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Actin-cytoskeleton polymerization differentially controls the stability of Ski and SnoN co-repressors in normal but not in transformed hepatocytes

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ABSTRACT

Background: Ski and SnoN proteins function as transcriptional co-repressors in the TGF-β pathway. They regulate cell proliferation and differentiation, and their aberrant expression results in altered TGF-β signalling, malignant transformation, and alterations in cell proliferation.
Methods: We carried out a comparative characterization of the endogenous Ski and SnoN protein regulation by TGF-β, cell adhesion disruption and actin-cytoskeleton rearrangements between normal and transformed hepatocytes; we also analyzed Ski and SnoN protein stability, subcellular localization, and how their protein levels impact the TGF-β/Smad-driven gene transcription.
Results: Ski and SnoN protein levels are lower in normal hepatocytes than in hepatoma cells. They exhibit a very short half-life and a nuclear/cytoplasmic distribution in normal hepatocytes opposed to a high stability and restricted nuclear localization in hepatoma cells. Interestingly, while normal cells exhibit a transient TGF-β-induced gene expression, the hepatoma cells are characterized by a strong and sustained TGF-β-induced gene expression. A novel finding is that Ski and SnoN stability is differentially regulated by cell adhesion and cytoskeleton rearrangements in the normal hepatocytes. The inhibition of protein turnover down-regulated both Ski and SnoN co-repressors impacting the kinetic of expression of TGF-β-target genes.
Conclusion: Normal regulatory mechanisms controlling Ski and SnoN stability, subcellular localization and expression are altered in hepatocarcinoma cells.
General significance: This work provides evidence that Ski and SnoN protein regulation is far more complex in normal than in transformed cells, since many of the normal regulatory mechanisms are lost in transformed cells.

1. Introduction

TGF-β is a pleiotropic cytokine that regulates a broad spectrum of cellular processes depending on cell type and context, such as proliferation, differentiation, migration and apoptosis [1]. The relevance of TGF-β/Smad signaling in controlling the liver homeostasis has long been recognized: TGF-β is a powerful cytostatic factor for hepatocytes, acting as an inhibitor of cell cycle and as an inducer of apoptosis, thereby controlling the liver growth [2,3]. Any deregulation within the pathway promotes an increased turnover of hepatocytes, and predisposes to liver fibrosis and/or hepatocarcinogenesis [3–8]. The versatility of TGF-β effects relies on the variety of Smad2, Smad3 and Smad4 heteromeric complexes formed, as well on Smad interaction with a plethora of transcription factors whose expression is cell context-specific [1,9].

Nuclear Ski and SnoN proteins are known as potent co-repressors of Smad transcriptional factors due to their capacity to interact with R-Smad2/3 and Smad4, and by recruiting elements of the repressor machinery (e.g. HDACs, mSIN3A, or NCoR) on TGF-β/Smad target gene promoters [10–15]. Importantly, TGF-β is the main known signal that controls Ski and SnoN protein stability, which uses activated Smad complexes to target Ski and SnoN proteins for polyubiquitination and proteasome-dependent degradation [16]. TGF-β also induces the up-regulation of SnoN mRNA and protein as part of a negative feedback loop that eventually turns off the TGF-β signal, restoring the basal conditions [17]. Despite the fact that there are many characterized TGF-β/Smad-regulated genes, so far, only the negative regulation of two

Abbreviations: Hep, HepG2 human hepatoma; AS, AS30D rat hepatoma; FH, freshly isolated rat hepatocytes; HCC, hepatocarcinoma; TGF-β, transforming growth factor beta1; TβR, TGF-β receptor I or II; pS2, phosphorylated Smad2; S2/3, Smad2/Smad3; CHX, cycloheximide; SB43, SB431542; ActD, actinomycin D; Jasp, jasplakinolide; CytD, cytochalasin D; LatB, latrunculin B; Colch, colchicine; WB, Western Blot; IP, immunoprecipitation; ChIP, Chromatin-immunoprecipitation

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genes, *smad7* and *sno/skil*, by Ski and SnoN co-repressors has been characterized [10,11,15].

Ski and SnoN proteins play important roles in regulating cell proliferation and differentiation [13,14]. Aberrant expression of these co-factors causes cell insensitivity to TGF-β signals, malignant transformation and alterations in cell cycle progression [14,18–21]. Likewise, it has been proposed that high levels of SnoN protein might lead to tumor growth whereas lower levels may favor tumor metastasis [20]. These findings strongly suggest that hyper- or hypo-expression of either Ski or SnoN may promote cell transformation.

Aside from the fact that Ski and SnoN can be transiently up-regulated during liver regeneration after partial hepatectomy or hepatic damage by carbon tetrachloride (CCl_4) treatment [22], little is known about their function and regulation in the context of a normal liver or in hepatocarcinoma (HCC). Herein, we set to characterize the regulation of Ski and SnoN protein stability in normal and transformed hepatocytes. We found major differences between the two cell contexts: in normal hepatocytes, Ski and SnoN proteins exhibit a short half-life, low stability, both nuclear and cytoplasmic subcellular localization, and are regulated by rearrangements of the cytoskeleton; importantly, TGF-β/Smad target genes show a transient up-regulation. Surprisingly, these features seen in normal cells are lost or deeply modified in hepatoma cells.

2. Material and methods

2.1. Material

Recombinant human TGF-β1 (TGF-β1) was obtained from PeproTech (Rocky Hill, NJ). MG132 (proteasome inhibitor) and SB431542 (SB43, TβRI receptor inhibitor) were obtained from Tocris Bioscience (Minneapolis, MN). Latrunculin B (LatB) and jasplakinolide (Jasp) compounds were obtained from Calbiochem-Millipore (Billerica, MA). Cycloheximide, actinomycin D and cytochalasin D were obtained from Sigma-Aldrich (St. Louis, MO). Anti-LaminB, anti-Ski (H-329), anti-SnoN (H-317), anti-Smad4 (C20) and anti-Smad2/3 (N-19) rabbit polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Smad2 rabbit polyclonal antibody was from Chemicon-Millipore (Billerica, MA). Anti-β-tubulin mouse antibody was from Cell Signaling (Danvers, MA). Secondary anti-rabbit or anti-goat HRP-coupled antibodies were from Zymed-Invitrogen (Waltham, MA), while secondary anti-mouse HRP-coupled antibody was from Santa Cruz Biotech. Culture media and reagents were obtained from GIBCO-Invitrogen (Waltham, MA).

2.2. Cell lines culture and treatment

Rat C9 cell line (normal hepatocyte-like phenotype) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and penicillin/streptomycin. Human hepatoma HepG2 cell line was maintained in minimal essential medium (MEM) supplemented with 10 % FBS, sodium pyruvate and penicillin/streptomycin. All cell lines were serum-starved for 12 h prior to each experiment; cells were treated with 0.3 nM TGF-β for indicated times. For acute Ca^{+2} removal, cells were pre-incubated for 10 min in an isotonic NaCl-based medium (140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 20 mM Hepes, pH 7.4 and 1 mM $CaCl_2$), and then incubated in the same medium without $CaCl_2$ plus 1 mM EGTA.

2.3. Rat hepatocyte primary cultures

Rat hepatocytes were isolated using the modified collagenase perfusion method from Berry and Friend as previously described [22]. Briefly, hepatocytes were isolated from collagenase-digested liver from Wistar rats and separated by centrifugation at 400 rpm for 2 min; viable hepatocytes (~95%) were isolated by iso-density percoll centrifugation (Amersham-GE Life Science, Pittsburg, PA), and cell viability was evaluated by trypan blue exclusion. Fresh hepatocytes (FH, cells in suspension) were resuspended in William's E medium (Sigma-Aldrich), maintained at 37 °C (5% CO_2) and used within 1–2 h after isolation. For primary culture, hepatocytes were seeded on plastic Petri dishes coated with 1% rat-tail collagen type 1 (Becton Dickinson, Franklin Lakes, NJ). Cultured hepatocytes (CH, adherent cells) were allowed to adhere for 4 h in the presence of attachment medium, and then cells were cultured for 24 h in feeding medium (serum-free attachment medium) [23].

2.4. AS30D rat ascites hepatoma cells

AS30D hepatoma cells were grown by intra-peritoneal inoculation in female Wistar rats as previously described [24]. The cells were isolated from the rat ascites fluid at 5 to 7 days after inoculation, and immediately counted, seeded ($20x10^6$ cells) in Petri dishes, and maintained at 37 °C (5% CO_2) for a maximum of 5 h in Williams E medium supplemented with 1 mM Hepes and penicillin/streptomycin.

2.5. Immunoprecipitation and Western blot

For protein analysis, cells were washed once with cold phosphate-buffered saline (PBS), and lysed with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet) plus a protease and phosphatase inhibitors cocktail. After protein quantification, 80 μg of protein was used for loading controls, and 1 to 3 mg of protein was used for the immunoprecipitation (IP) assays. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by Western blot (WB) using enhanced chemiluminescence assay (ECL) (Millipore) or SuperSignal West Pico kit (Pierce-Thermo Fisher Scientific, Waltham, MA). Densitometric analysis was carried out with ImageJ 1.47 free software and data expressed as fold change over control.

2.6. Cell fractionation

The protocol used for cellular fractionation was modified from Grewal *et al.* [25]. Briefly, AS30D, HepG2 or C9 cells were washed with cold phosphate-buffered saline (PBS) and resuspended in homogenization buffer (3 mM imidazole pH 7.4, 250 mM sucrose and protease inhibitors cocktail) followed by 20 passages through a 22-gauge needle. The homogenate was centrifuged at 707 ×*g* for 15 min a 4 °C and the cytoplasmic fraction (supernatant) was separated from the nuclear fraction (pellet). Each fraction was lysed in RIPA buffer for 1 h at 4 °C, and then centrifuged at 19,100 ×*g* for 5 min. Protein extracts from each fraction were analyzed by IP/WB.

2.7. RT-PCR

The *smad7*, *ski*, *snoN/snoN2*, *pai-1*, *gadd45b* and *β-actin* mRNA levels were detected by RT-PCR as previously described [11,15]. Briefly, total RNA was isolated using TRIzol (Invitrogen), and 2 μg of total RNA were used for cDNA synthesis using random hexamers (Roche, Basel, Switzerland) and M-MLV RT (Invitrogen). The PCR reaction was carried out using Taq PCR Master Mix kit (Qiagen, Venlo, The Netherlands) along with the specific primer sets described in the Supplementary Table S1, following the program: 95 °C for 5 min, 95 °C for 45 sec, 55 °C or 60 °C for 30 sec, 72 °C for 1 min. Products were analyzed by electrophoresis on 1.5 % agarose gels.

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out as previously described [15]. Briefly, cells were treated with 1 % formaldehyde at 37 °C for 15 min and then sonicated on ice for 8 cycles of 30 s each with a Fisher Sonic