RESEARCH COMMUNICATION

Mcl-1 deficiency results in peri-implantation embryonic lethality

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We disrupted the *Mcl-1* locus in murine ES cells to determine the developmental roles of this *Bcl-2* family member. Deletion of *Mcl-1* resulted in peri-implantation embryonic lethality. *Mcl-1*^{-/-} embryos do not implant in utero, but could be recovered at E3.5–4.0. Null blastocysts failed to hatch or attach in vitro, indicating a trophoblast defect, although the inner cell mass could grow in culture. Of note, *Mcl-1*^{-/-} blastocysts showed no evidence of increased apoptosis, but exhibited a delay in maturation beyond the precompaction stage. This model indicates that *Mcl-1* is essential for preimplantation development and implantation, and suggests that it has a function beyond regulating apoptosis.

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MCL-1 was identified in a screen for differentiation-induced genes activated in the human monocytic leukemia cell line, ML-1 (Kozopas et al. 1993). The *MCL-1* protein is a member of the *BCL-2* family displaying all four of the *BCL-2* homology domains (BH1–4) and has been localized to intracellular membranes, particularly the mitochondrial membrane (Kozopas et al. 1993; Yang et al. 1995). *MCL-1* is widely expressed in human and murine tissues and cell lines as well as in a wide variety of human tumors (Kozopas et al. 1993; Krajewski et al. 1994, 1995). *MCL-1* has been shown to delay cell death in selected cell lines, but in comparison, is not as potent as *BCL-2*, and has no effect in other systems in which *BCL-2* proves protective (Reynolds et al. 1994, 1996; Bodrug et al. 1995; Zhou et al. 1997). Transgenic mice that overexpressed *Mcl-1* displayed improved hematopoietic cell survival and enhanced outgrowth of myeloid cell lines (Zhou et al. 1998). *MCL-1* expression has been noted to be rapidly up-regulated in response to certain cytotoxic and differentiation stimuli, but the increased expression is often transient (Yang et al. 1996). These

results indicated that *MCL-1* functions to inhibit apoptosis in selected cell types but do not exclude that *MCL-1* could possess other nonapoptotic functions.

Whereas multiple *BCL-2* family members are often co-expressed in the same tissues, evolving evidence indicates a selectivity in their participation following specific death or survival signals. For example, although multiple proapoptotic members are present in superior cervical ganglion (SCG) cells, *BAX* is singularly required for their death following NGF deprivation (Deckwerth et al. 1996). Conversely, it is the proapoptotic *BAD* molecule that is inactivated by phosphorylation following exposure to the IL-3 survival factor (Zha et al. 1996). Mice deficient for individual family members also support distinct roles for each member. *Bcl-2* knockout mice proved viable but fail to maintain homeostasis with dramatic apoptosis of B and T lymphocytes and melanocytes. A loss of nephron units during embryogenesis manifests as polycystic kidney disease postnatally (Veis et al. 1993). *Bcl-x*-deficient mice are embryonic lethal with massive hematopoietic and neuronal cell death (Motoyama et al. 1995). Knockouts of the proapoptotic *Bax* (Knudson et al. 1995) as well as the antiapoptotic *Bcl-w* gene (Print et al. 1998; Ross et al. 1998) each manifest as testicular degeneration and male sterility. Consequently, we elected to generate an *Mcl-1*-deficient mouse to identify the tissues in which *Mcl-1* is most critical and to gain further insight into its functional role.

Results and Discussion

We constructed a deletional targeting vector for *Mcl-1* that eliminates most of exon I including the initiation codon (Fig. 1A). Subsequent chimeric mice transmitted the disrupted *Mcl-1* to the germ line. *Mcl-1*^{+/-} heterozygous males and females were grossly and microscopically normal and fertile. Matings of heterozygotes on C57BL/6 and 129Sv/J backgrounds produced 529 pups; 67.5% were *Mcl-1*^{+/-}, but none were *Mcl-1*^{-/-} homozygous nulls (Fig. 1b).

A nested PCR strategy (Fig. 1a) was developed to genotype embryos (Fig. 1c). Timed pregnancies of *Mcl-1* heterozygote matings were analyzed at E7.5–13.5 of gestation. No *Mcl-1* null pups were observed at any of these time points (Table 1). Dissection of embryos from implantation sites at E7.5 as well as histologic sections of uteri at E5.5 and E6.5 revealed no evidence of embryo resorption or empty decidua (not shown). A normal mouse blastocyst attaches to the uterus between E4.5 and E5.0 (Rinkenberger et al. 1997). Even embryos that die or are degraded shortly after attachment stimulate decidualization, a process whereby stromal cells expand, elaborate extracellular matrix, and form tight junctions (Welsh 1993). Consequently, we isolated and genotyped preimplantation embryos from timed heterozygote matings. *Mcl-1* null blastocysts and morula were identified, although the lower than expected frequency (16%) sug-

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Figure 1. (a) Map of the *Mcl-1* murine genomic locus and targeting vector. Exons are indicated by black boxes, and positions of internal and external probes are represented by gray boxes; PCR primers are denoted by arrows. The *pgk-neo* cassette is inserted in the opposite transcriptional orientation and replaces a *SacII* (S2) to *SacI* (S1) fragment of exon I containing the translation start site. *EcoRI* (R1), *ScaI* (Sc1). (b) Representative genomic blot probed with the external probe. A wild-type 12-kb *EcoRI* fragment is recognized; the rearranged homologous recombinant allele is 10-kb. (c) Representative nested PCR genotyping results from preimplantation embryos. The paired primers distinguished a 350-bp rearranged fragment of the homologous recombinant allele from a 168-bp fragment of the wild-type allele.

gested *Mcl-1* deficiency had impaired development by this stage (Table 1).

To determine the expression pattern of *Mcl-1* at pre-implantation time points, we performed RNA in situ

Table 1. Genotypes of embryos from heterozygous matings

Freshly isolated embryos from gestational day ^a	Genotype			
	Number of Embryos (percent of total)			
	+/+	+/-	-/-	not determined
13.5–14.0	4 (25)	12 (75)	0	0
9.5	6 (25)	13 (54)	0	5 (21)
7.5	11 (24)	31 (69)	0	3 (7)
4.0*	18 (30)	27 (44)	10 (16)	6 (10)
Embryos cultured on				
MEFs				
attached	6	15	0	0
nonattached	8	0	1	6
Vitronectin				
attached	24	30	0	2
nonattached	9	17	10	33

^a Preimplantation morula- and blastocyst-stage embryos.

hybridization on sections of wild-type embryos (Fig. 2). *Mcl-1* was expressed in both morula and blastocysts and was present in both trophectoderm (TE) and inner cell mass (ICM) (Fig. 2a,b), whereas the control sense probe exhibited no hybridization signal above background (Fig. 2c). *Mcl-1* null blastocysts, which approached the expected frequency from heterozygote matings, lacked a hybridization signal with the antisense probe (Fig. 2d). Most *Mcl-1* null blastocysts, whether identified by RNA in situ or PCR genotype, were morphologically indistinguishable from wild-type blastocysts (not shown). However, a subset of null embryos isolated at E4.0 exhibited delayed maturation resembling a precompaction 8- to 16-cell blastomere stage embryo instead of a morula or blastocyst (Fig. 2e). This apparent arrest of *Mcl-1* null embryos by the blastomere stage became consistent as

Figure 2. Representative *Mcl-1* RNA in situ hybridization results on preimplantation embryos. (a) Light- and dark-field images of a morula stage wild-type embryo hybridized with the *Mcl-1* anti-sense probe. (b) Light- and dark-field images of a blastocyst stage wild-type embryo with the *Mcl-1* antisense probe revealing signal in both TE and ICM. (c) The *Mcl-1* sense probe hybridized to wild-type blastocyst displayed no signal above background. (d) Light- and dark-field image of an *Mcl-1* null blastocyst hybridized with the *Mcl-1* antisense probe. (e) Representative noncompacted blastomere stage *Mcl-1* null embryo that fails to hybridize with the *Mcl-1* antisense probe. Magnification, 400x.

the mice were crossed further onto a C57BL/6 background.

Mcl-1 RNA in situ hybridization was also performed on fixed sections through blastocysts attached to uterine epithelium early in implantation at E6.5, and at later time points of E5.5 and E6.0 (Fig. 3). The intensity of *Mcl-1* expression was highest for the implanting blastocyst at E5.0 and was also strikingly prominent in the maternal decidua surrounding the embryo (Fig. 3a). The intensity of the *Mcl-1* signal within the embryo itself decreased as development proceeds beyond E5.5 (Fig. 3b) to E6.5 (Fig. 3c). In contrast, expression in the decidua intensifies and narrows to the stromal cells proximal to the embryo (Fig. 3c). Thus, the expression pattern of *Mcl-1* is consistent with a gene critical at the peri-implantation stage.

Because *Mcl-1* is expressed in both TE and ICM, it was uncertain whether either or both were responsible for the failure of *Mcl-1* null embryos to implant in utero. In vitro culture of whole blastocysts can clarify peri-implantation lethal phenotypes (Fassler and Meyer 1995; Stephens et al. 1995). Consequently, blastocysts from *Mcl-1*^{+/-} matings were cultured on murine embryonic fibroblast (MEF) feeder layers or vitronectin-coated microdrop cultures. Regardless of the substratum, *Mcl-1* null blastocysts failed to hatch from the zona pellucida or attach to the substrate (Table 1).

To assess whether *Mcl-1* null embryos display a growth disadvantage in culture, two- to four- or eight-cell-stage embryos from heterozygous matings were plated. Of these embryos, 66% progressed to the blasto-

cyst stage, and 88% of those attached and grew when replated onto a vitronectin substrate. However, none of the embryos that progressed to the blastocyst stage were *Mcl-1*^{-/-} indicating the development of nulls was arrested during in vitro culture.

The failure of *Mcl-1* null embryos to hatch or attach in vitro indicates that the TE may be defective. Culturing isolated ICM can likewise provide an estimate of whether embryonic progenitor cells are able to develop postimplantation. Isolated ICM were obtained following immunosurgical removal of the TE (Behrendtsen and Werb 1997) plated on fibronectin and cultured for 5 days. A few *Mcl-1*^{-/-} ICMs did attach and begin to develop as documented in Figure 4. Of 34 total ICMs from *Mcl-1*^{+/-} matings, 26 grew on the substrate, 2 were *Mcl-1*^{-/-} (8%), 14 were +/- (54%), and 7 were ++ (27%), whereas 3 (12%) could not be genotyped. The capacity of *Mcl-1* null ICM to develop in vitro, even though perhaps impaired, indicates that the embryonic portion of the blastocyst may be less dependent on *Mcl-1* than the TE. Complementmentation by tetraploid aggregation (Guillemot et al. 1994) was not feasible because of the limited *Mcl-1* null embryos that survive preimplantation development and their impaired in vitro culture.

To determine whether the developmental potential of *Mcl-1* null embryos was limited as a consequence of increased apoptosis, we modified the TUNEL assay for use on free-floating blastocysts that could be subsequently harvested for nested PCR genotyping. Several different approaches have estimated that ~10% of cells of the ICM and TE of an E4.0 unhatched blastocyst (~64 cell stage)

are undergoing apoptosis (El-Shershaby and Hinchliffe 1974; Handyside and Hunter 1986). The average number of TUNEL-positive cells per embryo did not vary substantially for 7 *Mcl-1*^{-/-} (2.6 ± 2.2 TUNEL-positive cells per blastocyst), 42 *Mcl-1*^{+/-} (2.3 ± 2.6), or 20 *Mcl-1*^{+/+} (3.3 ± 2.7) embryos. None of the *Mcl-1* null embryos displayed >10% apoptotic cells. In support of the TUNEL assay, DAPI and bis-benzimide staining revealed normal nuclear architecture and no increase in apoptotic cells in *Mcl-1* null embryos. These results indicate that *Mcl-1* deficiency does not result in catastrophic apoptosis at the last time embryos can be isolated from the uterus, ~24 hr prior to implantation. We cannot exclude massive cell death after that time point, including the induction of apoptosis in the trophectoderm upon contact with the uterine epithelium. However, the introduction of *p53* deficiency did not rescue the *Mcl-1* nulls as it had for *Mdm2* (Jones et al. 1995; Montes de Oca Luna et al. 1995) and *rad51* (Lim and Hasty 1996) nor did the introduction of *Bax* deficiency (Knudson et al. 1995), which eliminated that proapoptotic molecule.

The *Mcl-1* null phenotype is a combination of an embryonic developmental delay and an implantation defect so severe that there is no maternal decidualization. The peri-implantation lethality from knockouts of proliferation and DNA repair genes

Figure 3. *Mcl-1* RNA in situ hybridization of peri-implantation wild-type embryos. (a) Light- and dark-field images of E5.0 implanting blastocyst hybridized with the *Mcl-1* antisense probe. (b) Light- and dark-field images of E5.5 implantation site. (c) Light- and dark-field images of E6.5 implantation site. Arrow points to ICM of implanting embryo. Maternal decidua is marked with an asterisk in b and c. Magnification, 100 $\times$.

Figure 4. Light microscope images of inner cell mass outgrowths 5 days after immunosurgery and culture on fibronectin. Outgrowths were genotyped as *Mcl-1*^{+/+} (a), *Mcl-1*^{+/-} (b), and *Mcl-1*^{-/-} (c). Magnification, 200 $\times$.

varies in that *Mdm2* (Jones et al. 1995; Montes de Oca Luna et al. 1995), *Brca1* (Hakem et al. 1996; Liu et al. 1996), *Rad51* (Lim and Hasty 1996), *Ref-1* (Xanthoudakis et al. 1996), and thioredoxin (Matsui et al. 1996) all show empty decidua or degrading embryos. E-cadherin (Larue et al. 1994; Riethmacher et al. 1995) and α E-catenin (Torres et al. 1997) null embryos arrest as morula unable to form a blastocoel cavity. Although *Mcl-1* does not appear to be required for TE to be distinguished from ICM, the *Mcl-1*-deficient embryos are defective at multiple steps required for successful embryonic development. The inability of the TE of null blastocysts to attach and the delayed maturation beyond the noncompacted blastomere stage for others, suggest that *Mcl-1* null embryos are ill suited for the uterine environment. In addition to *Mcl-1*, it is remarkable how many *Bcl-2* family knockouts display infertility with *Bax* (Knudson et al. 1995) and *Bcl-w* (Print et al. 1998; Ross et al. 1998) nulls demonstrating aspermatogenesis, whereas *Bcl-2*-deficients (Ratts et al. 1995) have decreased numbers of oocytes. As many as 65% of natural pregnancies terminate during the peri-implantation period (Wilcox et al. 1988). These results indicate that *Mcl-1* is a critical determinant for the success of this highly vulnerable stage of embryonic development.

Materials and methods

Construction of an *Mcl-1* gene targeting vector

129Sv/J genomic phage clones were identified using a murine *Mcl-1* cDNA probe. Clones were restriction mapped and partially sequenced. The targeting vector's 5' arm of homology included part of the 5' UTR. A 500-bp *SacII*-*SacI* fragment that included most of exon 1 and the translation start site of the *Mcl-1* gene was deleted and replaced with the neomycin drug-resistance gene driven by the phosphoglycerate kinase gene promoter. The 3' arm of homology contained the remainder of exon 1 through intron 2 (Fig. 1A). The construct was electroporated into the RW4 ES cell line and homologous recombinant clones microinjected into C57BL/6 blastocysts.

An *Mcl-1* external probe located outside of the targeting construct was designed to distinguish germ line from homologous recombinant alleles and included the last 117 bp of *Mcl-1* coding sequence from exon 3 as well as 165 bp of the 3' UTR. The internal probe consisted of base pairs 508–847 from the cDNA, a portion of exon I present in the targeting vector.

PCR genotyping

Pre- and postimplantation embryos were genotyped with nested sets of primers. The sequence of the first primer set for the wild-type allele was JR26, 5'-aaagcgctgctgaatgcg-3', and JR27, 5'-aagtagcgcgagatgatccagc-3'. The second set of wild-type allele primers was JR28, 5'-gaggaggaactg-

gacggctg-3', and JR29, 5'-cgactggcggataggtcgtc-3'. The first set of rearranged allele primers was JR23, 5'-taccgccttcattgctcag-3', and JR24, 5'-tatagtcgtcctcttctcctcgc-3'. The second set of rearranged allele primers was JR25, 5'-tgctacttcattgtcagtc-3', and JR30, 5'-tggaggcagagagccgtc-3'. The PCR product obtained after 32 cycles using the second sets of primers yielded fragments of the following sizes: wild-type allele, 168 bp, and rearranged allele, 350 bp.

RNA in situ hybridization

Hybridization was performed on frozen (OCT) and paraffin-embedded tissue that had been preserved in 4% paraformaldehyde/PBS solution for 12–20 hr at 4°C (Wilkinson and Nieto 1993). Preimplantation embryos were fixed at room temperature for 30 min before OCT embedding. Slides were dipped twice in 0.1% gelatin solution and air-dried to prevent blastocyst sections from detaching during hybridization. Antisense and sense controls of the external *Mcl-1* probe were hybridized to tissue sections that were exposed to photographic emulsion for 7–21 days before development.

TUNEL analysis

Blastocysts were fixed in 4% paraformaldehyde/PBS for 30 min at room temperature followed by two washes in 3% BSA in PBS for 5–10 min each. The TACS 2 Tdt-based TUNEL assay kit (Trevigen) instructions were followed with the following exceptions: (1) The blastocysts were not fixed onto a slide to enable them to be collected individually for PCR analysis but, instead, were transferred between watchglasses (Thomas Scientific) containing reagent solutions; (2) proteinase K was not required; and (3) the TACSBlue stain was used to visualize apoptotic cells without counterstain to avoid embryo shrinkage. At the end of the assay, all embryos were transferred to a drop of distilled water under mineral oil and were photographed from multiple orientations to assure the visualization and counting of all blue-labeled apoptotic nuclei. After photography, individual embryos were collected for PCR analysis.

Inner cell mass culture

Immunosurgery and ICM culture were performed as described (Behrendtsen and Werb 1997).

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