Loss of p16/Ink4a drives high frequency of rhabdomyosarcoma in a rat model of Duchenne muscular dystrophy.

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Running head: RHABDOMYOSARCOMA IN MUSCULAR DYSTROPHY
ABSTRACT

Rhabdomyosarcoma (RMS) is an aggressive type of soft tissue sarcoma, and pleomorphic RMS is a rare subtype of RMS found in adult. p16 is a tumor suppressor which inhibits cell cycle. In human RMS, p16 gene is frequently deleted, but p16-null mice do not develop RMS. We reported that genetic ablation of p16 by the crossbreeding of p16 knock-out rats (p16-KO rats) improved the dystrophic phenotype of a rat model of Duchenne muscular dystrophy (Dmd-KO rats). However, p16/Dmd double knock-out rats (dKO rats) unexpectedly developed sarcoma. In the present study, we raised p16-KO, Dmd-KO, and dKO rats until 11 months of age. Twelve out of 22 dKO rats developed pleomorphic RMS after 9 months of age, while none of p16-KO rats and Dmd-KO rats developed tumor. The neoplasms were connected to skeletal muscle tissue with indistinct borders and characterized by diffuse proliferation of pleomorphic cells which had eosinophilic cytoplasm and atypical nuclei with anisokaryosis. For almost all cases, the tumor cells immunohistochemically expressed myogenic markers including desmin, MyoD, and myogenin. The single cell cloning from tumor primary cells gained 20 individual Pax7-negative MyoD-positive RMS cell clones. Our results demonstrated that double knock-out of p16 and dystrophin in rats leads to the development of pleomorphic RMS, providing an animal model that may be useful to study the developmental mechanism of pleomorphic RMS.

Key words: Desmin, Muscular dystrophy, MyoD, Rhabdomyosarcoma, skeletal muscle
INTRODUCTION

Skeletal muscle is composed of multinucleated and terminally differentiated myofibers. Skeletal muscle is a highly regenerative tissue and maintains its homeostasis by rapidly regenerating when the myofibers are injured. Regeneration of myofibers depends on muscle progenitor cells called satellite cells, which reside between sarcolemma and myofiber [28, 29, 44]. Muscle regeneration is regulated by four basic helix-loop-helix (bHLH)-type transcription factors, Myf5, MyoD, myogenin, and MRF4, which are essential for the expression of skeletal muscle-specific proteins [27]. Satellite cells are normally quiescent, but upon injury of myofibers, they are activated and proliferate, then differentiate to myoblasts [33]. Activated satellite cells become myoblasts expressing MyoD and/or Myf5, and divide several times [9, 33]. Subsequently, the myoblasts exit from cell cycle, and differentiate to myocytes expressing myogenin and MRF4 [9, 33]. These myocytes eventually fuse each other to form multinucleated myotubes [9, 33]. Then, muscle regeneration is completed by maturation of myotubes to myofibers [9, 33].

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma of childhood in humans [30]. RMS often occurs in various parts of the body such as head, neck, genitourinary system, and soft tissue regions [20]. Tumor cells which comprise RMS express myogenic markers including desmin, MyoD, myogenin [23, 38, 47], and their morphology varies from undifferentiated round cells to terminally differentiated myoblasts with cross striations [37]. RMS is pathologically classified into 4 subtypes: embryonal, alveolar, pleomorphic, and a newer rare entity, spindle/sclerosing RMS [2]. Embryonal and alveolar RMS are mainly developed in children, whereas pleomorphic RMS is common in adult [20]. The prognosis of embryonal RMS is favorable, while
those of alveolar and pleomorphic RMS are unfavorable [20]. Although what determine the subtype of RMS remains unclear, induction of mutations in various differentiation stage of myogenic cells; from satellite cells to maturing myoblasts, can result in the development of RMS of all histological types [43]. Although RMS cells are mainly derived from myogenic cells, recent reports revealed that RMS can also originate from other cell types of mesenchymal or endothelial lineages [14, 17]. In veterinary medicine, RMS have been most commonly diagnosed in dogs, but a variety of species is involved [11]. In mice and rats, although RMSs can be experimentally induced [62], spontaneous RMSs are very rare [40, 39, 52]. There are a few reported cases for RMSs in both young and aged rats [10, 21, 31, 53].

p16, also known as INK4a, is a cyclin-dependent kinase (CDK) inhibitor encoded by CDKN2A gene. In response to irreversible DNA damage, including oncogene activation or oxidative stress, p16 is upregulated and lead to cell cycle arrest [26]. p16 is also acting as a tumor suppressor. Germ line deletion of CDKN2A can cause familial melanoma in human and tumor-prone phenotype in mouse [18, 48]. It has also been reported that loss or inactivation of p16 expression occurs in many spontaneous human tumors as results of deletion, epigenetic changes in the promoter region, or abnormal splicing of CDKN2A gene [41, 42]. Although RMS cells in human often lack p16 expression [19, 54], p16-deficient mice do not develop RMS, whereas various types of tumors other than RMS could be developed [48]. Thus, it is thought that deletion of p16 is related to an onset of RMS but not sufficient to cause RMS.

Dystrophin is a protein encoded by DMD gene on the X chromosome. Dystrophin composes dystrophin-glycoprotein complex and links the cytoskeleton to the extracellular matrix in skeletal muscle and cardiac muscle [7]. Deletion of DMD gene
cause Duchenne muscular dystrophy (DMD) [32]. In DMD patients, the cell membrane of myofibers is fragile, and mechanical stress damages myofibers, resulting in their repeated necrosis and regeneration. When the rate of necrosis exceeds that of regeneration, muscle atrophy as well as loss of muscle strength occurs.

We previously generated Duchenne muscular dystrophy model rats (Dmd-KO rats) with out-of-frame mutations in *Dmd* gene [35]. The skeletal muscles of Dmd-KO rats show repeated necrosis and regeneration, and typical pathologies seen in DMD patients, such as progressive muscle atrophy, fibrosis, and adipogenesis in skeletal muscles [35, 51]. In addition, we found that p16, cellular senescent marker, was significantly upregulated after 6 months of age in Dmd-KO rats, and the *p16* and *Dmd* double knock-out rats (dKO rats) showed improved muscle regeneration, fibrosis, and adipogenesis, suggesting that suppressing cellular senescence by p16 deletion ameliorate the dystrophic phenotype of Dmd-KO rats [51]. However, some of dKO rats developed sarcoma at later stage of their life. In this study, we aimed to characterize the sarcoma observed in dKO rats.

**MATERIALS AND METHODS**

**Animals**

Male wild-type (WT), *Dmd* knock-out (Dmd-KO), *p16* knock-out (p16-KO), and double knock-out (dKO) rats of the Wistar Imamichi strain were generated as described in our previous studies [35, 51]. The numbers of rats used and analyzed in the present study are shown in Table 1. Animals were maintained under controlled environmental conditions at 23°C with a light/dark (12/12 h) cycle (lights on at 8 am), and food and water were provided ad libitum. Animals developing tumor were euthanized by exsanguination under deep anesthesia by isoflurane, and tumor tissues were excised.
All animal experiments performed in this study were in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tokyo and were approved (P18-125) by the Institutional Animal Care and Use Committee of the University of Tokyo.

Histological analyses

The tumor tissues were excised and fixed in 10% formalin. Paraffin-embedded sections (2-4 µm) were prepared with rotary microtome (PR-50; Yamato Kohki Industrial, Saitama, Japan), and stained with hematoxylin and eosin and phosphotungstic acid hematoxylin, or used for immunohistochemistry. Slides were observed under the microscope (BX53, Olympus, Tokyo, Japan) equipped with a digital camera (DP73, Olympus). Mitotic figures for each case were counted in randomly chosen 10 high-power fields (x 400, HPF) and expressed as sum of the counts.

Cell culture

The tumor tissues were excised and cut into approximately 1 mm cubes and digested with 0.05% Trypsin-EDTA (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) / Dulbecco’s Modified Eagle Medium (DMEM; Gibco™) at 37°C for 30 min. The digested cells were centrifuged at 2,150 x g for 3 min, and the supernatant was removed. The cell pellet was resuspended in DMEM supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France) (10% FBS/DMEM), 50 unit/ml penicillin, 50 µg/ml streptomycin (Penicillin-Streptomycin; Gibco™) and 50 µg/ml gentamicin (Gibco™), and filtered through a cell strainer (70 µm, BD Falcon). The cells were plated on poly-L-lysine- and fibronectin-coated 48-well plates (IWAKI, Chiba, Japan), and cultured at 37°C and 5% CO₂. Immunostaining was performed 1 day after seeding. Where applicable, some cells were stored at -80°C using CELLBANKER2® (ZENOAQ,
Cloning of tumor cells

RMS-derived primary cultured cells preserved at -80°C were plated on poly-L-lysine- and fibronectin-coated 48-well plates (IWAKI) and incubated for 2 days. After incubation, cells were detached off the plate by trypsinization and plated on 96-well plates (IWAKI) at a density of 1-2 cells per well. Wells with one cell per well were selected on the next day, and wells with one colony per well were further selected 4 days later. Selected cells were passaged when confluent and incubated until sufficient cells were obtained.

Immunostaining

For immunostaining of the tissue sections, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 5 min, and antigen retrieval was performed. The sections were blocked for 30 min in 8% skim milk/Tris-buffered saline (TBS) at 37°C. Then, they were incubated with primary antibodies diluted with TBS overnight at 4°C. The following primary antibodies were used: anti-MyoD antibody (1:100, mouse monoclonal, A5.8; Novocastra, Newcastle upon Tyne, UK), anti-desmin antibody (1:400, mouse monoclonal, DE-U-10; Sigma-Aldrich, St. Louis, MO, USA), and anti-myogenin antibody (1:100, mouse monoclonal, F5D; Developmental Studies Hybridoma Bank). After washing with TBS, the sections were incubated with Dako EnVision+ System- HRP Labelled Polymer anti-mouse secondary antibody (Dako, Tokyo, Japan) at 37°C for 40 min and washed with TBS. The signal was visualized by diaminobenzidine as a substrate and the cell nuclei were counterstained with hematoxylin.

For immunostaining of the cultured cells except for desmin, they were fixed with 4%
paraformaldehyde (PFA)/phosphatate-buffered saline (PBS) for 15 min. For immunostaining of desmin, the cells were fixed with methanol for 10 min. After washing with PBS, cells were blocked with 5% normal goat serum (NGS)/PBS containing 0.1% triton X-100 for 10 min. Then, they were incubated with primary antibodies diluted with 5% NGS/PBS overnight at 4°C. The following primary antibodies were used: anti-Pax7 antibody (1:100, mouse monoclonal; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), anti-MyoD antibody (1:100), anti-desmin antibody (1:400), and anti-myogenin antibody (1:100). To detect the labeled cells, they were incubated with Alexa-conjugated secondary antibodies (1:400).

RESULTS

All rats were maintained under normal conditions until 11 months of age and compared their tumorigenesis. None of WT, p16-KO, and Dmd-KO rats developed tumor, while 12 out of 22 dKO rats developed tumor (Table 1). Tumors from the 12 dKO rats were pathologically analyzed for diagnosis. Gross postmortem examination revealed that the majority of tumors were located in forelimbs or hindlimbs (7/12 cases), and others in neck, cheek, or peritoneal cavity (Table 2). Among 12 cases, all the tumors were located subcutaneously and connected to skeletal muscle (Fig. 1A) and were found at 9 months or older (Table 2). Histologically, diffuse proliferation of tumor cells replaced the skeletal muscle tissue (Fig. 1B). The tumor tissues were composed of highly pleomorphic cells with higher cellularity and irregular bundle arrangement (Fig. 1C). Sheets of round-to-ovoid or spindle cells with eosinophilic cytoplasm were observed, and the tumor cells displayed significant pleomorphism and atypia, such as anisokaryosis, multiple nuclei and prominent nucleoli (Figs. 1D, E). The average number of mitotic figures in 12 cases was $21 \pm 2.3$ (per 10 HPFs) (Table 2). Multinucleated giant cells and aberrant mitosis were occasionally observed (Figs. 1D,
Cross striations were rarely present in spindle cells in 5/12 cases (Fig. 1F). The results of immunohistochemical features are summarized in Table 3. The neoplastic cells were immunopositive for both desmin and myogenin in all cases, and immunopositive for MyoD in all cases except for case 10 (Fig. 2A, B, Supplementary fig. 1). Based on the aforementioned findings, the tumors were diagnosed as pleomorphic RMS in all 12 cases.

In order to gain insight to the nature of RMS developed in dKO rats, it is advantageous to isolate and characterize tumor cells in vitro. Cells were enzymatically isolated from the tumor tissues of case 1, which contained desmin-, MyoD-, and myogenin-positive cells (Fig. 2A). One day after cell isolation, immunocytochemistry was performed for myogenic markers, desmin, as a muscle-specific marker, and Pax7, MyoD, and myogenin, which are expressed in myogenic cells at early, middle, and late stages of myogenesis respectively [33]. Nearly 100% of the cells were desmin-positive, while the percentage of Pax7 and MyoD positive cells was about 40% to 50%. There were almost no myogenin-positive cells (Fig. 3A and 3B). For further characterization of RMS-derived myogenic cells, obtained cells were analyzed after establishing single cell-derived clones. Twenty individual clones were obtained after 3 passages, and all clones were positive for desmin and MyoD, while they were negative for Pax7 and myogenin (Fig. 4, Table 4).

**DISCUSSION**

In this study, more than 50% of dKO rats developed RMS until 11 months old, while none of p16-KO and Dmd-KO rats developed tumor, suggesting that lack of p16 or dystrophin alone does not induce tumor development, but defects in both increase the incidence of tumor formation.
Deletion of dystrophin might be the leading cause of the development of RMS observed in the present study. Interestingly, all of 12 tumors found in dKO rats were RMS. p16 is known as a tumor repressor [26]. In response to various stressors, loss of p16 function increases tumorigenesis [26, 48]. p16 is inactivated in most tumor including RMS [19, 42], and p16 knock-out mice develop not only soft tissue sarcomas, but also lymphomas and melanomas [49]. Thus, deficiency of p16 may not seem to be a leading cause that restricts the type of tumor to RMS in dKO rats. It has been reported that lack of dystrophin increases the incidence of RMS in mice [8, 16]. Ninety percent of mdx mice lacking p53, a cell cycle inhibitor, developed RMS by 5 months of age, and induction of recurring myofiber necrosis in p53-deficient mice by repeated injection of myotoxin, cardiotoxin, to their skeletal muscle also caused a high incidence of RMS [6]. In DMD, prolonged damage of skeletal muscle causes chronic inflammation [5, 60], and this can support tumorigenesis by inducing DNA damage [34]. In fact, previous studies suggest that the accumulation of inflammation and DNA damage attributes to RMS development in mdx mice [6, 45]. Thus, repeated myofiber necrosis and inflammation in skeletal muscle of dKO rats due to the lack of dystrophin [35, 51] would have caused RMS in the p16-deficient background in this study. It should be also mentioned that dystrophin regulates asymmetric division of satellite cell upon myogenesis, and the lack of dystrophin cause such abnormal division as centrosome amplification [15]. This report suggested that dystrophin has a role, additionally to its original role in maintaining membrane integrity, in regulating cell cycle. Supporting this hypothesis, dystrophin has been reported to act as a tumor suppressor in myogenic sarcomas [61]. Therefore, although it is unknown at present whether the absence of dystrophin itself or chronic inflammation caused by the lack of dystrophin attributes the incidence of RMS, the genetic background of dKO rats, in which both dystrophin and p16 are lacked, could be
associated with development of RMS in the present study.

As in humans, pleomorphic RMS appears to be the least common form in animals [11]. Although virtually all RMSs may show some cellular pleomorphism, only tumors lacking any areas of embryonal or alveolar pattern should be diagnosed as the pleomorphic variant in humans and animals [11]. In the present study, the embryonal or alveolar morphology was not observed in all cases, and thus, it was indicated that RMS observed in dKO rats is pleomorphic type. Based on the widely accepted categorization of RMS, embryonal RMS is thought to be both myogenic and non-myogenic origin, and genetically heterogeneous [2, 17, 43], while alveolar RMS is often driven by the expression of Pax3-FOXO1 or Pax7-FOXO1, produced by gene translocation [1, 2, 24, 12]. On the other hand, the driving mutations of pleomorphic RMS are almost completely unknown so far. Future studies such as tracing myogenic and non-myogenic progenitor cells during RMS development in dKO rats will be shed light on this issue.

Without exception, RMS development in dKO rats was observed after 9 months of age in the present study. Cellular senescence is considered to be a physiological system to repress tumor development. In our previous study, Dmd-KO rats showed persistently increased expression of p16 in skeletal muscle after 6 months of age, indicating the accumulation of senescent cells [51]. Since chronic inflammation is known to induce either cellular senescence or tumor development and is consistently seen in the skeletal muscle of Dmd-KO rats as early as from 1 month of age, cellular environment in their muscle could be preferable to induce both cellular senescence and tumor development. Thus, the expression of p16 is thought to be act as a key to specify the cells to either fate. As the dKO rats lack p16 expression which is seen after 6 months of age in Dmd-KO rats, this would have forced the fate of cells in their skeletal
muscle to tumor development, and, as a result, RMS development became apparent after 9 months of age.

RD cell line, which was established from human RMS, expresses MyoD and myogenin [56]. This contrasts with the results that the cells isolated from RMS of dKO rats in the present study were negative for myogenin expression. Although myogenin expression was confirmed in all 12 RMS analyzed by immunohistochemistry, RMS-derived cells did not express myogenin after cultured in vitro. Currently, the reason for the lack of myogenin-positive cells is unknown. One of the possibilities is that enzymatically isolated myogenin-positive cells may lose adhesive property and were detached from the surface of culture plate. It is known that myogenin expression is regulated by cell-to-cell contact [55]. Thus, alternatively, cells that had been positive for myogenin in vivo were not lost but the myogenin expression may have been downregulated upon culturing in vitro. If the latter is the case, it will be intriguing to see whether myogenin expression is regained when the cloned cells obtained in the present study are transplanted in vivo.

Several evidences indicate that most of the RMS originates from myogenic cells [3, 43, 57]. However, RMS can originate from endothelial cells through trans-differentiation induced by hyperactivation of Sonic hedgehog (SHH) signaling [14]. Activation of SHH signaling is also capable of driving cells of adipogenic lineage to RMS [17]. Although the present study highly suggested that RMS cells developed in dKO rats are of myogenic origin, we still cannot exclude the possibility that they are from non-myogenic lineages.

In multiple cancer types, small populations of self-renewing cancer stem cells (CSC) are thought to be present. They proliferate and differentiate to give rise to
heterogeneous cancer cells [22]. So far, CSC of RMS has been identified only in zebrafish [25]. In mammalian model, mouse muscle stem cells are shown to give rise to RMS in severe dystrophic mouse model [4]. These reports suggest the existence of stem cells in RMS. In the present study, Pax7-positive cells were present in isolated cells from RMS, but no Pax7-positive clones were obtained. Pax7 is expressed in myogenic cells with stem cell property such as satellite cells [46], and its expression is decreased along with the myogenic differentiation [58]. Thus, it is possible that the Pax7-positive cells observed in isolated cells from RMS had stem cell-like property but during cloning, they partially underwent myogenic differentiation process and lost Pax7 expression. If so, there might be a specific environment that maintains their stem cell-like property in vivo, and RMS in dKO rat model and cloned RMS cells will be a useful tool to identify and elucidate the nature of RMS stem cells.

It is obvious that more careful considerations are required to firmly conclude that the cloned cells are indeed representing the nature of tumor cells. Especially, the essential and basic features of tumor cells such as cell morphology, doubling time and karyotype should be examined. In addition, it would be needed to establish the appropriate condition to maintain the cloned cells including the requirement of supplement such as cytokines and growth factors. Furthermore, in vivo transplantation experiments to see whether the cloned cells indeed have a tumorigenicity are also awaited. These points would be clarified in our future study.

In dKO rats, spontaneous pleomorphic RMS developed at high frequency, and the neoplasms with pleomorphic RMS in human shared histopathological features of anaplastic and atypical cells with bizarre nuclei and very rare cross-striations. Pleomorphic RMS is very rare with limited cases, and typically has a bad prognosis [36].
Only a few mouse models are reported to develop pleomorphic RMS specifically [13, 50, 59]. Therefore, dKO rats and their derived RMS cell clone would be useful to gain deeper insights into pleomorphic RMS development and to establish effective treatment.

Potential conflicts of interest

The authors have nothing to disclose.

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FIGURE LEGENDS

Figure 1. Gross and histopathological appearance of rhabdomyosarcoma in double knock-out rats. (A) The subcutaneous tumors (arrows) are located in the cheek, neck, peritoneal cavity and forelimb region. All the tumors are connected to skeletal muscles. (B) Tumor cells invade the adjacent muscle tissue and are now diffuse. Hematoxylin and eosin stain. Bar = 250 \( \mu \)m. (C) Tumor cells showing indistinct cytoplasmic borders are arranged in an irregular bundle pattern. Hematoxylin and eosin stain. Bar = 100 \( \mu \)m. (D) Sheets of pleomorphic round-to-ovoid cells are observed. Multinucleated giant cells are occasionally present (arrow). Hematoxylin and eosin stain. Bar = 20\( \mu \)m. (E) Sheets of pleomorphic spindle cells are observed. Aberrant mitotic figures are occasionally present (arrow). Hematoxylin and eosin stain. Bar = 20 \( \mu \)m. (F) Striations are rarely present (arrow). Phosphotungstic acid hematoxylin stain. Bar = 20 \( \mu \)m.

Figure 2. Immunohistochemical characteristics of rhabdomyosarcoma in double knock-out rats. (A) The tumor cells show focal cytoplasmic immunolabeling with desmin. The nuclei of the tumor cells weakly to moderately label with MyoD and myogenin. Arrowheads indicate immune-positive cells. Bar = 50 \( \mu \)m. (B) The tumor cells in case 10 show focal cytoplasmic immunolabeling with desmin. The nuclei of the tumor cells weakly to moderately label with myogenin. The tumor cells are negative for MyoD. Bar = 50 \( \mu \)m.

Figure 3. Immunocytochemical characteristics of rhabdomyosarcoma-derived primary cells from case 1. (A) The cells express strongly desmin, but moderately Pax7 and MyoD. The cells are negative for myogenin. Yellow arrowheads indicate immune-positive cells, and white arrowheads indicate immune-negative cells. Scale bar=250 \( \mu \)m. (B) Quantification of desmin, Pax7, MyoD, and myogenin-positive cells.
relative to total cell count. Data are expressed as means of 5 middle-power fields ± SE.

**Figure 4.** Immunocytochemical characteristics of rhabdomyosarcoma-derived cloned cell lines from case 1. The cells express strongly desmin and MyoD. The cells are negative for Pax7 and myogenin. Scale bar=100 μm.

**Supplementary fig. 1.** Immunohistochemical characteristics of rhabdomyosarcoma in double knock-out rats. The tumor cells show focal cytoplasmic immunolabeling with desmin. The nuclei of the tumor cells are weakly to moderately labeled with anti-MyoD and myogenin. Arrowheads indicate immune-positive cells. Bar = 50 μm.
Table 1. Tumor incidence rate of wild-type (WT), \( p16 \) knock-out (p16-KO), \( Dmd \) knock-out (Dmd-KO) and double knock-out (dKO) rats until 11 months old.

| Genotype    | WT   | p16-KO | Dmd-KO | dKO  |
|-------------|------|--------|--------|------|
| Number of rats developed | 0/14 | 0/12   | 0/13   | 12/22 |
| tumor (until 11 months)    |      |        |        |      |
Table 2. Age at onset, tumor location, and mitotic figures of tumor found in double knock-out (dKO) rats

| Case No. | Age at onset (month) | Tumor location         | Mitotic figures<sup>a</sup> |
|----------|----------------------|------------------------|-----------------------------|
| #1       | 9                    | Neck                   | 26                          |
| #2       | 9                    | Forelimbs              | 21                          |
| #3       | 10                   | Hindlimbs              | 16                          |
| #4       | 10                   | Hindlimbs              | 17                          |
| #5       | 10                   | Peritoneal cavity      | 13                          |
| #6       | 10                   | Neck                   | 17                          |
| #7       | 10                   | Cheek                  | 13                          |
| #8       | 11                   | Cheek                  | 12                          |
| #9       | 11                   | Forelimbs              | 28                          |
| #10      | 11                   | Forelimbs              | 35                          |
| #11      | 11                   | Hindlimbs              | 19                          |
| #12      | 11                   | Hindlimbs              | 35                          |

<sup>a</sup> Mitotic figures are expressed as sum of the counts from randomly chosen different 10 high-power fields.
Table 3. Expression of myogenic markers (desmin, MyoD, myogenin) in 12 RMS cases.

| Case No. | Desmin | MyoD | Myogenin |
|----------|--------|------|----------|
| #1       | +      | +    | +        |
| #2       | +      | +    | +        |
| #3       | +      | +    | +        |
| #4       | +      | +    | +        |
| #5       | +      | +    | +        |
| #6       | +      | +    | +        |
| #7       | +      | +    | +        |
| #8       | +      | +    | +        |
| #9       | +      | +    | +        |
| #10      | +      | -    | +        |
| #11      | +      | +    | +        |
| #12      | +      | +    | +        |
Table 4. Expression of myogenic markers (desmin, Pax7, MyoD, myogenin) in 20 cell lines from case 1

| Clone No. | Desmin | Pax7 | MyoD | Myogenin |
|-----------|--------|------|------|----------|
| #1        | +      | -    | +    | -        |
| #2        | +      | -    | +    | -        |
| #3        | +      | -    | +    | -        |
| #4        | +      | -    | +    | -        |
| #5        | +      | -    | +    | -        |
| #6        | +      | -    | +    | -        |
| #7        | +      | -    | +    | -        |
| #8        | +      | -    | +    | -        |
| #9        | +      | -    | +    | -        |
| #10       | +      | -    | +    | -        |
| #11       | +      | -    | +    | -        |
| #12       | +      | -    | +    | -        |
| #13       | +      | -    | +    | -        |
| #14       | +      | -    | +    | -        |
| #15       | +      | -    | +    | -        |
| #16       | +      | -    | +    | -        |
| #17       | +      | -    | +    | -        |
| #18       | +      | -    | +    | -        |
| #19       | +      | -    | +    | -        |
| #20       | +      | -    | +    | -        |
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