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Disruption of Ini1 Leads to Peri-Implantation Lethality and Tumorigenesis in Mice

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SNF5/INI1 is a component of the ATP-dependent chromatin remodeling enzyme family SWI/SNF. Germ line mutations of INI1 have been identified in children with brain and renal rhabdoid tumors, indicating that INI1 is a tumor suppressor. Here we report that disruption of Ini1 expression in mice results in early embryonic lethality. Ini1-null embryos die between 3.5 and 5.5 days postcoitum, and Ini1-null blastocysts fail to hatch, form the trophectoderm, or expand the inner cell mass when cultured in vitro. Furthermore, we report that approximately 15% of Ini1-heterozygous mice present with tumors, mostly undifferentiated or poorly differentiated sarcomas. Tumor formation is associated with a loss of heterozygocity at the Ini1 locus, characterizing Ini1 as a tumor suppressor in mice. Thus, Ini1 is essential for embryo viability and for repression of oncogenesis in the adult organism.

The compact nature of chromatin structure presents a barrier to cellular processes that require access to DNA. A number of multiprotein complexes have been identified that share the ability to modify chromatin structure. These include the histone acetyltransferases and deacetylases, complexes which chemically modify the amino-terminal tails of histones by the addition or removal of acetyl groups, respectively, as well as a group of enzymes that utilize the energy derived from ATP hydrolysis to alter nucleosome structure (16, 20, 44, 50). Included among these ATP-dependent chromatin remodeling enzymes is the SWI/SNF family of chromatin modifiers.

SWI/SNF enzymes are large multisubunit enzymes of ~1 to 2 MDa. Yeast SWI/SNF genes were originally identified as being required for mating type switching or sucrose fermentation (4, 32, 42). Later work determined that SWI/SNF genes were required for the induction of a subset of yeast genes and that the SWI2/SNF2 protein possessed a DNA-stimulated ATPase activity (6, 22, 26, 33, 34, 54). Mutations in SWI/SNF genes could be suppressed by mutations altering histone gene expression, histone structure, or nonhistone chromatin proteins, leading to the suggestion that these gene products facilitated transcriptional activation by altering chromatin structure (15, 23, 24).

Human SWI/SNF (hSWI/SNF) complexes contain either the human BRM (hBRM) (hSNF2α) or BRG1 (hSNF2β) homologues of the yeast SWI2/SNF2 ATPase (7, 19, 30). Both yeast and human SWI/SNF complexes have been shown to possess nucleosome remodeling activity in vitro (8, 17, 25). Components of mammalian SWI/SNF complexes have been implicated in a variety of cellular processes, including gene activation and repression, development and differentiation, recombination and repair, and cell cycle control. There is evidence supporting a role for SWI/SNF in gene activation events mediated by nuclear hormone receptors, environmental stress, and viral infection (1, 7, 10, 13, 30). In contrast, SWI/SNF components also were shown to be involved in repression of c-fos and some E2F-regulated genes (31, 48). Both BRG1 and hBRM can interact with the retinoblastoma oncoprotein and induce cell cycle arrest, an effect that is abrogated by the association of BRG1 with cyclin E (11, 41, 45, 56). Evidence suggesting a role for hSWI/SNF in recombination and repair was provided by studies demonstrating an interaction of components of the hSWI/SNF complex with BRCA1, which is thought to be involved in DNA damage and repair pathways (3). Furthermore, members of the SWI/SNF complex are targets of viral regulatory proteins upon infection of cells by adenovirus, Epstein-Barr virus, human immunodeficiency virus, and human papillomavirus (18, 27, 28, 53).

The role of SWI/SNF enzymes in whole organisms is unclear. While homozygous disruption of Brm1 in mouse embryonic carcinoma cells resulted in lethality, disruption of Brm expression in mice produced only mild proliferative effects (35, 46). The upregulation of Brml in the Brm-deficient mice may provide a compensatory effect; however, one cannot rule out the possibility that these differences are due to distinct functions of Brm- or Brml-containing complexes.

SNF5/INI1 is a member of both BRG1- and BRM-containing SWI/SNF complexes (29, 51). INI1 was shown to interact with ALL-1, translocations of which are associated with several types of human acute leukemias (37). Furthermore, INI1 has been found to be altered in malignant rhabdoid tumors, chordoid plexus carcinomas, medullablastomas, and central primitive neuroectodermal tumors (2, 9, 39, 40, 49). Identification of constitutional mutations in a subset of these tumors indicates that INI1 is a tumor suppressor (2, 40). In an attempt to generate a mouse model that would allow further characterization of the mechanisms of Ini1 in tumorigenesis and to determine the role of the mammalian SWI/SNF complexes in development, we generated mice deficient for Ini1 expression.
FIG. 1. Disruption of \textit{Ini1}. (A) Targeting strategy for \textit{Ini1}. A retroviral promoter trap vector was inserted in intron 3 of \textit{Ini1}. sa and sd, the splice acceptor site in the \textit{\beta}-geo gene cassette and the splice donor site in the puromycin selection marker, respectively. Carets represent the site of alternate splicing. Positions of the primers used for PCR analysis are shown. LTR (del), long terminal repeat deleted; \textit{\beta}A, polyadenylation signal; Puro, puromycin. (B) Genotyping of \textit{Ini1}-targeted mice by PCR. Genomic DNA was harvested from tails and genotyped as described in Materials and Methods. The size and position of wild-type (WT) and targeted bands are indicated. (C) Genotyping of \textit{Ini1}-targeted embryos. Embryos 6.5 d.p.c. and younger were genotyped by nested PCR. The size and position of wild-type and targeted bands are indicated.

We show that \textit{Ini1}-deficient mice die early in embryogenesis, likely due to an inability of the blastocysts to hatch, implant in the uterus, and continue development. In addition, we report that a subset of the \textit{Ini1}-heterozygous mice present with a variety of tumors in the soft tissues of the head and neck and that loss of heterozygosity at the \textit{Ini1} locus is correlated with tumor formation.

MATERIALS AND METHODS

\textbf{Ini1 targeting.} Embryonic stem (ES) cells (Omnibank no. OST32815) bearing a retroviral promoter trap that functionally inactivates one allele of \textit{Ini1} were generated as described previously (55). Analysis by rapid amplification of cDNA ends also is described. The site of insertion was determined using sequence analysis.

\textbf{Creation of \textit{Ini1}-null mice.} \textit{Ini1}-targeted ES cells were injected into 3.5 days postcoitus (d.p.c.) C57BL/6 blastocysts. Male chimeric mice were mated with wild-type C57BL/6 or 129 females. Germ line transmission of the mutant allele was determined by PCR analysis of tail genomic DNA using the following primers: for \textit{Ini1} -heterozygous mice

\begin{align*}
\text{sa} & : 5'-GCAAGCCTCTGCACAAATTGCC-3' \\
\text{sd} & : 3'-\text{CACACCCTATTGTCACCTGGAA-5'} \\
\text{\beta}A & : 5'-CGGTATCTGAAGCTATGTA-3' \\
\textit{\beta}geo & : 3'-GTCAACGCGTCGGACTTACCGC-5'
\end{align*}

\textit{Ini1}-heterozygous mice were intercrossed to generate \textit{Ini1-null} mice. Embryos 6.5 d.p.c. and younger were prepared for genotyping by PCR as described previously (47). Nested PCR was done using the above primers for the first round of PCR (29 cycles) and the following intron 3-nested primers for the second round (29 cycles): 5'-GCGTGGCGCCAACCATGCTGGG-3' and 3'-CTCTCTGAACTCTACTTACGCC-5'. \textit{Ini1}-heterozygous mice were genotyped as described above.

\textbf{Blastocyst culture.} Blastocysts from heterozygous intercrosses were flushed from the uteri of \textit{Ini1}x\textit{Ini1} females 3.5 d.p.c. with M15 media (Dulbecco’s minimal essential medium, 15% fetal calf serum, 100 \mu M \beta-mercaptoethanol, 2 mM glutamine, and 1 \times penicillin-streptomycin) and cultured in tissue culture plates for 96 h. Embryo cultures were genotyped as described above.

\textbf{\beta-Galactosidase staining of cultured ES cells.} Wild-type AB2.2 ES cells and \textit{Ini1}x\textit{Ini1} ES cells were grown to near-confluency and fixed in 0.5% glutaraldehyde. Cells were then rinsed with phosphate-buffered saline and stained overnight in the dark at room temperature in a solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl\textsubscript{2}, and 1 mg of 5-bromo-4-chloro-3-indolyl-\beta-D-galactopyranoside (X-Gal)/ml.

\textbf{Whole mount staining of embryos for \beta-galactosidase activity.} Embryos were harvested at various time points postfertilization and fixed in 4% paraformaldehyde for 20 min at 4°C. Embryos were washed and then stained in an X-Gal histochemical reaction mixture (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM MgCl\textsubscript{2}, 1 mg of X-Gal/ml) overnight at room temperature. Following staining, embryos were rinsed in phosphate-buffered saline and cleared in 30% sucrose.

\textbf{Western analysis of tumor samples.} Control tissues and tumor samples were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, 20% glycerol, 1 mM dithiothreitold, 1 \mu g of pepstatin A/ml, 4 \mu g of leupeptin/ml, and 1 mM phenylmethylsulfonyl fluoride. Extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western analysis for \textit{Ini1} protein was performed as described previously (10).

\textbf{Histologic analysis of tumors in mice.} Tumor samples and selected tissues were fixed in 10% buffered formalin phosphate and processed for paraffin embedding as described previously (14). Sections were prepared, stained with hematoxylin and eosin, and examined under a microscope.

RESULTS AND DISCUSSION

Mouse ES cells bearing a retroviral promoter trap that functionally inactivates one allele of \textit{Ini1} were constructed as described previously (55). Sequence analysis revealed that the promoter trap was inserted within intron 3 of \textit{Ini1} (Fig. 1A). The beta-galactosidase-neomycin (\beta-geo) gene fusion cassette within the retroviral insertion has a 5' splice acceptor site; thus, \beta-geo expression is regulated by the native \textit{Ini1} promoter. We were able to utilize the \beta-geo gene cassette in a colorimetric assay to determine if \textit{Ini1} is normally expressed in ES cells. \textit{Ini1}-targeted cells stained positive for \beta-galactosidase activity, indicating that \textit{Ini1} is expressed in ES cells (Fig. 2A). Northern analysis of ES cell total RNA confirmed \textit{Ini1} expression (data
not shown). Furthermore, sequence data obtained from 5′ rapid amplification of cDNA ends analysis of the Ini1–b-galactosidase fusion mRNA revealed that transcripts utilizing either splice donor site in exon 2 spliced into the trap, indicating that both splice variants of Ini1 were inactivated (5).

To determine the role of Ini1 in mammalian development and tumorigenesis, we used the targeted ES cells in blastocyst injection experiments to generate Ini1-heterozygous (Ini1in3/1) mice. In order to monitor expression of Ini1 during embryogenesis, we performed whole mount staining for β-galactosidase activity in embryos harvested from Ini1in3/1 matings at various times during development. We found that Ini1in3/1 embryos stained positive in all tissues at all time points examined, including 6.5, 8.5, 9.5, and 10.5 d.p.c., indicating that Ini1 is ubiquitously expressed during embryogenesis (Fig. 2B). Ini1 expression was also detected by Northern analysis in a wide range of adult tissues (35) (data not shown).

Chimeric mice generated from C57BL/6 strain blastocyst injections of the 129 strain-derived ES cells were bred to wild-type C57BL/6 or 129 mice in order to obtain Ini1in3/1 mice on either a mixed (C57BL/6 × 129) or pure (129) background. Intercrosses of Ini1in3/1 mice in both backgrounds yielded Ini1in3/1 offspring and wild-type offspring at a 2:1 ratio (63:26 in the mixed background, 34:17 in the pure background) and no Ini1-null offspring, indicating that disruption of Ini1 induces embryonic lethality (Fig. 1B). Timed matings of Ini1in3/1 mice were performed, and embryos were harvested at various time points in gestation for genotyping via PCR. Ini1-null embryos could be isolated at 3.5 d.p.c. and were normal in appearance (Fig. 1C and 3). However, no Ini3/in3 embryos were detected at 6.5 d.p.c. or later (Table 1). Dissection of maternal deciduae at 6.0 to 6.5 d.p.c. revealed no significant increase in the number of embryo reabsorptions, suggesting that Ini3/in3 lethality occurred between days 3.5 and 5.5 of gestation. These results

FIG. 2. Ini1 is expressed in ES cells and ubiquitously throughout development. (A) β-galactosidase staining of targeted ES cells showing expression of Ini1. Wild-type (WT) AB2.2 ES cells were used as a control. (B) Whole mount staining of Ini1in3/1 embryos showing ubiquitous expression of Ini1 at indicated time points. Wild-type embryos at 6.5 and 10.5 d.p.c. are shown as controls.

FIG. 3. Ini1-null mice are early embryonic lethal and fail to hatch in vitro. Blastocysts were harvested from C57BL/6 Ini1in3/1 females and plated in culture for 96 h, at which time outgrowths were processed for PCR. Blastocysts are shown before and after culturing. TE, trophectoderm; ICM, inner cell mass.

<table>
<thead>
<tr>
<th>Table 1. Genotyping of embryos from heterozygous intercrosses</th>
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<tr>
<td>Embryo category</td>
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<tr>
<td>-----------------</td>
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<tr>
<td>3.5 d.p.c.</td>
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<tr>
<td>6.5 d.p.c.</td>
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<tr>
<td>8.5 d.p.c.</td>
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<tr>
<td>13.5 d.p.c.</td>
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* WT, wild type; Het, heterozygous.  
† The embryo was dead.

<table>
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<th>TABLE 2. Tumor occurrence</th>
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<tbody>
<tr>
<td>Mouse no.</td>
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<tr>
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</tr>
<tr>
<td>Ini 119</td>
</tr>
<tr>
<td>Ini 44</td>
</tr>
<tr>
<td>Ini 138</td>
</tr>
<tr>
<td>Ini 100</td>
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<tr>
<td>Ini 180</td>
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<td>Ini 264</td>
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<td>Ini 262</td>
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<td>Ini 29</td>
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<td>Ini 322</td>
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<td>Ini 95</td>
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<tr>
<td>Ini 127</td>
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<td>Ini 328</td>
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* 129 strain.
FIG. 4. Ini1-heterozygous mice present with various tumors. Microscopic features of different tumors from Ini1
tm/b-1 mice. Paraffin-embedded tumors were sectioned, stained with hematoxylin and eosin, and examined under a microscope at magnification $\times 75$. Mouse numbers corresponding to those presented in Table 2 are indicated in each panel.
into the uterine wall or were implanted and were reabsorbed blastocysts from Ini1in3
Ini1in3 implanted onto the tissue culture plastic. Both wild-type and the majority of the facial tumors were poorly differentiated or structures which contribute to formation of the face. While the arch and in the frontonasal and maxillary processes (Fig. 2B), the peri-implantation or preimplantation stage of embryogenesis. ICM of 3.5 d.p.c. blastocysts, is consistent with a gene crucial to the formation. Tumor samples and control tissue (wild-type [WT] male brain) were processed for Western analysis as detailed in Materials and Methods. The band corresponding to the Ini1 protein is indicated. NS represents a nonspecific band.

indicate that Ini1-null embryos either failed to be implanted into the uterine wall or were implanted and were reabsorbed shortly thereafter. In order to examine further the developmental defect of Ini1in3/in3 embryos, we analyzed the ability of blastocysts from Ini1in1 mice to expand in vitro. When 3.5-d.p.c. blastocysts were plated in culture, wild-type and Ini1in3/in3 blastocysts hatched from the zona pellucida and were implanted onto the tissue culture plastic. Both wild-type and Ini1in3/in3-implanted embryos formed the trophoderm and expanded their inner cell mass (ICM). In contrast, no Ini1in3/in3 blastocysts hatched and were implanted in culture (Fig. 3). The results of these experiments suggest that the peri-implantation embryonic lethality of Ini1-null mice may be due to a defect in the hatching of the blastocyst from the zona pellucida, an obligate step for implantation of the embryo into the wall of the uterus during normal development. Manual disruption of the zona pellucida of 19 (C57BL/6 × 129) blastocysts harvested from Ini1in1 intercrosses did not result in expansion of the Ini1-null trophectoderm or ICM during in vitro culture, suggesting that growth of these tissues also is compromised (data not shown). Expression of Ini1 in ES cells, which are derived from the ICM of 3.5 d.p.c. blastocysts, is consistent with a gene crucial to the peri-implantation or preimplantation stage of embryogenesis.

In humans, loss of Ini1 is correlated with a variety of tumors, the vast majority of which are neuronal or renal in nature. To date, most human malignant rhabdoid tumors and choroid plexus carcinomas examined have deletions and/or mutations in INI1, as do a subset of central primitive neuroectodermal tumors and medulloblastomas (39). In mice, we found that approximately 15% of Ini1 heterozygotes in both the mixed F1 (C57BL/6 × 129) or pure 129 backgrounds presented with tumors. All of these tumors arose in the head or neck regions of the mice, particularly in the soft tissue of the face (Table 2). While 2 of the 15 mouse tumors analyzed thus far had varying degrees of rhabdoid-like cells, none had the characteristic, monomorphous appearance of human rhabdoid tumors. Two Ini1in1 mice were found to have a lymphoproliferative disorder or lymphoma originating in an ill-defined region on the neck (Fig. 4). Two-thirds of the tumors originated on the faces of the mice. Interestingly, expression of Ini1 appears to be elevated during development in the branchial arch and in the frontonasal and maxillary processes (Fig. 2B), structures which contribute to formation of the face. While the majority of the facial tumors were poorly differentiated or undifferentiated sarcomas and not neuronal in origin, it is possible that the tumors arose in cells derived from neural crest progenitors, since neural crest cells, along with mesodermal cells, coordinate to form the facial primordia (12, 38).

We have analyzed tumors in three representative mice. Northern analysis of total RNA harvested from tumor tissue indicated the presence of wild-type-length Ini1 message (data not shown). However, Western blot analysis of proteins harvested from these tumors revealed the absence of Ini1 protein in all three samples (Fig. 5). This indicates that loss of heterozygosity at the Ini1 locus is responsible for tumor formation in the Ini1in1 mice.

The mechanism of Ini1-mediated tumor suppression is unclear. Other subunits of the human and mouse SWI/SNF chromatin remodeling complexes have been reported to associate with known tumor suppressors, including Rb and Bre1 (3, 11, 48, 56), and several of the SWI/SNF subunits appear to be molecular targets of viral regulators of cell proliferation (18, 27, 28, 53). In addition, one of these subunits, BRG1, recently has been reported to be missing or mutated in a variety of human tumor cell lines, and reintroduction of BRG1 into these tumor cells reverses their transformed morphology (52). These findings suggest a role for chromatin remodeling in regulation of cell growth and/or in tumor suppression.

While this report was in preparation, Roberts et al. and Klochendler-Yeivin et al. published data consistent with our findings (21, 36). The fact that these results are reproducible in knockout lines generated by different targeting strategies confirms the importance of Ini1 in development and tumorigenesis. Klochendler-Yeivin et al. (21) further report in their study that Ini1-deficient embryos can induce the formation of maternal decidua, suggesting that Ini1-deficient embryos undergo hatching and implantation prior to their demise. In contrast, Ini1in3/in3 embryos fail to hatch from the zona pellucida, suggesting that subtle strain variations may influence the precise timing of embryonic lethality. In agreement with these other groups, a percentage of the Ini1-heterozygous mice in our colony presented with tumors that contained variable numbers of rhabdoid cells. However, we are hesitant to classify these undifferentiated sarcomas as true rhabdoid tumors, which are described as monomorphic tumors in the human population. Discrepancies between tumor types associated with disruption of Ini1 in humans and in mice may be due to differences in species-specific differentiation pathways. Regardless, the Ini1-heterozygous mice should provide a useful model for studying the general mechanisms involved in tumor suppression by Ini1.

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