EUROPEAN JOURNAL OF CANCER 43 (2007) 2747–2756

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Adipogenic, osteogenic and myofibrogenic differentiations of a rat malignant fibrous histiocytoma (MFH)-derived cell line, and a relationship of MFH cells with embryonal mesenchymal, perivascular and bone marrow stem cells

Jyoji Yamate[a,*], *Keiko Ogata*[a], *Takahiro Yuasa*[a], *Mitsuru Kuwamura*[a], *Shigeo Takenaka*[b], *Daijirou Kumagai*[a], *Kazuyuki Itoh*[c], *Jonathan LaMarre*[d]

[a]*Laboratory of Veterinary Pathology, Life and Environmental Sciences, Osaka Prefecture University, Gakuencho 1-1, Sakai, Osaka 599-8531, Japan*
[b]*Laboratory of Molecular Science, Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka, Japan*
[c]*Department of Biology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan*
[d]*Department of Biomedical Sciences, Ontario Veterinary Collage, University of Guelph, Guelph, Ontario, Canada*

ARTICLE INFO

Article history:
Received 9 March 2007
Accepted 9 October 2007

Keywords:
Malignant fibrous histiocytoma
Histogenesis
Marrow stem cells
Pluripotential mesenchymal differentiation
Rat-MFH specific antibody
MFH model
c-kit

ABSTRACT

Malignant fibrous histiocytoma (MFH) is regarded as an undifferentiated pleomorphic sarcoma with unproven histogenesis. We investigated pathobiological characteristics of a rat MFH cell line (MT-9). Immunocytochemically, MT-9 cells and MT-9-induced tumours reacted to vimentin, A3 (rat MFH cell-specific antibody), macrophage markers and α-SMA (myofibroblastic marker), indicating that MT-9 showed both histiocytic and (myo)fibroblastic features. Adipogenic supplement-added MT-9 showed increased accumulation of lipid droplets. Addition of BMP-2 or osteogenic supplement to MT-9 enhanced osteoblastic markers (ALP activity, osteocalcin mRNA expression and calcification). TGF-β1-treated MT-9 revealed increased numbers of α-SMA-immunopositive cells, and enhanced protein levels of α-SMA and fibronectin, indicating myofibrogenesis. In rat tissues, A3 labelled with immature mesenchymal and perivascular cells in foetuses and neonates, and with marrow stem cells in adults. c-kit mRNA expression was seen in bone marrows and MT-9. Collectively, progenitors of MFH should be sought in lineage of marrow stem cells capable of differentiating into mesenchymal cells.

© 2007 Elsevier Ltd. All rights reserved.

1. Introduction

Malignant fibrous histiocytoma (MFH) has been reported to be the most common neoplasm of the soft-tissue in the elderly.[1,2] Although MFH is characterised histologically by the presence of histiocytic and fibroblastic cells arranged in a storiform pattern,[1–3] the precise histogenesis and derivation remain undetermined. Besides subcutis, sarcomas with MFH-like histology have been reported to occur in visceral organs and bones,[4,5] and MFH-like histological phenotypes have been seen in parts of fibrosarcomas, leiomyosarcomas, osteosarcomas, malignant schwannomas and liposarcomas of human clinical species.[1,2,6,7] MFH may merely represent a morphological pattern shared by a wide variety of poorly differentiated pleomorphic sarcomas.[2] Based on these observations, we drew a hypothesis that MFH may generate from

* *Corresponding author:* Tel.: +81 72 254 9482; fax: +81 72 250 7208.
E-mail address: yamate@vet.osakafu-u.ac.jp (J. Yamate).
0959-8049/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2007.10.021

mesenchymal stem cells with multidirectional differentiations.

Knowledge of pathobiological characteristics of cancers is essential to establish effective treatments. Previously, we established a cloned cell line (MT-9) from a spontaneous MFH found in an aged F344 rat.[8,9] The original tumour was demonstrated to consist of an admixure of histiocytic and fibroblastic cells in electron microscopy and enzyme histochemistry.[9] This study was conducted to clarify the differentiation potential of MFH cells which may be induced by stimulating factors already established as suitable inducers. Particularly, we focused on osteogenic, adipogenic and myofibrogenic differentiations of MT-9 cells. Furthermore, using a rat-MFH specific antibody (A3) that we have developed previously,[10] the distribution of A3-positive cells was immunocytochemically investigated in tissues from foetal, neonatal and adult rats, to explore the possible progenitor of MFH cells. This study clearly demonstrated that MFH cells have a capacity for mesenchymal differentiations, and that MFH cells, embryonal mesenchymal cells, perivascular cells and bone marrow stem cells have common antigens recognised by A3. It is likely that the progenitor of MFH cells is involved in the differentiating lineage of marrow stem cells.

2. Materials and methods

2.1. Rat MFH cell line (MT-9)

A parent cell line (MT-P) was prepared from a homotransplantable tumour which had been established from a spontaneous MFH arising in the subcutis of an aged F344 male rat.[8,9] MT-P was cloned twice by the limiting dilution technique, and a cloned cell line (MT-9) was isolated.[8] Eagle's minimum essential medium (E-MEM, Nissui, Tokyo, Japan) containing 10% foetal bovine serum (FBS, Bioserum, UBL, Japan), 0.03% L-glutamine (Nissui), penicillin (100 U/ml) and streptomycin (100 μg/ml) was used as the growth medium. MT-9 cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C as described previously.[8]

2.2. Animals

Pregnant F344 rats and 5-week-old F344 rats were obtained from Charles River Japan (Shiga, Japan). Samples were obtained from foetal rats at gestation days 18 and 21, post-natal rats aged 1, 3, 6, 9, 12 and 15 days, as well as adult rats over 6-weeks-old. These tissue samples and MT-9-induced tumours were fixed in 10% neutral buffered formalin, Zamboni's solution or periodate-lysine-paraformaldehyde (PLP) fixative.[11] The present experiments were conducted in full compliance with our institutional guidelines for animal care.

2.3. Histopathological analyses of MT-9 cells and MT-9-induced tumours

MT-9 cells cultured for 2 days in E-MEM on tissue culture chamber slides (LAB-TEK; Naperville, IL, USA) were fixed in formalin or cold acetone/ethanol (50:50). Formalin-fixed cells were stained with haematoxylin and eosin (HE), and acetone/ethanol-fixed cells were labelled immunocytochemically as described below. For *in vivo* observations, MT-9 cells (10^6/ml) were inoculated subcutaneously into syngeneic rats. Subcutaneous tumours developed were fixed in the above fixatives. Tumour tissue sections were stained with HE, and immunocytochemically labelled.

For immunocytochemical analysis, the avidin–biotin complex (ABC) method (Vectastain ABC Kit; Vector Laboratories Inc., Burlingame, CA, USA) was utilised with the primary antibodies as follows[11]: anti-vimentin (monoclonal; Dako, Corp., Carpinteria, CA, USA; ×200), anti-α-smooth muscle actin (α-SMA; monoclonal; Dako, ×100), ED1 (monoclonal; Chemicon International Inc., Temecula, CA, USA; ×500), ED2 (monoclonal; Serotec Ltd., Tokyo, Japan; ×500), anti-cytokeratin (monoclonal; DAKO, predilution), anti-glial fibrillary acidic protein (GFAP; polyclonal; Dako; × 1000), anti-S-100 protein (polyclonal; Dako; ×200), and the A3 (monoclonal; Trans Genic Inc., Kobe, Japan; ×100). ED1 and ED2 are rat macrophage/histiocyte specific antibodies,[11–14] and A3 antibody is highly specific for rat MFH cells.[10] Formalin-fixed deparaffinised sections were used for vimentin, cytokeratin, α-SMA, ED1, S-100 protein and GFAP.[11] Zamboni's solution-fixed deparaffinised sections and PLP-fixed deparaffinised sections were used for ED2 and A3, respectively.

The following procedures were applied in common to tissue sections and culture cell sections. The sections were incubated with 0.5–5% H_2O_2 for 10 min to quench endogenous peroxidase. The slides were then treated with 5% non-fat milk for 45 min, followed by incubation with each primary antibody for 14 h at 4 °C. Next, 30 min of incubation with biotinylated goat anti-mouse antibody for monoclonal antibodies or with goat anti-rabbit antibody for polyclonal antibodies was performed. Final incubation was carried out for 30 min with an avidin-biotinylated peroxidase complex, and positive reactions were visualised with 3,3′-diaminobenzidine (DAB). Non-immunised mouse or rabbit serum was used for negative controls.

2.4. Adipogenic differentiation

We used two different adipogenic inducers. The 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15-dPGJ_2) was obtained from BIOMOL International (PA, USA),[15] and adipogenic supplement (including insulin, 3-isobutyl-1-methylxanthin, indomethacin and dexamethasone (DMSO)), which was developed as an adipogenic stimulus for bone marrow stem cells,[16] was purchased from Dainippon Sumitomo Pharma Co. Ltd. (Esaka, Japan). For the experiment of 15-dPGJ_2, cells (0.5×10^4/ml) were incubated in E-MEM containing 15-dPGJ_2(5μM) (diluted in DMSO) or an equivalent DMSO (vehicle control). The E-MEM containing 15-dPGJ_2 or vehicle was changed every 2 days. After addition, cells were fixed in formalin at 4, 8 or 25 days, and stained with Oil-Red-O. For adipogenic supplement, cells (0.5×10^4/ml) were incubated in E-MEM containing the supplement (0 or 50 μl/ml);[16] after addition, cells were fixed in formalin at 4, 8 and 12 days, and stained with Oil-Red-O. The number of colonies of cells with Oil-Red-O-positive lipid droplets was counted in four different fields at 200×, and the ratio of lipid droplets per cytoplasm was evaluated in 10 randomly-se-

lected cells with lipid droplets by the colour image analyser (Mac SCOPE, Mitani Inc., Fukui, Japan).[15]

2.5. Osteogenic differentiation

We first used an osteogenic supplement (including beta-glycerophosphate, ascorbic acid phosphate and DMSO) obtained from Dainippon Sumitomo; the supplement was developed as an osteogenic inducer for marrow stem cells.[17] MT-9 cells (0.5×10^4/ml) were incubated in E-MEM containing the supplement (0 or 50 μl/ml). At 4, 8, 12 and 25 days after addition, culture supernatants were collected, and values of alkaline phosphatase (ALP) and calcium were measured by a Clinical Analyser (Hitachi 7170, Tokyo, Japan); cells fixed in formalin were stained with HE or von Kossa's method for calcification.[18] The magnitude of calcification was evaluated at scores of – to 4+: –, negative; +, faintly positive; 2+, moderately positive; 3+, strongly positive; 4+, strongly and diffusely positive.

Bone morphogenetic protein-2 (BMP-2) is a well-known inducer of osteogenesis.[18,19] Next, we added rhBMP-2 (generous gift from Astellas Pharmaceutical Co., Tokyo, Japan) to 10% serum-containing α-MEM (GIBCO, BRL, Gland Island, NY, USA), a medium that has been used in osteogenic differentiation.[20] MT-9 cells (3×10^4/ml) were incubated in α-MEM added rhBMP-2 (0, 30 or 100 ng/ml). The α-MEM including rhBMP-2 was changed every 3 days. ALP activity, ALP enzyme-staining and osteocalcin mRNA expression as well as calcium deposition were investigated. To measure the ALP activity, MT-9 cells cultured for 4 days with rhBMP-2 were lysed in M-Per™ Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) following its protocol.[20] ALP activity was assayed using *p*-nitrophenylphosphate as a substrate by Alkaline Phospha Test Wako (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) and the protein content was measured using the bicinchoninic acid Protein Assay Kit (Pierce). MT-9 cells incubated with rhBMP-2 (0 and 100 ng/ml) for 4 days were fixed in acetone/ethanol, and enzymatically stained for ALP by the naphthol AS (SIGMA) method (pH 9.0).[8] To measure mRNA expression of osteocalcin, MT-9 cells incubated with rhBMP-2 (0 and 100 ng/ml) for 7 days were used. Total RNA was isolated from the cultured cells by Trizol™ reagent (GIBCO).[11] Reverse transcriptase-polymerase chain reaction (RT-PCR) was conducted as described previously.[11] The following conditions were used for the amplification[21]: 31 (for osteocalcin) and 20 (for β-actin; control) cycles of 30 s of denaturation at 94 °C, 20 s of annealing at 58 °C and 30 s of synthesis at 72 °C. The oligonucleotide primers used for PCR were as follows: for rat osteocalcin,[21] forward primer 5′-GCACCACCGTTTAGGGCAT-3′ and reverse primer 5′-AGAGAGAGGGAACAGGGAG-3′: for β-actin, forward primer 5′-TAAAGACCTCTATGCCAACAC-3′ and reverse primer 5′-CTCCTGCTTGCTGATCCACAT-3′.[11] The PCR products were electrophoresed in 2% agarose gels and DNA was stained with ethidium bromide on the gel. The intensity of the osteocalcin bands was evaluated semiquantitatively using image analysis software (Image J, NIH), and compared to that of β-actin. Measurement was performed in triplicate using different samples. For calcification assays, MT-9 cells were incubated with rhBMP-2 (0 and 100 ng/ml) for 4, 7, 14 and 21 days, fixed in 10% formalin, and stained by von Kossa's method.

2.6. Myofibrogenic differentiation

Transforming growth factor-β1 (TGF-β1) plays a central role in the induction of myofibroblasts from pre-existing fibroblasts.[22] Immunophenotypical change for α-SMA was examined by the addition of rhTGF-β1 (R&D system, Minneapolis, MN, USA). α-SMA is a marker for the development of myofibroblasts.[23] MT-9 cells (3×10^4/ml) were incubated in 2% serum-containing E-MEM for 1 day. The medium then was changed to 2% serum-containing E-MEM added rhTGF-β1 (0, 1 and 5 ng/ml). In the same manner, other chambers containing 5 ng/ml of TGF-β1 were incubated with anti-TGF-β1 antibody (5 ng/ml) (R&D system). Twenty-four hours after addition, the cells were fixed in cold acetone/ethanol, and stained by immunocytochemistry with α-SMA antibody. The percentage of cells reacting with the antibody was assessed five times by examining 300 cells at each assessment.[24]

To further investigate the expression levels of proteins, the immunoblotting with monoclonal antibodies against α-SMA, vimentin (DAKO), and β-actin (control; SIGMA), and with a polyclonal antibody to fibronection (SIGMA) was conducted in MT-9 cells incubated with TGF-β1 (0, 1 and 5 ng/ml) or TGF-β1 (5 ng/ml)/anti-TGF-β1 antibody (5 ng/ml) in the same fashion described above. The cells were homogenised with CellLytic (SIGMA) for 30 min. The crude homogenate was centrifuged for 10 min at 13,000 rpm at 4 °C. The supernatant was precipitated with acetone and used as a sample. According to the methods described,[25] the sample (2.5 μg of protein/well) was subjected to SDS–PAGE in a 7% gel and proteins in the gel were transferred to a PVDF membrane by electronblotting. Membranes were incubated with an antibody to α-SMA, vimentin, fibronection or β-actin. Immunoreactive proteins were then detected by incubating for 30 min with Histofine simple stain Kit (Nichirei, Tokyo, Japan). The intensity of these bands was evaluated semiquantitatively using image analysis software (Image J), and compared to that of β-actin. Measurement was performed using three different samples.

2.7. Distribution of cells reacting to a rat MFH-specific antibody (A3) in rat tissues, and c-kit mRNA expression in MT-9 cells

A monoclonal antibody (A3) was generated using whole cell antigens prepared from rat MFH cells.[10] The antigen recognised by A3 is approximately 80 kDa in molecular weight, and the epitope is located on the plasma membrane of rat MFH cells. Major organ samples (lungs, kidneys, heart, liver, muscles, skin and bone marrow) from foetal, neonatal and adult rats were fixed in PLP fixative, and stained immunocytochemically by the ABC method with A3 as described above.

Bone marrow stem cells specifically express c-*kit* receptor tyrosine kinase (KIT).[26] To pursue the relationship between MT-9 cells and marrow stem cells, c-*kit* mRNA expression was investigated by the RT-PCR method. The preparations of total RNA from MT-9 cells and rat femur bone marrow tissues and single-strand cDNA are described above. The following conditions were used for amplification[27]: 34 cycles of 1 min of denaturation at 94 °C, 2 min of annealing at 55 °C and 3 min of synthesis at 72 °C. The oligonucleotide primers used for the PCR were as follows: for rat c-*kit*, forward primer

5′-GGCCCACCCTGGTCATTACAGAAT-3′ and reverse primer 5′-ACCTGGTAGGAAAAGCTCAGCAA-3′. The PCR conditions, and the forward and reverse primers used for β-actin (control) are described above.

2.8. *Statistical evaluation*

Obtained data represented means ± SD, and statistical analysis was performed using Student's *t*-test or analysis of variance. Significance was accepted at $P < 0.05$.

3. Results

3.1. *Immunocytochemical characteristics of MT-9 cells and MT-9-induced tumours*

Cultures of MT-9 cells consisted of polygonal or fusiform cells with a round or spindle nucleus arranged in an ambiguous interlacing pattern (Fig. 1A). Neoplastic cells reacted strongly to vimentin (Fig. 1B) and A3 (Fig. 1C), but negatively to cytokeratin. Occasional cells were positive for α-SMA (Fig. 1D), ED1 (Fig. 1E) and ED2 (Fig. 1F). Clusters of cells positive for S-100 protein (Fig. 1G) and GFAP (Fig. 1H) are present.

Tumours induced by MT-9 cells showed a storiform growth pattern composed of spindle-shaped cells and round cells supported by collagen fibres (Fig. 1I). Occasional giant cells with bizarre nuclei were present (Fig. 1I, arrow). Neoplastic cells reacted strongly to vimentin (Fig. 1J) and A3 (Fig. 1K), and cells reacting to α-SMA (Fig. 1L), ED1 (Fig. 1M), ED2 (Fig. 1N), S-100 protein (Fig. 1O) and GFAP (Fig. 1P) were seen sporadically or forming clusters. No cells reacting to cytokeratin were observed in the induced tumours.

3.2. *Adipogenic differentiation*

Cells with Oil-Red-O-positive lipid droplets tended to form colonies. However, there was no significant change in the colony number between controls and 15 d-PGJ_2- or adipogenic supplement-added samples. By contrast, we found that the size of lipid droplets increased and expanded in the cytoplasm (Fig. 2A). In HE-stained sections, such cells had variously-sized vacuoles in the cytoplasm (Fig. 2B). Thus, we

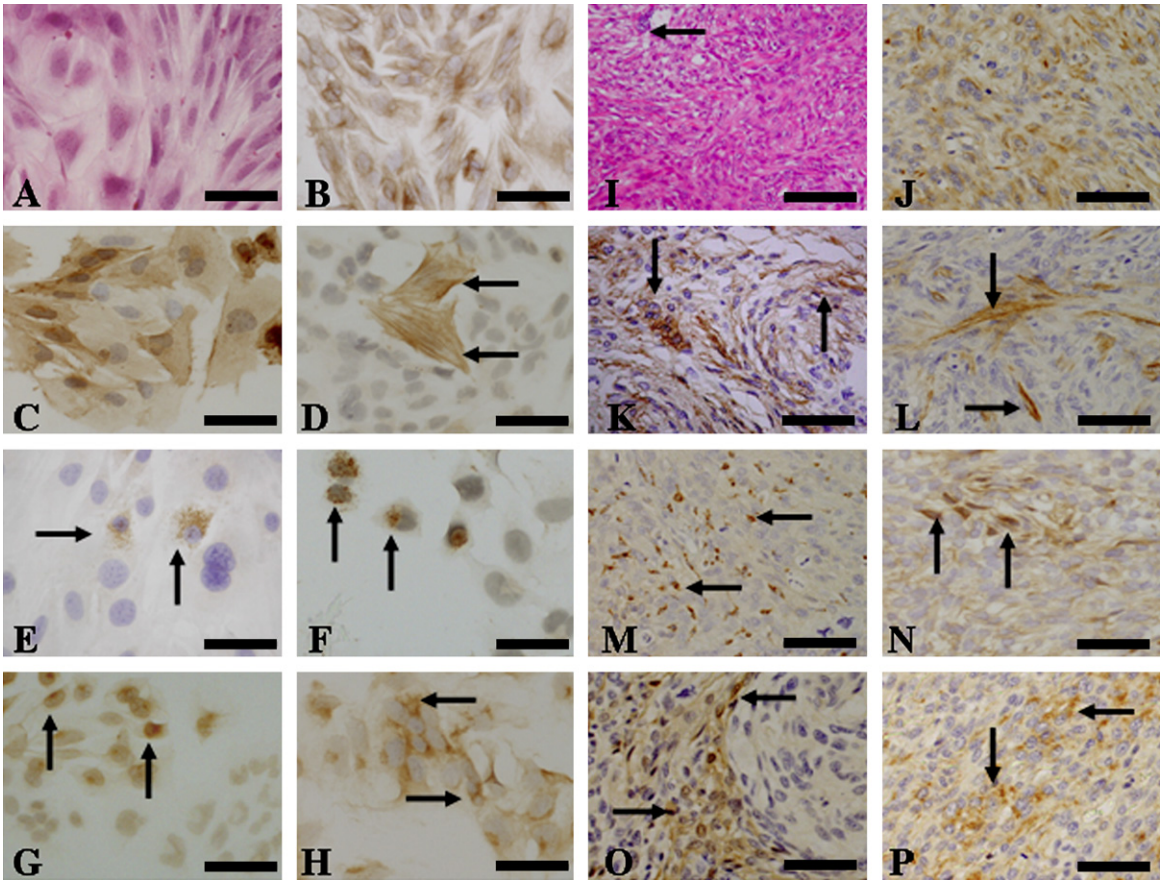

Fig. 1 – Histopathological findings of a rat MFH-derived cloned MT-9 line (A–H) and its induced tumours (I–P). Culture consists of polygonal and fusiform cells (A); neoplastic cells react to vimentin (B), A3 (rat MFH-specific antibody) (C), α-SMA (D, arrows), ED1 (E, arrows), ED2 (F, arrows), S-100 protein (G, arrows) and GFAP (H, arrows). The induced tumour shows a storiform growth pattern (I; arrow indicates a giant cell); neoplastic cells react to vimentin (J), A3 (K, arrows), α-SMA (L, arrows), ED1 (M, arrows), ED2 (N, arrows), S-100 protein (O, arrows) and GFAP (P, arrows). A and I, HE; B–H and J–P, immunocytochemistry; A–H, bar = 30 μm; I–P, bar = 50 μm.

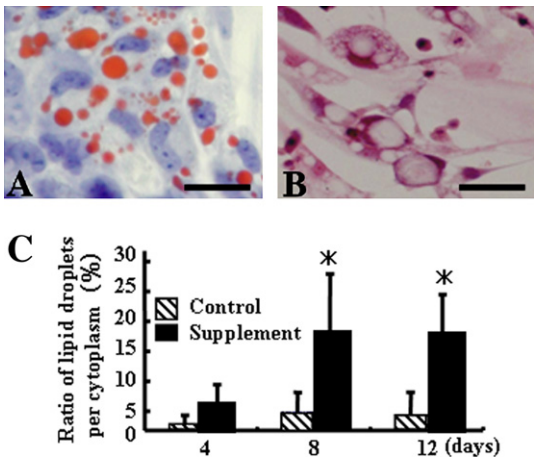

Fig. 2 – At day 12 after addition of adipogenic supplement, MT-9 cells increase the size of lipid droplets and have variously-sized vacuoles (A, B). The supplement addition significantly increases the ratio of lipid droplets per cytoplasm at 8 and 12 days (C). A, Oil-Red-O stain; B, HE; bar = 10 μm. *, significantly different from controls at $P < 0.05$.

evaluated the ratio of lipid droplets per cytoplasm. Although no significant change was seen between controls and 15d-PGJ_2-added samples, the addition of adipogenic supplement significantly increased the ratio at 8 and 12 days, as compared with that of controls (Fig. 2C).

3.3. *Osteogenic differentiation*

In the experiment using osteogenic supplement, ALP values were significantly greater than those of controls at 8, 12 and 25 days after addition (Fig. 3A). In contrast, calcium values were significantly decreased at each day (Fig. 3B). The supplement addition dramatically induced calcification at 8, 12 and 25 days; the degree increased with incubation time (Fig. 3C).

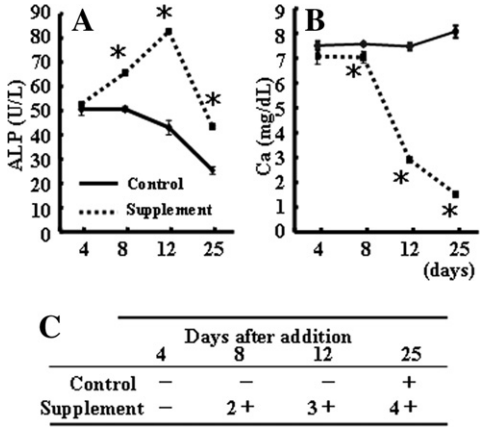

Fig. 3 – Values of supernatant ALP in MT-9 incubated with osteogenic supplement are significantly greater at days 8, 12 and 25 (A), whereas calcium (Ca) contents are significantly decreased (B). The supplement addition induces time-dependent calcification at 8 (2+), 12 (3+) and 25 (4+) days (C), (see Section 2.5 for the magnitude). *, significantly different from controls at $P < 0.05$.

In another experiment using rhBMP-2, ALP staining intensity was greater in MT-9 cells incubated for 4 days with rhBMP-2 (100 ng/ml) compared with controls (0 ng/ml) (Fig. 4A and B). ALP activity showed a significant increase at 30 and 100 ng/ml of rhBMP-2 (Fig. 4C). Osteocalcin mRNA expression at 100 ng/ml of rhBMP-2, measured at 7 days after addition, was significantly greater than that of 0 ng/ml (Fig. 4D and 4E). Calcification developed time-dependently in control (0 ng/ml) and rhBMP-2 (100 ng/ml) added MT-9 cells (Fig. 4F–M), however, the magnitude in rhBMP-2 (Fig. 4J–M) was much greater than that in 0 ng/ml (Fig. 4F–I) at each evaluation day.

3.4. *Myofibrogenic differentiation*

The positive reactions for α-SMA appeared filamentous in the cytoplasm (Fig. 5A). The addition of TGF-β1 increased the number of cells reacting to α-SMA at 1 and 5 ng/ml (Fig. 5A and B), and the increased number at 5 ng/ml of TGF-β1 was significantly suppressed by the addition of anti-TGF-β1 antibody (5 ng/ml) (Fig. 5B). By the immunoblot analysis, MT-9 cells expressed proteins of α-SMA, vimentin and fibronection prior to TGF-β1 treatment (Fig. 5C, 0 ng/ml), indicating fibroblastic nature of these cells. The expression of these proteins was significantly increased by the treatment with 5 ng/ml of TGF-β1, and the increased expression of each protein was suppressed by the addition of anti-TGF-β1 antibody (Fig. 5C–F).

3.5. *Distribution of cells reacting to A3 in rat tissues, and c-kit mRNA expression in bone marrow and MT-9 cells*

In foetuses at gestation days 18 and 21 and post-natal rats until 15-days-old, A3 labelling was present in perivascular cells (Fig. 6A) and spindle cells around hair follicles (Fig. 6B) in the subcutis. Cells in the perinurium (Fig. 6C), interstitial cells in the muscles (Fig. 6D), peribronchial interstitial cells (Fig. 6E), interstitial cells around renal tubules (Fig. 6F) and interstitial cells in the lamina propria of intestinal mucosa (Fig. 6G) reacted to A3. In the lungs and kidneys, spindle cells around arterioles strongly reacted to A3 (Fig. 6E and 6H). The number and reactivity of these A3-positive cells were most prominent in foetuses at gestation day 21, and post-natal rats aged 1–9 days, and then tended to gradually decrease as age progressed until 15-days-old. In adult rats over 6-weeks-old, there were no cells reacting to A3 in the subcutis and visceral organs.

In the bone marrow of foetal (Fig. 6I) and post-natal (Fig. 6J) rats, A3-reacting cells were sporadically present, whereas the positive cells were frequently observed in the bone marrow of adult rats (Fig. 6K). The positive cells in adults showed spindle or stellate shapes, and long branching processes were present, apparently forming networks with each other (Fig. 6L). *c-kit* mRNA expression was seen in samples of adult bone marrows, MT-9 cells and another rat MFH cell line (MT-8)[8]; the expression level of MFH cell lines was greater than that of marrow tissues (Fig. 6M).

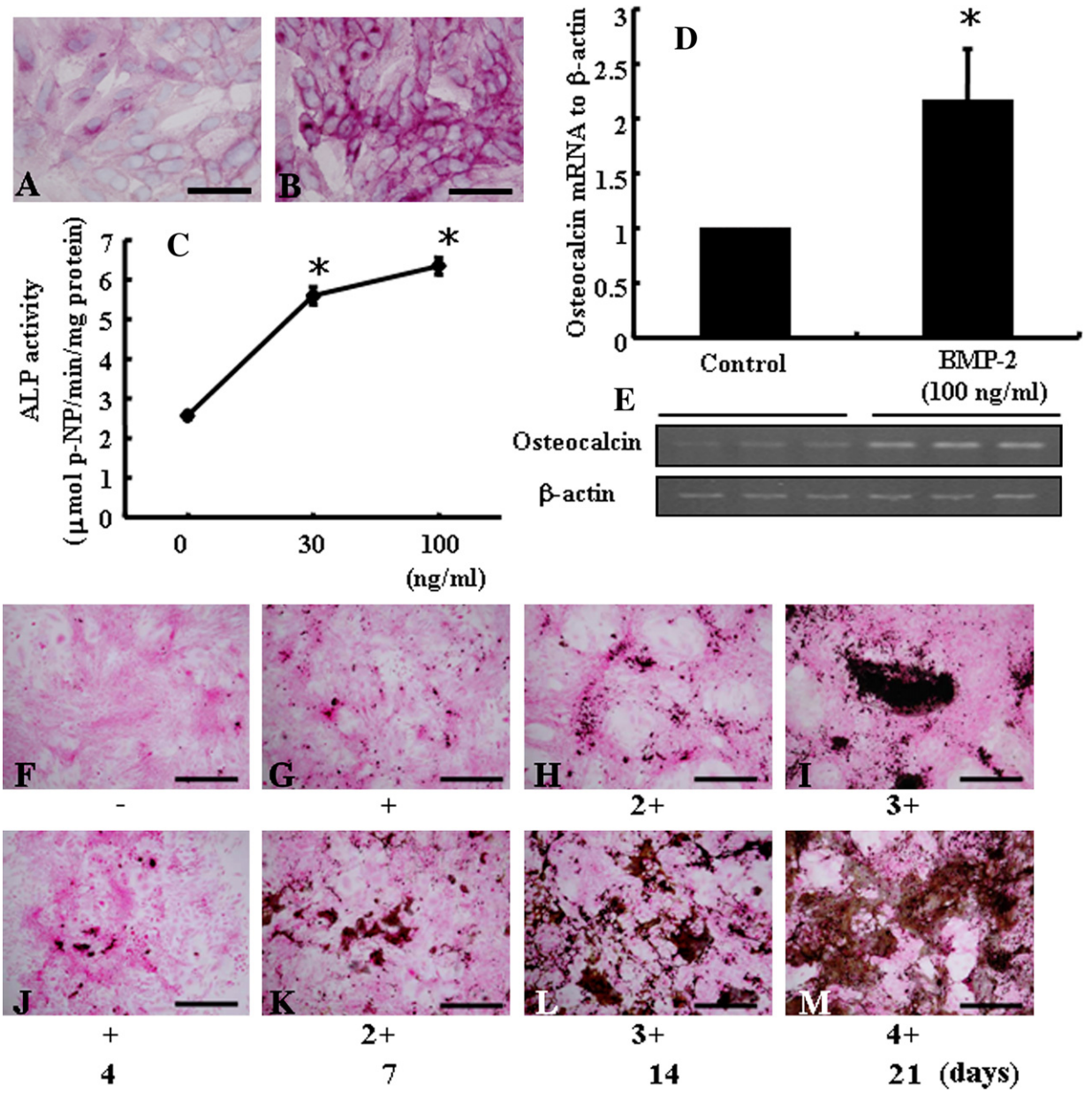

Fig. 4 – In contrast to control (0 ng/ml rhBMP-2) (A), ALP staining intensity is greater in MT-9 cells incubated for 4 days with rhBMP-2 (100 ng/ml) (B). ALP activity, measured at 4 days after addition, shows a significantly dose-dependent increase at 30 and 100 ng/ml of rhBMP-2 (C). rhBMP-2-treated MT-9 cells show increased mRNA levels of osteocalcin (D, E); the band of osteocalcin mRNA in three different samples (E) is normalised semiquantitatively by that for β-actin, and expressed relative to the intensity of control (0 ng/ml; level = one) (D). Calcification deposition in rhBMP-2 (100 ng/ml) (J–M) is much greater than that in 0 ng/ml (F–I) at each evaluation day (see Section 2.5 for the magnitude). A and B, ALP enzymatic staining, bar = 30 μm; F–M, von Kossa's stain, bar = 60 μm; *, significantly different from controls at $P < 0.05$.

4. Discussion

4.1. *Immunocytochemical characteristics of rat MFH-derived MT-9 cells*

The most common histological classification of human MFHs is the storiform-pleomorphic type.[1] MT-9-induced tumours also showed histological characteristics of storiform-pleomorphic type. In cultures and tumours of MT-9, all neoplastic cells reacted to vimentin, but not cytokeratin, indicating their mesenchymal nature. Occasional cells reacted to ED1 and ED2. ED1 is used to identify infiltrating macrophages in pathological lesions of rats.[11,12] The antigens recognised by ED1 are mainly on the membrane of cytoplasmic granules, especially phagolysosomes of macrophages.[23] ED2 recognises a scavenger receptor for haemoglobin–haptoglobin complexes of rat resident macrophages (e.g. Kupffer cells).[14] These observations indicated the presence of neoplastic cells with histiocytic/macrophage-like characteristics in MT-9. Some investigators have suggested that histiocytic cells in

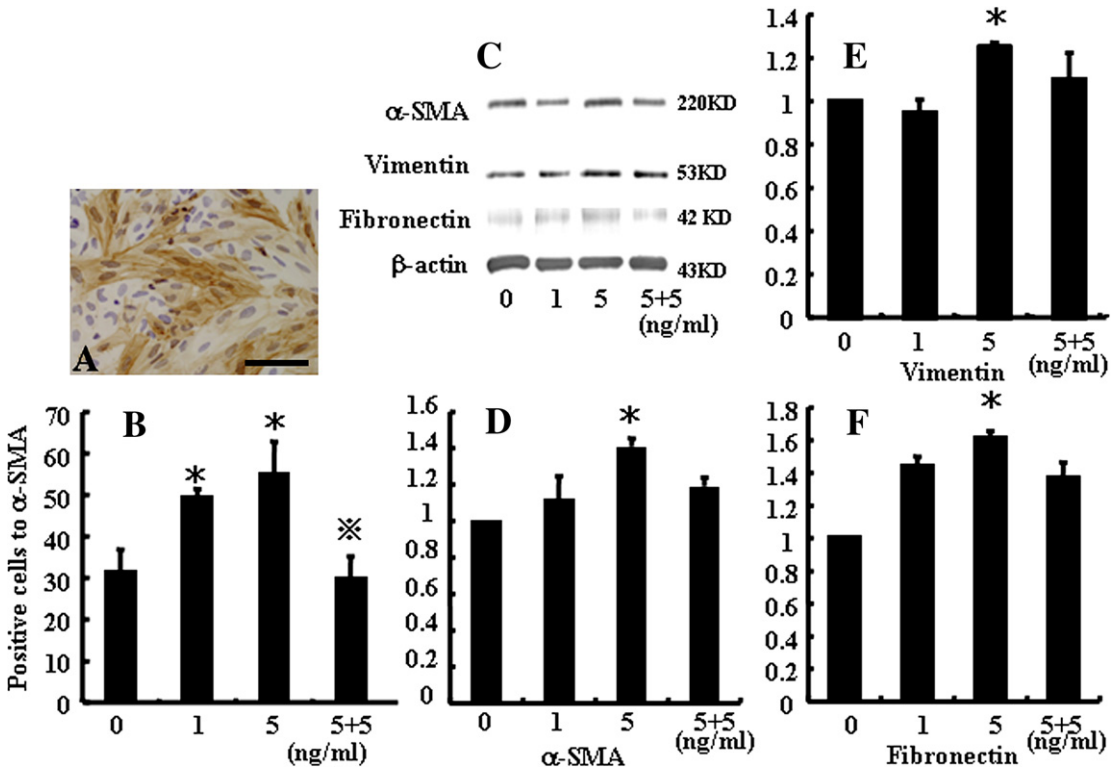

Fig. 5 – The addition of TGF-β1 increases the number of cells reacting to α-SMA at 1 and 5 ng/ml (A for 5 ng/ml of TGF-β1, compare Fig. 1D as non-added cells), and the increased number at 5 ng/ml is suppressed by anti-TGF-β1 antibody (5 ng/ml, expressed as 5 + 5) (B). By immunoblot analysis, expression levels of α-SMA, vimentin and fibronectin are significantly increased at 5 ng/ml of TGF-β1, and the increased expression is suppressed by anti-TGF-β1 antibody (D–F). Band of each protein (C shows a representative band) is normalised semiquantitatively by that for β-actin, and expressed relative to the intensity of control (0 ng/ml; level = one). A, immunocytochemistry, bar = 40 μm; *, significantly different from controls at $P < 0.05$; ※, significantly different from 5 ng/ml TGF-β1 at $P < 0.05$.

MFHs may be non-neoplastic, infiltrative macrophages induced by monocyte chemoattractant protein-1 (MCP-1) produced by neoplastic cells.[12,27] However, MT-P, the parent cell line of MT-9, did not produce MCP-1.[12] Positive reactions of MT-9 cells to these macrophage markers suggest that cells with histiocytic nature are neoplastic. In human MFHs, neoplastic cells stained positive for CD68, a monoclonal antibody specific for human histiocytes and macrophages.[28] α-SMA-positive cells that were occasionally seen in cultures and tumours of MT-9 were regarded as myofibroblasts. The myofibroblasts have been suspected to originate from pre-existing fibroblasts under pathological conditions.[23] Neoplastic cells reacting to α-SMA have also been reported in human MFHs.[29] Interestingly, many cells in MT-9 reacted strongly to A3. A3 did not label other rat tumours such as histiocytic sarcoma, fibrosarcoma, leiomyosarcoma, malignant meningioma, mononuclear cell leukaemia, malignant schwannoma and endometrial stromal sarcoma.[10] A3 is highly specific for rat MFH cells. Based on these findings, MT-9 cells retained the characteristics of MFH cells expressing both histiocytic and (myo)fibroblastic phenotypes.

Antibodies against S-100 protein and GFAP have been used for identification of neurogenic or astrocyte-derived tumours.[30,31] However, S-100 protein antibody labels with chondrocytic or lipogenic cells,[1,30] and the GFAP antibody reacts with the transformed hepatic stellate cells with myofibroblastic nature.[32] Neoplastic cells reacting to S-100 protein have been reported in human MFHs.[29] Some MT-9 cells reacted to these antibodies, indicating that besides histiocytic and fibroblastic phenotypes, MT-9 cells exhibited heterogeneous immunophenotypes. Diverse immunophenotypes have been also reported in human MFHs.[1,6,29]

4.2. *Adipogenic differentiation*

The 15-dPGJ$_2$ accelerates adipogenesis in pre-adipocytes through activation of peroxisome proliferator-activated receptor-γ2 (PPARγ2).[15] However, 15-dPGJ$_2$ addition to MT-9 cells did not increase the number of colonies of Oil-Red-O-positive cells or the ratio of lipid droplets in the cytoplasm. Generally, adipogenesis is a complex process including proliferation of precursors, their commitment to the adipogenic lineage and terminal differentiation.[16] The adipogenesis induced by 15-dPGJ$_2$ may be completed by very complicated mechanisms depending on cell types or differentiation stages.[15] Thus, we selected the adipogenic supplement by which adipogenesis has been showed in bone marrow stem cells.[16] The present study revealed that the ratio of lipid droplets in cytoplasm was significantly greater in the supplement-added MT-9 cells, indicating enhanced accumulation of lipid

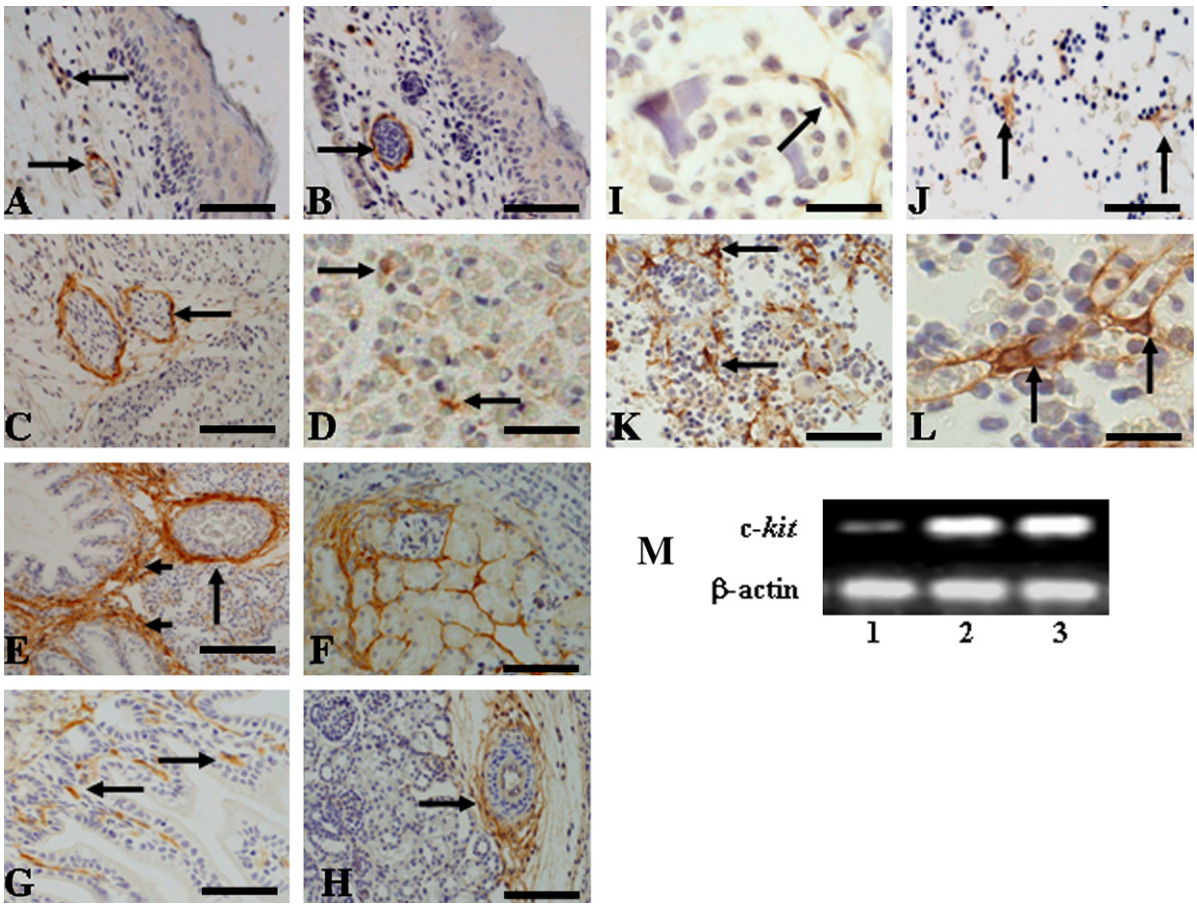

Fig. 6 – In the subcutis of foetuses at gestation days 18 (A) and 21 (B–E, I) and neonates under 15-days-old (F–H), A3 reacts with perivascular cells (A, arrows) and spindle cells around hair follicles (B, arrow). Cells in the perinurium (C, arrow), interstitial cells in the muscle (D, arrows), peribronchial interstitial cells (E, small arrows), interstitial cells around renal tubules (F) and interstitial cells in the lamina propria of intestinal mucosa (G, arrows) show a positive reaction to A3. Spindle cells around arterioles in the lung (E, large arrow) and kidney (H, arrow) react strongly to A3. Occasional marrow cells in foetuses (I, arrow) and neonates (J, arrows) react to A3, whereas A3-positive cells are frequently seen in the bone marrow of a 10-week-old adult rat (K and L, arrows). *c-kit* mRNA expression is seen in samples of an adult bone marrow (lane 1), MT-9 (lane 2) and another rat MFH line (lane 3)(MT-8).[8] A–L, immunocytochemistry; A, B, F, G, bar = 80 μm; D, I, L, bar = 50 μm; C, E, H, bar = 100 μm; J, K, bar = 70 μm.

droplets. The accumulation of large fat containing droplets within the stem cells has been used as an index of adipogenesis.[15,19] MT-9 cells possess the potential to differentiate into adipocytes.

4.3. *Osteogenic differentiation*

The osteogenic supplement used has been demonstrated to induce osteogenesis when added to human and rat marrow stem cells.[17] With increasing incubation time, the addition of the supplement to MT-9 cells significantly increased ALP values and developed calcification. Consistent with time-dependently increased calcification, calcium values were significantly decreased in the supplemented MT-9 cells; this suggests that calcium in the medium is probably consumed for increased calcification. The addition of rhBMP-2 to MT-9 cells also increased ALP activity and the intensity of ALP staining; furthermore, increased expression of osteocalcin mRNA and time-dependently increased deposition of calcium were seen in rhBMP-2-treated MT-9 cells. The expression of ALP, a membrane-bound glycoprotein, is an early marker of osteoblastic differentiation.[33] During the maturation of osteoblasts, ALP activity and osteocalcin expression are increased, and then the bone is formed by calcification.[18] These sequential events were confirmed in the supplement- or rhBMP-2-treated MT-9 cells. These experiments show that MT-9 cells are capable of differentiating into osteoblasts.

4.4. *Myofibrogenic differentiation*

The addition of TGF-β1 increased the number of α-SMA-positive cells in MT-9, and the addition of ant-TGF-β1 antibody decreased the number. Myofibrobastic cells are capable of producing extracellular matrix such as fibronectin.[22] Western blot analyses revealed that besides α-SMA protein, vimentin and fibronectin protein levels were increased in MT-9 cells treated with TGF-β1, and the levels were reduced by

anti-TGF-β1 antibody. These findings indicated that MT-9 cells underwent myofibrogenesis after TGF-β1 treatment.

4.5. *Distribution of A3-immunopositive cells in rat normal tissues, and the possible origin of MFH cells*

In foetuses and neonates, immature mesenchymal cells around bronchi and hair follicles, as well as interstitial cells in the muscles, intestinal mucosa, kidneys and perinurium were positive for A3. Additionally, spindle cells around arterioles in the subcutis, lungs and kidneys showed a strong positive reaction to A3. Previously, Iwasaki et al. generated two monoclonal antibodies using human MFH cells as antigens, and these antibodies produced intense immunolabelling of mesenchymal cells around arteries or arterioles in human connective tissues.[34] During embyrogenesis, primitive mesenchymal cells concentrated in organs/sites are an ancestral to all the cells of loose connective tissue.[19] It has been recently reported that perivascular cells underwent osteogenic or chondrocytic differentiation *in vitro*.[35] Although the biological functions of antigens recognised by A3 are under study, embryonal mesenchymal cells, perivascular cells and MFH cells have common antigens that are recognised by A3.

Importantly, mesenchymal marrow cells in adult rats strongly reacted to A3. Judging from the distribution and cellular shape, the A3-positive cells were considered to be stromal stem cells in the bone marrow. Bone marrow or tissue stem cells specifically express KIT.[26,36,37] By the RT-PCR method, mRNA expression of *c-kit* was seen in tissues from bone marrows and MT-9 cells. These findings suggest that MFH cells and bone marrow stem cells possess common origins.

Recent studies on the regenerative therapy have highlighted the potential role of perivascular stem cells as a source of undifferentiating mesenchymal cells.[35,36] Their role is to replenish multiple mature differentiated cell types and thereby achieve long-time reconstitution.[36] Perivascular stem cells present in various organs and sites are recruited from bone marrow stem cells.[26,36] Therefore, the progenitors of MFH are primarily derived from bone marrow stem cells, and MFH cells are in the developing lineage of marrow stem cells with mesenchymal differentiations. Bone marrow-derived stem cells have the capacity to differentiate not only into various kinds of mesenchymal cells, but also into cells with visceral mesoderm, neuroectoderm and endoderm characteristics.[26] Thus, it is not surprising that MFH-like tumours arise at various sites and in many organs, and that MFH-like histological phenotypes exist in parts of well-differentiated mesenchymal tumours as areas of de-differentiation. It has been reported that MFH cells produced inflammatory factors such as IL-6 and TGF-β.[38,39] Various factors released by neoplastic cells might be attributable to cellular differentiation.[38] Therefore, phenotypes of MFH cells are altered easily by microenvironmental conditions evoked by cell–cell or cell–matrix interaction.

In conclusion, this study has showed that MFH cells can undergo adipogenic, osteogenic or myofibrogenic differentiation under appropriate conditions. Furthermore, based on the distribution of cells labelled with the rat MFH-specific antibody (A3) in rat tissues, it was found that the progenitor of MFH may be involved in the lineage of marrow stem cells capable of differentiating into mesenchymal cells. Spontaneously occurring MFHs have rarely been detected in rats,[9] and to our knowledge, there are no cell lines derived from spontaneous MFHs in F344 rats. MT-9 should provide a valuable tool for investigating the pathobiology of MFHs and the mesenchymal differentiation.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported in part by a Grant-in-Aid (No. 18658110 to J. Yamate) for Exploratory Research, the Ministry of Education, Culture, Sports, Science and Technology, Japan. We thank Dr. O. Sawamoto, Division of Pharmacology, Drug Safety and Metabolism, Otsuka Pharmaceutical Factory, Tokushima, Japan, Dr. M. Kawashima, Department of Veterinary Sciences, Osaka Prefecture University, Sakai, Osaka, Japan and Dr. M. Nakasaki, Department of Biology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan, for their technical assistances.

REFERENCES

1. Weiss SW, Goldblum MD. Malignant fibrohistiocytic tumors. In: Enzinger FM, Weiss SW, editors. *Soft tissue tumors*. 4th ed. St. Louis: Mosby Company; 2002. p. 539–69.
2. Tos AP. Classification of pleomorphic sarcomas: where are we now? *Histopathlogy* 2006;**48**:51–62.
3. Fletcher CD. The evolving classification of soft tissue tumors: an update based on the new WHO classification. *Histopathology* 2006;**48**:3–12.
4. Mizukami H, Yajima N, Wada R, et al. Pancreatic malignant fibrous histiocytoma, inflammatory myofibroblastic tumor, and inflammatory pseudotumor related to autoimmune pancreatitis: characterization and differential diagnosis. *Virchows Arch* 2006;**448**:552–60.
5. Tarkkanen M, Larramendy ML, Bohling T, et al. Malignant fibrous histiocytoma of bone: analysis of genomic imbalances by comparative genomic hybridization and C-MYC expression by immunohistochemistry. *Eur J Cancer* 2006;**42**:1172–80.
6. Hashimoto H, Daimaru Y, Tsuneyoshi M, Enjoji M. Soft tissue sarcoma with additional anaplastic components: a clinicopathologic and immunohistochmeical study of 27 cases. *Cancer* 1990;**66**:1578–89.
7. Fabre-Guillevin E, Coindre JM, Somerhausen Nde S, Bonichon E, Stoeckle E, Bui NB. Retroperitoneal liposarcomas: follow-up analysis of dedifferentiation after clinicopathologic reexamination of 86 liposarcomas and malignant fibrous histiocytomas. *Cancer* 2006;**106**:2725–33.
8. Yamate J, Tajima M, Togo M, Shibuya K, Ihara M, Kudow S. Heterogeneity of cloned cell lines established from a transplantable rat malignant fibrous histiocytoma. *Jpn J Cancer Res* 1991;**82**:298–307.
9. Yamate J, Tajima M, Shibuya K, Ihara M, Kudow S. Morphologic characteristics of a transplantable tumor derived from a spontaneous malignant fibrous histiocytoma in the rat. *Jpn J Vet Sci* 1989;**51**:587–96.

10. Kumagai D, Yamate J, Tajima T, et al. Distribution of cells labelled by a monoclonal antibody (A3) against a cloned cell line derived from a rat malignant fibrous histiocytoma. *J Comp Pathol* 2000;**123**:77–87.
11. Yamate J, Kuriyama T, Kawashima M, et al. Establishment and characterization of transplantable tumor line (KB) and cell lines (KB-P and KB-D8) from a rat thymus-derived dendritic cell sarcoma. *Virchows Arch* 2002;**440**:616–26.
12. Yamashiro S, Takeya M, Nishi T, et al. Tumor-derived monocyte chemoattractant protein-1 induces intratumoral infiltration of monocyte-derived macrophage subpopulation in transplanted rat tumors. *Am J Pathol* 1994;**145**:856–67.
13. Damoiseaux JG, Döpp EA, Calame W, Chao D, MacPherson GG, Dijkstra CD. Rat macrophage lysosomal membrane antigen recognized by monoclonal antibody ED1. *Immunology* 1994;**83**:140–7.
14. Polfliet MM, Fabriek BO, Daniëls WP, Dijkstra CD, van den Berg TK. The rat macrophage scavenger receptor CD163: expression, regulation and role in inflammatory mediator production. *Immunobiology* 2006;**211**:419–25.
15. Lecka-Czernik B, Moerman EJ, Grant DF, Lehmann JM, Manolagas S, Jilka RL. Divergent effects of selective peroxisome proliferator-activated receptor-gamma 2 ligands on adipocyte versus osteoblast differentiation. *Endocrinology* 2002;**143**:2376–84.
16. Janderová L, Mcneil M, Murrell AN, Mynatt RL, Smith SR. Human mesenchymal stem cells as an in vitro model for human adipogenesis. *Obes Res* 2003;**11**:65–74.
17. Ohgushi H, Dohi Y, Katuda T, Tamai S, Tabata S, Suwa Y. In vitro bone formation by rat marrow cell culture. *J Biomed Mater Res* 1996;**32**:333–40.
18. Gilbert L, He X, Farmer P, et al. Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. *Endocrinology* 2000;**141**:3956–64.
19. Zur Nieden NI, Kempka G, Rancourt DE, Ahr H. Induction of chondro-, osteo-, and adipogenesis in embryonic stem cells by bone morphogenetic protein-2: effect of cofactors on differentiating lineages. *BMC Dev Biol* 2005;**5**:1–15.
20. Higuchi C, Myoui A, Hashimoto N, et al. Continuous inhibition of MARK signaling promotes the early osteoblastic differentiation and mineralization of the extracellular matrix. *J Bone Miner Res* 2002;**17**:1785–94.
21. Pirih FQ, Tang A, Ozkurt IC, Nervina JM, Teradis S. Nuclear orphan receptor Nurr1 directly transactivates the osteocalcin gene in osteoblasts. *J Biol Chem* 2004;**279**:53167–74.
22. Li Y, Foster W, Deasy BM, et al. Transforming growth factor-beta1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: a key event in muscle fibrogenesis. *Am J Pathol* 2004;**164**:1007–19.
23. Desmoulière A, Darby IA, Gabbiani G. Normal and pathological soft tissue remodeling: role of the myofibroblast, with special emphasis on liver and kidney fibrosis. *Lab Invest* 2003;**83**:1689–707.
24. Yamate J, Yokoyama Y, Kumagai D, et al. Tumour lines from a spontaneous rat endometrial stromal sarcoma, showing dendritic cell-like and myofibroblastic cell-like phenotypes. *J Comp Pathol* 2004;**131**:38–51.
25. Tanno T, Takenaka S, Tsuyama S. Expression and function of slit1 alpha, a novel alterative splicing product for slit 1. *J Biochem (Tokyo)* 2004;**136**:575–81.
26. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002;**418**:41–9.
27. Takeya M, Yamashiro S, Yoshimura T, Takahashi K. Immunophenotypic and immunoelectron microscopic characterization of major constituent cells in malignant fibrous histiocytoma using human cell lines and their transplanted tumors in immunodeficient mice. *Lab Invest* 1995;**72**:679–88.
28. Binder SW, Said JW, Shintaku IP, Pinkus GS. A histiocyte-specific marker in the diagnosis of malignant fibrous histiocytoma: use of monoclonal antibody KP-1(CD68). *Am J Clin Pathol* 1992;**97**:759–63.
29. Montgomery E, Fisher C. Myofibroblastic differentiation in malignant fibrous histiocytoma (pleomorphic myofibrosarcoma): a clinicopathological study. *Histopathology* 2001;**38**:499–509.
30. Plaza JA, Wakely Jr PE, Suster S. Lipoblastic nerve sheath tumors: report of a distinctive variant of neural soft tissue neoplasm with adipocyte differentiation. *Am J Surg Pathol* 2006;**30**:337–44.
31. Pina-Oviedo S, De Leon-Bojorge B, Cuesta-Mejias T, et al. Glioblastoma multiforme with small cell neuronal-like component: association with human neurotropic JC virus. *Acta Neuropathol (Berl)* 2006;**111**:388–96.
32. Olaso E, Salado C, Egilegor E, et al. Proangiogenic role of tumor-activated hepatic stellate cells in experimental melanoma metastasis. *Hepatology* 2003;**37**:674–85.
33. Suga K, Saitoh M, Fukushima S, et al. Interleukin-11 induces osteoblast differentiation and acts synergistically with bone morphogenetic protein-2 in C3H10T1/2 cells. *J Interferon Cytokine Res* 2001;**21**:695–707.
34. Iwasaki H, Isayama T, Johzaki H, Kikuchi M. Malignant fibrous histiocytoma: evidence of perivascular mesenchymal cell origin: immunocytochemical studies with monoclonal anti-MFH antibodies. *Am J Pathol* 1987;**128**:528–37.
35. Collett G, Wood A, Alexander MY, et al. Receptor tyrosine kinase Axl modulates the osteogenic differentiation of pericytes. *Circ Res* 2003;**92**:1123–9.
36. Fazel S, Cimini M, Chen L, et al. Cardioprotective c-kit+ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines. *J Clin Invest* 2006;**116**:1865–77.
37. Jackson KA, Mi T, Goodell MA. Hematopoietic potential of stem cells isolated from murine skeletal muscle. *Proc Natl Acad Sci USA* 1999;**96**:14482–6.
38. Nakanishi H, Yoshioka K, Joyama S, et al. Interleukin-6/soluble interleukin-6 receptor signaling attenuates proliferation and invasion, and induced morphological changes of a newly established pleomorphic malignant fibrous histiocytoma cell line. *Am J Pathol* 2004;**165**:471–80.
39. Yamamoto T, Akisue T, Marui T, et al. Expression of transforming growth factor beta isoforms and their receptors in malignant fibrous histiocytoma of soft tissues. *Clin Cancer Res* 2004;**10**:5804–7.