Potential role of LMP2 as an anti-oncogenic factor in human uterine leiomyosarcoma: morphological significance of calponin h1. (125 characters)

Takuma Hayashi[1,10], Akiko Horiuchi[2,11], Kenji Sano[3], Nobuyoshi Hiraoka[4], Mari Kasai[5], Tomoyuki Ichimura[5], Tamotsu Sudo[6], Ryuichiro Nishimura[6], Osamu Ishiko[5], Tanri Shiozawa[2], Yae Kanai[4], Nobuo Yaegashi[7], Hiroyuki Aburatani[8], Ikuo Konishi[9]

[1]Dept. of Immunology and Infectious Disease, Shinshu University Graduate School of Medicine, [2]Dept. of Obstetrics and Gynecology, Shinshu University School of Medicine, [3]Dept. of Laboratory Medicine, Shinshu University Hospital, Nagano 390-8621, Japan, [4]Pathology Division, National Cancer Center Research Institute, Tokyo 104-0045, Japan, [5]Dept. of Obstetrics and Gynecology, Osaka City University Graduate School of Medicine, Hyogo 673-8558, Japan, [6]Dept. of Gynecology, Hyogo Cancer Center, Hyogo 673-8558, Japan, [7]Dept. of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, Miyagi 980-8574, Japan, [8]The Cancer System Laboratory, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo 153-9804, Japan, [9]Dept. of Obstetrics and Gynecology, Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan, [10]Promoting Business Using Advanced Technology, Japan Science and Technology Agency (JST) Tokyo 102-0076, Japan, [11]Dept. of Obstetrics and Gynecology, Aizawa Hospital, Nagano 390-8510, Japan.

Correspondence and requests for materials should be addressed to T.H.
(takumah@shinshu-u.ac.jp)

ABSTRACT

Uterine leiomyosarcoma (LMS) is a highly metastatic smooth muscle neoplasm for which calponin h1 is suspected to have a biological role as a tumor-suppressor. We earlier reported that LMP2-null mice spontaneously develop uterine LMS through malignant transformation of the myometrium, thus implicating this protein as an anti-tumorigenic candidate as well. In the present study, we show that LMP2 may negatively regulate LMS independently of its role in the proteasome. Moreover, several lines of evidence indicate that although calponin h1 does not directly influence tumorigenesis, it clearly affects LMP2-induced cellular morphological changes. Modulation of LMP2 may lead to new therapeutic approaches in human uterine LMS. (104 words)

Keywords: LMP2, calponin h1, uterine leiomyosarcoma, cell morphology

1. Introduction

Smooth muscle tumors (SMTs) are commonly divided into benign leiomyoma (LMA) and malignant leiomyosarcoma (LMS) based on cytological atypia, mitotic activity, and other criteria. Uterine LMS is a rare gynecologic malignancy in the female genital tract, with an estimated annual incidence of 0.64 per 100,000 women [1]. LMS accounts for approximately one-third of uterine sarcomas, of which 53% are confined to the uterus [2-5]. Gynecological tumors are strongly promoted by female hormones. The rate of hormone receptor expression is reported to be significantly lower in human uterine LMS than in normal myometrium, but these low receptor levels were found to correlate neither with the promotion of initial disease development, nor with the overall survival of patients with uterine LMS.

Although uterine LMS is sensitive to certain types of chemotherapy with gemcitabine or docetaxel, it is resistant to hormone therapy and radiotherapy, and thus surgical intervention is virtually the only means of treatment at present [6-8]. It should be noted that when adjusted for stage and mitotic count, LMS has a significantly worse prognosis than carcinosarcoma; the 5-year survival rate for patients with uterine LMS is 15%-25%. The development of effective adjuvant treatments is expected to improve the outcome of this disease through the use of emerging molecular targeting therapies [4,5,9-11]. Determining the malignant potential of smooth muscle neoplasms also represents a significant diagnostic conundrum with important therapeutic ramifications. However, the genetic changes underlying the neoplastic transformation of uterine smooth muscle cells (SMCs) have not been fully characterized.

The ubiquitin-proteasome degradation pathway is essential for many cellular processes, including cell cycle and regulation of gene expression. Abnormal expression of low molecular weight protein (LMP)2, LMP7, and LMP10(MECL-1) subunits is therefore believed to contribute to the initiation and development of disorders. A recent study revealed a unique role for LMP7 in controlling pathogenic immune responses and provided a therapeutic rationale for targeting LMP7 in autoimmune disorders, especially rheumatoid arthritis [12]. It is also noteworthy that mice with a

targeted disruption of LMP2 exhibited defects in tissue- and substrate-dependent proteasomal function, and that female LMP2-null mice spontaneously developed uterine LMS with a disease prevalence of 40% by 14 months of age [13,14]. Furthermore, LMP2 expression was markedly down-regulated in human uterine LMS tissues in comparison with both LMA and normal myometrium. This defective LMP2 expression was attributable to somatic mutations in the catalytic region of JAK1 in tumor tissues from patients with uterine LMS [15], and so defective LMP2 expression is likely to be one of the risk factors in the development of human uterine LMS as it is in LMP2-null mice [14,15]. In addition, recent reports have shown an association between malignant transformation of the myometrium and reduced expression of calponin h1, which is specifically expressed in smooth muscle and binds calmodulin, actin, and tropomyosin [16,17]. Calponin h1 reportedly inhibits the ATPase activity of myosin and may play a role in smooth muscle contraction [18,19]. Although calponin h1 may function as a tumor-suppressor in uterine LMS, calponin h1-null mice do not exhibit uterine LMS. The biological characterization of human uterine LMS remains incomplete [16,17].

In the present study, we investigated the molecular mechanism behind the tumorigenesis of human uterine LMS involving defective LMP2 expression. Biological and histological findings showed that while calponin h1 expression was clearly induced by LMP2, defective calponin h1 expression contributed to abnormal cell morphology, but to not cell proliferation, which directly correlated to tumor progression. Thus, LMP2 expression appeared to be responsible for the calponin h1-mediated morphological phenotypes in human uterine LMS cells in an anti-oncogenic manner. Continued improvement of our knowledge of the molecular biology of uterine LMS may ultimately lead to novel diagnoses and therapies and improved outcome. (612 words)

2. Materials and Methods

2.1. Tissue Collection. A total of 51 patients aged between 32 and 83 years who were diagnosed as having smooth muscle tumors of the uterus were selected from pathological files. Serial sections were obtained from at least 2 tissue blocks from each patient for hematoxylin and eosin staining and immunostaining. All tissues were used with the approval of the Ethical Committee of Shinshu University after obtaining written consent from each patient.

2.2. Western Blotting. To detect the expression of LMP2, LMP7, cyclin B, calponin h1, SRF, and β-actin, whole cell lysates, nuclear extracts, or cytosolic extracts were resolved by 10% SDS-PAGE, and immunoblotting was performed using appropriate antibodies by standard procedures.

2.3. DNA Transfection and Isolation of Flat Revertants. Transfection of pCEM9-LMP2wt, pCEM9-LMPK33A, pCalponin h1shRNA, pScr.shRNA, or the empty pCEM9 vector was carried out with FuGENE6 Transfection Reagent according to the manufacturer's recommendations with 5 μg of plasmid DNA and 5 x 10^5 SKN cells plated onto 6-well tissue culture dishes on the previous day.

2.4. Xenograft Studies. Nude mice (BALB/cSlc-*nu/nu*, female, 7-8 weeks old, Japan SLC, Shizuoka, Japan) were injected intracutaneously with 1×10^7 cells of the SKN-CEM9 (T type) clone, SKN-LMP2wt (F type) clone, SKN-LMP2K33A (F type) clone, SKN-LMP2wt/Scr.shRNA (F type) clone, or SKN-LMP2wt/calponin h1 shRNA (T type) clone with BD Matrigel Matrix (BD Biosciences, MA, USA) in 5 mg/ml of culture medium containing 15% FCS plus SmGM-2 SingleQuots (CAMBREX, MD, USA) at a volume of 100 μl.

2.5. Reverse Transcription-polymerase Chain Reaction Analysis (RT-PCR) and Quantitative RT-PCR. The expression of LMP2, LMP7, calponin h1, SRF, and β-actin transcripts was examined using RT-PCR. Total RNA was prepared from human uterine LMS tissues and normal myometrium tissues with TRIzol reagent according to the manufacturer's protocol. RNA was reverse-transcribed with the Superscript II enzyme, and single stranded cDNA was used for amplification. A LightCycler and SYBRGreen system were used for quantitative real-time RT-PCR according to the manufacturer's recommendations.

2.6. Immunohistochemistry (IHC). IHC staining for LMP2, calponin h1, ER, PR, p53, and Ki-67 was

performed on serial human uterine LMS sections. IHC was performed using the avidin-biotin complex method as described previously.

2.7. Cell Transfections and Reporter Assays. SKN-transfectants, SKN-CEM9#2, SKN-LMP2wt#121, or SKN-LMP2wt#122 clones were seeded at 70% confluence in 6-well plates, and a total of 2 μg of luciferase reporter vectors (-549calponin h1 promoter-Luc. or Basic-Luc.) were transfected into SKN-transfectants with FuGENE6 Transfection Reagent according to the manufacturer's recommendations.

Details of methods and any associated references are available in the Supplementary information

3. Results

LMP2 and calponin h1 expression were evaluated using human tissue samples. IHC revealed a pronounced loss in the ability to induce LMP2 and calponin h1 expression in human uterine LMS tissue in comparison with normal myometrium located in the same section. Of the 32 patients with uterine LMS examined, 29 were negative for LMP2, 1 was focally positive, and 1 was partially positive (Supplementary Fig. 1). One LMS sample retained the expression of LMP2 (Supplementary Fig. 1). Of the 32 patients with uterine LMS examined, 31 were negative for calponin h1, and 1 was partially positive (Supplementary Fig. 1). All lymph nodes were negative for LMS metastases, and IHC showed positivity for Ki-67 and negativity for LMP2 and calponin h1. In both Western blotting and RT-PCR experiments, LMP2 and calponin h1 were expressed in normal myometrium but not in human uterine LMS, which were strongly supportive of the IHC findings. Although our research group previously demonstrated that the abnormal expression of the ovarian steroid receptors, TP53, and Ki-67, and mutations of TP53 were frequently associated with uterine LMS, defective LMP2 and calponin h1 expression appeared to be more characteristic of uterine LMS in our cohort (Supplementary Table 1).

The defective LMP2 expression was attributable to somatic mutations in the catalytic region of JAK1 in tumor tissues from our patients with uterine LMS [15] (Supplementary Table 1). Although cell proliferation has been demonstrated to be strongly inhibited by IFN-γ-induced JAK1 kinase activation [20], it is difficult to demonstrate tumorigenicity in JAK1-null mice because they die perinatally [21]. Therefore, the differential responsiveness to genetically modified stable LMP2 expression of the SKN human uterine LMS cell line was investigated to determine whether reintroducing LMP2 into a LMS cell line would affect its tumorigenic properties for development of uterine LMS and if the observed effect was due to the immunoproteosomal function of the protein. SKN cells were transfected with pCEM9, pCEM9-LMP2wt, or pCEM9-LMP2K33A, which has no effect on immunoproteasome function due to non-incorporation into the 20S proteasome [22] (Supplementary Fig. 2), and selected in medium containing G418. The efficiency of neo-marker

transfer of these three plasmids was comparable (Supplementary Table 2). However, in the case of pCEM9-LMP2wt, approximately 78% (LMP2wt) or 76% (LMP2K33A) of the total G418-resistant colonies were relatively compact and appeared dark when observed under a phase-contrast microscope after 6-7 days of selection (Supplementary Table 2). These partially flat (P/F type) colonies consisted of cells with increased attachment to the substrate, while the majority of the other transformed (T type) colonies looked similar in cell morphology to the cells observed in the control dishes, albeit slightly smaller in size (Fig. 1A, Supplementary Fig. 3). After 2 to 3 weeks when most of the colonies outgrew and detached from the substrate, colonies consisting of flatrevertant (F type) cells were found at frequencies of approximately 29.5% (pCEM9-LMP2wt) or 24.8% (pCEM-LMP2K33A) of the total number of G418-resistant colonies initially observed (Supplementary Table 2). No colonies of this F type morphology were observed in the cultures transfected with pCEM9 (Table 1, Supplementary Fig. 3, Table 2, and 3). We next isolated and expanded representative colonies of each type and analyzed their growth properties and the occurrence and expression of LMP2. The growth rate, expressed as doubling time, of typical SKN-LMP2wt (F type) colonies (8 clones) and SKN- LMP2K33A (F type) colonies (4 clones) was generally lower than that of SKN-LMP2wt (P/F type) colonies (2 clones) and control SKN-CEM9 (T type) colonies (4 clones) (Table 1, Supplementary Table 3).

In trials evaluating the anti-tumorigenic properties of LMP2wt and LMP2K33A, the efficiency of colony formation and colony size in soft agar were greatly reduced in the F type clones and P/F type clones, and significantly reduced in the morphologically similar T type SKN-LMP2wt clones, compared with those in the SKN-CEM9 (T type) clone (Table 1, Supplementary Table 3). Tumor growth was clearly observed in control mice inoculated with the SKN-CEM9 (T type) clone, whereas a reduction in tumor growth was observed in mice inoculated with the SKN-LMP2wt (F type) or SKN-LMP2K33A (F type) clones (Fig. 1A, Table 1, Supplementary Table 3). Since both wild type and mutant LMP2 blocked tumorigenesis, it became necessary to rule out a toxic effect of LMP2 overexpression in a control cancer cell line. Additional experiments demonstrated no toxic

effects of either wild-type LMP2 or mutant LMP2K33A overexpression in HeLa cervix cancer cells (data not shown).

The association between reduced expression of calponin h1 and malignant transformation of the myometrium suggests that re-expression of human calponin h1 can suppress cell proliferation and tumorigenicity in uterine LMS cells [16,17]. Calponin h1 was highly expressed in myometrium and leiomyoma of the uterus, but was very poorly expressed or negative in LMS [16,17] (Supplementary Fig. 1, Table 1). However, unlike mice lacking LMP2, calponin h1-null mice do not exhibit uterine LMS [14,15]. To examine the biological connection between LMP2 and calponin h1, we analyzed the expression pattern of calponin h1 in the SKN-CEM9 clone (2 clones), SKN-LMP2wt (F type) clone (8 clones), and SKN-LMP2K33A (F type) clone (4 clones) (Supplementary Fig. 3). Unexpectedly, our results revealed that calponin h1 was markedly expressed in both SKN-LMP2wt (F type) and SKN-LMP2K33A (F type) clones (Fig. 1B,C, Supplementary Fig. 4, Table 3). Calponin h1 expression depended on the presence of LMP2, suggesting that LMP2 may involve the function of calponin h1 in tumor suppression of human uterine LMS. In reporter assays, the calponin h1 promoter was markedly activated in SKN-LMP2 (F type), #121, and #122 clones, but not in SKN-CEM9#2, which was strongly supportive of the RT-PCR and Western blotting assays (Fig. 1). Recent papers have demonstrated serum response factor (SRF)-dependent regulation of the human smooth muscle *calponin h1* gene [23,24]. Here, mutation of SRF binding sites abolished the ability of stable LMP2 expression to induce SRF gene expression in SKN-LMP2wt (F-type), #121, and #122 clones (Fig. 1D). RT-PCR and Western blotting experiments showed that stable expression of LMP2 induced SRF expression, which may account for the activation of calponin h1 gene expression in SKN-LMP2wt (F-type) clones (Fig. 1, Supplementary Fig. 4).

To demonstrate the functional significance of calponin h1 on LMP2-induced differential cellular phenotypes, additional experiments were performed on cell morphology, cell cycle, and tumorigenesis of human uterine LMS. The SKN cell line was co-transfected with pCEM9-LMP2wt plus calponin h1shRNA or Scr.shRNA (negative- control vector) (Santa Cruz Biotechnology Inc.

CA, USA) and then selected in medium containing G418. The efficiency of neo-marker transfer with combinations of these plasmids was comparable (Fig. 2). However, in SKN-LMP2/Scr.shRNA colonies, approximately 96.4% of G418-resistant cells were relatively compact and appeared dark when observed under a phase-contrast microscope after 6-7 days of selection (Supplementary Table 4). After 2 to 3 weeks, when most of the colonies outgrew and detached from the substrate, colonies consisting of SKN-LMP2wt/Scr.shRNA flatrevertant (F type) cells (4 clones) were found at frequencies of approximately 59.1% of the total number of G418-resistant colonies initially observed (Fig. 2). Furthermore, colonies of P/T type or T type morphology were observed in cultures co-transfected with LMP2wt plus calponin h1shRNA (4 clones), which specifically prevents calponin h1 expression (Fig. 2, Table 2, Supplementary Fig. 5). No colonies with morphological changes were observed in the SKN cultures co-transfected with CEM9 plus calponin h1shRNA or Scr.shRNA (Fig. 2, Table 2, Supplementary Fig. 5). Western blotting and RT-PCR analysis demonstrated that although calponin h1 was clearly detected in the SKN-LMP2wt/Scr.shRNA colony (4 clones), calponin h1shRNA markedly prevented calponin h1 expression in the SKN-LMP2wt/calponin h1shRNA colony (4 clones) (Fig. 2, Table 2, Supplementary Fig. 4). These findings suggest that LMP2-induced cellular morphological phenotypes involved the biological function of calponin h1. We confirmed that the morphological change was due to the impaired expression of calponin h1 by using shRNAs against different calponin h1 sequences (Supplementary Fig. 6).

We next isolated and expanded representative colonies of each cell type and analyzed their growth properties. The growth rate of the SKN-LMP2wt/calponin h1shRNA (T type) colony (4 clones), measured as population doubling time (PDT), was slightly higher than that of the SKN-LMP2wt/Scr.shRNA colony (F type) (4 clones) (Fig. 2, Table 2). We also analyzed their tumorigenic properties as well as the occurrence and expression of LMP2 and calponin h1. The efficiency of colony formation and size of the clones in soft agar were not significantly reduced in

the SKN-LMP2wt/calponin h1shRNA (T type) colony (4 clones) as compared with those of the SKN-LMP2wt/Scr.shRNA (F type) colony (4 clones) (Table 2). Tumor growth was clearly observed in control mice inoculated with SKN-CEM9 (T type) (2 clones) (Fig. 1, Supplementary Table 3). A significant reduction in tumor growth was observed in mice inoculated with the SKN-LMP2wt/Scr.shRNA control (F type) clone (4 clones); tumors grew slowly in mice inoculated with the SKN-LMP2wt/calponin h1shRNA (T type) colony (4 clones) in comparison with those inoculated with the SKN-CEM9 colony (4 clones) (Fig. 1 and 2, Table 1, Table 2, Supplementary Table 3). The present experiments suggest that the expression of calponin h1 might affect cell morphology, but not suppression of tumorigenicity in the LMP2-induced differentiation of cellular phenotype.

4. Discussion

Recent molecular targeting therapies against tumors have achieved remarkable results. To improve the prognosis of human uterine LMS, studies are being performed to identify the roles of key pro- and anti-oncogenic factors that have important functions in tumor pathogenesis and may serve as molecular targets for treatment. For this purpose, several research groups have shown that cell cycle regulatory factors and known pro-oncogenic factors, such as the brain-specific polypeptide PEP-19 and c-kit, may be associated with the pathogenesis of human uterine LMS [25-28]. LMP2-null mice are reportedly prone to the spontaneous development of uterine LMS [14]. In the present study, histopathological experiments demonstrated a high correlation between a loss of LMP2 and calponin h1 and the malignancy of uterine tumors developing in the myometrium. It is interesting that only LMP2-null mice spontaneously developed uterine LMS, especially since the individual expression of LMP2, LMP7, and LMP10/MECL-1 subunits has been reported to contribute to the initiation and development of several disorders [12,14,15].

Calponin h1 most likely exerts its biologic effects through interactions with other cellular molecules that transduce signals from outside the cell. Calponin h1 can promote actin filament

stability *in vitro* by inhibiting depolymerization [29]. Cell attachment and spreading are essential for cells to traverse through the G_1 phase of the cell cycle [30]. The present observations on cell morphology following LMP2-induced calponin h1 expression suggest that calponin h1 may inhibit the signaling pathways concerned with the control of cellular morphology, including cell attachment, cell shape, and spreading. The *calponin h1* gene is expressed in resting aortic SMCs, but its expression rapidly decreases when growth-arrested cells re-enter the G_1 phase and proliferate. Thus, this protein inhibits proliferation in SMCs and fibroblasts [31,32]. Our findings show that calponin h1 expression under stable LMP2 expression affects cellular morphology, but does not markedly suppress cell proliferation and tumorigenicity in LMS cells.

The molecular mechanisms by which *calponin h1* gene-inducing SRF expression is induced by single-unit LMP2 are poorly understood in LMS. The functionally inactivated K33A mutant of LMP2 has the same morphology *in vitro* as the wt transfectant, suggesting that LMP2 acts not only through its role in proteasomes, but also as a single subunit. The relative amounts of LMP2 and LMP7 vary significantly among tissues and cell lines in mice and humans [33], and LMP2 levels in proteasomes show greater variation among tissues than do those of LMP7 [33]. Proteasome subunits that are not incorporated into complexes are believed to individually mediate gene transcriptional activation together with other co-factors [34]. For instance, LMP2 is also reportedly required for estrogen receptor-mediated gene transcription as well as for estrogen-stimulated cell cycle progression [35]. Other reports have demonstrated the nuclear localization of single-unit LMP2 in various mammalian tissues and cell types [33,36], where single-unit LMP2 may regulate *calponin h1* gene activation with cellular co-factors. Further experiments may provide a more complete understanding of the tumorigenic phenotypes of SKN cells by nonproteosomal LMP2.

LMP2 molecules reportedly associate with cellular factor(s) to regulate cellular processes, such as cell cycle and gene expression. The present observations on cell morphology following calponin h1shRNA expression suggest that single-unit LMP2-mediated cellular factors other than calponin h1 prevent cell proliferation and tumorigenicity in LMS cells. Elucidation of the mechanism by

which LMP2-induced biological events, including calponin h1 expression, are regulated may provide a great deal of information about the transformation of cellular phenotypes, the control of cell proliferative activity, and the pathogenesis of uterine smooth muscle neoplasias at the molecular level. In conclusion, we have shown that LMP2-induced calponin h1 expression plays a role in the cellular morphological change of LMS cells, and that LMP2 may be a tumor suppressor in human uterine LMS. (2124 words)

Acknowledgments

We sincerely appreciate the donation of LMP2-null breeding mice and scientific cooperation of Prof. Susumu Tonegawa (Picower Institute of Learning and Memory, M.I.T.). We thank Dr. Isamu Ishiwata (Ishiwata Clinic, Ibaraki, Japan) for generously providing the uterine LMS cell line and also thank Dr. Yoshi Adachi (Shinshu University, Nagano, Japan) for generously providing the uterine HeLa cell line. We also thank BEX Corporation (Tokyo, Japan) for assistance with constructing plasmid vectors. This work was supported by grants from the Ministry of Education, Culture, Science, and Technology, the Japan Science and Technology Agency, The Foundation of Takeda Medical Research, The Foundation for the Promotion of Cancer Research, Kanzawa Medical Research Foundation, and The Ichiro Kanehara Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version of this paper at doi:

References

[1] Zaloudek, C. & Hendrickson, M.R. Mesenchymal tumors of the uterus, in Kurman RJ.(ed):(2002) *Blaustein`s Pathology of the Female Genital Tract (ed 5)*. New York, Springer-Verlag 561-578.

[2] Gadducci, A. Landoni, F. Sartori, E. Zola, P. Maggino, T. Lissoni, A. Bazzurini, L. Arisio, R. Romagnolo, C. & Cristofani, R. (1996) Uterine leiomyosarcoma: analysis of treatment failures and survival. Gynecol. Oncol. 62, 25-32.

[3] Nordal. R. & Thoresen, S. (1997) Uterine sarcomas in Norway 1956–1992: incidence, survival and mortality. Eur. J. Cancer 33, 907-311.

[4] Brooks, S.E., Zhan, M., Cote, T. & Baquet, C.R. (2004) Surveillance, epidemiology, and end results analysis of 2677 cases of uterine sarcoma 1989–1999. Gynecol. Oncol. 93, 204-208.

[5] Dusenbery, K.E., Potish, R.A., Argenta, P.A. & Judson, P.L. (2005) On the apparent failure of adjuvant pelvic radiotherapy to improve survival for women with uterine sarcomas confined to the uterus. Am. J. Clin. Oncol. 28, 295-300.

[6] Wu, T.I. Chang, T.C. Hsueh, S. Hsu, K.H. Chou, H.H. Huang, H.J. & Lai, C.H. (2006) Prognostic factors and impact of adjuvant chemotherapy for uterine leiomyosarcoma. Gynecol. Oncol. 100, 166-172.

[7] Leitao, M.M. Soslow, R.A. Nonaka, D. Olshen, A.B. Aghajanian, C. Sabbatini, P. Dupont, J. Hensley, M. Sonoda, Y. Barakat, R.R. & Anderson, S. (2004) Tissue microarray immunohistochemical expression of estrogen, progesterone, and androgen receptors in uterine leiomyomata and leiomyosarcoma. Cancer 101, 1455-1462.

[8] Perez, E.A., Pusztai, L. & Van de Vijver, M. (2004) Improving patient care through molecular diagnostics. Semin. Oncol. 31, 14-20.

[9] Lin, J.F. & Slomovitz, B.M. (2008) Uterine sarcoma. Curr. Oncol. Rep. 10, 512-518.

[10] Amant, F., Coosemans, A., Debiec-Rychter, M., Timmerman, D. & Vergote, I. (2009) Clinical management of uterine sarcomas. Lancet Oncol. 10, 1188-1198.

[11] Miettinen, M. & Fetsch, J.F. (2006) Evaluation of biological potential of smooth muscle tumours. Histopathology 48, 97-105.

[12] Muchamuel, T. Basler, M. Aujay, M.A. Suzuki, E. Kalim, K.W. Lauer, C. Sylvain, C. Ring, E.R. Shields, J. Jiang, J. Shwonek, P. Parlati, F. Demo, S.D. Bennett, M.K. Kirk, C.J. & Groettrup, M. (2009) A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. Nature Med. 15, 781-788.

[13] Van Kaer, L. Ashtonrickardt, P.G. Eichelberger, M. Gaczynska, M. Nagashima, K. Rock, K.L. Goldberg, A.L. Doherty, P.C. Tonegawa, S. (1994) Altered peptidase and viral-specific T cell response in LMP2 mutant mice. Immunity 1, 533-541.

[14] Hayashi, T. & Faustman, D.L. (2002) Development of spontaneous uterine tumors in low molecular mass polypeptide-2 knockout mice. Cancer Res. 62, 24-27.

[15] Hayashi, T., Kobayashi, Y., Kohsaka, S. & Sano, K. (2006) The mutation in the ATP-binding region of JAK1, identified in human uterine leiomyosarcomas, results in defective interferon-gamma inducibility of TAP1 and LMP2. Oncogene 25, 4016-4026.

[16] Horiuchi, A. Nikaido, T. Ito, K. Zhai, Y.I. Orii, A. Taniguchi, S. Toki, T. & Fujii, S. (1998) Reduced

expression of calponin h1 in leiomyosarcoma of the uterus. Lab. Invest. 78, 839-846.

[17] Horiuchi, A., Nikaido, T., Taniguchi, S. & Fujii, S. (1999) Possible role of calponin h1 as a tumor suppressor in human uterine leiomyosarcoma. J. Natl. Cancer Inst. 91, 790-796.

[18] Winder, S.J. Walsh, M.P. Vasulka, C. & Johnson, J.D. (1993) Calponin-calmodulin interaction: properties and effects on smooth and skeletal muscle actin biding and actomyosin ATPases. Biochemistry 32, 13327-13333.

[19] Wills, F.L. McCubbin, W.D. & Kay, C.M. (1994) Smooth muscle calponin-caltropin interaction: effect on biological activity and stability of calponin. Biochemistry 33, 5562-5569.

[20] Sexl, V. Kovacic, B. Piekorz, R. Moriggl, R. Stoiber, D. Hoffmeyer, A. Liebminger, R. Kudlacek, O. Weisz, E. Rothammer, K. & Ihle, J.N. (2003) Jak1 deficiency leads to enhanced Abelson-induced B-cell tumor formation. Blood 101, 4937-4943.

[21] Rodig, S.J. Meraz, M.A. White, J.M. Lampe, P.A. Riley, J.K. Arthur, C.D. King, K.L. Sheehan, K.C.F. Yin, L. Pennica, D. Johnson, E.M. & Schreiber, R.D. (1998) Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. Cell 93, 373-383.

[22] Schmidtke, G. Kraft, R. Kostka, S. Henklein, P. Frommel, C. Lowe, J. Huber, R. Kloetzel, P.M. & Schmidt, M. (1996) Analysis of mammalian 20S proteasome biogenesis: the maturation of b-subunits is an ordered two-step mechanism involving autocatalysis. EMBO J.15, 6887-6898.

[23] Miano, J.M. Carlson, M.J. Spencer, J.A. & Misra, R.P. (2000) Serum response factor-dependent regulation of the smooth muscle calponin gene. J Biol Chem. 275, 9814-9822.

[24] Yamamura, H. Hashio, M. Noguchi, M. Sugenoya, Y. Osakada, M. Hirano, N. Sasaki, Y. Yoden, T. Awata, N. Araki, N. Tatsuta, M. Miyatake, S.I. & Takahashi, K. (2001) Identification of the transcriptional regulatory sequences of human calponin promoter and their use in targeting a conditionally replicating herpes vector to malignant human soft tissue and bone tumors. Cancer Res. 2001 61, 3969-3977.

[25] Kanamori, T. Takakura, K. Mandai, M. Kariya, M. Fukuhara, K. Kusakari, T. Momma, C. Shime, H. Yagi, H. Konishi, M. Suzuki, A. Matsumura, N. Nanbu, K. Fujita, J. & Fujii, S. (2003) PEP-19 overexpression in human uterine leiomyoma. Mol. Hum. Reprod. 9, 709-717.

[26] Wang, L. Felix, J.C. Lee, J.L. Tan, P.Y. Tourgeman, D.E. O'Meara, A.T. Amezcua, C.A. (2003) The proto-oncogene c-kit is expressed in leiomyosarcomas of the uterus. Gynecol. Oncol. 90, 402-406.

[27] Ylisaukko-oja, S.K. Kiuru, M. Lehtonen, H.J. Lehtonen, R. Pukkala, E. Arola, J. Launonen, V. & Aaltonen, L.A. (2006) Analysis of fumarate hydratase mutations in a population- based series of early onset uterine leiomyosarcoma patients. Int. J. Cancer 119, 283-287.

[28] Mittal, K.R. Chen, F. Wei, J.J. Rijhvani, K. Kurvathi, R. Streck, D. Dermody, J. & Toruner, G.A. (2009) Molecular and immunohistochemical evidence for the origin of uterine leiomyosarcomas from associated leiomyoma and symplastic leiomyoma-like areas. Mod. Pathol. 10, 1303-1311.

[29] Kake, T. Kimura, S. Takahashi, K. & Maruyama, K. (1995) Calponin induces actin polymerization at low ionic strength and inhibits depolymerization of actin filaments. Biochem J. 312, 587-592.

[30] Guadagno, T.M. Ohtsubo, M. Roberts, J.M. & Assoian, R.K. (1993) A link between cyclin A expression and adhesion-dependent cell cycle progression. Science 262, 1572-1575.

[31] Samaha F.F. Ip, H.S. Morrisey, E. Seltzer, J. Tang, Z. Solway, J. & Parmacek, MS. (1996) Developmental pattern of expression and genomic organization of the calponin-h1 gene. A contractile smooth muscle cell marker. J. Biol. Chem. 271, 395-403.

[32] Jiang, Z. Grange, R.W. Walsh, M.P. & Kamm, K.E. (1997) Adenovirus-mediated transfer of the smooth muscle cell calponin gene inhibits proliferation of smooth muscle cells and fibroblasts. FEBS Lett. 413, 441-445.

[33] Frentzel, S. Frentsel, S. Kuhnhartmann, I. Gernold, M. Gott, P. Seelig, A. & Kloetzel, P.M. (1993) The major histocompatibility complex encoded beta-type proteasome subunits LMP2 and LMP7. Evidence that LMP2 and LMP7 are synthesized as proproteins and that cellular levels of both mRNA and LMP-containing 20S proteasomes are differentially regulated. Eur. J. Biochem. 216, 119-126.

[34] Choi, H.S. Seol, W. & Moore, D.D. (1996) A component of the 26S proteasome binds on orphan member of the nuclear hormone receptor superfamily. J. Steroid Biochem. Biol. 56, 23-30.

[35] Zhang, H. Sun, L.Y. Liang, J. Yu, W.H. Zhang, Y. Wang, Y. Chen, Y.P. Li, R.F. Sun, X.J. & Shang, Y. (2006) The catalytic subunit of the proteasome is engaged in the entire process of estrogen receptor-regulated transcription. EMBO J. 25, 4223-4233.

[36] Reits, E.A. Benham, A.M. Plougastel, B., Neefies, J. & Trowsdale, J. (1997) Dynamics of proteasome distribution in living cells. EMBO J. 16, 6087-6094.

Figure 1 Biological activity of hLMP2 in uterine leiomyosarcoma (LMS). **(**A**)** Phase-contrast micrographs of the parental transformed SKN-CEM9#2 (T type) clone and flat revertants of the SKN-LMP2#122 (F type) clone (magnification x100). Changes in human uterine LMS cell line, SKN-transfectants, SKN-CEM9 (T type) clone, and SKN-LMP2wt (F type) clone xenograft volumes in mice (*n*=8). Representative photographs of xenografts in mice (Left). Tumor growth of SKN-LMP2 was markedly reduced in comparison with that of the control transfectant SKN-CEM9 (T type) clone. Tumor growth kinetics after subcutaneous injection of the SKN-CEM9 (T type) clone and SKN-LMP2 (F type) clone (Right). (B) RT-PCR experiments revealed *hLMP2, hLMP7, Calponin h1, SRF, cyclin B* and *β-actin* mRNA expression in tumors. Precursor LMP2 or LMP7 (pre-LMP2, pre-LMP7) and mature LMP2 or LMP7 (LMP2, LMP7) are shown. (C) Western blotting revealed LMP2, LMP7, calponin h1, SRF, cyclin B, and β-actin in SKN-transfectant clones. (D) The luciferase reporter vectors containing the hCalponin h1 promoter with wild type SRF binding sites (Calponin-wt-Luc.), mutant SRF binding sites (Calponin-mut-Luc.), or empty luciferase reporter vector (Basic-Luc.) [23] were transiently co-transfected with pSV-β-galactosidase in SKN-transfectants, SKN-CEM9#2, SKN-LMP2#121, or SKN-LMP2#122 clones for the final 48 h, and then luciferase activities were measured. Values were normalized to those obtained with the co-transfected pSV-β-galactosidase expression vector. Each assay was performed at least three times and in triplicate. Luciferase reporter assays showed that LMP2 expression markedly induced calponin h1 promoter activation. Data are presented as the mean from three independent experiments (*S.D.). The experiments were performed four times with similar results.
SKN transformants[a], CEM9 SKN-CEM9#2; LMP2, SKN-LMP2wt#121, SKN-LMP2wt#122. Detail is shown in SFig. 2, SFig. 3 and STable 3. RT-PCR[b] , total RNA samples were isolated from the individual xenografted-tumors, which were removed at 5 weeks after xenografting. W.B.[c], W.B. are performed with the total cell lysates from SKN transformants.

Figure 2 Biological activity of calponin h1 in uterine leiomyosarcoma (LMS). (A) Phase-contrast micrographs of the parental transformed SKN-CEM9#1Scr.shRNA (T type) clone, SKN-CEM9#2 calponin h1shRNA (T type) clone, SKN-CEM9#2 (T type) clone, SKN-LMP2#1Scr.shRNA (F type) clone, and SKN-LMP2#2Calponin h1shRNA (T type) clone of the SKN-LMP2 (F type) clone (magnification x60). The growth rates of the SKN-transfectant clones were measured as population doubling time (PDT). (B) Western blotting and RT-PCR experiments revealed calponin h1, precursor LMP2 (pre-LMP2), mature LMP2 (LMP2), and β-actin in SKN-transfectant clones. SKN transformants[a], CEM9#3 Scr.shRNA, CEM9#4 Calponin h1shRNA, LMP2#1 Scr.shRNA, LMP2#2 Calponin h1shRNA, Detail is shown in Table 1 and SFig. 5 and STable 3. (C) Changes in the human uterine LMS cell line, SKN-transfectant, SKN-CEM9#2 (T type) clone, SKN-LMP2wt#2/Calponin h1shRNA (T type) clone, and SKN-LMP2wt#1/ Scr.shRNA (F type) clone xenograft volumes in mice (*n*=3). Representative photographs of xenografts in mice (Left). Tumor growth of the SKN-LMP2wt#2/Calponin h1shRNA (T type) clone is mildly increased in comparison with that of the SKN-LMP2wt#1/Scr.shRNA (F type) clone. Tumor growth kinetics after subcutaneous injection

of the SKN-transfectant clones (Right). RT-PCR experiments revealed *hCalponin h1, hLMP2* and *β-actin* mRNA expression in tumors (Bottom). Experiments were performed three times with similar results. SKN-CEM9[c], SKN-CEM9#2; LMP2wt+Calponin h1shRNA[d], SKN-LMP2wt#2/ CalponinshRNA; LMP2wt/Scr.shRNA[e], SKN-LMP2wt#1/Scr.shRNA. Details of SKN transfectants are shown in Table 1, SFig. 5 and STable 3. RT-PCR[f], total RNA samples were isolated from the individual xenografted-tumors, which were removed from BALB/c *nu/nu* mice at 5 weeks after xenografting. Xenografts[g], BALB/c nu/nu mice were inoculated with SKN-CEM9#2, SKN-LMP2wt#2/CalponinshRNA or SKN-LMP2wt#1/Scr.shRNA.

Table 1. Biological/Growth Properties of Transfectants.

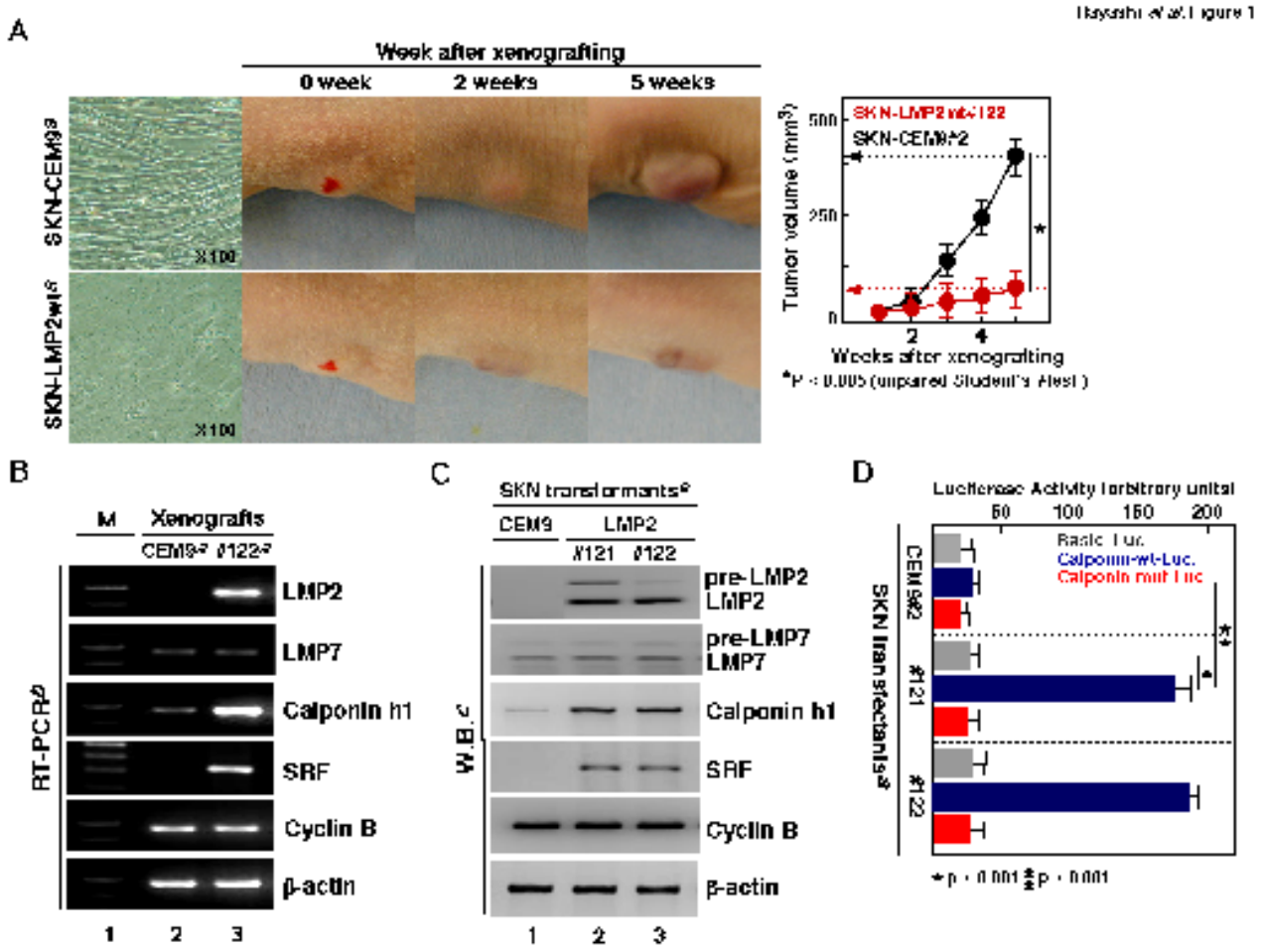
Average:SKN-CEM9[a], total 4 clones; SKN-LMP2wt[b], total 8 clones, SKN-LMP2K33A[c], total 4 clones. Details in Table 2 and STable 3. Cryo UtSMC[d] , Normal Human Uterine Smooth Muscle Cells (CambrexBioScience Walkerville Inc.). [1]Morphology. After 2 to 3 weeks, when most of the colonies outgrew and detached from the substrate, some colonies consisting of very flat cells were found at frequencies of around a few percent of the total number of G418-resistant colonies initially observed in Table S4. Microscopic characteristics such as flat cell morphology and transformed cell morphology were analyzed. The number of flat cells or transformed cells in 10 consecutive high-power-fields was counted. Transformed, no evidence about appearance of flat cells; Flatrevertant, transformed cell number is less than 10% of total cell number; P.Flat., Partially Flatrevertant, transformed cell number is less than 30% of total cell number; P.Tras., Partially Transformed, transformed cell number is higher than 30% of total cell number. [2]PDT: Population Doubling Time. [3]Ratio(%) of soft agar colonies/number of cells plated that have an ability to form colonies on plastic substrate. [4]Diameter of colonies as expressed by the number of cells lined up across the colonies; determined on day 21. The experiments were repeated three times with similar results. [5]Cells ($1x10^7$) were inoculated subcutaneously into 7-8-weeks old nude mice, and the mice were periodically examined for evidence of tumors, -, no evidence of tumors; +/-, tumors of <0.5 cm diameter; +, tumors of 0.5-1.0 cm diameter; ++, tumors of 1.0-1.5 cm diameter, +++, tumors of 1.5-2.0 cm diameter, in two inoculated mice. Experiments were terminated at 5 weeks after inoculation. [6]Estimated by immunoblot analysis. W, weak expression; L, low expression; M, medium expression; H, high expression; -, no evidence of expression. [7]Incorporation of LMP2 into proteasome complexes. Proteasome was isolated by GST-UbL beads in accordance with manufacturer's recomendation (Merck Ltd. Darmstadt, Germany). Immunoblot analysis was performed. -, no evidence of expression; +, Detectable expression of LMP2 by immunoblot analysis.

Table 2. Biological Properties of the SKN-Transfectants with shRNAs.

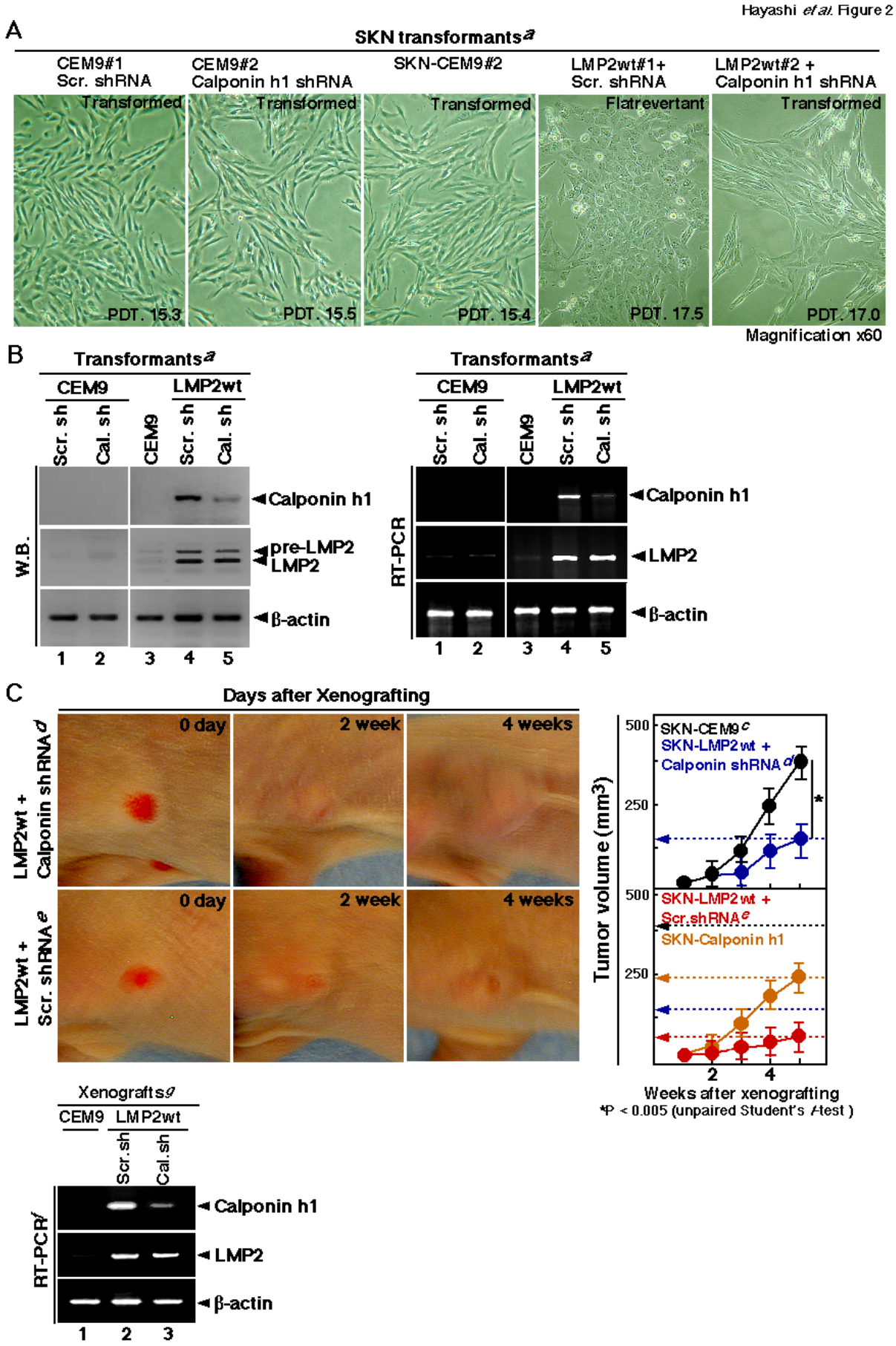
[a]Clone no. Details of SKN transtectant clones are in Table 1 and Table S5. [b]Vector DNA. LMP2 expression vector was co-transfected into SKN cells with shRNA vector. [c]shRNA, Calponin h1 shRNA or Scramble shRNA were co-transfected into SKN cells with LMP2wt expression vector

using manufacturer's recomendations (Santa Cruz Biotechnology, Inc., CA, USA). [d]Morphology. Microscopic characteristics such as flat cell morphology and transformed cell morphology were analyzed. The number of flat cells or transformed cells in 10 consecutive high-power-fields was counted. Transformed, no-evidence about appearance of flat cells; Flatrevertant, transformed cell number is less than 10% of total cell number; P.Flat., Partially Flatrevertant, transformed cell number is less than 30% of total cell number, P.Trans., Partially Transformed, flatrevertant cell number is less than 30% of total cell number. [e]Estimated by immunoblot analysis. +, high expression; -, no evidence of expression; MD, marked down-expression. [f]Cells ($1x10^7$) were inoculated subcutaneously into 7-8-weeks old nude mice, and the mice were periodically examined for evidence of tumors; -, no evidence of tumor; +/-, tumor of <0.5 cm diameter; +, tumor of 0.5-1.0 cm diameter; ++, tumor of 1.0-1.5 cm diameter; +++, tumor of 1.5-2.0 cm diameter; in two inoculated mice. Experiments were terminated at 5 weeks after inoculation. [g]Ratio(%) of soft agar colonies/number of cells plated that have an ability to form colonies on plastic substrate. [h]Diameter of colonies as expressed by the number of cells lined up across the colonies; determined on day 21. The experiments were repeated three times with similar results.

Hayashi *et al.* Figure 1.



Hayashi *et al.* Figure 2.



Hayashi et al. Table 1.

Hayashi *et al.* Table 1

Biological/Growth Properties of Transfectants										
Cell clones	Morphology[1]	Doubling time PDT[2]	SoftAgarCology Efficiency(%)[3]	Size[4]	Tumori-genecity[5]	Protein Expression[6] LMP2	LMP7	Calponin	Cyclin B	Inco. LMP2[7]
SKN-CEM9[a]	Transformed	15.3 ± 1	60 ± 7	11-27	+++	W	L	-	M	-
SKN-LMP2wt[b]	Flatrevertant	17.6 ± 3	5.2 ± 7	5-10	+/-	H	H/M	H/M	M	+
SKN-LMP2K33A[c]	Flatrevertant	17.2 ± 4	6.1 ± 3	5-11	+/-	H	M	M	M	-
Cryo UtSMC[d]	Flat	19.4	< 0.5	-	-	M	M	H	M	+

Hayashi et al. Table 2.

Hayashi *et al.* Table 2

Biological Properties of the SKN-transfectants with shRNAs

[a]Clone no. or [b]Vector DNA	[c]shRNA	[d]Morphology	PDT	Expression[e] LMP2	Calponin	Tumori-genicity[f]	Sof Agar Colony Efficiency(%)[g]	Size[h]
CEM9#1	Scr.	Tronsformed	15.2	-	-	+++	58	11-29
CEM9#2	Calponin	Tronsformed	15.3	-	-	+++	67	10-26
CEM9#3	Scr.	Tronsformed	15.5	-	-	++	63	11-28
CEM9#4	Calponin	Tronsformed	15.1	-	-	++	62	11-25
CEM9#5	Scr.	Tronsformed	15.3	-	-	+++	68	12-24
CEM9#6	Calponin	Tronsformed	15.5	-	-	+++	61	14-26
CEM9#7	Scr.	Tronsformed	15.3	-	-	+++	64	10-29
CEM9#8	Calponin	Tronsformed	15.1	-	-	+++	59	11-27
[b]LMP2wt#1	Scr.	Flatrevertant	17.5	+	+	+/-	5.9	5-10
LMP2wt#2	Calponin	Transformed	17.0	+	MD	++	6.7	6-12
LMP2wt#3	Scr.	Flatrevertant	17.6	+	+	+	5.6	7-11
LMP2wt#4	Calponin	Transformed	16.8	+	MD	++	6.8	6-10
LMP2wt#5	Scr.	Flatrevertant	17.5	+	+	+/-	6.4	7-14
LMP2wt#6	Calponin	Partially Trans.	16.9	+	MD	++	6.2	5-12
LMP2wt#7	Scr.	Flatrevertant	17.5	+	+	+	6.6	6-11
LMP2wt#8	Calponin	Transformed	16.7	+	MD	++	6.9	5-10