Oncogenic Property of Acrogranin in Human Uterine Leiomyosarcoma: Direct Evidence of Genetic Contribution in In vivo Tumorigenesis

Noriomi Matsumura, Masaki Mandai, Masanori Miyashita, Ken Fukuhara, Tsukasa Baba, Toshihiro Higuchi, Masatoshi Kariya, Kenji Takakura, and Shingo Fujii

Abstract

To identify potential oncogenes that contribute to the development of uterine leiomyosarcoma, we conducted a cDNA microarray analysis between normal uterine smooth muscle and uterine leiomyosarcoma. We found that acrogranin (also named PCDGF or progranulin) is overexpressed in uterine leiomyosarcoma. With immunohistochemical staining of 12 leiomyosarcoma cases, we verified acrogranin expression in tumor cells. Furthermore, the intensity of acrogranin expression correlated with high histologic grade and poor prognosis. To directly analyze the oncogenic properties of acrogranin, we established an immortalized uterine smooth muscle cell line by transfection of human telomerase reverse transcriptase into primary culture. This cell line retained the original characteristics of uterine smooth muscle cells, including spindle-shaped extension as well as expression of vimentin, estrogen receptor α, progesterone receptor, and α smooth muscle actin. Transfection of acrogranin into the immortalized uterine smooth muscle cells resulted in colony formation in soft agar, but the diameter of the colonies did not exceed 100 μm. Transfection of both acrogranin and SV40 early region (SV40ER) into the immortalized uterine smooth muscle cells resulted in an increased number of colonies and increased colony size in soft agar versus transfection of SV40ER alone. We show that only immortalized uterine smooth muscle cells expressing both acrogranin and SV40ER are capable of tumor formation in nude mice. Thus, acrogranin is overexpressed in uterine leiomyosarcoma cells, particularly in high-grade cases, and forced expression of acrogranin in immortalized uterine smooth muscle cells contributes to malignant transformation, which suggest that acrogranin plays an important role in the pathogenesis of uterine leiomyosarcoma.

Leiomyosarcoma comprises only 1% of all gynecologic malignancies and has an extremely poor prognosis (1). Although the majority of leiomyosarcomas are diagnosed at an early and resectable stage, 40% of these cases recur after therapy. Eighty-one percent of women with stage III tumors will have tumor recurrence, and only 8% of women with stages II to IV tumors will survive 5 years (2, 3). As uterine leiomyosarcoma is resistant to chemotherapy and radiotherapy (4, 5), developing an efficient adjuvant therapy is expected to improve the prognosis of the disease. Because a significant effect was achieved for breast cancer using antibody therapy directed against HER2, molecular targeting is regarded as a promising strategy for treatment of malignant tumors (6). In this regard, it is becoming increasingly important to identify targets that play an important role in the pathogenesis of specific tumors.

Our objective was to identify molecules involved in the pathogenesis of leiomyosarcoma that might serve as therapeutic targets. For this purpose, we conducted a cDNA microarray analysis and from these results we focused on acrogranin. Acrogranin (Genbank accession no. AF055008; National Cancer Institute of Canada abbreviation, GRN), also called PCDGF, progranulin, or proepithelin, is an 88 kDa glycoprotein that was identified as a growth factor secreted from prostate cancer cell, a human teratoma cell (7, 8). Physiologically, acrogranin is known to play important roles in development and wound repair (9, 10). It has also been recently reported that acrogranin is overexpressed in various malignant tumors, including gliomas; breast, ovarian, and prostate cancers; and hepatocellular carcinomas (11–16). In these tumors, the expression level of acrogranin was shown to be a prognostic factor. Experimentally, overexpression of acrogranin in SW-13 adrenal carcinoma cells and Madin-Darby canine kidney cells non-transformed renal epithelia results in the transfection-specific secretion of acrogranin, acquisition of clonogenicity in semisolid agar, and increased mitosis in monolayer culture (17). Acrogranin overproduction in SW-13 cells also increased tumorigenicity in nude mice (17). Another study showed...
diminution of acrogranin gene expression-impaired tumori-
genecity of the breast cancer cell line MDA-MB-468 in immu-
nedeficient mice (18). Thus far, it is unknown whether
overexpression of acrogranin can transform human primary cells.

Development of malignant tumor is a complicated process
involving multiple genetic and epigenetic alterations. Analyzing
the process of malignant transformation that is induced
experimentally in cell culture is undoubtedly a powerful
approach for uncovering the basic biological and biochemical
principles underlying development of malignant tumor. Be-
because the first report of in vitro malignant transformation
of primary human cells by transfection of defined genetic
elements (19), several groups, including us, reported in vitro
generation of human cancer cells in various organs (20–25).
However, there has been no report about in vitro malignant
transformation of uterine smooth muscle cells.

Herein, we show that acroganin is overexpressed in uterine
leiomyosarcoma compared with uterine smooth muscles and
leiomyomas and its expression is correlated with prognosis of
uterine leiomyosarcoma. We also show malignant transforma-
tion of uterine smooth muscle cells by transfection of human
telomerase reverse transcriptase (hTERT), acrogranin, and SV40
early region (SV40ER). These results suggest that acroganin
may play an important role in the pathogenesis of uterine
leiomyosarcoma and further indicate that acroganin may be a
useful target of molecular therapy.

### Materials and Methods

cDNA microarray analysis. cDNA microarray analysis was conducted
using tissues extracted from two cases of uterine leiomyosarcoma and
three cases of uterine smooth muscle as described previously (26).
Briefly, from histologically diagnosed tissues, polyadenylate+ RNA was
extracted from tissues using an mRNA purification kit (Amersham
Biosciences Corp., Piscataway, NJ). To analyze the average expression
values from different cases in one assay, the same amount of RNA extracted

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
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<td>Chitinase 3-like 1 (cartilage glycoprotein-39)</td>
<td>CHI3L1</td>
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<tr>
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<td>Fibrin, heavy polypeptide 1</td>
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<td>TKT</td>
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<td>L33930</td>
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<td>X52022</td>
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<td>Protease, serine, 11 (IGF binding)</td>
<td>PRSS11</td>
<td>D87258</td>
<td>3.92</td>
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</table>

(Continued on the following page)
from leiomyosarcoma cases and uterine smooth muscle cases, respectively, was mixed individually and served for further analysis. Then, RNA was labeled with Cy3-dCTP (leiomyosarcoma RNA) and Cy5-dCTP (uterine smooth muscle RNA). Labeled probes were mixed with microarray hybridization solution version 2 (Amersham Biosciences) and formamide to yield a final concentration of 50%. Each sample was hybridized onto the UniGEM V cDNA microarray (Incyte Genomics, Inc., Saint Louis, MO). The UniGEM V array contains 7,800 unique and sequence-verified cDNA or expressed sequence tag elements. The Cy3/Cy5 signal ratio (leiomyosarcoma/uterine smooth muscle) was calculated and analyzed by GEM tools 2.4 (Incyte Genomics, Palo Alto, CA).

Construction of retroviral vectors and production of retroviruses. The plasmid containing hTERT cDNA (pcDNA3-hTERTn2; ref. 20) was cut to produce the hTERT cDNA fragment and this was inserted into the retroviral vector pPGS-CITEneo. The plasmid containing acrogranin cDNA (pCMV-SPORT6-acrogranin) was obtained from Invitrogen Corporation (Carlsbad, CA: clone ID 3457813). We converted pLHCX retroviral vector (Clontech, Franklin Lakes, NJ) to the Gateway destination vector by using the Gateway Vector Converting System (Invitrogen). Then pLHCX-acrogranin plasmid was constructed using Gateway technology. The retroviral vector containing the SV40ER (pWB-SV40ER) was kindly provided by Dr. Jean J. Zhao (Department of Cancer Biology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA; ref. 22). The final construct was transduced by lipofection into the packaging cell line, Amphopack 293 (Clontech). Forty-eight hours later, the supernatants containing amphotropic viruses were collected. Virus was used to infect uterine smooth muscle cells with 5 ng/mL 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide (Nacalai Tesque; Kyoto, Japan).

Generation of uterine smooth muscle cell lines. Uterine smooth muscle tissue was obtained from a 48-year-old patient with regular menstrual cycles who underwent surgery for uterine leiomyomas at Kyoto University Hospital. Informed consent was obtained from the patient before surgery. Uterine smooth muscle tissue was minced and digested in DMEM (Nikken Biomedicals, Kyoto, Japan) containing 0.02% collagenase (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 4 hours at 37°C with agitation. The dispersed cells were plated in DMEM containing penicillin-streptomycin (2%, v/v; Nacalai Tesque).
and fetal bovine serum (10%, v/v, Asahi Technoglass, Funabashi, Japan) and maintained in a 37°C incubator ventilated with 5% CO2 in room air. The following day, uterine smooth muscle cells were infected for 24 hours with the amphotropic hTERT retroviral vectors followed by supplementation 48 hours later with G418 (500 µg/mL). G418 was used for 6 weeks to eliminate all noninfected cells and select for colony formation of the hTERT-infected cells. Cell lines were generated by clonal selection and were maintained in DMEM with 10% fetal colony serum and antibiotic. Among multiple colonies generated, one was isolated and maintained. It was defined as “population = 1” when cells become confluent in a 100 mm culture dish. When confluent, the cells were trypsinized and seeded at a 1:4 split ratio. The pLHCX retroviral vector containing acrogranin cDNA or the pLHCX retroviral vector only were transfected into the immortalized uterine smooth muscle cell line in the same way and selected by hygromycin B (200 µg/mL; Invitrogen). The pWB retroviral vector containing the SV40ER was similarly transfected into these cells and transfectants were selected and maintained using blasticidin (2 µg/mL; InvivoGen, San Diego, CA).

**Immunostaining of cultured cells.** The hTERT-transfected uterine smooth muscle cells were cultured for 3 days on multichamber culture slides (BD Biosciences, San Jose, CA) in a 37°C incubator ventilated with 5% CO2 in room air. Cells were fixed and permeabilized by incubation in −20°C methanol for 15 minutes and −20°C acetone for 1 minute. Blocking was done with normal rabbit serum (Nichirei, Tokyo, Japan). Samples were incubated with primary antibodies at 4°C overnight and washed. The cells were then incubated for 30 minutes with a 1:40 dilution of FITC-conjugated rabbit anti-mouse IgG secondary antibody (DakoCytomation, Glostrup, Denmark) and washed. Primary antibodies used to stain cultured cells were all anti-human mouse monoclonal antibodies: anti–estrogen receptor α (ERe; Nichirei), anti–progesterone receptor (PR; Nichirei), anti–α smooth muscle actin (α-SMA; DakoCytomation), anticytokeratin (Biomed, Foster City, CA), and antivimentin (Santa Cruz Biotechnology, Santa Cruz, CA).

**Immunostaining of tissue samples.** Tissues were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Sections were stained with H&E. For the immunohistochemical staining, they were deparaffinized in xylene and ethanol. Sections were blocked for endogenous peroxidase with 0.3% H2O2 followed by sequential incubation with normal goat serum, primary antibody, secondary antibody, and peroxidase-conjugated streptavidin. Slides were then stained with 3,3'-diaminobenzidine and hematoxylin. We used the following antibodies as primary antibodies: a goat polyclonal antiacrogranin antibody (Santa Cruz Biotechnology), a rabbit polyclonal antimlvimtin antibody (Santa Cruz Biotechnology), a mouse monoclonal anti-SV40 large T-antigen antibody (Santa Cruz Biotechnology), and a mouse monoclonal anti-cytokeratin antibody (Biomed). For acrogranin and vimentin staining, samples were retrieved with Target Retrieval Solution high H (DakoCytomation) for 15 minutes at 95°C. Then, SAB-PO kit (Nichirei), which contains blocking serum, secondary antibodies, and peroxidase-conjugated streptavidin, was used. For SV40 large T-antigen and cytokeratin staining, samples were soaked in citrate buffer and retrieved in a microwave for 15 minutes. To stain xenograft tumor tissues formed in nude mice with murine monoclonal antibodies, Mouse Stain kit was used according to the protocol of the manufacturer (Nichirei).

**Evaluation of acrogranin expression in human tissue samples.** The level of acrogranin protein expression was evaluated by immunohistochemical staining and defined as follows: +, stronger than smooth muscle but weaker than endometrial glands; ++, equivalent to endometrial glands; ++++, stronger than endometrial glands. Immunostaining of all human tissue samples was done simultaneously and under the same conditions. Three persons (gynecologic pathologists) examined each slide independently in a blind manner with final assignment of expression levels determined by consensus.

**Reverse transcription-PCR.** Total RNA was extracted from cultured uterine smooth muscle cell lines using Trizol reagent (Invitrogen). Reverse transcription-PCR (RT-PCR) was done with the One-Step RT-PCR kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. ERα, PR, hTERT, acrogranin, and actin cDNAs were amplified using specific primers for each transcript. PCR amplification was done as follows: denaturation at 94°C for 1 minute, annealing at primer-specific temperatures (ERα, PR, 52°C; acrogranin, hTERT, actin, 55°C) for 1 minute, and extension at 72°C for 2 minutes. The constitutively expressed actin transcript was used as a reference to evaluate transcription of ER, hTERT, and PR. For evaluation of acrogranin mRNA expression, actin was used as an internal control by multiplexing the acrogranin- and actin-specific primers in each reaction. PCRAs for ERα and PR were terminated at 30 cycles, PCR for hTERT at 28 cycles, and PCR for acrogranin at 26 cycles. PCR for actin was terminated at 25 cycles except for where actin was used as an internal control. Primers used were as follows: ERα, forward 5'-ACAAGCCGCAGAGAGATGAT-3', reverse 5'-VCAAGTTCATCATGCGGAACC-3'; PR, forward 5'-GACTCTCTCACCTGACA-3'; reverse 5'-TGTTTCACACCCCGCTGCA-3'; hTERT, forward 5'-CAGATTCATCATGCGGAACC-3'; reverse 5'-AGTCTGGA-3', acrogranin, forward 5'-TGTTTCACACCCCGCTGCA-3',
vector, colony formation in semisolid agar was assayed by suspending cells, we generated a uterine smooth muscle cell line by transfecting uterine smooth muscle cells with hTERT and acrogranin or empty vector, colony formation in semisolid agar was assayed by suspending 4 x 10^4 cells in 800 μL of 0.33% agarose and placing this suspension on the top of 800 μL solidified 0.5% agarose. Cultures were maintained at 37°C in a 5% CO2 atmosphere. Colonies larger than 50 μm in diameter were counted 10 days later. The experiments were repeated four times individually.

Tumorigenicity assays. Six-week-old female BALB/C nude mice were purchased from CLEA Japan (Tokyo, Japan). The animals received proper care according to the rules of the Kyoto University Committee on Animal Care. Animal experiments were done in compliance with the United Kingdom Coordinating Committee on Cancer Research Guidelines. In nude mice, 1 x 10^5 cells were injected s.c. per animal. If a s.c. mass was confirmed, the mice were sacrificed and the tumors were resected. Cells were considered nontumorigenic if the mouse failed to form a tumor within 4 months of injection.

Statistical analysis. For analysis of between-group differences, P values were determined using Mann-Whitney U test. P < 0.05 was considered significant.

Results

cDNA microarray analysis. Table 1 lists highly expressed genes in leiomyosarcoma from a cDNA microarray analysis of 7,800 genes between leiomyosarcoma and uterine smooth muscle. Acrogranin was the focus of the present study because of (a) the high ratio of acrogranin expression in leiomyosarcoma compared with smooth muscle, (b) a previous report concerning pathogenesis of cancers (17, 18), and (c) the fact that acrogranin may be a useful molecular tumor marker and therapeutic target in leiomyosarcoma, for which sensitive tumor markers and effective cytotoxic agents do not currently exist.

Immunohistochemical study for acrogranin expression. An immunohistochemical study was done among uterine leiomyosarcoma, leiomyoma, and smooth muscle tissues using an antiacrogranin antibody. Acrogranin protein expression was observed in uterine leiomyosarcoma, but expression was weak or undetectable in smooth muscles and leiomyomas (Fig. 1A-D). Strong expression was observed in endometrial glands, which were used as an internal control. Uterine leiomyosarcoma was diagnosed using standard criteria (27). Furthermore, histologic grading was done based on previously established criteria (28). Among leiomyosarcoma cases, the histologically high-grade group showed significantly stronger expression of acrogranin than the low-grade group and myxoid type (P < 0.05, χ² test; Fig. 1E-G; Table 2). Specifically, strong expression was observed in five of six high-grade cases (case 11 is of high grade but with low acrogranin expression). In contrast, strong expression was observed in only one case (case 4) of six low-grade/myxoid cases. Prognoses of high-grade cases were extremely poor (Table 2).

Generation of a uterine smooth muscle cell line. To study the effect of acrogranin overexpression in uterine smooth muscle cells, we generated a uterine smooth muscle cell line by hTERT gene transfection into primary culture cells (Fig. 2). It was previously reported by several groups that uterine smooth muscle cells could be immortalized by hTERT and acrogranin or empty vector, colony formation in semisolid agar was assayed by suspending 4 x 10^4 cells in 800 μL of 0.33% agarose and placing this suspension on the top of 800 μL solidified 0.5% agarose. Cultures were maintained at 37°C in a 5% CO2 atmosphere. Colonies larger than 50 μm in diameter were counted 10 days later. The experiments were repeated four times individually.

**Table 2. Summary of correlation among histologic grading, expression of acrogranin, and prognosis in uterine leiomyosarcoma cases**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age (y)</th>
<th>Size (cm)</th>
<th>Operation</th>
<th>Adjuvant therapy</th>
<th>Clinical stage</th>
<th>Grade</th>
<th>Follow-up</th>
<th>Acrogranin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51</td>
<td>12</td>
<td>TAH</td>
<td>ND</td>
<td>1</td>
<td>Low grade</td>
<td>NED 60 mo</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>4</td>
<td>TAH + BSO</td>
<td>CAP-F (CDDP-ADR-CPM-5FU) x2</td>
<td>1</td>
<td>Low grade</td>
<td>NED 60+ mo</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>49</td>
<td>16</td>
<td>TAH + BSO</td>
<td>ND</td>
<td>1</td>
<td>Low grade</td>
<td>NED 60+ mo</td>
<td>+</td>
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<tr>
<td>4</td>
<td>39</td>
<td>15</td>
<td>TAH + BSO</td>
<td>ND</td>
<td>1</td>
<td>Low grade</td>
<td>NED 60+ mo</td>
<td>++</td>
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<tr>
<td>5</td>
<td>65</td>
<td>10</td>
<td>TAH + BSO</td>
<td>ND</td>
<td>1</td>
<td>Myxoid</td>
<td>NED 51 mo</td>
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<tr>
<td>6</td>
<td>22</td>
<td>20</td>
<td>TAH + BSO</td>
<td>CDDP-THP-IFO x3</td>
<td>1</td>
<td>Myxoid</td>
<td>NED 50 mo</td>
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<td>7</td>
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<td>8</td>
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<td>CDDP-ADR x2, VP-16 x2</td>
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<td>High grade</td>
<td>DOD 8 mo</td>
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<td>DOD 14 mo</td>
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<td>10</td>
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<td>IV</td>
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<tr>
<td>11</td>
<td>47</td>
<td>9</td>
<td>Tumor resection + TAH + BSO</td>
<td>CDDP-ADR-VCX x2, CAP-F (CDDP-ADR-CPM-5FU) x2</td>
<td>III</td>
<td>High grade</td>
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<td>+</td>
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<tr>
<td>12</td>
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<td>Tumor resection+ TAH + BSO</td>
<td>CAP-F (CDDP-ADR-CPM-5FU) x2</td>
<td>III</td>
<td>High grade</td>
<td>DOD 60 mo</td>
<td>+++</td>
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NOTE: The strength of acrogranin expression was evaluated as described in Materials and Methods. Twelve cases of uterine leiomyosarcoma were treated in Kyoto University Hospital during the last 15 years. Abbreviations: TAH, total abdominal hysterectomy; BSO, bilateral salpingo-oophorectomy; LSO, left salpingo-oophorectomy; ND, not done; CDDP, Cisplatin; ADR, Adriamycin; CPM, Cyclophosphamide; 5FU, 5-Fluorouracil; THP, Therarubicin; IFO, Ifosfamide; VP-16, Etoposide; VCR, Vincristin; NED, no evidence of disease; DOD, died of disease.
expression of hTERT (data not shown). This cell line did not show acrogranin gene expression as a primary culture (Fig. 3A). On the contrary, SKN (uterine leiomyosarcoma cell line) showed acrogranin gene expression (Fig. 3A).

Impact of forced expression of acrogranin in uterine smooth cells on anchorage-independent cell growth. Acrogranin was retrovirally transfected into the immortalized smooth muscle cell line and transfected cells were selected with hygromycin B. Forced expression of acrogranin in the immortalized uterine smooth muscle cells was confirmed by RT-PCR (Fig. 3A). The immortalized uterine smooth muscle cell line (transfected only with hTERT gene) did not show increased cell proliferation compared with early primary culture of uterine smooth muscle cells as detected by WST-1 assay (data not shown). When this cell line was further transfected with acrogranin, it showed no change in cell proliferation, but showed anchorage-independent growth (Fig. 3B and C) to some extent. However, in this case, the colony size did not exceed 100 μm. The immortalized uterine smooth muscle cell line was also transfected with SV40ER (eventually transfected with hTERT + SV40ER). Transfection of SV40ER resulted in slight increase in cell proliferation and in more apparent anchorage-independent growth (some of the colonies exceeded 100 μm in size as shown in Fig. 3D). When the cell was eventually transfected with hTERT + SV40ER + acrogranin, anchorage-independent growth was markedly accelerated (Fig. 3D and E), but further stimulation of cell growth was not observed. Cells (5 × 10^4/mL) were seeded in soft agar and colonies larger than 100 μm were counted 10 days later. The cells transfected with hTERT + SV40ER + acrogranin formed significantly increased number of colonies in soft agar compared with cells transfected with hTERT + SV40ER (Fig. 3E).

Impact of forced expression of acrogranin in uterine smooth cells on tumor formation in nude mice. Cells used in Fig. 3 were inoculated s.c. into nude mice at 6 to 8 weeks of age (1 × 10^7 per animal). Tumor formation and growth was assessed until at least 4 months after the inoculation. Tumor formation in nude mice was observed when uterine smooth muscle cells transfected with hTERT, acrogranin, and SV40ER were inoculated (Fig. 4A; Table 3). In histologic analysis, tumor cells were arranged around tumor vessels and caused sudden massive hemorrhage necrosis, which are typical features of mesenchymal tumors (Fig. 4B). The mesenchymal character of the tumor was...
also confirmed by the immunohistochemical analysis: vimentin (+) and cytokeratin (−) (Fig. 4C). However, ERα, PR, and α-SMA were negative (data not shown). Acrogranin and SV40 expression were confirmed in all tumor cells (Fig. 4D).

**Discussion**

Uterine leiomyosarcoma is a disease with extremely poor prognosis, is highly aggressive, and is resistant to chemotherapy. At present, surgical intervention is virtually the only means of treatment for uterine leiomyosarcoma (4, 5). However, molecular-targeting therapies against malignant tumors have recently shown remarkable achievements (6). To improve the prognosis of uterine leiomyosarcoma, we aimed to search for key oncogenes that play an important role in their pathogenesis and that could serve as a molecular target for treatment. For this purpose, we conducted a cDNA microarray analysis between uterine leiomyosarcoma and normal uterine smooth muscle and showed that several factors, such as brain-specific polypeptide PEP-19, may be associated with the pathogenesis of leiomyosarcoma (26). However, in terms of tumorigenesis of leiomyosarcoma, merely comparing the expression of potential oncogenes between normal and malignant tissues is not sufficient because the results obtained may be the consequence of malignant transformation and, therefore, not necessarily the cause. To obtain more direct evidence for a genetic contribution to tumor occurrence, we established a method in which a candidate oncogene was transfected into phenotypically normal smooth muscle cells and oncogenicity was determined using soft agar assays or tumor formation in nude mice.

Among the various genes that exhibited differential expression between normal and malignant uterine smooth muscles, acrogranin, which is an autocrine/paracrine growth factor, was chosen as our research target. This was largely because, thus far,
clinically successful molecular targets include growth factors or their receptors, such as HER2 or vascular endothelial growth factor. Acrogranin is a pluripotent growth factor that mediates cell cycle progression and cell motility (32, 33). Structurally, it bears no homology to the well-established growth factor families (34). In vivo, acrogranin is expressed at a high level in epithelial cells that undergo rapid turnover, notably in the intestinal deep crypt and epidermal keratinocytes, suggesting a role of acrogranin in the regulation of epithelial proliferation (35). It is scarce in connective tissue, endothelia, and muscle in healthy adult tissues.

**Table 3. Summary of tumor formation in nude mice**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Number of Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth muscle cells + hTERT + empty vector</td>
<td>0/3</td>
</tr>
<tr>
<td>Smooth muscle cells + hTERT + acrogranin</td>
<td>0/4</td>
</tr>
<tr>
<td>Smooth muscle cells + hTERT + empty vector + SV40ER</td>
<td>0/7</td>
</tr>
<tr>
<td>Smooth muscle cells + hTERT + acrogranin + SV40ER</td>
<td>6/8</td>
</tr>
</tbody>
</table>

**NOTE:** 1 × 10⁷ cells were inoculated s.c. in nude mice at 6 to 8 weeks of age. Tumor formation and growth was not assessed until at least 4 months post-inoculation.

First, we examined the expression of acrogranin in uterine smooth muscle tumors and showed that it is highly expressed in uterine leiomyosarcoma and in the leiomyosarcoma cell line SKN (Figs. 1 and 3A; Table 2). In immunohistochemical studies, the intensity of its expression correlated with the histologic grade and prognosis although the number of samples analyzed are relatively few (Table 2). In other types of malignancies, acrogranin is highly expressed in aggressive cancer cell lines and clinical specimens, including breast, hepatocellular, ovarian, and prostatic cancers, as well as gliomas (11–16, 18). Accordingly, we hypothesized that acrogranin may play a critical role in tumorigenesis in uterine leiomyosarcoma.

We attempted to evaluate the oncogenic property of acrogranin in a more direct fashion, namely by transfecting its gene into phenotypically normal uterine smooth muscle cells. It has been reported that uterine smooth muscle cells are immortalized by transfection of hTERT cDNA and these immortalized cells retain their original characteristics (29–31). However, to our knowledge, there have been no attempts to transform immortalized uterine smooth muscle cells by transfecting potential oncopgenes. We first transfected hTERT cDNA into uterine smooth muscle cells and one of the selected colonies was maintained. These cells expressed ERα, PR, and α-SMA (Fig. 2C), showing the original character of primary uterine smooth muscle cells. This cell line did not show anchorage-independent growth or tumorigenicity in nude mice and, therefore, was suitable to examine the oncogenic function of acrogranin.

Although it was reported that overexpression of acrogranin in leiomyosarcoma cell line promote proliferation (35), it did not promote cell proliferation in immortalized uterine smooth muscle cells as detected by WST-1 assay. However, it resulted in colony formation in soft agar, which was only microscopically detected; three to four colonies of 4 × 10⁴ cells were obtained that were only ~50 μm in diameter and never exceeded 100 μm (Fig. 3B). These cells did not form tumors in nude mice (Table 3). These results may show a potential oncogenic function of acrogranin, but also suggest that coexpression of hTERT and acrogranin is not sufficient to transform uterine smooth muscle cells.

We then transfected the SV40ER into these uterine smooth muscle cells. When transfected with the SV40ER, hTERT-immortalized uterine smooth muscle cells showed obvious anchorage-independent growth but did not form tumors in nude mice (Fig. 3D; Table 3). On the other hand, coexpression of hTERT, acrogranin, and SV40ER resulted in more extensive anchorage-independent growth and tumor formation in nude mice (Figs. 3D and 4A; Table 3). This result indicates that...
coexpression of these three genes is necessary and sufficient to transform uterine smooth muscle cells. A number of nonmitogenic actions of acrogranin relevant to cancer are known, e.g., decreased susceptibility to anoikis, increased angiogenesis, invasion, and motility (10, 11, 18). As the expression of acrogranin did not accelerate cell proliferation in monolayer (data not shown), our results suggest oncogenic, but nonproliferative, actions of acrogranin.

Studies on in vitro malignant transformation have revealed that to transform primary human cells, the following four signals are thought to be necessary: (a) elongation of telomere length by hTERT expression, (b) perturbation of p53 and retinoblastoma by SV40LT expression or other methods, (c) perturbation of PP2A by SV40 small tumor antigen expression or other methods, and (d) constitutive RAS signaling by transfection of the oncogenic allele of RAS (36–39). In general, signals from growth factors are transmitted through tyrosine kinase type receptors to RAS (40). As such, constitutively activated RAS signaling can be used to substitute for persistent growth factor signaling. Actually, as we previously reported, ovarian surface epithelial cells immortalized by coexpression of hTERT and SV40 are transformed by c-ERBB2 instead of oncogenic RAS (20). However, there has been no report that overexpression of growth factors can substitute for transfection of oncogenic RAS.

Signals generated by acrogranin are thought to be strong. Embryonic fibroblasts from mice in which the insulin-like growth factor-I receptor was deleted (i.e., R− cells) fail to divide when stimulated by well-established growth factors. However, proliferate when exposed to acrogranin (41). Acrogranin activates the mitogen-activated protein kinase and phosphatidylinositol-3 kinase signaling cascades (42). Activated phosphatidylinositol-3 kinase can substitute, at least in part, for oncogenic RAS signal in de novo transformation (22). Hence, it is one of the possible mechanisms that acrogranin exert oncogenicity via phosphatidylinositol-3 kinase activation by substituting RAS signaling. However, we do not have direct evidence for that yet and other possibilities cannot be excluded.

In summary, we showed that acrogranin was overexpressed in uterine leiomyosarcoma and their higher histologic grade was correlated with higher expression of acrogranin. Transfection of hTERT, SV40ER, and acrogranin in uterine smooth muscle cells resulted in transformation of primary cultured cells. These data indicate that acrogranin may be a promising target for developing effective molecular targeting therapy for treatment of uterine leiomyosarcoma. In addition, acrogranin may also serve as a specific diagnostic marker to identify leiomyosarcomas of the uterus, the diagnosis of which is clinically difficult. Moreover, our study established a new method to evaluate the oncogenic property of specific genes in the process of malignant transformation of hTERT-immortalized and SV40-activated human smooth muscle cells.

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Oncogenic Property of Acrogranin in Human Uterine Leiomyosarcoma: Direct Evidence of Genetic Contribution in In vivo Tumorigenesis

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