FuGENE 6 had no significant effect on the expression of CTSK in the absence of specific siRNA. It is not clear as to why Lipofectamine 2000 effects CTSK gene expression. Regardless, FuGENE 6 was found to be a useful reagent in the context of primary human osteoclasts. Once the concentration of siRNA was optimized at 12.5nM, the expression of the interferon response gene OAS1 was not affected by siRNA. The absence of an interferon effect, and the lack of knock-down of cathepsin B, suggests a specific effect of the diced cathepsin K siRNA, targeting CTSK specifically.

We have further established that siRNA to CTSK is a potent inhibitor of bone resorption in osteoclasts, resulting in significant inhibition of the number of pits resorbed by osteoclasts and the area resorbed, without changing the visible appearance nor number of the cells. Usually bone resorption assays have a reasonably long time frame, in the order of weeks. The usual bone resorption time frame is not compatible with the shorter time frame of gene expression knock-down by transfected siRNA, which is thought to persist for several days. By predifferentiating osteoclasts on collagen, then plating onto dentine chips we observed surprisingly rapid resorption pit formation, bringing the time scale of the resorption assay into line with the expected time frame of transfected siRNA effects. Combining this technique with the optimal delivery agent, FuGENE 6, provides

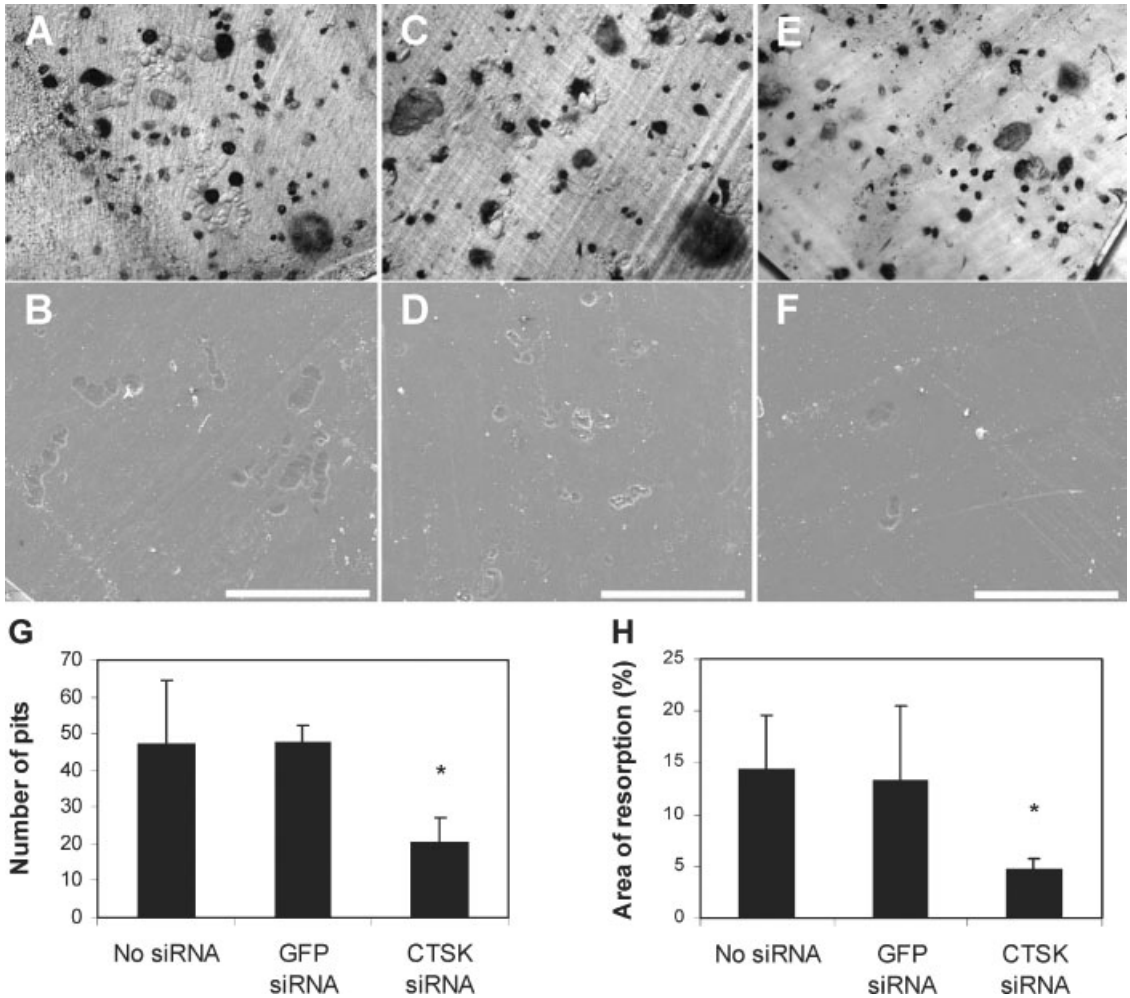

Fig. 3. Visualization and quantitative analysis of cell phenotype and resorption pit formation. Images **A**, **C**, and **E** are light micrographs of TRAP stained cells on dentine chips. TRAP+ cells stain purple and associated resorption pits are visible as translucent profiles adjacent to cells. Images **B**, **D**, and **F** are scanning electron micrographs of dentine chip surfaces (Bar represents 100 μm), but of different regions to those in A, C, and E. The phenotype of control untransfected osteoclasts is TRAP+ (A) with normal resorption pit formation (B). Transfection of GFP siRNA does not alter the visual appearance of osteoclasts (C) nor resorption pit formation (D). Transfection of specific CTSK siRNA does not alter the visual appearance of the cells (E), but does alter the number of resorption pits (F) and their size (seen in F and in E where the translucent pits are smaller). The reduced bone resorption observed in CTSK siRNA transfected cells was not due to decreased cell number, with total cell count not significantly different between CTSK siRNA and control ($P = 0.85$). (**G**) Average number of resorption pits observed in cultures of control untransfected osteoclasts, GFP siRNA transfected osteoclasts, and CTSK siRNA transfected osteoclasts. CTSK siRNA treatment significantly reduced resorption pit number ($P = 0.036$) (* denotes significance). (**H**) Average percentage resorption for untreated osteoclasts, GFP siRNA treated osteoclasts, and CTSK siRNA treated osteoclasts. CTSK siRNA significantly reduced the area resorbed by osteoclasts ($P = 0.032$) (* denotes significance). Error bars are the standard errors derived from three replicate experiments performed independently.

a method capable of examining gene-knock down effects on primary human osteoclasts cultured *in vitro*. Cathepsin K is a major target of pharmaceutical development, and a prime candidate target gene for siRNA knock-down. Furthermore, cathepsin K gene knock-out results in the syndrome of pycnodysostosis, characterised by osteopetrosis [Gowen et al., 1999] due to an inability to degrade the bone matrix. In our experiments, using the optimized protocol (at 12.5nM CTSK siRNA), where no off-target artefacts were detected, the extent of suppression of bone resorption mirrored the extent of CTSK knock-down. Since the most obvious effect of CTSK siRNA transfection was the suppression of the number of bone resorption pits, it seems likely that siRNA transfected cells are incapable of generating resorption pits

and that the resorption pits that were observed represent cells that escaped transfection. Further analysis is required to clarify this point.

This study provides an optimized method for the direct transfection of primary human osteoclasts on dentine chips, permitting the analysis of the contribution of particular genes to mature osteoclast function, through quantitative analysis of bone resorption. We confirm here that cathepsin K expression is necessary for bone resorption in mature osteoclasts. These facts suggest that multiple simultaneous siRNA experiments are possible with mature human primary osteoclasts, using siRNA directly on osteoclasts plated on dentine chips in order to analyze the function of the large number of candidate genes provided by gene array experiments on osteoclasts.

ACKNOWLEDGMENTS

We thank members of the laboratory for donating blood samples for these studies. Prof. Geoff Nicholson is kindly thanked for the kind gift of sperm whale dentine chips. Selinger and Day were supported by postgraduate scholarships from Griffith University and the Australian Research Council, respectively. Support from the NHMRC is gratefully acknowledged.

REFERENCES

Bertrand JR, Pottier M, Vekris A, Opolon P, Maksimenko A, Malvy C. 2002. Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo. Biochem Biophys Res Commun 296:1000–1004.

Day CJ, Kim MS, Stephens SR, Simcock WE, Aitken CJ, Nicholson GC, Morrison NA. 2004. Gene array identification of osteoclast genes: Differential inhibition of osteoclastogenesis by cyclosporin A and granulocyte macrophage colony stimulating factor. J Cell Biochem 91:303–315.

Drake FH, Dodds R, James IE, Connor JR, Debouck C, Richardson S, Lee-Rykaczewski E, Coleman L, Rieman D, Barthlow R, Hastings G, Gowen M. 1996. Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. J Biol Chem 271:12511–12516.

Garnero P, Borel O, Byrjalsen I, Ferreras M, Drake FH, McQueney MS, Foged NT, Delmas PD, Delaisse J-M. 1998. The collagenolytic activity of cathepsin K is unique among mammalian proteinases. J Biol Chem 273:32347–32352.

Gowen M, Lazner F, Dodds R, Kapadia R, Feild J, Tavaria M, Bertoncello I, Drake F, Zavarselk S, Tellis I, Hertzog P, Debouck C, Kola I. 1999. Cathepsin K knockout mice develop osteopetrosis due to a deficit in matrix degradation but not mineralization. J Bone Miner Res 14:1654–1663.

Hannon GJ. 2002. RNA interference. Nature 418:244–251.

Hayman AR, Jones SJ, Boyde A, Foster D, Colledge WH, Carlton MB, Evans MJ, Cox TM. 1996. Mice lacking tartrate-resistant acid phosphatase (Acp 5) have disrupted endochondral ossification and mild osteopetrosis. Development 122:3151–3162.

Hill PA, Buttle DJ, Jones SJ, Boyde A, Murata M, Reynolds JJ, Meikle MC. 1994. Inhibition of bone resorption by selective inactivators of cysteine proteinases. J Cell Biochem 56:118–130.

Inaoka T, Bilbe G, Ishibashi O, Tezuka K, Kumegawa M, Kokubo T. 1995. Molecular cloning of human cDNA for cathepsin K: Novel cysteine proteinase predominantly expressed in bone. Biochem Biophys Res Commun 206: 89–96.

Kafienah W, Bromme D, Buttle DJ, Croucher LJ, Hollander AP. 1998. Human cathepsin K cleaves native type I and II collagens at the N-terminal end of the triple helix. Biochem J 331:727–732.

Nicholson GC, Moseley JM, Sexton PM, Mendelsohn FA, Martin TJ. 1986. Abundant calcitonin receptors in isolated rat osteoclasts. Biochemical and autoradiographic characterization. J Clin Invest 78:355–360.

Nilsen TW, Weissman SG, Baglioni C. 1980. Role of 2′,5′-oligo(adenylic acid) polymerase in the degradation of ribonucleic acid linked to double-stranded ribonucleic acid by extracts of interferon-treated cells. Biochemistry 19:5574–5579.

Saftig P, Hunziker E, Wehmeyer O, Jones S, Boyde A, Rommerskirch W, Moritz JD, Schu P, von Figura K. 1998. Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin K-deficient mice. Proc Natl Acad Sci USA 95:13453–13458.