Co-localisation of intra-nuclear membrane type-1 matrix metalloproteinase and hypoxia inducible factor-2α in osteosarcoma and prostate carcinoma cells

COREY D. CHAN[1], EMMA J. HAAGENSEN[2], HAYEIT A. TENSAOUT[3], KATHERINE J. RENNIE[3], ZAKAREYA GAMIE[2], JAMES BARRY[2], MARK A. BIRCH[4], CRAIG H. GERRAND[5], SOHAIL NISAR[2], CRAIG N. ROBSON[2], JOHN LUNEC[2] and KENNETH S. RANKIN[2]

[1]Translational and Clinical Research Institute, Newcastle University Faculty of Medical Sciences, Newcastle upon Tyne NE2 4HH; [2]Northern Institute for Cancer Research, University of Newcastle upon Tyne; [3]Institute of Cellular Medicine, Musculoskeletal Research Group, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH; [4]School of Clinical Medicine, Addenbrooke's Hospital, Cambridge University, Cambridge CB2 2QQ; [5]Royal National Orthopaedic Hospital, Brockley Hill, Stanmore, Middlesex HA7 4LP, UK

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Abstract. Increased membrane type-1 matrix metalloproteinase (MT1-MMP) expression in osteosarcoma is predictive of poor prognosis and directs bone metastasis in prostate carcinoma. MT1-MMP subcellular localisation varies with oxygen tension, and, therefore, the aim of the present study was to assess protein interactions between MT1-MMP and the hypoxia inducible factors (HIF-1α and HIF-2α). MT1-MMP protein expression was investigated across a panel of cancer cell lines, including a positive and negative control. The hypoxia-induced alteration in subcellular location of MT1-MMP, HIF-1α and HIF-2α in the U2OS osteosarcoma cell line was assessed using subcellular fractionation. A proximity ligation assay was utilised to assess protein to protein interactions in the osteosarcoma U2OS and prostate carcinoma PC3 cell lines. U2OS and PC3 cells exhibited a significantly increased intra-nuclear interaction between MT1-MMP and HIF-2α in response to hypoxia. The role of this warrants further investigation as it may unveil novel opportunities to target MT1-MMP, which is of particular significance for osteosarcoma since current treatment options are limited.

Introduction

Osteosarcoma is the most prevalent malignant primary bone tumour in children, teenagers, and young adults (1). Due to limited novel therapeutic options, the five-year survival rate is 60-70% and has not improved over the last 30 years (2). Molecular mechanisms underlying osteosarcoma disease progression are unclear, however membrane type-1 matrix metalloproteinase (MT1-MMP; MMP-14) is likely to play a key role. MT1-MMP has been shown to direct cancer cell invasion in a wide variety of carcinomas and directs bone metastasis in prostate carcinoma (3,4). Due to their mesenchymal origin, HT1080 fibrosarcoma cells overexpress MT1-MMP which makes them useful as a positive control when studying the role of MT1-MMP in sarcoma and carcinoma. In osteosarcoma, overexpression of MT1-MMP in tumour tissues has been shown to correlate with poor survival (5), however the potentially important relevance of this proteinase to cancer progression in bone sarcoma has not been extensively studied. MT1-MMP is an important member of the matrix metalloproteinase family. As a zinc dependent endopeptidase that contains a transmembrane domain, its role in remodelling the extracellular matrix when localised to the cell surface membrane has been subject to extensive research (6). However, the cytoplasmic tail of MT1-MMP has been reported to regulate HIF-1α expression in cancer cells, suggesting that this protein has an additional intracellular function (7). Therefore, the aim of this study was to investigate the expression of MT1-MMP in osteosarcoma and prostate carcinoma cell lines in response to hypoxia and also to determine a potential protein interaction between MT1-MMP and the hypoxic inducible factors 1α and 2α.

Materials and methods

Cell culture. HT1080, U2OS, MDA-MB-231, MCF-7, PC3 and LNCaP cell lines were purchased from American Type Culture Collection (ATCC). The two sarcoma cell lines (U2OS and HT1080), two breast cancer cell lines (MDA-MB-231 and MCF-7) and two prostate carcinoma cell line (PC3 and LNCaP) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamate, 100 U/ml penicillin, and 10 μg/ml streptomycin. The human mesenchymal stem cell (MSCs) were

Correspondence to: Dr. Corey D. Chan, Translational and Clinical Research Institute, Newcastle University Faculty of Medical Sciences, Framlington Place, Newcastle upon Tyne NE2 4HH, UK
E-mail: corey.chan@newcastle.ac.uk

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grown in α-minimal essential medium (MEM), supplemented with 20% FBS, 5% L-glutamine and 8 ng/ml of basic fibroblast growth factor (bFGF).

MSC derivation and culture. Primary MSC cultures were established from 10 individuals aged between 36 and 75 years, undergoing hip replacement surgery for osteoarthritis. Samples were collected following appropriate consent and according to approval given by the Newcastle and North Tyneside 1 Research Ethics Committee (REC Reference no. 17/NE/0361) and processed within 24 h of surgery. Trabecular bone fragments were dissected out from the femoral head and processed over a 1.077 g/ml Lymphoprep™ density gradient medium (StemCell Technologies). Mononuclear cells including putative MSC were visible as an opaque band at the Lymphoprep™-marrow interphase. The mononuclear band was washed in MSC wash buffer (5 mM EDTA/0.2% BSA/1% penicillin-streptomycin) and resuspended in low glucose DMEM (1,000 mg/ml) (Sigma-Aldrich; Merck KGaA) reconstituted with 20% fetal calf serum (Gibco®; Life Technologies), 1% L-glutamine (Sigma-Aldrich; Merck KGaA) and 1% penicillin-streptomycin (Sigma-Aldrich; Merck KGaA). Healthy MSC adhere to plastic within 24 h. At this point the cells were washed with MSC wash buffer to avoid contaminants and fresh media was added, with the addition of bFGF (Gibco®; Life Technologies) at 8 ng/ml. MSC were used between passages 2-5 and within 40 days following surgery.

MSC characterisation. Cells were removed from the T75cm^3 flask with 2 ml Trypsin/EDTA, transferred into a universal container and diluted up to 20 ml with PBS. The cells were centrifuged at 1,100 rpm and the supernatant removed without disturbing the cell pellet. The cells were resuspended in 20 ml PBS and centrifuged again at 1,100 rpm. The supernatant was removed and the cells resuspended in 3 ml of flow cytometry buffer (500 ml PBS, 2.5 ml of 0.2 mmol/l EDTA prepared from anhydrous stock, 2.5 ml of MACS BSA Stock Solution) and transferred to a flow cytometry tube. The cells were centrifuged at 1,100 rpm, the supernatant was removed and the cells were resuspended in 100 μl of flow cytometry buffer. Cells were then blocked by adding 5 μl human IgG at room temperature for 10 min. Cells were then stained for MSC markers using the Miltenyi Human MSC Phenotyping Kit (130-125-285; Miltenyi Biotec Ltd.) by adding 5 μl of the MSC antibody cocktail at room temperature for 30 min. The cells were then washed twice by adding 3 ml flow cytometry buffer and centrifuging at 1,100 rpm. The cells were then run through a BD FACSCanto II flow cytometer and the flow cytometry plots analysed using FACSDiva software.

MSC differentiation assays. For all the below protocols, duplicate cells were grown in MSC Growth Medium 2 (C-28009; Merck Life Science UK, Ltd.) only in order to act as negative controls.

Adipogenesis differentiation. MSCs were grown in MSC Growth Medium 2 (C-28009; Merck Life Science UK, Ltd.) for up to 2 days in 6-well plates and allowed to reach 80-90% confluency. The cells were then induced with MSC Adipogenic Differentiation Medium 2 (C-28016; Merck Life Science UK, Ltd.) for 14 days. The medium was changed every third day taking care not to disturb the cell monolayer. The cells were gently washed with 1X PBS. The cells were then fixed with 4% paraformaldehyde for 30 min at room temperature and then washed twice with distilled water. The distilled water was aspirated and enough 60% isopropanol was added to cover the cell monolayer. The cells were incubated at room temperature for 5 min. The 60% isopropanol was aspirated and enough Oil Red O staining solution (O1391; Merck Life Science UK, Ltd.) was added to cover the cell monolayer. The cells were incubated at room temperature for 15 min. The staining solution was carefully aspirated and the cells washed several times with distilled water until the water became clear. The plate was blotted upside down on a paper towel to remove as much water as possible. The cells were covered with PBS and promptly analysed as the dye tends to fade upon prolonged light exposure. Intracellular lipid vesicles in mature adipocytes stained bright red and were photographed using a light microscope.

Osteogenesis differentiation. MSCs were grown in MSC osteogenic differentiation medium (C-28013; Merck Life Science UK, Ltd.) for 14 days in 24-well plates. The cells were removed from the incubator and the medium aspirated. The cells were gently washed with 1X phosphate buffered saline (PBS). The cells were then fixed with 4% paraformaldehyde for 30 min at room temperature and then washed with 1X PBS followed by a further wash with PBS Tween (1X PBS with 0.05% Tween-20). The PBS Tween was aspirated and the cells stained for alkaline phosphatase with 0.4 ml per well of StemTAG™ AP solution (CB-306; Cell Biolabs). Following incubation at room temperature for 30 min in the dark, the solution was aspirated and the cells washed twice with 1X PBS. The cells were then photographed under a light microscope.

Chondrogenesis differentiation. MSCs were grown in a 96-well U-bottom suspension culture plate for 2 days to allow spheroid formation. The spheroids were induced with Chondrogenic Differentiation Medium (C-28012; Merck Life Science UK, Ltd.) and incubated for 21 days. The medium was changed every 3 days. The cells were gently washed with 1X phosphate buffered saline (PBS). The cells were then fixed with 4% paraformaldehyde for 30 min at room temperature and then twice with washed distilled water. Immediately before use, Alcian Blue staining solution (TMS010; Merck Life Science UK, Ltd.) was passed through a 0.22 μm Millex PES filter. The distilled water was aspirated and enough filtered Alcian Blue staining solution was added to generously cover the cartilage spheroids. The cells were incubated in the dark for 45 min at room temperature. The Alcian Blue staining solution was aspirated and the cartilage spheroids washed with the destaining solution for 10 min. The wash step was repeated twice with distilled water. The spheroids were covered in PBS and photographed with a light microscope.

Hypoxia and normoxia treatment. Cells undergoing hypoxic treatment were grown in a 37˚C incubator set to maintain a hypoxic atmosphere of 1% O_2, 5% CO_2, and 94% N_2 by controlled injection of nitrogen. The normoxic condition was met with standard cell culture conditions (20% O_2 and 5% CO_2).

Antibodies. The same antibodies were used for western blot analysis and for the proximity ligation assay. Note that MT1-MMP is also classified as MMP14. The antibody to the catalytic domain of MT1-MMP was mouse monoclonal anti-MMP-14 clone LEM-2/15.8, MAB3328 (Merck Millipore). Rabbit monoclonal anti-HIF-1α (EP1215Y) and rabbit polyclonal anti-HIF-2α (ab199) were from Abcam. The reference antibodies were as follows: mouse monoclonal anti-GAPDH (MAB374) from

Merck-Millipore and mouse monoclonal anti-Lamin A/C clone 4C11 (4777) from Cell Signaling Technology.

Western blot analysis. Cells were harvested into lysis buffer [0.1 M Tris, pH 7.5-4 M NaCl-10 mM Na_3VO_4, pH 10 (phosphatase inhibitor) 1% TX-100-1 mM DTT-1 mM PMSF - 1:50 protease inhibitors cocktail (Sigma-Aldrich; Merck KGaA)]. Protein concentration was determined with a Bradford assay (Thermo Fisher Scientific, Inc.). The protein concentrations were equalized with lysis buffer as required. Total protein was subjected to SDS-PAGE using 10% polyacrylamide gels and transferred to a polyvinylidene fluoride (PVDF) membrane (Merck-Millipore). Secondary antibodies were polyclonal goat anti-mouse (for MT1-MMP) or anti-rabbit (for HIF-1α and 2α) IgG/HRP (horseradish peroxidase conjugated). Following the addition of the developer Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore), protein bands were visualised using Bio-Rad ChemiDoc Imaging System and analysed using Fiji ImageJ software.

Subcellular protein extraction and fractionation. In order to analyse protein subcellular localisation, cytoplasmic and nuclear fractions were isolated from U2OS cells using the subcellular fractionation kit catalogue no. 78840 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Exponentially growing cells were exposed to 1% oxygen (hypoxia) or 20% oxygen (normoxia) for 48 h. The cytoplasmic and nuclear fractions were separated by SDS-PAGE and analyzed by western immunoblotting with the mouse anti-MMP-14 and rabbit anti-HIF-1α, anti-HIF-2α antibodies as described above.

Proximity ligation assay (PLA). The *in situ* Proximity Ligation Assay (PLA) was performed using the Duolink II Green kit according to the manufacturer's instructions (Olink Bioscience). Cells were cultured in 8 well Lab-Tek chamber slides (Thermo Fisher Scientific, Inc.). Exponentially growing cells were then exposed to 1% oxygen (hypoxia) or 20% oxygen (normoxia) for 48 h. Following cell culture, media were removed and wells were washed with ice cold PBS/Tween (0.01%). Cells were fixed with 4% paraformaldehyde and blocked with goat serum solution (Sigma-Aldrich; Merck KGaA) at room temperature for 20 min. The slides were then incubated overnight at 4˚C with antibodies diluted in goat serum at 1:50. The reaction with probes, ligation, amplification, and detection were performed according to the manufacturer's instructions. The slides were mounted using Duolink *in Situ* Mounting Medium with DAPI (Olink) and then analysed using a laser scanning confocal microscope (Leica Lasertechnik). A 63X 1.4NA oil objective and sequential scanning with filters 360-460 nm for Dapi (blue) and 495-527 nm for FITC (green) were used.

Representative results and averaged quantitative values are shown from experiments repeated three times. To quantify the signal dots representative of protein interaction between MT1-MMP and HIF-1α or HIF-2α, the number and location (cytoplasmic or nuclear) of the dots were assessed using Volocity 3D Image analysis software (Perkin Elmer).

Immunofluorescence and confocal microscopy on patient sarcoma tissue. Sections were cut from the patient specimen Formalin-fixed paraffin-embedded (FFPE) tissue block to a thickness of 3 μm using a rotary microtome, floated on distilled water and mounted on electrostatically charged microscope slides. Slides were dried in an incubator at 37˚C overnight and left to cool at room temperature for 30 min before initiating the staining procedure. For antigen retrieval the slides were loaded into glass slide holders and dewaxed in 100% xylene (Fisher Scientific UK) followed by progressively decreasing quantities of ethanol (BDH; Poole) from 100% down to 50% and then distilled water. Antigen retrieval was then performed in citrate buffer in a decloaking chamber for 30 min at 125˚C. Slides were rinsed in running water and blocked in 4% BSA/PBS for 1 h. The MT1-MMP primary antibodies (mouse monoclonal anti-MMP-14 clone LEM-2/15.8, MAB3328 (Merck Millipore and rabbit polyclonal anti-HIF-2α (ab199) Abcam) were diluted to 1 in 50 in 4% BSA/PBS. Slides were subsequently incubated at 4˚C with the primary antibody overnight. The secondary antibodies [Invitrogen Alexa Fluor® 594 goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, A-11005 and goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 488, A-11034] were diluted to 1 in 100 in 4% BSA/PBS and added to the slide for further incubation at room temperature for 30 min in the dark. Nuclei were mounted and counterstained using Vectashield mounting medium with DAPI (Vector Laboratories) and visualised on a Leica SP2 confocal scanning microscope (Leica Lasertechnik).

Statistical analysis. A single comparison between 20% oxygen versus 1% oxygen was statistically analysed following the PLA to compare intranuclear interaction. The number and location of the signal dots were analysed and compared using the one sample Student's t-test (SPSS version 21). For ease of presentation the graph was generated with percentages on the y-axis.

Results

Protein expression of MT1-MMP in a panel of cell lines and subcellular localisation of MT1-MMP in the U2OS line. MT1-MMP expression in total cell lysate was assessed using western blotting in a panel of cell lines which included the HT1080 fibrosarcoma line, the U2OS osteosarcoma line, two breast carcinoma lines (MDA-MB-231 and MCF-7), two prostate carcinoma lines (PC3 and LNCaP) and a bone marrow derived mesenchymal stem cell population (Fig. 1A). The blot demonstrates high expression of MT1-MMP in the HT1080 cell line (positive control) and an absence of MT1-MMP protein expression in the MCF-7 line (negative control). A differing degree of MT1-MMP expression was shown across the rest of the cell lines in the panel, with the active 57 kDa form present in HT1080, U2OS, MDA-MB-231, PC3 and MSCs. Expression of the inactive 64 kDa pro-peptide is clearly evident in the HT1080 and MDA-MB-231 cell lines but not in U2OS, PC3 or MSCs (Fig. 1A).

Subcellular fraction analysis of the U2OS cell line (Fig. 1B) in 20% oxygen versus 1% oxygen demonstrates increased nuclear localisation of MT1-MMP and HIF-1α in hypoxia. HIF-2α expression is similar in all compartments regardless of oxygen tension.

Control proximity ligation assay. As a control for the proximity ligation assay increased number of foci and increased signal intensity in the nucleus of all three proteins was demonstrated for cells treated with 1% oxygen (Fig. 2). The MT1-MMP

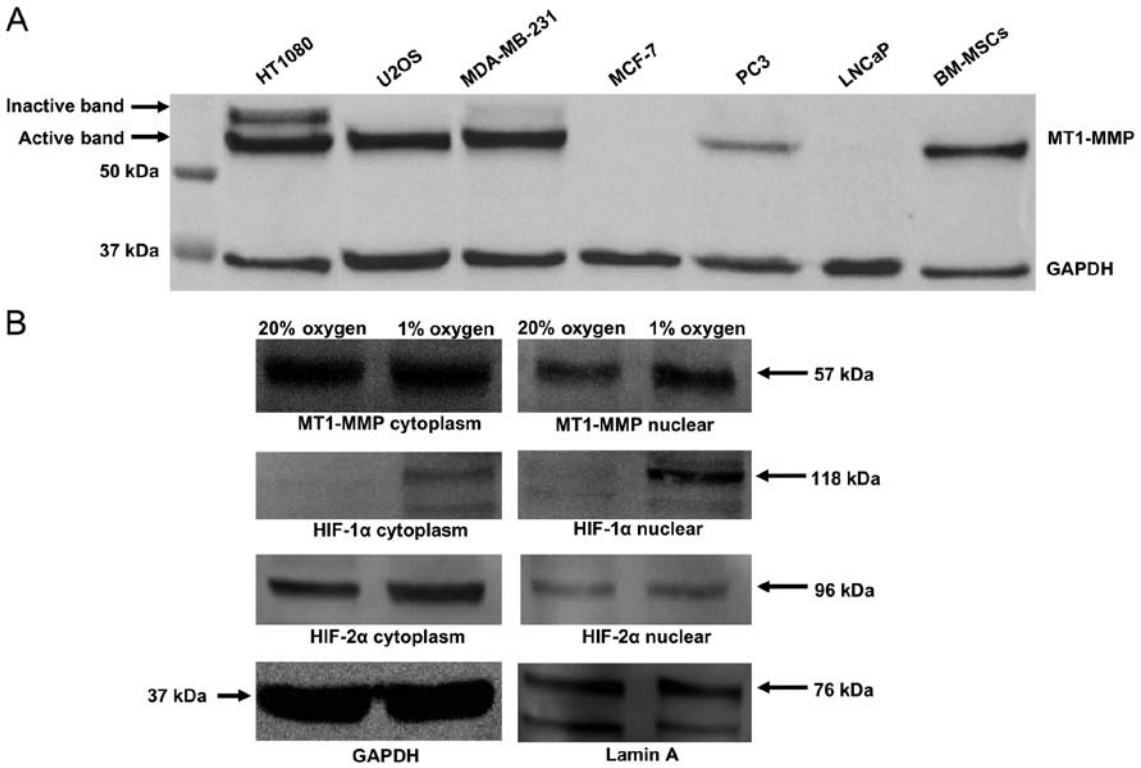

Figure 1. Western blotting to measure MT1-MMP expression in total cell lysate and subcellular fractions. (A) MT1-MMP protein expression in total cell lysate in the fibrosarcoma HT1080 (positive control), osteosarcoma U2OS, breast carcinoma MDA-MB-231 and MCF-7 (negative control), and prostate carcinoma PC3 and LNCaP cell lines and human-derived BM-MSCs. The cancer cell lines in this panel express mainly the active form of MT1-MMP (57 kDa). (B) Subcellular fractionation of U2OS cells treated in 1% oxygen demonstrated increased nuclear expression of MT1-MMP compared with control. BM-MSCs, bone marrow mesenchymal stem cells; HIF, hypoxia-inducible factor; MT1-MMP, membrane type-1 matrix metalloproteinase.

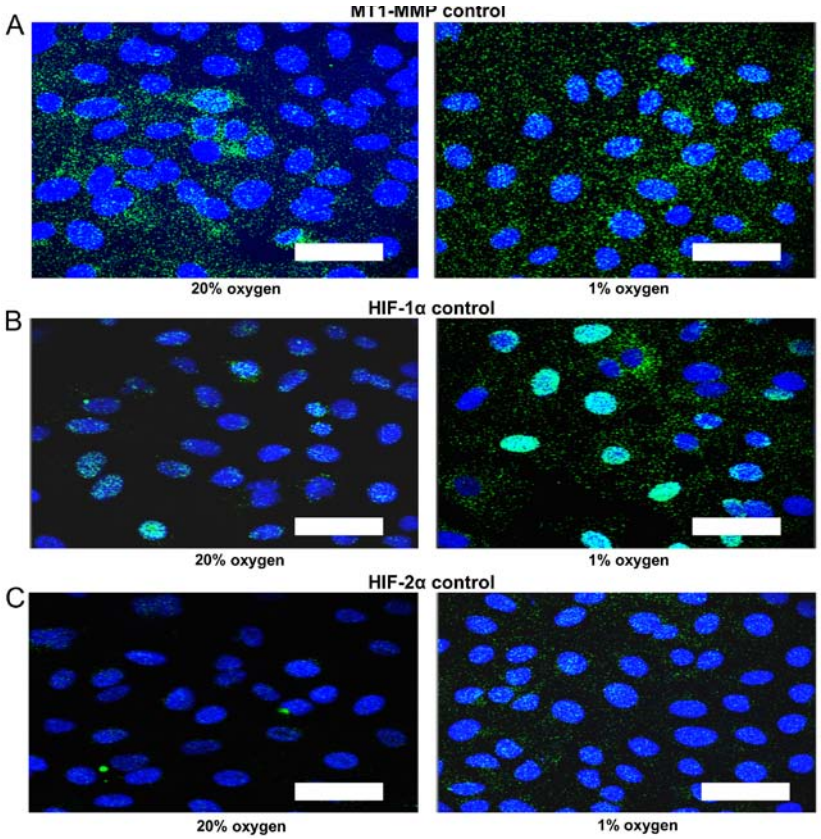

Figure 2. Confocal microscopy of U2OS cells following proximity ligation assays for MT1-MMP, HIF-1α and HIF-2α. (A) Demonstration of intra-nuclear presence of MT1-MMP. (B) There was strong signal for HIF-1α in 1% oxygen. (C) There was a slightly increased signal for HIF-2α in hypoxia. Scale bars, 50 μm. HIF, hypoxia-inducible factor; MT1-MMP, membrane type-1 matrix metalloproteinase.

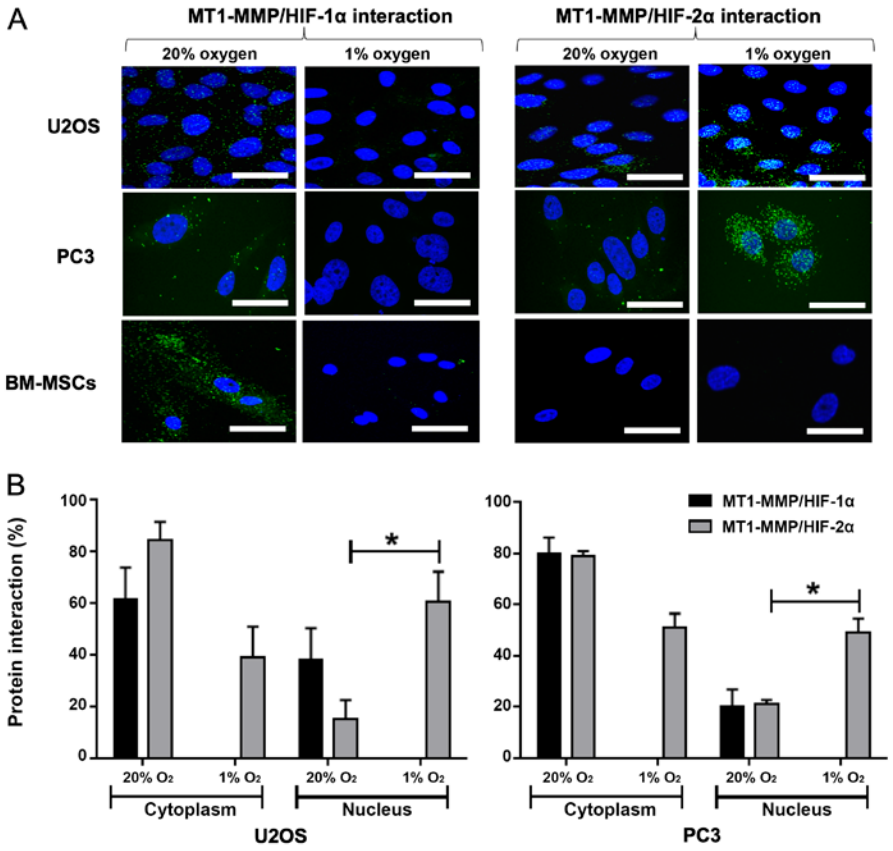

Figure 3. Confocal microscopy following proximity ligation assays to assess protein-protein interactions between MT1-MMP and HIFs in U2OS and PC3 cells with BM-MSCs as a control. (A) There was some interaction between MT1-MMP and HIF-1α in 20% oxygen in both U2OS cells and MSCs. There was no significant interaction between MT1-MMP and HIF-1α in either cell type in 1% oxygen. There was evidence of intra-nuclear interaction between MT1-MMP and HIF-2α in U2OS and PC3 cells in 1% oxygen. Scale bars, 50 μm. Magnification, x63. (B) Intra-nuclear interaction between MT1-MMP and HIF-2α in U2OS and prostate carcinoma PC3 cells treated with 1% oxygen was significant compared with control cells in 20% oxygen. *P=0.040 for U2OS and *P=0.028 for PC3 cells (Student's t-test). BM-MSCs, bone marrow mesenchymal stem cells; HIF, hypoxia-inducible factor; MT1-MMP, membrane type-1 matrix metalloproteinase.

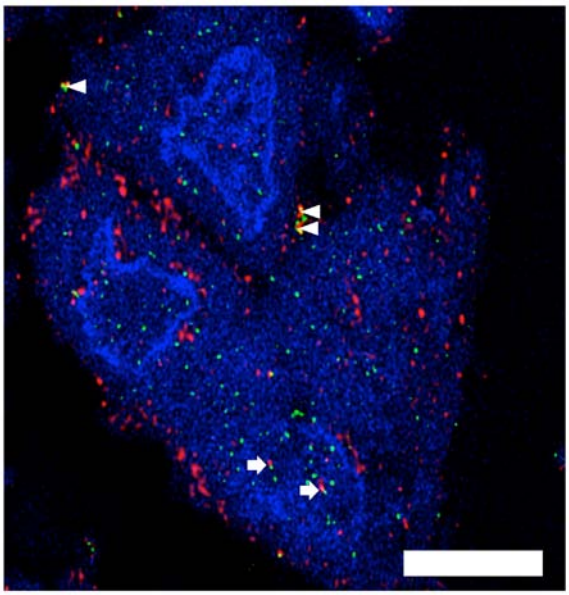

Figure 4. Confirmation of intra-nuclear MT1-MMP in patient tumour cells using immunofluorescence and confocal microscopy with evidence of co-expression of MT1-MMP and HIF-2α. MT1-MMP (red dots) and HIF-2α (green dots) were present in the nucleus and cytoplasm of the sarcoma cells. There was evidence of co-localisation of MT1-MMP and HIF-2α in the nucleus (white arrows) and in the cytoplasm (white arrowheads). Scale bar, 10 μm. HIF, hypoxia-inducible factor; MT1-MMP, membrane type-1 matrix metalloproteinase.

signal is distributed in both cytoplasmic and nuclear compartments and tends to increase in the nuclear compartment in 1% oxygen (Fig. 2A). The intra-nuclear signal is most intense for HIF-1α in 1% oxygen (Fig. 2B). There is also a trend towards an increase in intra-nuclear HIF-2α signal in hypoxia (Fig. 2C).

Proximity ligation assay indicates an interaction between MT1-MMP and HIF-2α in the nucleus in U2OS and PC3 cells. The interaction between MT1-MMP and HIF-1α in 20% oxygen demonstrates signal in the cytoplasm for U2OS, PC3 and MSCs (Fig. 3A). There is no evidence in either cell type of an interaction between MT1-MMP and HIF-1α in 1% oxygen. Furthermore, there is no evidence of interaction between MT1-MMP and HIF-2α in the MSCs regardless of oxygen tension. In U2OS and PC3 cells cultured in 20% oxygen, the signal indicates a low level interaction between MT1-MMP and HIF-2α, mainly within the cytoplasm. Compared with 20% oxygen, the MT1-MMP/HIF-2α interaction in cells cultured in 1% oxygen is markedly increased with a larger number and amplified intensity of interaction signals in the nucleus. Quantitative and statistical analysis using the Student's t-test showed this increase in intra-nuclear interaction to be significant; P=0.040 for U2OS and P=0.028 for PC3 (Fig. 3B).

Confirmation of intra-nuclear MT1-MMP in patient tumour cells using immunofluorescence and confocal microscopy.

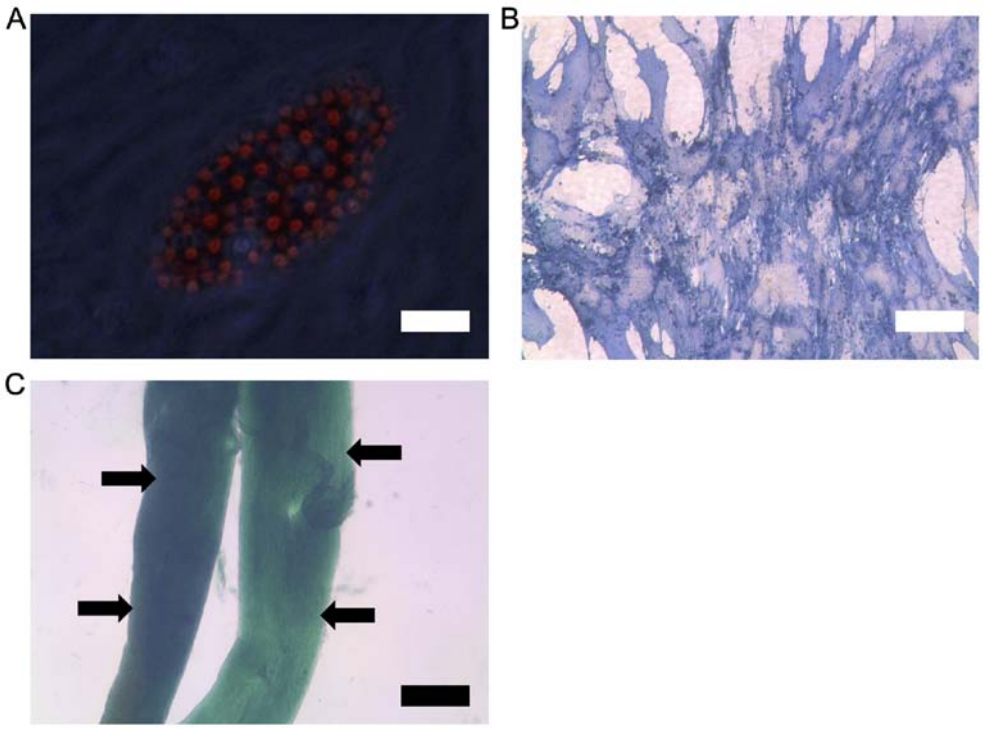

Figure 5. Confirmation of mesenchymal stem cell phenotype using tri-lineage differentiation assay. (A) Adipocyte differentiation was demonstrated by oil red staining of lipid vesicles. Scale bar, 20 μm. (B) Osteoblast differentiation was demonstrated by positive alkaline phosphatase staining (blue). Scale bar, 20 μm. (C) Chondrocyte differentiation was demonstrated by the formation of cartilage layers visualised with Alcianblue staining (black arrows). Scale bar, 50 μm.

A patient sarcoma specimen with evidence of possible intra-nuclear MT1-MMP on conventional immunohistochemistry was selected from our archive. Confocal microscopy demonstrates the presence of MT1-MMP in the nucleus of most of the tumour cells in the representative image along with evidence of MT1-MMP and HIF-2α co-expression in the cytoplasm and the nucleus (Fig. 4).

MSC characterisation using FACS to demonstrate positive surface expression of stem cell markers. FACS data showed that the human derived MSCs had positive surface expression for MSC-associated surface markers CD73, CD90 and CD105 (data readily available upon request).

Confirmation of stem cell phenotype using differentiation assays. The data demonstrates that the human derived MSCs were able to differentiate into adipocytes, osteoblasts and chondrocytes in culture. Successful tri-lineage differentiation was confirmed by the relevant staining for lineage markers followed by microscopy (Fig. 5). From the results, adipocytes show lipid vesicles upon oil red staining (Fig. 5A), osteoblasts show positive staining for alkaline phosphatase (Fig. 5B) and chondrocytes show clear formation of cartilage layers upon Alcian blue staining (Fig. 5C).

Discussion

MT1-MMP has been extensively investigated as a potential target for carcinoma therapy with several extracellular roles described including the facilitation of cancer cell invasion, migration, angiogenesis and metastasis (6,8,9). These features are all relevant to osteosarcoma, particularly as this type of cancer metastasises via the haematogenous route. Whilst the extracellular role of MT1-MMP is important and has been identified as a therapeutic target, the intra-nuclear presence of MT1-MMP has also previously been reported in hepatocellular carcinoma and therefore warrants further investigation (10). Our study confirms the intra-nuclear presence of MT1-MMP and provides evidence that there is a protein-protein interaction with HIF-2α in the nucleus, particularly in osteosarcoma and prostate carcinoma cells under hypoxic conditions. Furthermore, there is evidence that intra-nuclear MT1-MMP in macrophages results in transactivation of gene networks (11). The significance of this could be important because the nuclear presence of MT1-MMP in cancer cells may alter gene expression. Therefore, improved understanding of the intra-nuclear role of MT1-MMP may open up novel avenues for targeting the diverse activities of this enzyme. This is particularly relevant for osteosarcoma which severely lacks additional therapeutic options.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors of this paper contributed significantly to the work stated and in the writing of this manuscript. CDC, EJH, HAT, KJR and KSR drafted the manuscript, designed the study and planned the experiments. CDC, EJH, ZG, JB, MAB and SN were involved in undertaking the majority of the laboratory experiments and data collection, including the imaging and analyses. EJH, ZG, CHG, CNR and JL made substantial contributions to the analysis and interpretation of the data. CDC, CHG, CNR, JL and KSR were involved in writing, processing the figures and editing the manuscript. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Appropriate informed consent was obtained and approved by the Newcastle and North Tyneside 1 Research Ethics Committee (REC Reference no. 17/NE/0361).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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