Regulation of Ski co-repressor stability by TGF-β signaling in normal and tumorigenic hepatocytes

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Abstract

Transforming growth factor beta (TGF-β) is a multifunctional cytokine that regulates cell differentiation, proliferation and migration depending on cell context. TGF-β has a growth inhibitory effect on epithelial cells via activation of Smadproteins (R-Smad/Co-Smad complex). TGF-β signaling is negatively regulated by the proto-oncogene Ski (Sloan Kettering Institute). Ski in turn is degraded by TGF-β signaling (Smads) via ubiquitin proteasome system. TGF-β acts like a tumor suppressor in early stages of cancer due to its inhibitory growth effect, but in late stages it acts like a tumor promoter. The main aim of this thesis was to study the regulation of corepressor Ski stability in normal and transformed hepatic cells by TGF-β signaling and Smad proteins as well as determine Ski protein subcellular localization by using immunoprecipitation and Western blot assays for these studies. The obtained data showed that the TGF-β/Smad signaling pathway caused a downregulation of Ski protein through its degradation via proteasome in HepG2 cells. In addition, the Ski protein levels were restored when Smads were restored in C9 and hepatocytes even though the activated Smads were still present. In the subcellular fractionation studies it was observed that Ski protein was mainly localized in the nucleus of HepG2 cells, whereas it was localized in both nucleus and cytoplasm in hepatocytes. The presence of Ski in the cell cytoplasm could be explained because of low sensitivity in TGF-β signaling or translocation of Ski from nucleus to cytoplasm. C184M is a protein that binds to Ski in the cytoplasm and Ski in turn binds to Smads and inhibits their translocation from cytoplasm to nucleus, which in turn means inhibition of gene transcription that instead results in growth stimulation.
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1. Introduction

1.1 Liver

The liver is the largest gland in human body which is located under the diaphragm in the upper right part of the abdominal cavity. Connective tissues divide the liver into two lobes, a large right lobe and a smaller left lobe. The liver regulates many important metabolic processes, such as the carbohydrate metabolism, which is regulated in response to the carbohydrate levels in the bloodstream. Regulation of lipids is maintained via fatty acid oxidation. Protein metabolism is another important process that is maintained by liver, including amino acid conversion, urea production and formation of different types of bloods proteins (Hole et al. 1987). The liver is also an important organ for storage of different types of substances such as vitamin A, D, B_{12}, glycogen and iron. The liver is also responsible for secretion of bile, detoxification and blood filtration from damaged erythrocytes and foreign substances (Hole et al. 1987).

There are different types of cells in the liver: hepatocytes also called parenchymal cells or epithelial liver cells, endothelial cells, Kupffer cells, stellate cells and pit cells (large granular lymphocytes). Hepatocytes constitute the main population of cells in the liver, of around 80%, and the other 20% are the non-parenchymal cells (Gupta, 2007; Taub, 2004).

In different liver diseases changes in stable plasma proteins occurs such as accumulation and successively enhanced production of collagen and activation of hepatic stellate cells (HSCs). The HSCs normally are inactive but during injury they get active and lead to differentiation of myofibroblastic cells. Platelet-derived growth factor and transforming growth factor beta (TGF-β) are two major growth factors and cytokinases that stimulates kupffer cells to promote proliferation and transformation of HSCs (Gao et al. 2008).

The extra cellular matrix (ECM) of the liver encloses 3 % of its area. Gene expression, differentiation, migration and cell proliferation are examples of functions controlled by the ECM. Growth factors, cytokines and other types of factors are stored in the ECM in an inactive form and are activated in different processes like wound healing, and fibrogenesis (Bedossa and Paradis, 2003).

1.2 Transforming growth factor β (TGF-β)

An important growth factor stored in ECM is the TGF-β1, which regulates different cellular processes, playing different key roles in homeostasis and in different diseases (Bedossa and Paradis, 2003). TGF-β1 belongs to the multifunctional
cytokine superfamily of TGF-β. In mammals, TGF-β has three isoforms; TGF-β1, TGF-β2 and TGF-β3 (Clark and Coker, 1998).

To the TGF-β superfamily belongs also activin, anti Muellerian hormone (AMH), nodals and bone morphogenetic proteins (BMPs) and together they regulate cell differentiation, division, adhesion, localization, and death. These factors are active in different stages of cell growth and some of them are also active during development of embryo (Massagué et al. 2005).

TGF-β is produced as pre-pro-TGF-β with a length of 390-412 amino acids and by glycosylated it is converted to pro-TGF-β which in turn is cleaved to a latent complex by an endopeptidase called furin. The platelets contain granules, where the latent form of TGF-β can be stored and released from (Clark and Coker, 1998). Normally, TGF-β is bound to ECM as a latent inactive form (LTGF-β), its activation leads to its mobility toward an active profibrogenic structure, depending on changes in the microenvironment (Bedossa and Paradis, 2003).

1.3 TGF-β signaling pathway

TGF-β mediates its effect via serine/threonine kinase membrane receptor type I and type II (TRβI and TRβII respectively). Smads are proteins of approximately 500 amino acids. There are eight Smad proteins in humans and they are divided in three different categories depending on their action. Smads (1-3, 5 and 8) belongs to receptor regulated Smads (R-Smads). Common Smads (Co-Smads) is another group of Smad proteins, to this group belongs Smad number 4. Smad number 6 and 7 belong to the inhibitory Smads (I-Smads) group that negatively regulates the action of TGF-β (Massagué et al. 2005).

There are two main domains in Smad proteins, the C-terminal or Mad-homology 2 (MH-2) and the N-terminal, also called Mad-homology 1 (MH-1). MH-1 and MH-2 domains are connected by the linker region. MH-1 has the capacity to bind DNA since it contains a β-hairpin motif (Briones-Ortiz et al. 2011).

Activation of Latent TGF-β allows that its active form, TGF-β ligand, can bind the type II receptors (TβRII), that then recruits and phosphorylates the TβRI receptor at the GS domain. TβRI receptor in turn binds to and phosphorylates Smad2 and Smad3 (R-Smads). The activated R-Smads form a complex with the Co-Smad (Smad-4). This complex translocates to the nucleus, where it regulates gene transcription (Figure. 1).
Figure 1. TGF-β signaling pathway. TGF-β (Tβ) binds to TβRII that phosphorylate TβRI and together they phosphorylate R-Smads which in turn binds to Co-Smad. The R-Smad/Co-Smad complex translocates later to the nuclei. In the nuclei Smads exert their action as stimulators of gene transcription, with the help of coactivators and transcription factors.

Transcription factors such as the forkhead family members (FoxH and FoxO), RUNX, AP1 and E2F are necessary to complete the process of gene transcription. These transcription factors have different effects on a cell depending on the signal that is mediated by TGF-β. FoxO activates cyclin-dependent kinase inhibitor p21 Cip1/CDKN1A whereas FoxH have the opposite effect.

AP1 regulates interleukin11 and c-jun and the E2F family represses c-Myc, a proto-oncogene in epithelial cells. Coactivators and corepressors are other important factors that bind to Smads and transcription factors in order to inhibit or stimulate the gene expression. CBP and p300 are two main coactivators that bind to Smad proteins. Coactivators have the ability to acetylate the N-terminal of histones the consequence of this process is reduction of DNA compatibility (Massagué et al. 2005).

1.4 Dual role of TGF-β

TGF-β has a dual role in controlling the proliferation of different types of cells in the body. TGF-β has an inhibitory effect on growth of different cell types such as epithelial, lymphocytes and endothelial cells whereas it also has growth stimulating effects on fibroblastic cells (Sporn et al. 1986).

TGF-β controls the expression of proteins that are involved in growth inhibition such as p21, p15 and c-Myc. Stimulation of p21 and p15 is necessary to get an inhibitory effect on cell growth whereas c-Myc has to be repressed to achieve inhibition. Repression of c-Myc is performed by the TGF-β inhibitory element (TIE), that binds to promoter of c-Myc, this complex then binds to Smad 3 and 4, which in turn binds to the repression factor E2F and induces down regulation of c-
Myelinating to cell growth inhibition (Massagué et al. 2005). Cdc 25 A is a phosphatase that is involved in dephosphorylation of cyklin dependent kinases (cdks), which are activated by c-Myc. Cyclin/cdk complexes are important for a cell to continue the check points during the cell cycle. Interruption of a Cyclin/cdk complex by inactivation of cdks via repression of cdc 25A, which in turn is regulated by TGF-β, leads to cell cycle arrest (Liu et al. 2001). Some corepressors like transforming growth interacting factor (TGIF) and Sloan Kettering institute (Ski) can inhibit this process in a cell via their binding to Smad complexes and thereby deacetylate DNA in the cell nucleus. This gives Smads a dual role since it can acetylate the DNA by binding to coactivators and it can also deacetylate the DNA by binding to corepressors (Massagué et al. 2005).

1.5 Proteasome degradation

Smad levels are regulated by poly ubiquitination posterior degradation via proteasome. It’s a three step enzymatic process, which is dependent on the activity of E1 ubiquitin-activating enzyme, E2 ubiquitin- conjugating enzyme and E3 ubiquitin ligases (Zhang et al. 2000).

E3 Ubiquitin ligases are categorized into three groups: 1) Homologous to the E6-associated protein C-terminus (HECT also called WW or C2), 2) Really interesting gene (RING), and 3) U-box. Smadubiquitination-related factor 1/2 (Smurf 1/2) and Nedd4-2 belongs to the HECT family, they contain a WW domains that binds to a PY motif on the R-Smads and send them to degradation. Co-Smads can also be degraded by this system even though they lack a PY motif probably because they are attached to R-Smads, which have this motif. I-Smads are involved in regulation of TGF-β signaling via their interaction between the Serine/threonine receptors and Smurf 1/2 and Nedd4-2 via the PY domain that binds to the WW domain of members in the HECT family (Izzi and Attisano 2006). I-Smads act like adaptors that send the serine/threonine receptors to degradation (Izzi and Attisano 2006). The carboxyl terminus of the Hsc70-interacting protein (CHIP) is a member of the U-box family which has been shown to down regulate Smad 4 (Izzi and Attisano, 2006). I-Smads can also bind to DNA and inhibit the action of Co-/R-Smad complexes, which leads to inhibition of gene transcription (Briones-Ortaet al. 2011).

The growth inhibitory function of TGF-β is an important feature in cancer progression because in the early stages of a tumor, TGF-β acts like a tumor suppressor via its growth inhibitory pathways. TGF-β also causes growth inhibition by activation of apoptosis via p38 and p53 which activates the pro-apoptotic protein BIM in response to prevention of tumor progression (Heldin at el. 2009). This kind of TGF-β signaling can be inhibited by proto-oncogenes like Ski and SnoN (Schuster and Krieglstein, 2001).
1.6 Sloan-Kettering Institute (Ski)

Avian Sloan-Kettering retrovirus (v-Ski) was first discovered in chicken embryos as an oncogenic transforming protein (Luo, 2004). It occurs from cellular homologue ski (c-Ski) which is composed of 728 amino acids (Liu et al. 2001). It belongs to the proto-oncogenic Ski family. Another important protein that also belongs to this family is the Ski-like protein/ Ski related novel gene (Sno) (Macías-Silva and Briones-Orta, 2009). Ski have a C- and a N-terminal, the N-terminal contains a SAND domain which contain an interaction loop (I-loop) that binds to a L3 loop on the MH-2 domain in Smads (Luo, 2004). Once bound, Ski recruits the Nuclear Corepressor (N-CoR), that binds to mSin3A and this complex binds to histone deacetylase (HDAC) and represses gene transcription (Figure 2). Ski inhibits the acetylation effect of Smads by disturbing the interaction between Smads andcoactivators like P300 and their interaction with histone acetyltransferase (HAT) in the nuclei (Liu et al. 2001).

![Figure 2. Inhibition of gene transcription by Ski corepressor. To repress gene transcription, Ski can bind to the phosphorylated R-Smad/Co-Smad complex and other transcription factors. In order to recruit histone deacetylases (HDAC) and repressors.](image)

Ski is highly expressed in different carcinoma cell lines such as those from lung, stomach, melanoma, breast and prostate. Subcellular localization of Ski seems to have an important function during development of cancer. Malignant melanoma cells express high levels of Ski protein in the cytoplasm, in contrast to Ski levels during early stages of tumors and in normal cells were cytoplasmic Ski is found at low levels. Some other studies have also shown that the Ski and SnoN proteins can also act like tumor suppressors because their deficiency in heterozygous mice provoke that these mice to be more sensitive to develop tumors induced by chemical substances(Luo, 2004).

TGF-β regulates Ski levels via Smad proteins. Down regulation of Ski is mediated by TGF-β via Smads. The R-/Co-Smad complex ubiquitinate Ski by E3 ligases and send Ski for degradation by the proteasome (Figure 4).
Figure 4. Downregulation of Ski protein by TGF-β signaling. Ski binds to R-Smad/Co-Smads which recruit E3 ubiquitin ligases that ubiquitinate Ski and sends it to degradation via proteasome.

1.7 TGF-β and diseases

One of the main cytokines that is involved in fibrosis is TGF-β, which its levels are enhanced, leading to overproduction of extracellular matrix components like fibronectin and collagen. This state appears due to a not successful normal tissue repair system (Border and Noble, 1994).

In the late stages of a tumor, when it becomes invasive, the mode of TGF-β switches from a tumor suppressor to a tumor promoter. TGF-β activates the oncogenes like Ras and Raf, which promotes cell division and survival. TGF-β also causes acceleration of cancer metastasis by a biological process named epithelial mesenchymal transition (EMT), which consists in the loss of adhesion between epithelial cells. Loss of cell adhesion is due to downregulation of E-cadherin and delocalization of beta-catenin (β-catenin), which are important proteins in the tight junction of epithelial cells. Later, cells also lose the polarity and acquire a fibroblast-like morphology. EMT allows the translocation of the tumor cells from one part of the body to another part, a process named metastasis (Heldin et al. 2009).

2. Aim of thesis

The general aim of this thesis is to study the Ski corepressor subcellular localization and stability in normal and transformed hepatic cells by the TGF-β/Smad signaling.
3. Methods

Different types of cell lines were used in this thesis in the effect of TGF-β signaling on Ski subcellular localization and stability. Immortalized rat hepatocyte cell line C9, Human hepatoma cell line HepG2 (ATCC, U.K.) (see table 1) and primary cultured rat hepatocytes were used in this study. The normal hepatocytes were obtained from Wistar rats livers and primary cultured. The rats were anesthetized with chloroform in a gas chamber before the surgery of the rat was started. The isolated liver was washed with a Kreb-Ringer (see table 3) buffer (37°C) to remove the blood. The liver was then perfused with 20-30 mg collagenase type IV (Gibco, Sweden) to isolate the hepatocytes. The hepatocytes were separated from non-parenchymal cells by centrifugation and the cell viability was evaluated by trypan blue exclusion, i.e. dead cells had blue color of the nuclei staining. Hepatocytes were seeded on culture plates treated with collagen type I in the presence of attachment media. The attachment media was replaced by feeding media (Gibco, Sweden) and the cells were incubated with 5% CO2 at 37°C, with 95% humidity over night. Stimulation of the cells was carried out the day after with 0.3 nM of TGF-β (Gibco, Sweden). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Sweden) plus 10% fetal bovine serum (FBS) (Gibco, Sweden) and penicillin and streptomycin (Gibco, Sweden).

3.1 Immunoprecipitation (IP)

For detection of the Smads activity and Ski protein levels, immunoprecipitation followed by Western blot were used. Immunoprecipitation (IP) is used for detection of a protein of interest by identifying that protein through a specific antibody added to the protein mixture. The cells were lysed by adding lysis buffer (RIPA with protease and phosphatase inhibitor) (see table 4 and 5). The cells were then scraped from the petri dishes, and collected into microtubes. The tubes were incubated for 30 minutes at 4°C on a rotator so that the RIPA buffer with the inhibitors would insert its action. The protein-antibody reaction was performed by adding the antibody to a settled protein concentration (3-8 mg/ml) of the mixture. Another protein (G protein, Gibco, Sweden) was added; it bound to the antibody and made the mixture insoluble. The cells were incubated with G protein for 1-2 h. The G protein was diluted 1:5 in 0.1% TNTE buffer. Protein concentration was quantified by Bradford method. Primary α-ski rabbit polyclonal antibody (2μl) (Santa Cruz, USA) (see table 2) was added to the samples.

Subcellular fractionation of the cells was carried out to separate the nucleus from cytoplasm in order to detect the localization of Ski protein. Cells were lysed in homogenation buffer (1 ml) containing 0.5 % detergent Nonidet-P40 and protease and phosphatase inhibitor mixture. The proteins from the cell cytoplasm and nucleus were separated by centrifugation (3400 r.p.m.) at 4°C for 20 minutes.
3.2 Western blot

The immunoblotting/ Western blot technique was used to detect a protein by its binding to specific antibodies. The proteins from IP(3-8 mg/ml) samples were separated by SDS-PAGE(10 % gel). The proteins were then transferred to PVDF (Millipore, USA) membranes by electrotransferring chambers at 4°C for 2 h at 100 V.

The membranes were then blocked with 5% milk and the primary antibody was added and incubated O.N. Then, a secondary antibody coupled to HRP was added for 1 h. Chemiluminescence (Millipore, USA) was used for detection of protein signals on the PVDF membranes.

Table 1. List of cell lines that were used in the experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Species</th>
<th>Origin</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG 2</td>
<td>Human</td>
<td>Liver</td>
<td>Cancer</td>
</tr>
<tr>
<td>C9</td>
<td>Rat</td>
<td>Liver</td>
<td>Immortalized</td>
</tr>
<tr>
<td>Normal hepatocytes</td>
<td>Rat</td>
<td>Liver</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Table 2. List of anti-body’s that were used in the experiment.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Source</th>
<th>Secondary antibody</th>
<th>Company</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-β-tubulin</td>
<td>mouse</td>
<td>goat, anti-Mouse</td>
<td>Sigma</td>
<td>USA</td>
</tr>
<tr>
<td>α-Smad 2/3</td>
<td>rabbit</td>
<td>goat, anti-Rabbit</td>
<td>Millipore</td>
<td>USA</td>
</tr>
<tr>
<td>α-Ski</td>
<td>rabbit</td>
<td>goat, anti-Rabbit</td>
<td>Santa Cruz</td>
<td>USA</td>
</tr>
</tbody>
</table>

Table 3. Kreb-Ringer-solution.

<table>
<thead>
<tr>
<th>NaCl</th>
<th>7.33 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl (5.75%)</td>
<td>6.51 ml</td>
</tr>
<tr>
<td>K2HPO4 (10.55%)</td>
<td>1.63 ml</td>
</tr>
<tr>
<td>MgSO4 (19.1%)</td>
<td>1.63 ml</td>
</tr>
<tr>
<td>CaCl2 (7.85%)</td>
<td>2.67 ml</td>
</tr>
<tr>
<td>NaHCO3 (6%)</td>
<td>~14-18 ml</td>
</tr>
<tr>
<td>dH2O</td>
<td>Full up to 1 liter</td>
</tr>
<tr>
<td>Adjust pH</td>
<td>7.34–7.4</td>
</tr>
</tbody>
</table>
Table 4. RIPA buffer.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl pH 7.4, 50 mM</td>
<td></td>
</tr>
<tr>
<td>NaCl, 150 mM</td>
<td></td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td></td>
</tr>
<tr>
<td>0.5% DOC (Deoxycholic acid, sodium salt)</td>
<td></td>
</tr>
<tr>
<td>1% NP-40 (Nonidet P-40)</td>
<td></td>
</tr>
<tr>
<td>0.1% SDS (Sodium dodecylsulfate)</td>
<td></td>
</tr>
<tr>
<td>Tris-HCl pH 7.4, 50 mM</td>
<td></td>
</tr>
<tr>
<td>NaCl, 150 mM</td>
<td></td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td></td>
</tr>
<tr>
<td>0.5% DOC (Deoxycholic acid, sodium salt)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. RIPA buffer with inhibitors (lysing buffer).

<table>
<thead>
<tr>
<th>Phosphatase inhibitors</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaF (40X)</td>
<td>25μl/ml</td>
</tr>
<tr>
<td>NaPPi (100X)</td>
<td>10μl/ml</td>
</tr>
<tr>
<td>NaVO4(100X)</td>
<td>10μl/ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protease inhibitors</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF(200X)</td>
<td>5μl/ml</td>
</tr>
<tr>
<td>Trypsin inhibitor(500X)</td>
<td>2μl/ml</td>
</tr>
<tr>
<td>Pepstatin A(500X)</td>
<td>2μl/ml</td>
</tr>
<tr>
<td>Leupeptin(500X)</td>
<td>2μl/ml</td>
</tr>
<tr>
<td>Benzamidine(500X)</td>
<td>2μl/ml</td>
</tr>
<tr>
<td>Beta-glycerophosphate(500X)</td>
<td>2μl/ml</td>
</tr>
</tbody>
</table>

4. Results and Discussion

4.1 In normal hepatocytes, the downregulated Ski levels by TGF-β signal were shortly restored even in the presence of the activated Smads

TGF-β is a multifunctional cytokine that has different effects in different types of cells. TGF-β has a growth inhibitory effect on epithelial cells, the benefit of this cytokine is important especially in cancer, were in the early stages it have an inhibitory role on tumor growth. A balance in the expression of this protein in cells is also important since it has been shown that irregular amounts of this protein leads to development of different diseases like fibrosis and cancer. TGF-β together with the corepressor Ski have important roles in cancer, were Ski protein levels are very high compared with its levels in normal cells. In this study, three types of cells were used: normal hepatocytes from primary culture of Wistar rat liver, immortalized rat
hepatocytes C9 and hepatoma cell line HepG2 in order to understand the regulation of Ski corepressor by TGF-β/Smad signaling.

In order to detect the Ski protein levels regulation and the activation of Smads by TGF-β signaling in normal cells (hepatocytes), immortalized (cell line, C9) and tumorigenic (hepanonoma cell line HepG2) epithelial cells, an immunoprecipitation (IP) of proteins followed by Western blot analysis were performed.

A time course (0.5, 2, 6, 12, 20, 24, 30 and 48 h) for HepG2 cells was performed, were the cells were stimulated with 0.3 nm of TGF-β for up to 48 hours.

In Figure 5, it was observed that basal protein levels of Ski in hepatoma are higher than in normal cells, before cell stimulation with TGF-β (0 h, not treated cells/ control). Levels of Ski protein are downregulated, almost totally disappeared after stimulation with TGF-β in different time points (0.5, 2, 6, 12, 20, 24, 30 and 48 h). During the period of time where Ski was downregulated (30 min - 20 hours), phosphor Smad levels were increased. At 24 h after TGF-β stimulation, phosphor Smad levels decreased and then Ski protein levels were increased. The Smads proteins (p-Smads) are not found in the control (see Figure 1) whereas the Ski protein levels were high.

It is possible that activated Smad (phosphorylated Smad/ p-Smad) downregulated Ski protein levels by sending it for degradation via proteasome.

![Figure 5](image)

**Figure 5.** Time course analysis of the Ski protein levels regulated by TGF-β signaling in HepG2 cells (human hepatoma). The cells were treated with 0.3 nM of TGF-β at different time points (0.5, 2, 6, 12, 20, 24, 30 and 48 hours). In the control/untreated cells, Ski protein levels are highly expressed, and the cell treatment with TGF-β leads to Ski protein degradation. P-smad2 levels were also detected. Ski protein levels were restored at the basal level when the p-smad2 levels decreased.

Ski protein levels were also analyzed in C9 cells and in rat hepatocytes in order to study the regulation of Ski protein levels by TGF-β signaling in different cell lines. First, C9 cells were stimulated with 0.3 nM of TGF-β for 1 and 3 h (see Figure 6). Upon TGF-β stimulation, Ski protein was degraded after 1 h of treatment but it shortly restored its protein levels after 3 hours of treatment. The levels of p-Smad can be seen during 1 and 3 hours after TGF-β stimulation, interestingly Ski proteins levels
were restored even though the Smad proteins were still active. The levels of p-Smad2 in the controls were low comparing to the treated cells were the p-Smad2 levels increases (see Figure, 6).

Comparing the results from C9 cells with HepG2, it can be seen that independently of Smad activity and TGF-β signaling, Ski proteins are restored after 3 hours of stimulation in C9 cells. This means that TGF-β is signaling is active but that the activated Smad are not sending Ski for degradation, which is the case in HepG2 cells.

![Figure 6. Time course analysis (1 and 3 h) of the Ski protein levels regulated by TGF-β signaling in C9 cells. The cells were treated with 0.3 nM of TGF-β at different time points. In the control/untreated cells, Ski protein levels are highly expressed, and the cell treatment with TGF-β leads to Ski protein degradation. P-smad2 levels were also detected. Ski protein levels were restored at the basal level when the p-smad2 levels decreased.](image)

Another experiment was also performed for detection of Smad activity and Ski behavior in response to TGF-β signaling in normal hepatocytes from primary culture of Wistar rat liver. A time course of treatment was performed to compare the results from hepatocytes with C9 and HepG2 cells. The cells were stimulated with 0.3 nm TGF-β in different time points (0.5, 1, 3 and 6 h). The behavior of Ski in hepatocytes is very similar to its behavior in C9 cells, were cells treated with TGF-β shows that p-Smad2 are active and downregulates Ski after 30 minutes and after 6 hours Ski restores its levels (Figure 6 and 7). Comparing with HepG2 cells, the behavior of Ski and activated p-Smad2 are different from both C9 and hepatocytes because they have similar effect of TGF-β signaling and the remaining Smad activity independently of Ski restored levels. In the control the p-Smad2 is not presence comparing to its levels in treated cells.
Figure 7. Time course analysis (0.5, 1, 3 and 6 h) of the Ski protein levels regulated by TGF-β signaling in C9 cell line (rat hepatocytes). The cells were treated with 0.3 nM of TGF-β at different time points. In the control/untreated cells, Ski protein levels are highly expressed, and the cell treatment with TGF-β leads to Ski protein degradation. P-smad2 levels were also detected. Ski protein levels were restored at the basal level when the p-smad2 levels decreased.

4.2 Smads remain active for several hours after TGF-β stimulation

Hepatocytes were lysed to detect the lasting of Smad activity after TGF-β treatment. In Figure 8, a 36 hours time course is shown (0.5, 6, 12, 24 and 36 h) the cells were treated with 0.3 nM TGF-β from 30 min and up to 36 h. The results that were obtained where not expected, in Figure 8 it can be seen that Smads are active during all this time detecting that the TGF-β is signaling. In the controls the p-Smads are not present.

Figure 8. Time course (0.5, 3, 6, 12, 24 and 36 h) analysis of the phospho-Smad2 levels regulated by TGF-β signaling in hepatocytes. Cells were treated with 0.3 nM of TGF-β at different time points. Phospho-Smad2 protein levels were detected by IP/WB.

To understand the behavior of Ski protein levels depending on Smad activity and TGF-β signaling in hepatocytes, C9 and HepG2, the study was continued with experiment of time course of cell fractioning. Cell fractioning was carried out to separate the nuclear fraction from cytoplasm in order to study the localization of Ski, which was important to evaluate in normal cells as ski is a nuclear protein in cancer cells.
4.3 In normal hepatocytes, Ski showed a subcellular localization in both nucleus and cytoplasm. In contrast, Ski is in nucleus in hepatoma cells

A time course treatment (1, 2 and 4 h) for HepG2 was performed (see Figure 9), both nuclei and cytoplasm fractions was treated with 0.3 nm of TGF-β. It can be seen that Ski is not found in the cytoplasm but it appears in the nuclei while the cytoplasmic control (B-tubulin) is only found in the cytoplasm fraction. That in turn means that Ski is nuclear in this sample. In the nucleus cells treated with TGF-β leads to Ski degradation compared to the control were it is strongly appeared (Figure 9)

Treatment with TGF-β leads to down regulation of Ski by proteasome ubiquitin ligases (Ub), Arkadia, Smurf1/2 and APC via smads that acts like an adaptor between Ski and Ub. Beta- tubulin is a control which shows if the fractioning have worked or not since β- tubulin is normally found in the cytoplasm but not in nuclei.

![Figure 9](image)

Figure 9. Western blot of HepG2 cell fractions. The cells were treated with 0.3 nm of TGF-β in different time points (1, 2 and 4 hours). In the cytoplasm Ski cannot be seen. In the nuclei its found in the control. Treatment with TGF-β leads to down regulation of ski.

The experiment for cell fractioning was also performed in normal hepatocytes. The fractions (cytoplasm and nuclei) were treated with 0.3 nm of TGF-β for 3 hours (Figure 10). Ski is found both in cytoplasm and nuclei. Ski levels in the nuclei are higher than its levels in the cytoplasm and compared to the control,Ski is less expressed in cytoplasm than in nuclei. Ski is downregulated after 3 hour of TGF-β stimulation (Figure 10). Ski levels in the cytoplasm remains unchanged compared to Ski levels in the nuclei and the Ski found in the cytoplasm is not responding to TGF-β signaling, and is not controlled by Smads to be sent for degradation.
Figure 10. Western blot of hepatocyte cell fractions. The cells were treated with 0.3 nm of TGF-β for 3 hours. In the cytoplasm and in nuclei Ski is present. Treatment with TGF-β leads to Ski degradation in the nuclei but not in cytoplasm.

The reason for Ski appearing in the cytoplasm could be that the Ski protein is translocated from the nuclei to the cytoplasm. Another theory could be that ski in cytoplasm is not as sensitive to TGF-β signals as in the nuclei. In Figure 10, it can be seen that small amount of β-tubulin is found in the nucleus that means that the fractioning is not 100% clear. The reason could be due to cytoplasm residues that have been ended in the nucleus during the fractionation process. However, these founding may have been affecting the results of this experiments. The most important issue in this, Figure 10 is that β-tubulin highly expressed in the cytoplasm sense the observations were to find the Ski protein in the cytoplasm.

Kokura et al, reported that when Ski is found in cytoplasm it is bound to a protein called C184M and this complex inhibits translocation of R-Smads into the nuclei. Ski acts as an adaptor between C184M and Smads and inhibits their translocation (Kokura et al. 2003).

Localization of Ski in different parts of the cell (in nucleus and cytoplasm) is important because it indicates that Ski is not only inhibiting TGF-β signaling in the nucleus but also exert its action in cytoplasm. Ski found in cytoplasm can inhibit TGF-β signaling by recruitment to phosphorylated Smads which means that the Smads cannot enter the nuclei. Recruitment of Ski to Smads occurs when SAND domain of Ski binds to MH-2 domain of Smads(Luo, 2004).

Smad proteins are coactivators of gene transcription by acetylating DNA, which is the main factor for cell growth inhibition (Massagué et al. 2005). Coactivators are important for gene transcription because they activate different proteins like p21 and p15 which are active during inhibition of cell growth. Growth inhibitory proteins can be inactivated like in the case of ski inhibiting TGF-β signaling by binding to phosphorylated Smad and inhibiting them from translocating to the nuclei, which leads to cell growth. TGF-β affects oncogene activation, c-Myc which in turn affects the process of cell growth. Thus Ski localization is important for inactivation of the antiproliferative action of TGF-β in cells, both in cytoplasm and in nucleus (Massagué et al. 2005).
Comparing Ski localization in HepG2 and hepatocytes the conclusion is that in HepG2 Ski is nuclear and it is down regulated by TGF-β signaling via p-Smad2.

In hepatocytes Ski can be seen both in the nuclei and in cytoplasm but it is only downregulated in the nucleus. That means that Smad can send Ski to degradation and TGF-β is signaling, whereas in cytoplasm, Ski is not responding to TGF-β due to its binding to C184M (Kokura et al. 2003). Ski appearance in cytoplasm can also lead to blockage of translocation of phosphorylated Smads, which means inhibition of gene transcription and stimulation of cell growth.

Corepressors are also proteins that inhibit TGF-β signaling. TGIF and Ski are two main corepressors that inhibit TGF-β signaling. In this experiment Ski was studied, and it was found that Ski negatively regulates TGF-β signaling in the nucleus by binding to Smads and deacetylate DNA, leading to inhibition of gene transcription and cell growth. This behavior is important in cancer cells since when ski inhibits TGF-β signaling, cells continue to grow, leading to stimulation of tumor growth. In this study, ski was also detected in cytoplasm in hepatocytes, indicating that ski may have a negative feedback on TGF-β signaling in cytoplasm were it inhibits Smads to translocate to the nuclei and exerting their action. That in turn leads to stimulation of cell growth.

The ability of Ski to inhibit Smads from translocation to the nuclei could be due to the Ski-C184M protein complex that is found in cytoplasm (Kokura et al. 2003). Another theory is that Ski is not as sensitive to TGF-β signaling in the nuclei as in cytoplasm. Translocation of Ski from the nuclei to cytoplasm could be another reason for why the protein is found in cytoplasm of hepatocytes compared to HepG2 where it can only be found in the nuclei but not in cytoplasm. The Ski protein appearance in only nuclei but not in the cytoplasm in HepG2 cells could be that the Ski is only nuclear/ exerting its effect only in nucleus in tumorogenic cells.
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References


