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Analysis of the E-Cadherin Repressor Snail in Primary Human Cancers

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Abstract
Epithelial-mesenchymal transition (EMT), a normal developmental process, is known to play a crucial role in tumor progression. Molecules involved in this process, such as the E-cadherin repressor Snail, facilitate migration and invasion of carcinoma cells. A growing number of studies addressing the expression of Snail in clinical samples have been reported and are discussed in this review. A total of 2,112 cases from 9 different tumor types were evaluated. So far, a clear picture has emerged only in some cancer types analyzed with regard to overexpression of Snail and clinical-pathological parameters. Currently, it seems that Snail may play a role in hormone-dependent carcinomas but may be of minor importance in gastrointestinal cancers for tumor dedifferentiation and the maintenance of the invasive phenotype. It should be kept in mind, however, that the threshold for Snail activity does not have to be the same in every tumor type analyzed. The recent introduction of well-characterized novel monoclonal antibodies reacting with the short-lived nuclear Snail protein may help to establish a potential clinical usefulness for this master molecule of EMT, at least for certain types of cancer.

Abbreviations used in this paper

EMT	epithelial-mesenchymal transition
GSK3beta	glycogen synthase kinase 3 beta
HNSCC	head and neck squamous cell carcinoma
PAK1	p21-activated kinase 1
qRT-PCR	quantitative real-time reverse transcription PCR
Sip1	smad-interacting protein 1

Introduction

A highly controlled key process in normal development is epithelial-mesenchymal transition (EMT). During EMT a loss of intercellular adhesion is observed together with extensive remodeling of the cytoskeleton. Epithelial cells undergoing the transition to a mesenchymal phenotype display an increased propensity for mi-

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gration. Generally, EMT can be induced by a variety of molecules that also play major roles during tumor invasion and metastasis [Thiery, 2002; Barrallo-Gimeno and Nieto, 2005; Thiery and Sleeman, 2006]. A hallmark for EMT is the loss of the cell adhesion molecule and invasion suppressor E-cadherin. Downregulation of E-cadherin can arise – amongst other mechanisms – through transcriptional repression. Several EMT regulators have been identified as E-cadherin repressors, including the zinc finger transcription factors Snail [Batlle et al., 2000; Cano et al., 2000; Nieto, 2002; de Craene et al., 2005], Slug [Snail2; Hajra et al., 2002] and Sip1 (smad-interacting protein 1) [Comijn et al., 2001].

Since EMT is frequently observed in human tumors, Snail has been analyzed in carcinoma cells in culture to determine if there is evidence for a functional role during tumor invasion and metastasis. Indeed, it has been shown that Snail directly binds to the E-boxes present in the E-cadherin promoter resulting in downregulation of promoter activity in vitro. Moreover, transfection of Snail in E-cadherin-positive carcinoma cells induced a full EMT with downregulation of E-cadherin and other epithelial marker genes, such as occludin, and upregulation of mesenchymal markers, including vimentin and fibronectin [reviewed by Barrallo-Gimeno and Nieto, 2005]. Importantly, Snail-expressing cells became invasive after transfection, supporting its role in tumor progression [Batlle et al., 2000; Cano et al., 2000; Barbera et al., 2004]. Snail not only induces invasion but also blocks the cell cycle and confers resistance to cell death [Vega et al., 2004].

Snail Expression in Various Types of Cancers

We review and discuss here studies for Snail (recently termed Snail1) expression in a variety of primary human cancers (table 1). Up to now, a total of 2,112 cases of 9 different tumor types reported in 21 studies were evaluated, including carcinomas from breast (10 studies), stomach (2 studies), colon (3 studies), liver (1 study), ovary (1 study), esophagus (1 study), head and neck (1 study), endometrium (1 study), and synovial sarcomas (1 study). These studies have provided a wealth of information on the biology of EMT in human tumors and point out a critical role for Snail in tumor dedifferentiation, at least in some cancers.

There is a great spectrum of tissue sources and methods used, making it difficult to exactly compare the results of these studies. These variations in study design include the following points: some studies used (semi-quantitative) reverse transcription PCR (RT-PCR); others used quantitative real-time RT-PCR (qRT-PCR) or in situ hybridization to determine Snail mRNA expression. Different primer and probes were used in the experiments. Frozen tissues or formalin-fixed and paraffin-embedded tissues were analyzed, with or without tissue microdissection. Publicly available gene expression datasets were examined for Snail mRNA expression or institutional cases were analyzed. Self-made or commercial tissue microarrays were examined for immunohistochemistry, using antibodies reacting with different epitopes and specificities. Despite these limitations we provide an overview of studies for analyzing Snail expression in human primary cancer samples, and try to summarize the critical findings.

Breast Cancer

The first studies analyzing Snail in primary human tumor tissues were reported by Cheng et al. [2001] and Okubo et al. [2001]. These researchers could demonstrate that Snail was expressed in frozen breast cancer tissue samples by semiquantitative RT-PCR. However, there was a discrepancy in interpretation of the results. Cheng et al. suggested that Snail may act to reduce E-cadherin levels in tumorous tissues. They found that almost all tumors displaying reduced/negative E-cadherin expression showed increased Snail expression. Of the tumors with positive E-cadherin expression, only 5.3% were found to have increased Snail mRNA levels. Because no Snail-specific antibodies were available at that time, these researchers raised a cautionary note and suggested that future studies should use immunohistochemistry to confirm the results at the cellular level. In sharp contrast, Okubo et al. [2001] suggested that Snail may be normally expressed in nontumorous breast tissues and may be reduced in tumor tissues as only 16 (29%) out of 55 cancer samples showed Snail expression. They suggested a cancer protective role for Snail but they mentioned that more extensive analyses of 'normal' breast tissues were needed.

One year later, in 2002, Blanco et al. used in situ hybridization to demonstrate Snail mRNA expression in breast carcinomas. They found that Snail mRNA was not detectable in normal breast epithelium. Snail was found to be expressed in 47% (8/17) of infiltrating ductal carcinomas and correlated with a dedifferentiated phenotype ($p = 0.034$). Furthermore, a higher percentage of tumors with reduced E-cadherin expression was observed among the Snail-positive cancers. In addition to tumor cells, a subset of stromal fibroblasts showed Snail mRNA expres-

Table 1. Snail expression in primary human tumors

Tumor type (number of cases)	Method of analysis	Major results	Reference
Breast cancer (n = 51)	RT-PCR from frozen tissues	increased Snail mRNA expression was associated with reduced E-cadherin levels	Cheng et al., 2001
Breast cancer (n = 55)	RT-PCR from frozen tissues	Snail mRNA was detected in 29% of breast cancer tissues	Okubo et al., 2001
Breast carcinoma (n = 21)	in situ hybridization	inverse correlation between Snail mRNA expression and grade of differentiation	Blanco et al., 2002
Breast carcinoma (n = 115)	analysis of a publicly available gene expression dataset	negative correlation between estrogen receptor alpha and Snail mRNA expression	Fujita et al., 2003
Breast cancer (n = 129)	immunohistochemistry (commercial antibody)	Snail immunoreactivity (no nuclear staining) correlated with GSK3beta inhibition and E-cadherin reduction; no association with tumor grade	Zhou et al., 2004
Breast carcinomas (n = 23)	RT-PCR and in situ hybridization from fresh effusions	high Snail mRNA expression predicts shorter disease-free survival	Elloul et al., 2005
Breast cancer (four datasets with 465 tumors in total)	analysis of publicly available gene expression datasets	Snail mRNA expression predicts shorter relapse-free survival	Moody et al., 2005
Breast carcinoma (n = 114)	qRT-PCR from frozen tissues and immunohistochemistry (commercial antibody)	no significant Snail overexpression, reduced Snail expression in poor-outcome patients	Martin et al., 2005
Breast carcinoma (n = 128)	qRT-PCR from frozen tissues and immunohistochemistry (antibody: Sn9H2)	Snail overexpression in invasive tumors	Côme et al., 2006
Node-negative invasive ductal breast carcinomas (n = 86)	qRT-PCR from frozen tissues	Snail mRNA expression correlated with disease-free and overall survival	Toyama et al., 2006
Ovarian carcinomas (n = 78)	RT-PCR and in situ hybridization from fresh effusions	high Snail mRNA expression predicts shorter effusion-free survival	Elloul et al., 2005
Gastric cancer (n = 48)	qRT-PCR from formalin-fixed tissues	reduced E-cadherin levels are accompanied by Snail mRNA overexpression in diffuse-type but not in intestinal-type tumors	Rosivatz et al., 2002
Esophagus (n = 154), cardia (n = 102), and stomach (n = 84); total carcinomas: n = 340	immunohistochemistry, formalin-fixed tissues (antibody: Sn9H2)	Snail immunoreactivity was generally rare; significantly more frequent in esophageal adenocarcinomas than in cardia or gastric carcinomas; no correlation with clinicopathological parameters	Rosivatz et al., 2006
Colon cancer (n = 32)	qRT-PCR from frozen tissues	overexpression of Snail mRNA in 69% of the cases, correlation with downregulation of vitamin D receptor	Palmer et al., 2004
Colon cancer (n = 25)	qRT-PCR from formalin-fixed tissues	no Snail mRNA expression in any of the 25 cases analyzed	Rosivatz et al., 2004
Colon cancer (n = 59)	immunohistochemistry, formalin-fixed tissues (commercial tissue array; commercial antibody)	Snail overexpression in 78% of cases, no immunoreactivity in stromal cells	Roy et al., 2005

Table 1 (continued)

Tumor type (number of cases)	Method of analysis	Major results	Reference
Endometrial cancer (n = 87) and metastases (n = 26)	Immunohistochemistry, formalin-fixed tissues (antibody: Sn9H2)	Snail immunoreactivity seen in 29% and 54% of the primary tumors and metastases, respectively; correlation to higher tumour grade and reduced E-cadherin expression in metastases	Blechschmidt et al., 2006, submitted
Head and neck, squamous cell carcinoma (n = 147)	immunohistochemistry, formalin-fixed tissues; commercial antibody	Snail immunoreactivity correlated with cervical node and distant metastasis	Yang et al., 2006
Synovial sarcoma (n = 40)	qRT-PCR from frozen tissues	elevated E-cadherin levels are associated with reduced Snail mRNA expression	Saito et al., 2004
Variety of cancers (n = ?)	immunohistochemistry, formalin-fixed tissues (antibody: EC3)	low percentage of Snail immunoreactive tumor cells, mostly detected in stromal cells close to tumor cells	Franci et al., 2006
Total cases evaluated: n = 2,112.			

sion. Additional studies, including those from Fujita et al. [2003], Zhou et al. [2004], Martin et al. [2005], Moody et al. [2005], Elloul et al. [2005], Côme et al. [2006], and Toyama et al. [2006], are generally supportive for a critical role of Snail in this disease.

Some results are difficult to interpret, e.g. Zhou et al. [2004] reported Snail immunoreactivity in breast cancer; however, the immunoreactivity was seen exclusively in the cytoplasm, at least in the photograph provided. To be active as a transcription factor, Snail has to be located in the cell's nucleus. Maybe the technique, immunohistochemistry, is not sensitive enough to detect Snail in the nucleus or the antibody used may cross-react with another protein. Thus, the meaning of these findings is unclear at present, although our own data suggest that it may be possible to detect nuclear Snail in breast cancer (fig. 1). From 106 tumor samples analyzed, we found nuclear Snail immunoreactivity in 33% [Becker, unpubl. observation].

Gastric Cancer

Gastric cancer, the second most frequent cause of cancer death in the world, can be divided into two major subtypes, diffuse-type and intestinal-type carcinomas. Intestinal-type carcinomas, the predominant type of tumor in high-risk areas, have a glandular pattern and are usually accompanied by papillary formation or solid components. The diffuse type, in contrast, consists of poorly cohesive cells diffusely infiltrating the gastric wall with little or no gland formation. In 2002, our group analyzed Snail in a series of gastric carcinomas using qRT-PCR from microdissected formalin-fixed tissues. For every patient, tumorous and nontumorous tissues were compared. We could show that Snail mRNA was expressed in the diffuse-type and associated with reduced E-cadherin levels. In the intestinal-type, Snail mRNA could not be detected [Rosivatz et al., 2002].

In a later study, we established a novel Snail-specific monoclonal antibody, termed Sn9H2, and analyzed gastric cancer samples by immunohistochemistry (n = 84). On the protein level, there was no association with nuclear Snail immunoreactivity and the diffuse type, indicating that Snail mRNA and protein levels may not correlate well. To provide additional evidence for the specificity of our novel hybridoma supernatant Sn9H2, we analyzed GC2957 gastric cancer cells. Using anti-Snail antibody Sn9H2 we could detect a weak band of about 32 kDa in GC2957 cells, suggesting expression of low levels of endogenous Snail (fig. 2). Several groups reported that Snail is affected by glycogen synthase kinase-3 beta (GSK3beta) which phosphorylates Snail and decreases its stability [Zhou et al., 2004; Yook et al., 2005] and inhibits its transcription [Bachelder et al., 2005]. We tested if treatment with LiCl, a known inhibitor of the kinase, results in stabilization of the protein that is recognized

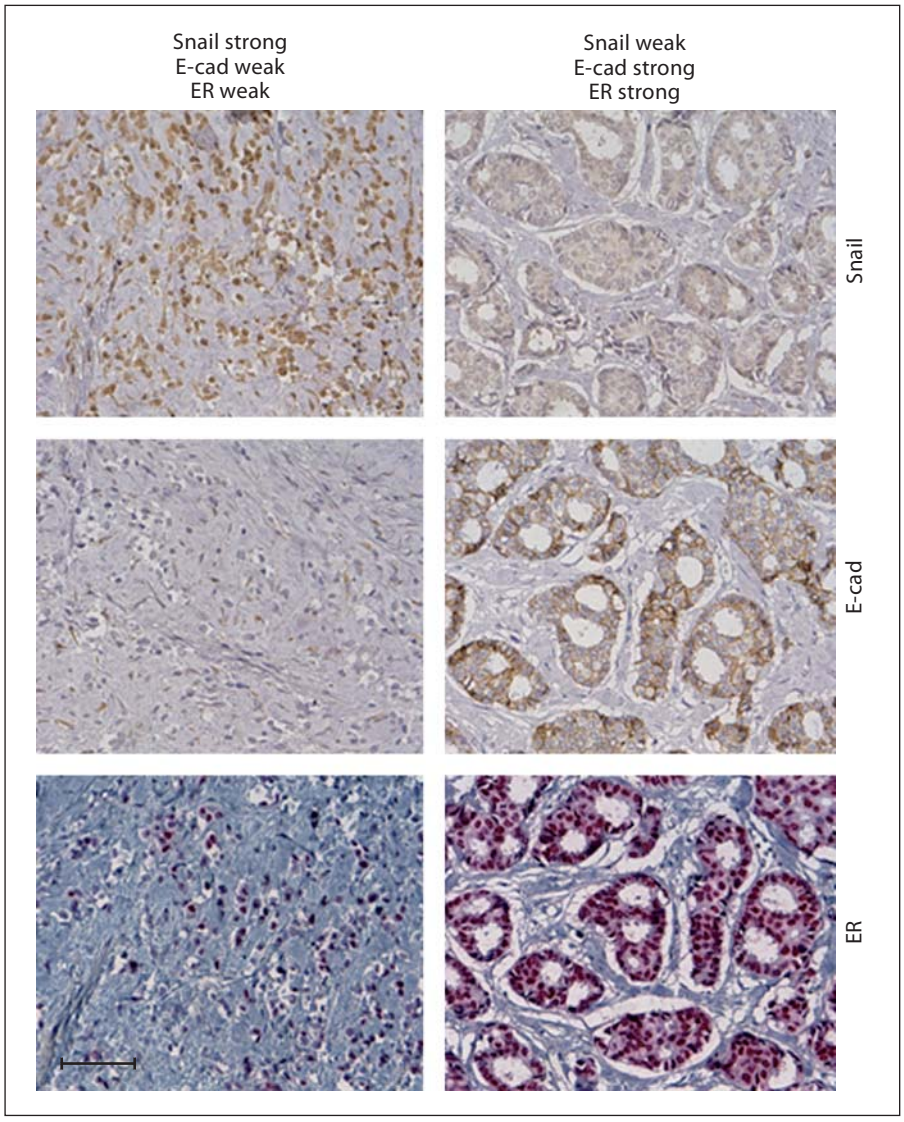

Fig. 1. Snail nuclear immunoreactivity in a breast carcinoma: invasive lobular carcinoma (grade G2) (left side) and invasive ductal carcinoma (grade G2) (right side). Strong nuclear Snail immunoreactivity is seen in the left example, E-cadherin (E-cad) and estrogen receptor alpha (ER) show weak immunoreactivity in this case. Scale bar = 100 μm.

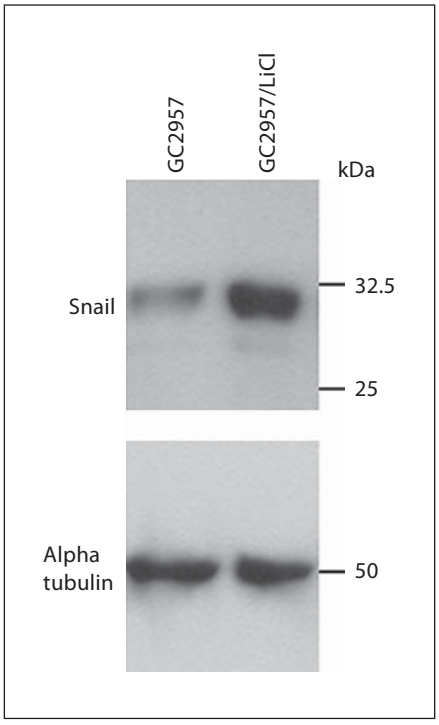

Fig. 2. Western blot analysis with our novel Snail-specific monoclonal antibody Sn9H2. LiCl treatment (inhibition of kinase GSK3beta) of GC2957 gastric cancer cells results in stabilization of a protein that reacts with our new hybridoma Sn9H2. Alpha tubulin (control) levels remained unchanged.

by our hybridoma Sn9H2. Indeed, after LiCl treatment the protein at about 32 kDa is much more prominent. Levels of alpha tubulin as a control did not change (fig. 2). Thus, this finding provides strong additional evidence that hybridoma Sn9H2 reacts with human Snail protein.

Colon Cancer

Colon cancer is one of the most frequent cancers in the world. In Europe, it is the second leading cause of cancer death, with 218,000 new cases diagnosed each year [Ferlay et al., 1999]. Several groups analyzed Snail in colon cancers, including our group. The first studies used qRT-PCR and showed a very low percentage of

Snail-positive cases in one report [Rosivatz et al., 2004], whereas a higher percentage of Snail-positive cases was seen in another report [Palmer et al., 2004]. The reason for this discrepancy could be that the precise contribution of stromal and carcinoma cells was not always assessed. In two subsequent studies, immunohistochemistry was performed using two different antibodies. The results, however, were again very different, which is probably due to the different antibodies used. In one study [Franci et al., 2006], most of the cases showed a very low percentage of Snail-positive cells. Snail immunoreactivity was mostly detected in stromal cells close to tumor cell islands. Some tumor cells also showed nuclear Snail immunoreactivity. Franci et al. [2006] intensively verified the specificity of their in-house made novel mouse monoclonal antibody EC3 against Snail. They could demonstrate that a 32-kDa protein band was seen in Western blots only when Snail was overexpressed. There was no cross-reactivity with Slug (Snail2). In contrast, Roy et al. [2005] reported that 78% of their series of colon cancers showed Snail immunoreactivity. They did not detect Snail positivity in the nontumorous mucosa; the tumor stroma was also negative. Own data for specificity of the commercial antibody used, however, were not provided by Roy et al. [2005].

Hepatocellular Carcinoma

Snail mRNA was quantified using real-time RT-PCR in 43 hepatocellular carcinomas and found to be overexpressed in 16% compared with adjacent noncancerous liver tissue. Those cases with Snail overexpression showed significantly downregulated E-cadherin protein expression, as detemined using immunohistochemsitry. Snail mRNA expression correlated with tumor invasiveness [Sugimachi et al., 2003].

Tumors of the Upper Gastrointestinal Tract (Esophagus, Cardia, and Stomach)

Besides 84 cases of stomach cancers mentioned above, esophagus (n = 154) and cardia (n = 102) carcinomas, arranged in tissue microarrays, were also examined for Snail expression by our group and correlated to E-cadherin expression and clinicopathological parameters [Rosivatz et al., 2004]. Nuclear Snail immunoreactivity was seen in 27 (7.9%) tumors and tended to be more frequent in esophageal adenocarcinomas (11.1%) than in cardiac (6.9%) or in gastric carcinomas (3.6%). In 35% of Snail-positive cases, E-cadherin immunoreactivity was lost. No correlation was found for nuclear Snail expression and tumor grade, Lauren's classification, WHO classification, tumor stage and tumor size. Besides in cancer cells, Snail immunoreactivity was found consistently in a small subset of stromal cells, i.e. fibroblasts and endothelial cells, inside and outside of the tumors. The pattern of Snail expression observed with our new hybridoma Sn9H2 suggests only a minor role for Snail in tumors of the upper gastrointestinal tract. This finding is in line with the results reported by Franci et al. [2006] for colon cancer mentioned above, suggesting that Snail protein is probably not responsible for the maintenance of the invasive phenotype of gastrointestinal tumors.

Head and Neck Squamous Cell Carcinoma

Head and neck squamous cell carcinoma (HNSCC) represents the sixth most frequent type of cancer worldwide. Major causes of HNSCC-related deaths are cervical lymph node and distant metastasis. In order to evaluate the clinical significance of Snail expression in HNSCC, Yang et al. [2006] analyzed 147 tissue samples arranged in tissue microarrays by immunohistochemistry. Increased Snail immunoreactivity was seen in 37% of the cases and was correlated with cervical node and distant metastasis.

Ovarian Cancer

Elloul et al. [2005] not only analyzed Snail expression in breast cancer (see above) but also in ovarian carcinoma, a major contributor to tumor-related morbidity and mortality in women. The objective of the study of Elloul et al. [2005] was to analyze Snail mRNA expression in ovarian carcinoma effusions. RT-PCR showed Snail mRNA expression in 87% (68/78) of ovarian carcinomas. There were extreme differences in mRNA expression between the cases (range 1–1059% of control value), which may be due to the sensitivity of the densitometric analysis used. Very low expression levels could be detected that may have been scored as negative by the naked eye, although the data were not correlated to results from real-time qRT-PCR.

Endometrial Cancer

Endometrial cancer is the most common gynecological cancer in the developed world. A recent study from our group aimed to investigate the expression of E-cadherin, Snail and estrogen receptor alpha in 87 primary tumors and 26 metastases of endometroid endometrial carcinomas [Blechschmidt et al., 2006]. Reduced E-cadherin immunoreactivity was seen in 44.8% of primary tumors and 65.4% of the metastases with a statistical correlation to higher tumor grade only in metastatic lesions.

28.7% of primary tumor specimens showed a positive Snail immunoreactivity which was correlated to reduced estrogen receptor alpha expression. Positive Snail immunoreactivity was also seen in 53.8% of the metastases where it was correlated to higher tumor grade and abnormal E-cadherin expression. The results of our study indicate that Snail plays a role in endometrial tumor progression and suggest that loss of estrogen receptor alpha signaling may lead to initial Snail expression.

Synovial Sarcoma

The only noncarcinoma study discussed here reported Snail expression in synovial sarcoma, which accounts for about 10% of all soft-tissue sarcomas. It typically arises from the para-articular region in adolescents and young adults. The origin of synovial sarcomas is uncertain. Saito et al. [2004] analyzed Snail in a series of synovial sarcomas by qRT-PCR. E-cadherin-positive cases showed statistically significant lower Snail expression than E-cadherin-negative cases. There was, however, no correlation between the histologic subtype (biphasic vs. other) and Snail expression.

Conclusions and Perspectives

In almost 20 years after the first description of Snail in *Drosophila melanogaster* [Boulay et al., 1987], only the last few years have demonstrated an increased interest in the study of Snail in clinical specimens. Now Snail comprises a superfamily of zinc-finger transcription factors whose best known function is the induction of EMT. For vertebrates three Snail gene family members have been identified: Snail1, Snail2 (Slug), and Snail3 [Barrallo-Gimeno and Nieto, 2005]. Only Snail1 was discussed here.

Snail genes are a convergence point in EMT induction and are activated by numerous signaling pathways. Snail activation induces the loss of epithelial markers and the gain of mesenchymal markers. Besides being tightly regulated at the transcriptional level, Snail's activity is also influenced by its subcellular localization. Snail can cycle between the nucleus and the cytosol by virtue of a nuclear export sequence. At least two kinases, GSK3beta and p21-activated kinase 1 (PAK1), are known to govern Snail's localization [Dominguez et al., 2003; Yang et al., 2005]. In addition, the zinc-finger transporter LIV-1 seems to be involved in this level of regulation, as shown for zebrafish [Yamashita et al., 2004]. The complexity of Snail's functional activation may explain the fact that in several tumors co-expression of Snail mRNA and E-cadherin protein has been observed. In addition, there may have been a lack of efficient translation despite increased Snail mRNA expression in some tumor samples. As mRNA levels may not correspond to the active Snail protein, which has a very short half-life, the recent establishment of Snail-specific monoclonal antibodies [Franci et al., 2006; Rosivatz et al., 2006] will be very useful to reveal protein expression and localization in additional tumor samples. Phospho-specific monoclonal antibodies, although not yet available for Snail, may be generated in the future for analysis of the functional status of the protein.

The current views of regulation of the Snail gene family [Thiery, 2002; Barrallo-Gimeno and Nieto, 2005; Radisky, 2005; Thiery and Sleeman, 2006] integrate results from cell culture studies, model organisms, and the analysis of healthy or diseased human tissues. It should be emphasized, however, that not all signalling pathways and all levels of regulation may be active in a given cell type or tissue. Moreover, it needs to be demonstrated that molecules identified to be important for the regulation of Snail in cells or tissues from model organisms have a similar function in human tissues. As more and more information is available for EMT in cells in culture and from model organisms, the molecules involved can be analyzed in human tissues. As mentioned above and taken as an example, in zebrafish embryogenesis recent work reported a link between the zinc transporter LIV-1 and Snail. Yamashita et al. [2004] demonstrated that LIV-1 is essential for nuclear localization of Snail in invasive organizer cells during gastrulation. LIV-1 was originally identified as an estrogen-regulated breast cancer protein with possible involvement in metastatic spread [Manning et al., 1994]. A putative network of ER → LIV-1 → Snail → E-cadherin → EMT → metastasis is thus inferred but has not been reported so far and is, therefore, currently being investigated in cancer tissues of hormonally regulated cancers, such as breast cancer.

Although much has been learned about Snail expression and its regulation with regard to tumor progression, very little is known about how other Snail family members and other EMT regulators (e.g. Sip1, Twist) work together and which signal pathways are activated in a certain type of cancer. Instead of the examination of only single molecules, e.g. Snail, the future will see analyses of complex signaling pathways in diseased human tissues, integrating all relevant players of EMT. Thus, signaling pathways, activated critical transcription factors, and their downstream targets will all be analyzed at the same time in a variety of well-characterized clinical tissues at

the protein level, including posttranslational modifications, such as phosphorylation. Recent developments for high-throughput techniques, such as protein lysate microarrays [Becker et al., 2006; Wulfkuhle et al., 2006], have the potential to unravel the role of Snail gene family members and EMT in the progression of cancer for the discovery of novel disease markers for clinical use.

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