Animal Model

A Mouse Model of Uterine Leiomyosarcoma

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We are using an approach that is based on the *cre/loxP* recombination process and involves a binary system of Cre-producing and Cre-responding transgenic mice to achieve ubiquitous or tissue-specific expression of oncoproteins. To develop mouse models of tumorigenesis, Cre-producers are mated with responder animals carrying a dormant oncogene targeted into the 3' untranslated region of the locus encoding cytoplasmic β -actin (actin cassette). Production of oncoprotein from a bicistronic message is accomplished in bitransgenic progeny by Cre-mediated excision of a segment flanked by *loxP* sites that is located upstream from the oncogenic sequence. Widespread Cre-dependent activation and expression of an actin-cassette transgene encoding the T antigens of the SV40 early region (SVER) commencing in embryos was compatible with normal development and did not impair viability. However, at ~3 months of age, all female animals developed massive uterine leiomyosarcomas, whereas practically all males exhibited enormously enlarged seminal vesicles because of pronounced hyperplasia of the smooth muscle layers. In addition, because of smooth muscle hyperproliferation, marked dilation of the gallbladder was observed in mice of both sexes. To begin exploring aberrant signaling events in the SVER-triggered tumorigenic pathways, we analyzed the expression profile of leiomyosarcomas by DNA microarray analysis. (*Am J Pathol* 2004, 164:325–336)

The most common neoplasms of the female genital tract are uterine leiomyomas (ULM; "fibromas"). Although the exact incidence of these benign, monoclonal smooth-muscle tumors is uncertain, it has been estimated that they occur in ~20 to 30% of women older than 30 years of age.^{1,2} This, however, could be an underestimate, as serial sections of hysterectomy specimens have demon-

strated that, regardless of the indication for surgery, leiomyomas were present in up to 77% of the cases.³

In contrast to the high frequency of ULM, malignant uterine leiomyosarcomas (ULMS) are rare neoplasms, representing only 1 in 200 to 800 smooth muscle tumors or ~1% of all malignancies of the uterus.^{4,5} Although the reported 5-year ULMS survival rates are variable, these tumors are clinically aggressive and have a high risk of recurrence and an overall poor prognosis.⁶ It is notable that firm diagnosis of ULMS cases among the large number of benign ULMs could be challenging for the pathologist because, in addition to the unambiguous ULM and ULMS extremes, a spectrum of intermediate forms with overlapping features ("smooth muscle tumors of uncertain malignant potential" or STUMP and other variants) can be encountered.^{7,8} It is unknown, however, whether the lesions in this histopathological spectrum have any relationships in pathogenesis and could represent stages of tumor evolution. Moreover, despite some reports⁹ suggesting that ULMS could arise not only *de novo* but also from pre-existing ULM, conclusive evidence documenting the transformation of ULM to ULMS is lacking in humans and it is thought that, if it occurs, the incidence is <0.1%.^{2,10} Nevertheless, it seems that such malignant conversion is possible in Eker rats,¹¹ which carry an insertional germline mutation of the tuberous sclerosis 2 (*Tsc2*) gene. Loss of heterozygosity in this animal model results in the development of ULM and other reproductive tract leiomyomas in ~65% of the female rats (in addition to renal carcinomas), but rare ULMS are also seen.¹¹

Whereas transgenic mouse models of ULM also exist,^{12–15} genetically modified mice developing ULMS have not been described thus far, although they could be valuable for studying the pathobiology of the tumors and for eventually providing clinically important information,

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especially for diagnostic purposes. In problematic STUMP cases, for example, which necessitate a detailed assessment of multiple histological variables, diagnosis and prognosis could be significantly facilitated by establishing molecular criteria. Such information can be potentially based on data derived by studying murine tumors with the advanced analytical approach of expression profiling using DNA microarrays in combination with detailed histopathological analysis.

Here we report the characterization of a mouse model of ULMS that was developed in the context of a broader research program aiming to construct genetically modified mice forming tumors at various anatomical sites.

Materials and Methods

Mice

Mice expressing SV40 T antigen oncoproteins (see Results) were generated as described¹⁶. In addition, we used our *Hs-cre*¹⁷ strain of transgenic mice, in which *cre* is expressed constitutively under the control of a heat-shock gene promoter.

Molecular Analyses

For genotyping, DNA was prepared from tails and tissues of mice and analyzed by Southern blotting. Northern analysis was performed using total RNA (10 μ g per lane) that was extracted from tissues with TRIzol reagent (Invitrogen, Carlsbad, CA). The blots were hybridized with probes recognizing the sequences encoding SV40 large T antigen or β -actin. For protein analysis, immunoprecipitation and immunoblotting were performed by standard procedures using cell lysates from various tissues. To assay for SVLT, a monoclonal antibody (DP02; Oncogene Research Products, San Diego, CA) was used to immunoprecipitate 100 μ g of tissue extract followed by immunoblotting with the same antibody. Antibodies against Twist (sc-15393; Santa Cruz Biotechnology, Santa Cruz, CA) and Cdc7 (sc-13010) were also used for immunoblotting.

Histological Analysis

Mice exhibiting overt pathological signs were sacrificed and autopsied. Dissected tissue samples from all major organs were fixed in 10% formalin for at least 24 hours, dehydrated, and embedded in paraffin. Paraffin blocks were sectioned and stained with hematoxylin and eosin. Immunophenotyping was performed according to standard procedures using primary antibodies against SVLT (DP02; Oncogene Research Products), estrogen receptor (Santa Cruz Biotechnology), progesterone receptor (Santa Cruz Biotechnology), androgen receptor (Novocastra, Newcastle, UK), smooth muscle actin (DAKO, Carpinteria, CA) and desmin (DAKO).

Microarray Analyses

For expression profiling of uterine leiomyosarcomas, total RNA was extracted from tumor-bearing uteri of five transgenic mice and from normal (control) uteri of four wild-type virgin littermates. The samples were profiled individually.

For each assay, 7 μ g of total RNA were used to generate biotinylated cRNA, as described.¹⁸ Fragmented cRNA (15 μ g) was then hybridized to MGU74Av2 DNA chips (Affymetrix, Santa Clara, CA) with $\sim$ 12,000 gene entries. The microarrays were scanned (Affymetrix Scanner) and expression values for the genes were determined using Affymetrix Microarray Suite version 5.0. A gene-by-gene analysis was then pursued, rather than a pattern discovery procedure, in an attempt to extract detailed information. Before statistical analysis, the data were filtered to exclude genes with very low or undetectable absolute expression values in all samples (a value <1000 was chosen as a cutoff point; although arbitrary, this value was used on the basis of our experience that hybridization signal intensities with readings <1000 tend to fluctuate and are unreliable). The filtered data were then analyzed using GeneCluster2 software,¹⁹ to determine the statistical significance of the expression differences observed. In the final list of differentially expressed genes, we included entries that met a criterion of significance at the $P < 0.05$ level in the permutation analysis of the GeneCluster2 program and had a t -test score >2 . Using then the list of entries that had passed both of these tests, ratios of averages of experimental and control values were computed and genes that showed a fold difference of two or greater (246 up-regulated and 238 down-regulated transcripts) were studied in detail through extensive literature searches. A ratio of ≥ 2 as the cutoff point indicating a significant difference is an arbitrary, but widely used conventional setting in the vast majority of microarray analyses.

Results and Discussion

Experimental System

The murine model of ULMS that we describe below arose during development of an approach to trigger tumorigenic events by expressing oncoproteins either widely (in all or many tissues) or specifically at a chosen anatomical site¹⁶. This scheme, which involves a binary system of transgenic mice, is a novel application of the features of the *cre/loxP* site-specific recombination process. The ability of the recombinase (Cre) to excise segments of DNA flanked by *loxP* sites (floxed) has been used extensively for conditional modifications of mouse genomic loci, including expression of activatable alleles.²⁰

In our method, the genetic components of the recombination system, ie, the Cre-encoding transgene and the *loxP*-modified locus that also carries a dormant oncogene, reside in different mouse strains (Cre-producers and Cre-responders, respectively), and are brought together in the progeny after mating. As a consequence,

the oncogene is activated by removal of a floxed sequence. Therefore, the wide or narrow specificity of the promoter driving *cre* expression determines the tissue in which the oncoprotein will be produced. This provides great experimental flexibility to the system, because a single line of responders can be used with various activating strains of Cre-producers to study the oncogenic process in a variety of tumors.

For the generation of responder mice, it is advantageous to insert oncogenes (usually in the form of cDNAs) into loci that are expressed highly and ubiquitously using an embryonic stem (ES) cell targeting (knock-in) protocol. Currently, we use as a host locus the cytoplasmic β -actin gene and insert by knock-in oncogenic transgenes into the 3'-noncoding region downstream from the translational terminator, to generate bicistronic messages (Figure 1A). In the targeting vector, the 5' and 3' regions of homology to the actin locus flank a segment consisting of an internal ribosome entry site (IRES) for cap-independent translation of a floxed *neo* gene used for selection of targeted ES cells, which is linked to a polyadenylation signal and is followed by the oncogene (for details, see legend to Figure 1). The DNA segment consisting of these three elements is positioned between very rare restriction sites. Thus, the vector serves as a cassette (actin cassette) because any oncogenic sequence can be easily removed and replaced by another.

Among several strains with actin-cassette constructs that we generated were mice carrying a transgene encoding the three T antigens of the SV40 early region (Figure 1, A and Ba). These proteins, large T (SVLT), small t (SVST), and 17kT,²¹ collectively referred to here as SVER, are products of differentially spliced mRNAs. Although the protein that is essential for cell transformation is SVLT, we decided to also use the other antigens that stimulate its actions, considering that the spectrum of tumors developing in transgenic mice expressing either SVER or SVLT alone could be different.^{22,23}

Unexpectedly, in addition to Cre-dependent DNA excision as planned (see next section), we encountered deletion of the *neo* sequence and consequent SVER expression occurring in responder mice without the involvement of Cre. Although the frequency of such spontaneous deletion events was extremely low (they occurred in $<1/10^6$ cells), SVER activation resulted through clonal growth in the appearance of disseminated high-grade undifferentiated sarcomas that involved various abdominal and/or thoracic organs (T_{50} , the time for 50% tumor-free survival was ~ 8 months).¹⁶

Ubiquitous Cre-Dependent SVER Expression

To examine the consequences of expression of SVER in all tissues, as a means of revealing particular tissue susceptibilities to these oncoproteins, we used for Cre-mediated activation our *Hs-cre1* transgenic line.¹⁷ In this strain of mice, a *cre* transgene driven by the *Hsp70-1* heat-shock promoter is expressed in two-cell stage embryos because of constitutive transcription.

We observed that expression of SVER in β -actin^{SVER/+}/*Hs-cre1* bitransgenic progeny was compatible with via-

bility, at least at the levels attained by the encoded oncoproteins. In these animals, *neo* excision had occurred in all tissues (Figure 1Bb) and resulted in ubiquitous, albeit variable, expression of SVLT, as shown by Northern and Western analyses (Figure 1B). Immunohistochemically detectable SVLT was observed in a wide spectrum of tissues including cells of the hematopoietic lineage, immature neurons, mesenchymal cells, myoepithelial cells, and mammary epithelial cells (not shown).

Unexpectedly, despite this broad expression pattern, the manifested tumorigenic phenotype was narrowly tissue-specific. Thus, when the animals reached adulthood, they developed very large abdominal tumors and were sacrificed. Survival analysis showed that the T_{50} was 83 days for females (range, 61 to 114 days) and 107 days for males (range, 59 to 176 days).

Dissection and macroscopic examination showed the presence of large masses originating from the uterus in females; enormously enlarged seminal vesicles and excretory ducts in males; and dilated gallbladders in both sexes (Figure 2 A, B, J, and M). The uterine/gallbladder phenotype was manifested in 24 of 24 females that were monitored, whereas 22 of 24 males exhibited the seminal vesicle and gallbladder abnormalities. Of the affected animals, four males and four females developed, in addition, undifferentiated sarcomas identical to those recorded as Cre-independent events (see above). Such sarcomas were the only phenotypic manifestation in 2 of 24 mice in the male cohort. Histopathological analysis and immunophenotyping indicated that all tumors, other than undifferentiated sarcomas, originated from smooth muscle cells (SMCs) of the affected organs (Figure 2). The uterine tumors were classified as leiomyosarcomas. They were solid neoplasms with a diameter ~ 20 times larger than that of normal myometrium (Figure 2, C and D; 5.9 ± 2.2 versus 0.28 ± 0.07 mm; $P < 0.0001$; $n = 21$ and 13, respectively) involving the corpus of the uterus and also the cervix and the uterine horns (parallel examination of the oviducts revealed smooth muscle hyperplasia, but not neoplasia). In the uterine tumors, the spiral cell arrangement of the normal muscle layers was completely disrupted and replaced by long fascicles of atypical, densely packed cells with large dark nuclei crossing at various angles (Figure 2F). Indicative of the proliferative activity in the tumors, which resulted in the observed marked hypercellularity, was the detection of mitotic figures (MF) in high number (31 ± 16 MF per 10 high-power fields on average; range 10 to 60; Figure 2G), in contrast to the quiescent SMCs of the normal myometrium (absence of mitotic figures). The MF number in the murine ULMS was three times higher than the threshold of 10 MF/10 high-power fields distinguishing ULM from (malignant) ULMS in humans. Frequent invasion of the endometrial stroma and ulceration of the epithelial lining, revealed further the malignant character of the mouse tumors (Figure 2E; leiomyosarcomas, rather than leiomyomas). The fibrovascular tumor stroma, containing mitotically active fibroblasts and endothelial cells suggestive of neovascularization, was also expanded, in comparison with its normal counterpart, and contained a

variable amount of inflammatory infiltrate. In addition to the expected positive immunostaining for SVLT (Figure 2H), the uterine leiomyosarcomas were positive for smooth muscle markers (α -actin and desmin; Figure 2I) and also for estrogen and progesterone receptors. Preliminary ovariectomy experiments using two females at the age of 1 month suggested a potential involvement of ovarian hormones in the growth of these tumors. When these animals were sacrificed 2 months after the operation because of enlarged gallbladders, the sizes of the detected uterine smooth muscle tumors were very small. In fact, histopathological analysis of one of them showed moderate neoplastic thickening (1.5 mm) without discernible mitotic activity.

The enlargement of the seminal vesicles (Figure 2J) was due to pronounced hyperplasia of the smooth muscle layers that were approximately nine times thicker than normal (Figure 2, K and L; 246 ± 87 versus $28 \pm 5 \mu\text{m}$; $P < 0.0001$; $n = 16$ and 5, respectively) and exhibited significant mitotic activity (33 ± 27 MF per 10 high-power fields, in contrast to the cellular quiescence in control animals). Analogous hyperplasia was observed in the vas deferens (and to some degree in the epididymis and occasionally in the anterior lobe of the prostate). The

arrangement of the hyperplastic muscle in longitudinal and transverse layers and the amount of stroma were as in control animals, and no signs of neoangiogenesis were evident. Immunostaining was positive for SVLT, smooth muscle markers and androgen receptor, and negative for estrogen and progesterone receptors. Despite a high level of mitotic activity, we favor the interpretation that the condition of the seminal vesicles is hyperplastic, rather than neoplastic on the basis of two criteria: we found no evidence for invasion and were unable to detect distortions in the shape and contour of these organs.

Smooth muscle hyperproliferation resulting in ductal obstruction and marked dilation was observed in the gallbladders of transgenic animals regardless of sex (Figure 2; M, N, and O). With the exception of rare SMCs immunoreactive for the estrogen receptor, the immunophenotyping for steroid hormone receptors was overall negative in the enlarged gallbladders, whereas the immunostaining for SVLT and smooth muscle markers and also the degree of hyperplasia were similar to those described for male genital tracts (average thickness of $60.6 \pm 15 \mu\text{m}$ versus $14.4 \pm 2 \mu\text{m}$ in controls; $P < 0.0001$; $n = 37$ and 17, respectively), despite a low level of mitotic activity (<3 MF/10 high-power fields).

A. Experimental Design

B. Molecular Analyses

Figure 1. Experimental design and molecular analyses of β -actin^{SVER/+} mice. **A:** Schematic representation of the experimental strategy used for conditional expression of SVER in mice. SVER is targeted to the mouse β -actin locus (**top**; the six exons are shown as **black rectangles**) by homologous recombination in ES cells and is inserted between rare *PacI* (*Pc*) and *NofI* (*N*) sites engineered downstream of the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) and floxed neomycin resistance marker (*neo*) present in the 3' UTR of the gene (targeted β -actin locus). A bicistronic actin-*neo* message is expressed in these mice. Upon Cre-mediated *neo* excision, a single *loxP* site is left behind and a new bicistronic mRNA (actin-SVER) is generated (targeted β -actin locus after Cre-mediated recombination). The position of the probe used for Southern analysis and the sizes of the wild-type (11.1 kb), targeted (8.2 kb), and recombined (7.0 kb) DNA fragments recognized by this probe are indicated. Additional restriction sites shown are: *PstI* (*P*), *EcoRI* (*R*), *Pml* (*Pm*), and *SalI* (*S*). **B:** Molecular analyses of SVER-induced tumorigenesis in β -actin^{SVER/+}; *HS-cre1* mice. For Southern analysis, DNA was digested with *PacI* and *EcoRI*. The results of genotyping using tail DNA from mice that turned out to be wild type (Wt) or heterozygotes carrying the SVER transgene (Tg) are shown in **a**. In **b**, the results of the displayed Southern analysis indicate that Cre-mediated recombination occurred in all of the examined tissues [kidney (Ki), liver (Li), lung (Lu), heart (He), pancreas (Pa), spleen (Sp), and brain (Br)] of a female animal with a β -actin^{SVER/+}; *HS-cre1* genotype, which developed a uterine leiomyosarcoma (Ls). The results of the Northern analysis shown indicate that SVER-containing RNA is produced only after Cre-mediated recombination in the examined tissues. The kidney (Ki), spleen (Sp), and brain (Br) samples shown on the **left** are from an animal with a β -actin^{SVER/+} genotype, whereas the samples from the same tissues shown on the **right** are from an animal with a β -actin^{SVER/+}; *HS-cre1* genotype. The membrane was first hybridized with an SVLT-specific probe (**top**) and subsequently rehybridized with a β -actin cDNA probe (**bottom**). The transcripts from the intact β -actin allele and the bicistronic actin-*neo* and actin-SVLT mRNAs are indicated. Western analysis to detect SVLT expression was performed either directly (**top**) or after immunoprecipitation (**bottom**) with an antibody against SVLT using protein extracts from various tissues [kidney (Ki), heart (He), lung (Lu), spleen (Sp), brain (Br), striated muscle (Mu), seminal vesicle (Sv), pancreas (Pa), and liver (Li)] of a male animal with a β -actin^{SVER/+}; *HS-cre1* genotype (**top**) or a female animal (**bottom**) that had the same genotype and developed a leiomyosarcoma (Ls). SVLT was present in all of the examined tissues, although at variable amounts, as indicated by a comparison after analysis of the same membrane with an actin antibody. Proteins extracted from targeted ES cells before (Tr) and after (Rc) *neo* excision by transiently expressed *cre* were used, respectively, as negative and positive controls. Examples of validation of ULMS microarray data were provided by Western analysis of the levels of Cdc7 and Twist proteins present in extracts from normal uterus (Wt) and leiomyosarcomas (Ls). The same membranes were analyzed using an antibody against actin (control).

Figure 2. Smooth muscle tumors and hyperplasias developing in β -actin^{SVL/+}; HS-cre1 mice. **A**: A normal uterus from a virgin animal (**A**) is compared to the grossly enlarged uterus of a bitransgenic female that has developed a leiomyosarcoma (**B**). The histological sections in **C** and **D** correspond to uteri as those shown in **A** and **B**, respectively. The width of the normal smooth muscle layers (**A**) has been greatly expanded in the leiomyosarcoma (**B**), as indicated by the sizes of the black bars. **E**: Bundles of tumor cells invading the adjacent endometrial stroma (arrow) are indicative of the malignant character of the uterine leiomyosarcomas. The tumors are composed of cells with large dark nuclei (**F**), have a high mitotic index (**G**, arrow), and exhibit positive immunostaining for SVLT (**H**) and desmin (**I**). **J**: Normal seminal vesicles (left) are compared with enormously hyperplastic seminal vesicles from a bitransgenic male (right), whereas the sizes of the testes (**J**, bottom) are similar in both cases. The sizes of the bars in the histological sections shown in **K** and **L** show the difference in thickness between the normal and hyperplastic smooth muscle layers of the seminal vesicles, respectively. **M**: A normal gallbladder, photographed attached to a piece of liver for display, is indicated by an arrow on the left, whereas a distended gallbladder from a bitransgenic animal is shown on the right. In the histological sections, the bars indicate the difference in width between the normal (**N**) and hyperplastic (**O**) smooth muscle layers of the gallbladder walls. Original magnifications: $\times 10$ (**C**, **D**, **K**, **L**); $\times 20$ (**E**, **N**, **O**); $\times 40$ (**F**, **H**, **I**); $\times 100$ (**G**).

Comparison of SVL-Induced Uterine Leiomyomas and Leiomyosarcomas

Noninvasive ULM of variable (sometimes large) size also developed with high or complete penetrance in transgenic mice carrying either SVLT driven by the promoter of

the rat *Calb3* gene (encoding the cytosolic calcium-binding protein calbindin-D9K)¹³ or SVL driven by the promoters of the genes encoding mouse oviduct glycoprotein 1 (*Ovgp1*),¹⁴ mouse smooth muscle actin (*Acta2*),¹⁵ or rabbit smooth muscle myosin heavy chain (*Myh11*).¹⁶ In the latter two cases, smooth muscle hyperplasia of the seminal vesicles was also observed. In the *Ovgp1*-SVL case, the endometrium was involved, in addition to the myometrium, while the oviductal and vaginal epithelia exhibited some hyperplasia and atypia. Interestingly, despite SVL expression in the ovary, tumors did not appear in this tissue. Thus far, development of a leiomyosarcoma in a uterine horn was described only in a single transgenic female carrying SVLT under the human mineralocorticoid receptor gene (*Nr3c2*) promoter.²⁴ Therefore, the female mice that we have described here, manifesting ULMS with complete penetrance, constitute the first animal model for this type of malignancy. It is notable that leaky expression of a polyomavirus large T-antigen transgene driven by the MMTV LTR (in addition to mammary glands, transcripts were detected in several other tissues) resulted in the development of ULMs and seminal vesicle hyperplasias.²⁵ It is likely, therefore, that this oncoprotein and SVL are deregulating similar or overlapping signaling pathways.

The ability of SV40 oncoproteins to trigger development of ULM or ULMS in different mouse models strongly suggests that the open question of potential transformation of ULM to ULMS, regardless of frequency, merits re-examination. In this regard, it is highly suggestive that, when our microarray analysis data for murine ULMS (see next section) were compared with analogous gene expression results for human ULM,²⁶ some entries could be identified that were common and mostly concordant between the two data sets. Thus, of 53 reported transcripts found up-regulated (more than twofold-increase) in human ULM, 10 (~19%) were also up-regulated in mouse ULMS (*Actc1*, *Col5a2*, *Gria2*, *Igf1*, *Igf1bp5*, *Mmp9*, *Nptx2*, *Pik3r1*, *Thbs2*, and *Tgfb3*), whereas only 2 of 53 entries (~4%) were discordant (*Bcat1* and *Pcp4*; down-regulated in ULMS). Similarly, of 52 reported transcripts found down-regulated (more than twofold decrease) in human ULM, 7 (13.5%) were also down-regulated in mouse ULMS (*Aldh1a1*, *Dpt*, *Kit*, *Krt19*, *Map3k5*, *Rnase4*, *Tnxb*), whereas only 2 of 52 entries (~4%) were discordant (*Cdh13* and *Gbp2*; up-regulated in ULMS). The probability that the overall common entries are fortuitously concordant is <0.003 .

The SVL-induced ULMS that we have studied are signature tumors (ie, tumors exhibiting reproducibly a characteristic histopathological pattern). Importantly, however, in addition to their stereotypical appearance, these neoplasms involve the entire myometrium, instead of being simply multifocal, ie, all smooth muscle cells have undergone malignant transformation and there is no remnant of the normal myometrial layers. Thus, the oncogenic process appears to be strictly deterministic. Although uniform benign leiomyomas of the uterus induced by SVLT have been described,¹³ it is impossible to posit that analogous tumors evolved in our case and became polyclonal malignant signature leiomyosarcomas by

Table 1. Genes Differentially Expressed Between Uterine Leiomyosarcomas and Normal Uterus

Up-regulated genes			Down-regulated genes		
Gene symbol	Gene name	LMS/WT	Gene symbol	Gene name	WT/LMS
Transcription factors			Transcription factors		
E2f1	E2F transcription factor 1	3.2	Myb	Myeloblastosis oncogene	4.1
Twist	Twist gene homolog, (Drosophila)	3.2	Lmyc1	Lung carc. myc rel. onc. 1	7.7
Irb4	Inhibitor of DNA binding 4	3.5	Nmyc1	Neuroblas. myc-rel. onc. 1	4
Nfe2	Nuclear factor, erythroid derived 2	3.8	Hoxb5	Homeobox B5	2.9
Signaling molecules			Hoxb6	Homeobox B6	3.8
Igf1	Insulin-like growth factor 1	3.2	Hoxb7	Homeobox B7	2.4
Cdc711	Cell division cycle 71 hom.-like 1	4.0	Msx1	Homeobox, msh-like 1	12.8
Sfrp2	Sec. frizzled-related seq protein 2	3.4	Dlx5	Distal-less homeobox 5	12.4
Wisp1	WNT1 ind. sig. pathway protein 1	5.0	Pax8	Paired box gene 8	24
Rb1	Retinoblastoma 1	2.1	Foxa2	Forkhead box A2	15.7
Rb11	Retinoblastoma-like 1 (p107)	2.5	Gata6	GATA binding protein 6	3
Myc	Myelocytomatosis oncogene	2.4	Klf5	Kruppel-like factor 5	3.3
Map3k8	Mitogen act, protein 3 kinase 8	5.5	Sox17	SRY-box containing gene 17	7.4
Rac2	RAS-rel C3 botulinum substrate 2	4.5	Ctr1-pending	Tcfcp2-rel. repressor 1	13.1
Rrad	Ras-rel. associated with diabetes	5.0	Eya2	Eyes absent 2 homolog	5.6
Gdf10	Growth differentiation factor 10	3.7	Elf3	E74-like factor 3	5.6
Sh3d2c2	SH3 domain protein 2C2	3.6	Lisch7-pending	Liver-spec. bHLH-Zip fac	5.6
Ltbp1	Latent Tgf beta binding protein 1	3.4	Signaling molecules		
Csrp2	Cysteine-rich protein 2	3.4	Wnt4	Wingless-rel. MMTV int. site 4	3.1
Fhl1	Four and a half LIM domains 1	3.2	Efnb2	Ephrin B2	5.1
Pscdbp	PH, Sec7, coil./coil dom bind. prot.	3.1	Grb7	Growth fac. rec. bound pr. 7	3.1
Adhesion and ECM			Eps8	Egfr pathway substrate 8	4.2
Mmp8	Matrix metalloproteinase 8	14.1	Gpr56	G protein-coupled receptor 56	3.1
Mmp9	Matrix metalloproteinase 9	7.0	Tec	Tyr. kinase, Dscr28C related	3.5
Tnc	Tenascin C	5.0	Rgnf	Rho-guanine nuc. exch. fac.	3.2
Thbs2	Thrombospondin 2	4.0	Adhesion and ECM		
Itgb2	Integrin beta 2	5.9	Itga6	Integrin alpha 6	4.4
Cdh13	Cadherin 13	4.2	Itgb4	Integrin beta 4	3.4
Selp	Selectin, platelet	5.4	Lamc2	Laminin, gamma 2	3.9
Ncam1	Neural cell adhesion molecule 1	4.8	Tnxb	Tenascin XB	3.4
Gjb6	Gap junction mem. channel prot. β 6	8.7	Cldn3	Claudin 3	8.3
Sgcg	Sarcoglycan, gamma	5.5	Cldn7	Claudin 7	8
Other genes			Icam2	Intercellular adh. molecule 2	3.6
Myla	Myosin light chain, alk. card. atria	18.5	Krt1-19	Keratin complex 1, acidic, 19	4.6
Myom1	Myomesin 1	4.6	Krt2-8	Keratin complex 2, basic, 8	7.2
Acta2*	Actin, alpha 2, sm. muscle, aorta	1.6	Krt1-12	Keratin complex 1, acidic, 12	3.5
Actg2*	Actin, gamma 2, smooth muscle	1.5	Clu	Clusterin	3.5
Acta1*	Actin, alpha 1, skeletal muscle	1.8	Selenbp1	Selenium binding protein 1	3.1
Cnn1*	Calponin 1	1.9	Celsr1	Cadherin EGF LAG receptor 1	3.1
Cnn2	Calponin 2	3.5	Cftr	Cystic fibrosis transmem. reg.	4.1
Gria2	Glutamate rec., ionotropic, AMPA2	11.9	Other genes		
S100a4	S100 calcium binding protein A4	3.3	Casp6	Caspase 6	3.1
Ltb4r1	Leukotriene B4 receptor 1	4.7	Gadd45a	Growth arrest & DNA-dam45 α	3.3

The genes are functionally categorized (related genes are grouped). Only a subset of the genes listed is discussed in the text, including certain ones that are below the two-fold cutoff point (indicated by asterisks). LMS/WT is the fold difference of the average expression values for the leiomyosarcoma samples over normal uterus (values are not listed in order of magnitude). WT/LMS is the reciprocal ratio.

stepwise occurrence of identical and independently selected secondary mutational events. Accordingly, we think that the diverse actions of the SVER-encoded oncoproteins caused the recruitment and combinatorial engagement of deranged signaling pathways involved in apoptosis and proliferation control. Such abnormalities eventually culminated in the development of full-fledged malignancy with the participation of the cellular microenvironment consisting of the tumor stroma, including the extracellular matrix (ECM), and potentially the neighboring endometrial stroma as well. We propose, in this regard, that the particular tissue susceptibility to SVER-induced malignant oncogenicity that we have encountered should be attributed to a distinct interplay of aberrant signaling between the uterine smooth muscle cells and their environment.

Microarray Analysis of Uterine Leiomyosarcomas

Analyses of histopathological patterns in combination with expression profiling of signature tumors can provide important clues for initiating studies to address mechanistic questions in regard to aberrant signaling in tumorigenic pathways.

In our microarray analysis of SVER-induced leiomyosarcomas (Table 1; for details of our approach, see Materials and Methods), we attempted to gain some insights on affected pathways by considering the functions of the tumor-inducing viral oncoproteins on one hand, and the particular tumor type (smooth muscle sarcoma) on the other. We chose, however, to comment selectively on

apparent candidate mechanistic relationships by presenting literature-based annotations, instead of providing an exhaustive and tedious account of the entire set of profiling data (our results are presented in detail at http://icg.cpmc.columbia.edu/efstratiadis/2003_AJP.htm).

A caveat concerning the evaluation of such data is that comments about correlations between microarray entries are based on extrapolations from fragmented and heterogeneous molecular/cell-biological information mainly derived from experiments using different types of cultured cells under a variety of conditions. In the case of ULMS, additional caution is needed for oncogenesis-related extrapolations because most of the available information concerns epithelial tumors and may not be directly relevant. Moreover, considering that aberrant events at any level, other than that of transcription, remain invisible in a microarray analysis, the comparison of expression profiles between ULMS and whole normal uterus should be viewed as a coarse, overall index of the tumorigenic process. Nevertheless, in the few cases that we have tested, we observed a correspondence between differences in transcript levels and amounts of cognate proteins, as assayed by Western analysis (Figure 1B, microarray verification). Still, abnormal protein interactions cannot be assessed by this approach.

Our discussion of the microarray data is focused predominantly on SVLT, considering that very little is known about 17kT and that most of the SVST activity²⁷ is attributed to its inhibition of protein phosphatase 2A (PP2A). We note that through PP2A-related and unrelated mechanisms, SVST can induce the transcriptional activity of Ap1, Nfkb, Sp1 and other transactivators.²⁸ Such functions could augment the tumorigenicity of SVLT (see below).

As an effector, SVLT is a multifunctional protein,^{29–31} which interacts with various host proteins and through some of these interactions ultimately induces or represses the transcription of cellular genes (the DNA-binding ability of SVLT itself does not appear to be essential for modulating its effects on transcriptional activity). Some of the SVLT inhibitory interactions include those with the CBP/p300 co-activators and with the tumor suppressors p53 and pRb (and also p107 and p130 pocket proteins). Whereas the SVLT amino-terminal region (consisting of the J domain and pRb-binding motif) appears in particular transgenic settings to be necessary and sufficient for tumor induction,³² the blocking of p53 action by the SVLT carboxy-terminal segment may be predominantly involved in tumor progression. Inactivation of pRb releases E2F transactivators promoting the expression of S-phase genes required for cellular proliferation. An interesting example, in this regard, is the up-regulation in ULMS of *Cdc71* (Table 1 and Figure 1B). This gene encodes a serine/threonine kinase that appears to be essential for G₁/S transition (it serves as the ultimate trigger of the S phase). The *Cdc71* promoter contains three putative E2F-binding sites that are indispensable for transcriptional activity, as shown by deletion analysis.³³ This gene is overexpressed in transformed cell lines and in 31% (15 of 48) of examined diverse tumors, including a ULM.³⁴

Interestingly, our microarray data indicated that the expression of E2F1 itself was increased in ULMS, perhaps as a consequence of its positive autoregulatory transcriptional activity³⁵ once it was released from Rb. Moreover, it is possible that the up-regulation of Map3k8 in ULMS may have contributed to E2F1-induced transcription, as can be inferred from *in vitro* results.³⁶ *Map3k8* (also known as *Tpl2* or *Cot* oncogene) encodes a serine/threonine protein kinase that controls various signal transduction pathways converging into the activation of several MAP kinases.³⁷ Apparently, the involvement of Map3k8 in cell-cycle progression is related to its transforming and tumorigenic action.³⁸

Despite the presumptive inhibitory action of SVLT on pRB, the level of *Rb1* transcripts in ULMS was twofold higher than in control uterus [with a concomitant 2.5-fold increase in *Rb1* (p107)]. An analogous fourfold increase of *Rb1* mRNA above the normal level was previously observed in SV40-transformed monkey kidney cells,³⁹ but the mechanism and significance of this observation remain unknown. Moreover, the picture is complicated by the fact that, although Rb is overexpressed in some examined human neoplasms, it is underexpressed in others.⁴⁰

A marginal increase in the level of p53 transcripts (1.4-fold greater than normal) was also observed in ULMS. Whether or not this is significant, it must at least be taken into account that p53 was not decreased perhaps because its presence stabilizes SVLT, which in some cases is less tumorigenic when p53 is absent.⁴¹ Reciprocally, SVLT stabilizes the p53 protein but, at the same time, prevents activation of p53-target promoters and, thus, blocks cell-cycle arrest or apoptosis.

The participation of the aforementioned SVLT inhibitory activities in the development of ULMS is likely, but the phenotype cannot be interpreted exclusively on the basis of Rb and p53 inactivations, according to the following genetic evidence. First, only 0.6% (2 of 337) of p53 nullizygous mice and 2.4% (6 of 249) of p53^{+/-} heterozygotes developed leiomyosarcomas.^{42–47} This type of tumor never appeared in Rb^{+/-} mice (*n* = 210) or in Rb^{+/-}/p53^{-/-} double mutants, whereas only a single animal (1 of 113; 0.9%) with an Rb^{+/-}/p53^{+/-} compound genotype developed a leiomyosarcoma.^{48,49} Similarly, ULMS do not appear in mouse mutants carrying a deletion of the second and third exons of *INK4a*, despite disruptions of both the Rb and p53 pathways resulting from the absence of the indirect regulators p16^{INK4a} and p19^{ARF}.⁵⁰

Considering that SVLT inactivates two tumor suppressors, we searched the ULMS expression profile to determine whether up-regulation of oncogenes, potentially collaborating in the tumorigenic process, could be identified. We found, however, that most of the known oncogenes represented in the microarray were expressed at normal levels in ULMS, including the members of the ras family (*Hras1*, *Kras2*, and *Nras*), although some others, such as *Myb*, *Lmyc*, and *Nmyc* were drastically down-regulated. Among the few exceptions, such as the *Cot* oncogene mentioned above, was *Myc*, which, although not dramatically up-regulated, was expressed

at a higher level than in control uterus (2.4-fold). Transcriptional activation of the *Myc* promoter by SVLT has been described and is probably E2F-dependent.^{51,52} Interestingly, overexpression of *Myc* has been reported in 50% of human ULMS (6 of 12) and ULMS (11 of 23).⁵³ The same percentage of human ULMS (50.9%; 29 of 57) exhibited p53 overexpression, whereas leiomyomas lacked this feature.^{54,55} Among the cell-cycle arrest genes known to be suppressed by *Myc*, *Gadd45a* was found to be down-regulated (3.3-fold). Although *Myc* promotes proliferation, it also induces apoptosis, at least under certain conditions. This apoptotic function, if concomitant with promotion of proliferation, must be suppressed by survival factors.⁵⁶ A key anti-apoptotic pathway suppressing *Myc*-induced apoptosis involves insulin-like growth factor (IGF) signaling.⁵⁶ Consistent with this relationship, we observed an increase in *Igf1* expression in the SVLT-induced ULMS. A similar increase in IGF1 levels was previously noted in human ULM,²⁶ in Eker rat ULM⁵⁷ and in ULM induced by *Calb3*-driven SVLT.¹³ It is notable, in regard to IGF signaling, that fibroblasts lacking the type 1 IGF receptor (IGF1R) cannot be transformed by SVLT.⁵⁸ It is also notable that IGF1 can induce expression of the transcription factor Twist, which becomes involved in IGF1R-mediated anti-apoptotic effects.⁵⁹ It is interesting, therefore, that up-regulation of *Twist* expression was detected in ULMS. However, expression of *Twist* in the uterus had not been examined previously. Thus, we used Western analysis to confirm that the microarray results were reflected in the relative amounts of assayable Twist protein in normal uterus and in the tumor (Figure 1B, microarray verification).

Twist, which appears to be involved in the development of human rhabdomyosarcoma,⁶⁰ is a basic helix-loop-helix (bHLH) protein that interacts directly with the HAT (histone acetyltransferase) domains of p300 and PCAF (CBP/p300-associated factor) and inhibits their activities.⁶¹ Thus, Twist could act similarly to SVLT, which, in part, exerts its transforming activity by perturbing CBP/p300 functions. In *Drosophila*, Twist is an activator for genes in the pathway involved in the development of body-wall muscles,⁶² whereas in mouse cells, it serves as a repressor of the striated muscle myogenic program⁶³ brought about by the MyoD family of bHLH proteins (MyoD, Myf5, myogenin, and MRF4). Our results provide a strong indication for studying closely the function(s) of Twist in uterine smooth muscle, especially because our evidence shows that this transcription factor does not act as a repressor of the myogenic program in this particular tissue. In smooth muscle, there is still no known counterpart of the MyoD class of transcription factors, which act in striated muscle, but are absent from SMC. However, whatever the key components of the SMC myogenic program may be, its execution, albeit perturbed, is not eradicated in ULMS in terms of differentiation.

Smooth muscle cells, although mostly quiescent, do not attain a state of absolute terminal differentiation, but maintain low proliferation capacity that is augmented during tumorigenic progression without loss of expression of contractile proteins characteristic of smooth muscle. In

fact, the levels of uterine transcripts encoding the smooth muscle-specific myosin heavy chain (Myh11) and the myosin regulatory light chain were found to be normal in ULMS (Table 1). On the other hand, the detection of myomesin (*Myom1*) transcripts, encoding a structural component of the sarcomeric M band present in striated but not in smooth muscle cells,⁶⁴ and also the significant overexpression (18.5-fold) of atrial type myosin alkali light chain (*Myla*) were indicative of a perturbed differentiation program. Also increased, but only to a slight or moderate degree, were the transcript levels of actin isoforms (α - and γ -smooth muscle actin, and cardiac and skeletal α -actin; Table 1), all of which are expressed in the uterus (the levels of ubiquitous cytoplasmic β - and γ -actin mRNAs were normal).

Notable among additional transcriptional modulators found up-regulated in ULMS is Id4 (Idb4). This factor belongs to a group of four related HLH proteins (Id1 to Id4) that lack a basic DNA-binding domain and, thus, by forming heterodimers with bHLH transcription factors deprive them of their ability to bind DNA. Id4 can also sequester Rb family members through direct binding⁶⁵ with a consequent positive effect on cell-cycle progression. It is possible, therefore, that SVLT and Id4 act synergistically in ULMS by inhibiting the action of pocket proteins. Id4 is highly expressed in malignant seminoma cells,⁶⁶ but its potential involvement in the development of other human tumors has not been examined.

The observed reduced expression of the *Gata6* zinc-finger transcription factor in ULMS appears to be congruent with a tumor growth phenotype, considering that *Gata6* is involved in the maintenance of a quiescent, differentiated state and exhibits anti-proliferative action at least in vascular smooth muscle cells (VSMCs). Thus, *Gata6* is down-regulated in proliferating VSMCs, whereas its overexpression in VSMCs results in cell-cycle arrest.⁶⁷ However, detailed information about the expression pattern of *Gata6* in visceral smooth muscle is currently lacking, although transcripts have been detected in the uterus by Northern analysis.⁶⁸

Although still fragmentary, the expression profiling data also underscored the fact that during tumor progression there is a complex interplay between neoplastic cells and their microenvironment (tumor stroma) composed of fibroblasts, endothelial cells of the vasculature, and immune cells, all embedded in the ECM that consists of insoluble structural proteins and soluble components, such as growth factors and cytokines. In addition, the available information suggested that there is some signaling communication between the tumor stroma and the residual mesenchymal component of the endometrium (uterine stroma) that is still morphologically normal, but diminished in size because of the expansion of neoplastic tissue. Apparently, the transcriptional program of the endometrial epithelium, which remains histologically intact, can also be altered. For example, the homeobox-containing *Msx1* gene that is expressed exclusively in the uterine luminal and glandular epithelial cells⁶⁹ was down-regulated in ULMS.

It would be interesting to eventually examine whether the down-regulation of *Msx1* is the result of altered Wnt

signaling during tumor progression because members of the Wnt family of secreted ligands are centrally involved in mesenchymal-epithelial patterning in various organs, including the uterus,⁷⁰ and can play a role in cancer.⁷¹ Whereas *Msx1* is a transcriptional target of Wnt3a in human embryonic carcinoma cells,⁷² its relationship to the particular *Wnt* genes expressed in the uterus, such as *Wnt4* that is down-regulated in ULMS, is unknown. Nevertheless, *Wnt4* is expressed in the uterine stroma,⁷³ and it may be relevant that expression of *Msx1*, considered as a marker of epithelial cytodifferentiation, depends on induction by factors present in the stromal mesenchyme.⁶⁹ On the other hand, the epithelium apparently exerts negative control on the smooth muscle cell lineage (during postnatal development, uterine mesenchyme gives rise to both smooth muscle and stroma). Thus, uterine mesenchyme grown in a nude mouse in the absence of epithelium becomes a mass of disorganized bundles of smooth muscle associated with stromal cells expressing *Wnt4* at lower levels than in intact uterus or in analogous grafts using both mesenchyme and epithelium.⁷³ It is possible, therefore, that during ULMS progression similar negative signals from the overgrown smooth muscle cells and/or their neoplastic stroma down-regulate *Wnt4* expression. Reduced transcription of the *Wnt4* gene may be a more general feature of the neoplastic process because the expression of *Wnt4* mRNA was significantly reduced in endometrial carcinoma in comparison with normal endometrium.⁷⁴

Wnt ligands bind to Frizzled receptors and induce an intracellular signaling cascade that can be modulated by Sfrp's,⁷⁵ such as Sfrp2. This soluble inhibitor of Wnt activity increases the resistance of cells to apoptotic stimuli when overexpressed,⁷⁶ and is able to promote the proliferation of malignant glioma cells.⁷⁷ Sfrp2 interacts physically with Wnt4⁷⁸ and antagonizes its activity.⁷⁹ Sfrp2, which is expressed in the mouse uterine stroma,⁸⁰ is up-regulated in ULMS. Overexpression of another member of the Sfrp family (*Sfrp1*) was observed in human ULMS.⁸¹ It is notable that the *Wisp1* gene, which was up-regulated in ULMS, is induced by Wnt1 in a mouse mammary epithelial cell line.⁸² *Wisp1* has transforming activity,⁸³ potentiates Myc-induced oncogenesis by inhibiting its apoptotic action,⁸⁴ and is overexpressed in human cancers.^{82,85}

Other significant alterations observed in ULMS concerned the amounts of transcripts encoding several stromal components, ECM molecules, and integral cell membrane adhesion receptors mediating cell-cell and cell-matrix interactions (listed in Table 1). Tumor invasion requires proteolysis of ECM proteins mainly effected by metalloproteinases.⁸⁶ The transcripts for two of these enzymes, Mmp8 and Mmp9, were significantly up-regulated in ULMS, and this might be indicative of the malignancy of the tumor, because Mmp9 did not exceed normal levels in human leiomyomas.⁸⁷ Expression of Mmp9 is low, but detectable in normal myometrium, whereas Mmp8 is not expressed in the virgin uterus.^{88,89} Mmp9 may be further involved in tumor progression because it promotes angiogenesis.⁹⁰

Mmps are often co-expressed with tenascin C (Tnc), a glycoprotein of the ECM that is expressed pericellularly in normal mouse myometrium⁹¹ and in human ULMS.⁹² Up-regulation of *Tnc* in the murine ULMS that we have examined is presumably related to the adhesion-modulatory activity of the encoded protein. It has been suggested that Mmps may lie upstream from Tnc,⁹³ as it is thought that they could release or process soluble growth factors, such as transforming growth factor- β 1, from the ECM, which in turn could induce Tnc production. On the other hand, *in vitro* data suggest that Tnc is itself able to induce production of Mmps, such as Mmp9.⁹⁴ In contrast to Tnc, Tnxb was decreased in murine ULMS and also in human ULM.²⁶ Opposite expression levels between Tnc and Tnxb in tumors have been previously noted.^{95,96} Interestingly, tumor invasion was augmented in Tnx-null mice and was correlated with an increase in Mmp9 and an adhesion defect in ECM.⁹⁷ We finally note that expression of transcripts encoding thrombospondin-2 (Thbs2) was increased in murine ULMS compared to normal uterus (a similar observation was made in human ULM).²⁶ Thbs2 is an ECM protein exhibiting anti-angiogenic activity. Thus, its frequently observed up-regulation in the stroma of malignant tumors^{98,99} was unexpected and has been attributed to a host-defense response to tumor development.¹⁰⁰

A more detailed discussion of currently available profiling data should await further experimentation, including extensive verifications by immunohistochemical and real-time polymerase chain reaction analyses, as well as initiation of work to address mechanistic questions.

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