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The Silencing of N-myc Downstream-Regulated Gene-1 in an Orthotopic Pancreatic Cancer Model Leads to More Aggressive Tumor Growth and Metastases

Corina Kim-Fuchs[a] Sebastian Winterhalder[a] Annja Winter[a] Thomas Malinka[a] Diana Born[b] Stephan Schäfer[b] Deborah Stroka[a] Beat Gloor[a] Daniel Candinas[a] Eliane Angst[a, c]

[a]Department of Visceral Surgery and Medicine, Inselspital and [b]Institute of Pathology, University of Bern, Bern, and [c]Clinic of Surgery, Cantonal Hospital, Schaffhausen, Switzerland

Key Words
Pancreatic cancer · Orthotopic xenograft animal model of recurrence · N-myc downstream-regulated gene-1

Abstract
Background: The understanding of molecular mechanisms leading to poor prognosis in pancreatic cancer may help develop treatment options. N-myc downstream-regulated gene-1 (NDRG1) has been correlated to better prognosis in pancreatic cancer. Therefore, we thought to analyze how the loss of NDRG1 affects progression in an orthotopic xenograft animal model of recurrence. ***Methods:*** Capan-1 cells were silenced for NDRG1 (C^{sil}) or transfected with scrambled shRNA (C^{scr}) and compared for anchorage-dependent and anchorage-independent growth, invasion and tube formation in vitro. In an orthotopic xenograft model of recurrence tumors were grown in the pancreatic tail. The effect of NDRG1 silencing was evaluated on tumor size and metastasis. ***Results:*** The silencing of NDRG1 in Capan-1 cells leads to more aggressive tumor growth and metastasis. We found faster cell growth, double count of invaded cells and 1.8-fold increase in tube formation in vitro. In vivo local tumors were 5.9-fold larger ($p = 0.006$) and the number of metastases was higher in animals with tumors silenced for NDRG1 primarily (3 vs. 1.1; $p = 0.005$) and at recurrence (3.3 vs. 0.9; $p = 0.015$). ***Conclusion:*** NDRG1 may be an interesting therapeutic target as its silencing in human pancreatic cancer cells leads to a phenotype with more aggressive tumor growth and metastasis.

Introduction

Pancreatic cancer is still the 4th leading cause of cancer death in men and women, with 43,920 new cases in the USA in 2012 [1]. The overall 5-year survival rate is less than 5% and effective therapies are largely lacking. Improved 5-year survival over 20–30% has been reported in surgically resected patients in specialized centers. However, many eligible patients are not offered this option and the majority of patients are diagnosed with advanced-stage pancreatic cancer which prohibits surgical treatment [2–3]. Current chemotherapeutic treatment in locally advanced and metastatic pancreatic cancer patients involves multimodal regimen (FOLFIRINOX) superior to the treatment with gemcitabine, but the overall prolongation of survival is disappointingly small [4]. The resistance of pancreatic cancers to chemotherapeutic agents is

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Eliane Angst
Department of Visceral Surgery and Medicine
Inselspital, University of Bern
CH–3010 Bern (Switzerland)
E-Mail eliane.angst@dkf.unibe.ch

thought to be caused by antiapoptotic protection [5]. New and improved therapeutic strategies to treat pancreatic cancer require a better, detailed understanding of the molecular mechanisms underlying the survival pathways in pancreatic cancers. In this context, N-myc downstream-regulated gene-1 (NDRG1) has been suggested to be a tumor suppressor gene, but its precise biological function is not yet known [6–7].

NDRG1 has been linked to many cellular processes including cell cycle, apoptosis and cellular differentiation [8–11]. Differentiation of several cancer cell lines in vitro induced the expression of NDRG1 [10–15]. Furthermore, silencing of NDRG1 prevented differentiation of leukemic cells, suggesting a crucial role in cellular differentiation [16]. Consistent with a role in cell differentiation, NDRG1 expression in pancreatic cancer has been associated with better outcome [6, 17]. NDRG1 is upregulated in early pancreatic lesions (PanIN), and then lost in advanced cancer [17]. Consistent with these findings, we showed in a model of orthotopic pancreatic cancer that NDRG1 overexpression was associated with slower tumor progression through tumor cell apoptosis [18]. Therefore, it has been postulated that the loss of NDRG1 is the cause of poor prognosis in pancreatic cancer. However, the loss of NDRG1 expression and its impact on growth and metastatic behavior have not been investigated up to now.

Recent studies have shown that iron chelators upregulate NDRG1 expression and inhibit the epithelial-mesenchymal transition in prostate and colon cancer cells [19]. Given the need for new therapeutic strategies against pancreatic cancer, it is critical to know how the loss of NDRG1 affects growth and progression. To address this, we used an orthotopic xenograft model of recurrence at a late tumor stage with liver metastases.

Materials and Methods

Stable Lines

The human pancreatic cancer cell line Capan-1 was from the American Type Culture Collection (Rockville, Md., USA) and cultured as previously described [17]. Stable lines were established as already specified [20]. Briefly, Capan-1 cells were transfected with either silencing (5-CGC TGA GGC CTT CAA GTA CTT-3) or scrambled (5-GGA ATC TCA TTC GAT GCA TAC-3) plasmid and selected with 250 μg/ml neomycin. Successful transfection and RNA silencing was confirmed by Western blotting (online suppl. fig.1a; see www.karger.com/doi/10.1159/000363065 for all online suppl. material) and qRT PCR for each clone [17]. For subsequent experiments the silenced clones 3, 6 and 7 (sil3, sil6 and sil7) and the scrambled clones 1, 3 and 4 (scr1, scr3 and scr4) were used.

Cell Growth Assays

For anchorage-dependent cell growth three individual clones of either C^{sil} or C^{scr} were analyzed using cell count and Thiazolyl Blue Tetrazolium Bromide (MTT) assay [21]. Briefly, subconfluent cells were plated in 6-well plates for cell counts and 96-well plates for MTT. After 24, 48, 72 and 96 h the cells were counted in a hemocytometer. Alternatively, MTT was added for 4 h and the formazan crystals were dissolved in 0.01 M HCl/10% SDS. Absorbance was determined at 562–750 nm. For anchorage-independent cell growth colony formation assays were performed as follows: cells were suspended in a 0.4% top agar and overlaid on a 0.8% base agar. After 4 weeks the colonies were stained with MTT, photomicrographed and counted.

Cell Cycle Analysis

The cells were harvested, washed in PBS and then stained with hypotonic propidium iodide buffer for 30 min at 4°C. The cells were analyzed by flow cytometry (FACScan BD LSR; BD Biosciences, San Jose, Calif., USA) and analyzed by ModFit LT Mac 3.1 SP3.

Matrigel™ Invasion Assay

BD BioCoat Matrigel invasion chamber 24-well plates (8.0 micron; BD Biosciences) were used according to the manufacturer's instructions. After rehydration of the insert, the cells were put in serum-free media at a density of 2×10^5 per insert. The lower chamber contained media with 20% FBS as chemoattractant. After 96 h the invaded cells were fixed with methanol and stained with hematoxylin and eosin. They were photographed at a 100× magnification and pictures were analyzed with Photoshop CS2 (Adobe Systems GmbH, Germany), measuring the percentage area of invaded cells.

Tube Formation Assay

For conditioned media C^{sil} or C^{scr} cells were plated in full media, which was replaced after 24 h by RPMI with 2% FBS. After a further 48 h the supernatant was cleared of particles at 1,500 rpm for 2 min, sterilized through a 0.2-μm filter (Nalgene, Switzerland) and kept at –80°C until use. The plates were coated with growth factor-reduced Matrigel (BD Biosciences). Human umbilical vein endothelial cells (HUVECs) were seeded in the respective conditioned media. Tube formation was measured after 4 h at 40× magnification in 5 fields of the wells. The tube length was measured with the free ImageJ software for every condition.

Orthotopic Xenograft Mouse Model

Animal studies were approved by the Animal Care Committee of the Canton of Bern, Switzerland. Animals were housed at 22°C in the animal facility of the University of Bern with a 12-hour light/dark cycle. The orthotopic xenograft model was modified to establish a model of recurrence [22]. 2×10^6 cells were injected subcutaneously into the flank of nude mice (Nu/Nu; Harlan Laboratories, Venray, The Netherlands). After 3 weeks the donor tumor was harvested and minced to fragments of approximately 1 mm^3. One tumor fragment was placed into the tail of the pancreas of recipient nude mice (n = 10). After 6 weeks the primary tumors and metastases were resected. The pancreas was oversown with an absorbable monofilament 6-0 suture. After 2 further weeks the animals were sacrificed and evaluated for recurrence size and the presence and number of metastases. The tumors and metastases were conserved in formalin.

Immunohistochemistry

NDRG1 was stained and scored as previously described using the Kinasource antibody 1:200 (Kinasource AB-160 Ltd., Scotland, UK) [17].

Statistical Analysis

Experiments were performed in three biological replicates. Data are presented as mean ± standard deviation. Comparisons of two groups were made by t test. Comparisons of several groups were made by a one-way analysis of variance (ANOVA). Categorical data were compared by the z test. An α value of 0.05 was used to determine significant differences. All statistics were done in SigmaStat 3.1 (Systat Software, Inc., Richmond, Calif., USA).

Results

The Loss of NDRG1 Leads to Enhanced Cell Growth and a Prometastatic Phenotype in vitro

To assess the role of NDRG1 silencing in anchorage-dependent and anchorage-independent contexts, we examined the growth of cells on solid surface and in soft agar. Silenced clones (C^{sil}) grew faster than clones expressing endogenous NDRG1 (C^{scr}) on solid surfaces over 96 h ($p < 0.001$). C^{sil} cells showed an almost 3.5-fold increase in cell number at 96 h versus only a 2.5-fold increase in C^{scr} cells (fig. 1). The MTT assay confirmed an increased metabolism in C^{sil} cells compared to C^{scr} cells. In accordance with these results the anchorage-independent assay showed that C^{scr} cells formed only very few detectable colonies in soft agar. Quantification of the colonies demonstrated an 18-fold higher count in C^{sil} than in C^{scr} clones (fig. 2). To determine whether the cell cycle was affected we performed a cell cycle analysis. There was no difference in the percentage of cells in the S phase, but there was a trend towards less apoptosis (online suppl. fig. 1b). Local tumor growth causes pain and bowel obstruction, but metastatic disease is fatal for the patient. Therefore, we investigated whether the loss of NDRG1 determines a prometastatic phenotype. As invasion is an important step in the spread of tumor cells, we showed that the silencing of NDRG1 not only influenced local tumor growth but also increased the propensity of cells to invade and therefore potentially metastasize (fig. 3). We used two clones each of silenced cells (C^{sil6} and C^{sil7}) and two clones of cells expressing endogenous NDRG1 (C^{scr3} and C^{scr4}). Cells of silenced clones invaded twice as much surface as cells of clones with endogenous NDRG1 expression ($p < 0.001$; $n = 3$). We used the model of tube formation from HUVECs to determine whether the loss of NDRG1 affects angiogenesis. We compared all the selected colonies with silenced NDRG1 (C^{sil}) to the colonies

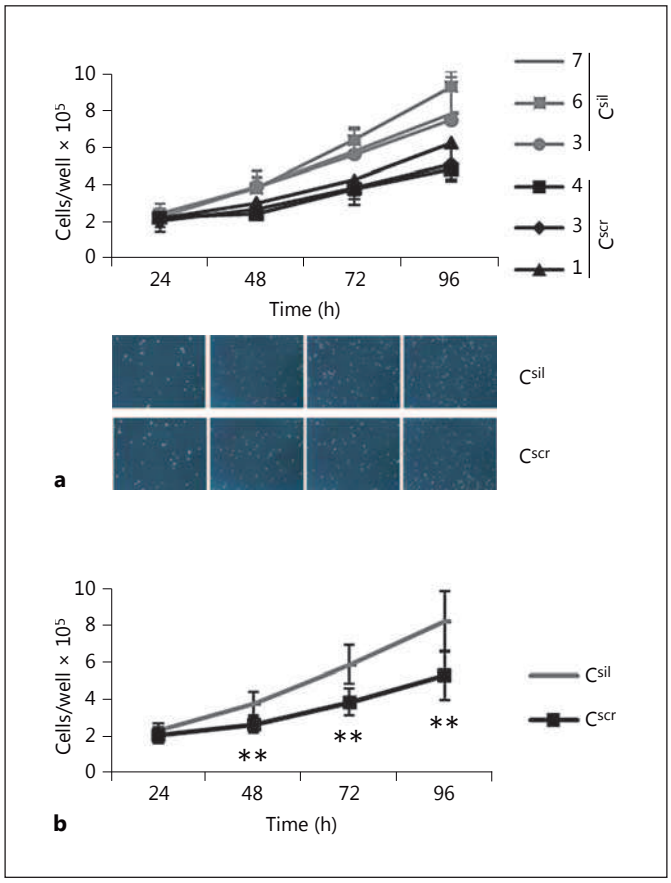


Fig. 1. Capan-1 cells silenced for NDRG1 (C^{sil}) or with endogenous NDRG1 expression (C^{scr}) were grown for 96 h on a solid surface. Three clones of each stable transfection were compared. **a** Growth of single clones and representative photographs of one series each. **b** Results of all clones ($n = 3$). ** $p < 0.01$.

with endogenous NDRG1 expression (C^{scr}) and found that the loss of NDRG1 increased the formation of tubes 1.8-fold, suggesting an increase of angiogenesis ($p = 0.016$; fig. 4).

NDRG1 Silencing Leads to More Aggressive Tumors in vivo

To confirm the in vitro phenotype of NDRG1 silencing in the microenvironment of the pancreas, we developed an orthotopic animal model. We analyzed tumor size, metastasis and recurrences, depending on the status of NDRG1 silencing. To mimic the clinical setting we established an orthotopic animal model of recurrence. Although in clinical practice we rarely resect metastases, the model was also applied to metastatic mice. After 6 weeks of orthotopic tumor growth in the pancreatic tail, we re-

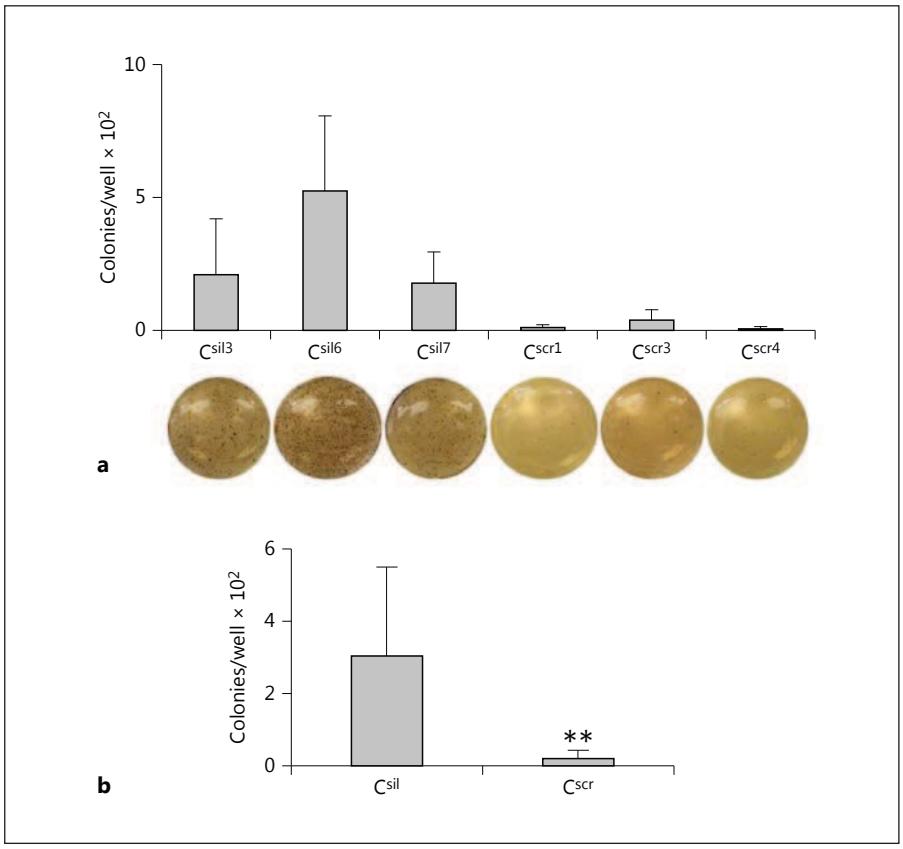


Fig. 2. C^{sil} or C^{scr} clones were grown for 4 weeks in soft agar. The colonies were stained with MTT, photographed and counted. Three clones of each stable transfection were compared. **a** Results of each single clone with photographs. **b** Results of all the clones (n = 3). ** $p < 0.01$.

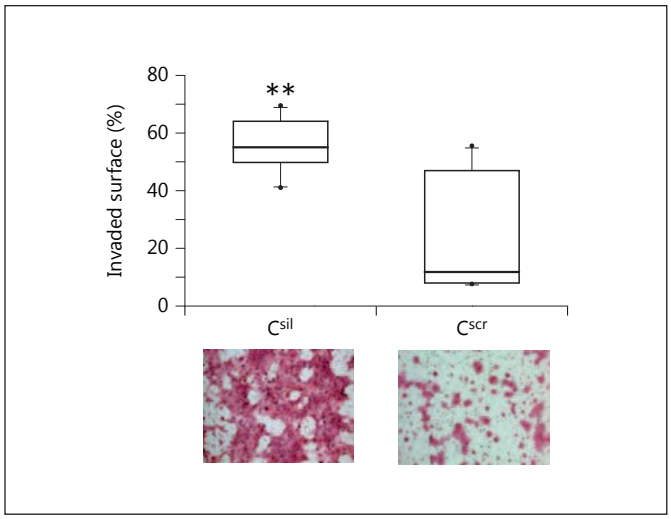


Fig. 3. Invasion assay of Capan-1 cells. C^{sil} or C^{scr} clones were kept in Matrigel-coated invasion chambers for 96 h. The invaded cells were stained and the invaded surface was measured. Two clones of each stable transfection were compared. Representative membranes are shown. The silencing of NDRG1 doubled the invaded surface in Capan-1 cells (n = 3). ** $p < 0.01$.

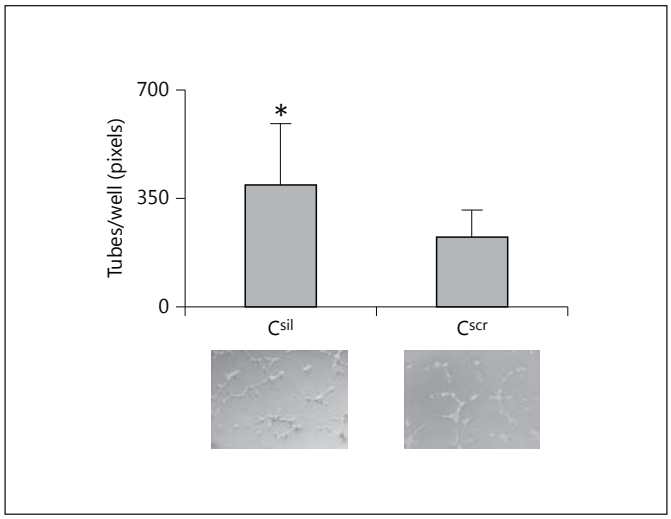


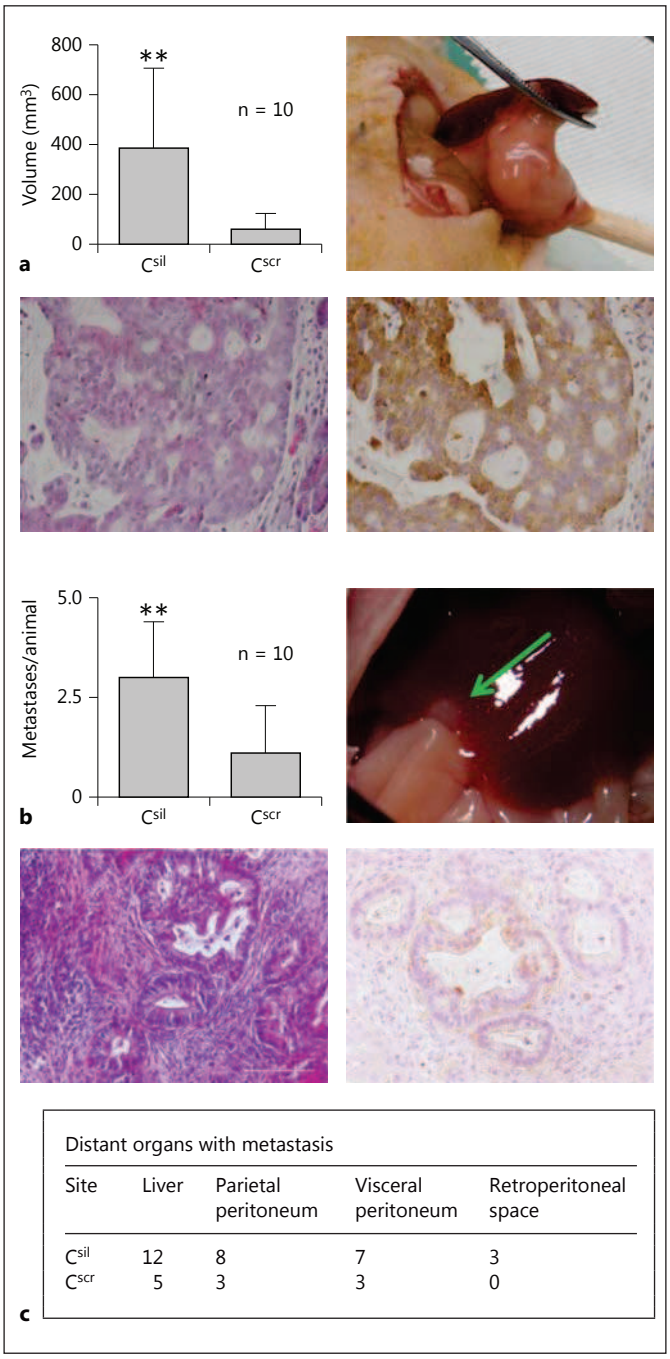
Fig. 4. Tube formation. Supernatant of C^{sil} or C^{scr} clones was used for a tube formation assay. HUVECs were seeded on growth factor-reduced Matrigel in the conditioned media. Tube formation was measured after 4 h. Representative examples are shown. The silencing of NDRG1 induced a 1.8-fold increase in tube formation (n = 3). * $p < 0.05$.

moved local tumors and metastases surgically, so that the mice were clear of tumor. All the mice developed tumors in the pancreatic tail, as expected by this transplantation model. The tumors originating from cells with silenced NDRG1 (C^{sil}) were 5.9-fold larger than tumors originating from cells with endogenous NDRG1 expression (C^{scr}; p = 0.006; fig. 5a). In these animals (C^{sil}) we found a mean of 3 (±1.4) compared to 1.1 (±1.2) metastases per animal in C^{scr} (p = 0.005; fig. 5b). Furthermore, 9/10 animals bore metastases in C^{sil} animals compared to 6/10 for C^{scr} animals, showing a trend towards more animals with metastases (p = 0.302; fig. 5c; online suppl. fig. 2a; table 1). We lost animals for extended surgery after the first harvest: 4 animals in the C^{sil} group and 3 animals in the C^{scr} group. Although the tumor load was very heavy in some animals they did not deteriorate in there feeding and general condition. There was no difference in animal weight between the two groups at any time (p = 0.404; online suppl. fig. 2b).

To ascertain stable silencing of NDRG1 in vivo we stained the harvested samples for NDRG1. We compared cancer originating from cells transfected with NDRG1-silencing shRNA (C^{sil}) to cancer originating of cells transfected with scrambled shRNA (C^{scr}). The tumors arising from cells transfected with silencing shRNA (C^{sil}) showed no staining or only weak staining in a small section of the tumor. In contrast, the majority of the tumors arising from cells transfected with scrambled shRNA (C^{scr}) showed staining, though it was only weak. This confirmed that the silencing of NDRG1 was stable in vivo. The mean intensity score from the samples of the first harvest was 0.01 for C^{sil} and 1.03 for C^{scr} (p < 0.001; online suppl. table 1).

More Aggressive Recurrence in NDRG1-Silenced Tumors

The animals were kept for 2 more weeks before sacrifice. To correlate the pattern of recurrence to the level of NDRG1 expression we compared the expression of NDRG1 in all the harvested samples. In our model NDRG1 expression was stable in both groups over time and also in metastases (online suppl. table 1). We found a trend towards larger local recurrences in C^{sil} animals compared to C^{scr} animals (p = 0.066; online suppl. fig. 2c). Again, the number of metastases was higher, with 3.3 (±1.5) in C^{sil} animals compared to 0.9 (±1.6) in C^{scr} animals (p = 0.015). Furthermore, all the C^{sil} mice (6/6) developed local recurrence and metastases, whereas only 5 of 7 C^{scr} mice developed local recurrence (p = 0.514) and 2 of 7 developed metastases (p = 0.039; fig. 6a, b; table 1).



c

Distant organs with metastasis				
Site	Liver	Parietal peritoneum	Visceral peritoneum	Retroperitoneal space
C^{sil}	12	8	7	3
C^{scr}	5	3	3	0

Fig. 5. After transplantation of subcutaneous tumors into the pancreatic tail of nude mice, tumors were resected after 6 weeks. **a** Orthotopic tumors. In animals with silenced NDRG1 (C^{sil}) the tumors were larger than in animals with endogenous NDRG1 expression (C^{scr}). ** p < 0.01. Photograph of the exteriorized spleen with the adjacent pancreatic tumor and a 40× magnification of HE and NDRG1 IHC of the tumor. **b** Number of metastases. In these animals we also found more metastases per animal. ** p < 0.01. Photograph and 40× magnification of a liver metastasis (arrow). **c** Number of metastases in each site.

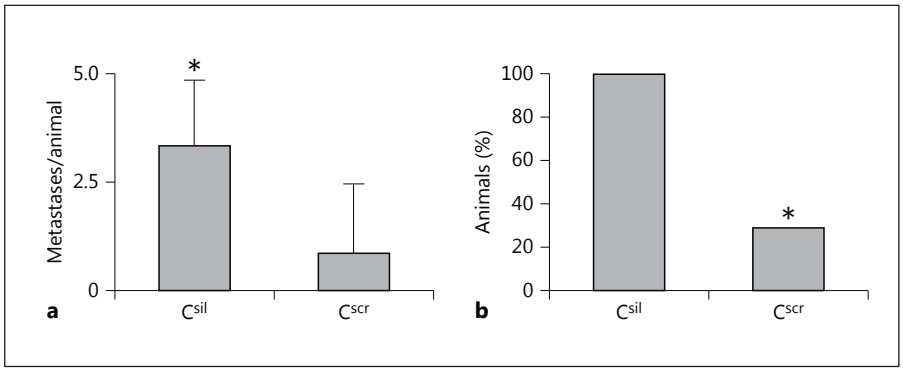


Fig. 6. After resection of the tumors and metastases, the mice were kept for another 2 weeks before sacrifice. **a** The number of metastases was higher in C^{sil} animals than in C^{scr} animals. * $p < 0.05$. **b** 100% of C^{sil} animals bore metastasis compared 28% of C^{scr} animals.

Table 1. Number of metastases after 6 weeks and recurrences and metastases after 8 weeks for C^{sil} and C^{scr}

	C^{sil} mice	C^{scr} mice	p value (z test)
Orthotopic tumor growth	10/10	10/10	
Metastasis after 6 weeks	9/10	6/10	p = 0.302
Orthotopic tumorrecurrence	6/6	5/7	p = 0.514
Metastasis after 8 weeks	6/6	2/7	p = 0.039

Discussion

In this study we show for the first time that the silencing of NDRG1 in human pancreatic cancer cells leads to enhanced tumor growth and metastasis in vitro and in vivo. These data confirm that the loss of NDRG1 is an important negative prognostic factor in pancreatic cancer.

Previous studies showed that in human pancreatic cancer the protein expression of NDRG1 as measured by immunohistochemistry is correlated with better prognosis and better differentiation of human pancreatic cancer [6, 17]. Therefore, it was suggested that the loss of NDRG1 is causal for poor prognosis, but no study analyzed the effect of NDRG1 silencing on growth and metastasis. We showed previously in vitro that the human pancreatic cancer cell line BxPC-3 silenced for NDRG1 grew faster than BxPC-3 cells with endogenous NDRG1 expression [18]. Although originating from human pancreatic cancer the BxPC-3 cell line is not mutated for Kras, which is uncommon for pancreatic cancer. Most pancreatic cancers bear mutated Kras, leading to increased activity of the gene [23]. Therefore, we switched to a Kras-mutated cell line for the further studies presented here. The Capan-1 cell line was also chosen for its high endogenous expression of NDRG1. Lastly, we used Capan-1 cells previously in our orthotopic pancreatic cancer model and it showed moderate tumor growth and metastases. The orthotopic animal model is well suited to analyze the effect of genetic changes on tumor growth in the pancreatic microenvironment. It mimics the human disease and we showed previously that its results are highly reproducible [24]. We thought to use a model of recurrence, as in the clinical setting recurrences are common even after radical surgery – even after R0 resections the median survival in several studies ranged between 12 and 55 months and patients died of tumor recurrences [25]. Several animal models have been described previously in SCID and athymic mice. We based our model on a study by Eibl and Reber [26] in which the pancreas was ligated instead of oversown. Although the mice lost 1 g of their body weight 3 days after surgery, both groups recovered from the weight loss after 1 week (online suppl. fig. 2).

Although the level of NDRG1 expression was similar in the three clones of C^{sil} and C^{scr} (online suppl. fig. 1a), the growth kinetics differed slightly in the anchorage-dependent and anchorage-independent assays. However the overall reduction in growth was shown in vitro and confirmed in the in vivo model.

For clinical outcome, invasion and metastasis are even more important as they contraindicate surgical therapy. We found that cells silenced for NDRG1 exhibited a more metastatic phenotype in vitro and in vivo, as shown by increased invasion and tube formation in vitro, as well as higher numbers of metastasis in vivo. This is in accordance with previous studies where NDRG1 over-expression in MiaPaCa-2 cells inhibits the expression of chemokines through the suppression of NFκB binding activity. These cells grew smaller subcutaneous tumors with less angiogenesis in a mouse model [27]. The process of inva-

sion and metastasis is highly complex. It involves many phenotypic changes like breaching of the basal membrane, angiogenesis, epithelial-mesenchymal transition, inflammation and tumor-associated macrophages accompanied by many molecular changes. These include changes in chemokine expression, matrix metalloproteinases, vimentin, cadherin and microRNA amongst others [28]. Further studies are needed to analyze where NDRG1 is involved in this complex process.

Several mechanisms may lead to changes in NDRG1 expression in pancreatic cancer cells. We have previously shown that although the promoter region of NDRG1 contains multiple CpG sites forming a large CpG island, the promoter was not found to be methylated [20]. Several transcription factors including HIF-1α and p53 upregulate NDRG1. HIF-1α is usually stabilized in pancreatic cancer, as these are hypoxic and the tumor suppressor gene p53 is commonly mutated in pancreatic cancer [9]. Chromosomal aberrations may lead to differential expression of NDRG1, as amplifications and deletions of chromosome 8, where NDRG1 (8q24.3) is located, are common in pancreatic cancer [29].

Pancreatic cancer has very heterogeneous tumors and several genetic changes may change the cells to a more aggressive phenotype, but our data suggest for the first time that the loss of NDRG1 is causal for more aggressive tumors. Therefore, therapies that enhance NDRG1 expression may be successful. A recent study has shown that the pharmacological treatment of prostate and colon cancer cells with iron chelators upregulates NDRG1 expression and inhibits the epithelial-mesenchymal transition [19]. This treatment may be successful in pancreatic tumors with low NDRG1 expression.

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