Published in final edited form as:

Biochem Biophys Res Commun. 2009 October 2; 387(4): 641–645. doi:10.1016/j.bbrc.2009.07.029.

IDENTIFICATION OF CITED2 AS A NEGATIVE REGULATOR OF FRACTURE HEALING

Jonathan Y. Lee^{*,1,2}, Peter J. Taub^{*,2}, Liang Wang³, Amelia Clark¹, Ling L. Zhu^{1,4}, Edward R. Maharam¹, Daniel J. Leong^{1,5}, Melissa Ramcharan^{1,5}, Zhengzhi Li¹, Zhonghou Liu³, Yuan-Zheng Ma³, Li Sun⁴, Mone Zaidi⁴, Robert J. Majeska^{1,5}, and Hui B. Sun¹

¹Leni and Peter W. May Department of Orthopaedics Mount Sinai School of Medicine, New York, USA

²Division of Plastic Surgery Mount Sinai School of Medicine, New York, USA

³Department of Orthopaedics 309 Clinical Center of the General Hospital of PLA, Beijing, China

⁴Mount Sinai Bone Program, Mount Sinai School of Medicine, New York, USA

⁵Department of Biomedical Engineering City College of New York New York, USA

Abstract

The transcription regulator CITED2 (CBP/p300-Interacting-Transactivator-with-ED-rich-tail-2) is known to suppress genes mediating angiogenesis and extracellular matrix (ECM) remodeling. However, it is unclear whether CITED2 has a role in controlling skeletal repair or remodeling. We tested the hypothesis that CITED2 functions in bone fracture healing by suppressing the expression of genes critical to ECM remodeling, angiogenesis and osteogenesis, importantly the matrix metalloproteinases (MMPs). Three hours following mandibular osteotomy or sham surgery of adult rats, osteotomy fronts were harvested and the expression of CITED2 and genes associated with fracture healing was quantitated by quantitative PCR. In parallel, gain-of-function studies examined the effect of overexpressing CITED2 on the expression and activity of several MMPs. In the fractured mandible, CITED2 expression was inversely related to the expression of MMP-2, -3, -9, -13, VEGF, HIF-1 α , M-CSF, RANK-L, and OPG. Consistent with this, the over-expression of CITED2 in osteoblasts inhibited the expression and activity of MMP-2, -3, -9 and -13. Taken together, the studies suggest that CITED2 is a critical upstream regulator of fracture healing. The suppression of CITED2 early after fracture may allow an optimal initiation of the healing response.

Keywords

CITED2; bone fracture healing; ECM remodeling; angiogenesis; osteogenesis; matrix metalloproteinases

© 2009 Elsevier Inc. All rights reserved.

Address correspondence to: Hui B. Sun, Ph.D. One Gustave L. Levy Place, Box 1188 Department of Orthopaedics Annenberg Building 20-66 New York, NY 10029 Phone – (212) 241-3767 Fax – (212) 867-3168 Herb.Sun@mssm.edu.

*These authors contributed equally.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

Trauma or surgical osteotomy of the craniofacial bone often results in poor or delayed healing. While mechanisms underlying craniofacial fracture healing remain poorly understood, optimal bone regeneration in any post-fracture setting depends upon integrated molecular signals that regulate extracellular matrix (ECM) remodeling, angiogenesis and osteogenesis.

ECM remodeling is mediated predominately by matrix metalloproteinases (MMPs), a family of proteases that cleave components of the ECM[1]. Angiogenesis, in contrast, occurs in response to hypoxia in which HIF-1 α expressed initially[2] stimulates VEGF production that, in turn, initiates new vessel formation[3,4]. Within this microenvironment, osteogenesis induced by different cytokines[5], such as bone morphogenetic proteins, can be monitored using markers, such as M-CSF[6], RANK-L[7] and osteoprotegerin (OPG)[7]. The regulation of each of these processes is complex and diverse; however, determining a common regulatory mechanism may highlight a critical molecular target for future treatment strategies.

CBP/p300-Interacting Transactivator with ED-rich tail (CITED2) is a transcriptional co-regulator[8] that has been implicated in controlling, at least in part, genes related to both ECM remodeling[9] and angiogenesis[10]. CITED2 binds to the transcriptional factors CBP and p300[11], both of which can be recruited by other factors to activate a variety of genes. Specifically, HIF-1 α can bind to CBP and p300 to promote the downstream expression of angiogenesis-related genes[12]. Likewise, the transcription factor ETS-1 can bind to p300 to activate MMP gene expression[13]. CITED2 competes with HIF-1 α and ETS-1 for CBP and p300 binding, in essence inhibiting the expression of the respective remodeling and angiogenesis genes[9,12,14]. CITED2 expression in bone following fracture might therefore limit fracture healing. However, the role of CITED2 in bone and specifically in fracture healing has not been examined previously.

Here, we report for the first time an inverse relationship between CITED2 and genes related to ECM remodeling, angiogenesis and osteogenesis in the setting of early craniofacial fracture repair. A mandibular fracture model, shown in Figure 1, was utilized because the mandible is commonly fractured in trauma[15] and undergoes multiple reconstructions in patients with congenital abnormalities[16], with a high variation in healing rates (2%-32%) [17-20].

MATERIAL AND METHODS

Animals

All experiments were performed in accordance with the Mount Sinai School of Medicine Animal Care and Use Committee. Adult female Spague Dawley rats (5 to 6 month-old) were purchased from Charles River Laboratories (Wilmington, MA).

Surgery

Animals were anesthetized with intraperitoneal injections of ketamine (7.5mg/kg) and xylazine (1.5mg/kg). Following incision in the skin along the inferior border of the mandible and division of the masseter muscle, a full thickness surgical osteotomy was made between the coronoid process and the condyle using a 1.6 mm drill bit and Dremel® hand drill (Racine, WI) (Figure 1). Buprenorphine (0.1mg/kg) was administered subcutaneously for pain relief. Three hours post-surgery, the animals were sacrificed using CO₂ inhalation, and both osteotomy fronts of the left hemimandible were promptly harvested. All soft tissue and blood clots were carefully dissected from the bone, and the bone segments were rinsed in

PBS three times to remove any remaining blood and soft-tissue debris. After rinsing, the bone segments were immediately flash frozen in liquid nitrogen to minimize the insults from tissue harvest. Sham-operated animals which underwent skin and muscle incisions above the periosteum, but without osteotomy, were sacrificed 3 hours postoperatively and the left hemimandible was harvested and processed similarly to experimental rats.

Quantitative PCR

Harvested bony tissue was pulverized using the Mikro-Dismembrator S (B. Braun Biotech International), and total RNA was extracted using RNeasy Minikit (Qiagen). Quantitative PCR was performed, as described previously *per* manufacturer's instructions[9].

Western Blot

Western blotting was used to confirm the over-expression of CITED2 in transfected MC3T3 cells using a method described previously[9]. A polyclonal CITED2 antibody against a specific CITED2 sequence was generated at Sigma. The expected molecular size for CITED2 was 28 kDa. β -actin protein (42 kDa) was detected using a mouse β -actin specific antibody (Sigma).

MMP Activity Assay

MMP activities on protein extracted from culture supernatants were determined using fluorogenic substrates specific for MMP-2, MMP-3, MMP-9, or MMP-13 (Molecular Probes). Total MMP activity, contributed by both pro-form and active form MMPs, was assayed in the presence of 4-aminophenylmercuric acetate (APMA; Sigma-Aldrich), while endogenous MMP activity was assayed in samples without APMA. APMA-activated and un-activated samples were then incubated with the fluorogenic substrates (Molecular Probes) consisting of 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM CaCl_2 , and 0.2 mM sodium azide at room temperature for 2 hours. Fluorescent intensity was measured using FluoroMax-2 spectrofluorometer (Instruments S.A., Inc.), as described previously[9].

RESULTS

Figure 2 shows that three hours after mandibular osteotomy in adult rats, CITED2 expression at the fracture site, as measured by qPCR, declined significantly by ~40% compared with that of sham controls ($p=0.012$). In contrast, genes related to ECM remodeling, angiogenesis and osteogenesis were either upregulated or remained unchanged. Specifically, the expression of the metalloproteinases MMP-2, -3, -9 and -13 increased by 1.96-, 2.88-, 3.29- and 4.29-fold, respectively. The angiogenesis-related genes VEGF and HIF-1 α were likewise upregulated by 2.72- and 3.96-fold, respectively. Consistent with optimal fracture repair, expression of marker genes for early osteoblast differentiation, namely M-CSF, RANK-L and OPG, increased by 1.52-, 3.15- and 2.16-fold, respectively. Importantly, early fracture repair was expectedly not associated with increased levels of BMP-4 expression ($p = 0.95$), a gene that regulates late osteogenesis. The latter finding testifies that our osteotomy model displays early, rather than late fracture healing.

To confirm the inverse relationship between CITED2 and the expression of ECM genes at the fracture site, we conducted *in vitro* gain-of-function studies by transiently transfecting MC3T3.E1 osteoblast-like cells with CITED2 cDNA. Figure 3a shows a Western blot confirming that CITED2 was indeed over-expressed in CITED2-transfected cells, but not in untransfected cells or cells transfected with vector only. qPCR showed that CITED2 over-expression down-regulated MMP expression in osteoblasts within 48 hours. Thus, MMPs 2, 3, 9 and 13 were inhibited to 0.59-, 0.67-, 0.64- and 0.59-fold, respectively (Figure 3b).

In addition to the reductions in MMP *expression* noted above, impaired fracture healing in the face of a high CITED2 expression could also result from reduced MMP *activity*. For this, the activity of each individual MMP was determined by using fluorogenic substrates specific for the respective MMPs. Of note is that MMPs are initially in a pro-form, and their activation requires additional post-translational modification[21]. Thus, total MMP activity, contributed to by both pro- and active forms, was assayed in the presence of 4-aminophenylmercuric acetate (APMA). In addition, endogenous MMP activity was measured without APMA. Proteolysis of the exogenous fluorogenic substrate (Molecular Probes) provided a spectrofluorimetric measure of MMP activity[9].

Figure 4 shows that CITED2 over-expression resulted in a decrease in both total and endogenous activity of all four MMPs. Relative to vector-transfected MC3T3 cells, total activities for MMPs 2, 3, 9, and 13 were reduced to 0.52-, 0.45-fold, 0.69, and 0.48-fold, respectively (Figure 4A). Likewise, endogenous activities for the respective MMPs were reduced to 0.46-, 0.57-, 0.69- and 0.55-fold (Figure 4B). While the total and endogenous activities for each MMP declined upon CITED2 over-expression, it is important to stress that this reduction was due to the down-regulation of MMP expression, rather than a reduction in enzymes' activity. This is testified by the observation that there was no significant difference between the total and endogenous relative activity in CITED2 transfected cells related to that in vector-transfected cells (Figure 4C). Thus, we conclude that CITED2 over-expression in osteoblasts results in a down-regulation of MMP expression, and hence, the cell's total and endogenous MMP activity, but without affecting the enzyme's catalytic activity *per se*.

DISCUSSION

Optimal bone repair is a critical to successful craniofacial reconstruction. Although the natural progression of fracture healing has been well described, the underlying cellular and molecular mechanisms are just beginning to unravel. The primary objective of this study was to determine whether the transcriptional co-regulator CITED2 controls genes related to ECM remodeling, angiogenesis, and osteogenesis.

CITED2, previously identified as MRG1 or p35srj, is induced by various interleukins, PGDF, insulin, and hypoxia[8,10] and binds to CBP and p300[11]. CITED2^{-/-} mouse embryos do not survive gestation and have multiple developmental defects[11,22]. We show for the first time, using a rat model of early mandibular fracture healing, that osteotomy attenuates CITED2 expression, while genes involved in ECM remodeling, angiogenesis and osteogenesis increase or show no change. Furthermore, we find that the expression of the matrix metalloproteinases MMP-2, -3, -9 and -13 is down-regulated upon CITED2 over-expression in osteoblasts, attesting to the negative regulation of ECM remodeling by CITED2.

Early fracture healing is characterized by the initial formation of cartilage tissue in the callus, which is then resorbed by MMPs to allow for vascular invasion with the eventual replacement of cartilage with osseous tissue[23]. MMPs 9 and 13 are critical to normal skeletal development[24,25]; most notably, MMP-9 deficiency delays fracture healing with poor cartilage resorption and impaired capillary and chondroclast invasion[26]. MMP-2, in contrast, participates in cartilage degradation[27], while MMP-3 activates pro-MMP-9[28] during wound repair[29].

We have shown that in chondrocytes, the transcription of the MMP genes is regulated by the binding of the co-activator p300 to ETS-1, which interacts directly with the MMP gene promoter. Our work also suggests that ETS-1-p300 binding can be competitively inhibited

by CITED2[30], which, in essence, will negatively regulate MMP gene transcription[9]. The present study clearly demonstrates CITED2-dependent inhibition of MMP gene expression, essentially recapitulating our earlier chondrocyte data. Our current studies are focused at pinning down the mechanism of this action in osteoblasts.

MMP activation and cartilage resorption facilitates vascular invasion and angiogenesis, which are both triggered by hypoxia[23,31,32]. Hypoxia directly induces HIF-1 α [2], which then promotes VEGF expression to enable angiogenesis during fracture repair[3,4]. VEGF also facilitates osteogenesis by promoting the recruitment of osteoblasts and osteoclasts toward ultimate osteogenesis[3]. For these actions, the CAD (carboxyl-terminal activation domain) of HIF-1 α interacts with the TAZ1 domain of CBP and p300[10], which can both interact with CITED2. Thus, CITED2 competitively inhibits HIF-1 α -induced transactivation[12]. Testifying to this, the forced expression of CITED2 suppresses VEGF promoter activity. Conversely, siRNA knockdown of CITED2 increases VEGF promoter activity regardless of the oxemic state[33]. Our *in vivo* results demonstrating that CITED2 and HIF-1 α expression are inversely related at the fracture site are thus consistent mechanistically with previous *in vitro* studies, and suggest strongly that CITED2 negatively regulates angiogenesis during fracture repair.

Finally, osteoblast and osteoclast invasion is necessary for the creation of osseous tissue. M-CSF, RANK-L and OPG are all osteoblast derived cytokines that regulate osteoclast formation, function and/or fate[6]. Hence, they have been considered as functional markers for osteogenesis at fracture sites. That CITED2 is down-regulated during early fracture repair when the aforementioned osteogenesis markers are stimulated points towards, but is not proof, for the involvement of CITED2 in regulating osteogenesis. It is not clear from our studies whether this relationship points to a direct inhibitory effect of CITED2 on osteoblast formation, or whether CITED2 primarily inhibits vascular invasion that, in turn, prevents osteoblast and osteoclast recruitment.

CONCLUSION

We report a novel role for the *hitherto* poorly characterized transcriptional regulator CITED2 in bone repair. Using a rat fracture model, we show that CITED2 expression declines rapidly within 3 hours of osteotomy. This is associated with a dramatic increase in essential matrix metalloproteinases, namely MMPs 2, 3, 9 and 13, as well as genes regulating angiogenesis and osteogenesis. In parallel studies, we find that CITED2 over-expression in osteoblasts *in vitro* down-regulates MMP expression, proving that at least one component of early fracture healing, notably ECM remodeling, is negatively regulated by CITED2. Further studies will not only focus on the mechanism through which CITED2 inhibits MMP, HIF-1 α and VEGF expression, but are likely also to utilize CITED2 as a new molecular target for intervention in non-healing craniofacial defects.

Acknowledgments

This study was supported by grants to H.S. from the National Institutes of Health (AR050968, AR047628). L.S. and M.Z. acknowledge the support of the National Institutes of Health (AG23176, DK70526 and DK80459).

REFERENCES

1. Wojtowicz-Praga SM, Dickson RB, Hawkins MJ. Matrix metalloproteinase inhibitors. Invest. New Drugs 1997;15:61–75. [PubMed: 9195290]
2. Semenza GL. HIF-1 and human disease: One highly involved factor. Genes Dev 2000;14:1983–1991. [PubMed: 10950862]

3. Maes C, Carmeliet P, Moermans K, Stockmans I, Smets N, Collen D, Bouillon R, Carmeliet G. Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Mech. Dev* 2002;111:61–73. [PubMed: 11804779]
4. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat. Med* 1999;5:623–628. [PubMed: 10371499]
5. Chen D, Zhao M, Mundy GR. Bone morphogenetic proteins. *Growth Factors* 2004;22:233–241. [PubMed: 15621726]
6. Sarma U, Flanagan AM. Macrophage colony-stimulating factor induces substantial osteoclast generation and bone resorption in human bone marrow cultures. *Blood* 1996;88:2531–2540. [PubMed: 8839845]
7. Boyce BF, Xing L. Biology of RANK, RANKL, and osteoprotegerin. *Arthritis Res. Ther* 2007;9(Suppl 1):S1. [PubMed: 17634140]
8. Sun HB, Zhu YX, Yin T, Sledge G, Yang YC. MRG1, the product of a melanocyte-specific gene related gene, is a cytokine-inducible transcription factor with transformation activity. *Proc. Natl. Acad. Sci. U. S. A* 1998;95:13555–13560. [PubMed: 9811838]
9. Yokota H, Goldring MB, Sun HB. CITED2-mediated regulation of MMP-1 and MMP-13 in human chondrocytes under flow shear. *J. Biol. Chem* 2003;278:47275–47280. [PubMed: 12960175]
10. Bhattacharya S, Michels CL, Leung MK, Arany ZP, Kung AL, Livingston DM. Functional role of p35srj, a novel p300/CBP binding protein, during transactivation by HIF-1. *Genes Dev* 1999;13:64–75. [PubMed: 9887100]
11. Bamforth SD, Braganca J, Eloranta JJ, Murdoch JN, Marques FI, Kranc KR, Farza H, Henderson DJ, Hurst HC, Bhattacharya S. Cardiac malformations, adrenal agenesis, neural crest defects and exencephaly in mice lacking Cited2, a new Tfp2 co-activator. *Nat. Genet* 2001;29:469–474. [PubMed: 11694877]
12. De Guzman RN, Martinez-Yamout MA, Dyson HJ, Wright PE. Interaction of the TAZ1 domain of the CREB-binding protein with the activation domain of CITED2: Regulation by competition between intrinsically unstructured ligands for non-identical binding sites. *J. Biol. Chem* 2004;279:3042–3049. [PubMed: 14594809]
13. Borden P, Heller RA. Transcriptional control of matrix metalloproteinases and the tissue inhibitors of matrix metalloproteinases. *Crit. Rev. Eukaryot. Gene Expr* 1997;7:159–178. [PubMed: 9034720]
14. Freedman SJ, Sun ZY, Kung AL, France DS, Wagner G, Eck MJ. Structural basis for negative regulation of hypoxia-inducible factor-1alpha by CITED2. *Nat. Struct. Biol* 2003;10:504–512. [PubMed: 12778114]
15. Haug RH, Prather J, Indresano AT. An epidemiologic survey of facial fractures and concomitant injuries. *J. Oral Maxillofac. Surg* 1990;48:926–932. [PubMed: 2395044]
16. Fritz MA, Sidman JD. Distraction osteogenesis of the mandible. *Curr. Opin. Otolaryngol. Head Neck Surg* 2004;12:513–518. [PubMed: 15548910]
17. Kellman RM. Repair of mandibular fractures via compression plating and more traditional techniques: A comparison of results. *Laryngoscope* 1984;94:1560–1567. [PubMed: 6503575]
18. Ellis E 3rd, Karas N. Treatment of mandibular angle fractures using two mini dynamic compression plates. *J. Oral Maxillofac. Surg* 1992;50:958–963. [PubMed: 1506972]
19. Jaques B, Richter M, Arza A. Treatment of mandibular fractures with rigid osteosynthesis: Using the AO system. *J. Oral Maxillofac. Surg* 1997;55:1402–6. discussion 1406–7. [PubMed: 9393399]
20. Anderson T, Alpert B. Experience with rigid fixation of mandibular fractures and immediate function. *J. Oral Maxillofac. Surg* 1992;50:555–60. discussion 560–1. [PubMed: 1593314]
21. Tschesche H, Knäuper V, Krämer S, Michaelis J, Oberhoff R, Reinke H. Latent collagenase and gelatinase from human neutrophils and their activation. *Matrix* 1992;(Suppl. 1):245–55. [PubMed: 1480034]
22. Yin Z, Haynie J, Yang X, Han B, Kiatchoosakun S, Restivo J, Yuan S, Prabhakar NR, Herrup K, Conlon RA, Hoit BD, Watanabe M, Yang YC. The essential role of Cited2, a negative regulator

- for HIF-1 α , in heart development and neurulation. *Proc. Natl. Acad. Sci. U. S. A* 2002;99:10488–10493. [PubMed: 12149478]
23. Weiss S, Zimmermann G, Pufe T, Varoga D, Henle P. The systemic angiogenic response during bone healing. *Arch. Orthop. Trauma. Surg.* 2008
 24. Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, Werb Z. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* 1998;93:411–422. [PubMed: 9590175]
 25. Stickens D, Behonick DJ, Ortega N, Heyer B, Hartenstein B, Yu Y, Fosang AJ, Schorpp-Kistner M, Angel P, Werb Z. Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. *Development* 2004;131:5883–5895. [PubMed: 15539485]
 26. Colnot C, Thompson Z, Miclau T, Werb Z, Helms JA. Altered fracture repair in the absence of MMP9. *Development* 2003;130:4123–4133. [PubMed: 12874132]
 27. Duerr S, Stremme S, Soeder S, Bau B, Aigner T. MMP-2/gelatinase A is a gene product of human adult articular chondrocytes and is increased in osteoarthritic cartilage. *Clin. Exp. Rheumatol* 2004;22:603–608. [PubMed: 15485014]
 28. Ramos-DeSimone N, Hahn-Dantona E, Siple J, Nagase H, French DL, Quigley JP. Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. *J. Biol. Chem* 1999;274:13066–13076. [PubMed: 10224058]
 29. Subramaniam K, Pech CM, Stacey MC, Wallace HJ. Induction of MMP-1, MMP-3 and TIMP-1 in normal dermal fibroblasts by chronic venous leg ulcer wound fluid*. *Int. Wound. J* 2008;5:79–86. [PubMed: 18336381]
 30. Vo N, Goodman RH. CREB-binding protein and p300 in transcriptional regulation. *J. Biol. Chem* 2001;276:13505–13508. [PubMed: 11279224]
 31. Peng H, Usas A, Olshanski A, Ho AM, Gearhart B, Cooper GM, Huard J. VEGF improves, whereas sFlt1 inhibits, BMP2-induced bone formation and bone healing through modulation of angiogenesis. *J. Bone Miner. Res* 2005;20:2017–2027. [PubMed: 16234975]
 32. Warren SM, Steinbrech DS, Mehrara BJ, Saadeh PB, Greenwald JA, Spector JA, Bouletreau PJ, Longaker MT. Hypoxia regulates osteoblast gene expression. *J. Surg. Res* 2001;99:147–155. [PubMed: 11421617]
 33. Agrawal A, Gajghate S, Smith H, Anderson DG, Albert TJ, Shapiro IM, Risbud MV. Cited2 modulates hypoxia-inducible factor-dependent expression of vascular endothelial growth factor in nucleus pulposus cells of the rat intervertebral disc. *Arthritis Rheum* 2008;58:3798–3808. [PubMed: 19035510]

Figure 1. Full thickness osteotomy of the mandible, leaving 1.6 mm fracture gap, is shown (A), together with the schematic highlighting of spatial relationships (B). “CP” denotes the mandibular coronoid process, and “C” denotes the mandibular condyle.

Figure 2.

Expression by quantitative PCR (relative expression) of CITED2, MMP-2, -3, -9 and -13, VEGF and HIF-1 α , and M-CSF, RANK-L, osteoprotegrin (OPG) and BMP-4. Statistics: significance at $p \leq 0.05$, based on ANOVA and Turkey post-hoc testing, shown as mean $\pm$ SEM, $n = 10$ rats per group.

Figure 3.

Western blot using whole cell lysates from MC3T3.E1 osteoblasts transiently transfected with CITED2 for 48 hours (C) showing abundant expression of CITED2 protein in transient transfection experiments (48 hours) (A), compared with no CITED2 expression in either untransfected cells (U) or those transfected with vector only (V). Actin is the loading control. Quantitative PCR was utilized to examine the expression of MMPs 2, 3, 9 and 13 in the aforementioned cell groups (B). Statistics: comparisons with group V; significance at $p \leq 0.05$, based on ANOVA and Turkey post-hoc testing, shown as mean $\pm$ SEM, $n = 5$ replicates per group.

Figure 4.

Metalloproteinase activity in MC3T3.E1 osteoblasts transiently transfected with CITED2 for 48 hours (C) *versus* untransfected cells (U) or those transfected with vector only (V). A fluorescent-labeled substrate (Molecular Probes) was utilized to provide spectrofluorimetric measures of the proteolytic activity of MMPs. Total activity, contributed to by both pro- and active forms, was assayed in the presence of 4-aminophenylmercuric acetate (APMA) (A). Endogenous activity was measured without APMA (B). The endogenous and total proteolytic activity of MMP-2, -3, -9, and -13 in CITED2 transfected cells relative to that in vector-transfected cells did not show significant differences (C). Statistics: comparisons with group V; significance at $p \leq 0.05$, based on ANOVA and Turkey post-hoc testing, shown as mean $\pm$ SEM, $n = 5$ replicates per group.

TABLE 1**Real-time PCR Primer Table**

Gene	Sense Primer Sequence	Anti-Sense Primer Sequence	Sequence AC Number
Rat CITED2	TCTTGGCTGCATGAACTTTG	GGGAGACAGCCAACCTTGAAA	NM_053698
Mouse CITED2	AGGAAAAGAAAAGGGAACGG	CAGCAGCACATAGAGGGACA	NM_010828
Rat MMP-2	AGCTCCCGGAAAAGATTGAT	TCCAGTTAAAGGCAGCGTCT	NM_031054
Rat MMP-3	TCAGCGGATCTTCACAGTTG	TGAGGTTGACTGGTGCCATA	NM_133523
Rat MMP-9	TAATAAACACGGATCCCCCA	ATGAGTGGATAGCTCGGTGG	NM_031055
Rat MMP-13	GCATGAAAACGTGTGGGGAGT	CAGCCACACTGTCCTCAGAA	XM_343345
Rat HIF1 α	TTGCTCATCAGTTGCCACTTC	CATGGTCACATGGATGGGTA	NM_024359
Rat VEGFA	AGGTTGCTCCTTCACTCCCT	CGACTGGTCCGATGAAAGAT	BC168708
Rat M-CSF	TAACAGACACCCCGTTGACA	CTTGCTGGTCCTCCTTTCAG	NM_023981
Rat RANKL	ACCAGCATCAAAATCCCAAG	GAAAGCCCCAAAGTACGTC	NM_057149
Rat OPG	GGGAATGAAGATCCTCCAG	CCTCTTTCTCAGGGTGCTTG	U94330
Rat BMP-4	TGATACCTGAGACCGGAAG	TCCTCACAGTGTTGGCTCTG	NM_012827
Mouse MMP-2	CACACCAGGTGAAGGATGTG	GCCCTCCTAAGCCAGTCTC	NM_008610
Mouse MMP-3	CCAGGTGTTGACTCAAGGGT	AGGATGCCTTCCTTGGATCT	NM_010809
Mouse MMP-9	CGTCGTGATCCCCACTTACT	ACACACAGGGTTTGCCTTC	NM_013599
Mouse MMP-13	ATCCTGGCCACCTTCTTCTT	TTTCTCGGAGCCTGTCAACT	NM_008607
Rat GAPDH	GACAATGCCTGGATCCCTAA	TGATGGCAACAATGTCCACT	AF106860
Mouse GAPDH	GTCCTACCCCAATGTGT	TGTGAGGGAGATGCTCAGTG	NM_008084

Sequences of PCR primers and sequence accession number from the NCBI Entrez search system.