



MMP and TIMP temporal gene expression during osteocytogenesis

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ABSTRACT

Osteocytes within bone differentiate from osteoblast precursors which reside in a mineralised extracellular matrix (ECM). Fully differentiated osteocytes are critical for bone development and function but the factors that regulate this differentiation process are unknown. The enzymes primarily responsible for ECM remodelling are matrix metalloproteinases (MMP); however, the expression and role of MMPs during osteocytogenesis is undefined. Here we used MLO-A5 cells to determine the temporal gene expressions of the MMP family and their endogenous inhibitors – tissue inhibitors of metalloproteinases (TIMPs) during osteocytogenesis. RT-qPCR revealed expression of 14 *Mmps* and 3 *Timps* in MLO-A5 cells. *Mmp2*, *Mmp23* and *Mmp28* were decreased concurrent with mineralisation onset ($P < 0.05^*$). *Mmp14* and *Mmp19* mRNAs were also significantly increased at day 3 ($P < 0.05^*$) before returning to baseline levels at day 6. Decreased expressions of *Timp1*, *Timp2* and *Timp3* mRNA were observed by day 6 compared to day 0 ($P < 0.05^*$). To examine whether these changes are linked to osteocytogenesis, we determined *Mmp/Timp* mRNA expressions in mineralisation-limited conditions. RT-qPCR revealed that the previously observed decreases in *Mmp2*, *Mmp23* and *Mmp28* were not observed in these mineralisation-limited cultures, therefore closely linking these MMPs with osteocyte differentiation. Similarly, we found differential expression of *Timp1*, *Timp2* and *Timp3* mRNA in mineralisation-restricted cultures ($P < 0.05^*$). In conclusion, we have identified several members of the MMP/TIMP families as regulators of ECM remodelling necessary for the acquisition of the osteocyte phenotype.

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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 (Bonewald, 2002; Franz-Odenaal et al., 2006; Pitsillides et al., 1995; Quarles, 2003). 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 (Franz-Odenaal et al., 2006; Nefussi et al., 1991; Palumbo et al., 2004). 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-Odenaal 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, 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 pro-enzymes and are activated in the tissue through the cleavage of pro-peptide (Krane, 1994).

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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 (Eisenach et al., 2010; Karelina et al., 1995; Raza and Cornelius, 2000). Elegant studies in mice, 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, 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 (Blaber et al., 2013; Holmbeck et al., 2005; Inoue et al., 2006; Itoh and Seiki, 2006; Mosig et al., 2007; Rocha et al., 2014; Tang et al., 2012; Zhou et al., 2000). 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 (Barragan-Adjemian et al., 2006; Kato et al., 2001b; Prideaux et al., 2012). Our studies identify several members of both families as potentially key regulators of ECM remodelling necessary for osteoblast–osteocyte transition.

1. Results

1.1. Matrix mineralisation in MLO-A5 cells

MLO-A5 cells cultured in the presence of ascorbic acid (AA) and β -glycerophosphate (β GP) 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, in comparison to AA only treated cells, from day 7 of culture onwards ($P < 0.001$; Fig. 1A and B, E and F). Despite the lack of mineral formation in MLO-A5 cells treated with AA only, these cells were still healthy and produced a matrix similar to that observed by the cells treated with both AA and β GP (Fig. 1C and D).

These data confirm our previous results with this cell line (Prideaux et al., 2012) in which we found that MLO-A5 cells cultured with AA and β GP progressively expressed osteocyte-selective markers such as *E11*, *Dmp1*, *Sost* and *Cd44*, however expression of these was significantly decreased in the AA only cultures (Prideaux et al., 2012). This therefore suggests that matrix mineralisation is required for osteoblast-to-osteocyte transition and as such confirms the suitability of this model to report the gene expression patterns of *Mmps* and *Timps* during late-stage osteoblast differentiation and osteocytogenesis.

1.2. 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 *Mmps*, 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 and 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 and 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).

1.3. 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 GP there was a marked ablation of matrix mineralisation by MLO-A5 cells over the 10 day culture period ($P < 0.001$) (Fig. 1A and 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 and 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 are 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 GP and, unlike MLO-A5 cells in differentiating medium, these remained elevated throughout the culture period ($P < 0.05$) (Fig. 2B, C and F). Interestingly, our studies also found that the expression of *Timp3* mRNA was also affected by the omission of β GP 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 expressions of *Timp1* and *Timp2* were also seen in the mineralisation-inhibited cultures after 9 days, compared to the control cultures (Fig. 3A and B).

2. 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 *Timps* 1–3, 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 GP 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

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