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Caspase-8 deficiency facilitates cellular transformation *in vitro*

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Caspase-8 is frequently deficient in several kinds of human tumors, suggesting that certain effects of this enzyme restrict tumor development. To examine the nature of the cellular function whose regulation by caspase-8 contributes to its antitumor effect, we assessed the impact of caspase-8 deficiency on cell transformation *in vitro*. Caspase-8-deficient mouse embryonic fibroblasts immortalized with the SV40 T antigen did not survive when cultured in soft agar, and were nontumorogenic in nude mice. However, the rate of transformation of these cells during their continuous growth in culture, as reflected in the observed emergence of cells that do grow in soft agar and are able to form tumors in nude mice, was far higher than that of cells expressing caspase-8. These findings indicate that caspase-8 deficiency can contribute to cancer development in a way that does not depend on the enzyme's participation in killing of the tumor cells by host immune cytotoxic mechanisms, or on its involvement in the cell-death process triggered upon detachment of the cells from their substrate, but rather concerns cell-autonomous mechanisms that affect the rate of cell transformation.
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Several kinds of human tumors, including small cell lung carcinoma, neuroblastoma, hepatocellular carcinoma, and others, are frequently deficient in caspase-8.[1–3] This deficiency is also common in hepatocellular carcinomas generated experimentally in mice by transgenic expression of oncogenes.[4] The deficiency was found to result either from hypermethylation of regulatory regions in the *caspase-8* gene or (less frequently) from gene mutation. The fact that it occurs by more than one kind of mechanism suggests that it is a causal factor in the oncogenic transformation rather than a consequence of it.

There are several possible ways in which caspase-8 deficiency might contribute to tumor development. The enzyme plays a key role in the initiation of the extrinsic cell-death pathway, a process triggered by ligands of the TNF family that are mainly expressed by cytotoxic immune cells.[5,6] Its deficiency might therefore help tumor cells to evade immune surveillance. Caspase-8 also contributes to death processes triggered in epithelial cells upon their detachment from the extracellular matrix, raising the possibility that deficiency of caspase-8 in tumors increases their invasive capacity.[7–9] Human neuroblastoma cells deficient in caspase-8 were indeed shown to penetrate chicken chorioallantoic membranes and generate metastases in chick embryos more effectively when they were deficient in caspase-8.[10] Caspase-8 also contributes, by mechanisms not yet well understood, to various nonapoptotic cellular functions (see, e.g., Salmena *et al.*[11] and Kang *et al.*[12]). Such effects might well also contribute to tumor development.

To explore the mechanisms by which caspase-8 deficiency contributes to tumor development, we sought to determine whether this contribution operates in the context of destruction of the tumor cells by host immune-surveillance mechanisms or through a role of caspase-8 in the process of the oncogenic transformation itself.

Results

Caspase-8 plays a crucial role in the initiation of the extrinsic cell-death pathway.[5,6] In mice, which do not express the related enzyme caspase-10, this role of caspase-8 is also nonredundant.[13] Fibroblasts generated from caspase-8-deficient mouse embryos are therefore resistant to death induction by ligands of the TNF family such as Fas ligand, even when sensitized to this cytotoxic effect by protein-synthesis blockers such as cycloheximide. In the absence of such death ligands, however, these fibroblasts do not appear to differ from fibroblasts derived from normal (i.e., caspase-8-expressing) embryos. They have similar morphology and grow at similar rates (Figure 1, data not shown).

To determine whether, despite this resemblance, there are certain functional consequences of caspase-8 deficiency that can contribute to tumor development in a cell-autonomous manner, we compared the rates of spontaneous transformation of caspase-8-deficient and normal fibroblasts in culture. Fibroblast strains were generated from caspase-8-deficient and normal embryos, and were then immortalized by

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Abbreviations: CHI, cycloheximide; DMEM, Dulbecco's modified Eagle's minimal essential medium; FCS, fetal calf serum; DMEM–FCS, DMEM supplemented with 10% FCS; KO, knockout; MEF, mouse embryonic fibroblasts; PBS, phosphate-buffered saline; WT, wild type
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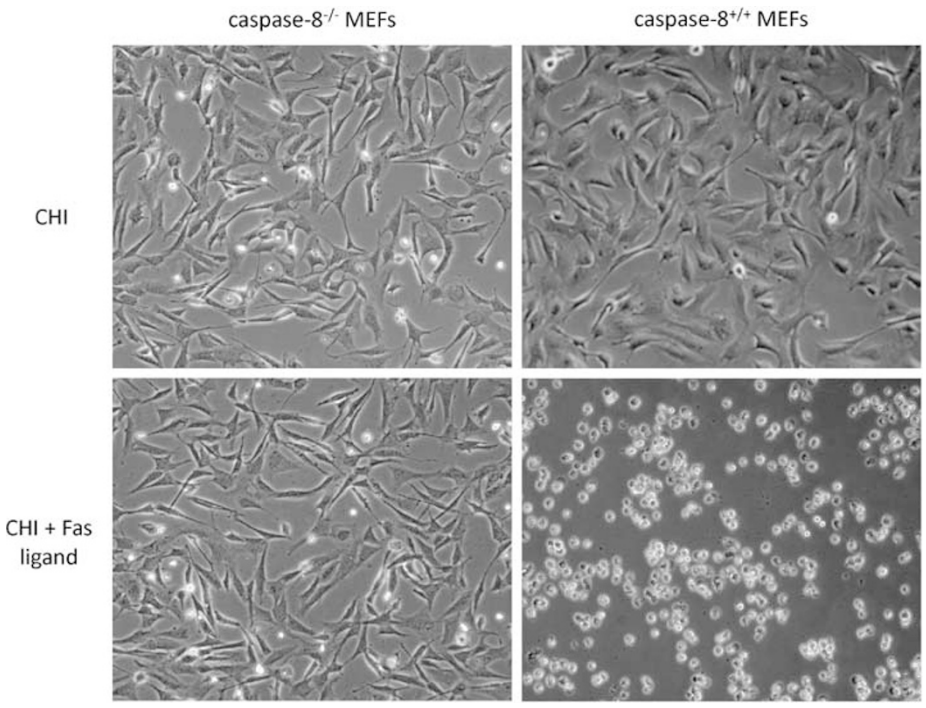

Figure 1 Appearance of the caspase-$8^{+/+}$ and caspase-$8^{-/-}$ mouse embryonic fibroblasts (MEFs) and their response to Fas ligand. The cells were treated for 8 h with cycloheximide (CHI, 10 μg/ml) alone, or with CHI (10 μg/ml) and Fas ligand (1%)

expression of the SV40 large T antigen in them. The cells were passaged repeatedly in culture and in each passage the extent of oncogenic transformation of the cells was assessed by determining their ability to generate solid tumors when injected subcutaneously into nude mice. Also assessed was their ability to form colonies in soft agar (Figures 2 and 3).

In line with earlier reports (see, e.g., Suzuki *et al*.[13] and May *et al*.[14]), we found that immortalization of normal mouse embryonic fibroblasts (MEFs) with the T antigen did not suffice to endow them with the ability to form tumors or to grow in soft agar, and their rate of spontaneous transformation upon passaging, as reflected in the emergence of cells that did have these abilities, was very low. Transformation occurred in only a few and at rather late passages of the caspase-8-expressing MEF strains that we generated, as manifested in a low percentage of mice with tumors (Figure 2a and b, left panels) and the presence of only a few small colonies, if any at all, in soft agar (Figure 3a, left panel and Figures 3b and c).

The caspase-8-deficient cells were initially also unable to form colonies in soft agar or tumors in mice. However, unlike in the normal MEFs, the rate of spontaneous transformation of the caspase-8-deficient cells was high. Some of the caspase-8-deficient cells had already acquired the ability both to form colonies in agar and to form tumors in nude mice by about their tenth passage, and from then onward the extent and frequency of their transformation progressively increased, exceeding by far the meager transformation observed in the normal MEFs (Figures 2a and 3a, right panels).

Still, even in cultures of MEF strains in which the generation of colonies in the soft agar test was high, the number of these colonies barely corresponded to 1% of the initially seeded cells. To find out what happened to those cultured cells that did not form colonies in soft agar, we applied the calcein-AM conversion test to assess their viability. In the soft agar cultures of both the normal and the caspase-8-deficient cells, the numbers of viable cells slowly decreased over a period of several days after seeding (Figure 4a). When we assessed the growth of the cells in semi-solid agar, which allows the cells to be recovered from the culture at various times after their seeding,[15] we observed a gradual increase in the numbers of cells that stain with annexin-V, a quantitative indicator of cell death (Figures 4b and c). We therefore concluded that the cells which did not form colonies in the soft agar died in it, irrespective of whether they had expressed caspase-8 or not.

Discussion

Our findings indicate that cellular transformation *in vitro* is suppressed by caspase-8. Of the various tests used for assessing transformation of cultured cells, the two applied in this study, formation of colonies in soft agar and formation of tumors in nude mice, are considered reliable indicators of genetic and epigenetic changes that contribute to the emergence of cancer.[16] It therefore seems likely that the mechanisms responsible for enhancement of the cellular transformation process monitored by these tests in caspase-8-deficient fibroblasts also account, at least in part, for the frequent deficiency of caspase-8 in certain tumors in humans.

Complementing these cell-culture studies by determining how *caspase-8* deletion affects the rate of tumor emergence in specific tissues in mice should provide a clue to the identities of the cell-type-specific mechanisms that dictate

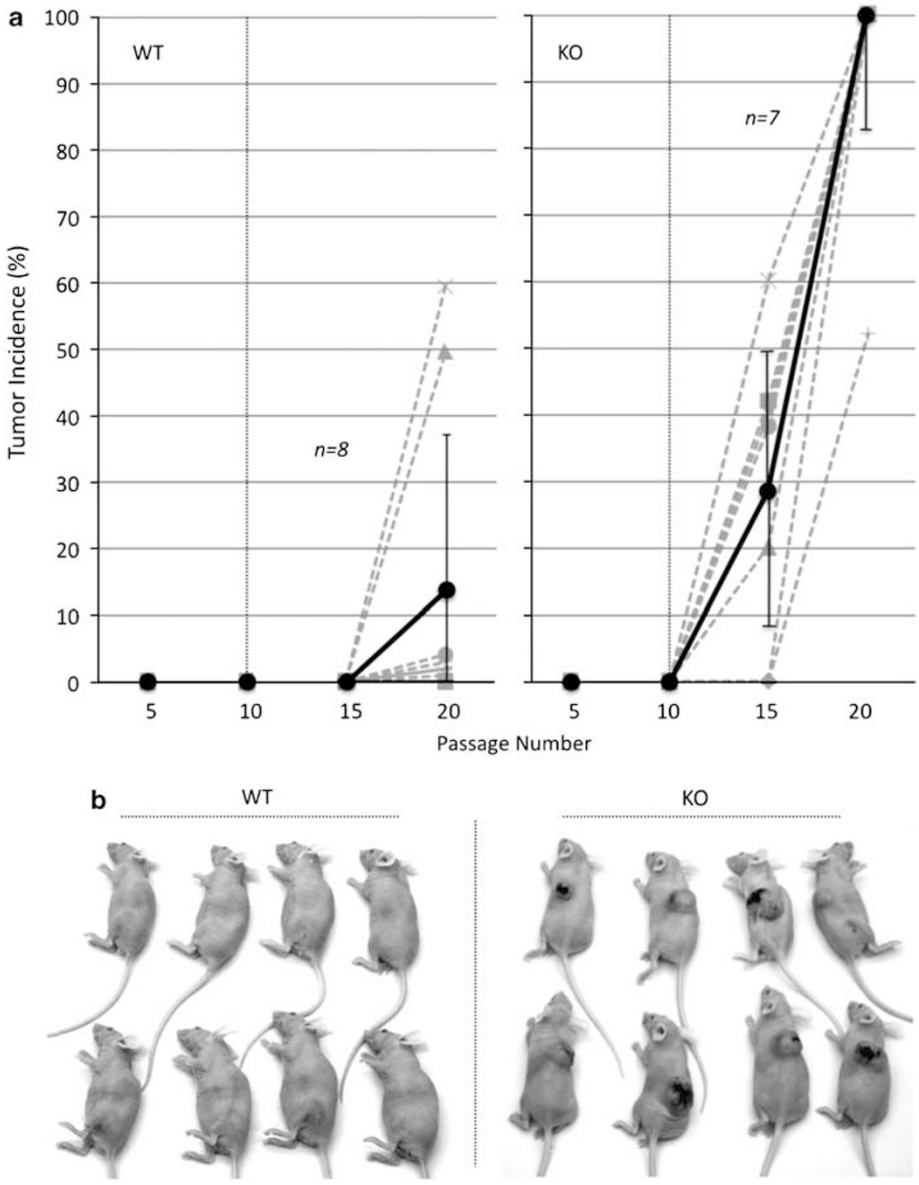

Figure 2 Comparison of the rates of transformation of caspase-$8^{+/+}$ (wild type, WT) and caspase-$8^{-/-}$ (knockout, KO) mouse embryonic fibroblasts (MEFs) in culture by an *in vivo* tumorogenicity test. Figures 2 and 3 record representative data from four independent series of MEF generation followed by assessments of their *in vitro* transformation rates, which yielded similar results. In each of these series, MEFs were established from several E9.5 WT and KO embryos obtained from several pregnant mice. To assess their tumorogenicity *in vivo*, cells of the indicated numbers of independent strains ('*n*') of either the WT or the KO phenotype were injected to nude mice. (**a**) Tumor incidence (%) is plotted as a function of passage number for each of the MEF clones (broken gray lines). The solid line shows the mean value ± S.D. The perpendicular dotted lines in this figure and in Figure 3a were drawn to point out the difference between the times at which transformation was first noticed in the WT and in the KO strains. (**b**) Typical gross appearance, 60 days post-inoculation, of representative groups of nude mice injected with WT or KO MEFs

deficiency of caspase-8 in some kinds of cancer but not in others.

The molecular basis for the suppression of cellular transformation by caspase-8 remains to be educidated. Because the cells used in this study were kept *ex vivo* both during their transformation and in the colony-formation test for assessing the transformation, it is clear that these mechanisms are independent of 'immune-surveillance' cytotoxic functions, or any other kind of host response to the tumor cells. Moreover, our findings suggest that these mechanisms are unrelated to the cell-death process triggered upon detachment of the cells from their substrate. It still seems possible, however, that cell death, triggered by caspase-8 in a cell-autonomous manner, accounts for the suppression of cell transformation by this enzyme. This may occur, for example, as a result of the expression of TNF, or of another ligand of the TNF family, by the transformed fibroblasts themselves. Alternatively, caspase-8 might suppress cell transformation by exerting some nonapoptotic effect. We recently found that mutation of *caspase-8* at the site of initiation of the enzyme's self-processing blocks activation of the extrinsic cell-death pathway, but not various nonapoptotic functions of the

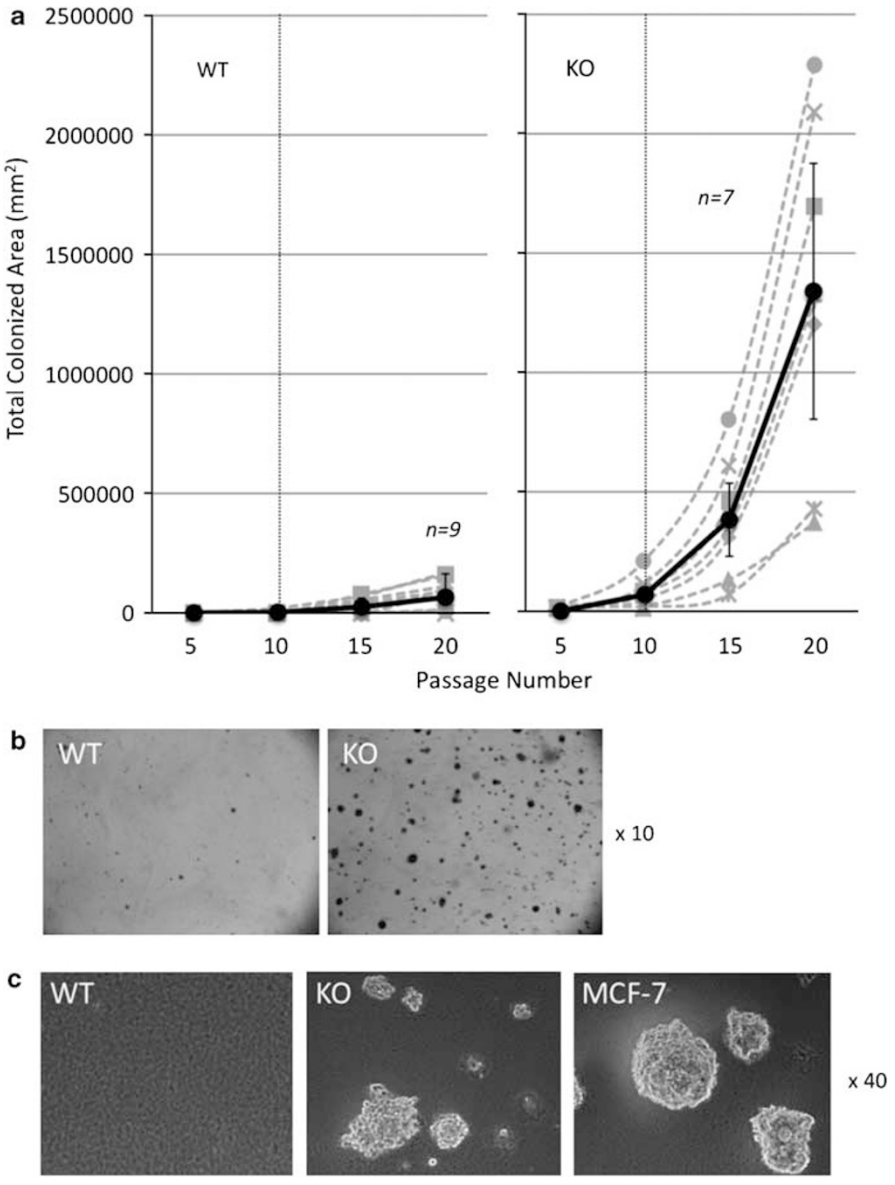

Figure 3 Comparison of the rates of transformation of caspase-$8^{+/+}$ and caspase-$8^{-/-}$ mouse embryonic fibroblasts (MEFs) in culture by a test of soft agar colony formation. Aliquots of the indicated numbers ('*n*') of independent MEF strains were examined. (**a**) Total colony size is plotted as a function of the passage number for each of the MEF strains (broken gray lines). Mean values ± S.D. (solid lines) are also shown. (**b**, **c**) Typical microscopic appearance, 21 days after plating, of representative fields of the agar layer inoculated with WT and KO MEFs, or, for comparison, with cells of the MCF-7 breast carcinoma line

enzyme (Kang *et al*., submitted). By assessing the impact of this mutation on the rate of cell transformation, it should be possible to determine which of the two kinds of caspase-8 activities accounts for its antitumor effect.

The fibroblasts used in this study expressed the SV40 large T antigen that interferes with the function of p53 and the Rb proteins.[17] Mutation or shut-down of these tumor-suppressor genes, which occurs frequently in various types of cancer including some that are often deficient in caspase-8,[18,19] results in immortalization of cells, but does not suffice for their transformation.[20] In fibroblasts at an early stage of passaging, expression of the T antigen did not suffice to endow the cells with the transformed phenotype even when the cells were deficient in caspase-8. Therefore, the gradual emergence in caspase-8-deficient cultures of cells that did exhibit the transformed phenotype probably reflects an accumulation of additional genetic or epigenetic changes. By identifying these additional genetic changes and identifying the particular structural features of caspase-8 required for the arrest of cellular transformation, it may be possible for us to determine whether this effect of caspase-8 reflects its role in restricting the occurrence of mutations or in affecting the survival or the proliferation of cells once they have accumulated such mutations.

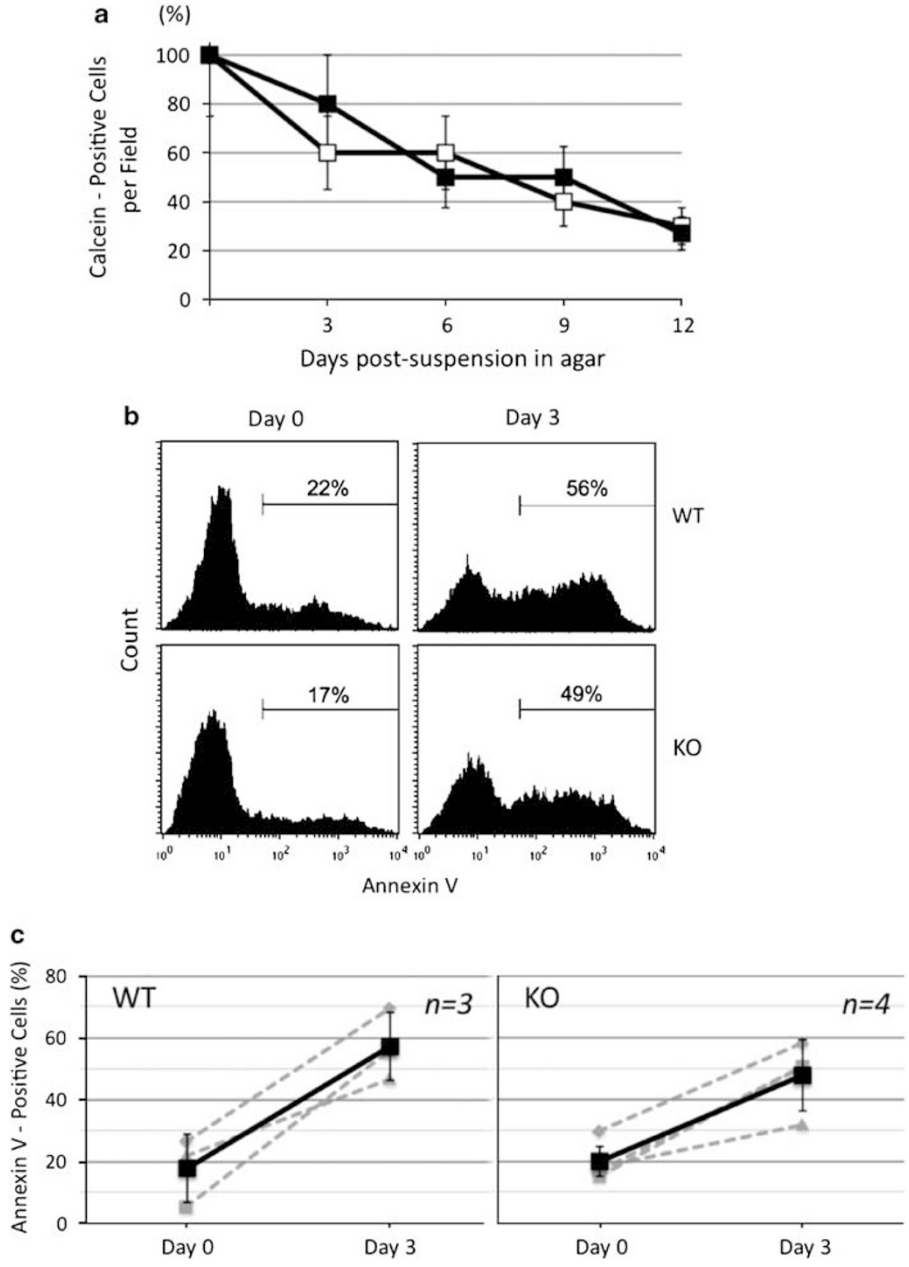

Figure 4 Assessment of the viability of the caspase-$8^{+/+}$ and caspase-$8^{-/-}$ mouse embryonic fibroblasts (MEFs) in agar tests. (**a**) Cell viability in the soft agar test. MEFs that were suspended in agar as described for Figure 3, and then showed positive staining with the vital dye calcein-AM in a representative pair of WT (empty squares), and KO (filled squares) MEFs in at least four randomly chosen microscopic fields ($\times$ 10) were counted. In each case the value (mean $\pm$ S.D.) of the obtained count, relative to that determined immediately after plating (day 0), was plotted as a function of the time following seeding in agar. (**b**, **c**) Cell viability in a semi-solid agar test. (**b**) Representative histogram of annexin-V staining of a pair of WT and KO MEFs, before and after growth for 3 days in semi-solid agar culture. The extent of cell death (% of annexin-V-positive cells) is indicated at the top of the histograms. (**c**) Quantification of the annexin-V-staining level, assessed as in (**b**), for a number of independent MEF strains (indicated by '*n*') having either the WT (empty squares) or the KO (filled squares) genotype, grown in semi-solid soft agar cultures (broken gray lines). Mean values $\pm$ S.D. are indicated by a solid line

Materials and Methods

Reagents. Human Fas ligand fused to a leucine-zipper and the FLAG tag was generated by its transient expression in HEK293T cells, as described for several other ligands of the TNF family.[21,22]

Establishment of mouse embryonic fibroblast cell strains. The strain of mice carrying a knocked out *caspase-8* allele (*Caspase-*$8^{-/+}$) have been described previously.[23] These mice were mated to obtain wild type (WT, caspase-$8^{+/+}$) and caspase-8 knockout (KO, caspase-$8^{-/-}$) E9.5 embryos. Fibroblasts were derived from these embryos by trypsinization[24] and grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), nonessential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin (DMEM–FCS). The cells were passaged four times to eliminate cells that are not fibroblasts and were then immortalized by retroviral infection with a recombinant virus expressing the temperature-sensitive mutant of the large T antigen of the SV40 virus.[25] Following selection with G418 for T-antigen-expressing cells, the established MEF strains were repeatedly passaged by replating of the cells every second day at a density of 1.2×10^6 cells per 100-mm dish. At the

indicated passage number the cells were expanded to log phase, rinsed twice with phosphate-buffered saline (PBS), and subjected to tumorogenicity tests. All the experiments presented in this study were carried out with cells derived from mice of pure C57Bl/6 background, obtained by 12–14 backcrossings with mice of that strain. A few experiments performed with cells derived from mice of the original mixed (129/Sv and MF1),[23] genetic background yielded similar findings (data not shown).

Assessment of *in vivo* tumorogenicity of the MEFs. For each MEF strain, five 7-week-old CD1-nude mice were injected subcutaneously in the back with a dose of 2×10^6 cells in 400 μl of PBS. Tumor occurrence ($\geqslant 0.1\,cm^3$) was evaluated 60 days post-inoculation. Tumor volume was calculated according to the following equation: tumor volume $=$ (length $\times$ width^2)/2.

Assessment of colony formation in soft agar. Aliquots of 10^4 MEFs were resuspended in 1 ml of 0.35% agar (w/v) in DMEM–FCS. The aliquots were poured into six-well dishes on top of a 1.5-ml layer of 0.5% agar (w/v) in DMEM–FCS, allowed to solidify, and incubated for 21 days at 37 °C in the presence of 5% CO_2. The dishes were photographed and the colony sizes estimated, using the Image-pro Plus® 4.1 software package, by determining the total area that the colonies occupied in the photographs.

Assessment of the viability of MEFs in agar tests. To assess the viability of the MEFs in the soft agar test, the cells were stained with the vital dye calcein-AM, a component of the Live/Dead Viability/Cytotoxicity Assay Kit (Molecular Probes), as specified by the manufacturer, and visualized by standard fluorescence microscopy using a band-pass filter. To assay cell death in a semi-solid agar test,[15] the cells were suspended in aliquots of 5×10^4 in 5 ml of DMEM–FCS and plated over a nutrient agar phase (3% agar in water, mixed with DMEM–FCS and 10 $\times$ PBS, in a ratio of 10 : 50 : 1). After the indicated number of days the cells were recovered from the cultures, filtered through a cell strainer (75 μm; BD Biosciences), and stained with APC-annexin V (BD Pharmingen) according to the manufacturer's instructions. Flow cytometry was performed with a FACSort machine (BD Biosciences) as described,[12] and the results were analyzed with the CELLQUEST software.

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