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Expression of the Shwachman-Bodian-Diamond syndrome (SBDS) protein in human pancreatic cancer and chronic pancreatitis

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Summary. Background: The Shwachman-Bodian-Diamond syndrome (SBDS) protein is a member of a highly conserved family which influences RNA activation and is associated with pancreatic, skeletal and bone marrow deficiencies, as well as hematological malignancies. Methods: In this study, the expression and localization of SBDS were investigated in normal human pancreatic tissues, chronic pancreatitis (CP) tissues, primary and metastatic pancreatic ductal adenocarcinoma (PDAC) tissues, as well as in cultured pancreatic cancer cell lines by immunohistochemistry, immunoblotting and immunocytochemistry. Results: In the normal pancreas, SBDS was localized in the cytoplasm of islet cells and ductal cells. In CP tissues, SBDS was found in the cytoplasm of ductal cells, tubular complexes, stromal fibroblasts and in PanIN1-2 lesions. In PDAC tissues, SBDS exhibited cytoplasmic and occasionally nuclear localization in tubular complexes, PanIN1-3 lesions, cancer cells, and stromal fibroblasts. Different levels of SBDS protein were detected in cultured pancreatic cancer cell lines. Conclusion: SBDS is expressed in normal, CP, and PDAC tissues, as well as in pancreatic cancer cell lines. The different expression and localization patterns suggest a role of SBDS in the pathogenesis of, or response to, inflammatory and neoplastic pancreatic diseases.

Key words: SBDS, Pancreatic adenocarcinoma, Chronic pancreatitis

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Introduction

The Shwachman-Bodian-Diamond syndrome (SBDS) gene is a member of a highly conserved family (Boocock et al., 2003). The predicted protein size is 28.8 kDa and the amino acid sequence has no homology to any known functional domain. Nevertheless, data suggest that the SBDS protein may play a role in chemotaxis (Wessels et al., 2006) and cell proliferation during early mammalian development (Zhang et al., 2006). Most recently, the function of the yeast ortholog (Sdo1) of SBDS has been reported to be critical for the assembly of ribosomes (Menne et al., 2007). In human cells, SBDS associates with the 60S ribosomal subunit and binds to 28S rRNA (Ganapathi et al., 2007). SBDS gene mutations can lead to a deficiency of a full-length SBDS protein (Woloszynek et al., 2004; Austin et al., 2005), and consequently to impaired protein stability and function (Erdos et al., 2006). The resulting syndrome is characterized by pancreatic exocrine insufficiency, hematological dysfunction and skeletal abnormalities (Bodian et al., 1964; Shwachman et al., 1964; Ginzberg et al., 1999, 2000). The pancreatic dysfunction is a result of a small, fatty pancreas with impaired enzyme output and low serum amylase and trypsinogen levels (Mack et al., 1996; Ip et al., 2002). In contrast to normal individuals, in young SBDS patients serum trypsinogen values are low and tend to increase with age, whereas serum isoamylase values remain low at all ages (Ip et al., 2002). Moreover, compound heterozygous mutations of the SBDS gene are associated with type 1 diabetes mellitus (Rosendahl et al., 2006) and with some malignancies, such as acute myeloid leukemia, myelodysplasia and acquired aplastic anemia (Mellink et al., 2004; Majeed et al., 2005; Shammas et al., 2005;

Maserati et al., 2006; Calado et al., 2007).

The *SBDS* gene is ubiquitously expressed in different tissues, including the pancreas (Boocock et al., 2003). SBDS intracellular localization is dynamic. SBDS is localized in both the nucleus—particularly within the nucleolus—and the cytoplasm of normal fibroblasts and hematopoietic cells (Austin et al., 2005), and its localization is cell cycle dependent. SBDS nucleolar localization (the primary cellular site of ribosome biosynthesis) is observed during the processing of ribosomal RNA (rRNA) in the G1 and G2 phases (Ganapathi et al., 2007), and diffuse nuclear localization is seen during the S phase of the cell cycle (Austin et al., 2005; Savchenko et al., 2005). Inhibition of rRNA transcription by actinomycin D results in the exit of SBDS from the nucleolus (Ganapathi et al., 2007).

The role of SBDS in pancreatic disease is poorly understood. So far, the exact localization of SBDS in the normal adult pancreas, as well as in chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC), has not been analyzed.

Materials and methods

Tissue sampling

Pancreatic tissue specimens were obtained from 10 CP and 20 PDAC patients with a mean age of 62.5 years (range: 41–78 years) in whom pancreatic resections were performed. Normal human pancreatic tissue samples were obtained through an organ donor program from 10 previously healthy individuals (mean age: 45 years; range: 18–76 years) whenever there was no suitable recipient for organ transplantation. Freshly removed tissues were either fixed in paraformaldehyde solution for 12–24 h and then paraffin-embedded for histological analysis, or immediately snap-frozen in liquid nitrogen. Histopathology was assessed in all samples using H&E staining. The Human Subjects Committee of the University of Heidelberg, Germany, approved all studies.

Cell culture

ASPC-1, BxPc-3, Capan-1, Colo-357, SU8686, and T3M4 pancreatic cancer cells were routinely grown in RPMI 1640 medium (Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS (Sigma-Aldrich, St. Louis, MO) and 100 U/ml penicillin/streptomycin (Invitrogen GmbH, Karlsruhe, Germany). Mia-PaCa-2 and Panc-1 were routinely grown in DMEM medium (Invitrogen GmbH) supplemented with 10% FCS and 100 U/ml penicillin/streptomycin.

Immunohistochemistry and immunocytochemistry

Immunohistochemistry was performed as previously described (Michalski et al., 2007). Consecutive tissue sections (5 µm; paraffin-embedded) were deparaffinized and rehydrated in progressively decreasing concentrations of ethanol. After antigens were retrieved by boiling tissue sections in 10 mM citrate buffer for 10 min, endogenous peroxidase activity was quenched by incubation in deionized water containing 3% hydrogen peroxide at room temperature for 10 min. The slides were then washed in washing buffer (10 mM Tris-HCl, 0.85% NaCl, 0.1% bovine serum albumin, pH 7.4) and incubated with mouse or rabbit anti-SBDS antibodies (Austin et al., 2005) diluted 1:500 in a universal blocking reagent (DAKO Corporation, Carpentaria, CA) for 18 h at 4°C. In consecutive sections, the specificity of the primary antibody was confirmed using the corresponding normal mouse or rabbit IgGs (DAKO). Specificity of the mouse anti-SBDS antibody was further confirmed using a rabbit anti-SBDS antibody. The slides were next rinsed with washing buffer and incubated with anti-mouse or anti-rabbit HRPO-labeled IgG (Amersham International, Buckinghamshire, UK) diluted in a universal blocking reagent (DAKO) for 1 h at room temperature. The tissue sections were then washed in washing buffer and each section was subjected to 100 µL of DAB-chromogen/substrate reagent (DAKO) and counterstained with Mayer's hematoxylin.

Immunocytochemistry was performed as described previously (Keleg et al., 2007). Briefly, pancreatic cancer cells were cultured on Super Frost microscope slides (Menzel GmbH & Co KG, Braunschweig, Germany) overnight until they were adherent, and then washed with phosphate buffered saline (PBS), fixed with 3.5% para-formaldehyde for 25 min, and quenched with 30 mM glycine/PBS for 5 min, followed by permeabilization of the cell membrane with 0.1% Triton x-100 for 5 min at room temperature. Next, slides were incubated with the mouse SBDS antibody and immunostaining was performed as described above. Slides were analyzed using the Axioplan 2 imaging microscope (Carl Zeiss light microscope, Göttingen, Germany).

Immunoblot analysis

Pancreatic cancer cells were seeded in 10 cm cell culture plates in 10% FCS growth medium until 50–70% confluence was reached. Next, cells were washed in phosphate-buffered saline (PBS), pH 7.4, lysed and homogenized, as previously described (Erkan et al., 2005; Michalski et al., 2007). The homogenized material was collected and centrifuged at 4°C for 30 min at 16,000 g to remove insoluble material. The protein concentration of the supernatant was measured with a spectrophotometer using the BCA protein assay method (Pierce, Rockford, IL). A total of 20 µg of protein/lane was separated by SDS-polyacrylamide gel electrophoresis. After transfer to nitrocellulose membranes, blots were incubated with the mouse anti-SBDS antibody. After washing, blots were incubated with anti-mouse HRPO-labeled IgG (Amersham International, Buckinghamshire, UK). Equal loading was confirmed by reprobing membranes with an anti-ERK2

antibody. Visualization was performed using the enhanced chemiluminescence method (Amersham International).

Results

Localization of SBDS in human pancreas

The localization of SBDS in normal pancreatic (n=10), CP (n=10), and PDAC (n=20) tissues as well as in liver (n=5), lymph node (n=1) and peritoneal (n=3) metastases was determined by immunohistochemistry. In normal pancreatic tissues, SBDS was localized mainly in the cytoplasm of the islet cells in 9/10 samples (Figure 1A), and in normal ductal cells in 6/10 samples (Figure 1B). Furthermore, nuclear staining was also observed in some cases (arrow, Fig. 1B). No acinar staining was detected but there was some stromal staining in normal pancreatic tissues. In CP tissues, SBDS was localized in the cytoplasm and nuclei of ductal cells in 8/10 samples, in tubular complexes in 5/10 samples (Fig. 1C), in PanIN1-2 lesions (Fig. 1D), in enlarged nerve fibers in 6/10 samples (Fig 1E), in walls of blood vessels in 5/10 samples (Fig. 1F), and in stromal fibroblasts in 1/10 samples (Fig. 1G).

In PDAC tissues, SBDS was strongly localized in the cytoplasm and – less prominently – in the nuclei of tubular complexes (Fig. 2A) and PanIN1-3 lesions (Fig. 2B). Interestingly, cancer cells in 14/20 PDAC tissues exhibited both cytoplasmic and nuclear SBDS staining (Fig. 2C-F), while 3/20 PDAC tissues displayed only cytoplasmic SBDS staining and 3/20 PDAC tissues only nuclear SBDS staining. The specificity of SBDS staining was confirmed using two different SBDS antibodies. Moreover, further confirmation was achieved using the corresponding negative control on consecutive sections (Fig. 2F, inset). Additionally, 3/5 samples of PDAC liver metastasis demonstrated cytoplasmic SBDS staining (Fig. 2G), and 2/5 samples exhibited combined cytoplasmic and nuclear SBDS staining in the metastatic cells. Similarly, one PDAC peritoneal metastasis sample demonstrated cytoplasmic SBDS staining (Fig. 2I) whereas 2/3 exhibited combined cytoplasmic/nuclear SBDS expression. The metastatic lymph node sample demonstrated combined cytoplasmic/nuclear SBDS staining (Fig. 2H).

SBDS was also detected in 8 cultured pancreatic cancer cell lines at the protein level by immunoblotting. This analysis revealed expression of SBDS in all pancreatic cancer cell lines as a single band of approximately 30 kDa (Fig. 3A). In order to localize SBDS in the 8 tested pancreatic cancer cell lines, immunocytochemistry was performed. This analysis demonstrated SBDS localization in the cytoplasm of Panc-1, SU8686 and T3M4 pancreatic cancer cell lines. Both cytoplasmic and nuclear staining were detected in ASPC-1, BxPc-3, Colo-357 and MiaPaCa-2 pancreatic cancer cell lines (Fig. 4B). The intensity of SBDS staining was in line with the expression levels of SBDS in pancreatic cancer cell lines as judged by immunoblotting. Specifically, BxPc-3 and Capan-1 cells expressed high SBDS levels and displayed strong staining, whereas SU8686 cells expressed the lowest SBDS levels and the weakest staining. Interestingly, cells that were grown to full confluence demonstrated low to absent SBDS expression, whereas low-density pancreatic cancer cells demonstrated high expression of SBDS (Fig. 3C).

Discussion

Both chronic pancreatitis and pancreatic ductal adenocarcinoma tissues are characterized by extensive fibrosis, ductal proliferation, extensive acinar cell degeneration and acinar conversion into tubular complexes (Bockman et al., 1982, 2003; Kloppel and Luttges, 2004) that can transform into PanIN lesions (Esposito et al., 2007). These changes stop at this stage in CP but further progress towards malignant transformation into cancer cells in PDAC (Bockman et al., 1997, 2003). Thus, the normal pancreatic morpho-histology that consists mainly of acinar structures is lost in CP and PDAC.

The Shwachman-Bodian-Diamond syndrome (SBDS) is an autosomal recessive disease (Ginzberg et al., 2000) characterized primarily by exocrine pancreatic, skeletal and bone marrow insufficiencies (Robberecht et al., 1985), as well as hematological malignancies/dysfunctions (Smith et al., 1996; Dokal et al., 1997). SBDS has been linked to mutations in the SBDS gene, which encodes a protein that has been shown to be involved in ribosomal activation. In the present study, the SBDS protein was detected by immunohistochemistry in the ductal cells and islets but not in the acini of normal pancreatic tissues. Furthermore, SBDS expression levels were increased in the tubular complexes, in deformed neurovascular elements of CP and PDAC, as well as in the cancer cells in PDAC. This indicates that SBDS expression is stimulated after acinar conversion into tubular complexes and PanIN lesions in CP and PDAC, suggesting that SBDS plays a role during the pathogenesis of both diseases. It is also possible that SBDS expression is increased in response to these pathological processes. In addition, the continued expression of SBDS in PDAC cells that metastasize to the liver, lymph node and peritoneum would be consistent with potential involvement in the progression of PDAC.

Furthermore, SBDS exhibited a cytoplasmic and occasionally nuclear localization in some PDAC tissues, as well as in cultured pancreatic cancer cell lines. This is in line with recent reports demonstrating that SBDS is localized both in the cytoplasm and in the nucleus—and especially within the nucleolus—of normal fibroblasts, lymphoblasts, and myeloid cell lines (Austin et al., 2005). Together, the nuclear localization of SBDS in a subset of PDAC tissues and pancreatic cancer cell lines

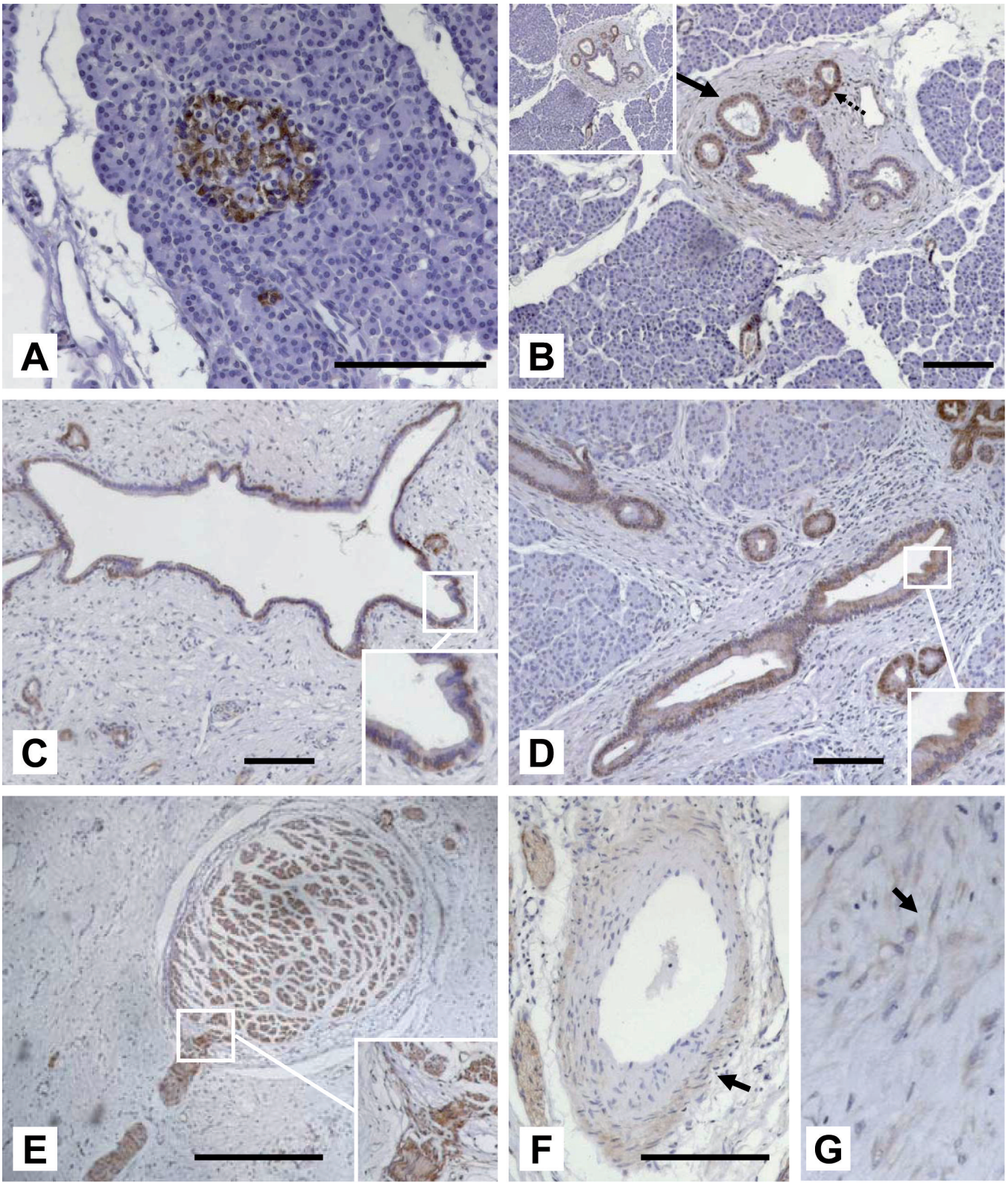

Fig. 1. Localization of SBDS in normal pancreatic tissues and CP tissues. Immunohistochemistry was performed using a specific mouse anti-SBDS antibody, as described in the Methods section. SBDS was localized in the cytoplasm of the normal islet cells (**A**) and in the cytoplasm and the in some cases also in the nucleus of normal ductal cells (**B**, arrow). In CP tissues, SBDS was localized in the cytoplasm of ductal cells (**C**), PanIN1-2 lesions (**D**), nerves (**E**) and blood vessels (**F**), as well as in fibroblasts (**G**, arrow). Insets show high magnification of structures indicated by arrows. Scale bar: 100 μm.

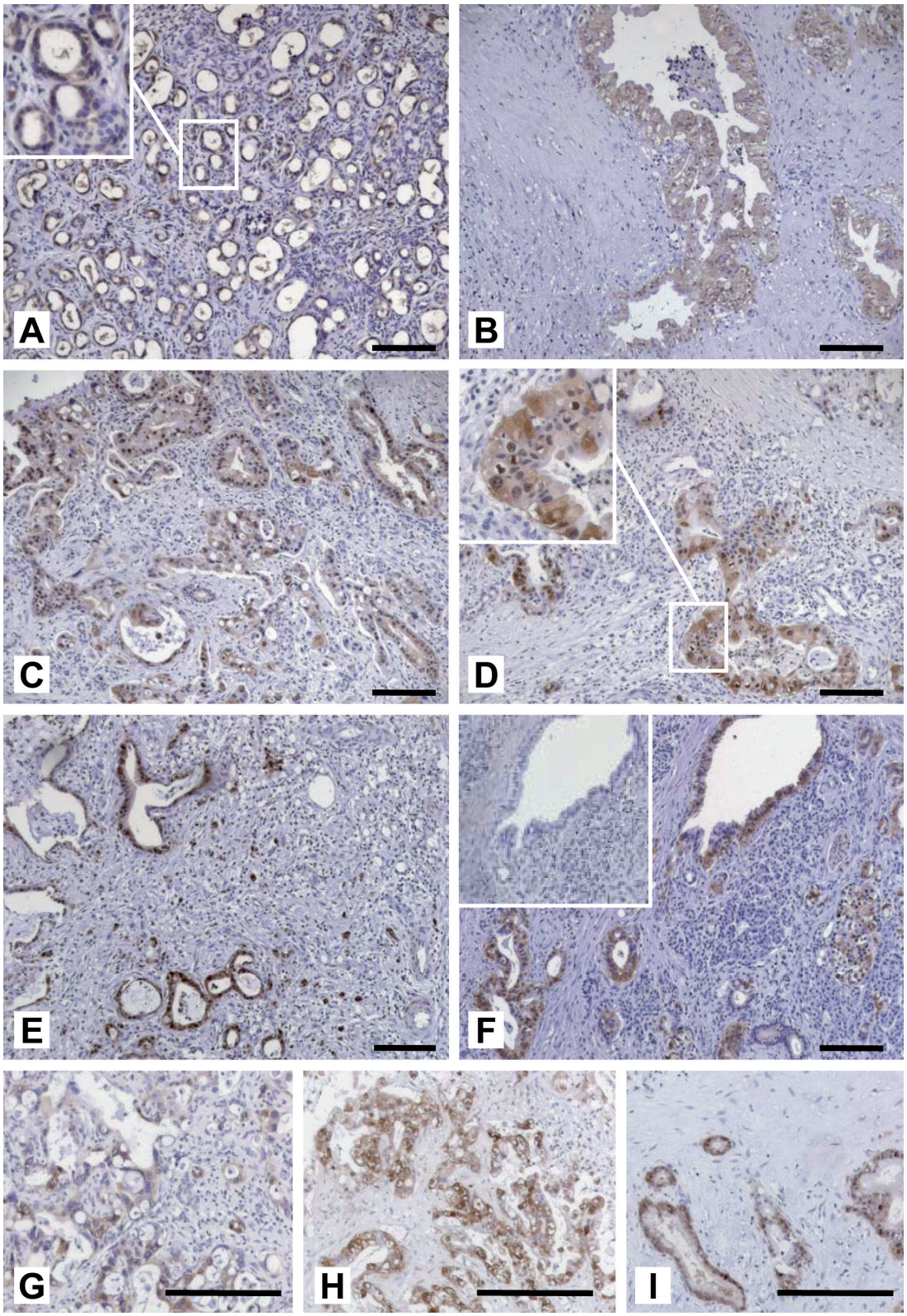

Fig. 2. Localization of SBDS in PDAC tissues. SBDS exhibited cytoplasmic and nuclear localization in tubular complexes (**A**), PanIN2 lesions (**B**), cancer cells (**C-F**), and metastases of PDAC to the liver (**G**), lymph nodes (**H**), and peritoneum (**I**). The specificity of the staining was confirmed using control IgG (**F**, inset). Scale bar: 100 μm.

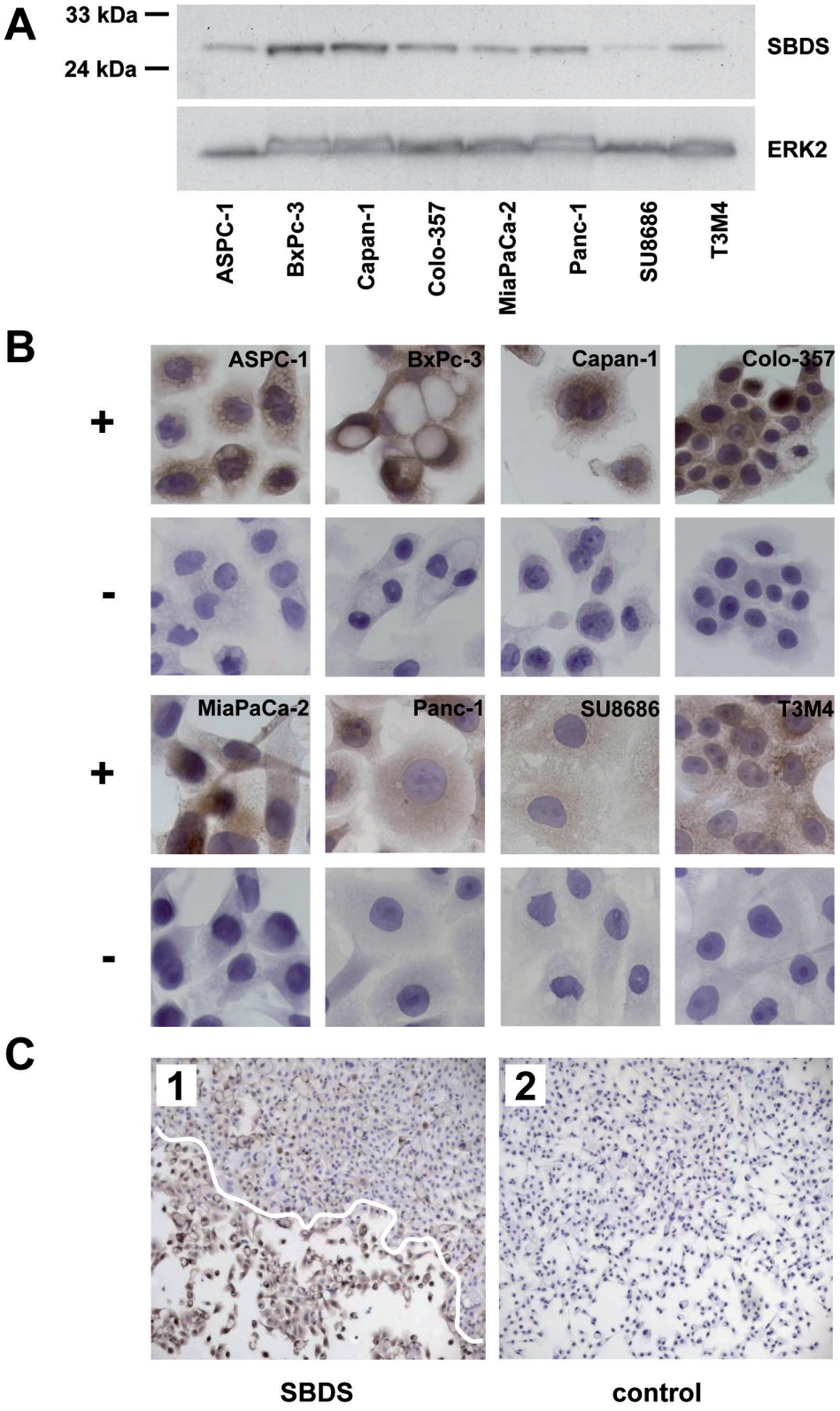

Fig. 3. Expression and localization of SBDS in cultured pancreatic cancer cell lines. **A.** Immunoblotting of cell lysates from cultured pancreatic cancer cells was performed using a specific mouse anti-SBDS antibody, as described in the Methods section. SBDS was expressed in 8 pancreatic cancer cell lines as an approximately 30 kDa protein (upper band). Equal loading was confirmed using an ERK2 antibody (lower band). **B.** Localization of SBDS in 8 cultured pancreatic cancer cell lines was performed by immunocytochemistry, as described in the Methods section, using a specific SBDS antibody (first and third panels, +). The specificity of the staining was confirmed using control IgG (second and fourth panels, -). **C1.** Immunocytochemistry demonstrating the difference of SBDS expression in fully confluent (upper right corner) cultured pancreatic cancer cells compared to non-confluent pancreatic cancer cells (lower left corner). The specificity of the staining was confirmed using control IgG (**C2**).

suggests that SBDS plays a role in rRNA processing (Austin et al., 2005; Ganapathi et al., 2007) during the progression of PDAC.

It is known that SBDS localization is cell-cycle dependent, with nucleolar localization during G1 and G2 and diffuse nuclear localization during S phase (Austin et al., 2005). SBDS nucleolar localization also appears to be dependent on active ribosomal RNA transcription (Ganapathi et al., 2007). Thus, a possible shuttling of SBDS expression between the cytoplasm and nucleus might correlate with the phase of the cell cycle in a subset of PDAC tissues as well as in pancreatic cancer cell lines. This would be supported by our observation of low to absent levels of SBDS in the center of fully confluent cultured pancreatic cancer cells; whereas in the periphery of the same culture or in non-confluent pancreatic cancer cells, strong SBDS expression was observed. It is known that fully confluent cells undergo contact inhibition (Blagosklonny, 2003), where the proliferation of the terminally differentiated cells is arrested in a quiescent (senescence-like) state. In contrast, the non-confluent cancer cells show increased proliferative activity, and therefore increased SBDS expression.

In conclusion, SBDS is expressed in normal pancreatic ductal and islet cells. The expression of SBDS in the pathologically altered cells in CP and PDAC tissues increased during acinar conversion into tubular complexes and PanIN lesions during the pathogenesis of both diseases. The nuclear and/or cytoplasmic expression of SBDS, as well as the level of SBDS expression in pancreatic cancer cells, might be related to the phase of the cell cycle and rRNA processing during the pathogenesis of CP and PDAC.

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