Expression of pericyte, mesangium and muscle markers in malignant rhabdoid tumor cell lines: Differentiation-induction using 5-azacytidine

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Malignant rhabdoid tumor (MRT) has been considered to have multiphenotypic diversity characteristics. Some MRTs exhibit a neural phenotype. However, it is still unclear whether MRT cells can display a skeletal muscle, smooth muscle or smooth muscle-like cell phenotype, like those of pericytes and mesangial cells. To determine if MRTs exhibit skeletal muscle cell or smooth muscle-like cell phenotypes, six MRT cell lines (TM87-16, STM91-01, TTC549, TTC642, YAM-RTK1 and TTC1240) were examined for markers of skeletal muscle (MyoD, myogenin, myf-5, myf-6, acetylcholine receptor-α, -β and -γ), smooth muscle (α-smooth muscle actin, SM-1 and SM22), and smooth muscle-like cells, such as pericytes (angiopoietin-1 and -2) and mesangial cells (megsin), using conventional RT-PCR, semi-quantitative PCR, western blotting and immunocytochemistry before and after differentiation-induction with 5-azacytidine. α-Smooth muscle actin and SM22 were detected in all six MRT cell lines, while MyoD and myf-5, crucial markers for skeletal myogenic determination, were not. The TM87-16 cell line expressed SM-1 and angiopoietin-1. TTC1240 also expressed angiopoietin-1. Interestingly, STM91-01 expressed megsin, a novel marker for mesangial cells, in addition to angiopoietin-1. Our results indicated that some MRTs exhibited smooth muscle and/or smooth muscle-like cell phenotypes and some renal MRTs might be of mesangial origin. Recently, smooth muscle and also smooth muscle-like cells have been considered to be of neuroectodermal origin. MRT can thus considered to belong to the category of primitive neuroectodermal tumors (PNETs) in the broad sense. (Cancer Sci 2003; 94: 1059–1065)

Malignant rhabdoid tumor (MRT) was initially reported in the kidney as a rare variant of Wilms' tumor with 'rhabdomyosarcomatoid' features, and had a particularly poor outcome. However, MRT has been described as a primary tumor in a variety of extrarenal sites, including the liver, pancreas, heart, orbit, mediastinum, retroperitoneum, pelvis, gastrointestinal tract, uterus, urinary bladder, skin, soft tissue, neck, extremities, chest wall and the central nervous system.[1–4] MRT usually consists of large, round, polygonal tumor cells with eccentric nuclei and characteristic eosinophilic inclusions. The cells look like rhabdomyoblasts, and hence were named accordingly. In some primary tumors, focal positivity for desmin or muscle-specific actin has been described.[4–7] However, MRTs have been considered to have no obvious evidence of a rhabdomyoblastic phenotype.[1,2,8–10] Cytogenetically, MRTs frequently have chromosome 22 alterations.[11,12] Truncated mutations or a homozygous deletion of *hSNF5/INI1*, located in 22q11.2, have been identified in MRT.[13]

Various cellular origins have been proposed for MRT, including neuroectodermal,[3,9,10] myogenic,[7] histiocytic,[14] neural[15] and epithelial.[16] Various authors have reported MRT as having multiphenotypic characteristics,[3,7,17–21] but the true origin of this enigmatic tumor remains unknown. Neural phenotypes of some MRTs have been shown by our laboratory.[10,17–19] In the present study, the expression of myogenic regulatory factors, such as MyoD, myogenin, myf-5 and myf-6, and skeletal muscle nicotinic acetylcholine receptor subunits, acetylcholine receptor-α (AchR-α), acetylcholine receptor-β (AchR-β), and acetylcholine receptor-γ (AchR-γ), were examined before and after 5-azacytidine (5-aza-CR)-induced differentiation in six MRT cell lines. Then, the expression of smooth muscle markers, α-smooth muscle actin (α-SMA), SM22 and SM-1, was investigated. We also examined the smooth muscle-like cell phenotype, using angiopoietin-1 (Ang-1), and angiopoietin-2 (Ang-2) as markers of pericytes, and megsin as a novel marker of mesangial cells.

Materials and Methods

Cell culture and differentiation. Six MRT cell lines were utilized in this study (TM87-16, STM91-01, TTC549, TTC642, YAM-RTK1 and TTC1240). Five of these (TM87-16, STM91-01, TTC549, TTC642 and TTC1240) were provided by Dr. Hiroyuki Shimada and Dr. Timothy J. Triche (Childrens Hospital Los Angeles, Los Angeles, CA). YAM-RTK1 was provided by Dr. Kanji Sugita (Yamanashi Medical University, Kofu). The diagnosis of primary MRT was confirmed with histopathology and electron microscopy. Aberrations of the *hSNF5/INI1* gene were detected in all six MRT cell lines.[22] TM87-16 was established from a pleural effusion, STM91-01 from the pulmonary metastasis of a renal MRT, TTC549 and TTC642 from extrarenal primary tumors, YAM-RTK1 from ascites, and TTC1240 from a brain rhabdoid tumor from a patient with renal MRT. Clinical data of the patients relevant to the establishment of the tumor-derived cell lines are summarized in Table 1. The MRT cell lines were passaged 16 to 23 times. The neuroblastoma cell line IMR-32 and the rhabdomyosarcoma cell line RD were used as controls and were purchased from the Health Science Research Resources Bank (Japan).

The cell lines were cultured in RPMI-1640 (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (ICN Biomedicals, Inc., Aurora, OH). To induce differentiation, cells were treated for 48 h with the same medium containing 10 μ*M* 5-aza-CR (Nacalai Tesque, Inc., Kyoto). After 5-aza-CR removal, the medium was changed to RPMI-1640 with 5% horse serum (HS) (Gibco) because it was previously found that low concentrations of HS enhanced myogenic differentiation.[23,24] Cells used for mRNA expression analysis were obtained on day 0 (before differentiation induction), 2 (2 days after 5-aza-CR addition), 4 (2 days after drug removal) and 6 (4 days after drug removal).

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Table 1. Clinical data of patients relevant to the establishment of the tumor-derived cell lines

	Age (month)	Sex	Primary site	Outcome	Origin of cell line	*hSNF5/INI1* gene
TM87-16	21	M	Retroperitoneal	Died	Pleural effusion	Homozygous deletion
STM91-01	8	M	Left kidney	Died	Lung metastasis	Partial deletion
TTC549	6	F	Hepatic mass	Died	Primary site	Homozygous deletion
TTC642	5	F	Neck mass	Died	Primary site	Nonsense mutation
YAM-RTK1	7	M	Left kidney	Died	Ascites	Partial deletion
TTC1240	9	F	Right kidney	Died	Brain tumor	Nonsense mutation

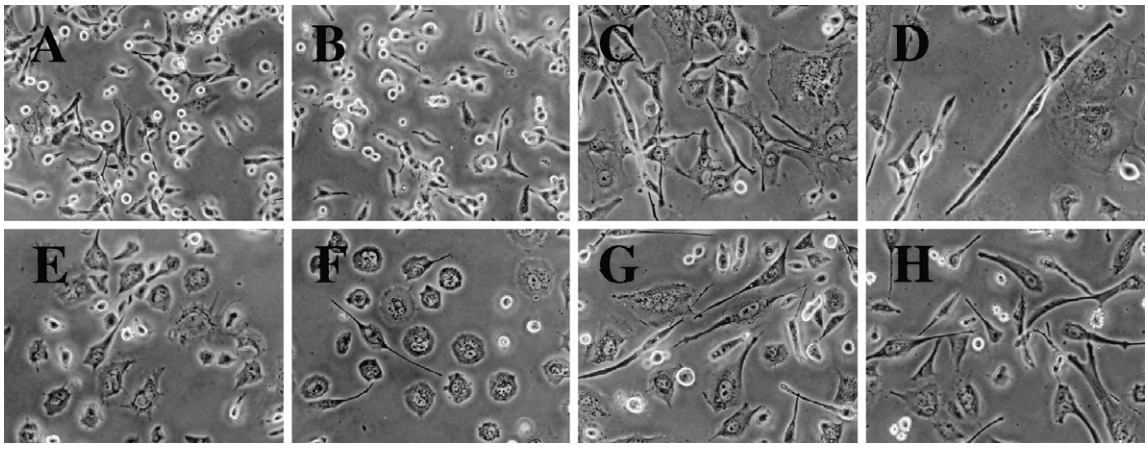

Fig. 1. Light microscopy of RD and TM87-16 cells before and after differentiation-induction with 5-aza-CR. RD cells on day 0 (A), 2 (B), 4 (C) and 6 (D) after differentiation-induction. TM87-16 cells on day 0 (E), 2 (F), 4 (G) and 6 (H) after differentiation-induction. Light microscopy demonstrated the production of elongated cytoplasmic processes after differentiation-induction in both cell lines.

Morphological analysis. All cells were rinsed and fixed with 4% paraformaldehyde for light microscopy. Cells were also fixed in 2% glutaraldehyde with 0.1 *M* phosphate-buffered saline (PBS, pH 7.4), post-fixed in 1% phosphate-buffered osmium tetroxide, dehydrated in an ethanol series, and embedded in Epon 812 (Polysciences, Inc., Warrington, PA) for electron microscopy.

RNA preparation and RT-PCR. Total RNA from each cell line was isolated using TRIzol (Gibco). Total RNA (10 μg) was incubated for 1 h at 37°C with 10 U of RNase-free DNase I (Stratagene, La Jolla, CA). Reverse transcription (RT) with random hexadeoxynucleotide primers (TaKaRa Shuzo, Shiga) was performed using 200 U of MMLV Reverse Transcriptase RNaseH (ReverTra Ace, Toyobo Co., Ltd., Osaka) at 42°C for 1 h after incubation at 30°C for 10 min. Diluted RT solutions were used as templates for PCR.

PCR primers. Forward (f) and reverse (r) primers were designed according to published sequences. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. All primers were obtained from Pharmacia (Tokyo). Primer sequences were: MyoD f (5′-CCAAATGTAGCAGGTGTAAC-3′) and r (5′-AGAGATAAATACAGCCCCAG-3′); myogenin f (5′-GTGGGCGTGTAAGGTGTGTA-3′) and r (5′-TGGTTGGGGTTGAGCAGGGT-3′); myf-5 f (5′-AAGTCCCAAACCAAGACAAC-3′) and r (5′-TCGAACAAGCTACCCTCAAT-3′); myf-6 f (5′-AGGAAGTGGTGGAGAAGTAA-3′) and r (5′-AAATAAAAGCCCAAAGCCGA-3′); AchR-α f (5′-AGTGCTGTGCCCTTGATTGG-3′) and r (5′-CTTGCTTTTCTCTGGATGGT-3′); AchR-β f (5′-GGGGAGGGAGGGAAGGACAG-3′) and r (5′-GAACACAGTAAGGGTCAGCA-3′); AchR-γ f (5′-CAGACCTACAGCACCAATGA-3′) and r (5′-GCTACGGAGGAGATGAGGAC-3′); α-SMA f (5′-GTGGCTATTCCTTCGTTACT-3′) and r (5′-GGCAACTCGTAACTCTTCTC-3′); SM-1 f (5′-CCGTCAAGTCCAAGTTCAAG-3′) and r (5′-GGTCTCGTTTCCTCGTCTGA-3′); SM22 f (5′-TCAGATGGGCAGCAACAGAG-3′) and r (5′-GGCTGGTTCTTCTTCAATGG-3′); Ang-1 f (5′-CCTACACTTTCATTCTTCCA-3′) and r (5′-GGTTTCTCTTCCTCTCTTTT-3′); Ang-2 f (5′-GAAGAAAGAAATGGTAGAGA-3′) and r (5′-TAGTTGGATGATGTGCTTGT-3′); megsin f (5′-ATGATCTCAGCATTGTGAATG-3′) and r (5′-ACTGAGGGAGTTGCTTTTCTAC-3′); and GAPDH f (5′-GCCAAAAGGGTCATCATCTCTG-3′) and r (5′-CATGCCAGTGAGCTTCCCGT-3′).

RT-PCR. PCR amplification was performed using *Taq* DNA polymerase (Toyobo) with the following PCR cycle conditions: denaturation at 94°C for 60 s, then 33 cycles of annealing for 90 s at 52°C (for myf-5, myf-6, AchR-α, AchR-β, Ang-1 and Ang-2), 54°C (for MyoD and myogenin), 56°C (for AchR-γ and α-SMA), or 58°C (for SM-1, SM22 and megsin), followed by 72°C for 60 s. Aliquots of PCR reaction products were separated by electrophoresis in 1.5% agarose gels (Nakalai Tesque) containing 0.2 mg/ml ethidium bromide (Sigma).

Semi-quantitative PCR. Band densities from each amplified product sample and GAPDH were determined by measuring fluorescence intensity using an AIC Epi-Light UV FA1100 (Aisin Cosmos R&D, Tokyo) and the accompanying Luminous Imager software (Aisin Cosmos). Density ratios (sample/GAPDH) were then calculated and used as relative values with respect to results for RD cells before differentiation induction.

PCR product sequencing. PCR-amplified products were directly sequenced using an ABI Prism 310 Genetic Analyzer and a "DYEnamic" ET terminator cycle sequencing kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Western blot analysis. Whole-cell extracts were prepared by direct lysis. Protein concentrations were measured using a Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Aliquots of total protein (50 μg) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene difluoride (PVDF) mem-

branes and probed with anti-Ang-1 goat polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-megsin mouse monoclonal antibody provided by Dr. Miyata (Tokai University School of Medicine, Kanagawa). Membranes were then incubated with alkaline phosphatase (AP)-conjugated secondary antibodies. The immunoblots were visualized using the CDP-star detection reagent (Amersham International plc, Little Chalfont, UK).

Immunocytochemistry. Cells were attached to silanized slides, which were then rinsed and fixed with a solution of 4% paraformaldehyde, 0.5% glutaraldehyde and 0.2% picric acid in 0.1 *M* PBS. Immunocytochemical staining was performed using 1:500 dilutions of anti-myf-6 protein rabbit polyclonal antibody (Santa Cruz), mouse monoclonal antibody to the smooth muscle myosin heavy chain (Santa Cruz), anti-Ang-1 goat polyclonal antibody (Santa Cruz), or anti-megsin mouse monoclonal antibody (Tokai University School of Medicine). Cells were incubated with peroxidase conjugated anti-rabbit, anti-goat or anti-mouse immunoglobulin (Envision+; Dako Co., Carpinteria, CA) for 30 min. Reaction products were visualized with 0.05% diaminobenzidine solution containing 0.01% hydrogen peroxide for 5 min at room temperature. All slides were then observed under a laser scanning confocal fluorescence microscope (BioRad MRC 600, Bio-Rad Microscience, Ltd., Hemel Hempstead, UK).

Results

Light microscopy. Light microscopy of TM87-16 and RD cells after differentiation-induction with 5-aza-CR demonstrated the production of elongated cytoplasmic processes (Fig. 1). Light microscopy of TTC642 and TTC1240 after 5-aza-CR differentiation-induction also demonstrated elongated cytoplasmic process production, while the cell lines STM91-01, TTC549, YAM-RTK1 and IMR-32 showed no marked change following 5-aza-CR treatment (data not shown).

Electron microscopy. None of the six MRT cell lines exhibited skeletal muscle structures by electron microscopy, even after

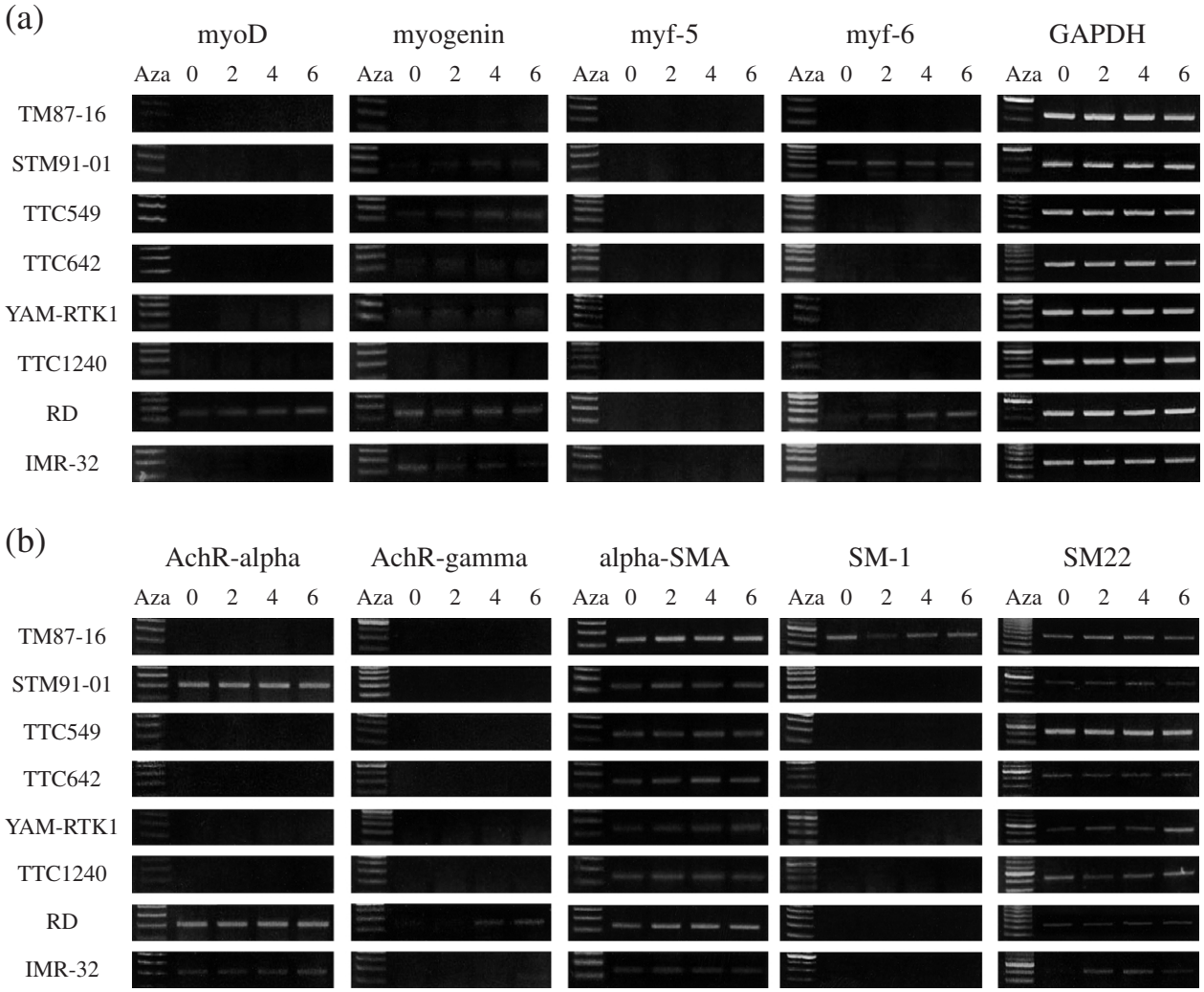

Fig. 2. (a) RT-PCR analysis of expression of the MyoD family and the housekeeping gene *GAPDH* in six MRT cell lines on days 0, 2, 4 and 6 after differentiation-induction with 5-aza-CR. None of the six MRT cell lines expressed MyoD or myf-5 mRNA. MyoD mRNA expression and up-regulation were observed only in RD cells. Myogenin mRNA was expressed in RD cells. Low expression of myogenin mRNA was observed in STM91-01, TTC549, YAM-RTK1 and IMR-32 cells. Myf-6 mRNA expression was detected in STM91-01 and RD cells, and myf-6 mRNA up-regulation was observed in RD cells. (b) RT-PCR analysis of AchR subunit genes (*AchR-α* and *AchR-γ*), α-SMA, SM-1 and SM22 in six MRT cell lines on days 0, 2, 4, and 6 after differentiation-induction with 5-aza-CR. AchR-α mRNA expression was observed in STM91-01, RD and IMR-32 cells (low expression). AchR-γ mRNA up-regulation was observed in RD cells. All MRT and RD cells expressed α-SMA and SM22 mRNA. Low expression of α-SMA and SM22 mRNA was observed in IMR-32 cells. SM1 mRNA was only observed in TM87-16 cells.

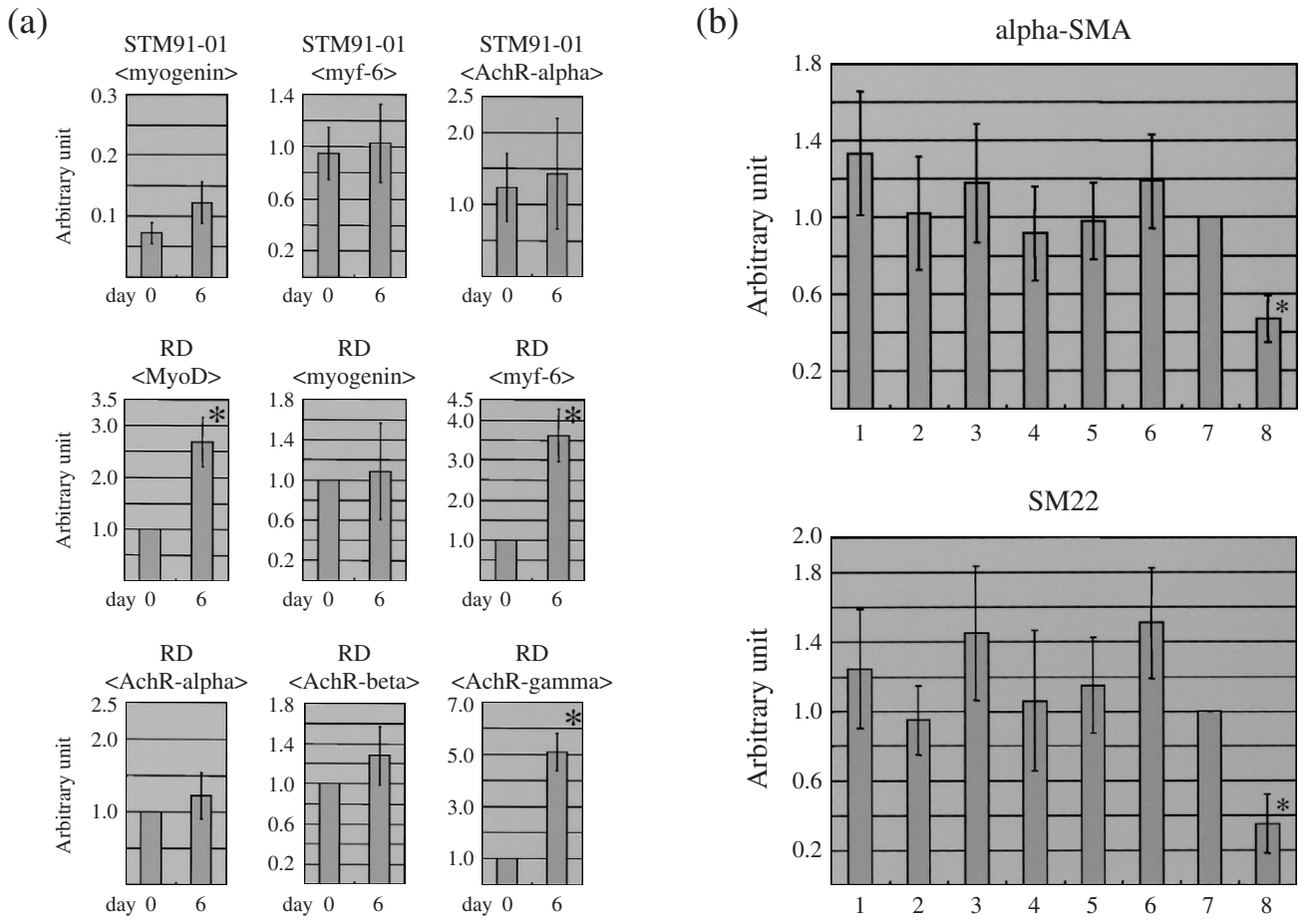

Fig. 3. (a) Semi-quantitative RT-PCR analysis before and after differentiation-induction. Representative results from experiments using STM91-01 and RD cell lines are shown. Band density ratios (sample/GAPDH) were calculated as relative values with respect to RD cells before differentiation-induction. * $P<0.05$ relative expression of MyoD, myf-6 or AchR-γ mRNA in RD on day 0 versus day 6 after 5-aza-CR treatment. (b) Semi-quantitative RT-PCR analysis of α-SMA and SM22. Representative results from experiments using the six MRT, RD and IMR-32 cell lines are shown. Lane 1, TM87-16; lane 2, STM91-01; lane 3, TTC549; lane 4, TTC642; lane 5, YAM-RTK1; lane 6, TTC1240; lane 7, RD; lane 8, IMR-32. Band density ratios (sample/GAPDH) were calculated relative to RD cells before differentiation-induction. * $P<0.05$ relative expression of the six MRT cell lines or the RD cell line versus the IMR-32 cell line.

differentiation-induction with 5-aza-CR (data not shown).

mRNA for myogenic regulatory factors. mRNA for MyoD or myf-5 was not detected in any of the MRT cell lines. Low myogenin mRNA expression was observed in STM 91-01, TTC549 and YAM-RTK1 cell lines. After differentiation with 5-aza-CR, mild up-regulation of myogenin mRNA expression was observed in STM91-01 and TTC549 cells. However, the increase in fluorescence intensity was not significant. Myf-6 mRNA expression was also detected in STM91-01 cells. The rhabdomyosarcoma cell line RD expressed MyoD, myogenin and myf-6 mRNA, with significant up-regulation of MyoD and myf-6 after 5-aza-CR treatment. Myf-5 mRNA expression was not detected. The neuroblastoma cell line IMR-32 weakly expressed myogenin mRNA, but not any of the other tested markers (Fig. 2a and Fig. 3a).

mRNA for AchR subunits. AchR-β and AchR-γ mRNA was not detected in any of the six MRT cell lines and AchR-α mRNA expression was observed only in STM91-01 cells. AchR-α and AchR-β mRNA were expressed in RD and IMR-32 cells, with significant up-regulation of AchR-γ mRNA in RD cells after 5-aza-CR treatment (Fig. 2b [data for AchR-β not shown] and Fig. 3a).

mRNA for smooth muscle markers. All six MRT cell lines expressed mRNA for α-SMA and SM22, as did the RD and IMR-32 cell lines, with IMR-32 cells showing significantly weaker α-SMA and SM22 mRNA expression compared to the other cell lines. SM-1 mRNA was observed only in the TM87-16 cell line (Fig. 2b and Fig. 3b).

mRNA for markers of pericytes and mesangial cells. Ang-1 mRNA was expressed in the TM87-16, STM91-01, TTC1240 cell lines and in mesangial cells (control). Ang-1 mRNA was not detected in TTC549, TTC642, YAM-RTK1, RD or IMR-32 cells. Ang-2 mRNA was not detected in any of the six MRT cell lines or in mesangial cells, but it was expressed in RD and IMR-32 cells. Megsin mRNA was only expressed in STM91-01 cells and positive control mesangial cells. In contrast, YAM-RTK1, RD, Wilms' tumor tissue, and fibroblasts did not express detectable levels of megsin mRNA (Fig. 4).

Western blotting. Western blotting demonstrated the presence of Ang-1 protein in TM87-16, STM91-01 and TTC1240, and the expression of megsin in STM91-01 (Fig. 5).

Immunocytochemistry. A positive result was defined as expression in more than 80% of the cells.[3] Immunocytochemistry results correlated well with the RT-PCR findings. Immunocytochemical staining using the rabbit polyclonal antibody to myf-6 protein demonstrated the presence of myf-6 protein in STM91-01 and RD cells, while smooth muscle myosin heavy chain protein was detected in TM87-16 cells (Fig. 6a). Ang-1 protein was expressed in TM87-16, STM91-01 and TTC1240 cells, with megsin protein detected only in STM91-01 cells (Fig. 6b).

Discussion

MRT has been reported to have characteristics of multiphenotypic diversity.[7, 17–21] Recently, Sugimoto *et al.*[21] reported a smooth muscle phenotype in an MRT cell line. Steahelin *et al.*[25] reported MyoD expression in bone marrow metastasis of extrarenal MRT. However, it is difficult to evaluate the significance of a muscle phenotype in MRT using a bone marrow sample. Thus, it is still controversial whether MRT cells have a skeletal muscle, smooth muscle or smooth muscle-like cell phenotype, as found in pericytes and mesangial cells. To determine the skeletal muscle phenotype, markers for MyoD, myogenin, myf-5, myf-6, AchR-α, -β and -γ were utilized. To investigate the smooth muscle phenotype, expression of α-SMA, SM-1 and SM22, was examined. Among smooth muscle-like cell phenotypes, we chose Ang-1 and Ang-2 for the pericyte phenotype

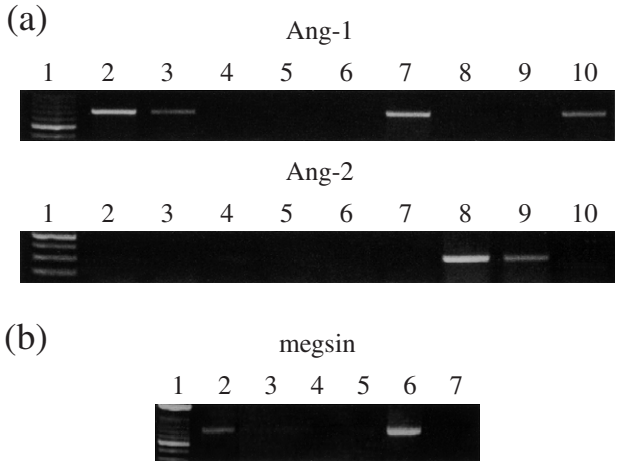

Fig. 4. A. RT-PCR analysis of Ang-1 and Ang-2 mRNA expression. Lane 1, marker; lane 2, TM87-16; lane 3, STM91-01; lane 4, TTC549; lane 5, TTC642; lane 6, YAM-RTK1; lane 7, TTC1240; lane 8, RD; lane 9, IMR-32; lane 10, mesangial cells. Ang-1 mRNA was expressed in TM87-16, STM91-01, TTC1240 and mesangial cells. Ang-2 mRNA was expressed only in RD and IMR-32 cells. B. RT-PCR analysis of megsin mRNA expression. Lane 1, marker; lane 2, STM91-01; lane 3, YAM-RTK1; lane 4, RD; lane 5, Wilms' tumor tissue; lane 6, mesangial cells; lane 7, fibroblasts. Megsin mRNA was expressed in STM91-01 and mesangial cells.

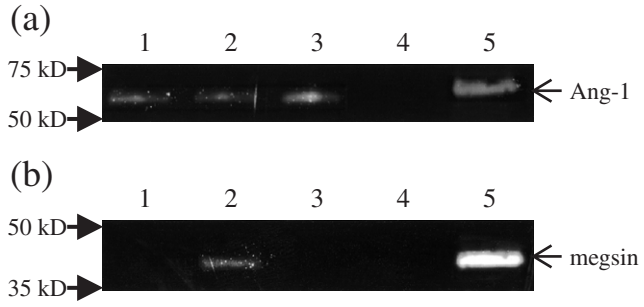

Fig. 5. Western blotting of Ang-1 protein (A) and megsin protein (B). Lane 1, TM87-16; lane 2, STM91-01; lane 3, TTC1240; lane 4, RD; lane 5, mesangial cells. Ang-1 protein was detected in TM87-16, STM91-01, TTC1240 and mesangial cells. Megsin protein was detected in STM91-01 and mesangial cells.

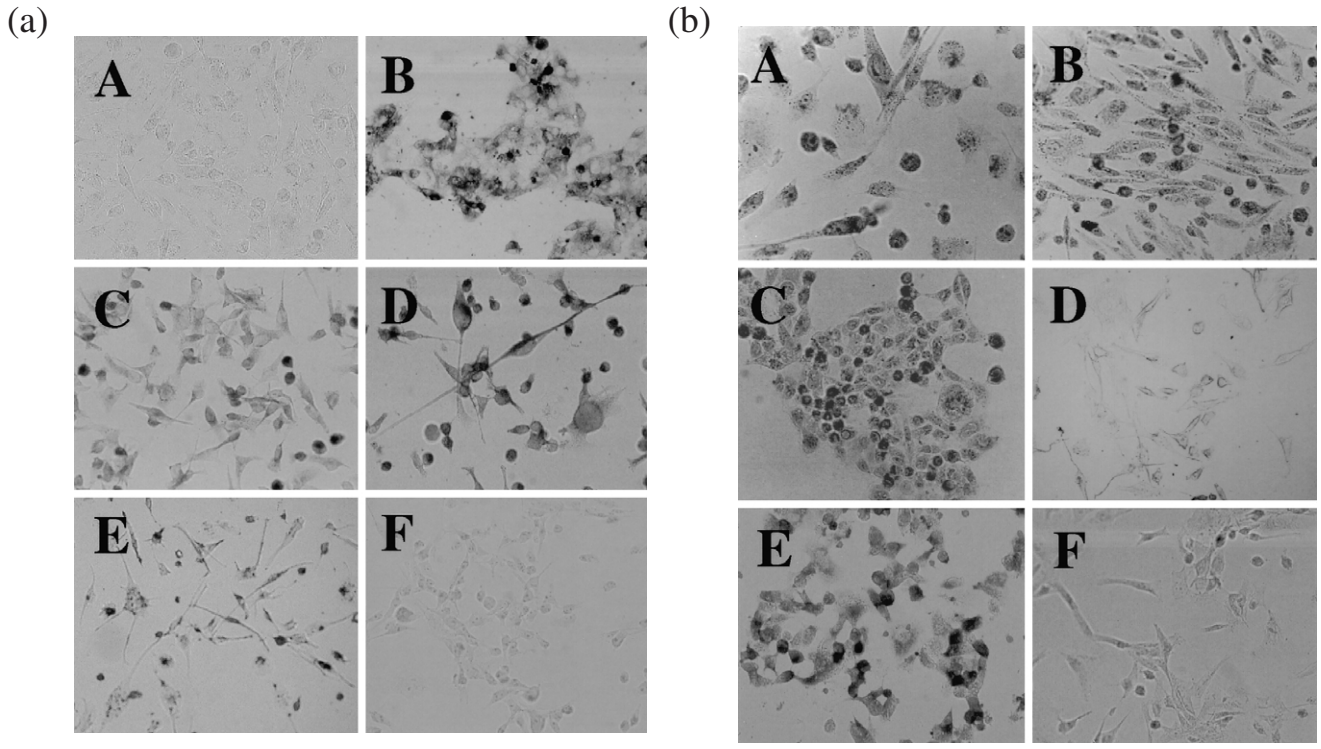

Fig. 6. (a) Immunocytochemistry of the myf-6 protein in TM87-16, STM91-01 and RD cells on day 0 and day 6 after 5-aza-CR treatment. A. TM87-16 on day 0, B. STM91-01 on day 0, C. RD on day 0, D. RD on day 6. Immunocytochemistry for smooth muscle myosin heavy chain protein in TM87-16 and RD cells on day 6 after 5-aza-CR treatment. E. TM87-16, F. RD. STM91-01 and RD cells expressed myf-6 protein. Myf-6 protein was not detected in TM87-16 cells. TM87-16 cells expressed smooth muscle myosin heavy chain protein on day 6. Smooth muscle myosin heavy chain protein was not detected in RD cells on day 6. (b) Immunocytochemistry for Ang-1 protein in TM87-16 (A), STM91-01 (B), TTC1240 (C) and RD (D) cells, and for megsin protein in STM91-01 (E) and RD (F) cells. TM87-16, STM91-01 and TTC1240 cells expressed detectable Ang-1 protein, while RD cells did not. STM91-01 cells expressed detectable levels of megsin protein, while RD cells did not.

and megsin for the mesangial phenotype. In this study, six MRT cell lines were used to reduce the possibility of contamination with connective tissue. The results for these six MRT cell lines was confirmed with both histopathology and electron microscopy.[3, 10, 17–20] Aberration of the *hSNF5/INI1* gene was also detected in the six MRT cell lines.[22]

5-Aza-CR was utilized to examine the expression of several muscle markers. 5-aza-CR is a potential muscle inducer and has been used on a variety of cell types, including mouse embryo fibroblasts, rhabdomyosarcoma cells, and some mesenchymal cells in several *in vitro* studies.[23, 24, 26–28]

Myogenic regulatory factors (MyoD, myogenin/myf-4, myf-5 and myf-6/MRF4/herculin) are usually detected during the course of normal muscle development. MyoD and myf-5 have a crucial role in the determination step for commitment of proliferating somatic cells to the myogenic lineage.[29, 30] These committed cells then proliferate and further differentiate into myocytes or myofibers based on myogenin and myf-6 activity.[29, 30] MyoD family members were considered to regulate AchR expression in muscle cells.[31, 32] The AchR protein is a pentamer composed of four subunits. In mammalian muscle development, AchR contains α-, β-, γ- and δ-subunits.[33]

The results of our differentiation study showed that three of the MRT cell lines, STM91-01, TTC549 and YAM-RTK1, expressed low levels of myogenin mRNA. STM91-01 also expressed myf-6 and AchR-α. However, MyoD and myf-5 were not detected in the six MRT cell lines, even after 5-aza-CR differentiation-induction. Thus, our study indicated that MRT cells lack definitive skeletal muscle phenotypes due to the lack of MyoD and myf-5 expression. In accordance with this observation, there was no evidence of skeletal muscle structures by electron microscopy, even after cellular differentiation.

α-SMA is a well-characterized smooth muscle marker, although it is occasionally expressed in non-smooth muscle cells, including striated muscle cells, myofibroblasts, and some tumors (e.g., gastrointestinal stromal and solitary fibrous tumors).[34–37] SM22 is considered to be a more exclusive smooth muscle marker, with the exception of its temporary expression in striated muscle.[34] SM-1 is a smooth muscle myosin heavy chain isoform and one of the most reliable smooth muscle markers.[34] However, SM-1 expression is restricted to slightly mature smooth muscle.[34] In this study, α-SMA and SM22 were significantly expressed in all six MRT cell lines, as well as RD. Considering the positive expression of the smooth muscle phenotype in some neuroblastoma cell lines,[38] MRT cell lines could have a smooth muscle phenotype of the very early developmental stage. However, SM-1 mRNA expression was detected only in the TM87-16 cell line. TM87-16 and TTC642 had been reported to show an immature neural phenotype.[3, 12, 17–19] As a result, TM87-16 has dual phenotypes of neural and slightly mature smooth muscle cells.

A unique group with an immature smooth muscle phenotype has been reported over the last decade.[39–41] These smooth muscle-like cells include pericytes, mesangial cells, hepatic stellate cells, and other cell types.[39–41] They have some similarity in their morphology, function and biochemical repertoire, regardless of their location.[39–41] It is known that smooth muscle-like cells express α-SMA, SM22 and platelet-derived growth factor receptor (PDGFR).[34, 39, 42] Nonetheless, in our preliminary study, PDGFR-α and -β were expressed in all the tumor cell lines and were considered to be not specific for smooth muscle-like cells.

Although smooth muscle-like cells lack a common and specific marker in the strict sense,[39, 43, 44] expression of Ang-1, but not Ang-2, was recently reported as a marker of microvessel pericytes or large vessel smooth muscle cells.[42] Ang-1 stimulates endothelial cell sprout formation *in vitro* and increases girth and endothelial stability, whereas Ang-2 antagonizes Ang-1 signaling and destabilizes the endothelium.[43] As Ang-2 induces destabilization of vessels, rendering them plastic and more responsive to VEGF-mediated growth, Ang-2 is generally thought to contribute to tumor angiogenesis.[43] In our preliminary study, all of the two rhabdomyosarcoma cell lines and the five neuroblastoma cell lines expressed Ang-2, while none expressed Ang-1 (data not shown). In this study, Ang-1 mRNA was expressed in TM87-16, STM91-01 and TTC1240 cells, while none of the six MRT cell lines expressed Ang-2 mRNA. Therefore, STM91-01 and TTC1240 were considered to have a possible pericyte phenotype.

Megsin was recently identified by Miyata *et al*.[45] as a novel marker for glomerulus mesangial cells. Previous studies involving STM91-01 cells failed to show expression of any specific gene suggesting a particular phenotype.[3, 10, 17–20] We examined megsin expression in the STM91-01 cell line, which was established from renal MRT, expressing myf-6 and having a possible pericyte phenotype. An important relationship between myf-5 or myf-6 mRNA expression and mesangial cell development was recently reported.[40] Our results showed that STM91-01 expressed megsin, which was detected by RT-PCR, western blotting, and immunocytochemistry. These data support the possibility that STM91-01 has a mesangial cell phenotype, corresponding to a type of pericyte.[39, 41] However, YAM-RTK1, also established from renal MRT, did not express Ang-1 or megsin mRNA. As there are many types of cells classified as smooth muscle-like cells even in the kidney, YAM-RTK1 may have the phenotype of another renal smooth muscle-like cell, such as a renal interstitial cell.

The six MRT cell lines expressing α-SMA and SM22 have a possible smooth muscle or smooth muscle-like cell phenotype. We have proposed that MRT is of neuroectodermal origin in a broad sense.[3, 10] Once smooth muscle cells and smooth muscle-like cells were considered to be of mesodermal mesenchyme origin.[34, 44, 46] However, recently a neuroectodermal origin has been proposed.[21, 46–48] Expression of a neural, smooth muscle and/or smooth muscle-like cell phenotype in MRT would support the neuroectodermal origin of MRT.[3, 9, 10, 21]

In conclusion, our study suggested that MRT cells did not have a definitive skeletal muscle phenotype, even after differentiation-induction. However, TM87-16 appeared to have a neural/smooth muscle dual phenotype, and STM91-01 has a mesangial phenotype. Our data showed that some MRT cell lines exhibit a smooth muscle or smooth muscle-like cell phenotype, like that of pericytes and/or mesangial cells. Neural, smooth muscle and/or smooth muscle-like cell phenotypes in MRTs supports our proposal that MRT can be regarded as a primitive neuroectodermal tumor (PNET) in the broad sense.[3, 10]

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