Identification of MMP-15 as an Anti-apoptotic Factor in Cancer Cells

Reimar Abraham§*, Juliane Schäfer¶, Mike Rothe§, Johannes Bange§, Pjotr Knyazev‡, and Axel Ullrich¶2

From the §Department of Molecular Biology, Max-Planck-Institute for Biochemistry, 82152 Martinsried, Germany, the ¶U3 Pharma AG, 82152 Martinsried, Germany, the ‡Venetian Institute of Molecular Medicine, Via Orus 2, 35129 Padova, Italy, and the ¶Department of Statistics, Ludwig-Maximilians-Universität, 80539 München, Germany

We have performed an in vitro selection for an anti-apoptotic phenotype that resembles the selection process that pre-malignant cells undergo in the initial phase of carcinogenesis in vivo. Using the cervical carcinoma cell line HeLa S3 as a model system, the selection procedure yielded cell clones that displayed increased resistance to apoptosis induced by Fas, tumor necrosis factor-related apoptosis-inducing ligand, and serum starvation. Gene expression profiling using gene family focused cDNA arrays revealed numerous genes that are differentially expressed in HeLa S3 and the resistant sub-clones and therefore are potentially involved in the definition of sensitivity to apoptotic stimuli. From the genes identified in this functional genomics approach we validated the anti-apoptotic activity of the membrane-anchored matrix metalloproteinase 15 (MMP-15) by means of small interfering RNA-mediated knockdown and ectopic expression in parental HeLa S3 cells and, to confirm a more general significance of our findings, in other cancer cell lines. The in vivo relevance of these findings is supported by the overexpression of MMP-15 in human lung adenocarcinoma compared with normal lung. Because MMP-15 is known to promote invasion, our results suggest that this protease connects metastasis and apoptosis resistance by an unknown regulatory mechanism. Our findings therefore strongly suggest that cancer characteristics such as metastatic potential, which are thought to evolve late in cancer progression, could be manifested early on by selection for an anti-apoptotic phenotype.

The inhibition of apoptosis has been generally recognized as a major hallmark of cancer (1). Oncogenic activation of proto-oncogenes by gene amplification or mutation normally induces apoptosis in the affected cells. Hence, apoptosis serves as a safeguard against cellular transformation. To allow for oncogenesis to occur, apoptosis has to be suppressed concomitantly with the activation of oncogenes and several apoptotic stimuli have to be bypassed later on in tumor evolution. These stimuli include attacks of the immune system, lack of growth factors, or detachment from the basal membrane. Finally, a number of chemotherapeutic agents such as doxorubicin and taxol act through inducing apoptosis. The inhibition of apoptosis has been generally recognized as a major hallmark of cancer (1). Oncogenic activation of proto-oncogenes by gene amplification or mutation normally induces apoptosis in the affected cells. Hence, apoptosis serves as a safeguard against cellular transformation. To allow for oncogenesis to occur, apoptosis has to be suppressed concomitantly with the activation of oncogenes and several apoptotic stimuli have to be bypassed later on in tumor evolution. These stimuli include attacks of the immune system, lack of growth factors, or detachment from the basal membrane. Finally, a number of chemotherapeutic agents such as doxorubicin and taxol act through inducing apoptosis. Notably, two ligands are best characterized for their ability to predominantly induce apoptosis: Fas ligand and TRAIL (6, 7). These ligands play a role in the immune surveillance of tumor cells by natural killer cells and cytotoxic lymphocytes which express them on the cell surface and induce apoptosis by binding to their receptors on target tumor cells (8–10). Fas ligand binds to the Fas receptor, whereas TRAIL mediates its effects through the receptors DR4 and DR5 (6, 11, 12). TRAIL has been found to induce apoptosis preferentially in tumor cells (7). Fas ligand and TRAIL may also be cleaved off the cell surface by matrix metalloproteinases or cysteine proteases, respectively resulting in soluble ligands (13, 14).

Signaling by death receptors in tumors is inhibited at numerous points in the signaling cascade. Decoy receptors that lack a functional death domain or soluble receptors reduce the effective ligand concentration (11, 15, 16). FADD-homologous ICE/CED-3-like protease (or FLICE)-like inhibitory proteins, which resemble caspase-8 but lack catalytic activity, can bind to FADD instead of caspase-8 end thereby prevent the initiation of the caspase cascade (17). Additionally, inactivating deletions and mutations of Fas, DR4, and DR5 have been found in tumor samples (18, 19). Further downstream and not limited to death receptor signaling, various components of the apoptosis machinery are differentially expressed or mutated in tumors (8).

We set out to attempt to identify as many genes as possible that are involved in the mediation of anti-apoptotic mechanisms in cancer cells. Here we focused on three gene families that play key roles in the cellular signal transduction network: protein kinases, protein phosphatases, and matrix metalloproteinases (MMPs). To this end, we took advantage of the genetic instability intrinsic to cancer cells and chose a tumor-derived cell line that is still sensitive to apoptosis induction by Fas. The cervical carcinoma cell line HeLa S3 fulfilled these criteria. Our functional genomics strategy involved the exposure of HeLa S3 cells to the Fas-activating antibody CH-11 and selection of resistant clones. This approach yielded 15 clones that were more resistant to Fas, TRAIL, and starvation-induced cell death than the parental cell line. Subsequently, through the use of cDNA arrays, we identified genes that are differentially expressed or mutated in tumors (8).

The abbreviations used are: FADD, Fas-associated via death domain; DR, death receptor; MMP, matrix metalloproteinase; siRNA, small interfering RNA; TIMP, tissue inhibitor of matrix metalloproteinases; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; ERK, extracellular signal-regulated kinase.

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tually expressed in the resistant clones and HeLa S3 and therefore are likely to function as apoptotic regulators. We discovered a novel anti-apoptotic function of the membrane-spanning metalloproteinase MMP-15. Taken together, our results implicate MMP-15 in the manifestation of the anti-apoptotic phenotype in human cancer and validate this enzyme as a novel cancer drug target.

MATERIALS AND METHODS

Reagents—The cDNA arrays were manufactured in our laboratory. Each array represented 666 genes: 413 protein kinases, 98 protein phosphatases, 31 ligands, 21 matrix metalloproteinases, and a group of 103 genes comprising “housekeeping” genes, various other signal transduction-related genes as well as some viral genes. The cDNA fragments ranging in length from 200 to 2000 bp had been cloned in pBluescript and spotted on nylon membranes (Amersham Biosciences) using a spotter from BioRobotics. As a background control, empty pBluescript was used. Each plasmid was spotted twice. Chemiluminescence reagents and [α-33P]ATP were purchased from PerkinElmer Life Sciences. The nitrocellulose membrane was from Schleicher & Schüll. The monoclonal α-myc antibody was purified from the hybridoma 9E102 obtained from the American Type Culture Collection (ATCC), the α-ERK-1/2 antibody (K-23) was from Santa Cruz Biotechnology, the α-Fas antibody (UB-2) was from Beckman-Coulter, and all secondary antibodies were from Dianova. The siRNA sequence targeting luciferase (GL2) was described earlier (20), the siRNA target sequence for MMP15 was UGA CGC AGC CUA CAC CUA C; the siRNA oligonucleotides were supplied by Dharmacon. Agonistic α-Fas antibody CH-11 was purchased from Beckman-Coulter and TRAIL from TEBU. The oligonucleotide primers and TaqMan probe for quantitative real-time PCR were supplied as “Assays-on-Demand” by Applied Biosystems (ABI).

Cell Culture and Cell Death Induction—The cell lines HeLa S3, PC-3, and MCF-7 were purchased from the ATCC and cultivated according to the supplier’s recommendations. Mel Juso was from the German Collection of Microorganisms and Cell Cultures and grown in RPMI 1640 supplemented with 10% fetal calf serum. The retroviral packaging cell line Phoenix A was a kind gift by Gary Nolan (Stanford University) and was cultured in Dulbecco’s modified Eagle’s medium, 4500 mg/liter glucose, supplemented with 10% fetal calf serum. All cell culture reagents were purchased from Invitrogen.

For cell death assays establishing the apoptosis sensitivity of HeLa S3 and the resistant clones as well as HeLa S3 ectopically expressing MMP-15, cells were seeded at 50% confluency, grown for 2 days, and then treated depending on the apoptotic stimulus. For CH-11-induced cell death, cells were starved without serum for 10 h, CH-11 was added at 100 ng/ml, and cell death was measured after an additional 14 h. For TRAIL-induced cell death, cells were starved for 36 h, TRAIL was added at 100 ng/ml, and cell death was measured after an additional 12 h. For starvation-induced cell death, the cells were starved for 3 days before measuring cell death. For cell death assays after MMP-15 knock-down in HeLa S3 and the resistant clones, siRNA-transfected cells were seeded at 50% confluency and grown for 1 day. Then, cells were starved for 10 h, CH-11 or TRAIL was added at 200 ng/ml, and cell death was measured after further 14 h. Starvation-induced cell death was measured after a total of 3 days of starvation.

For cell death assays after MMP-15 knock-down in the other cancer cell lines, siRNA-transfected cells were seeded at 50% confluency and, depending on the cell line, were grown for 1–2 days and treated with 20–300 ng/ml TRAIL for 1 day. Cell death was measured by propidium iodide dye exclusion.

Cell Death Assays—Cell death was measured by counting the percentage of hypodiploid cells using flow cytometry as described previously (21). Alternatively, cell death was measured by dye exclusion by determining the percentage of dead cells that took up propidium iodide. Flow cytometry was used by gating for the cells that were bigger than the smallest cells (by forward scatter) of the healthy cell population, thus excluding cell debris. Propidium iodide fluorescence was measured in the FL3 channel on a FACSCalibur (BD Biosciences) or EpicsXL (Beckman-Coulter) flow cytometer. For both assays, cell culture supernatant and trypsinized cells were pooled. Background values without cell death stimulus were subtracted for CH-11- and TRAIL-induced cell death.

The correction factor for the siRNA experiments in HeLa S3 and the resistant clones (Fig. 3) was calculated by dividing the -fold down-regulation (i.e. expression level of MMP-15 with GL2 siRNA divided by expression level with MMP-15 siRNA) of HeLa S3 by the -fold down-regulation of the respective clone. This yielded factors 1.5, 1.3, and 1.6 for clones 14, 36, and 58, respectively.

Transfection of siRNA and Viral Infection—All cells were seeded at 90% confluency in 12 wells. The next day, 100 pmol of siRNA duplexes per well were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. After 14 h the siRNA duplexes were removed, and the cells were trypsinized and seeded at 50% confluency in 12 wells for further assays. Preparation of viral supernatant and infection of HeLa S3 cells was carried out as described on the Nolan laboratory website (www.stanford.edu/group/nolan/protocols/pro_helper_free.html). After infection, the cells were selected with 0.5 mg/ml G418 for 2 weeks.

Cloning of MMP-15—MMP-15 was amplified from HeLa S3 cDNA by PCR using primers flanking the coding region and sequenced. The protein sequence contained a Gly → Arg substitution at position 609 as published previously (22). MMP-15 was cloned into pcDNA 3.1/myc-His (Invitrogen) and subcloned into pLXSN (Clontech) to yield myc- and His-tagged MMP-15.

Preparation of RNA and cDNA—For cDNA array experiments, the following methods were used: total RNA was extracted from exponentially growing cells kept under normal growth conditions with fetal calf serum using guanidinium thiocyanate as described before (23). Afterward, mRNA was isolated with the help of oligo-dT cellulose and reverse transcribed using oligo-dT primers (24). Single-stranded cDNA was purified with the help of the PCR purification kit from Qiagen.

For quantitative real-time PCR, total RNA was isolated using the RNeasy Kit (Qiagen), and cDNA was prepared using ABI Reverse Transcription Reagents with random hexamer primers according to the manufacturer’s protocols. This cDNA was used without further purification in quantitative real-time PCR.

Quantitative Real-time PCR and Immunoblotting—Real-time PCR was performed using the PCR Master Mix (ABI) and “Assays on Demand” (ABI) for MMP-15 and β-Actin as a loading control according to the supplier’s instructions on an ABI Prism 7000. Expression levels were calculated according to the following formula: 1000* 2(Ct Actin − Ct MMP-15). Cell lysis and immunoblotting were performed as described previously (25).

cDNA Array Hybridization—Radioactive labeling of the cDNAs was achieved using the Megaprime-DNA labeling kit (Amersham Biosciences) and 50 μCi of [α-33P]ATP per reaction. The labeled cDNA was purified via the Nucleotide Removal Kit from Qiagen and incubated with 0.5 mg/ml COT-DNA (Invitrogen) in hybridization buffer (5× SSC, 0.1% SDS) for 5 min at 95 °C and 30 min at 68 °C to block repetitive sequences in the cDNA. The cDNA was added to pre-warmed (68 °C) hybridization buffer containing 100 μg/ml tRNA (baker’s yeast, Roche Applied Science).
The cDNA arrays were incubated in pre-hybridization buffer (5× Denhardt’s, 5× SSC, 100 mM NaPO₄, 2 mM Na₂HPO₄, 100 μg/ml tRNA) for 4 h and subsequently with the labeled cDNA in hybridization buffer for 16 h. The cDNA was removed, and the cDNA arrays were washed with increasing stringency, dried, and exposed on phosphorimaging plates (Fujifilm). The plates were read on a Fuji BAS2500 phosphorimaging device, and the raw spot values (volume) were determined using ArrayVision (RayTest, Canada).

**Statistical Analysis**—The raw DNA microarray intensity data were pre-processed using robust estimation of calibrating and variance-stabilizing transformations (26). After this, systematic array biases should be removed, and the variance should be approximately independent of the mean intensity. This is useful for subsequent t tests, which were conducted to evaluate the significance level of differences between the gene expression levels in HeLa S3 versus cell death resistant clones. The problem of multiplicity was addressed using the False Discovery Rate criterion (27, 28). Class prediction from gene expression profiling was analyzed using the method of “nearest shrunken centroids” (29). This supervised approach identifies subsets of genes that best characterize the two groups HeLa S3 and resistant clones. All these analyses were performed using the statistical computing language “R” and packages implemented for its programming environment freely available under the terms of the Free Software Foundation’s GNU General Public License.

**FIGURE 1. Apoptosis sensitivity of the resistant clones and Fas expression.**

A, cell death was measured counting the percentage of hypodiploid cells upon treatment with 100 ng/ml CH-11. Values from two independent experiments. B, cell death was measured as in A upon treatment with the indicated concentrations of CH-11. Values from three independent experiments in duplicates. C, cell surface expression of Fas. Cells were stained with α-Fas UB-2 and FITC-labeled α-mouse or with α-mouse alone as a control and analyzed by flow cytometry. Ab, antibody; red, HeLa S3; magenta, Clone 14; blue, Clone 36; green, Clone 58. D, cell death was measured by propidium iodide dye exclusion upon treatment with 100 ng/ml CH-11, 100 ng/ml TRAIL or 3 days serum starvation expressed in percent of HeLa S3 values. Results from three independent experiments in duplicates. Error bars represent standard error of the mean. All values for the resistant clones were significantly different from HeLa S3 values (p < 0.05; t test).
Literature Search—The literature search was carried out with the help of PubMed at the National Library of Medicine using “/H11021 gene name/H11022 AND apoptosis” as a search string.

RESULTS

Selection of CH-11 Resistant Clones—To select for cells that could resist cell death induced by Fas, we incubated HeLa S3 for 3 days with the agonistic antibody CH-11. To increase the effect, the cells were kept without serum during that time. After 3 days, the vast majority of the cells had died. The medium was exchanged for growth medium without CH-11, containing 10% fetal calf serum, and the remaining single cells were allowed to grow clonally. About 40 clones were obtained per 100,000 seeded cells.

Sensitivity of the Clones to Apoptotic Stimuli—We chose to test 15 clones for their resistance to CH-11. We reasoned this number of clones should yield enough statistical power for the following cDNA array analysis, which was intended to find genes that are differentially expressed in all of these clones with respect to parental HeLa S3. As shown in Fig. 1A, all clones were less sensitive to Fas-induced cell death than parental HeLa S3. These data are corroborated by a dose-response study using HeLa S3 and a subset of three clones (Fig. 1B). The resistance to Fas-mediated cell death was not merely caused by down-regulation of Fas, because cell surface expression of Fas as assessed by flow cytometry was even higher in the resistant clones (Fig. 1C). We also asked whether these clones were similarly resistant to other apoptosis inducers. Therefore, we tested the same subset of

| Category | Gene symbol | GenBank™ accession number | Pro-or anti-apoptotic according to literature | Reference | Adjusted p value | -Fold up-regulation |
|----------|-------------|--------------------------|-----------------------------------------------|-----------|-----------------|-------------------|
| (A)      | OT          | β-Tubulin | NM_178014 | Pro- and anti-apoptotic | (74,75) | 0.0007 | 1.4 |
|          | MP          | MMP-15    | Z48482 | Anti-apoptotic | (77,78) | 0.0014 | 1.4 |
|          | STK         | MAPKK3    | NM_002756 | Pro- and anti-apoptotic | (74,75) | 0.0014 | 1.4 |
|          | OT          | EF-2      | Z11692 | Pro-apoptotic | (76) | 0.0105 | 2.3 |
|          | STK         | GRK6      | L16862 | Pro-apoptotic | (76) | 0.017 | 1.5 |
|          | MP          | TIMP-1    | X03124 | Anti-apoptotic | (76) | 0.0105 | 2.3 |
|          | STK         | MAPKK6    | U39657 | Pro-apoptotic | (76) | 0.0117 | 1.5 |
|          | MP          | MMP-2     | J03210 | Pro-apoptotic | (76) | 0.0137 | 2.1 |
|          | GP          | α2         | NM_002070 | Pro-apoptotic | (79) | 0.0149 | 1.4 |
|          | MP          | MMP-3     | X05232 | Pro-apoptotic | (79) | 0.0158 | 1.3 |
|          | STK         | Bcr       | X02596 | Pro-apoptotic | (79) | 0.0181 | 1.6 |
|          | STK         | MAST205   | NM_008641 | Pro-apoptotic | (79) | 0.0181 | 1.4 |
|          | STK         | Akt/Rac-α | M63167 | Anti-apoptotic | (77,78) | 0.0220 | 1.7 |
|          | PP          | P2B-R     | M30773 | Anti-apoptotic | (77,78) | 0.0273 | 1.1 |
|          | STK         | IRAK      | L76191 | Anti-apoptotic | (79) | 0.0275 | 1.7 |
|          | LIG         | VEGF      | AF022375 | Anti-apoptotic | (80) | 0.0297 | 1.3 |
|          | OT          | 90k       | X97809 | Anti-apoptotic | (81,82) | 0.0345 | 1.9 |
|          | MP          | TIMP-2    | S48568 | Pro- and anti-apoptotic | (81,82) | 0.0372 | 1.4 |
| (B)      | STK         | MARK      | NM_018650 | Pro-apoptotic | (83) | 0.0001 | 1.3 |
|          | STK         | ERK-3     | X80692 | Pro-apoptotic | (84,85) | 0.0007 | 2.8 |
|          | STK         | RICK      | AF027706 | Pro- and anti-apoptotic | (84,85) | 0.0007 | 2.8 |
|          | STK         | NDR       | Z35102 | Pro-apoptotic | (86) | 0.0007 | 1.8 |
|          | STK         | hUBR1     | AF068760 | Pro-apoptotic | (86) | 0.0007 | 1.8 |
|          | PP          | hVH5      | U27193 | Anti-apoptotic | (87) | 0.0007 | 1.5 |
|          | OT          | BRCA1     | U14680 | Pro- and anti-apoptotic | (88,89) | 0.0008 | 2.6 |
|          | STK         | Hdc7      | AF015592 | Pro-apoptotic | (83) | 0.0008 | 2.0 |
|          | PP          | TC-PTP     | M25393 | Pro-apoptotic | (90) | 0.0009 | 2.4 |
|          | STK         | ROCK      | U43195 | Pro-apoptotic | (91,92) | 0.0011 | 2.9 |
|          | STK         | CHK1      | AF016582 | Anti-apoptotic | (93) | 0.0011 | 1.9 |
|          | PP          | YVH1      | AF119226 | Anti-apoptotic | (94) | 0.0014 | 2.2 |
|          | STK         | Nlk       | AF036332 | Pro-apoptotic | (94) | 0.0014 | 1.9 |
|          | STK         | CK-1α      | X80693 | Anti-apoptotic | (95) | 0.0015 | 1.9 |
|          | STK         | RPS6KC1   | AF037447 | Pro-apoptotic | (96) | 0.0015 | 1.6 |
|          | STK         | CDK-6     | X66365 | Pro-apoptotic | (96) | 0.0015 | 1.6 |
|          | UK          | PB-11     | AF061944 | Pro-apoptotic | (97) | 0.0016 | 2.4 |
|          | YK          | Pyk2      | U33284 | Pro-apoptotic | (97) | 0.0017 | 2.4 |

a STK, serine/threonine protein kinase; YK, tyrosine-protein kinase; UK, unknown kinase; PP, protein phosphatase; MP, matrix metalloproteinase; MP-I, matrix metalloproteinase inhibitor; GP, involved in G-protein signaling; LIG, ligand; OT, other.

Genes that are differentially expressed in the resistant clones with respect to parental HeLa S3

(A) Genes that are up-regulated in the clones. (B) The first 18 genes that are down-regulated in the clones. The fourth column shows the result of the unbiased literature search. Genes were classified as anti-apoptotic, pro-apoptotic, or pro- and anti-apoptotic if reports for both actions were found. p values were adjusted for multiple hypotheses testing (see “Materials and Methods”).

MMP-15 in Apoptosis Control

Sensitivity of the Clones to Apoptotic Stimuli—We chose to test 15 clones for their resistance to CH-11. We reasoned this number of clones should yield enough statistical power for the following cDNA array analysis, which was intended to find genes that are differentially expressed in all of these clones with respect to parental HeLa S3. As shown in Fig. 1A, all clones were less sensitive to Fas-induced cell death than parental HeLa S3. These data are corroborated by a dose-response study using HeLa S3 and a subset of three clones (Fig. 1B). The resistance to Fas-mediated cell death was not merely caused by down-regulation of Fas, because cell surface expression of Fas as assessed by flow cytometry was even higher in the resistant clones (Fig. 1C). We also asked whether these clones were similarly resistant to other apoptosis inducers. Therefore, we tested the same subset of
clones for their sensitivity toward TRAIL- and serum starvation-induced cell death. Fig. 1D shows that these clones were also more resistant to TRAIL-induced cell death, and two of the clones (clones 14 and 58) were more resistant to serum starvation. However, clone 36 was even more sensitive to starvation than the parental cell line HeLa S3. The different apoptosis sensitivities of the clones toward the various inducers show that the clones are indeed different from each other and not mere derivatives of one clonal cell population.

**Characterization of Resistant Clones by Gene Expression Array Analysis**—To investigate the genetic basis of the apoptosis-resistance phenotype in the selected clones, we used cDNA array hybridization to determine the expression level of 666 signal transduction related genes in parental HeLa S3 cells and the apoptosis-resistant clones. These included 413 protein kinases, 98 protein phosphatases, and 21 matrix metalloproteinases. Poly(A)$^+$ RNA was isolated from each clone, converted into radioactively labeled cDNA, and hybridized to the macro arrays. Because HeLa S3 was the control for further analysis, we used two independently generated mRNA preparations from HeLa S3, converted the mRNA into $[^{33}P]$cDNA, and hybridized each cDNA in separate experiments to two cDNA arrays. Together with the double spots of each probe on the arrays (see "Materials and Methods") this yielded eight data points per gene probe for HeLa S3 and two data points for each clone.

We were most interested in the genes that showed a significantly different expression level in all clones compared with HeLa S3. Therefore, we applied a series of t-Tests to detect differentially expressed genes and controlled for multiplicity using the false discovery rate criterion, i.e. the expected ratio of erroneously discovered differentially expressed genes among all the discovered differentially expressed genes. Using this method, we identified 18 genes that were significantly up-regulated in the resistant clones and 90 genes that were significantly down-regulated (TABLE ONE and supplemental data) at a false discovery rate of 5%. The up-regulated genes should enhance the resistant phenotype of the clones and hence act anti-apoptotically, whereas the down-regulated genes should act pro-apoptotically. To verify that the employed strategy works in identifying genes that can influence apoptosis, we performed an unbiased literature search of the first 18 genes in the lists of up-regulated and down-regulated genes, respectively. For some genes, an anti-apoptotic as well as a pro-apoptotic role was reported, depending on the experimental conditions used by the investigators. As shown in the fourth column of TABLE ONE, A, all genes that are up-regulated in the resistant clones and that are known to influence apoptosis in one way only (enhance or suppress apoptosis) act anti-apoptotically. **Vice versa**, the known pro-apoptotic genes outnumber the known anti-apoptotic genes by 7:3 in the list of down-regulated genes (TABLE ONE, B). These results suggest a similar distribution among the genes that had not been linked to apoptosis before, and it confirmed the validity of our strategy.

We also wanted to know the minimal set of classifier genes whose expression can distinguish between the resistant clones and HeLa S3. The gene expression levels of these classifier genes would collectively act to differentiate between apoptosis-sensitive (HeLa S3) and apoptosis-resistant (clones) properties of the cell lines tested. The gene products of the classifiers could therefore also act together in common signaling pathways. We performed a supervised classification using the method of nearest shrunken centroids (29). As shown in TABLE TWO, a group of 19 genes is sufficient to classify the resistant clones and HeLa S3. Interestingly, three of the four up-regulated classifiers, MMP-15, MMP-2, and TIMP-1 belong to the group of MMPs and their regulators. Additionally, MMP-15 is second on the list of significantly up-regulated genes (TABLE ONE, A), but is so far not known to play a role in apoptosis. For these reasons, we decided to further validate the anti-apoptotic function of MMP-15.

**Verification of MMP-15 Gene Up-regulation**—First, we verified the up-regulation of MMP-15 gene expression in the resistant clones using a second methodology. We used quantitative real-time PCR to determine the relative abundance of MMP-15 mRNA. As can be seen in Fig. 2, the expression level of MMP-15 is higher in all resistant clones compared with HeLa S3.

On the protein level, we were unable to detect endogenous MMP-15 in HeLa S3 or the resistant clones by immunoblot analysis of both total cell lysates or immunoprecipitates of MMP-15. Likewise, we could not detect MMP-15 by Flow cytometry (data not shown).
Knock-down of MMP-15 Increases the Cell Death Rate—Next, we asked whether siRNA-mediated knock-down of MMP-15 could influence the cell death rate after CH-11 treatment. If MMP-15 indeed inhibits cell death, the number of dead cells should increase upon knock-down of MMP-15 in the resistant clones. Fig. 3 demonstrates that the MMP-15-directed siRNA works in reducing the MMP-15 mRNA level compared with control firefly luciferase-directed siRNA (GL2). Fig. 3B shows that this reduction of the MMP-15 level increased the cell death rate in clones 14 and 36. The marginal enhancement of cell death in HeLa S3 is statistically not significant (at $p < 0.05$, t test). Given the higher MMP-15 expression in the clones, the effect should be bigger in the clones than in HeLa S3, and thus the result fell within the expectations. However, in clone 58 knock-down of MMP-15 did not result in an enhancement of cell death. This shows that the anti-apoptotic function of MMP-15 depends on other genetic factors that are currently unknown.

We also examined the influence of MMP-15 on TRAIL- and starvation-induced cell death. Fig. 3 (C and D) shows that a decrease of MMP-15 level led also to an increase of the cell death rate upon these two inducers. The induction of cell death was again stronger in the clones than in HeLa S3. Remarkably, there was no effect on starvation-induced cell death in clone 36, which was more sensitive to starvation than HeLa S3.

Ectopic Overexpression of MMP-15 in HeLa S3 Cells Decreases the Cell Death Rate—There are concerns that siRNA oligonucleotides can have off-target effects (30). Hence, usage of only one siRNA does not guarantee that the observed phenotype is truly generated by knock-down of the siRNA target. To address this concern, and as a proof of principle, we simulated the up-regulation of MMP-15 in the clones by generating HeLa S3 stably overexpressing ectopic MMP-15. We examined whether induction of cell death would be inhibited in MMP-15 expression vector-infected HeLa S3 cells in comparison to empty vector control-infected cells. Fig. 4 shows that cell death induced through FAS, TRAIL, and serum starvation could indeed be inhibited by MMP-15 overexpression. Hence, MMP-15 overexpression alone is sufficient to protect from apoptosis.

Knock-down of MMP-15 in Other Cancer Cell Lines—To examine the wider significance of the anti-apoptotic action of MMP-15, we sought to determine if this protease also protects other cancer cell lines from apoptosis. We chose TRAIL as inducer of cell death, because this ligand is effective in a variety of tumor cell lines (7). Four cell lines of different cancer types, breast cancer (MCF-7), prostate cancer (PC-3), lung cancer (Calu-6), and melanoma (Mel Juso), were used for these experiments. All cell lines showed a lower endogenous MMP-15 expression level than HeLa S3 (Figs. 2, 3A, and 5A). As can be seen in Fig. 5B, siRNA-mediated knock-down of MMP-15 enhanced cell death induced by TRAIL in all cell lines, but Mel Juso where the increase in cell death rate did not achieve statistical significance (at $p < 0.05$, t test). Incidentally, this cell line also has the lowest MMP-15 expression of all studied cell lines.

Expression of MMP-15 in Tumor Samples—Although we have established that MMP-15 can protect cells of various cancer types from cell
were resolved by SDS-PAGE followed by immunoblot analysis using α-myc antibodies. The membrane was re-probed using α-Erk1/2 antibodies as a loading control. B, cells were treated with CH-11 at 100 ng/ml, TRAIL at 20 ng/ml, or starved for 3 days. Cell death was measured by propidium iodide dye exclusion. Background values without CH-11 or TRAIL were subtracted. Results are from two independent experiments in triplicates, and error bars represent standard error of the mean. *, significant difference (\( p < 0.05 \), t test).

FIGURE 4. Effect of ectopic MMP-15 expression in HeLa S3 on cell death. A, expression control of myc-tagged MMP-15 in total protein lysates of the infected HeLa S3 cells. HeLa S3 cells infected with empty vector pLXSN or pLXSN-MMP-15 were lysed, and the lysates were resolved by SDS-PAGE followed by immunoblot analysis using α-myc antibodies. The membrane was re-probed using α-Erk1/2 antibodies as a loading control. B, cells were treated with CH-11 at 100 ng/ml, TRAIL at 20 ng/ml, or starved for 3 days. Cell death was measured by propidium iodide dye exclusion. Background values without CH-11 or TRAIL were subtracted. Results are from two independent experiments in triplicates, and error bars represent standard error of the mean. *, significant difference (\( p < 0.05 \), t test).

FIGURE 5. Effect of MMP-15 knock-down in other cancer cell lines. A, expression of MMP-15 by quantitative real-time PCR using RNA isolated at the day of the assay and normalized to β-actin expression. B, siRNA transfected cells were treated with TRAIL as indicated under “Experimental Procedures.” Cell death was measured by propidium iodide dye exclusion. Background values without CH-11 or TRAIL were subtracted. Results are from two independent experiments in duplicates, and error bars represent standard error of the mean. *, significant difference (\( p < 0.05 \), t test).

Whereas 18 genes were significantly up-regulated in the resistant clones, 90 genes were down-regulated suggesting some of the up-regulated genes act as transcriptional repressors or that transcriptional activators are down-regulated. Similar findings had been reported for prostate cancer, where progression of the disease is accompanied by major transcriptional repression (39) and in several leukemia where chromosomal translocations lead to repression of genes required for cellular differentiation (40).

All genes that were known to be implicated in apoptosis and found to be up-regulated in the resistant clones had anti-apoptotic activity, strongly suggesting that the other, in regard to apoptosis, as yet unknown genes on the list also act anti-apoptotically.

Among the first 18 down-regulated genes there are not only 7 previously characterized pro-apoptotic genes but also 3 known anti-apoptotic genes. The presence of anti-apoptotic genes may be explained in different ways. (i) If the large number of down-regulated genes is indeed caused by transcriptional repression, it can be expected that the repressed amplicons contain both pro- and anti-apoptotic genes. (ii) The selection process during cancer development toward an apoptosis-resistant phenotype very likely is accompanied by compensatory mechanisms that favor an apoptosis-sensitive phenotype, the net outcome being apoptosis resistance. (iii) The anti-apoptotic genes may be inactivated by mutation, thus even acting as dominant negative inhibitors. Down-regulation would relieve this inhibition.

The real power of DNA microarrays lies in the identification of gene networks that may form also functional networks. The gene
classification that we performed found a minimal set of 19 genes whose collective expression can distinguish between the apoptosis-sensitive phenotype of HeLa S3 and the apoptosis-resistant phenotype of the clones. This strongly supports a role for these genes in apoptotic network.

Remarkably, three of the four classifier genes that are up-regulated in the resistant clones, namely MMP-15, MMP-2, and TIMP-1, belong to the group of matrix metalloproteinases and their regulators. Matrix metalloproteinases play diverse roles in cellular processes ranging from degradation of extracellular matrix to cleavage of cell surface proteins and receptor ligands. They are well characterized for their contribution to the metastatic phenotype of cancer cells and can be inhibited by tissue inhibitors of matrix metalloproteinases, TIMPs (41). Paradoxically, TIMPs can also play a role in the activation of MMPs. The MMP-15-related protease MMP-14 activates pro-MMP-2 by cleavage in a complex with TIMP-2 (42, 43). Interestingly, MMP-15 has also been shown to mediate cleavage and activation of pro-MMP-2, but independently of TIMP-2. Instead, it was suggested to activate MMP-2 with the help of claudin, a component of endothelial tight junctions (44, 45). So far it has not been established whether TIMP-1 could participate in the activation of MMP-2. Based on our findings here, it is tempting to speculate that MMP-15 activates MMP-2 with the help of TIMP-1, which in turn leads to inhibition of apoptosis.

The most striking feature of many down-regulated classifier genes is their nuclear localization attributing an important role in apoptosis regulation to this organelle. Of the 15 down-regulated classifier genes, five, DRAX, WEE1, NLK, BRCA1, and MSK1, are known to be localized exclusively in the nucleus (46–50), and ERK3 and YVH1 are predominantly nuclear (51–53). Interestingly, some of those genes play a role in chromosome remodeling. MSK1 has been shown to activate transcription by phosphorylating histone 3, and this phosphorylation is necessary for the induction of immediate early genes (54). Hence, down-regulation of MSK1 could contribute to the observed transcriptional repression. Additionally, the well studied tumor suppressor BRCA1 is down-regulated. BRCA1 is also involved in overall transcriptional activation and can take part in chromosome remodeling (49, 55). Down-regulation of BRCA1 could therefore also repress the transcription of many genes at once.

Down-regulation of NLK may serve a dual effect, the inhibition of the tumor suppressor transforming growth factor-β (56) and the activation of the tumor activator Wnt (57). Additionally, transforming growth factor-β activated kinase (TAK), an activator kinase for NLK and negative regulator of Wnt-signaling (58, 59), is down-regulated in the resistant clones (supplemental data).

Another common theme among the down-regulated classifiers is the decreased expression of protein tyrosine phosphatases potentially leading to increased signaling by receptor tyrosine kinases. These enzymes are intensively studied because of their well established involvement in tumorigenesis (60). Both the low molecular weight protein tyrosine phosphatase and the T-cell protein tyrosine phosphatase can dephosphorylate and inactivate the platelet-derived growth factor and insulin receptors (61–64), and T-cell protein tyrosine phosphatase may additionally dephosphorylate the epidermal growth factor receptor (65, 66).

Finally, another fraction of the down-regulated classifiers is implicated in cell cycle regulation. The protein kinases WEE1 (67, 68) and ERK3 (51) may block the cell cycle, whereas the yeast homologue of YVH1 stimulates cell cycle progression (69). Whether these genes enhance cell death through their role in cell cycle regulation or independent from their cell cycle function has to be investigated further.

As a representative gene of the identified potential apoptosis regulators, we further investigated and confirmed the newly discovered anti-apoptotic function of MMP-15. The significance of MMP-15 in apoptosis control was not only suggested by the up-regulation of the MMP-15 gene in all resistant clones but also by its identification as a classifier gene that distinguishes between HeLa S3 and the resistant clones. Both knock-down of endogenous MMP-15 in several cancer cell lines and ectopic expression in HeLa S3 support an anti-apoptotic function for this MMP. We therefore provide multiple evidences for a general inhibitory role of MMP-15 in cancer cells. This is underscored by the up-regulation of MMP-15 in small studies of various human cancer tissues (29–31). Furthermore, 3 independently published DNA microarray
datasets generated with different microarray platforms and comprising a total of 254 tumor samples (36–38) when combined, convincingly demonstrate enhanced expression of MMP-15 in primary lung adenocarcinoma relative to normal lung tissue. Hence, therapeutic blockade of MMP-15 may both inhibit its invasive conferring properties as well as lower the anti-apoptotic threshold of cancer cells. This would allow the immune system to more effectively combat tumors using TRAIL- and Fas-mediated signaling.

Recently, matrix metalloproteinases were shown to cleave death receptors off the cell surface resulting in a lower concentration of death receptors in the plasma membrane and an inhibition of cell death (70, 71). However, in our experiments the cell surface expression of Fas is even higher in the resistant clones compared with Hela S3. Therefore, the anti-apoptotic mechanism by which MMP-15 exerts its effect must be different from a simple removal of cell surface death receptors.

Remarkably, the selection for an anti-apoptotic phenotype also affected gene expression changes that appear to be connected to other hallmarks of cancer cells. This includes the up-regulation of the angiogenic factor vascular epithelial growth factor, the metastasis-promoting metalloproteinases MMP-15, MMP-2 and MMP-3 as well as down-regulation of the BRCA-1 and transforming growth factor-β tumor suppressor pathways and low molecular weight and T-cell protein tyrosine phosphatases. This is reminiscent of an earlier report that showed enrichment for transformed cells in NIH-3T3 cells selected for tumor necrosis factor-α-resistance (72). Recently, Green and Evan (73) postulated that deregulation of proliferation and inhibition of apoptosis are necessary and sufficient to transform cells and later on evoke all hallmarks of cancer. As an extension to that notion, our findings suggest that other cancer characteristics may already co-evolve with an anti-apoptotic phenotype.

Our results characterize MMP-15 as a new element in the anti-apoptotic pathway network of cancer cells and suggest an important role of this protease in cancer progression. The discovery of novel anti-apoptotic genes presented here opens new avenues for the development of more effective strategies for targeted therapy of cancer.

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