MMP and TIMP temporal gene expression during osteocytogenesis

Prideaux M[1], Jones ER[2], Riley GP[2], Staines KA[3], Pitsillides AA[4], Farquharson C[3]

[1]The University of Adelaide, North Terrace, Adelaide, SA, 5005, Australia [2] University of East Anglia, Norwich, NR4 7TJ [3] Roslin Institute and R(D)SVS, University of Edinburgh, Easter Bush, Midlothian, EH25 9RG [4]Royal Veterinary College, Royal College Street, London, NW1 0TU

Corresponding author

Katherine Staines

Roslin Institute and R(D)SVS

University of Edinburgh

Easter Bush

Midlothian, EH25 9RG

Email: katherine.staines@roslin.ed.ac.uk

Tel: 0131 651 9176

1 Introduction

Osteocytes within bone differentiate from osteoblast precursors. They are the longest-lived and the most numerous cell type in bone (>90%), and are fundamental to its structure and function. Their characteristic dendritic morphology allows them to retain direct contact with bone-forming osteoblasts and to create multicellular networks permeating the entire bone matrix (Dallas et al., 2013). Osteocytes are now known to play key roles in calcium and phosphate homeostasis and are versatile orchestrators of bone remodelling in response to load-bearing (Pitsillides et al., 1995; Bonewald, 2002; Quarles, 2003; Franz-Odendaal et al., 2006). Most models of osteocyte formation (osteocytogenesis) propose a predominantly passive role, during which an osteoblast destined for osteocytogenesis slows extracellular matrix (ECM) production and becomes surrounded by the osteoid synthesised by neighbouring osteoblasts (Nefussi et al., 1991; Palumbo et al., 2004; Franz-Odendaal et al., 2006). Evidence, however, indicates that the initial 'embedding' stage involves a dynamic progression and that the essential genetic and dramatic morphological transformations taking place are part of an active process, closely regulated by the cells involved; not just simple entrapment of defunct osteoblasts within the matrix (Franz-Odendaal et al., 2006).

Osteoblast-to-osteocyte transition occurs as the local ECM mineralises (Mikuni-Takagaki et al., 1995; Palumbo et al., 2004) and blockade of ECM mineralisation is accompanied by both lower expression of the earliest osteocyte marker E11, and by restricted formation of both osteocyte dendrites and sclerostin positive osteocytes (Irie et al., 2008; Prideaux et al., 2012). These and other studies confirm ECM mineralisation as a key player in pre-osteocyte maturation (Atkins et al., 2009a; Atkins et al., 2009b; Prideaux et al., 2012).

Additional changes to the surrounding ECM have also been implicated in actively driving osteocytogenesis. As osteocytes are encased within the bone matrix there is a requirement for

collagen remodelling and mice rendered resistant to collagenase activity exhibit increased numbers of empty lacunae and apoptotic osteocytes (Zhou et al., 2000). The primary enzymes responsible for the remodelling of ECM proteins are matrix metalloproteinases (MMP) which are a family of metal-dependent endopeptidases. MMPs are secreted in the form of inactive proenzymes and are activated in the tissue through the cleavage of pro-peptide (Krane, 1994).

MMPs have long been recognised to be essential for embryonic and post-natal bone development, and are required for angiogenesis and vascular endothelial growth factor (VEGF) expression (Karelina et al., 1995; Raza and Cornelius, 2000; Eisenach et al., 2010). Elegant studies combining osteoblast lineage tracing with monitoring of the vascular endothelium have shown that osteoblast precursors at E12.5 within the perichondrium translocate to the nascent primary ossification centre giving rise to trabecular osteoblasts and cortical osteocytes by E16.5 (Maes et al., 2010). Throughout this translocation the osteoblast precursors are intimately associated with invading blood vessels; processes that require MMP activity for ECM remodelling.

The importance of cleavage of collagen type 1 by MMP14 in particular has been demonstrated by the generation of mice lacking this enzyme (Holmbeck et al., 1999; Holmbeck et al., 2005). The osteocytes from these mice had a decreased number of cellular processes which were shorter and failed to achieve communication with neighbouring osteocytes. MMP14-null mice also had a ten-fold increase in apoptotic osteocytes which maybe via a decrease in the release of matrix bound TGF-β by the proteolytic activity of MMP14 (Karsdal et al., 2004). Similarly, MMPs 2, 13 and 10 have been localised to osteocytes (Zhou et al., 2000; Holmbeck et al., 2005; Inoue et al., 2006; Itoh and Seiki, 2006; Mosig et al., 2007; Tang et al., 2012; Blaber et al., 2013; Rocha et al., 2014). However, little is known about their precise role, and the expression patterns of other members of the extensive MMP family, during osteocytogenesis.

Therefore in this study we have used the murine MLO-A5 late osteoblastic cell line, which both synthesizes a mineralised matrix and expresses osteocyte markers, to describe the temporal changes in the transcriptional profile of members of the MMP family and their endogenous inhibitors – tissue inhibitors of metalloproteinases (TIMPs) during osteocytogenic transition (Kato et al., 2001b; Barragan-Adjemian et al., 2006; Prideaux et al., 2012). Our studies identify several members of both families as potentially key regulators of ECM remodelling necessary for osteoblast – osteocyte transition.

2 Results

2.1 Matrix mineralisation in MLO-A5 cells

MLO-A5 cells cultured in the presence of ascorbic acid and β-glycerophosphate achieved mineralisation of their associated matrix that was initially evident from day 3 of culture as shown by alizarin red staining. Quantification of eluted alizarin red revealed this mineralisation to be significantly increased from day 7 of culture onwards onwards ($P<0.001$; Fig. 1A & B). These data confirm our previous results with this cell line (Prideaux et al., 2012) in which we found that these cells also progressively expressed osteocyte-selective markers such as *E11*, *Dmp1, Sost* and *Cd44*. This confirms the suitability of this model to report the gene expression patterns of *Mmps* and *Timps* during late-stage osteoblast differentiation and osteocytogenesis.

2.2 Expression of Mmps and Timps during MLO-A5 cell differentiation

We found that 14 *Mmps* and 3 *Timps* were expressed by MLO-A5 cells cultured in differentiating medium, whereas *Mmps 1a, 1b, 7, 8, 12, 24, 25, 27* and *Timp4* were not found to be expressed. We also observed weak and variable expression of *Mmps 3, 9, 13* thus precluding them from temporal quantification. Of the expressed *Mmp*s, several failed to show

any significant temporal changes in expression at any stage throughout the culture period (*Mmp10*, *Mmp15*, *Mmp16*, *Mmp17* and *Mmp21*) whereas others showed clear and significant modulation. Our studies revealed that mRNA expression of both *Mmp14* and *Mmp19* were significantly increased at day 3 ('a'; P<0.05) before returning to baseline levels at day 6 (Fig. 2B & C). The mRNA expression of *Mmp11* was also modestly increased, although not significant, at day 3 of culture, but in this case remained increased thereafter throughout the culture period (Fig. 2F). We also found that *Mmp2*, *Mmp23* and *Mmp28* had decreased expression concurrent with the onset of mineralisation at day 3 of culture (P<0.05) (Fig. 2A, D & E). Expression of *Timp1*, *Timp2* and *Timp3* mRNA each followed a similar pattern, with *Timp2* analyses revealed significantly decreased levels of expression by day 6 compared to day 0 (P<0.05; Fig. 3A - C).

2.3 The effects of blocking MLO-A5 cell differentiation on Mmp and Timp expression

To examine whether these changes are linked to the process of osteocytic differentiation during matrix mineralisation, we also determined *Mmp* and *Timp* mRNA expression levels in conditions where mineralisation did not occur. Thus, in the absence of β-glycerophosphate there was a marked ablation of matrix mineralisation by MLO-A5 cells over the 10 day culture period (P<0.001) (Fig. 1A & B).

In these conditions not favouring full osteocytic differentiation of MLO-A5 cells or mineralisation, the decreases in *Mmp2*, *Mmp23* and *Mmp28* expression previously noted in mineralising cultures by day 6 were not observed (Fig. 2A, D & E). Indeed, *Mmp28* expression levels were instead increased at days 3 and 9 of culture compared to day 0 (P<0.001, Fig. 2E), suggesting that the initiation and continued mineralisation of the MLO-A5 cells is linked with the down-regulation of these MMPs and therefore closely associated with osteocyte differentiation. We also found that levels of *Mmp14*, *Mmp19* and *Mmp11*

mRNA were also increased at day 3 compared to day 0 in the absence of β-glycerophosphate and, unlike MLO-A5 cells in differentiating medium, these remained elevated throughout the culture period ($P<0.05$) (Fig. 2B, C & F). Interestingly, our studies also found that the expression of *Timp3* mRNA was also affected by the omission of βGP from MLO-A5 cultures, with increased expression observed on days 6 and 9 ($P<0.001$) of culture compared to cultures maintained in differentiating medium (Fig. 3C). Increased mRNA expression of *Timp1* and *Timp2* were also seen in the mineralisation-inhibited cultures after 9 days, compared to the control cultures (Fig. 3A & B).

3 Discussion

These gene expression patterns identify selective modulation of particular MMPs and their TIMP regulators during osteocyte differentiation and matrix mineralisation. In particular, they highlight that *Mmp14* and *Mmp19* show acute and transient up-regulation that coincides with MLO-A5 osteocytic differentiation, as shown by our previous profiling of osteocyte-selective markers such as *E11*, *Dmp1, Sost* and *Cd44* (Prideaux et al., 2012). We also show that *Mmp2, Mmp23* and *Mmp28,* as well as the *Timps1-3*, in particular *Timp3,* show suppressed levels of expression upon osteocytogenic differentiation that are retained at low levels during matrix mineralisation by MLO-A5 cells. The reversal of these changing profiles of transcription in the absence of β-glycerophosphate links them closely with the mineralisation and osteocytogenesis of these cells.

Remodelling of the ECM is pivotal for the development and maintenance of structural integrity of many tissues and key proteinases in this process are the MMPs; a family of secreted and cell surface enzymes known for their proteolytic activity (Sternlicht and Werb, 2001; Itoh and Seiki, 2006). They can be divided into two subgroups: membrane-tethered MMPs, which are expressed on the cell surface and soluble MMPs, which are secreted into

the extracellular matrix. MMPs are expressed in a variety of cell types and are essential for embryonic and post-natal bone development, as well as angiogenesis and VEGF expression (Karelina et al., 1995; Raza and Cornelius, 2000; Eisenach et al., 2010). The actions of MMPs are moderated by their natural specific inhibitors – TIMPs which are required to work together in a balanced manner to remodel the ECM of a variety of tissues (Riley et al., 2002). To date the TIMP family consists of four members which have been localised to chondrocytes and osteoblasts and have a temporospatial expression during development of the mouse skeleton (Bord et al., 1999; Joronen et al., 2000).

The importance of members of the MMP family of proteinases during osteocytogenesis has been demonstrated by the generation of *Mmp*14 (MT1-MMP) and *Mmp*2 knockout mice (Holmbeck et al., 1999; Zhou et al., 2000; Inoue et al., 2006; Mosig et al., 2007). These mice show defects in skeletal remodelling, reduced mineralisation and also show a profound effect on osteocyte formation characterised by a lack of cellular processes (Holmbeck et al., 2005; Inoue et al., 2006). These changes were particularly apparent in the MMP14 knockout mouse and identifies this member of the MMP family as playing a key regulatory role during osteoblast to osteocyte differentiation. MMP14 has also been implicated in mediating the osteocyte response to mechanical stimuli (Kulkarni et al., 2012). These mutant mice, along with MMP9 knockout mice, have also been shown to have defects in angiogenesis to varying degrees; in MMP14 knockout mice blood vessel growth is not observed where as it is simply reduced in MMP2 and MMP9 mutant mice (Vu et al., 1998; Zhou et al., 2000; Kato et al., 2001a). Despite these associations between these MMPs and the skeleton, the expression patterns of MMPs during osteocytogenesis, however remains relatively unexplored and was the focus of this present study.

Several MMPs were expressed in MLO-A5 cell cultures, with *Mmp2* and *Mmp14*, as expected, amongst them (Fig. 4). Our data suggest that *Mmp2* expression is decreased with

cell differentiation towards an osteocyte-like phenotype. In contrast, *Mmp14* is increased, concurrent with initiation of mineralisation. However, its decreased expression at day 6 suggests a role for *Mmp14* in mineral deposition initiation and the formation of osteocytic processes, further enhancing the link between these two events. *Mmp19* also followed a similar pattern of expression to that of *Mmp14* in differentiating MLO-A5 cells. This is despite the fact that no role for MMP19 within the bone environment has so far been established and *Mmp*19 knockout mice do not display the gross abnormalities in bone phenotype exhibited by mice deficient in other MMPs (e.g. *Mmp*14) (Pendas et al., 2004). Two other MMPs, *Mmp23* and *Mmp28*, were also decreased in expression during MLO-A5 cell differentiation, with *Mmp23* showing the greatest change in expression of all the MMPs analysed. Although the roles of both MMP23 and MMP28 in bone are currently unclear, *Mmp23* mRNA has previously been detected in osteoblasts and chondrocytes (Clancy et al., 2003) (Zaman et al., 2010) and microarray analysis revealed that its expression levels are significantly lower in osteocytes compared to osteoblasts (Paic et al., 2009). *Mmp28* mRNA has previously been detected in rat bone (Bernal et al., 2005) but the specificity of expression within the bone environment in unknown. These results suggest that like *Mmp*23, *Mmp*28 is down-regulated during osteoblast to osteocyte differentiation. Further analysis of enzyme activity would be of importance to more fully delineate the precise role of these proteins in osteocytogenesis.

The expression and activity of TIMPs, which act as endogenous inhibitors of MMP activity (Visse and Nagase, 2003) is unknown during osteocytogenesis. *Timp1*, *Timp2* and *Timp3* mRNA expression has been observed in both osteoblasts and osteocytes (Hatori et al., 2004), which is in confirmed in the MLO-A5 cells of this study. Expression of these inhibitors was observed to decease with differentiation into an osteocyte-like phenotype. This is consistent

with the notion that a reduced blockade upon MMP activity is required during osteocytogenesis. No expression of *Timp4* was detected in the MLO-A5 cell cultures.

The inhibition of matrix mineralisation was also found to have a significant effect on *Mmp* mRNA expression by MLO-A5 cells. Indeed, the down- regulation of *Mmp2*, *Mmp23* and *Mmp28* expression which was observed during MLO-A5 cell differentiation under mineralising conditions, was lacking when mineralisation was inhibited, implying that these cells were not differentiating normally. *Mmp14* expression in the mineralisation-inhibited cultures increased at day 3, similar to that in the control cultures, but then remained elevated even at the later timepoints. *Mmp19* also showed a similar effect. These data indicate that mineralisation of the ECM which helps to drive osteocytogenesis is required for maintaining the correct MMP expression patterns associated with this process (Irie et al., 2008; Prideaux et al., 2012). The MMPs are known to be important in regulating cellular events such as adhesion, differentiation and morphology (Nagase et al., 2006; Page-McCaw, 2008). This disruption of MMP expression is likely to contribute to the detrimental effects on osteoblast-to-osteocyte differentiation observed previously in both *in vitro* and *in vivo* studies (Irie et al., 2008; Prideaux et al., 2012).

Interestingly, forced expression of *Mmp*14 greatly reduced the mineralisation of rat osteoblast cultures *in vivo*, as did inhibition of MMP14 activity (Manduca et al., 2009). Mineralisation of MC3T3 osteoblast cultures was also significantly inhibited by the addition of a MMP14-neutralising antibody before the initiation of mineralisation (Nakano et al., 2010). Addition of the MMP14-neutralising antibody after the initial onset of mineralisation had no effect on subsequent mineral deposition. Together with our data from MLO-A5 cells, these findings suggest that a transient peak in *Mmp*14 expression is required for mineralisation and osteocyte process formation to occur. Expression of *Timp1*, *Timp2* and *Timp3* mRNA was increased at day 9 in the mineralisation-inhibited MLO-A5 cultures compared to control

cultures. *Timp1* and *Timp2* have previously been shown to be down-regulated during late mineralisation (Filanti et al., 2000). This is supported by the present data which also show that the inhibition of mineralisation in MLO-A5 cells appears to maintain TIMP and reduce the down-regulation of their expression.

4 Materials and methods

4.1 Differentiation and mineralisation of MLO-A5 cell line

MLO-A5 cells (a generous gift from Prof. Lynda Bonewald, University of Missouri-Kansas City, USA; passage number 21-22) were plated at a density of $3.5x10^4$ cells/cm^2 in maintenance media (α-MEM containing L-glutamine, ribonucleosides and deoxyribonucleosides, 0.5% gentamicin and 5% calf serum and 5% fetal bovine serum (FBS), (Invitrogen, Paisley, UK)) (Kato et al., 2001b). At confluency, the media was replaced with differentiation media (α-MEM containing 0.5% gentamicin and 10% FBS) and mineral deposition was induced by supplementing the cultures with 100μg/ml ascorbic acid (AA) and 5mM β-glycerophosphate (βGP) (Prideaux et al., 2012). To inhibit matrix mineralisation, cells were cultured in (i) differentiation medium alone, (ii) differentiation medium lacking βGP (100μg/ml AA alone), and (iii) differentiation medium lacking AA (5mM βGP alone).

4.2 Quantification of ECM mineralisation

Cells were fixed with 4% paraformaldehyde (PFA) for 5 min at 4°C. Cell layers were stained with aqueous 2% (w/v) Alizarin red solution (Sigma) at pH 4.2, for 5min at room temperature as previously described (Newton et al., 2012; Staines et al., 2012). The bound stain was subsequently solubilized in 10% cetylpyridinium chloride (Sigma) and the optical density of

the resultant solution determined at 570 nm by spectrophotometry (Thermo Multiskan Ascent).

4.3 RNA extraction and reverse transcription

Cultures were stopped at confluency (day 0) and thereafter, every 3 days and total RNA was extracted using Tri-Reagent (Ambion, Huntingdon UK) according to the manufacturer's instructions. Purified RNA was treated with DNase (Ambion, Paisley, UK). RNA samples were reverse-transcribed using Superscipt II reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

4.4 RT-qPCR of osteoblast/osteocyte markers

RT-qPCR was carried out in a Stratagene Mx3000P cycler with each reaction containing 50ng template DNA, 250nM forward and reverse primers (Primer Design, Southampton UK) (Table 1) and PrecisionPlus Mastermix (Primer Design). The Ct values for the samples were normalised to that of *Gapdh* and the relative expression was calculated using the ΔΔCt method (Livak and Schmittgen, 2001). The amplification efficiencies of all the primers were between 90-100%.

4.5 *Mmp and Timp mRNA profiling*

Mmp and *Timp* mRNA expression profiling was carried out by TaqMan qPCR analysis**.** Briefly, 50ng cDNA derived from 3 individual cultures at each timepoint was amplified in duplicate using ProbeFast 2x qPCR MasterMix (Kapa Biosystems), 200nmol/l forward and reverse primer and 100nm/l probe (Table 1) (Wilkinson et al., 2012). Reactions were cycled on an Applied Biosystems 7500 Real Time PCR system under the following conditions: 50°C, 2min; 95°C, 1min; 40 cycles of 95°C, 15sec and 60°C, 1min. The results were

normalized to 18S and the relative expression was calculated using the ΔΔCt method (Livak and Schmittgen, 2001).

5 Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis was performed by two way analysis of variance (ANOVA). P<0.05 was considered to be significant and noted '*'; P values of <0.01 and <0.001 were noted as '**' and '***' respectively.

6 Conclusions

In conclusion, this study has profiled the temporal expression patterns of members of the MMP and TIMP families of proteins during osteoblast differentiation. This approach has identified several members of both families as potentially key regulators of ECM remodelling necessary for osteoblast – osteocyte transition.

Acknowledgements

The authors wish to acknowledge Elaine Seawright (Roslin Institute, University of Edinburgh) for her technical assistance. This project was funded by the Biotechnology and Biological Sciences Research Council (BBSRC).

References

Atkins, G.J., K.J. Welldon, P. Halbout, and D.M. Findlay. 2009a. Strontium ranelate treatment of human primary osteoblasts promotes an osteocyte-like phenotype while eliciting an osteoprotegerin response. *Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA*. 20:653-664.

Atkins, G.J., K.J. Welldon, A.R. Wijenayaka, L.F. Bonewald, and D.M. Findlay. 2009b. Vitamin K promotes mineralization, osteoblast-to-osteocyte transition, and an anticatabolic phenotype by {gamma}-carboxylation-dependent and -independent mechanisms. *American journal of physiology. Cell physiology*. 297:C1358-1367.

Barragan-Adjemian, C., D. Nicolella, V. Dusevich, M.R. Dallas, J.D. Eick, and L.F. Bonewald. 2006. Mechanism by which MLO-A5 late osteoblasts/early osteocytes mineralize in culture: similarities with mineralization of lamellar bone. *Calcified tissue international*. 79:340-353.

Bernal, F., H.P. Hartung, and B.C. Kieseier. 2005. Tissue mRNA expression in rat of newly described matrix metalloproteinases. *Biological research*. 38:267-271.

Blaber, E.A., N. Dvorochkin, C. Lee, J.S. Alwood, R. Yousuf, P. Pianetta, R.K. Globus, B.P. Burns, and E.A. Almeida. 2013. Microgravity induces pelvic bone loss through osteoclastic activity, osteocytic osteolysis, and osteoblastic cell cycle inhibition by CDKN1a/p21. *PloS one*. 8:e61372.

Bonewald, L.F. 2002. Osteocytes: a proposed multifunctional bone cell. *Journal of musculoskeletal & neuronal interactions*. 2:239-241.

Bord, S., A. Horner, C.A. Beeton, R.M. Hembry, and J.E. Compston. 1999. Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) distribution in normal and pathological human bone. *Bone*. 24:229-235.

Clancy, B.M., J.D. Johnson, A.J. Lambert, S. Rezvankhah, A. Wong, C. Resmini, J.L. Feldman, S. Leppanen, and D.D. Pittman. 2003. A gene expression profile for endochondral bone formation: oligonucleotide microarrays establish novel connections between known genes and BMP-2-induced bone formation in mouse quadriceps. *Bone*. 33:46-63.

Dallas, S.L., M. Prideaux, and L.F. Bonewald. 2013. The osteocyte: an endocrine cell ... and more. *Endocrine reviews*. 34:658-690.

Eisenach, P.A., C. Roghi, M. Fogarasi, G. Murphy, and W.R. English. 2010. MT1-MMP regulates VEGF-A expression through a complex with VEGFR-2 and Src. *Journal of cell science*. 123:4182-4193.

Filanti, C., G.R. Dickson, D. Di Martino, V. Ulivi, C. Sanguineti, P. Romano, C. Palermo, and P. Manduca. 2000. The expression of metalloproteinase-2, -9, and -14 and of tissue inhibitors-1 and -2 is developmentally modulated during osteogenesis in vitro, the mature osteoblastic phenotype expressing metalloproteinase-14. *J Bone Miner Res*. 15:2154-2168.

Franz-Odendaal, T.A., B.K. Hall, and P.E. Witten. 2006. Buried alive: how osteoblasts become osteocytes. *Developmental dynamics : an official publication of the American Association of Anatomists*. 235:176-190.

Hatori, K., Y. Sasano, I. Takahashi, S. Kamakura, M. Kagayama, and K. Sasaki. 2004. Osteoblasts and osteocytes express MMP2 and -8 and TIMP1, -2, and -3 along with extracellular matrix molecules during appositional bone formation. *The anatomical record. Part A, Discoveries in molecular, cellular, and evolutionary biology*. 277:262-271.

Holmbeck, K., P. Bianco, J. Caterina, S. Yamada, M. Kromer, S.A. Kuznetsov, M. Mankani, P.G. Robey, A.R. Poole, I. Pidoux, J.M. Ward, and H. Birkedal-Hansen. 1999. MT1-

MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell*. 99:81-92.

Holmbeck, K., P. Bianco, I. Pidoux, S. Inoue, R.C. Billinghurst, W. Wu, K. Chrysovergis, S. Yamada, H. Birkedal-Hansen, and A.R. Poole. 2005. The metalloproteinase MT1-MMP is required for normal development and maintenance of osteocyte processes in bone. *Journal of cell science*. 118:147-156.

Inoue, K., Y. Mikuni-Takagaki, K. Oikawa, T. Itoh, M. Inada, T. Noguchi, J.S. Park, T. Onodera, S.M. Krane, M. Noda, and S. Itohara. 2006. A crucial role for matrix metalloproteinase 2 in osteocytic canalicular formation and bone metabolism. *The Journal of biological chemistry*. 281:33814-33824.

Irie, K., S. Ejiri, Y. Sakakura, T. Shibui, and T. Yajima. 2008. Matrix mineralization as a trigger for osteocyte maturation. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*. 56:561-567.

Itoh, Y., and M. Seiki. 2006. MT1-MMP: a potent modifier of pericellular microenvironment. *Journal of cellular physiology*. 206:1-8.

Joronen, K., H. Salminen, V. Glumoff, M. Savontaus, and E. Vuorio. 2000. Temporospatial expression of tissue inhibitors of matrix metalloproteinases-1, -2 and -3 during development, growth and aging of the mouse skeleton. *Histochemistry and cell biology*. 114:157-165.

Karelina, T.V., G.I. Goldberg, and A.Z. Eisen. 1995. Matrix metalloproteinases in blood vessel development in human fetal skin and in cutaneous tumors. *The Journal of investigative dermatology*. 105:411-417.

Karsdal, M.A., T.A. Andersen, L. Bonewald, and C. Christiansen. 2004. Matrix metalloproteinases (MMPs) safeguard osteoblasts from apoptosis during transdifferentiation into osteocytes: MT1-MMP maintains osteocyte viability. *DNA and cell biology*. 23:155-165.

Kato, T., S. Kameoka, T. Kimura, T. Nishikawa, and T. Kasajima. 2001a. Angiogenesis and blood vessel invasion as prognostic indicators for node-negative breast cancer. *Breast cancer research and treatment*. 65:203-215.

Kato, Y., A. Boskey, L. Spevak, M. Dallas, M. Hori, and L.F. Bonewald. 2001b. Establishment of an osteoid preosteocyte-like cell MLO-A5 that spontaneously mineralizes in culture. *J Bone Miner Res*. 16:1622-1633.

Krane, S.M. 1994. Clinical importance of metalloproteinases and their inhibitors. *Annals of the New York Academy of Sciences*. 732:1-10.

Kulkarni, R.N., A.D. Bakker, E.V. Gruber, T.D. Chae, J.B. Veldkamp, J. Klein-Nulend, and V. Everts. 2012. MT1-MMP modulates the mechanosensitivity of osteocytes. *Biochemical and biophysical research communications*. 417:824-829.

Livak, K.J., and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 25:402-408.

Maes, C., T. Kobayashi, M.K. Selig, S. Torrekens, S.I. Roth, S. Mackem, G. Carmeliet, and H.M. Kronenberg. 2010. Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. *Developmental cell*. 19:329-344.

Manduca, P., A. Castagnino, D. Lombardini, S. Marchisio, S. Soldano, V. Ulivi, S. Zanotti, C. Garbi, N. Ferrari, and D. Palmieri. 2009. Role of MT1-MMP in the osteogenic differentiation. *Bone*. 44:251-265.

Mikuni-Takagaki, Y., Y. Kakai, M. Satoyoshi, E. Kawano, Y. Suzuki, T. Kawase, and S. Saito. 1995. Matrix mineralization and the differentiation of osteocyte-like cells in culture. *J Bone Miner Res*. 10:231-242.

Mosig, R.A., O. Dowling, A. DiFeo, M.C. Ramirez, I.C. Parker, E. Abe, J. Diouri, A.A. Aqeel, J.D. Wylie, S.A. Oblander, J. Madri, P. Bianco, S.S. Apte, M. Zaidi, S.B. Doty, R.J. Majeska, M.B. Schaffler, and J.A. Martignetti. 2007. Loss of MMP-2 disrupts skeletal and craniofacial development and results in decreased bone mineralization, joint erosion and defects in osteoblast and osteoclast growth. *Human molecular genetics*. 16:1113-1123.

Nagase, H., R. Visse, and G. Murphy. 2006. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovascular research*. 69:562-573.

Nakano, Y., J. Forsprecher, and M.T. Kaartinen. 2010. Regulation of ATPase activity of transglutaminase 2 by MT1-MMP: implications for mineralization of MC3T3-E1 osteoblast cultures. *Journal of cellular physiology*. 223:260-269.

Nefussi, J.R., J.M. Sautier, V. Nicolas, and N. Forest. 1991. How osteoblasts become osteocytes: a decreasing matrix forming process. *Journal de biologie buccale*. 19:75-82.

Newton, P.T., K.A. Staines, L. Spevak, A.L. Boskey, C.C. Teixeira, V.E. Macrae, A.E. Canfield, and C. Farquharson. 2012. Chondrogenic ATDC5 cells: An optimised model for rapid and physiological matrix mineralisation. *International journal of molecular medicine*. 30:1187-1193.

Page-McCaw, A. 2008. Remodeling the model organism: matrix metalloproteinase functions in invertebrates. *Seminars in cell & developmental biology*. 19:14-23.

Paic, F., J.C. Igwe, R. Nori, M.S. Kronenberg, T. Franceschetti, P. Harrington, L. Kuo, D.G. Shin, D.W. Rowe, S.E. Harris, and I. Kalajzic. 2009. Identification of differentially expressed genes between osteoblasts and osteocytes. *Bone*. 45:682-692.

Palumbo, C., M. Ferretti, and G. Marotti. 2004. Osteocyte dendrogenesis in static and dynamic bone formation: an ultrastructural study. *The anatomical record. Part A, Discoveries in molecular, cellular, and evolutionary biology*. 278:474-480.

Pendas, A.M., A.R. Folgueras, E. Llano, J. Caterina, F. Frerard, F. Rodriguez, A. Astudillo, A. Noel, H. Birkedal-Hansen, and C. Lopez-Otin. 2004. Diet-induced obesity and reduced skin cancer susceptibility in matrix metalloproteinase 19-deficient mice. *Molecular and cellular biology*. 24:5304-5313.

Pitsillides, A.A., S.C. Rawlinson, R.F. Suswillo, S. Bourrin, G. Zaman, and L.E. Lanyon. 1995. Mechanical strain-induced NO production by bone cells: a possible role in adaptive bone (re)modeling? *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 9:1614-1622.

Prideaux, M., N. Loveridge, A.A. Pitsillides, and C. Farquharson. 2012. Extracellular matrix mineralization promotes E11/gp38 glycoprotein expression and drives osteocytic differentiation. *PloS one*. 7:e36786.

Quarles, L.D. 2003. FGF23, PHEX, and MEPE regulation of phosphate homeostasis and skeletal mineralization. *American journal of physiology. Endocrinology and metabolism*. 285:E1-9.

Raza, S.L., and L.A. Cornelius. 2000. Matrix metalloproteinases: pro- and anti-angiogenic activities. *The journal of investigative dermatology. Symposium proceedings / the Society for Investigative Dermatology, Inc. [and] European Society for Dermatological Research*. 5:47-54.

Riley, G.P., V. Curry, J. DeGroot, B. van El, N. Verzijl, B.L. Hazleman, and R.A. Bank. 2002. Matrix metalloproteinase activities and their relationship with collagen remodelling in tendon pathology. *Matrix biology : journal of the International Society for Matrix Biology*. 21:185-195.

Rocha, C.A., T.M. Cestari, H.A. Vidotti, G.F. de Assis, G.P. Garlet, and R. Taga. 2014. Sintered anorganic bone graft increases autocrine expression of VEGF, MMP-2 and MMP-9 during repair of critical-size bone defects. *Journal of molecular histology*. 45:447-461.

Staines, K.A., N.C. Mackenzie, C.E. Clarkin, L. Zelenchuk, P.S. Rowe, V.E. MacRae, and C. Farquharson. 2012. MEPE is a novel regulator of growth plate cartilage mineralization. *Bone*. 51:418-430.

Sternlicht, M.D., and Z. Werb. 2001. How matrix metalloproteinases regulate cell behavior. *Annual review of cell and developmental biology*. 17:463-516.

Tang, S.Y., R.P. Herber, S.P. Ho, and T. Alliston. 2012. Matrix metalloproteinase-13 is required for osteocytic perilacunar remodeling and maintains bone fracture resistance. *J Bone Miner Res*. 27:1936-1950.

Visse, R., and H. Nagase. 2003. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circulation research*. 92:827-839.

Vu, T.H., J.M. Shipley, G. Bergers, J.E. Berger, J.A. Helms, D. Hanahan, S.D. Shapiro, R.M. Senior, and Z. Werb. 1998. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell*. 93:411-422.

Wilkinson, J.M., R.K. Davidson, T.E. Swingler, E.R. Jones, A.N. Corps, P. Johnston, G.P. Riley, A.J. Chojnowski, and I.M. Clark. 2012. MMP-14 and MMP-2 are key metalloproteases in Dupuytren's disease fibroblast-mediated contraction. *Biochimica et biophysica acta*. 1822:897-905.

Zaman, G., L.K. Saxon, A. Sunters, H. Hilton, P. Underhill, D. Williams, J.S. Price, and L.E. Lanyon. 2010. Loading-related regulation of gene expression in bone in the contexts of estrogen deficiency, lack of estrogen receptor alpha and disuse. *Bone*. 46:628-642.

Zhou, Z., S.S. Apte, R. Soininen, R. Cao, G.Y. Baaklini, R.W. Rauser, J. Wang, Y. Cao, and K. Tryggvason. 2000. Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I. *Proceedings of the National Academy of Sciences of the United States of America*. 97:4052-4057.

Figure Legends

Figure 1. Alizarin red staining **(A)** and relative quantification of absorbance of eluted alizarin red staining **(B)** in mineralising MLO-A5 cells cultured with 100µg/ml ascorbic acid ± 5mM β-glycerophosphate. Data are represented as mean ± S.E.M. of 3 individual cell cultures. ‘a’ = P<0.001*** in comparison to ascorbic acid only cultures.

Figure 2. Effects of inhibition of mineralisation on *Mmp* expression in MLO-A5 cell cultures. RT-qPCR analysis of **(A)** *Mmp2* **(B)** *Mmp14* **(C)** *Mmp19* **(D)** *Mmp23* **(E)** *Mmp28* **(F)** *Mmp11* mRNA expression in mineralising MLO-A5 cells ± βGP over a 9-day culture period. Data are represented as mean ± S.E.M. of 5 individual cell cultures. P<0.05*, P<0.01**, P<0.001***. ‘a’ = P<0.05* in comparison to day 0 of culture.

Figure 3. Effects of mineralisation inhibition on *Timp* expression in MLO-A5 cell cultures. RT-qPCR analysis of **(A)** *Timp1* **(B)** *Timp2* **(C)** *Timp3* mRNA expression in mineralising MLO-A5 cells ± βGP over a 9-day culture period. Data are represented as mean ± S.E.M. of 5 individual cell cultures. P<0.001***. ‘a’ = P<0.05* in comparison to day 0 of culture.

Figure 4. Hypothetical schematic representation of the temporal expression of *Mmp* and *Timp* mRNA during differentiation and matrix mineralisation of MLO-A5 cells.