Cellular Oncology 31 (2009) 41–51
DOI 10.3233/CLO-2009-0460
IOS Press

TGF-β repressors SnoN and Ski are implicated in human colorectal carcinogenesis

Vasiliki Bravou [a], Anna Antonacopoulou [a], Helen Papadaki [b], Konstantina Floratou [a], Michalis Stavropoulos [c], Vasso Episkopou [d,e], Chariklia Petropoulou [e,*] and Haralabos Kalofonos [a]

[a] *Clinical Oncology Laboratory, School of Medicine, University of Patras, Rio Patras, Greece*
[b] *Department of Anatomy, School of Medicine, University of Patras, Rio Patras, Greece*
[c] *Department of Surgery, School of Medicine, University of Patras, Rio Patras, Greece*
[d] *Medical Research Council, Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital, London, UK*
[e] *Institute of Immunology, Biomedical Sciences Research Center "Alexander Fleming", Vari, Greece*

Abstract. *Background*: The TGF-β signaling repressors SnoN and Ski have been critically implicated in human cancer.

Methods: To explore the role of SnoN and Ski in the development and progression of colorectal cancer we examined their protein expression profile by immunohistochemistry in a series of human colorectal adenomas, carcinomas and lymph node metastases. The mRNA expression of SnoN was also quantified by Real-Time RT-PCR.

Results: SnoN and Ski were overexpressed both in adenomas with severe dysplasia and colorectal carcinomas. Protein expression was cytoplasmic and nuclear with predominant cytoplasmic localization. The subcellular localization was related differently to pathologic variables of colorectal carcinomas. Although there was no significant association of protein levels with tumor invasion and metastasis, a significant correlation of nuclear SnoN and Ski with β-catenin pathway was observed. Moreover, SnoN mRNA did not differ in carcinomas as compared to normal control and there was no correlation between SnoN protein and mRNA levels.

Conclusion: Our findings suggest that SnoN and Ski exert oncogenic effects in human colorectal carcinogenesis and their overexpression is implicated in early stage disease.

Keywords: Colorectal cancer, SnoN, Ski, TGF-β, β-catenin

1. Introduction

Colorectal cancer is a major cause of morbidity and mortality worldwide. The process of malignant transformation from normal tissue to invasive carcinoma during colorectal carcinogenesis involves the accumulation of genetic alterations in proliferating cells as well as the accumulation of chromosomal aberrations [1]. Transforming growth factor-beta (TGF-β) signaling pathway is known to play a central but complex role in the development and progression of colorectal cancer [2]. TGF-β induces growth arrest of colon epithelial cells and exerts tumor suppressing activity in early stages of colorectal carcinogenesis. However, it is also known to induce epithelial to mesenchymal transition (EMT) and to promote colorectal cancer invasion and metastasis [3]. Understanding how specific alterations in TGF-β signaling pathway contribute to the development and progression of colorectal cancer could provide novel opportunities for anticancer therapies.

SnoN and Ski are important negative regulators of TGF-β signaling since they interact with Smad proteins and repress their transcriptional activity [4]. They are considered to be oncoproteins based on their ability to induce oncogenic transformation of chicken and quail embryo fibroblasts when overexpressed [5,6]. Moreover, overexpression of SnoN and Ski inhibits

*Corresponding author: Chariklia Petropoulou, Institute of Immunology, Biomedical Sciences Research Center "Alexander Fleming", 34 Al. Fleming Street, 16672 Vari, Greece. Tel.: +30 210 9654465; Fax: +30 210 9653934; E-mail: petropoulou@fleming.gr.

TGF-β-induced growth arrest [7,8]. In accordance to these, up-regulation of SnoN and/or Ski expression has been detected in melanoma [9], breast cancer [10], pancreatic cancer [11] and esophageal squamous cell carcinoma (SCC) [12]. However, it has also been shown that SnoN and Ski may act as tumor suppressors since *ski* and *sno* deficient mice show increased susceptibility to tumorigenesis [13,14].

In colorectal cancer, gene amplification of Ski has been identified as a negative prognostic marker in early-stage disease [15], while SnoN mRNA expression has been found to be differentially regulated depending on the microsatellite status of the tumor [16]. Amplification and deletion of *SnoN* and *Ski* genes, as well as up- or downregulation of SnoN mRNA in colorectal cancer specimens suggests that they exert both oncogenic and tumor suppressive functions in colorectal carcinogenesis. However, the protein expression profile of the TGF-β repressors in human colorectal cancer has never been addressed and their precise role in colorectal carcinogenesis remains elusive.

This study aimed to elucidate the role of SnoN and Ski in human colorectal cancer development and progression. Therefore, we examined, in a series of primary human colorectal adenomas, carcinomas (CRCs) and concurrently excised lymph node (LN) metastases the protein levels and cellular localization of SnoN and Ski. We also studied the correlation of SnoN and Ski protein expression with clinicopathological variables of colorectal cancer as well as with the expression of β-catenin that is strongly linked to colorectal carcinogenesis [17,18]. We finally quantified SnoN mRNA by Real-Time RT-PCR to see whether transcript expression is related to SnoN protein levels in CRCs.

2. Materials and methods

2.1. Tissue specimens

The study was performed in accordance with the Institutional ethical guidelines and has been approved by the Committee on Research and Ethics and the Scientific Committee of the University Hospital of Patras, Greece. Formalin-fixed, paraffin-embedded (FFPE) tissue samples from 87 primary human CRCs and 23 concurrently excised nodal metastases as well as 20 paired normal colorectal tissue samples were obtained from the Departments of Pathology, "Agios Andreas" General Hospital and University Hospital of Patras, Greece. According to the American Joint Committee on Cancer Criteria (AJCC), from the 87 CRCs, 19 were classified as stage 0, 13 as stage I, 26 as stage II and 29 as stage III. Based on the WHO classification of tumors, from the 87 carcinomas, 30 were grade I, 28 grade II and 29 grade III. Clinicopathological parameters of the paraffin-embedded tissue specimens are shown in Tables 1 and 2.

Seven of the above and 4 extra primary CRC specimens were also available as fresh-frozen tissue. For these 11 fresh-frozen specimens, paired normal colorectal tissue was also available.

2.2. Immunohistochemistry

Immunohistochemistry was performed as previously described [17]. Rabbit polyclonal anti-SnoN and anti-Ski antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used in dilutions 1:80 and 1:60, respectively, overnight at 4°C. Bound primary antibodies were detected with the biotin–streptavidine–peroxidase method (Envision detection kit, DAKO, Hamburg, Germany). For negative controls, blocking solution was added instead of the primary antibody. Melanoma and breast carcinoma were used as positive controls for Ski and SnoN respectively.

2.3. Immunohistological evaluation

All slides were assessed by two pathologists (HP and VB) independently and blinded to the case. Cytoplasmic expression of SnoN and Ski was homogeneously distributed and intensity of staining was scored as follows: 0 – negative staining, 1 – weak staining, 2 – moderate staining and 3 – strong staining. For nuclear expression of SnoN and Ski both intensity of staining and percentage of positive cells were taken into account and the following scoring system was used: 0 – no staining or weak staining in less than 10% of tumor cells, 1 – weak staining in 10–70% of tumor cells or moderate staining in $<$40% of tumor cells, 2 – weak staining in $>$70% of cells, moderate staining in 40–70% of tumor cells or strong staining in 10–40% of tumor cells and 3 – moderate staining in $>$70% or strong staining in more than 40% of tumor cells.

2.4. Immunoblotting

Fresh-frozen tissues were lysed with buffer containing 1% Triton-X, 10 mM EDTA pH 8.0 and protease

Table 1
SnoN expression in human colorectal carcinomas and lymph node metastases. Correlation with clinicopathological parameters

	N	SnoN[a]	Cytoplasmic SnoN[b]				p value[b]	Nuclear SnoN[b]				p value[b]
			0	1	2	3		0	1	2	3	
			n (%)					n (%)				
Carcinomas	87	83	6	19	37	25		45	24	10	8	
		(95.4)	(6.9)	(21.8)	(42.5)	(28.7)		(51.7)	(27.6)	(11.5)	(9.2)	
In situ[c]	19	19	1	4	12	2	0.324	8	8	1	2	0.579
		(100)	(5.3)	(21.1)	(63.2)	(10.5)		(42.1)	(42.1)	(5.3)	(10,5)	
Invasive	68	64	5	15	25	23		37	16	9	6	
		(94.1)	(7.4)	(22.1)	(36.8)	(33.8)		(54.4)	(23.5)	(13.2)	(8.8)	
Depth of invasion[d]							0.595					0.139
Tis	19	19	1	4	12	2		8	8	1	2	
		(100)	(5.3)	(21.1)	(63.2)	(10.5)		(42.1)	(42.1)	(5.3)	(10.5)	
T1 + T2	13	13	0	2	8	3		10	2	1	0	
		(100)	(0)	(15.4)	(61.5)	(23.1)		(76.9)	(15.4)	(7.7)	(0)	
T3 + T4	55	51	5	13	17	20		27	14	8	6	
		(92.7)	(9.1)	(23.6)	(30.9)	(36.4)		(49.1)	(25.5)	(14.5)	(9.2)	
Grade							0.599					0.012
I	30	29	2	6	16	6		18	8	2	2	
		(96.7)	(6.7)	(20)	(53.3)	(20)		(60)	(26.7)	(6.7)	(6.7)	
II	28	27	1	7	9	11		17	5	4	2	
		(96.4)	(3.6)	(25)	(32.1)	(39.3)		(60.7)	(17.9)	(14.3)	(7.1)	
III	29	27	3	6	12	8		10	11	4	4	
		(93.1)	(10.3)	(20.7)	(41.4)	(27.6)		(34.5)	(37.9)	(13.8)	(13.8)	
Presence of LN metastasis							0.103					0.109
No	59	58	2	11	28	18		34	15	5	5	
		(98.3)	(3.4)	(18.6)	(47.5)	(30.5)		(57.6)	(25.4)	(8.5)	(8.5)	
Yes	28	25	4	8	9	7		11	9	5	3	
		(89.3)	(14.3)	(28.6)	(32.1)	(25)		(39.3)	(32.1)	(17.9)	(10.7)	
AJCC stage							0.032					0.198
0	19	19	1	4	12	2		8	8	1	2	
		(100)	(5.3)	(21.1)	(63.2)	(10.5)		(42.1)	(42.1)	(5.3)	(10.5)	
I + II	39	38	1	6	16	16		25	7	4	3	
		(97.6)	(2.6)	(15.4)	(41)	(41)		(64.1)	(17.9)	(10.3)	(7.7)	
III	29	26	4	9	9	7		12	9	5	3	
		(89.7)	(13.8)	(31)	(31)	(24.1)		(41.4)	(31)	(17.2)	(10.3)	
PT-LN[e]							0.631					0.105
Primary	23	20	4	7	7	5		9	8	4	2	
tumors		(87)	(17.4)	(30.4)	(30.4)	(21.7)		(39.1)	(34.8)	(17.4)	(8.7)	
LN	23	21	4	4	10	5		7	4	4	8	
metastases		(91.3)	(17.4)	(17.4)	(43.5)	(21.7)		(30.4)	(17.4)	(17.4)	(34.8)	

[a]Positive SnoN protein expression regardless of subcellular localization (includes all cases with only cytoplasmic, only nuclear or both cytoplasmic and nuclear SnoN expression). [b]Cytoplasmic and nuclear SnoN expression was scored as described in Section 2.3. [b]Kruskal–Wallis or Mann–Whitney test. p value < 0.05 was considered statistically significant. [c]*In situ* is referred to the Tis staging category of TNM classification and includes intraepithelial and intramucosal carcinomas. [d]According to TNM classification. [e]PT-LN: primary tumors (PT) and corresponding lymph node (LN) metastases.

Table 2
Ski expression in human colorectal carcinomas and lymph node metastases. Correlation with clinicopathological parameters

	N	Ski[a]	Cytoplasmic Ski[b]				p value[b]	Nuclear Ski[b]				p value[b]
			0	1	2	3		0	1	2	3	
			n (%)					n (%)				
Carcinomas	70	53	17	17	28	8		56	3	9	2	
		(75.7)	(24.3)	(24.3)	(40)	(11.4)		(80)	(4.3)	(12.9)	(2.9)	
In situ[c]	11	6	5	3	3	0	0.039	9	1	1	0	
		(54.5)	(45.5)	(27.3)	(27.3)	(0)		(81.8)	(9.1)	(9.1)	(0)	0.781
Invasive	59	47	12	14	25	8		47	2	8	2	
		(79.7)	(20.3)	(23.7)	(42.4)	(13.6)		(79.7)	(3.4)	(13.6)	(3.4)	
Depth of invasion[d]							0.068					0.787
Tis	11	6	5	3	3	0		9	1	1	0	
		(54.5)	(45.5)	(27.3)	(27.3)	(0)		(81.8)	(9.1)	(9.1)	(0)	
T1 + T2	13	13	0	4	5	4		11	1	1	0	
		(100)	(0)	(30.8)	(38.5)	(30.8)		(84.6)	(7.7)	(7.7)	(0)	
T3 + T4	46	34	12	10	20	4		36	1	7	2	
		(73.9)	(26.1)	(21.7)	(43.5)	(8.7)		(78.3)	(2.2)	(15.2)	(4.3)	
Grade							0.189					0.490
I	18	12	6	5	5	2		16	1	1	0	
		(66.7)	(33.3)	(27.8)	(27.8)	(11.1)		(88.9)	(5.6)	(5.6)	(0)	
II	24	19	5	3	11	5		18	1	5	0	
		(79.2)	(20.8)	(12.5)	(45.8)	(20.8)		(75)	(4.2)	(20.8)	(0)	
III	28	22	6	9	12	1		22	1	3	2	
		(78.6)	(21.4)	(32.1)	(42.9)	(3.6)		(78.6)	(3.6)	(10.7)	(7.1)	
Presence of LN metastasis							0.027					0.349
No	42	34	8	8	19	7		32	2	7	1	
		(81)	(19)	(19)	(45.2)	(16.7)		(76.2)	(4.8)	(16.7)	(2.4)	
Yes	28	19	9	9	9	1		24	1	2	1	
		(67.9)	(32.1)	(32.1)	(32.1)	(3.6)		(85.7)	(3.6)	(7.1)	(3.6)	
AJCC stage							0.001					0.522
0	11	6	5	3	3	0		9	1	1	0	
		(54.5)	(45.5)	(27.3)	(27.3)	(0)		(81.8)	(9.1)	(9.1)	(0)	
I + II	31	28	3	5	16	7		23	1	6	1	
		(90.3)	(9.7)	(16.1)	(51.6)	(22.6)		(64.1)	(3.2)	(10.3)	(3.2)	
III	28	19	9	9	9	1		24	1	2	1	
		(67.9)	(32.1)	(32.1)	(32.1)	(3.6)		(74.2)	(3.6)	(19.4)	(3.6)	
PT-LN[e]							0.066					0.934
Primary	21	12	9	8	4	0		18	0	2	1	
tumors		(57.1)	(42.9)	(38.1)	(19.0)	(0)		(85.7)	(0)	(9.5)	(4.8)	
LN	21	15	7	2	10	2		19	2	0	1	
metastases		(71.4)	(33.3)	(9.5)	(47.6)	(9.5)		(85.7)	(9.5)	(0)	(4.8)	

[a]Positive Ski protein expression regardless of subcellular localization (includes all cases with only cytoplasmic, only nuclear or both cytoplasmic and nuclear Ski expression). [b]Cytoplasmic and nuclear Ski expression was scored as described in Section 2.3. [b]Kruskal–Wallis or Mann–Whitney test. p value < 0.05 was considered statistically significant. [c]*In situ* is referred to the Tis staging category of TNM classification and includes intraepithelial and intramucosal carcinomas. [d]According to TNM classification. [e]PT-LN: primary tumors (PT) and corresponding lymph node (LN) metastases.

inhibitors. Lysates were then sonicated 3 times for 10 s and centrifuged to obtain supernatants. Prior to immunoblotting protein lysates were mixed with loading buffer containing 160 mM Tris (pH 6.8), 20% glycerol, 4% SDS, 0.01% bromophenol blue and 200 mM dithiothreitol. Immunoblotting was performed using standard procedures and proteins were detected using the ECL detection system (GE Healthcare UK Ltd., Buckinghamshire, UK). Rabbit polyclonal anti-SnoN (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse monoclonal anti-Actin (Abcam plc, Cambridge, UK) primary antibodies were used.

3. RNA extraction and real-time RT-PCR

Total RNA was extracted from formalin-fixed paraffin embedded tissue specimens as previously described [19]. For RNA isolation from CRCs, FFPE blocks that contained only tumor were selected after careful examination of hematoxylin and eosin stained sections so as to diminish contamination with adjacent normal colon tissue. Similarly for RNA isolation from normal tissues, FFPE blocks that contained only normal colon tissue (from free of disease surgical margins) were selected. From the fresh-frozen tissue samples, total RNA was extracted using the Absolutely RNA RT-PCR kit (Stratagene, La Jolla, USA) according to the manufacturer's instructions. For the samples that both formalin-fixed paraffin-embedded and fresh-frozen tissue was available, the fresh-frozen tissue was used for RNA extraction. DNA-free total RNA was quantified using Ribogreen (Molecular Probes, Leiden, The Netherlands) and the MX3000p (Stratagene, La Jolla, USA) according to the manufacturer's instructions.

Quantitative RT-PCR was performed with SnoN-specific primers (forward: 5′-GGCTGAATATGCAGG ACAG-3′, reverse: 5′-TGAGTTCATCTTGGAGTTC TTG-3′), as previously described [20].

4. Statistical analysis

Statistical analysis was performed with the SPSS for Windows, release 12.0 (SPSS Inc., Chicago, IL, USA). Correlations of clinicopathological parameters with protein or mRNA expression levels were analyzed with the non-parametric Kruskal–Wallis or Mann–Whitney tests for ordinal or continuous data. Differences between related groups were tested with Wilcoxon test. Correlations between expression of proteins (immunohistochemical scores) as well as between protein and mRNA levels were evaluated by the Spearman rank-order correlation coefficient. All ranking tests were performed with correction for ties. The significance level was defined as $p < 0.05$.

5. Results

5.1. SnoN protein is overexpressed in human colorectal tumors

To address the role of the oncoprotein SnoN in colorectal carcinogenesis we used immunohistochemical staining on 87 primary colorectal carcinomas and 23 lymph node metastases. In 19 cases of CRCs, precancerous lesions (adenomas) were present adjacent to the tumor and they were also evaluated for SnoN expression.

In adjacent normal colon mucosa expression of SnoN protein was either absent or weak cytoplasmic (Fig. 1A). In adjacent adenomas, intense cytoplasmic and nuclear immunoreactivity for SnoN was observed in areas with severe dysplasia, while mild or moderate dysplasia showed negative or weak cytoplasmic SnoN expression (Fig. 1(B and C)). Immunoreactivity for SnoN in CRCs was observed in the cytoplasm and nucleus of cancer cells (Fig. 1D) and variations were observed both in the SnoN expression levels and localization patterns. Eighty three out of 87 (95.4%) primary CRCs overexpressed SnoN either in the cytoplasm or nucleus. Cytoplasmic localization of SnoN was found in $81/87$ carcinomas (93.1%), while nuclear staining was detected in $42/87$ (48.3%) cases. Both cytoplasmic and nuclear SnoN expression was significantly higher in carcinomas compared to normal colon mucosa ($p = 0.002$ and $p < 0.001$, respectively) while no significant difference was observed between invasive and *in situ* carcinomas ($p = 0.324$ and $p = 0.579$ for cytoplasmic and nuclear SnoN, respectively). Cytoplasmic SnoN correlated significantly with disease stage ($p = 0.032$) with AJCC I and II carcinomas expressing higher levels of SnoN in the cytoplasm compared with tumors of stage 0 or III (Fig. 2(A–C)). A statistical significant correlation was also found between nuclear SnoN and tumor grade ($p = 0.012$) with poorly differentiated carcinomas showing higher levels of nuclear SnoN compared to well differentiated tumors as shown in Fig. 2(D–F). However, there were no significant correlations between cytoplasmic or nuclear

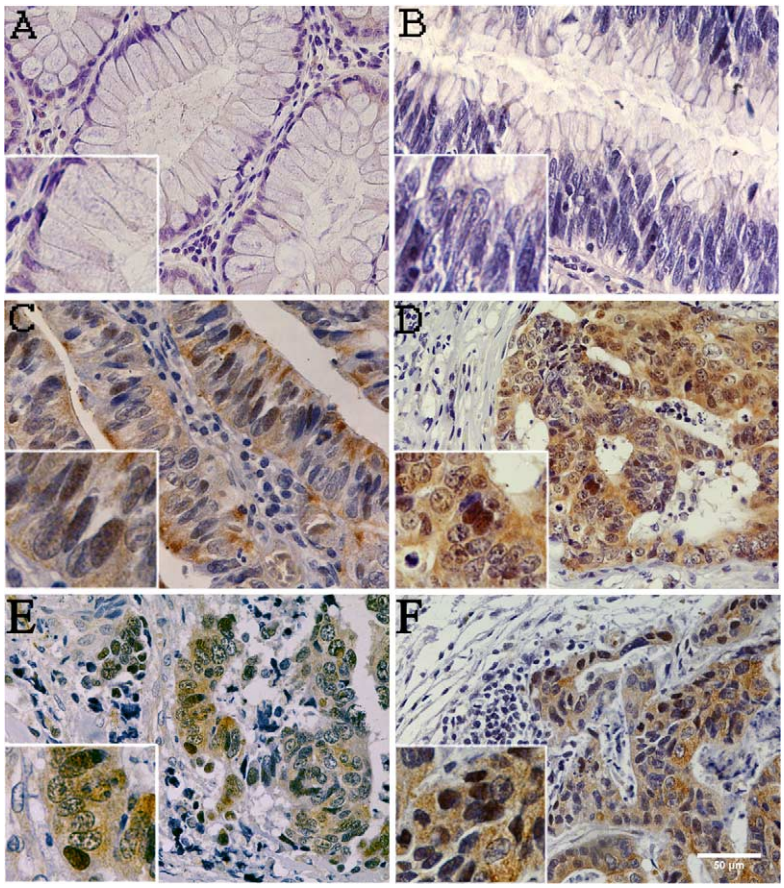

Fig. 1. SnoN expression in human colorectal cancer. (A) Normal colon mucosa with negative SnoN expression. (B) Negative SnoN staining in an adenoma with mild dysplasia. (C) Adenoma with high grade dysplasia showing cytoplasmic and nuclear immunoreactivity of SnoN. (D) Strong cytoplasmic and nuclear SnoN expression in an invasive carcinoma. (E, F) Representative cases of a primary tumor (E) and corresponding lymph node metastases (F) showing similar cytoplasmic and nuclear expression levels of SnoN (400× magnification). Inserts in lower left corner show the subcellular localization of immunostaining in each case at higher magnification (2× original objects). Bar corresponds to 50 μm.

SnoN expression and other clinicopathological parameters such as depth of invasion or presence of LN metastases (Table 1).

Furthermore, 21 out of 23 LN metastases (91.3%) were positive for SnoN either in nucleus or cytoplasm. Cytoplasmic and nuclear SnoN expression was found in 19/23 (82.6%) and 16/23 (69.6%) cases, respectively. However, there was no statistical significant difference in SnoN cytoplasmic or nuclear expression levels between primary tumors and lymph node metastases ($p = 0.631$ and $p = 0.105$, respectively) (Fig. 1(E and F)) (Table 1).

Immunoblotting in 11 cases of paired primary CRCs and normal colon specimens revealed SnoN protein expression in 9 out of 11 neoplastic tissue samples (81.8%) but only in 1 out of the 11 (9.09%) normal tissue samples. SnoN protein, in the rest 10 normal tissue samples, was absent (Fig. 3 and data not shown).

5.2. *Ski protein is overexpressed in human colorectal tumors*

As Ski is functionally related to SnoN we also examined its levels in a representative subset of our tumors. Specifically, Ski protein expression was studied by immunohistochemistry in 70 primary human CRCs and 21 LN metastases. Expression of Ski in normal colon mucosa was negative or weak cytoplasmic, while 53/70 (75.7%) carcinomas overexpressed Ski. In adjacent adenomas, Ski expression was cytoplasmic and nuclear and it was higher in areas of severe dysplasia compared to areas with lower degree of dysplasia. In carcinomas, staining for Ski was only cytoplasmic (39/70, 55.7%) or cytoplasmic and nuclear (14/70, 20%) (Fig. 4(A–D)). Cytoplasmic Ski expression was overall found in 53/70 carcinomas (75.7%) while nu-

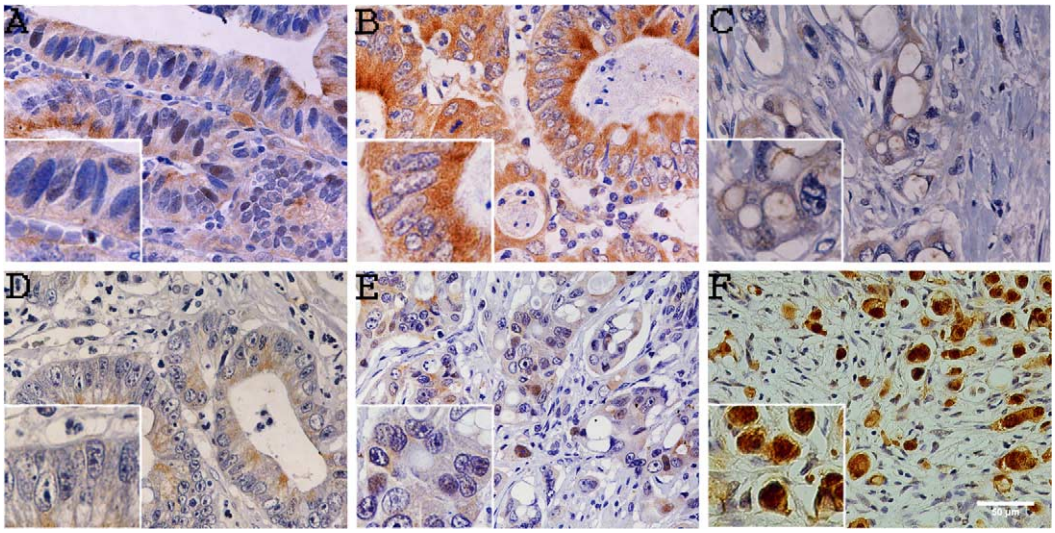

Fig. 2. Subcellular SnoN localization differentially correlates to tumor stage and grade. (A–C) Cytoplasmic SnoN expression correlates with disease stage. Higher cytoplasmic expression of SnoN in a stage II tumor (B) compared to tumors of stage 0 (A) or III (C). (D–F) Nuclear SnoN correlates with tumor grade. Negative nuclear SnoN staining in a grade I tumor (D). Weak SnoN nuclear expression in a grade II carcinoma (E). A grade III tumor showing strong nuclear immunoreactivity for SnoN (F) (400× magnification). Inserts in lower left corner show the subcellular localization of immunostaining in each case at higher magnification (2× original objects). Bar corresponds to 50 μm.

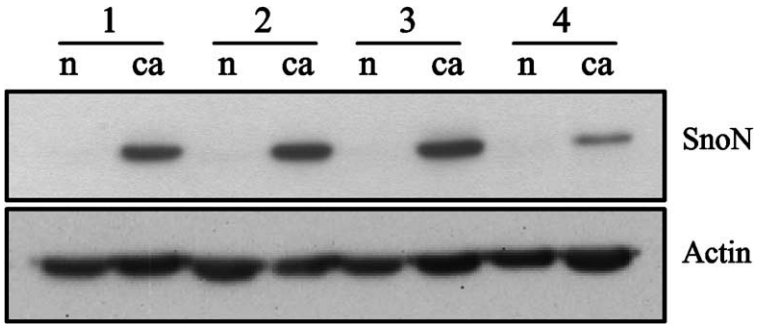

Fig. 3. SnoN expression by immunoblotting in four representative surgical specimens of human primary CRCs. Protein expression is absent in matched normal tissue. Actin antibody was used as a loading control. n – normal tissue, ca – neoplastic tissue.

clear localization was detected in only 14 cases (20%). Cytoplasmic and nuclear Ski expression was significantly higher in carcinomas compared to normal control ($p = 0.008$ and $p < 0.001$, respectively). Invasive tumors showed higher expression of Ski in the cytoplasm compared to *in situ* carcinomas ($p = 0.039$). There was also a significant correlation of cytoplasmic Ski with disease stage ($p = 0.001$) and, similar to SnoN, AJCC I and II carcinomas showed higher levels of Ski compared to tumors of stage 0 or III. However, there was no significant correlation between cytoplasmic or nuclear Ski with other clinicopathological parameters under evaluation (Table 2).

Furthermore, Ski was expressed in either the cytoplasm or nucleus in $15/21$ (71.4%) LN metastases. Cytoplasmic and nuclear staining was observed in $14/21$ (66.7%) and $3/21$ (14.3%) of cases, respectively. No significant difference was found in the cytoplasmic or nuclear expression levels of Ski between primary tumors and nodal metastases ($p = 0.066$ and $p = 0.934$, respectively) (Table 2, Fig. 4(E and F)).

5.3. SnoN and Ski expression in human colorectal cancer correlates with activation of the β-catenin pathway

We next examined the potential correlation of SnoN and Ski protein expression with the expression of β-catenin that is known to be critically implicated in colorectal carcinogenesis [17,18]. We have previously

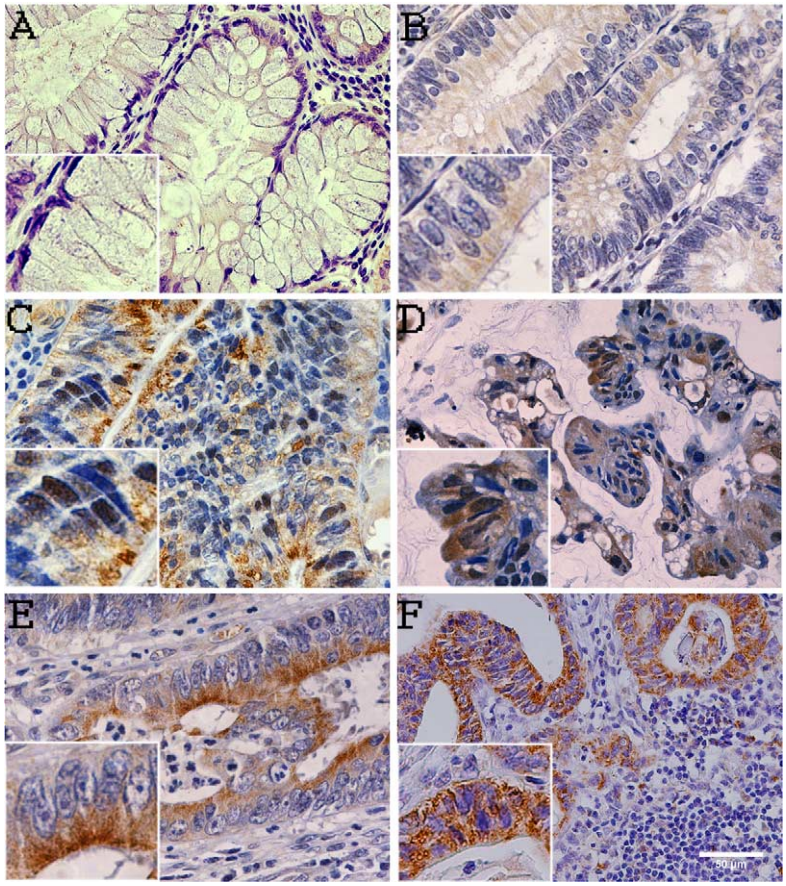

Fig. 4. Ski expression in human colorectal cancer. (A) Normal colon mucosa with negative Ski expression. (B) Weak Ski staining in an adenoma. (C) Adenoma with high grade dysplasia showing intense cytoplasmic and nuclear expression of Ski. (D) Cytoplasmic and nuclear Ski expression in an invasive mucinous carcinoma. (E, F) Comparable cytoplasmic expression levels of Ski in a case of nodal metastasis (F) compared to the corresponding primary tumor (E) (400× magnification). Inserts in lower left corner show the subcellular localization of immunostaining in each case at higher magnification (2× original objects). Bar corresponds to 50 μm.

shown that activation of β-catenin is frequently observed in colorectal cancer and correlates with tumour progression [17]. In 80 CRCs that were identical in both studies nuclear staining for β-catenin was observed in 92.5% of cases. Interestingly, both nuclear SnoN and nuclear Ski correlated with nuclear expression of β-catenin ($r = 0.269$, $p = 0.020$ and $r = 0.260$, $p = 0.024$, respectively) (Fig. 5).

5.4. *Transcriptional upregulation is not the underlying cause of high SnoN protein expression in human colorectal carcinomas*

To distinguish whether the SnoN protein overexpression in colorectal cancer is due to transcriptional upregulation, we examined SnoN transcript levels by real-time RT-PCR in 45 paraffin-embedded CRCs and 20 paired normal tissue specimens as well as in 11 paired fresh-frozen CRCs and normal samples.

SnoN mRNA was expressed in $20/20$ (100%) normal tissues and in $42/45$ (93.3%) carcinomas from paraffin-embedded specimens with median values 0.875 and 0.772, respectively. When SnoN mRNA expression was examined in the 20 paraffin embedded CRCs relative to matched normal specimens, no statistical significant difference was found (median fold difference: 1.125, range: 0.13–3.33).

In the fresh-frozen samples SnoN mRNA was detected in all tissue specimens, both neoplastic and normal. There was no statistical significant difference in the mRNA levels of SnoN in CRCs as compared to normal control (median fold difference: 1.36, range: 0.43–3.39).

While SnoN mRNA expression was found in all normal colon tissue specimens, SnoN protein expression

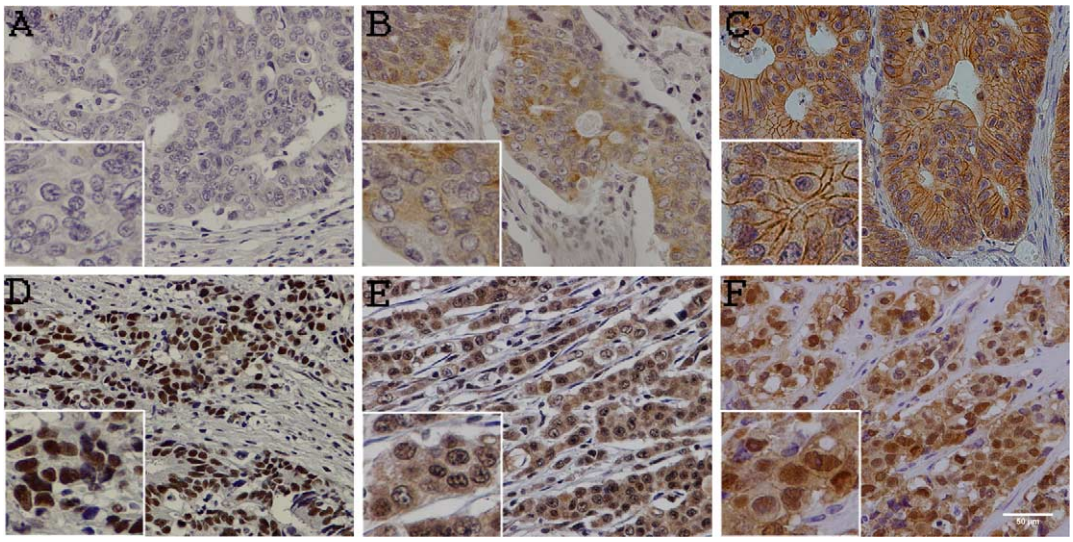

Fig. 5. Nuclear SnoN and Ski expression correlates with activation of β-catenin in colon cancer. (A–C) Adjacent sections of a colon carcinoma showing negative nuclear expression of SnoN (A) and Ski (B) and normal membranous expression of β-catenin (C). (D–F) Adjacent sections of a tumor showing intense nuclear localization of SnoN (D), Ski (E) and β-catenin (F) (400× magnification). Inserts in lower left corner show the subcellular localization of immunostaining in each case at higher magnification (2× original objects). Bar corresponds to 50 μm.

was absent or very low. Moreover, in the 45 paraffin-embedded and 11 fresh frozen carcinomas SnoN protein levels did not correlate with SnoN mRNA levels.

6. Discussion

Defects in TGF-β signal transduction are common in colorectal cancer indicating an important role of the TGF-β pathway in colorectal carcinogenesis. While previous studies suggest a critical involvement of the TGF-β repressors Ski and SnoN in human cancer, their role in colorectal carcinogenesis has not been fully addressed. The current study reports that SnoN and Ski protein overexpression is implicated in the development of human colorectal cancer and suggests that the subcellular localization, correlation with β-catenin activation and increased protein stability significantly contribute to the oncogenic role of the TGF-β repressors in colorectal carcinogenesis.

In the present study, SnoN and Ski proteins were found to be overexpressed in CRCs compared to normal control indicating their oncogenic role in colorectal carcinogenesis. While previous analyses of gene copy status and mRNA expression of SnoN and Ski in CRCs suggest a dual tumor promoting and tumor suppressive role of the TGF-β repressors in colorectal cancer [15,16], protein overexpression of Ski and SnoN has been reported in other human malignancies [9–12]. Consistent to their role as oncoproteins, overexpression of the SnoN and Ski has been also shown to inhibit TGF-β-induced growth arrest. In addition, we showed that SnoN and Ski protein accumulation is evident from early stages of colorectal carcinogenesis such as adenomas and it did not correlate with tumor invasion and metastases. Our findings indicate that overexpression of the TGF-β repressors is not implicated in CRC progression and metastasis but it is rather important for colorectal cancer development. Collectively, overexpression of SnoN and Ski seem to contribute to the resistance of colon cancer cells to the tumour suppressive functions of TGF-β, exerted in early stage disease, adding to the complexity of the TGF-β signaling pathway deregulation observed in human colorectal cancer.

We also showed that cytoplasmic levels of SnoN and Ski while high in stage I–II carcinomas they significantly decrease in stage III carcinomas and LN metastases. Interestingly, it has been previously suggested that SnoN expression may be regulated differently at distinct stages of carcinogenesis [21]. This regulated expression of SnoN may contribute to the dual role of TGF-β signaling in cancer: early in the course of tumor development, increased levels of SnoN or Ski protein could mediate resistance to TGF-β growth inhibition, while at later stages of cancer progression lower levels of SnoN and Ski may allow TGF-β to promote cancer cell invasion and metastasis.

An important finding of our study was that nuclear SnoN expression in CRCs correlated with ad-

vancing tumor grade. The TGF-β pathway is known to be critically involved in intestinal epithelial cell differentiation processes [22,23]. Since it negatively regulates TGF-β, SnoN may interfere with the normal differentiation process of the intestinal epithelial cells thus contributing to CRC development and tumor de-differentiation. Moreover, increased nuclear SnoN seems to mark tumors with poor differentiation and thus a more aggressive behavior suggesting its potential prognostic significance in the disease.

We also demonstrated a significant correlation of SnoN and Ski nuclear expression with the activation of β-catenin that is strongly linked to colorectal carcinogenesis [18]. In line with our finding, TGF-β signaling inactivation has been shown to interact with β-catenin pathway to drive intestinal carcinogenesis in transgenic mice [24]. Ski has been also shown to be a potent inducer of β-catenin signaling in melanoma [25]. It seems likely that the TGF-β repressors and the β-catenin pathway functionally interact and cooperate to promote colorectal carcinogenesis.

It has also become evident from our study that not only the protein levels but also the intracellular localization of the TGF-β repressors may be of importance for colorectal carcinogenesis. SnoN and Ski in CRCs were localized in both the cytoplasm and the nucleus of cancer cells with predominance of cytoplasmic localization. While Krakowski et al. have previously reported that SnoN is nuclear in malignant tumors [26], other studies have shown both cytoplasmic and nuclear localization of the TGF-β repressors in human malignancies [9–12]. In addition, our findings show that cytoplasmic and nuclear SnoN and Ski related differently to pathologic variables of CRCs such as stage and grade and only nuclear expression correlated to β-catenin signaling. Distinct localization patterns in relation to different tumour characteristics have been also reported for SnoN in breast carcinomas [10]. It is also known that while nuclear SnoN represses Smad transcriptional activity, cytoplasmic SnoN antagonizes TGF-β signaling by sequestering the Smad proteins in the cytoplasm [26]. Taken together, these data suggest that the subcellular localization of the TGF-β repressors in CRCs may produce different patterns of downstream responses, thus differentially affecting colorectal cancer biology.

In contrast to the overexpression of SnoN protein observed in all tumors compared to adjacent normal colon, SnoN mRNA did not differ significantly between CRCs and controls. Furthermore, there was no statistical significant correlation between SnoN protein and mRNA expression levels. Overexpression of SnoN in human cancer could occur by different mechanisms such as gene amplification, transcriptional upregulation or increased protein stability. However, the distinct mRNA and protein expression profile suggests that the excess of SnoN protein we observed in colorectal cancer may be due to dysregulation of proteasome degradation rather than transcriptional upregulation. This hypothesis is supported by recent evidence in esophageal cancer cells where abrogation of SnoN degradation caused resistance to TGF-β-mediated growth arrest [27]. Mutations that could render inert the proteins which mediate SnoN protein degradation [28–31] or mutations that could confer degradation-resistance characteristics to SnoN leading to colorectal cancer is an interesting possibility that needs to be investigated.

Collectively, the above findings implicate SnoN and Ski protein overexpression in early stages of human colorectal carcinogenesis and provide evidence that increased nuclear SnoN represents a potential marker of poorly differentiated tumors. The subcellular localization of the TGF-β repressors and their correlation with the β-catenin pathway seem also to be important for their oncogenic role in colorectal cancer. The distinct protein and mRNA expression profile, finally, indicates that increased protein stability may account for the protein accumulation of SnoN in colorectal cancer.

Acknowledgements

We would like to thank Prof. D. Bonikos, Prof. C.D. Scopa and Dr. M. Repanti for kindly providing tissue samples. This work was supported by the EPAN-Excellence Grant 2006–2008 from the Greek Secretariat for Research and Technology to V.E.

References

[1] M. Grade, H. Becker, T. Liersch, T. Ried and B.M. Ghadimi, Molecular cytogenetics: Genomic imbalances in colorectal cancer and their clinical impact, *Cell. Oncol.* **28** (2006), 71–84.

[2] R.L. Elliott and G.C. Blobe, Role of transforming growth factor Beta in human cancer, *J. Clin. Oncol.* **23** (2005), 2078–2093.

[3] K. Pardali and A. Moustakas, Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer, *Biochim. Biophys. Acta* **1775** (2007), 21–62.

[4] K. Luo, Ski and SnoN: negative regulators of TGF-beta signaling, *Curr. Opin. Genet. Dev.* **14** (2004), 65–70.

[5] C. Colmenares, P. Sutrave, S.H. Hughes and E. Stavnezer, Activation of the c-ski oncogene by overexpression, *J. Virol.* **65** (1991), 4929–4935.

[6] P.L. Boyer, C. Colmenares, E. Stavnezer and S.H. Hughes, Sequence and biological activity of chicken snoN cDNA clones, *Oncogene* **8** (1993), 457–466.

[7] S.L. Stroschein, W. Wang, S. Zhou, Q. Zhou and K. Luo, Negative feedback regulation of TGF-beta signaling by the SnoN oncoprotein, *Science* **286** (1999), 771–774.

[8] Y. Sun, X. Liu, E.N. Eaton, W.S. Lane, H.F. Lodish and R.A. Weinberg, Interaction of the Ski oncoprotein with Smad3 regulates TGF-beta signaling, *Mol. Cell* **4** (1999), 499–509.

[9] J.A. Reed, E. Bales, W. Xu, N.A. Okan, D. Bandyopadhyay and E.E. Medrano, Cytoplasmic localization of the oncogenic protein Ski in human cutaneous melanomas *in vivo*: functional implications for transforming growth factor beta signaling, *Cancer Res.* **61** (2001), 8074–8078.

[10] F. Zhang, M. Lundin, A. Ristimaki, P. Heikkila, J. Lundin, J. Isola, H. Joensuu and M. Laiho, Ski-related novel protein N (SnoN), a negative controller of transforming growth factor-beta signaling, is a prognostic marker in estrogen receptor-positive breast carcinomas, *Cancer Res.* **63** (2003), 5005–5010.

[11] T.R. Heider, S. Lyman, R. Schoonhoven and K.E. Behrns, Ski promotes tumor growth through abrogation of transforming growth factor-beta signaling in pancreatic cancer, *Ann. Surg.* **246** (2007), 61–68.

[12] M. Fukuchi, M. Nakajima, Y. Fukai, T. Miyazaki, N. Masuda, M. Sohda, R. Manda, K. Tsukada, H. Kato and H. Kuwano, Increased expression of c-Ski as a co-repressor in transforming growth factor-beta signaling correlates with progression of esophageal squamous cell carcinoma, *Int. J. Cancer* **108** (2004), 818–824.

[13] T. Shinagawa, H.D. Dong, M. Xu, T. Maekawa and S. Ishii, The sno gene, which encodes a component of the histone deacetylase complex, acts as a tumor suppressor in mice, *EMBO J.* **19** (2000), 2280–2291.

[14] T. Shinagawa, T. Nomura, C. Colmenares, M. Ohira, A. Nakagawara and S. Ishii, Increased susceptibility to tumorigenesis of ski-deficient heterozygous mice, *Oncogene* **20** (2001), 8100–8108.

[15] M. Buess, L. Terracciano, J. Reuter, P. Ballabeni, J.L. Boulay, U. Laffer, U. Metzger, R. Herrmann and C. Rochlitz, Amplification of SKI is a prognostic marker in early colorectal cancer, *Neoplasia* **6** (2004), 207–212.

[16] J.A. Chia, L.A. Simms, S.J. Cozzi, J. Young, J.R. Jass, M.D. Walsh, K.J. Spring, B.A. Leggett and V.L. Whitehall, SnoN expression is differently regulated in microsatellite unstable compared with microsatellite stable colorectal cancers, *BMC Cancer* **6** (2006), 252.

[17] V. Bravou, G. Klironomos, E. Papadaki, S. Taraviras and J. Varakis, ILK over-expression in human colon cancer progression correlates with activation of beta-catenin, down-regulation of E-cadherin and activation of the Akt-FKHR pathway, *J. Pathol.* **208** (2006), 91–99.

[18] M. Bienz and H. Clevers, Linking colorectal cancer to Wnt signaling, *Cell* **103** (2000), 311–320.

[19] M. Taron, R. Rosell, E. Felip, P. Mendez, J. Souglakos, M.S. Ronco, C. Queralt, J. Majo, J.M. Sanchez, J.J. Sanchez and J. Maestre, BRCA1 mRNA expression levels as an indicator of chemoresistance in lung cancer, *Hum. Mol. Genet.* **13** (2004), 2443–2449.

[20] P.D. Grivas, A. Antonacopoulou, V. Tzelepi, G. Sotiropoulou-Bonikou, Z. Kefalopoulou, A.G. Papavassiliou and H. Kalofonos, HER-3 in colorectal tumourigenesis: From mRNA levels through protein status to clinicopathologic relationships, *Eur. J. Cancer* **43** (2007), 2602–2611.

[21] Q. Zhu, A.R. Krakowski, E.E. Dunham, L. Wang, A. Bandyopadhyay, R. Berdeaux, G.S. Martin, L. Sun and K. Luo, Dual role of SnoN in mammalian tumorigenesis, *Mol. Cell. Biol.* **27** (2007), 324–339.

[22] J.C. Fleet, L. Wang, O. Vitek, B.A. Craig and H.J. Edenberg, Gene expression profiling of Caco-2 BBe cells suggests a role for specific signaling pathways during intestinal differentiation, *Physiol. Genomics* **13** (2003), 57–68.

[23] K.M. Juuti-Uusitalo, K. Kaukinen, M. Maki, J. Tuimala and H. Kainulainen, Gene expression in TGFbeta-induced epithelial cell differentiation in a three-dimensional intestinal epithelial cell differentiation model, *BMC Genomics* **7** (2006), 279.

[24] N.M. Munoz, M. Upton, A. Rojas, M.K. Washington, L. Lin, A. Chytil, E.G. Sozmen, B.B. Madison, A. Pozzi, R.T. Moon, H.L. Moses and W.M. Grady, Transforming growth factor beta receptor type II inactivation induces the malignant transformation of intestinal neoplasms initiated by Apc mutation, *Cancer Res.* **66** (2006), 9837–9844.

[25] D. Chen, W. Xu, E. Bales, C. Colmenares, M. Conacci-Sorrell, S. Ishii, E. Stavnezer, J. Campisi, D.E. Fisher, A. Ben-Ze'ev and E.E. Medrano, SKI activates Wnt/beta-catenin signaling in human melanoma, *Cancer Res.* **63** (2003), 6626–6634.

[26] A.R. Krakowski, J. Laboureau, A. Mauviel, M.J. Bissell and K. Luo, Cytoplasmic SnoN in normal tissues and nonmalignant cells antagonizes TGF-beta signaling by sequestration of the Smad proteins, *Proc. Natl. Acad. Sci. USA* **102** (2005), 12437–12442.

[27] J.S. Edmiston, W.A. Yeudall, T.D. Chung and D.A. Lebman, Inability of transforming growth factor-beta to cause SnoN degradation leads to resistance to transforming growth factor-beta-induced growth arrest in esophageal cancer cells, *Cancer Res.* **65** (2005), 4782–4788.

[28] S.L. Stroschein, S. Bonni, J.L. Wrana and K. Luo, Smad3 recruits the anaphase-promoting complex for ubiquitination and degradation of SnoN, *Genes Dev.* **15** (2001), 2822–2836.

[29] S. Bonni, H.R. Wang, C.G. Causing, P. Kavsak, S.L. Stroschein, K. Luo and J.L. Wrana, TGF-beta induces assembly of a Smad2–Smurf2 ubiquitin ligase complex that targets SnoN for degradation, *Nat. Cell Biol.* **3** (2001), 587–595.

[30] L. Levy, M. Howell, D. Das, S. Harkin, V. Episkopou and C.S. Hill, Arkadia activates Smad3/Smad4-dependent transcription by triggering signal-induced SnoN degradation, *Mol. Cell. Biol.* **27** (2007), 6068–6083.

[31] Y. Nagano, K.J. Mavrakis, K.L. Lee, T. Fujii, D. Koinuma, H. Sase, K. Yuki, K. Isogaya, M. Saitoh, T. Imamura, V. Episkopou, K. Miyazono and K. Miyazawa, Arkadia induces degradation of SnoN and c-Ski to enhance transforming growth factor-beta signaling, *J. Biol. Chem.* **282** (2007), 20492–20501.