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Transcriptional Regulation of Seprase in Melanoma by the Canonical Transforming Growth Factor-β (TGF-β) Signaling Pathway

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Abstract of the Dissertation

Transcriptional Regulation of Seprase in Melanoma by the Canonical Transforming Growth Factor-β (TGF-β) Signaling Pathway

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The tumor invasive phenotype driven by seprase expression/activity has been widely examined in an array of malignant tumor cell types, however very little is known about the regulatory mechanisms governing this critical protease. Human seprase (also named fibroblast activation protein-alpha (FAPα); antiplasmin-cleaving enzyme (APCE); dipeptidyl prolyl peptidase 5 (DPP5) is a 170 kDa homodimeric glycoprotein consisting of two 97 kDa subunits. Seprase is expressed at high levels by tumor cells and stromal fibroblasts in a variety of invasive carcinomas, but is essentially absent or undetectable in normal tissues except during active tissue remodeling in embryogenesis, and in the early stages of wound healing. Despite such confined expression to only highly invasive cell types, essentially nothing is known about the

transcriptional regulation of the seprase gene. The specific aims of this dissertation were firstly to determine if seprase was transcriptionally regulated in metastatic melanoma cell lines, and secondly to decipher which signaling pathway(s) and/or transcription factor(s) were involved in said regulation should it be occurring.

In my dissertation research, a significant portion of the human seprase promoter has been cloned. The transcriptional regulation of the gene has also been demonstrated in a pair of invasive melanoma cell lines with differential expression of the gene. In addition, a crucial TGF-β-responsive *cis*-regulatory element has been identified in the proximal seprase promoter region, which enabled robust transcriptional activation of the promoter as determined in promoter mutagenesis/reporter studies. Furthermore, treatment of melanoma cell lines with TGF-β1 caused a rapid and profound up-regulation of endogenous seprase mRNA, which coincided with an abolishment of the negative regulator c-Ski, and an increase in binding of Smad3/4 to the seprase promoter *in vivo*. Genetic blockade of TGF-β signaling by either stable overexpression of the transcriptional repressor c-Ski, or transient expression of a dominant negative form of the TGF-β Type II receptor in melanoma cells significantly reduced seprase mRNA levels. Consistent with genetic blockade of TGF-β/Smad signaling, treatment of cultured cell lines with the TGF-β RI chemical inhibitor SB-431542, or with a neutralizing antibody against the TGF-β ligand also severely impaired TGF-β-dependent seprase transcription.

Together, these data suggest that seprase is transcriptionally regulated in human melanoma cells via the canonical TGF-β signaling pathway. Our findings support the roles of both TGF-β and seprase in tumor invasion and metastasis.

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List of Abbreviations

- ANOVA: analysis of variance
- APCE: antiplasmin cleaving enzyme (also: DPP5, FAP-α, seprase)
- bp: base pair
- BP: Babe puro
- CCC medium: cancer cell culture medium
- DMEM: Dulbecco's modified Eagle's medium
- DMSO: Dimethyl sulfoxide
- DNA: deoxyribonucleic acid
- DPP4: dipeptidyl peptidase 4 (also: CD26)
- DPP5: dipeptidyl peptidase 5 (also: APCE, FAP-α, seprase)
- ECM: extracellular matrix
- EDTA: ethylenediaminetetraacetic acid
- ELISA: enzyme-linked immunosorbent assay
- kb: kilobase
- kDa: Kilo Dalton
- FAP-α: fibroblast activating protein (also: APCE, DPP5, seprase)
- FBS: fetal bovine serum
- mAb: monoclonal antibody
- mRNA: messenger RNA
- MMP: matrix metalloproteinase

- PBS: phosphate buffered saline

- RNA: ribonucleic acid

- qRT-PCR: quantitative Real-time polymerase chain reaction

- SBE: Smad-binding element

- SD: standard deviation

- SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

- TGF-β1: Transforming growth factor beta-1

- 5′ UTR: five prime untranslated region

I sincerely wish to thank my dissertation advisor and mentor, Dr. Wen-Tien Chen, whose advice, guidance, and support were critical in the completion of my studies and my dissertation project in its entirety for that matter. He gave me the freedom to explore and to grow independently as a scientist, yet was always there to keep me focused and productive. I am forever indebted to him for his support and mentorship during my graduate career.

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CHAPTER I: BACKGROUND

Tumor cell invasion involves the induction of the cell surface serine protease seprase (also named fibroblast activation protein-alpha (FAPα); antiplasmin-cleaving enzyme (APCE); dipeptidyl prolyl peptidase 5 (DPP5) in a variety of cancer tissues but the regulatory mechanisms governing the gene in metastatic tumor cells remain to be determined (O'Brien and O'Connor, 2008). The human gene encoding FAPα was originally cloned in 1994 (Scanlan et al., 1994), and was mapped to chromosome band 2q23 by fluorescence *in situ* hybridization (Mathew et al., 1995). Independently, seprase was identified and cloned from the LOX human metastatic melanoma cell line, and in fact deduced to be the same gene as FAPα (Goldstein et al., 1997; Aoyama and Chen, 1990).

Seprase was initially described in the LOX melanoma cell line and found to be a 760 amino acid type II transmembrane glycoprotein whose 97 kDa monomers can dimerize to form a 170 kDa enzymatically active gelatinase/dipeptidyl peptidase (DPP) complex (Aoyama and Chen, 1990; Goldstein et al., 1997; Pineiro-Sanchez et al., 1997). Although physiologic function(s) and substrate(s) of seprase have remained obscure for the most part, its gelatinase activity suggests a role in active tissue remodeling and invasion. Seprase has the ability to cleave gelatin and human collagen type I as observed in gelatin zymography experiments, but is unable to cleave other ECM proteins such as laminin, fibronectin, or collagen type IV (Pineiro-Sanchez et al., 1997). Seprase can work in concert with other proteases such as metalloproteases to cleave partially denatured collagen type I and type III in the process of ECM invasion (Christiansen et al., 2007). The DPP activity of seprase has been examined to a greater extent since it is the defining feature of DPP family members (DPP4, DPP2, DPP7, DPP8, DPP9)

(Kelly, 2005), which are all highly capable of cleaving the N-terminus of peptides with proline as the penultimate amino acid position (Park et al., 1999).

FAPα protein expression and proteolytic activity were independently identified in reactive tumor stromal fibroblasts, but not in tumor or endothelial cell types tested (Garin-Chesa et al., 1990; Park et al., 1999; Rettig et al., 1986). Seprase functions as a serine integral membrane protease and has been implicated in the cellular invasiveness of tumor cells, endothelial cells and fibroblasts of various human tumors (Goldstein et al., 1997; O'Brien and O'Connor, 2008; Pineiro-Sanchez et al., 1997; Chen et al., 2006; Huber et al., 2003; Iwasa et al., 2003; Kennedy et al., 2009; Goscinski et al., 2008; Jin et al., 2003). Specifically, seprase is up-regulated in infiltrating ductal carcinomas of the breast and in resulting tumor metastases (Kelly et al., 1998), as well as in peritoneal metastases in ovarian cancer (Kennedy et al., 2009; Zhang et al., 2007). Increased seprase expression has also been associated with a more aggressive disease state in colon cancer (Henry et al., 2007), and with lymph node metastases in human colorectal (Iwasa et al., 2003), pancreatic (Cohen et al., 2008), and gastric cancers (Mori et al., 2004).

Recently, the mouse FAPα promoter was cloned, shown to have some conserved regions as compared to the human seprase promoter, and basal transcription found to be regulated by EGR1 in a panel of human cancer cell lines (Zhang et al., 2010). In addition, *in silico* electronic northern blot has been carried out. Results indicated that normal tissues generally lack FAPα RNA signal, apart from the endometrium, whilst the majority of tumor tissues express FAPα RNA (Dolznig et al., 2005). FAPα gene expression was found to be up-regulated by a combination of interleukin-1 and oncostatin M in both chondrocytes and cartilage explant cultures (Milner et al., 2006). FAPα protein levels were found to be induced in FB20

leptomeningeal fibroblasts upon addition of either TGF-β1, 12-O-tetradecanoyl phorbol-13-acetate (TPA), retinoids, or a combination of TGF-β1 and TPA (Rettig et al., 1994). TGF-β1 was also able to induce FAPα protein expression in immortalized human dermal fibroblasts (Denys et al., 2008), as well as in the NIH3T3 fibroblast cell line (Chen et al., 2009b). Taken together, these results suggested to me the possibility that the TGF-β pathway may regulate seprase in some direct capacity.

TGF-β signaling controls a wide range of cellular processes, including differentiation, proliferation, embryonic development, tissue regeneration, apoptosis, cellular homeostasis, and regulation of the immune system (Shi and Massague, 2003). In the canonical pathway, the TGF-β ligand signals through a protein complex consisting of two type II receptors and two type I receptors, both of which are serine/threonine kinases. Upon binding of the ligand to this complex, the type II receptors phosphorylate and ultimately activate the type I receptors. Once activated, the type I receptors can phosphorylate and activate the so called receptor-regulated Smads (R-Smads), namely Smad2 and Smad3. The now activated R-Smads can form complexes and bind to the co-Smad, Smad4, forming heteromeric complexes, which can accumulate in the nucleus. These Smad complexes act as transcription factors in the regulation of target gene expression and can do so in both a positive or negative manner (Feng and Derynck, 2005; Massague et al., 2005).

Since the TGF-β pathway normally functions in an anti-proliferative, tumor suppressive fashion, alterations in components of the pathway such as deletions, mutations, down-regulation of positive regulators, and overexpression or amplification of negative regulators are observed in many types of human cancer (Levy and Hill, 2006; Kang, 2006). Contradictory to this, TGF-β can control processes such as tumor cell invasion, modification of the tumor microenvironment,

and modulation of immune system surveillance, all of which promote tumor progression (Massague, 2008; Korpal and Kang, 2010). In fact, TGF-β over-expression in some tumor types is correlated with tumor vascularization, metastatic progression, and poor patient prognosis (Derynck et al., 2001). However, precise elements involved in the induction of tumor cell invasiveness remain to be investigated.

Being that the majority of my studies were carried out using two metastatic melanoma cell lines (A375 and LOX) it is of interest to discuss TGF-β signaling in melanoma to some degree. Although there are no known genetic mutations in the TGF-β signaling pathway in melanoma the majority of studies point toward an increase in TGF-β ligand expression and production which correlates with tumor progression (Hussein, 2005; Javelaud et al., 2008; Rodeck et al., 1991; Rodeck et al., 1994; Rodeck et al., 1999). In addition, although normal melanocytes are quite sensitive to the anti-proliferative effects of TGF-β, melanoma cells are resistant to the growth inhibitory effects proportional to tumor progression stage, with metastatic melanoma cell populations being the least responsive to TGF-β-dependent growth inhibition as compared to melanoma cells originating from primary tumors (Krasagakis et al., 1999). Interestingly, despite being resistant to TGF-β-dependent growth inhibition, TGF-β is still quite capable of inducing Smad signaling and Smad-dependent transcription in melanoma cells (Rodeck et al., 1999). A possible explanation for such observations comes from the fact that melanoma cells typically express higher levels of two negative regulators of TGF-β signaling, SKI (c-Ski) and SnoN when compared with normal melanocytes (Fumagalli et al., 1993; Poser et al., 2005; Reed et al., 2001), and possibly that these repressors block transcription of negative cell cycle regulators such as p21.

Of particular interest for my studies is the SKI repressor protein, as the two melanoma cell lines used in my studies have differential expression and/or stability of the SKI transcriptional repressor. Interestingly, and as is the case for the Smad transcription factors, and for the TGF-β signaling pathway in general for that matter, there seems to be dual transcriptional activities of SKI. That is, SKI can coactivate or corepress transcription depending on its interactions with other transcription factors and the cellular context (Nicol et al., 1999; Nicol and Stavnezer, 1998b; Chen et al., 2003). Similarly, SKI can dually function either in a tumorigenic capacity as seen in melanoma cells (Chen et al., 2009a; Reed et al., 2005), whilst seemingly playing an antitumorigenic role in breast and lung cancer cells for instance (Le et al., 2008b). SKI has been shown to interact with a variety of proteins including Smad2, Smad3, Smad4, MeCP2, mSin3, N-CoR and SKIP (Medrano, 2003). SKI seems to be transcriptionally upregulated in melanoma (Fumagalli et al., 1993) and SKI protein levels have been shown to correlate with melanoma tumor progression (Reed et al., 2001). In melanocytes and primary noninvasive melanomas, SKI localizes predominantly in the nucleus. In primary invasive melanomas SKI can localize to both the nucleus and cytoplasm, and in metastatic melanoma tumors SKI localizes to both nuclear and cytoplasmic, or predominantly to the cytoplasmic compartment (Reed et al., 2001).

Extremely pertinent to my studies is that the SKI transcriptional repressor can exert its inhibitory effects in a variety of ways such as through the stabilization of inactive Smad complexes on the Smad-binding element, by blocking Smad-recruitment of transcriptional co-activators such as p300/CBP, or by recruitment of N-CoR co-repressor complexes (Akiyoshi et al., 1999; Luo et al., 1999; Suzuki et al., 2004; Wu et al., 2002). In addition, c-Ski protein could prevent transcription by direct interactions with Smad3 or Smad4, by disruption of the formation

of crucial R-Smad/co-Smad complexes (Ueki and Hayman, 2003; Wu et al., 2002), or by interacting directly with the activated TGF-β type I receptor (Ferrand et al., 2010).

The main objective of my dissertation was to determine if seprase is a transcriptionally regulated gene, more specifically in several metastatic tumor cell lines, and if so to decipher which signaling pathway(s) and/or transcription factor(s) are involved in this regulation. This is of great interest to our lab because data from our lab indicated that reducing/abolishing seprase expression in LOX melanoma cells led to a significantly diminished capability of the cells to metastasize to organs such as the lungs and liver in a melanoma xenograft animal model (D.Chen, W.T. Chen, unpublished data). To this end, a significant portion of the human seprase promoter was cloned and gene regulatory analyses were conducted to conclude that seprase is indeed a bona fide transcriptional target of the canonical TGF-β/Smad pathway in a pair of metastatic melanoma cell lines with differential expression of the gene.

CHAPTER II: MATERIALS AND METHODS

Cell Culture and Retroviral Infection:

The human malignant melanoma cell line A735 and human fibroblast cell line CCD-28SK were obtained from American Type Culture Collection (Manassas, VA). The HaCat keratinocyte cell line was originally described in Boukamp et al. JCB, 1988 (Boukamp et al., 1988), and the LOX human amelanotic melanoma cell line supplied by Dr. O. Fodstad (Fodstad et al., 1988). Cells were grown in Cancer Cell Culture (CCC) media which is a 1:1 mixture of DMEM (Gibco, Grand Island, NY) and RPMI 1640 (Gibco) supplemented with 10% Fetal Calf Serum (Gibco), 10% Nu-Serum (BD Biosciences, Bedford, MA), 1% L-glutamine (Gibco), 1% Penicillin-Streptomycin (Gibco), and 0.2% Fungizone (Gibco). The retroviral packaging cell line GPG29, supplied by Dr. M. Sadelain, was cultured and used as previously described (Ory et al., 1996). Briefly, transfections of retroviral plasmids were performed using Lipofectamine 2000 (Invitrogen). Post-transfection, GPG29 cells were cultured in CCC media. Virus-containing supernatants were harvested 24 and 48 hrs later and added to 70% confluent cultures of target cells in the presence of 8 μg/ml polybrene (Sigma) and incubated overnight. Cells were allowed to recover 24 hrs in CCC media. Stable cell lines were generated by selecting with 2μg/ml puromycin. All cell lines were maintained in a humidified 37°C incubator with 5% CO_2.

Plasmids:

Three wild-type (WT) human seprase promoter fragments of differing lengths: 2.637kbp (−2432 to +205), 1357bp (−1152 to +205), and 674bp (−469 to +205) were generated by PCR using the HotStar HiFidelity Polymerase Kit (Qiagen, Valencia, CA) according to manufacturer specifications, and C.H.O.R.I. BAC clone RP11-576I16 (Children's Hospital Oakland Research Institute, Oakland, CA) as template. Each promoter fragment was individually cloned into the KpnI and HindIII sites of the promoterless pGL4.15[luc2P/Hygro] (pGL4) luciferase reporter vector (Promega, Madison, WI). Site-directed mutagenesis to inactivate nine putative Smad binding sites, a TATA box, and a non-binding site identified in the WT-674bp promoter was carried out according to the DpnI-based QuikChange Site-Directed Mutagenesis Kit protocol (Stratagene, La Jolla, CA). Sequences of site-directed mutagenesis primers are located below in Table 1. All resulting plasmids were sequence verified and contained only the desired mutations. pBabe-Puro and pBabe-Puro/c-Ski retroviral constructs (He et al., 2003) were provided by Dr. K. Luo. 4x-SBE-Luc (Zawel et al., 1998) and $(CAGA)_9$-MLP-Luc (Dennler et al., 1998) are previously described TGF-β responsive reporters. pCMV5-TβRIIΔcyt is dominant negative version of the TGF-β Type II receptor and was provided by Dr. J. Massague. The pRL-TK control plasmid (Promega) contains the herpes simplex virus thymidine kinase promoter to provide low to moderate levels of *Renilla* luciferase expression in co-transfected mammalian cells and served as a transfection control in all luciferase experiments.

Table 1. Site-directed mutagenesis primers. List of Primers used to mutate various binding sites of the pGL4-Seprase 674bp WT promoter plasmid. All mutated bases are underlined and new base pair sequences written in place of WT sequences.

Site Mutated	Primer Sequence
SBE #1 mutant	
Forward	5′GCAGCCGTGGGTTTAAATAGTTGAATTTTTAAAC 3′
Reverse	5′GTTTAAAAATTCAACTATTTAAACCCACGGCTGC 3′
SBE #2 mutant	
Forward	5′GAAAAACATTTCTTTTTGCAATACCTC 3′
Reverse	5′ GAGGTATTGCAAAAAGAAATGTTTTTC 3′
SBE #3 mutant	
Forward	5′GCAATACCTCATAATCTTCTTTAGGAAAAAAAAGTGCAGTTA 3′
Reverse	5′TAACTGCACTTTTTTTTCCTAAAGAAGATTATGAGGTATTGC 3′
SBE #4 mutant	
Forward	5′AAAAAGTAGATATATGTTTAATGTAAAAACCTGCAAGTTTCATTATTTTA 3′
Reverse	5′TAAAATAATGAAACTTGCAGGTTTTTACATTAAACATATATCTACTTTTT 3′
SBE #5 mutant	
Forward	5′CCTCTGTATGTCAACGTAAGTTTTTGTTGGTGTAGTTACAAGG 3′
Reverse	5′CCTTGTAACTACACCAACAAAAACTTACGTTGACATACAGAGG 3′
SBE #6 mutant	
Forward	5′CTGTTTCTAATTTTAAAAAAATCTTTTGAAACTTGGC 3′
Reverse	5′GCCAAGTTTCAAAAGATTTTTTTAAAATTAGAAACAG 3′
SBE #7B mutant	
Forward	5′ CCAACTACAAAGACTTTTTTGGTCCTTTTCAACG 3′
Reverse	5′ CGTTGAAAAGGACCAAAAAAGTCTTTGTAGTTGG 3′
SBE #7A mutant	
Forward	5′ CCAACTACAATTTTAGACTTGGTCC 3′
Reverse	5′ GGACCAAGTCTAAAATTGTAGTTGG 3′
SBE #7AB mutant	
Forward	5′GCTTCAGCTTCCAACTACAATTTTTTTTTTGGTCCTTTTCAACGG 3′
Reverse	5′CCGTTGAAAAGGACCAAAAAAAAAATTGTAGTTGGAAGCTGAAGC 3′
SBE #8 mutant	
Forward	5′CCTTTTCAACGGTTTTCATTTTTCCAGTGACCCACGC 3′
Reverse	5′GCGTGGGTCACTGGAAAAATGAAAACCGTTGAAAAGG 3′
SBE #9 mutant	
Forward	5′CCAGTGACCCACGCTCTGATTTTAGAATTAGCTAACTTTCAAA 3′
Reverse	5′TTTGAAAGTTAGCTAATTCTAAAATCAGAGCGTGGGTCACTGG 3′
TATA mutant	
Forward	5′GTTACAAGGATGAGAAGGCTCCGAAACTTCCCTTGAGTCACTC 3′
Reverse	5′GAGTGACTCAAGGGAAGTTTCGGAGCCTTCTCATCCTTGTAAC 3′
Non-site mutant	
Forward	5′GAAACTTGGCACGGTATTTGGGAGTCCGTGGAAAGAAAAAAA 3′
Reverse	5′TTTTTTTCTTTCCACGGACTCCCAAATACCGTGCCAAGTTTC 3′

Quantitative real-time RT-PCR (qRT-PCR):

Total RNAs were purified from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the standard kit protocol. The following primer pairs were used for qRT-PCR: seprase (forward: 5′-TTGCCATCTAAGGAAAGAAAGG -3′ and reverse: 5′-TTTTCTGACAGCTGTAATCTGG-3′), c-Ski (forward: 5′-GAGGTGGAAGTTGAAAGCAGG-3′ and reverse: 5′-TCATGCAGGAACTTCTCTTTGG-3′ and the endogenous control β-actin (forward: 5′-AGATGACCCAGATCATGTTTGA-3′ and reverse: 5′-GCACAGCTTCTCCTTAATGTCA-3′). The QuantiTect SYBR Green RT-PCR Kit (Qiagen) was used to prepare PCR reactions according to manufacturer specifications. Serial dilutions of LOX cell total RNA were used to generate standard curves for every experiment on seprase expression. Inter-experimental data for seprase mRNA levels were comparable since LOX cell RNA standard was purified from a 500 cm^2 cell culture dish, pooled, quantified, and frozen as -80°C aliquots. RT-PCR conditions were: 50°C for 30 min, 95°C for 15 min; 40 cycles of 94°C for 15 sec, 59°C for 30 sec, 72°C for 30 sec and 76°C for 10 sec; 10 min at 72 °C. qRT-PCR was conducted on the Opticon II (MJ Research, Watertown, MA) and analyzed using OpticonMONITOR™ Analysis Software Version 3.0 (MJ Research). All samples were run in triplicate and non-template controls were included in each run. The RNA levels of seprase and c-Ski were normalized against β-actin in every case and experiments were repeated at least three times.

Luciferase Assays:

Assays were performed using the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer instructions and a SPECTRAmax M5 plate reader (Molecular Devices, Sunnyvale, CA) equipped with SoftMax Pro Software (Molecular Devices). Briefly, cells cultured in 12 well plates were transfected as indicated with .5 µg of seprase promoter plasmid (WT or mutant), 0.3 µg of the 4x-SBE-Luc plasmid, or 0.3 µg of the $(CAGA)_9$-MLP-Luc construct, respectively, and 50 ng of pRL-TK vector as an internal control. All transfections were carried out under standard protocols using Lipofectamine (Invitrogen), except in experiments comparing all 4 cell lines in which case Lipofectamine 2000 was used. Twenty-four hours post-transfection, cells were harvested and assayed for luciferase activity, normalizing reporter activity to *Renilla* luciferase control. In experiments with TGF-β1 addition, 2 ng/ml were added for the final six, or twelve hours of incubation depending on the experiment. All experiments were performed in triplicate and independently repeated at least three times.

Chromatin immunoprecipitation (ChIP):

ChIP assays were conducted using the Chromatin Immunoprecipitation Assay Kit (Upstate, Lake Placid, NY) following the provided standard protocol. Cells were sonicated on ice 5 times, 30 sec each time using a Kontes micro-ultrasonic cell disruptor with AS1 probe and an output of 55. This was optimized to consistently generate 200 - 500 bp fragments of cross-linked DNA. The following polyclonal antibodies were used for this assay: 4 µg anti-Smad2/3 (E-20, Santa Cruz, Santa Cruz, CA), 4 µg anti-Smad 4 (C-20, Santa Cruz), 4 µg anti-Ski (H-329, Santa Cruz), and for use as a negative control, 4 µg normal rabbit IgG (Cell Signaling Technology, Beverly, MA). The following primer pairs (flanking binding sites of interest) were used for real-time PCR: Smad site #5 (forward: 5′-GCAACATAAACCTGAACTGG-3′ and reverse: 5′-GCCCTCAAATGAACTGTGAG-3′): 134 bp amplicon, Smad site #7 (forward: 5′-ATTCAAAAGTCCGTGGAAAG and reverse: 5′-GCTAATTCTGTCTTCAGAGCG-3′): 125 bp amplicon, and control primers flanking a distal promoter region with no putative binding sites: Non-site (forward: 5′- TCACTCTCATCATTTCCCAC-3′ and reverse: 5′-GGATTCTATGACTGTGTGTGG-3′): 125 bp amplicon. The QuantiTect SYBR Green PCR Kit (Qiagen) was used to prepare PCR reactions according to manufacturer specifications. Dilutions of experimental input DNA were used for comparative analysis. Real-time PCR (QPCR) conditions were: 95°C for 15 min; 40 cycles of 94°C for 15 sec, 50°C for 30 sec and 72°C for 30 sec. (See qRT-PCR section for PCR run and analysis.) All samples were run in triplicate, non-template controls were included in each run, and experiments were independently repeated at least three times.

Enzyme-linked Immunosorbent Assay (ELISA):

To quantitatively determine the amount of TGF-β1 produced by melanoma cell lines, the Quantikine Human TGF-β1 ELISA kit (R&D Systems) was used according to kit instructions. Cell lines cultured in 12 well plates in full CCC media were grown to 70% confluency. Cells were then washed 3 times with 1X PBS and media changed to serum free CCC. 24 hr later conditioned media was collected and processed according to kit instructions. Only activated (bioactive) TGF-β1 is recognized by the kit, so samples must be acid activated beforehand, the readout indicating total TGF-β1 produced. To determine any bioactive TGF-β1 secreted, acid activation was skipped before conducting ELISAs. Values were normalized to account for differences in cell number at time of assay. In all cases readings were done in triplicate and experiments were performed at least three times.

Western Blotting:

Western blotting was carried out as previously described (Freudenberg and Chen, 2007) with minor modification. Whole-cell extracts were lysed in RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with PhosSTOP phosphatase inhibitor cocktail tablets (Roche, Mannheim, Germany), and Complete protease inhibitor cocktail tablets (Roche). Lysates were run on 10% SDS-PAGE gels. The following antibodies were used: antibodies against actin (AC-40, Sigma), TGFβ RII (H-567), TGFβ RI (R-20), Smad4 (C-20), Smad2/3 (E-20), and Ski (H-329), all from Santa Cruz, phospho-Smad2 (138D4) and phospho-Smad3 (C25A9) from Cell Signaling, and seprase (D8) generated in our laboratory and described previously (Pineiro-Sanchez et al., 1997).

Cell Treatments:

Cells were treated for the indicated amount of time with either 2 ng/ml or 5 ng/ml recombinant human TGF-β1 (R&D Systems), 20 ng/ml recombinant human latent TGF-β1 (R&D Systems), various concentrations of an anti-TGF-β neutralizing antibody (R&D Systems), 10 μM of the proteasomal inhibitor MG132 (Sigma), 10 μM of the TGF-β RI inhibitor SB-431542 (Sigma), or 100 μM of the RNA polymerase II inhibitor 5,6-dichlorobenzimidazole (DRB) (Sigma). Proper vehicle controls (water or DMSO) were used in all cell treatments that required it.

Statistical Analysis:

Statistical analyses were performed using GraphPad Prism 5 Software (GraphPad Software Inc, San Diego, CA). Two-way ANOVA and unpaired t tests were used to determine statistical significance. Values shown are the means ± SD. Statistical significance was defined as $P < 0.05$.

CHAPTER III: RESULTS

Identification of the Human Seprase Gene Promoter:

To identify and clone the human seprase promoter, the genomic sequence 5′ of the initiation codon was determined using GenBank™ (NCBI), and putative promoter fragments PCR-amplified starting from within the five prime untranslated region (5′ UTR) to upwards of about 2.6 kbp relative to ATG. Three fragments were generated: 2.637 kbp, 1357 bp, and 674 bp (Figure 1), all of which were identical in sequence to a region of chromosome 2 located 5′ of the seprase coding sequence (Genbank™ NT_005403.17). An Incyte (Open Biosystems) complete full-length human seprase cDNA sequence was available for analysis, although it had no accession number. The sequence was an exact match to human FAPα mRNA; GenBank™ sequence NM_004460.2, with the exception of being 1 bp longer at the 5′ end, and what we designate as the transcriptional start site (+1) (Figure 2). In comparison, this sequence is 168 bp longer on the 5′ end than the Mammalian Gene Collection seprase cDNA; GenBank™ accession BC026250.1, and 55 bp longer on the 5′ end than the most abundant transcript found in the HeLa cell line identified by 5′-RACE (Zhang et al., 2010). The full 5′ UTR of the human seprase gene identified spans 209 bp in length.

Putative seprase promoter fragments were cloned upstream of the luciferase gene into pGL4 and labeled as follows: pGL4-Sep2.637 (−2432 to +205), pGL4-Sep1357 (−1152 to +205), and pGL4-Sep674 (−469 to +205) and were used in subsequent reporter assays. A

canonical TATA box (−41 to −36) was readily identified 36 bp 5′ upstream of +1 (Figures 1 and 2), and found to be conserved between rat and human sequences (Figure 3). MatInspector™ bioinformatics software (Genomatix) was used to identify an array of putative *cis*-acting promoter elements. I systematically analyzed only the 674 bp putative seprase promoter since the transcriptional activity of this minimal 674 bp reporter was essentially equal to that of the larger versions (Figure 5). Putative canonical *cis*-elements found were an E-Box (−331 to −326), a c/EBP site (−180 to −176), 2 Ets family sites (−53 to −50; +75 to +78), an AP1 site (−27 to −21), and an LEF/TCF site (+113 to +120) (Figure 2).

I intentionally placed the MatInspector™ threshold low so as to identify any sites resembling known Smad3/4 binding sequences, in essence screening the entire 674 bp putative promoter for possible TGF-β-responsive elements. As a result, nine putative Smad sites were identified: #1 (−458 to −455), #2 (−368 to −365), #3 (−345 to −342), #4 (−245 to −241), #5 (−73 to −70), #6 (+34 to +37), #7A/B (+118 to +121/+122 to +125), #8 (+149 to +152), and #9 (+173 to +179) (Figure 2). The identification of putative Smad3/4 binding sites would be consistent with the induction of seprase expression in fibroblasts by TGF-β1 (Denys et al., 2008; Rettig et al., 1994), and further suggested that TGF-β may regulate the seprase gene at the transcriptional level.

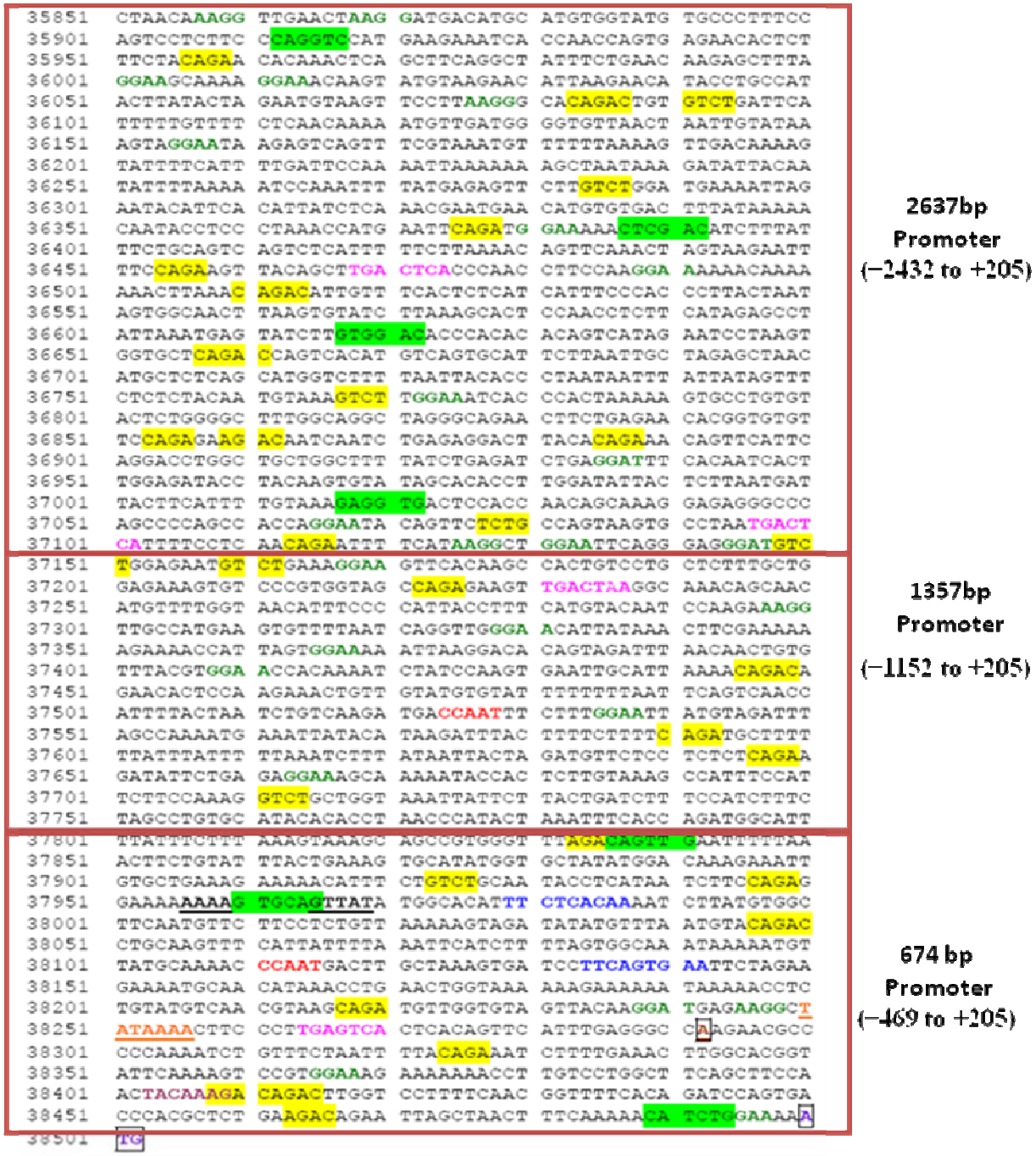

Figure 1. Cloning of human seprase promoter regions. Three wild-type seprase promoter fragments of varying sizes: pGL4-Sep2.637 (−2432 to +205), pGL4-Sep1357 (−1152 to +205), and pGL4-Sep674 (−469 to +205) were cloned into pGL4.15[luc2P/Hygro] empty reporter vector.

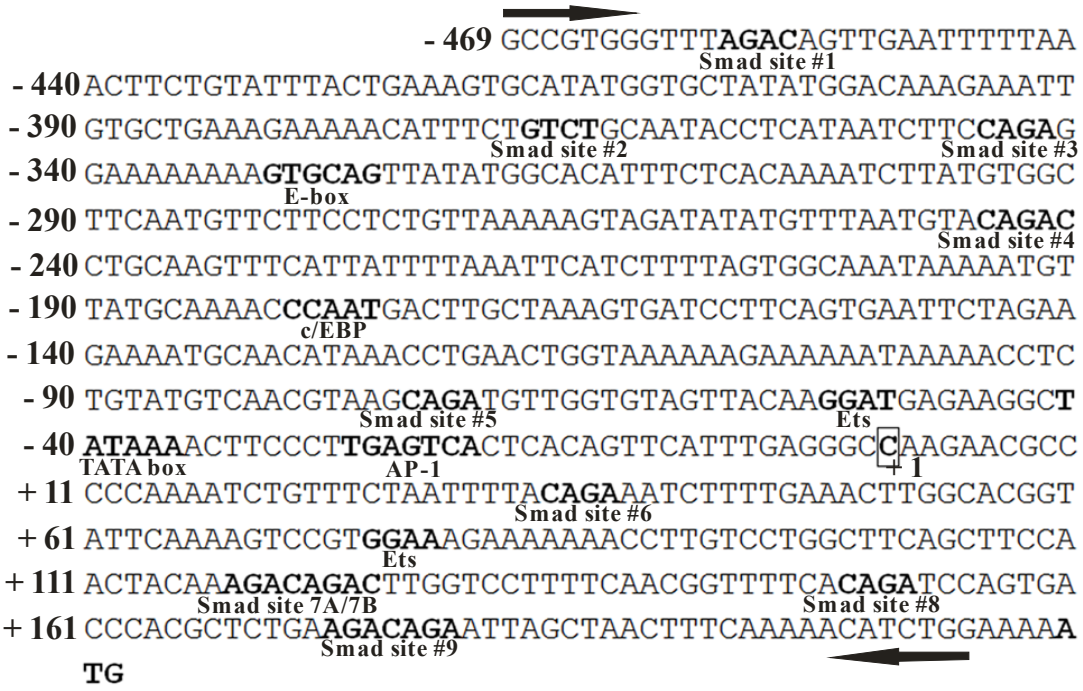

Figure 2. Characterization of the human seprase gene promoter (5′ regulatory region). DNA sequence of the 674 bp human seprase proximal promoter region spanning −469 to +205 and cloned upstream of the luciferase reporter gene. Black arrows designate the PCR amplified region. Essential promoter elements identified were the transcriptional start site (+1) based on the complete full-length human seprase cDNA sequence available, a TATA box at position −41 to −36, and a 209 bp 5′ UTR. Putative *cis*-regulatory elements are in bold and labeled according to which transcription factors putatively bind them. Putative Smad binding elements are labeled #1 through #9 starting from 5′ end.

Figure 3. ClustalW2 was used to align the 674 bp human seprase promoter with rat and mouse sequences. Putative Smad *cis*-binding elements are outlined and labeled #1 through #9. Other regions of interest are a TATA box (TATAAA) conserved between human and rat sequences, the human transcriptional start site designated by the C, and the translational start site designated by the ATG.

Endogenous Seprase Expression/Seprase Promoter Activity in a Panel of Four Human Cell Lines and Possible Implication of Transcriptional Regulation of the Seprase Gene.

To test the hypothesis that the seprase gene is transcriptionally regulated I conducted qRT-PCR for seprase gene expression using a panel of 4 human cell lines, including two metastatic melanoma (A375 and LOX). I found that both LOX and CCD-28SK cell lines had high levels of seprase mRNA relative to the A375 cell line, which had a moderate level, and to the HaCat cell line which was devoid of seprase mRNA (Figure 4). Parallel transient transfections in these cells comparing pGL4-Sep2.637, pGL4-Sep1357, and pGL4-Sep674 reporters to the empty pGL4-vector demonstrated that all 3 cloned putative seprase promoter fragments allowed for very high transcriptional activity in 3 out of the 4 cell lines tested (Figure 5), validating their ability to function as true gene promoters. Even the most minimal 674 bp promoter had ~45-, 100-, and 92-fold increases in activity above pGL4 in A375, LOX, and CCD-28SK cell lines, respectively. More importantly, the pattern of expression of exogenous luciferase transcribed off of the cloned seprase promoters was quite similar to the endogenous seprase mRNA level in that same cell line tested (moderate/high/high/no) (Figures 4 and 5). Most intriguing to me was the differential expression of seprase and promoter activity in the more closely matched A375 and LOX melanoma cell lines, so I focused on these cell lines for all subsequent studies.

Since in these promoter transfection experiments, differences in transfection efficiency were normalized for, and having observed that reporter levels expressed to essentially the same level as that of endogenous seprase mRNA in the very same cell type tested, I determined that the seprase gene might very well be regulated at the transcriptional level in these cells. However,

these experiments alone did not definitively prove that seprase is a transcriptionally regulated gene in these cells, as the seprase promoter regions I cloned encompass roughly 200 bp of seprase gene 5′ UTR. This 5′ UTR is in all likelihood being transcribed right ahead of the luciferase reporter gene mRNA and could therefore affect the stability of the nascent reporter transcripts, as might be the case with endogenous seprase mRNAs transcribed in these cells.

Support for my hypothesis that seprase is a transcriptionally regulated gene in cancer cells comes from a recent study that demonstrated the basal transcriptional regulation of the mouse seprase promoter by EGR-1 in six different human cancer cell lines (Zhang et al., 2010). Although this study failed to demonstrate *in vivo* binding of EGR-1 to the putative EGR-1 site, mutating the binding site showed a reduction in basal promoter activity in reporter studies, and was capable of binding to EGR-1 from protein lysates as seen in gel-shift experiments.

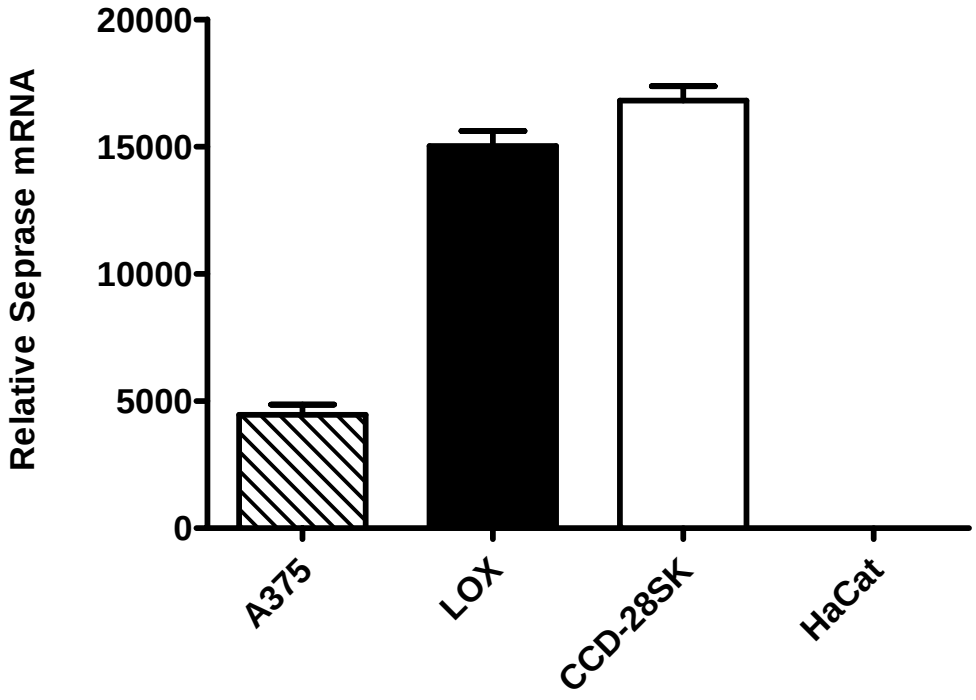

Figure 4. Endogenous seprase expression in panel of four human cell lines. Two metastatic melanoma cell lines: A375 and LOX, one non-transformed fibroblast cell line: CCD-28SK and one non-transformed keratinocyte cell line: HaCat were analyzed for seprase expression. Relative seprase mRNA levels were determined by qRT-PCR in the panel of cell lines with seprase mRNA levels normalized against β-actin levels for all samples. Results are the mean ± S.D. of three qRT-PCR results conducted in triplicate, n=9.

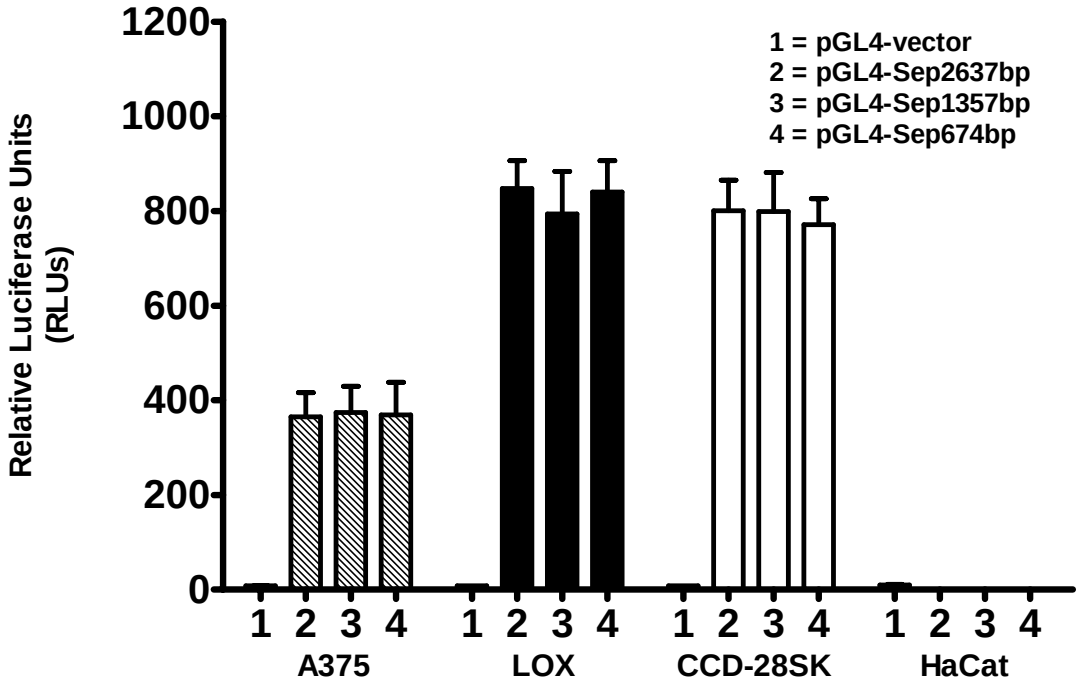

Figure 5. Wild type (WT) human seprase promoter activity in four human cell lines. Parallel transient transfections comparing reporter activity of WT seprase promoter regions pGL4-Sep2.637 (−2432 /+205), pGL4-Sep1357 (−1152/+205), pGL4-Sep674 (−469/+205) and the pGL4-vector in the indicated cell lines. All samples were normalized for transfection efficiency by co-transfection with pRL-TK vector. Reporter activity is expressed as relative luciferase units (RLUs). Results are the mean ± S.D. of triplicate samples from two of three experiments, n=6.

Additionally, A375 and LOX cell lines were treated with 100 μM of the protein kinase inhibitor 5,6-dichlorobenzimidazole riboside (DRB). DRB, a nucleoside analog, is an inhibitor of RNA synthesis by causing premature termination of transcription, as well as functioning as a casein kinase-2 inhibitor. DRB is a specific inhibitor of RNA polymerase II elongation and of certain protein kinases that may be involved in the phosphorylation of C-terminal domain of RNA polymerase II. Application of the drug significantly reduces the synthesis of mRNA and eliminates phosphorylated RNA polymerase II from cells (Dubois et al., 1994; Sehgal et al., 1976b; Sehgal et al., 1976a).

Treatment of A375 and LOX cells with 100 μM DRB showed significant and sustained decreases in the level of seprase mRNA in as little as 30 min of treatment ($P < .01$, n=6), with continual decreases throughout the entire time course of 24 hrs ($P < .01$, n=6) (Figure 6) as compared to vehicle treated control cell lines. In contrast, the β-actin housekeeping gene had no significant decrease in mRNA levels as compared to control non-treated cells, until 12 hrs of treatment and was a result of obvious cell death following such a prolonged treatment of the cells to the inhibitor (Figure 7).

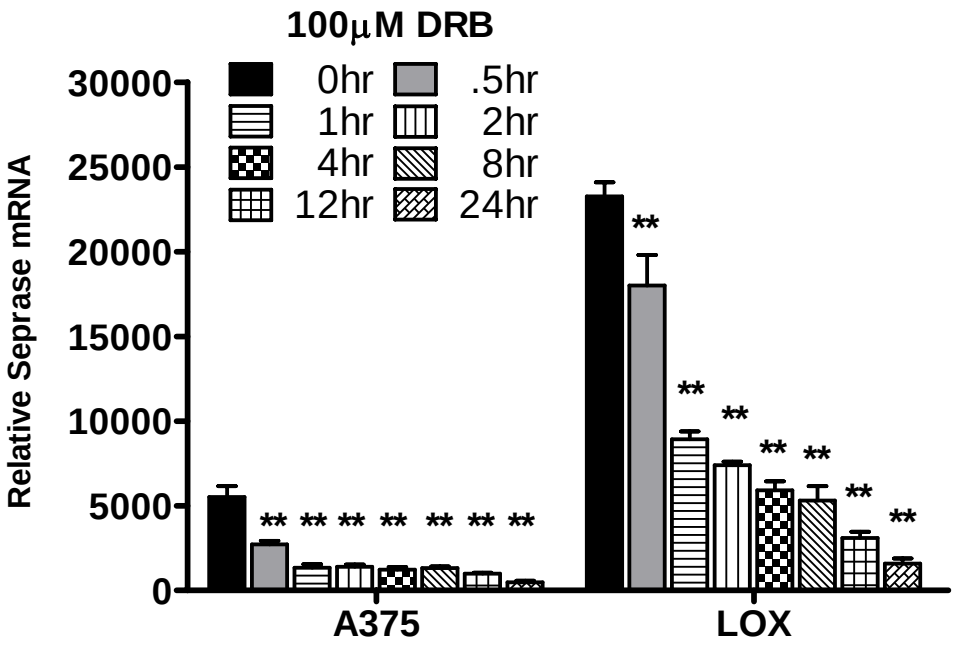

Figure 6. Effect of treatment of melanoma cell lines with 100 μM of the RNA polymerase II inhibitor 5,6-dichlorobenzimidazole (DRB) on seprase mRNA levels. A375 and LOX cell lines cultured in full CCC media were washed three times in PBS and treated or not with 100 μM of the RNA polymerase II inhibitor 5,6-dichlorobenzimidazole (DRB) in fresh CCC over a 24 hr time course. RNAs were harvested and seprase levels determined by qRT-PCR. Total input RNA was analyzed in parallel for normalization. Results are the mean ± S.D, n=6 of two of three experiments conducted, $P < .01$, indicated by **.

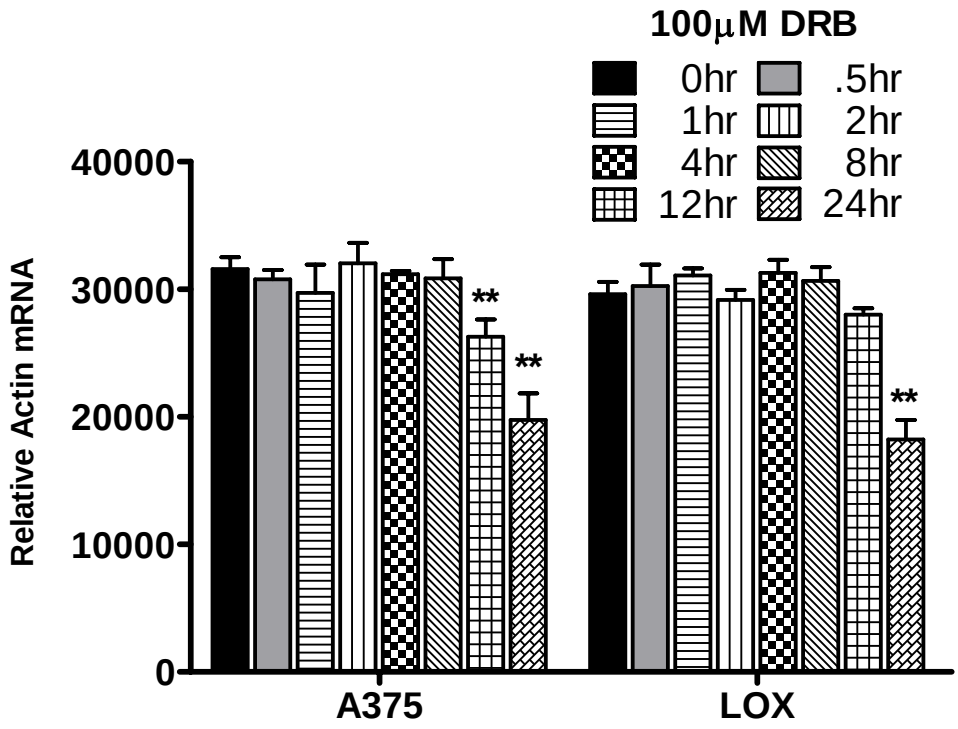

Figure 7. Effect of treatment of melanoma cell lines with 100 μM of the RNA polymerase II inhibitor 5,6-dichlorobenzimidazole (DRB) on β-actin mRNA levels. A375 and LOX cell lines cultured in full CCC media were washed three times in PBS and treated or not with 100 μM of the RNA polymerase II inhibitor 5,6-dichlorobenzimidazole (DRB) in fresh CCC over a 24 hr time course. RNAs were harvested and β-actin levels determined by qRT-PCR. Total input RNA was analyzed in parallel for normalization. Results are the mean ± S.D, n=6 of two of three experiments conducted, $P < .01$, indicated by **.

Taken together, all of the above results highly suggested to me the possibility that the transcriptional activity of the seprase promoter, as opposed to seprase mRNA stability, turnover, or negative regulation by siRNAs/miRNAs is the driving force behind the differential level of endogenous seprase mRNA observed although it remained to be more definitively proved at this point.

Expression and Functionality of Components of the Canonical TGF-β Signaling Pathway in A375 and LOX Melanoma Cell Lines

With my focus on a possible TGF-β-seprase connection, it was important to assess both the expression and functionality of the core components of the canonical TGF-β signaling pathway in the more closely matched A375 and LOX melanoma cell lines. If seprase was being regulated by TGF-β-Smad signaling in either of the cell lines they clearly should be expressing at least the most minimal and crucial proteins in the signal transduction machinery. In addition, since both melanoma cell lines express seprase, TGF-β signaling should be functioning and capable of driving TGF-β-dependent transcription should this signaling cascade truly be the major positive regulatory pathway governing the gene. To this end, western blotting using protein lysates from both A375 and LOX cell lines indeed revealed that all core proteins of the TGF-β signaling machinery were being expressed (Figure 8). Apparently equal levels of Smad2/3, Smad4, and TGF-β RI were expressed by each cell line. LOX cells expressed a higher level of TGF-β RII and had higher amounts of activated (phospho) Smad2 (P-Smad2), indicative of higher TGF-β signaling and in line with the increased seprase mRNA/protein observed, should TGF-β signaling truly up-regulate the gene. In contrast, A375 cells expressed the TGF-β signaling transcriptional repressor c-Ski in a considerably higher amount as compared to LOX cells which express almost no c-Ski protein (Figure 8) further supporting my hypothesis.

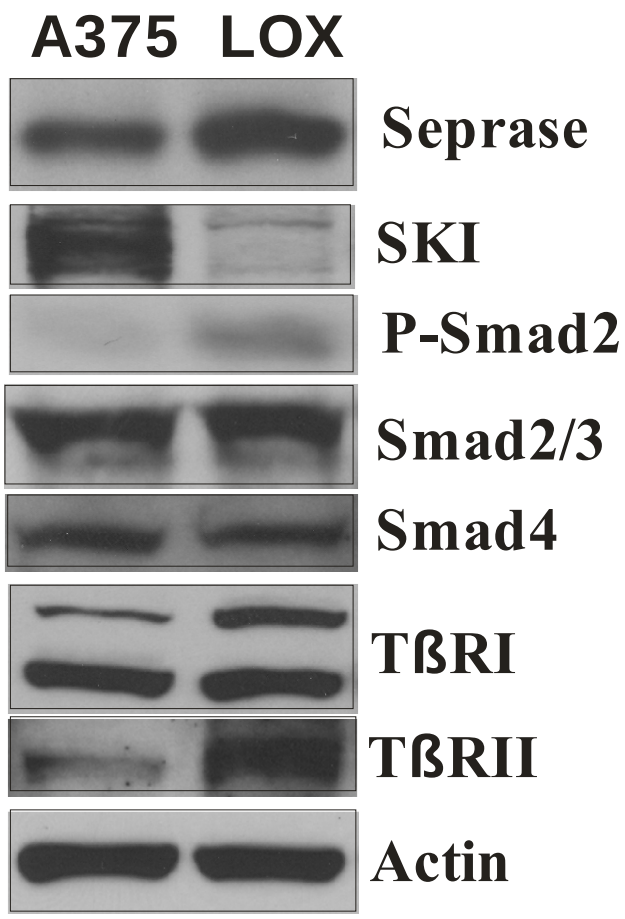

Figure 8. Analysis of the canonical TGF-β signaling pathway signaling proteins in A375 and LOX melanoma cell lines. Western blotting was performed using whole-cell extracts from A375 and LOX melanoma cell lines. The major signaling molecules in the canonical TGF-β signaling pathway were analyzed for their expression using the specific antibodies indicated. Note: c-Ski blot was overexposed to detect the low/no c-Ski in LOX cell line.

Conditioned media from cells cultured in serum free CCC for 24 hrs were measured for total TGF-β1 (Figure 9) as well as activated TGF-β1 production (Figure 10). LOX cells secreted a remarkable amount of TGF-β1, roughly 1500 pg/ml (~3-fold higher than A375 cells), and approximately 25 pg/ml of bioactive TGF-β1 (~3-fold higher than A375 cells). These results corroborate the higher levels of activated R-Smads found in LOX versus A375 cells, i.e. a more robust signal transduction due to higher amounts of TGF-β1 ligand available for signaling.

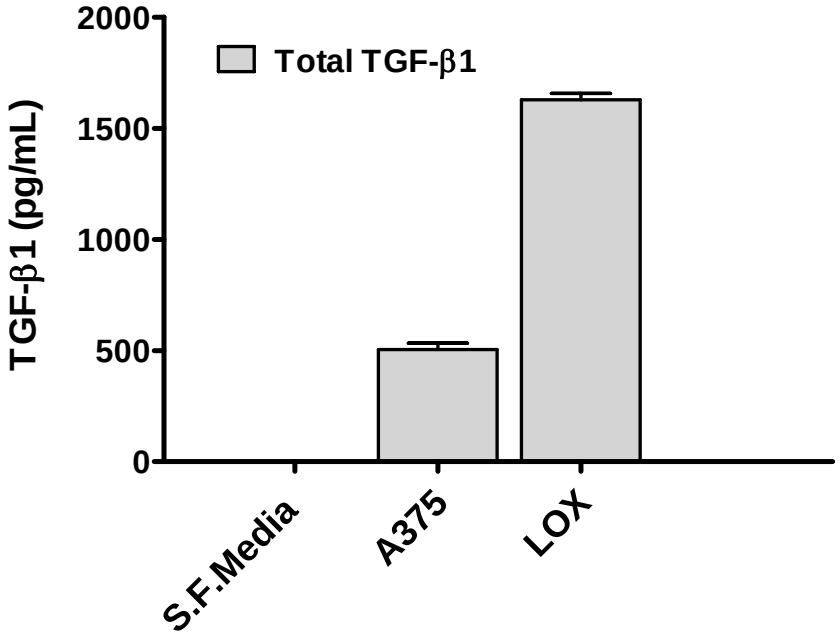

Figure 9. Total TGF-β1 production in A375 and LOX melanoma cell lines. TGF-β1 production by A375 and LOX cell lines was assessed by sandwich ELISA. Cells were cultured in CCC media at equal cell densities, washed three times in 1X PBS, and allowed to condition serum free CCC for 24 hr. Samples were acid activated prior to ELISA to measure total TGF-β1 production in pg/ml. Values were normalized to account for differences in cell number at time of assay. Results depicted are the mean ± S.D. of three ELISA assays, n=9.

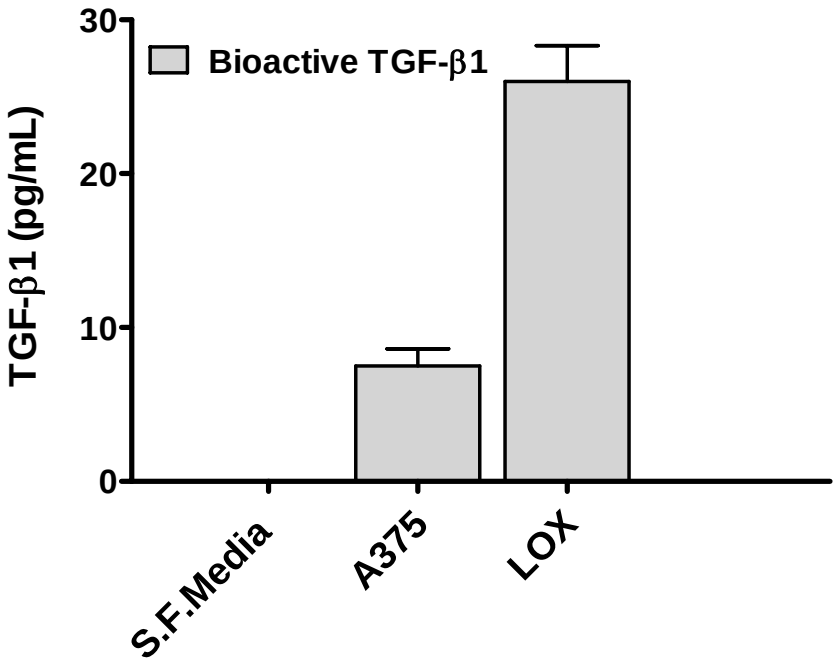

Figure 10. Bioactive TGF-β1 production in A375 and LOX melanoma cell lines. TGF-β1 production by A375 and LOX cell lines was assessed by sandwich ELISA. Cells were cultured in CCC media at equal cell densities, washed three times in 1X PBS, and allowed to condition serum free CCC for 24 hr. When using the kit samples are typically acid activated prior to ELISA to measure total TGF-β1 production in pg/ml. To measure bioactive TGF-β1 levels the acid activation step was skipped and bioactive TGF-β1 represented as pg/ml. Values were normalized to account for differences in cell number at time of assay. Results depicted are the mean ± S.D. of three ELISA assays, n=9.

To assess the functionality of TGF-β signaling, cell lines were tested for their ability to transactivate two different minimal TGF-β-responsive luciferase reporters in transient cell transfections upon stimulation with TGF-β1. As seen in Figures 11 and 12, transcriptional activity was observed for both reporters in both cell lines (untreated condition), the activity approximately 2-fold higher for each reporter in LOX versus A375 cells. These results are again in agreement with the higher activity of TGF-β signaling detected in the LOX cell line by western blotting.

A stimulatory effect of TGF-β was observed with both reporters in both A375 and LOX cells upon addition of exogenous 2 ng/ml TGF-β1 for 6 hr (~4- and 2-fold increases in reporter activity respectively) (Figures 11 and 12). Transcriptional activity of reporters in cells under treated conditions was essentially the same in each cell line indicating that both cell lines are equally equipped to activate gene promoters in a TGF-β-dependent context.

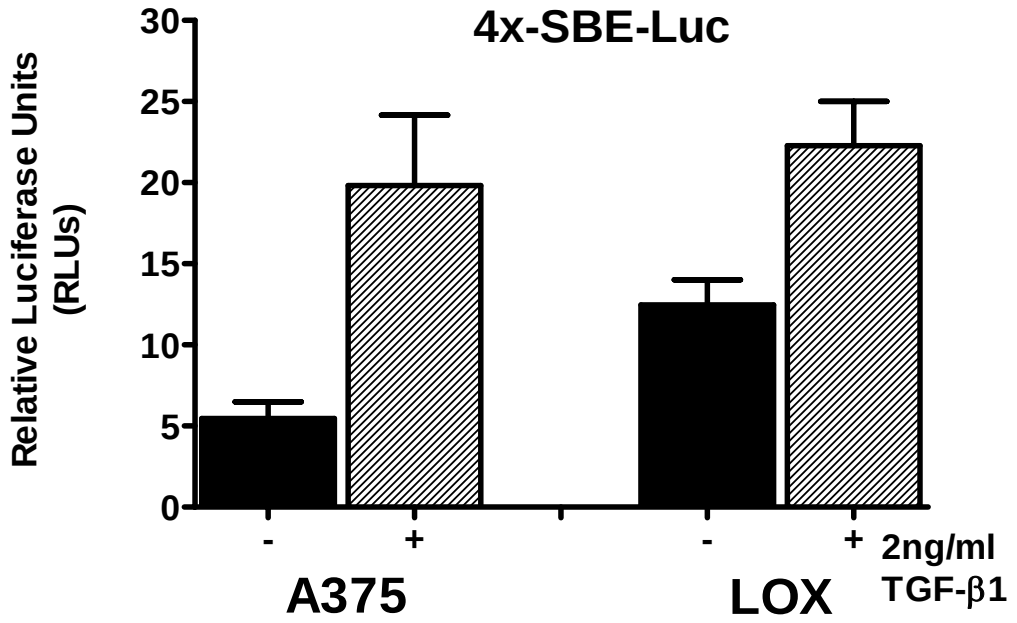

Figure 11. Functionality of TGF-β signaling in A375 and LOX cell lines. Activity of TGF-β signaling in A375 and LOX cells was determined by parallel transient transfection of the TGF-β-responsive reporter plasmid 4x-SBE-Luc into each cell line. Cells were untreated or treated with 2ng/ml TGF-β1 for 6 hr before luciferase activities were determined. Samples were normalized for transfection efficiency by co-transfection with pRL-TK vector. Reporter activity is expressed as relative luciferase units (RLUs). Results are the mean ± S.D. of triplicate samples from two of three experiments, n=6.

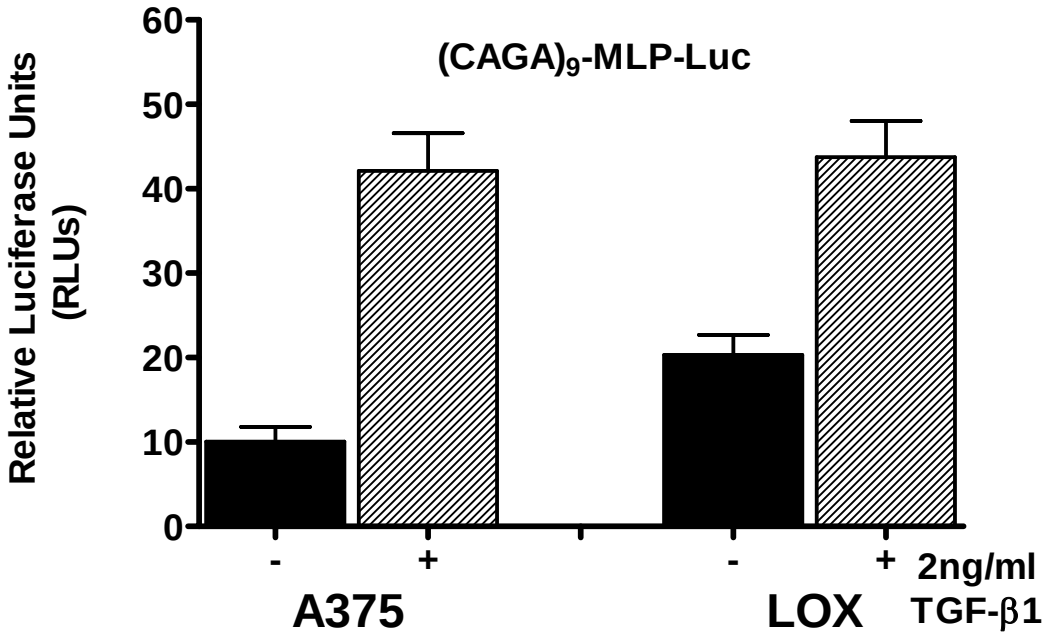

Figure 12. Functionality of TGF-β signaling in A375 and LOX cell lines. Activity of TGF-β signaling in A375 and LOX cells was determined by parallel transient transfection of the TGF-β-responsive reporter plasmid $(CAGA)_9$-MLP-Luc into each cell line. Cells were untreated or treated with 2ng/ml TGF-β1 for 6 hr before luciferase activities were determined. Samples were normalized for transfection efficiency by co-transfection with pRL-TK vector. Reporter activity is expressed as relative luciferase units (RLUs). Results are the mean ± S.D. of triplicate samples from two of three experiments, n=6.

TGF-β1 Increases Endogenous Seprase mRNA Levels and Seprase Promoter Activity

Treatment of A375 and LOX cell lines with exogenous TGF-β1 led to rapid and significant ($P < 0.05$, n=6) increases in the level of seprase mRNA in 1 hr (~2.5-fold) of treatment for both cell lines (Figure 13). Seprase mRNA levels remained significantly higher ($P < 0.05$, n=6 all time points) than baseline expression and continually increased over time, displaying apparently similar seprase mRNA levels by 12-24 hr of treatment (Figure 13). Accompanying this increase in seprase mRNA was a concomitant abolishment of the c-Ski repressor protein in A375 cells (Figure 14).

TGF-β-induced degradation of c-Ski in melanoma (including the A375 cells), as well as other cell lines has been described and is mediated by the ubiquitin-proteasomal degradation pathway (Sun et al., 1999; Le et al., 2008a; Nagano et al., 2007). I inferred that the removal of c-Ski, a known Smad transcriptional repressor, allows for this increased production of seprase mRNA, i.e. a higher amount of TGF-β-dependent transcription to take place. This differential expression/stability of the c-Ski repressor would explain the differential seprase mRNA levels observed in the A375 and LOX cell lines, and increases in seprase mRNA observed for both cell lines upon additional TGF-β1 treatment should this truly be a transcriptionally driven process.

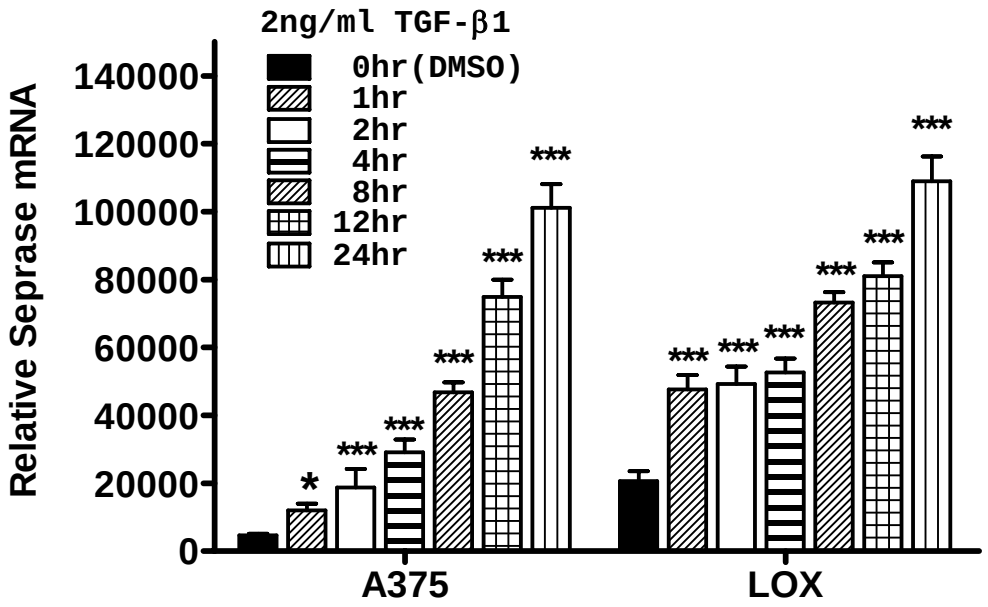

Figure 13. TGF-β1 treatment of melanoma cell lines increases endogenous seprase mRNA levels. A375 and LOX cell lines were serum starved for 8 hr and then untreated, or treated with 2ng/ml TGF-β1 over a 24 hr time course. Relative seprase mRNA levels were determined by qRT-PCR for all time points as indicated. Seprase mRNA levels were normalized to β-actin levels for all samples for comparison. Results are the mean ± S.D. of triplicate samples from two of at least three experiments, n=6. $P < 0.05$, indicated by *, $P < .001$, indicated by ***.

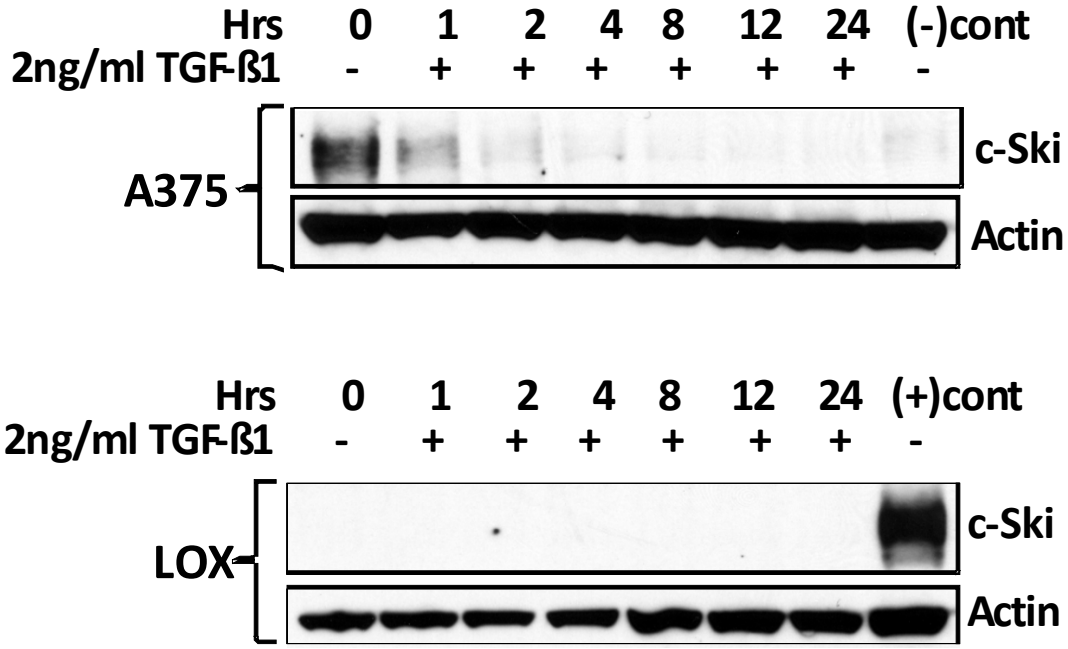

Figure 14. TGF-β1 treatment of melanoma cell lines induces degradation of the transcriptional co-repressor c-Ski. A375 (top) and LOX (bottom) cell lines were serum starved for 8 hr and then untreated, or treated with 2ng/ml TGF-β1 over a 24 hr time course. Western blotting was carried out using protein lysates from TGF-β1 time course experiment to assess c-Ski repressor protein levels in both A375 and LOX cell lines as is shown. (−) control is untreated LOX protein lysate for visualization of the reduction in c-Ski protein in A375 cells treated with TGF-β1. (+) control is untreated A375 protein lysate to validate that anti-c-Ski antibody/western was working due to the very low/no levels of c-Ski protein in the LOX cell line.

In addition, the LOX cell line was capable of processing latent TGF-β into bioactive TGF-β as treatment of cells with 20 ng/ml latent TGF-β1 led to significant increases in seprase mRNA by 12 hr ($P < .05$, n=3) which continued to increase upwards of 24 hr ($P < 0.001$, n=3) of treatment (Figure 15). No significant increases in seprase mRNA were observed in A375 cells upon treatment with latent TGF-β1 indicating that these cells seemed to be incapable of processing latent TGF-β1 outside the cell.

These observations could again explain the higher amount of TGF-β activity observed in the LOX cell line as compared to the A375 cell line. That is, the LOX cell line not only produces and secretes higher levels of the TGF-β1 ligand, but can also more efficiently process any latent TGF-β in the localized environment that might be produced but not activated within the cell so as to increase the pool of available bioactive TGF-β1.

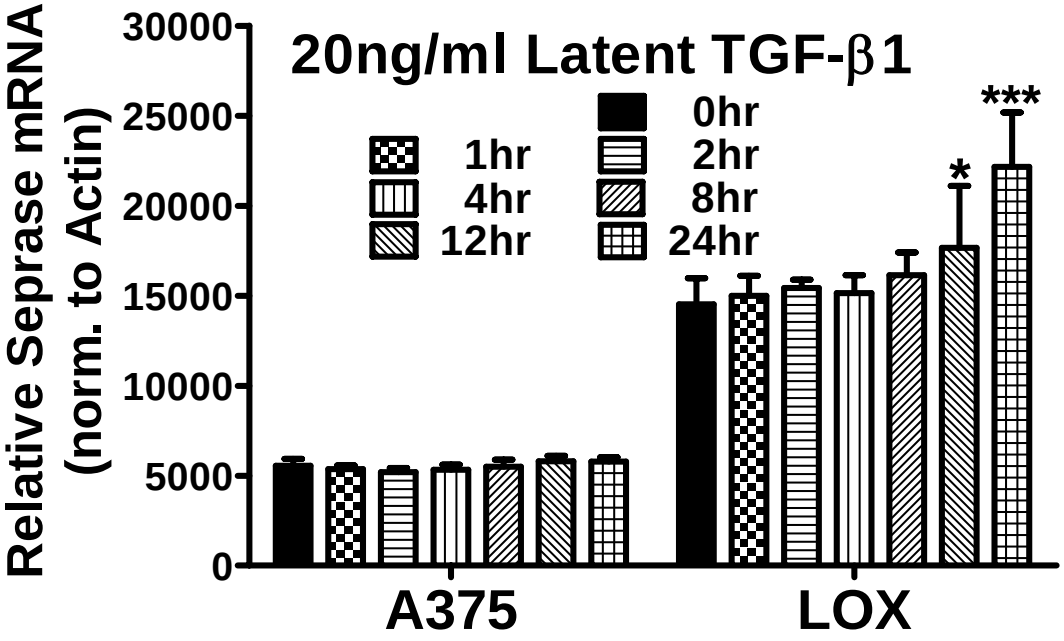

Figure 15. Treatment of melanoma cell lines with latent TGF-β1 increases seprase mRNA production in only the LOX cell line. A375 and LOX cell lines were serum starved for 8 hr and then untreated, or treated with 20 ng/ml latent TGF-β1 over a 24 hr time course. Relative seprase mRNA levels were determined by qRT-PCR for all time points as indicated. Seprase mRNA levels were normalized to β-actin levels for all samples for comparison. Results are the mean ± S.D. of triplicate samples from one representative of at least three experiments, n=3. $P < 0.05$, indicated by *, $P < .001$, indicated by ***

To determine if the seprase promoter regions cloned upstream of the luciferase gene were TGF-β-responsive, parallel transient cell transfection experiments were conducted in both A375 and LOX cell lines. I found that pGL4-Sep2.637, pGL4-Sep1357, and pGL4-Sep674 reporters all had robust transcriptional reporter activity as compared to empty pGL4-vector, and were all activated ~1.5- to 2-fold higher in LOX versus A375 cells in the untreated condition (Figure 16). A stimulatory effect of TGF-β was observed for pGL4-Sep2.637, pGL4-Sep1357, and pGL4-Sep674 plasmids, and not seen with pGL4, in both cell lines, in response to 12 hr of treatment with 2ng/ml TGF-β1 as compared to untreated cells (Figure 16). Seprase promoter plasmids were induced approximately 6-fold in A375 cells and 4-fold in the LOX cell line in response to treatment respectively.

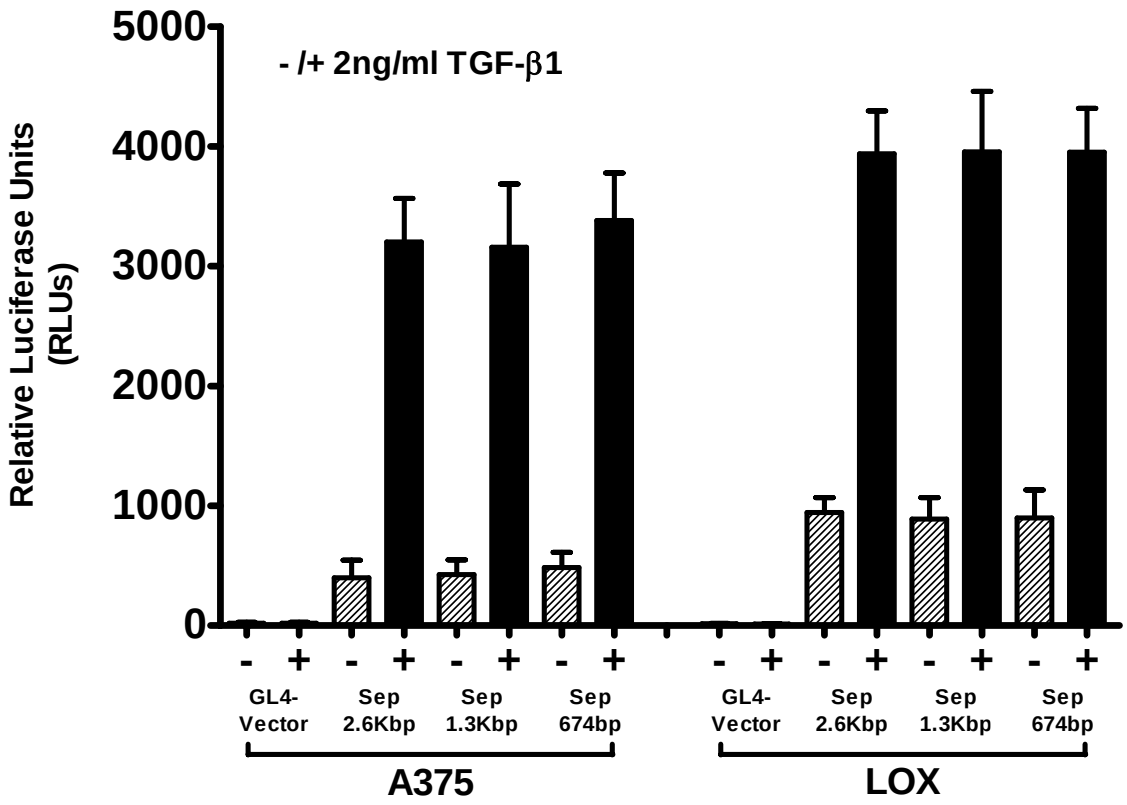

Figure 16. TGF-β1 increases human seprase promoter activity in melanoma cell lines. The human seprase promoter was assessed for TGF-β-responsiveness in both A375 and LOX cell lines by parallel transient transfection of pGL4-Sep2.637, pGL4-Sep1357, pGL4-Sep674, or GL4-vector into each cell line. Cells were untreated or treated with 2ng/ml TGF-β1 for 12 hr before luciferase activities were determined. Samples were normalized for transfection efficiency by co-transfection with pRL-TK vector. Reporter activity is expressed as relative luciferase units (RLUs). Results are the mean ± S.D. of triplicate samples from two of three independent experiments, n=6.

Substantial increases in both endogenous seprase expression and human seprase promoter activity in response to TGF-β1 treatment strongly suggested that if indeed the seprase gene is regulated at the level of transcription, it is done so in a TGF-β-dependent manner. It therefore became evident that the human seprase promoter must contain TGF-β-responsive region(s) which would allow for efficient seprase promoter transactivation in the melanoma cell lines tested. In my analysis of the 674 bp proximal seprase promoter I identified 9 putative TGF-β-responsive DNA sequences (putative Smad binding sites). The binding specificity of recombinant Smad proteins was initially defined as 5′-GTCTAGAC-3′ (Zawel et al., 1998) and later shown to be 5′-AGAC-3′, or its complement 5′-GTCT-3′ the so called Smad-binding element (SBE). In some instances an extra base is found 5′-CAGAC-3′. Another TGF-β-inducible DNA sequence found in some gene promoters, such as that of the human plasminogen activator inhibitor-1 (PAI-1) promoter for example, is called the CAGA box with the sequence 5′-AG(C/A)CAGACA-3′ (Dennler et al., 1998).

Functional Analysis of the Human Seprase Promoter

Of the nine putative Smad3/4 *cis*-elements identified in the proximal 674 bp human seprase promoter (Figures 2 and 3), five had consensus SBEs (Zawel et al., 1998): #1, #2, #4, #7AB (2 SBEs), and #9. Putative Smad site #3, #6, and #8 were non-consensus, but had the core (C/A)CAGA of the CAGA box element (Dennler et al., 1998), and site #5 had only the core CAGA. Comparing the human, rat, and mouse 674 bp regions, I found that putative Smad site #1, #5, #6, #7AB, and #8 were conserved across species (Figure 3). Putative Smad site #1 and/or #7AB therefore seemed likely to encompass the TGF-β-responsive region(s), as these sites were conserved across species and encompassed consensus SBE sequences.

WT pGL4-Sep674 and Smad site mutant reporters were assessed for transcriptional activity in transient cell transfection experiments in the LOX cell line. WT pGL4-Sep674 and pGL4-Sep674-mNon-site control (mutated at +64 to +67, no binding site) had comparable transcriptional activities that were well above pGL4-vector (~50-fold) (Figure 17). The mutant TATA reporter had a ~4-fold reduction in activity as compared to the WT promoter suggesting its function as a true TATA box (Figure 17). Interestingly, pGL4-Sep674-Smad promoter mutant sites: #1, #2, #3, #4, #8, and #9 all consistently demonstrated no apparent loss in transcriptional activity as compared to pGL4-Sep674 (Figure 17). Smad site mutant #5 and #6 had modest albeit consistent reductions in promoter activity. Both of the pGL4-Sep674 Smad site #7 mutants (#7A/#7B) displayed ~4-fold reductions in transcriptional activity as compared to pGL4-Sep674 (Figure 17), indicating that this region contains the major positive regulatory input for effective seprase promoter transactivation.

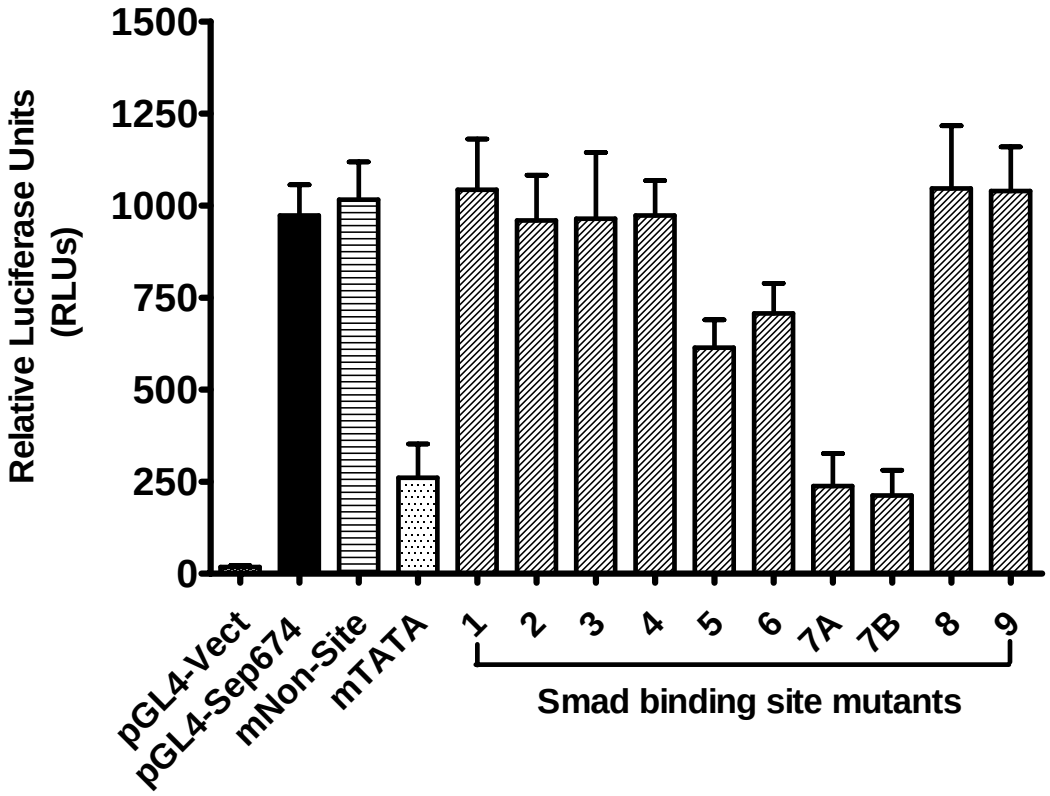

Figure 17. Functional analysis of putative Smad binding elements (SBEs) within the 674 bp human seprase promoter region. Site-directed mutagenesis was carried out to individually mutate all nine putative SBEs in the 674 bp (−469 to +205) seprase promoter. To assess the transcriptional activity of resultant mutants LOX cells were transfected with either the pGL4-Sep674 (WT) reporter construct or with pGL4-Sep674 plasmids harboring individual mutations in SBEs #1 through #9 or TATA box mutant and luciferase assays conducted. Controls were pGL4-Sep674 mNon-site (no putative binding site in region mutated) and pGL4-vector. All samples were normalized for transfection efficiency by co-transfection with pRL-TK vector. Reporter activity is expressed as relative luciferase units (RLUs). Results are the mean ± S.D. of triplicate samples from one (representative) of three independent experiments.

All of the multiple binding site mutants (double/triple/quadruple) in which Smad site #7 (A/B/AB) were mutated had similar reductions in activity as to that of site #7AB mutant alone (Figure 18), suggesting that Smad site #7AB is necessary and sufficient to drive high level transcriptional activity in these melanoma cell lines.

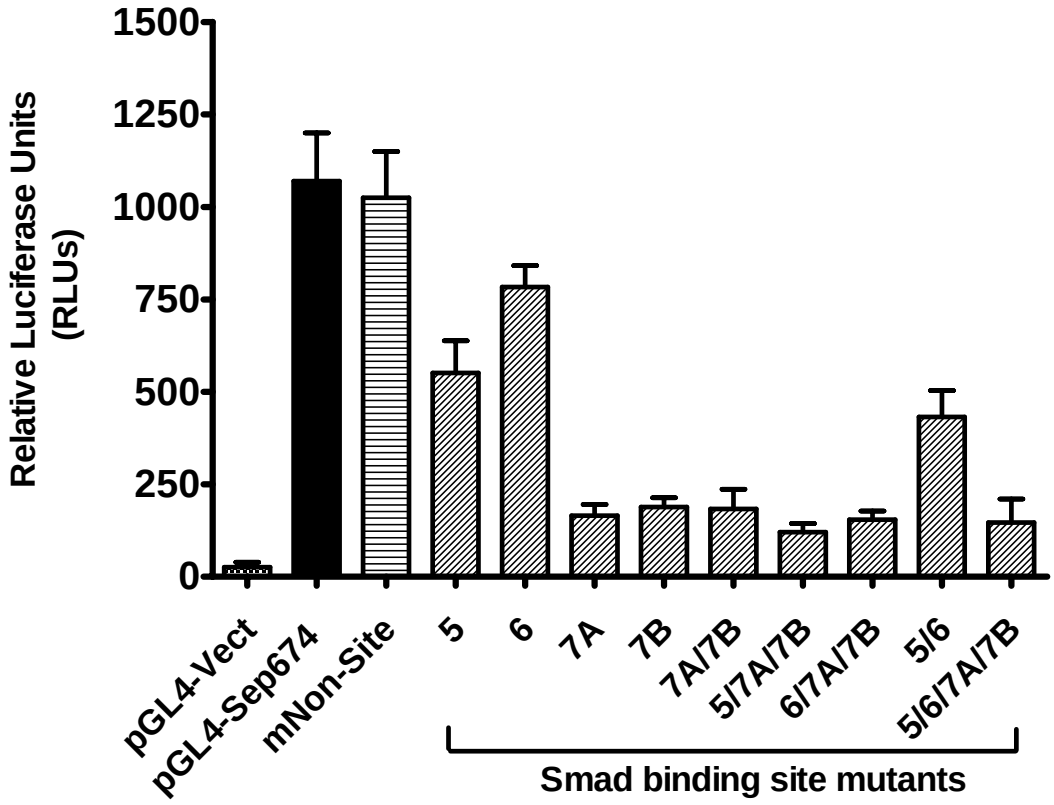

Figure 18. Functional analysis of putative Smad binding elements (SBEs) #5, #6, #7 within the 674 bp human seprase promoter region. Site-directed mutagenesis was carried out to mutate three SBEs deemed important for overall transcription (#5, #6, #7) in the 674 bp (−469 to +205) seprase promoter. To assess the transcriptional activity of resultant mutants LOX cells were transfected with either the pGL4-Sep674 (WT) reporter construct or with pGL4-Sep674 plasmids harboring mutations in SBEs and luciferase assays conducted. Controls were pGL4-Sep674 mNon-site (no putative binding site in region mutated) and pGL4-vector. All samples were normalized for transfection efficiency by co-transfection with pRL-TK vector. Reporter activity is expressed as relative luciferase units (RLUs). Results are the mean ± S.D. of triplicate samples from one (representative) of three independent experiments.

To determine if the TGF-β-induced increase in seprase promoter activity (Figure 16) mapped to the putative Smad binding sites deemed important for overall transcription (sites #5, #6 and #7), I conducted parallel transient cell transfection experiments in LOX cells, with or without 2 ng/ml TGF-β1 addition for 12hr. A stimulatory effect of TGF-β1 (~4- to 5-fold) was observed for both WT pGL4-Sep674 and pGL4-Sep674-mNon-site promoters, but not with pGL4 (Figure 19). The Smad site #5 mutant reporter was still induced very strongly in response to TGF-β1 (~3.5-fold), albeit to a lesser extent than the WT promoter, suggestive of low TGF-β-responsiveness (Figure 19). The Smad site #6 mutant promoter was strongly activated (~5-fold) upon TGF-β1 treatment, very similar to the WT promoter, thus ruling this site out as a TGF-β-responsive region.

The most dramatic effect was observed for the Smad site #7 promoter mutants, as mutation of these sites (#7A/#7B/#7AB) abolished the TGF-β-induced transcriptional activation of the seprase promoter, reducing levels to near baseline activity (Figure 19). Both site #7A and #7B are necessary for TGF-β-responsiveness, as mutation of either abolishes TGF-β-induced activation to the same degree as the #7A/#7B double mutant. All double/triple/quadruple mutants that contained the Smad site #7 mutation were incapable of transactivating the seprase promoter in response to TGF-β (Figure 19). Similar results were obtained in the A375 cell line (data not shown). The major TGF-β-responsive region in the human seprase promoter is therefore: putative Smad binding site #7AB (+118 to +125) with the sequence 5′-AGACAGAC-3′.

Since both putative Smad binding sites #5 and #7AB demonstrated low and high TGF-β-responsiveness respectively, I next wanted to determine whether or not the Smad transcription factors actually bind to these putative Smad binding sites. Namely, I wanted to look at the possible binding of Smad3, Smad 4, and the transcriptional repressor c-Ski to sites #5 and #7.

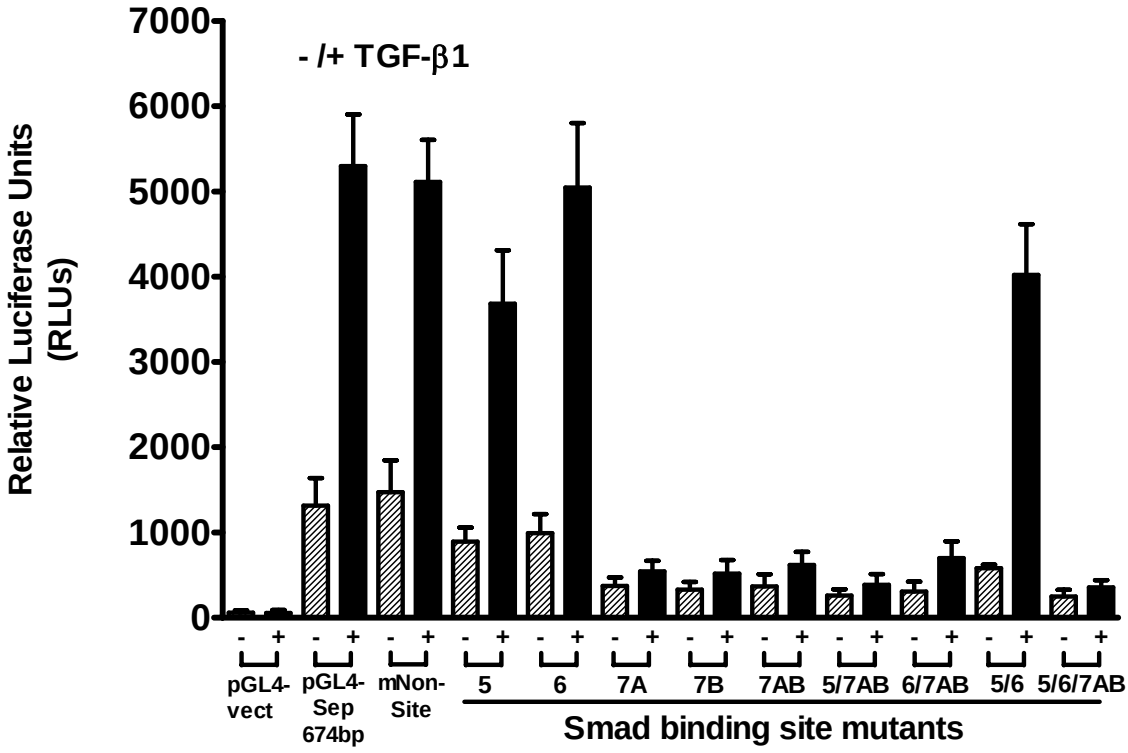

Figure 19. Determination of the Smad-binding element(s) responsible for the TGF-β-responsiveness of the 674 bp human seprase promoter. To map out the TGF-β-responsive region of the seprase promoter LOX cells were transfected with either the pGL4-Sep674 (WT) reporter plasmid, SBE mutants #5, #6, #7, or multiple SBE mutant versions. Controls were pGL4-Sep674 mNon-site and pGL4-vector. Cells were untreated or treated with 2ng/ml TGF-β1 for 12 hr before luciferase activities were determined. Samples were normalized for transfection efficiency by co-transfection with pRL-TK vector. Reporter activity is expressed as relative luciferase units (RLUs). Results are the mean ± S.D. of triplicate samples from one (representative) of three independent experiments.

TGF-β1-Induced Binding of Smad3/4 to the TGF-β-responsive Region of the Human Seprase Promoter *in vivo*

ChIP assays were conducted to determine Smad3, Smad4, and c-Ski binding to the TGF-β-responsive regions of the proximal seprase promoter. A375 and LOX cell lines were serum starved 8 hr followed by re-addition of CCC media treated, or not, with 5 ng/ml TGF-β1 for an additional 8 hr. The distal seprase promoter region (Non-site) had no enrichment of Smad3/4 or c-Ski binding compared to IgG in either cell line in either condition (Figures 20 and 21). Despite somewhat low TGF-β-responsiveness in reporter experiments, no enrichment of Smad 3/4 binding to the putative Smad site #5 in either treated or untreated cells was observed as compared to the IgG control (Figures 20 and 21). I therefore concluded that putative Smad site #5 is not a true Smad3/4 binding site and low level TGF-β-responsiveness observed to be Smad-independent. Putative Smad site #7AB was highly enriched in binding for Smad3 and Smad4 as compared to IgG control in non-stimulated A375 and LOX cell lines, and more so in LOX cells by comparison (Figures 20 and 21). Putative Smad site #7AB is thus a bona fide Smad3/4 binding element (SBE), both highly capable of binding Smads *in vivo*, and of driving TGF-β-dependent transcription of the human seprase gene in these melanoma cell lines. Additional TGF-β1 treatment increased binding of both Smad3 and Smad4 to the SBE in both cell lines showing that this region is a TGF-β-inducible promoter *in vivo* (Figures 20 and 21). An enrichment of c-Ski binding to Smad site #7AB was found in A375 cells as compared to the IgG control in the untreated condition, however no c-Ski binding was observed in the LOX cell line (Figures 20 and 21). Furthermore, c-Ski binding was lost (reduced to IgG levels) in A375 cells

treated with TGF-β1 (Figure 20), consistent with western data showing the TGF-β-driven abolishment of c-Ski in these cells (Figure 14).

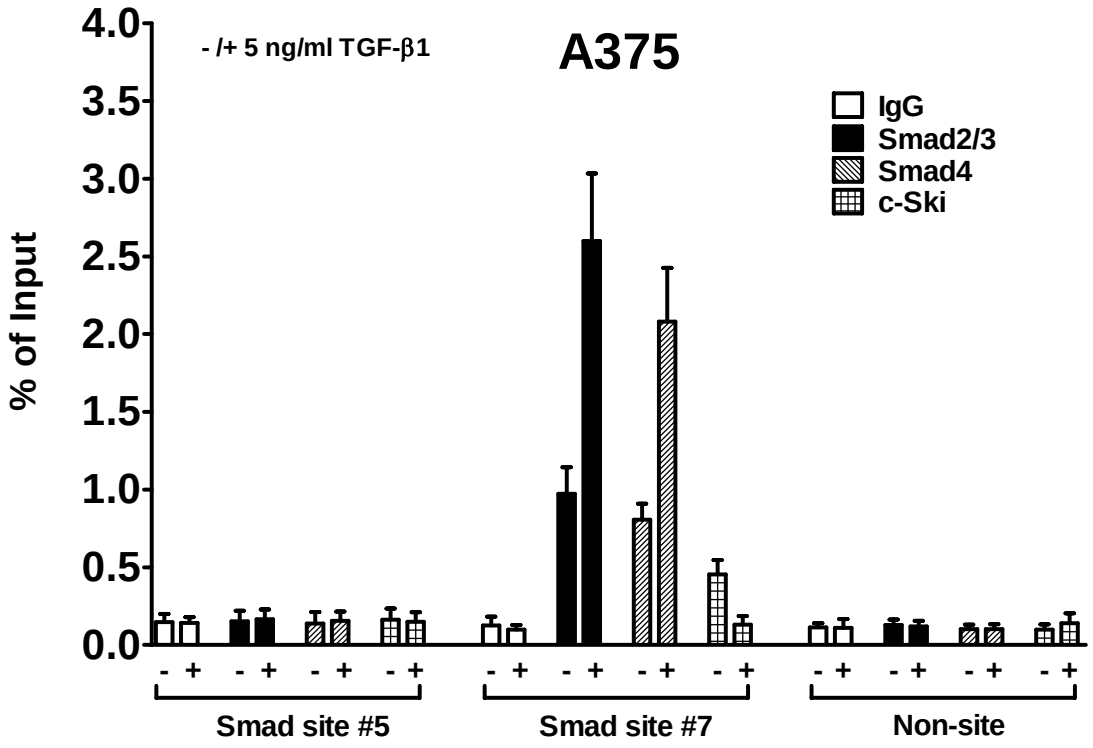

Figure 20. Assessment of Smad3/4 and c-Ski binding to the TGF-β-responsive element(s) of the human seprase promoter *in vivo*. A375 melanoma cells were serum starved for 8 hr followed by treatment with or without 5 ng/ml TGF-β1 in CCC media for an additional 8 hr, followed by collection and processing for ChIP analyses. Antibodies used for ChIP were anti-Smad2/3, anti-Smad4, ant-c-Ski, and anti-IgG. Promoter regions examined were putative SBE #5 (low TGF-β responsiveness), putative SBE #7 (high TGF-β responsiveness), and Non-site (negative control, distal promoter region encompassing no putative binding sites). Enrichment was assessed by Real-time PCR (QPCR) and represented as % of input. Results are the mean ± S.D. of triplicate samples from two of three independent experiments, n=6.

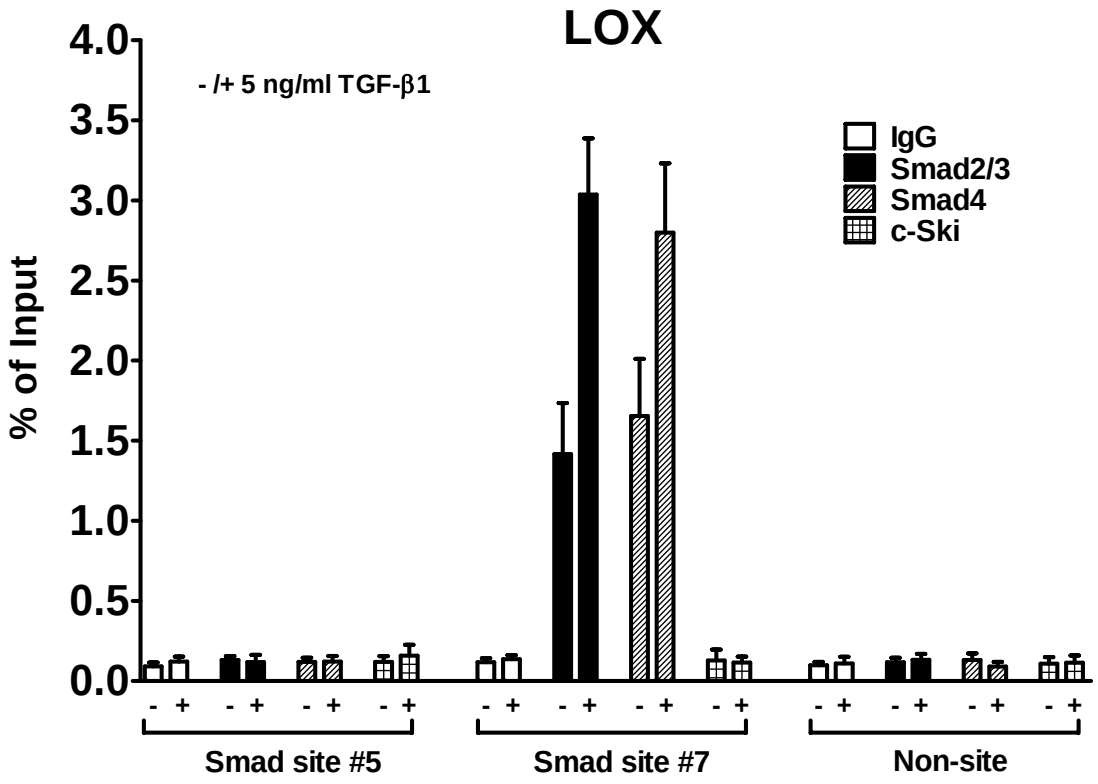

Figure 21. Assessment of Smad3/4 and c-Ski binding to the TGF-β-responsive element(s) of the human seprase promoter *in vivo*. LOX melanoma cells were serum starved for 8 hr followed by treatment with or without 5 ng/ml TGF-β1 in CCC media for an additional 8 hr, followed by collection and processing for ChIP analyses. Antibodies used for ChIP were anti-Smad2/3, anti-Smad4, ant-c-Ski, and anti-IgG. Promoter regions examined were putative SBE #5 (low TGF-β responsiveness), putative SBE #7 (high TGF-β responsiveness), and Non-site (negative control, distal promoter region encompassing no putative binding sites). Enrichment was assessed by Real-time PCR (QPCR) and represented as % of input. Results are the mean ± S.D. of triplicate samples from two of three independent experiments, n=6.

ChIP results could account for the differences in seprase expression observed in A375 and LOX cells. That is, a higher amount of Smad3/4 binding *in vivo* to SBE #7AB (indicative of a more robust TGF-β activation) in LOX cells as compared to A375 cells, in conjunction with the absence of c-Ski repressor binding *in vivo* to the SBE in LOX cells, but a fair amount of binding of c-Ski repressor to the SBE *in vivo* in A375 cells. These experiments also demonstrated that in all likelihood complexes of c-Ski and Smads are formed on SBE #7AB of the seprase promoter *in vivo*. I presume this because c-Ski has no intrinsic DNA binding capability, however c-Ski protein has been shown to be capable of binding Smads bound to SBEs (Luo et al., 1999; Nagase et al., 1990; Nicol and Stavnezer, 1998a). Having observed a significant amount of binding of c-Ski to SBE #7AB in A375 cells in the untreated condition highly suggested that c-Ski-Smad-DNA interactions on the seprase promoter were occurring, although other protein-protein (transcription factor) interactions cannot definitively be ruled out. I next tested what the forced expression of the transcriptional repressor c-Ski in both A375 and LOX cell lines would do to seprase transcription since the differential expression/stability of c-Ski as well as the binding of the molecule to the seprase promoter seemed likely to be playing a role in the differential transcription of the seprase gene observed in each of the cell lines.

Genetic Inhibition of Seprase Transcription by Stable Overexpression of the Transcriptional Co-Repressor c-Ski in A375 and LOX Cell Lines

As was mentioned in the background section, upon binding to the seprase promoter, c-Ski could exert its inhibitory effects in a variety of ways such as through the stabilization of inactive Smad complexes on the SBE, by blocking Smad-recruitment of transcriptional co-activators such as p300/CBP, or by recruitment of N-CoR co-repressor complexes (Akiyoshi et al., 1999; Luo et al., 1999; Suzuki et al., 2004; Wu et al., 2002). In addition, c-Ski protein could prevent transcription by direct interactions with Smad3 or Smad4, or by disruption of the formation of crucial R-Smad/co-Smad complexes (Ueki and Hayman, 2003; Wu et al., 2002). To determine if c-Ski was truly repressing seprase transcription, A375 and LOX cell lines were infected with either pBabe puro (BP) or pBabe Puro/Ski retrovirus and pooled stable lines selected for further characterization.

Results of qRT-PCR indicated that c-Ski mRNA levels for both A375 BP-Ski and LOX BP-Ski cell lines were quite comparable (Figure 22). Endogenous c-Ski mRNA levels were much higher in the A375 cell line as compared to the LOX cell line (Figure 22). When I looked at corresponding c-Ski protein levels I found that A375 BP-Ski cells expressed extremely high amounts of protein as compared to LOX BP-Ski cells which produced very limited amount of the protein (Figure 23). LOX BP-Ski cells were however expressing much higher c-Ski than LOX BP cells, and slightly higher than the levels typically found in A375 BP cells (Figure 23). I therefore expected that a reduction in seprase transcription should be occurring in both BP-Ski cell lines as compared to the BP counterparts if c-Ski was truly repressing the gene as I proposed.

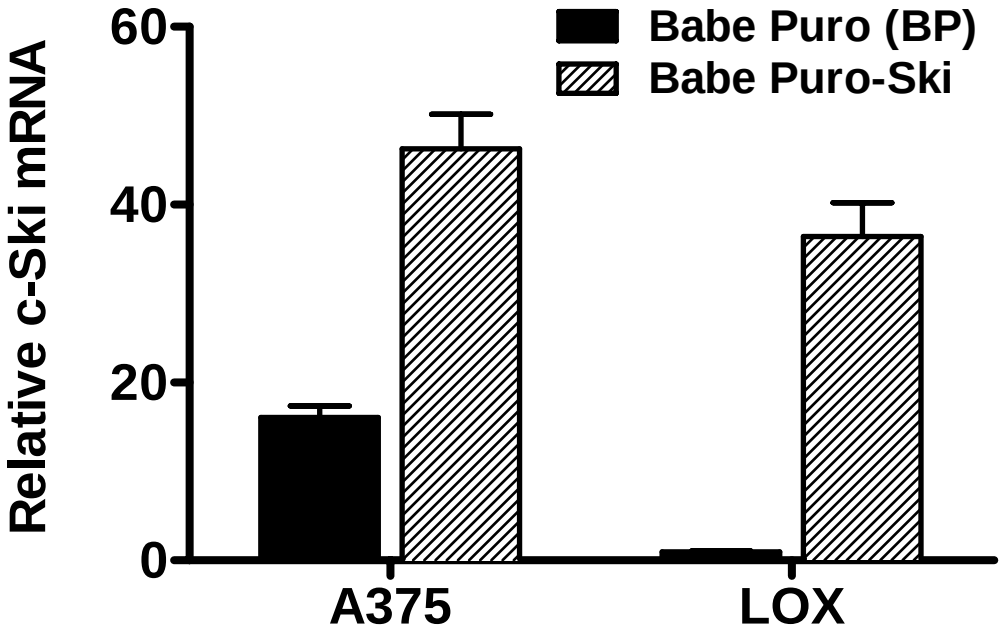

Figure 22. c-Ski mRNA levels in empty vector (BP) and c-Ski overexpressing (BP-SKI) stable A375 and LOX cell lines. A375 and LOX cell lines were selected to stably overexpress empty vector (BP) or exogenous c-Ski (BP-Ski). Relative c-Ski mRNA levels in these cell lines were determined by qRT-PCR and values were normalized to β-actin levels. Results are the mean ± S.D, n=3 of one representative of three independent experiments.

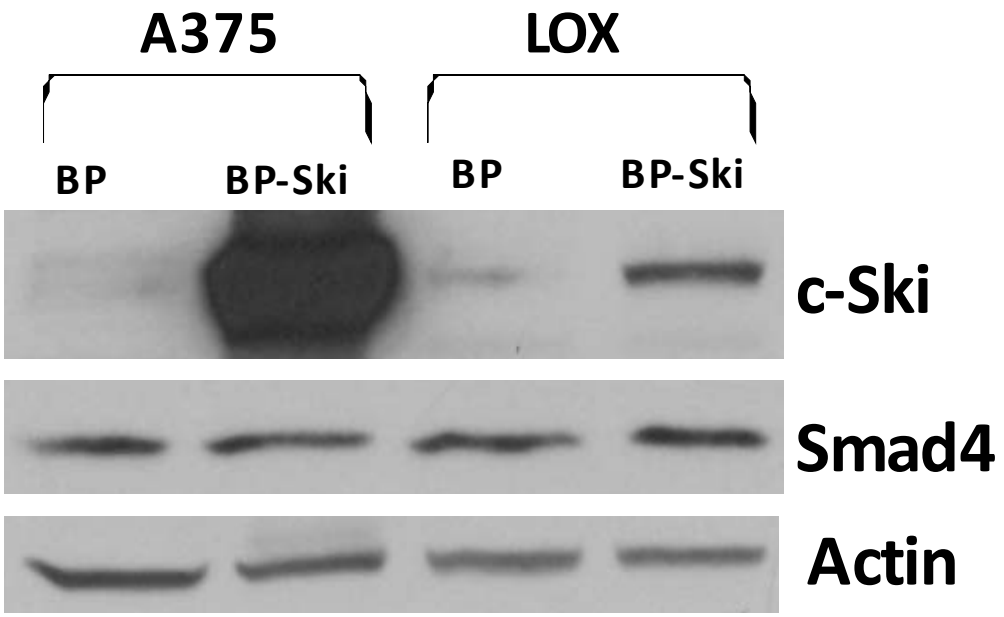

Figure 23. c-Ski protein levels in empty vector (BP) and c-Ski overexpressing (BP-Ski) stable A375 and LOX cell lines. Western blotting was carried out using whole-cell extracts of the stably overepxressing LOX BP/BP-Ski cells and A375 BP/BP-Ski cells. Blots were probed with anti-c-Ski to confirm overexpression. Smad4 and β-actin blots served as loading controls. Note: although the c-Ski band in A375 looks less abundant than in Figure 8. The levels are quite comparable a less exposed film was needed here do to the high levels of c-Ski in BP-Ski A375 cell line.

I next carried out qRT-PCR to determine if overexpression of c-Ski in A375 and LOX cell lines reduced levels of seprase mRNA (blocked seprase transcription). Indeed, both A375 BP-Ski and LOX BP-Ski cell lines had significant reductions in seprase mRNA levels as compared to their BP counterparts (Figure 24). Seprase mRNA levels in A375 BP-Ski cells were reduced ~11-fold ($P < .001$, n=6) to basal state level, whilst seprase mRNA levels in LOX BP-Ski cells were reduced ~1.9-fold (P=.0032, n=6) (Figure 24). The amount of seprase mRNA in LOX BP-Ski cells was reduced comparably to the levels seen in A375 BP cells.

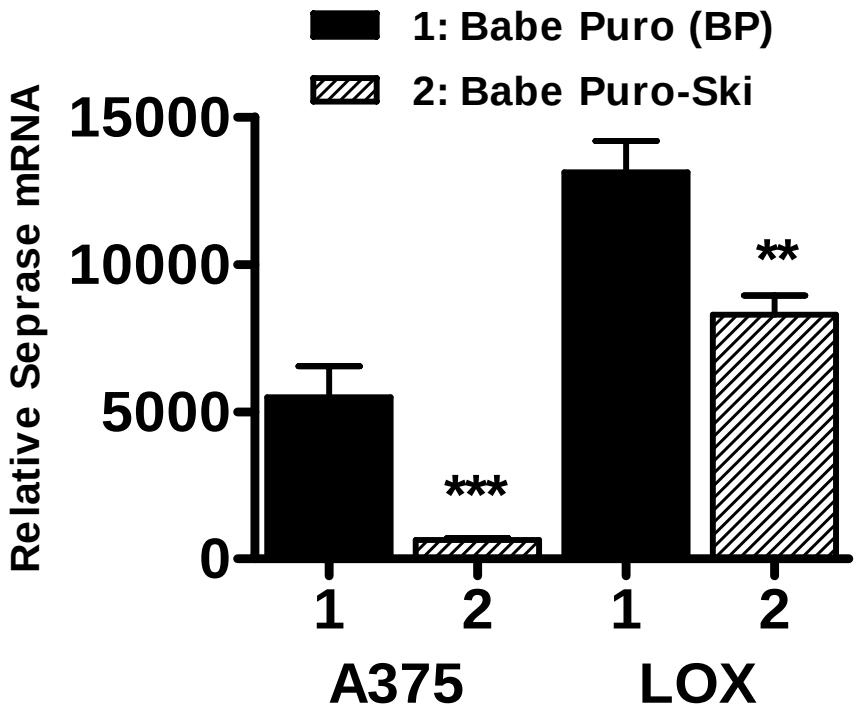

Figure 24. c-Ski mediated repression of seprase transcription in A375 and LOX melanoma cell lines. Effect of c-Ski overexpression on seprase mRNA levels was determined by qRT-PCR for seprase for both empty vector (BP) and c-Ski overexpressing (BP-Ski) A375 and LOX cell lines. All seprase values were normalized to β-actin level. Results are the mean ± S.D. of two qRT-PCR results conducted in triplicate, n=6. $P < 0.01$, indicated by **, $P < .001$, indicated by ***.

To confirm that the c-Ski-mediated reduction in seprase mRNA was at the level of the seprase promoter, i.e. transcriptional repression, I conducted ChIP assays using the A375 BP-Ski and LOX BP-Ski cell lines to determine if the exogenously expressed c-Ski was directly targeting the seprase promoter *in vivo*. Results demonstrated that A375 BP-Ski cells had enriched Smad3 binding to the seprase promoter SBE #7 as compared to control IgG pull-down (Figure 25). The SBE site #7 was also highly occupied by c-Ski protein as compared to IgG in these cells and equally to that of the amount of Smad3 binding (Figure 25). These results indicated that on essentially every SBE that was bound by Smad3, the site was also bound by c-Ski rendering the promoter almost completely inactive. LOX BP-Ski cells were also highly enriched for Smad3 binding on SBE site #7 as compared to the IgG control (Figure 25). In addition, c-Ski binding was enhanced ~3.5-fold on the seprase promoter SBE as compared to IgG pull-down (Figure 25). The overexpression of c-Ski was high enough of a level such that we observed a previously unseen occupancy on the seprase promoter SBE by the repressor (Figure 25), and a concomitant ~1.9-fold reduction in seprase mRNA in LOX BP-Ski cells accordingly (Figure 24).

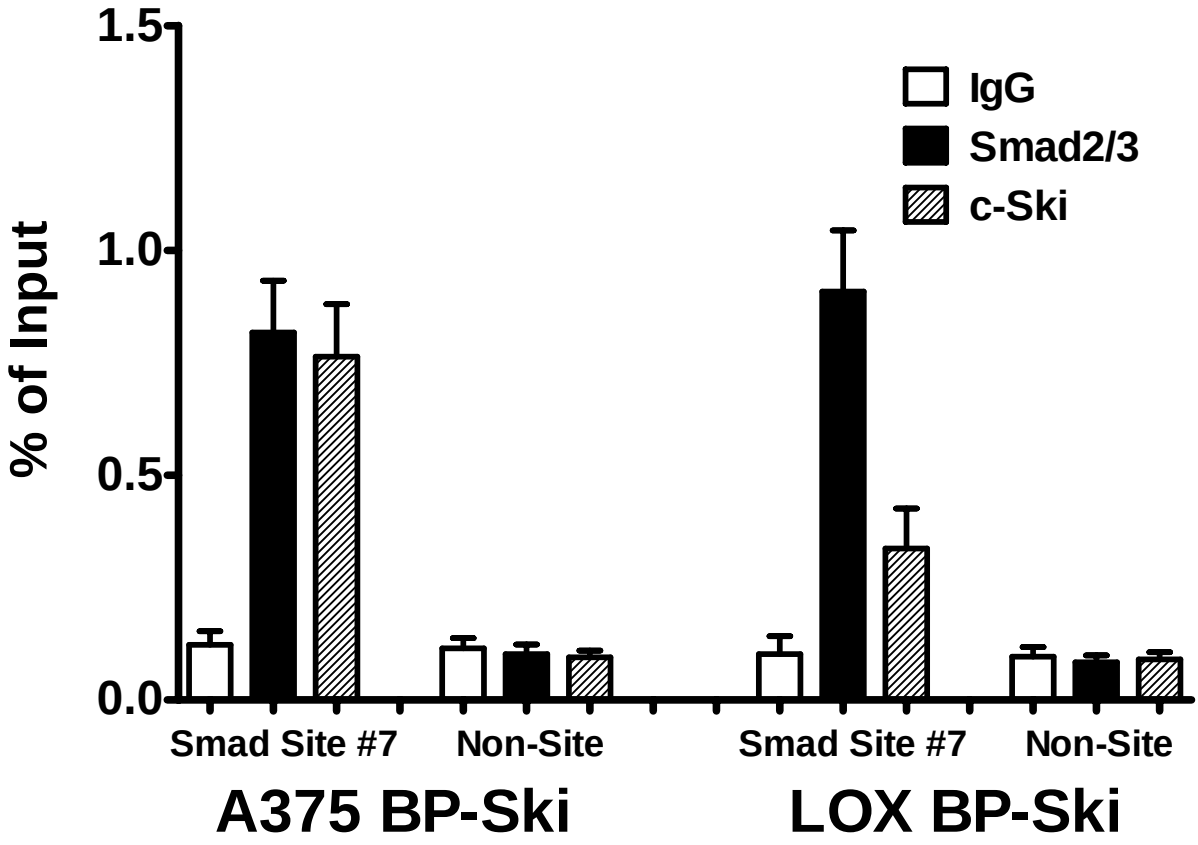

Figure 25. Smad2/3 and c-Ski binding to SBE #7 of the seprase promoter in c-Ski overexpressing A375 and LOX melanoma cell lines. ChIP assays were conducted to determine Smad3 and c-Ski binding to the major TGF-β-responsive region (SBE #7), or control distal promoter region with no putative transcription factor binding site (Non-site) in both A375 BP-Ski and LOX BP-Ski cell lines. Enrichment of binding was assessed via Real-time PCR (QPCR) and represented as % of input. IgG pull-down served as negative control. Results are the mean ± S.D. of triplicate samples from two of three independent experiments, n=6.

I next tested whether or not c-Ski protein was degraded in the LOX cell line via proteasomal degradation mediated by TGF-β signaling as was seen in other melanoma cell lines (Le et al., 2008a) including A375 cells which I used in my studies. If c-Ski protein was being degraded in such a manner, it would account for differences found in the level of c-Ski mRNA and corresponding amount of c-Ski protein in LOX BP-Ski cells. To test this, 10 μM of the proteasome inhibitor MG132 was added or not to both LOX BP and LOX BP-Ski cell lines in culture for 2 hr. Accumulation of c-Ski protein upon MG132 treatment profoundly inhibited seprase transcription, reducing levels of seprase mRNA ~10-fold in LOX BP-Ski cells as compared to LOX BP cells ($P < .001$, n=6) in the treated condition (Figure 26). A far more severe repression of seprase mRNA production was found in LOX BP-Ski cells in the treated versus untreated condition as was expected given the considerable stabilization of c-Ski repressor protein in the treated condition. In the untreated condition there is still some overexpression of c-Ski as I previously indicated so even in the absence of MG132 seprase mRNA levels were lowered in LOX BP-Ski versus LOX BP cells (~1.7-fold decrease, $P = .0044$, n=6) (Figure 26). Western blotting showed extremely high levels of stabilized c-Ski protein in the LOX BP-Ski overexpressor in the treated condition (Figure 27), similar to levels found in A375 BP-Ski cells, and much more in line with the c-Ski mRNA level produced in these cells.

These results suggested that the LOX cell line, which produces an abundance of TGF-β1, has all the necessary machinery for constant and efficient TGF-β-induced c-Ski degradation. This coupled with the already lower mRNA production/stability of c-Ski in LOX versus A375 cells demonstrates very clearly why c-Ski protein levels are so low in LOX cells as compared to A375 cells and is highly suggestive as to why seprase is being transcribed so readily in LOX cells; high amounts of TGF-β1 produced and the absence of the c-Ski transcriptional repressor protein.

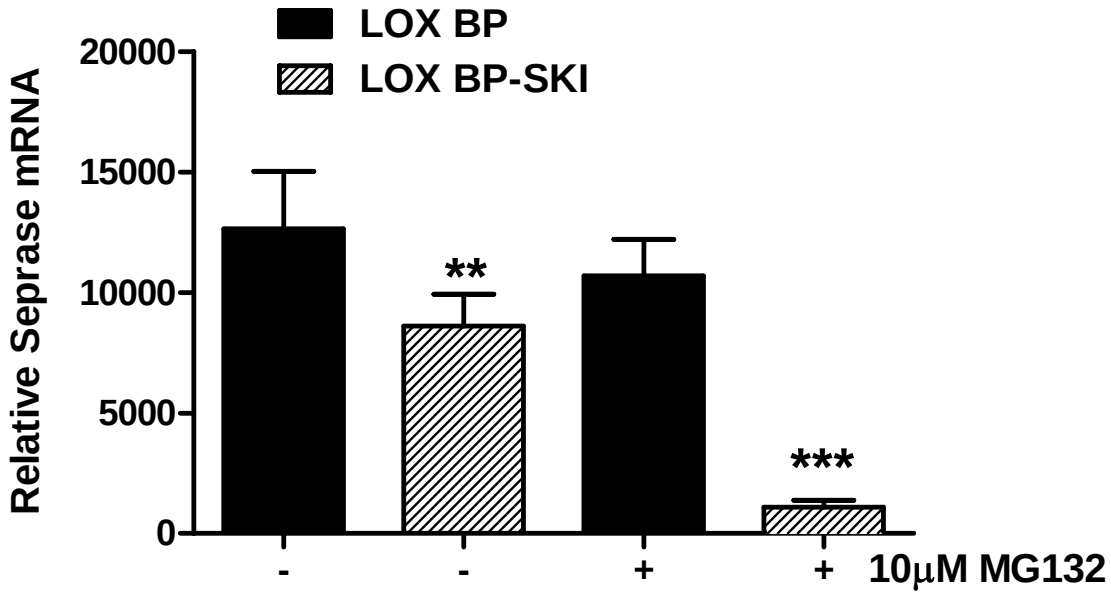

Figure 26. c-Ski stabilization and subsequent c-Ski mediated repression of seprase transcription in the LOX melanoma cell line. To test if c-Ski is degraded in the LOX cell line via the proteasomal degradation pathway, LOX BP and BP-Ski cell lines were treated, or not, with the proteasomal inhibitor MG132 for 2 hr. Effect of the stabilization of c-Ski on seprase mRNA levels was determined by qRT-PCR for seprase. All values were normalized to β-actin level. Results are the mean ± S.D. of two quantitative RT-PCR results conducted in triplicate $P < .05$, indicated by **, $P < .001$, indicated by ***, n=6 for all.

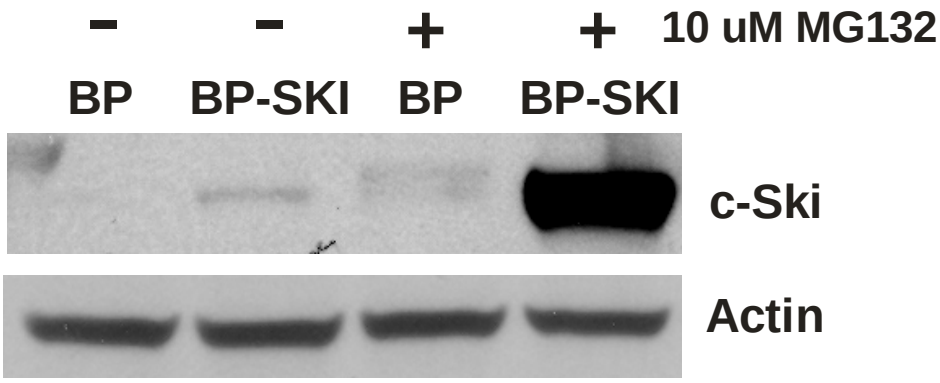

Figure 27. c-Ski protein stabilization in the LOX BP-Ski melanoma cell line upon treatment with MG132 proteasome inhibitor. To test if c-Ski is degraded in the LOX cell line via the proteasomal degradation pathway, LOX BP and BP-Ski cell lines were treated, or not, with the proteasomal inhibitor MG132 for 2 hr. Western blotting was conducted to assess c-Ski proteins levels. β-actin was used as the loading control.

I also genetically targeted TGF-β Type II receptor function by transient transfection and overexpression of a dominant-negative form of the TGF-β Type II receptor (TGF-β RII Δcyt), which lacks the cytoplasmic portion (kinase region) in both A375 and LOX cell lines. This shortened dominant-negative version of the protein is still highly capable of binding TGF-β ligand and so its forced expression sequesters ligand away from endogenous TGF-β Type II receptor expressed on the cell surface. TGF-β RII Δcyt is incapable of transactivating the TGF-β Type I receptor due to its lack of the cytoplasmic portion containing the crucial kinase domain. As a result there is a greatly diminished activity of the TGF-β Type I receptor and a subsequent blockade in the activation of the R-Smads from the TGF-β Type I receptor. With the activity of the R-Smads diminished it was expected that seprase mRNA levels should also go down, since all data to this point strongly suggested that seprase is not only transcriptionally regulated, but done so in a TGF-β-dependent fashion in the melanoma cell lines examined.

Cells cultured in 10 cm dishes were transfected with 15 μg of pCMV5-TGF-β RIIΔcyt or pCMV5 empty vector and allowed to express for 24 hr at which time cells were collected and RNA harvested for qRT-PCR analysis. Results of qRT-PCR for seprase indicated that A375 cells transfected with pCMV5-TGF-β RIIΔcyt had ~2.75-fold decrease in seprase mRNA, (P = .0048, n=3) as compared to pCMV5 transfected control cells, and LOX cells had a ~1.8-fold reduction (P = .0161, n=3) in seprase mRNA as compared to control counterparts.

Results of both genetic analyses conducted (stable overexpression of the co-repressor c-Ski or transient overexpression of a dominant-negative form of the TGF-β Type II receptor) indicate that targeting Smads (direct interaction/co-repression or blockade of Smad activation)

ultimately prevents their ability to transactivate the seprase promoter in a TGF-β-dependent manner, highlighting the importance of TGF-β-Smad signaling in the gene regulation of seprase.

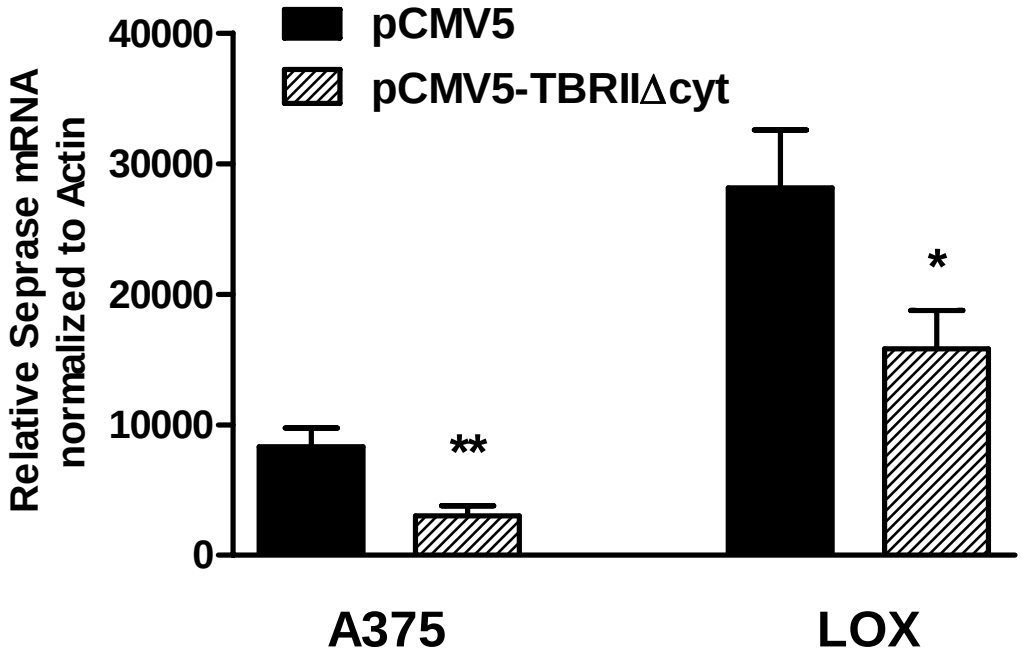

Figure 28. Exogenous overexpression of dominant negative TGF-β Type II receptor reduces TGF-β-dependent seprase transcription in melanoma cells. A375 and LOX cell lines were transiently transfected with either empty vector (pCMV5) or with a truncated TGF-β Type II receptor plasmid (pCMV5-TβRIIΔcyt) and allowed to express for 24 hr. Seprase mRNA levels was determined by qRT-PCR and all values were normalized to β-actin levels. Results are the mean ± S.D. of one representative of three quantitative RT-PCR results conducted in triplicate $P < .01$, indicated by **, $P < .05$, indicated by *, n=3 for all.

In addition to genetic inhibition, I found significant decreases in TGF-β-driven seprase transcription in both A375 and LOX cell lines by blocking the activity of TGF-β Type I receptor (TGF-β RI) using the specific TGF-β RI receptor chemical inhibitor SB-431542 (Figure 29). SB-431542 acts as a competitive ATP binding site kinase inhibitor (Callahan et al., 2002) and has been shown to potently inhibit TGF-β signaling at a concentration of 10 μM without causing any appreciable cell death in a variety of human and mouse cell lines, and without effect on the related Bone Morphogenetic Protein (BMP) receptor superfamily members (Laping et al., 2002; Inman et al., 2002).

A375 and LOX melanoma cell lines were cultured in full CCC media and in the presence or absence (DMSO alone) of 10 μM SB-431542 for 24 hr. After 24 hr cells were harvested, RNAs were purified and then examined by qRT-PCR for seprase mRNA levels. I found that seprase mRNA levels were reduced ~11-fold in A375 cells, and ~7-fold in LOX cells ($P < 0.001$, n=3 for all) in treated versus untreated cells (Figure 29). These experiments complemented the genetic inhibitory studies and further validated the importance of the canonical TGF-β/Smad signaling pathway in the transcriptional activation of the seprase gene.

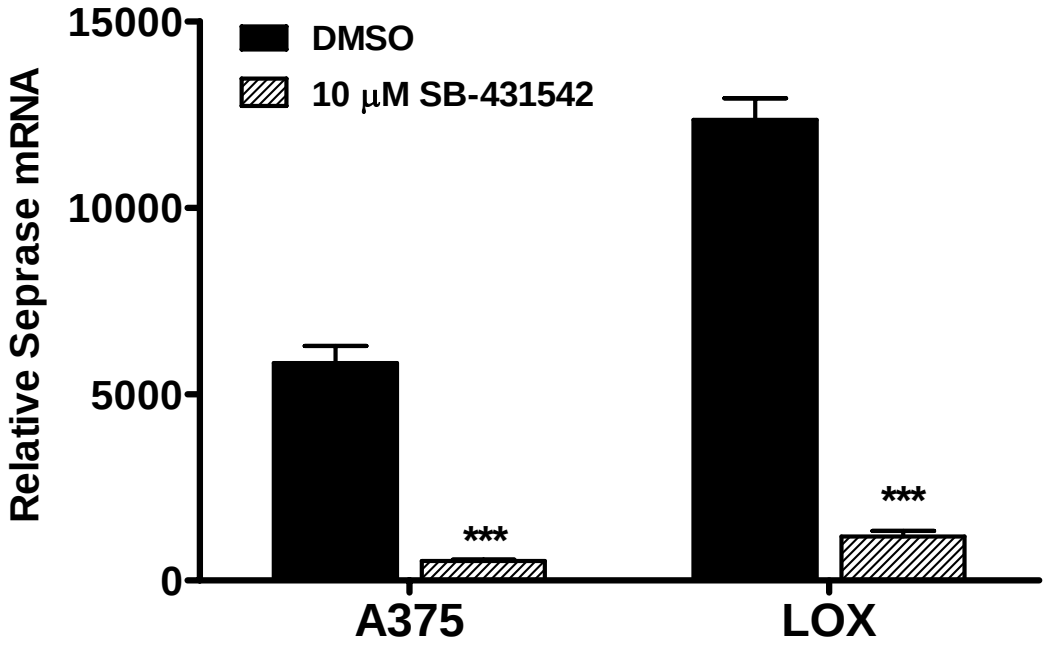

Figure 29. Chemical inhibition of the TGF-β Type I receptor prevents TGF-β-dependent seprase transcription in A375 and LOX melanoma cell lines. Cells were treated with either DMSO vehicle or with the specific TGF-β Type I receptor inhibitor SB-431542 for 24 hr and seprase levels determined by qRT-PCR. Values were normalized to β-actin. Results are the mean ± S.D, n=3 of one representative experiment of three conducted, $P < .001$, indicated by ***.

Seprase transcription was also significantly reduced in both A375 and LOX cell lines by depleting cells in culture of the autocrine TGF-β1 they produce using increasing concentrations (.5 - 10 μg/ml) of an anti-TGF-β neutralizing antibody for 24 hr. The concentration of anti-TGF-β1 neutralizing antibody needed to yield one-half maximal inhibition of the cytokine on a responsive cell line was determined to be ~.6 μg/ml in the presence of .25 ng/ml recombinant human TGF-β1 for one hour with this lot of neutralizing antibody. As is described in the product page and of particular relevance here is the fact that the exact concentration of antibody required to neutralize TGF-β1 activity is highly dependent on the TGF-β cytokine concentration. It is important to remember that LOX cells produce roughly three times the amount of TGF-β1 when compared to A375 cells and therefore should require more antibody for optimal neutralization which is exactly what I observed.

A375 and LOX cell lines were cultured in CCC media in 10 cm plates, washed three times in 1X PBS and neutralizing antibody then added or not with fresh CCC media for 24 hr. Cells were harvested after 24 hr and RNA purified for subsequent qRT-PCR. I found that A375 cells had significant reductions in seprase mRNA using as little as .5 μg/ml of the antibody ($P < 0.01$, n=3) (Figure 30). Levels were further reduced in cells treated with higher concentrations ($P < 0.001$, n=3 for all) (Figure 30). Significant reductions in seprase mRNA were not seen using .5 μg/ml of antibody, but were however observed in LOX cells using 2 μg/ml of the antibody, and were further reduced in cells treated with higher concentrations ($P < 0.01$, n=3 for all) (Figure 30).

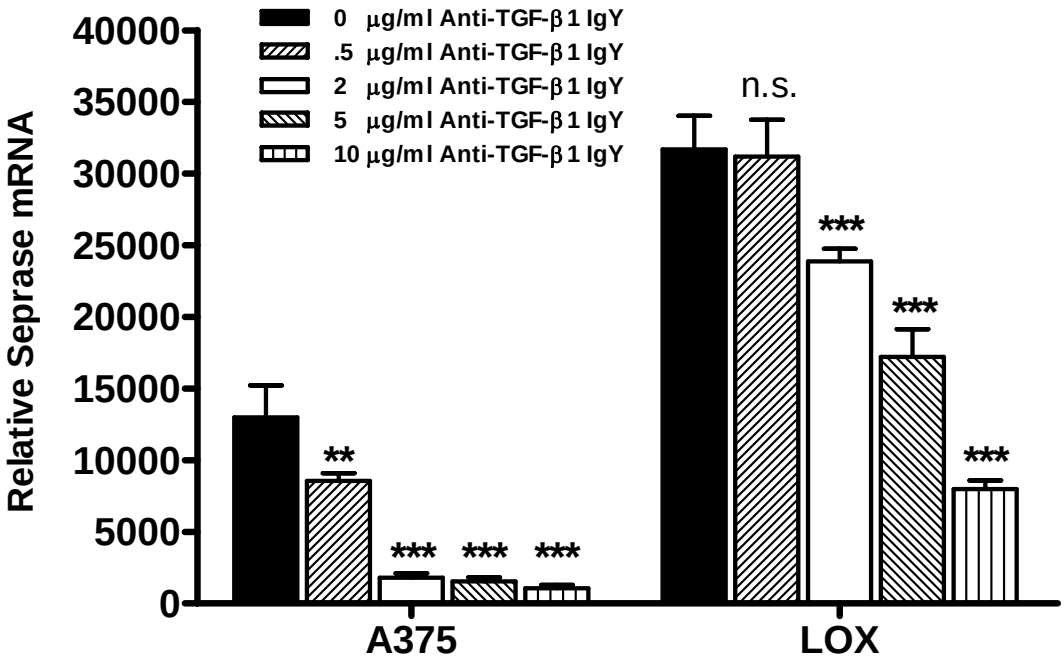

Figure 30. Treatment of melanoma cell lines with TGF-β1 neutralizing antibody reduces seprase mRNA production. A375 and LOX cell lines cultured in full CCC were washed three times in 1X PBS and treated or not with a neutralizing antibody against TGF-β1 in fresh CCC for 24 hr. Seprase levels were then determined by qRT-PCR. Values were normalized to β-actin. Results are the mean ± S.D, n=3 of one representative experiment of three conducted, $P < .01$, indicated by **, $P < .001$, indicated by ***.

Taken together, the results of the various inhibition studies shown above indicate that TGF-β signaling is the crucial pathway for seprase transcription in these metastatic melanoma cell lines and that blocking TGF-β signaling at essentially any level (ligand, receptor(s), Smads) leads to profound reductions in seprase transcription accordingly. In every instance the outcome is either the prevention of the activation of the Smad transcription factors, or a diminished ability of the Smads to function as positive regulatory transcription factors in the up-regulation of seprase transcription.

CHAPTER IV: DISCUSSION

In this study, I have cloned the human seprase promoter and identified key features of the core promoter such as a TATA box 36 bp upstream of the transcriptional start site (+1), a 209 bp 5′-UTR, and numerous putative *cis*-regulatory elements. Zhang et al (Zhang et al., 2010) were unable to identify a TATA box in the mouse seprase promoter, as it is not conserved between mouse and human sequences which they aligned, but is in fact conserved between rat and human sequences as I found. In addition I classify the transcriptional start site based on the Incyte complete full-length human seprase cDNA sequence, as opposed to the most abundant transcript found in HeLa cells (Zhang et al., 2010), and therefore were able to locate the TATA box in the expected 30 – 40 bp range upstream of (+1). It is also noteworthy to mention that although Zhang et al cloned the mouse seprase promoter, the region they identified as important for basal seprase transcription has 80% sequence homology between the human and mouse seprase gene, and contains a conserved EGR1 binding site (Zhang et al., 2010). In my studies, the main *cis*-regulatory element identified is a crucial Smad3/4 SBE which is both necessary and sufficient for TGF-β-dependent seprase transcription, and is in agreement with several independent studies showing that TGF-β1 induced seprase protein expression (Denys et al., 2008; Rettig et al., 1994).

I provide evidence that the seprase gene is transcriptionally regulated in a pair of human malignant melanoma cell lines with differential levels of seprase mRNA. A375 cells have a moderate level of seprase mRNA whilst the LOX cell line has an approximately 3-fold higher amount. Similarly, transfected seprase promoter reporters had ~2.5-fold higher activities in the LOX cell line as compared to the A375 cell line. In addition, HaCat keratinocytes expressed no

detectable seprase mRNA, and also had no seprase promoter activity. CCD-SK28 fibroblast cells expressed high levels of seprase mRNA and had high seprase promoter activity. Although the results of these experiments suggested the possibility that seprase is transcriptionally regulated and in a variety of cell types in no way does it definitively mean that transcriptional activation of seprase was the driving force behind the differential levels of seprase mRNA observed in A375 and LOX cell lines. This is due to the fact that the cloned seprase promoter regions contain about 200 bp of seprase gene 5′ UTR. This 5′ UTR sequence which is transcribed along with luciferase reporter mRNA could in theory be allowing for the same stability/instability for reporter mRNA as the endogenous seprase mRNA in each cell line tested. However, it cannot be excluded that transcription, i.e. promoter activity is actually behind the differential seprase mRNA/promoter activity observed.

Consistent with the increased level of seprase mRNA observed in LOX cells is an increase in seprase protein expression, invadopodia formation, gelatinase activity and resulting invasiveness in the LOX cell line as compared to the A375 cell line (Monsky et al., 1994). So, if seprase transcription is truly being driven by the TGF-β pathway as I propose, then this signaling pathway is indeed directly influencing the invasiveness and migratory capabilities of melanoma cells, as the levels of seprase mRNA/protein levels seem to be crucial for such an invasive phenotype.

Several studies have demonstrated the increased expression and secretion of TGF-β isoforms in melanoma cell lines in comparison to normal melanocytes (Rodeck et al., 1994; Krasagakis et al., 1994; Albino et al., 1991; Rodeck et al., 1991; Krasagakis et al., 1999). In addition, human metastatic melanoma cell lines were found to be more resistant to TGF-β-

dependent growth inhibition as compared to cells from primary tumors (Krasagakis et al., 1999). Despite resistance to TGF-β-induced growth arrest, TGF-β-resistant melanoma cell lines still have intact Smad-dependent regulation of gene expression in response to TGF-β, and can do so in an autocrine manner (Rodeck et al., 1999). In my studies I found that both A375 and LOX cell lines secrete fairly high amounts of TGF-β1; however LOX cells produce ~3-fold more than A375 cells do. In addition to producing more of the ligand, LOX cells also express ~4-fold more of the TGF-β type II receptor protein than A375 cells. LOX cells which produce higher amounts of TGF-β1 and express higher levels of the TGF-β type II receptor as compared to A375 cells would in theory have a more robust autocrine signal transduction capability. In accordance with these observations, the LOX cell line had higher levels of both phospho-Smad2 and phospho-Smad3 validating that TGF-β signaling is more active in LOX cells as compared to A375 cells. Another striking difference was in the level of the c-Ski transcriptional repressor protein, which was expressed much more abundantly in A375 cells as compared to LOX cells which express almost none of the protein. The presence of the TGF-β c-Ski repressor in one cell line but not the other would most certainly allow for the differential transactivation of the seprase promoter if it was under transcriptional regulation by the TGF-β signaling machinery which is what I found to be the case.

I also found that both A375 and LOX cell lines had functional TGF-β signaling as determined by their ability to transactivate two different minimal Smad-responsive reporters, validating that each cell line could signal in an autocrine manner. LOX cells induced higher expression of the reporters than A375 cells, as was expected given the higher production of TGF-β1 by this cell line, although both cells transactivated the reporters equally well in response to TGF-β1 treatment. A 674 bp region of the seprase promoter (−469 to +205) was sufficient to

drive high level expression of the reporter in cell lines tested, and was also found to be highly responsive to TGF-β1 in A375 and LOX cells. In agreement with these reporter results, treatment of A375 and LOX cells with TGF-β1 for 1 hr led to rapid and significant increases in the level of endogenous seprase mRNA, in both A375 and LOX cell lines, and a decrease in the amount of c-Ski repressor protein in the A375 cell line (LOX cells already express low/no c-Ski). These results again demonstrate that the amount of TGF-β1 cells are exposed to determines how much seprase mRNA is produced and/or stabilized in these melanoma cell lines. The TGF-β-dependent reduction in c-Ski protein might just be coincidental to the higher levels of seprase mRNA observed in the A375 cell line upon TGF-β1 treatment, but it seemed more likely to me, given c-Skis function as a transcriptional co-repressor, that this abolishment of the repressor was of biological relevance to the changes in seprase mRNA observed.

Since TGF-β1 signaling ultimately results in the activation of the Smad transcription factors which are then able to translocate to the nucleus and alter gene expression (Massague et al., 2005; Shi and Massague, 2003; Feng and Derynck, 2005), I interrogated nine different putative Smad binding elements in the cloned seprase promoter by mutagenesis and subsequently determined promoter activities of the resulting mutants as compared to the WT seprase promoter. Using this approach, I mapped the TGF-β responsive region of the promoter to the +118 to +125 portion of the gene promoter which encompassed two putative and consecutive SBEs, #7AB: 5′-AGACAGAC-3′. Mutation of either one these SBEs reduced the activity of the promoter to similar low/basal levels suggesting that they are in fact functionally redundant sites. Furthermore, mutation of the SBEs rendered the promoter completely insensitive to TGF-β1 stimulation, indicating that SBE #7AB is both necessary and sufficient for TGF-β-dependent seprase transcription. However, since SBE #7AB is found in the 5′ UTR it cannot definitively be

ruled out that mutating SBE #7AB might have contributed to a reduction in seprase-5′ UTR-luciferase reporter mRNA stability. It again seems much more likely to me a transcriptionally driven process as SBE #7AB encompasses two consensus SBEs that are conserved across species, and given the extremely high transactivation off of the seprase promoter in response to TGF-β1. To rigorously test out either possibility (active transcription versus mRNA stability of endogenous seprase mRNA), I needed to look at possible binding of Smad transcription factors and the transcriptional repressor c-Ski, to the seprase promoter in both A375 and LOX cell lines. If Smad binding was observed on SBE #7AB, it would very strongly point to the active transcriptional regulation of seprase by TGF-β, and as a direct transcriptional target, in essence ruling out the decreased stability of seprase promoter reporters mutated at SBE #7AB compared to the WT seprase promoter reporter. I also expected to observe differential levels of either Smad3/4 and/or c-Ski in A375 and LOX cell lines given their differential seprase mRNA levels, again assuming that transcription is behind such differences observed.

Indeed, a significant amount of Smad3 and Smad4 binding to SBE #7AB was observed in A375 and LOX cells, as both cells had active TGF-β signaling and expressed seprase mRNA. As I expected there was a more enhanced binding of the Smads to SBE #7AB in LOX cells than in A375 cells, suggestive of the increased transcription of seprase in LOX cells. In addition, I consistently found binding of the c-Ski repressor protein to SBE #7AB in A375 cells but not in LOX cells. The binding of c-Ski was not enriched to the same degree as Smad3/4 in these cells, suggesting that not all seprase gene promoters occupied by Smad3/4 on SBE #7AB were equally occupied by c-Ski. The very low level expression of the c-Ski repressor and lack of binding to the SBE #7AB in LOX cells again fully explains higher seprase mRNA produced in LOX cells than in A375 cells. Addition of exogenous TGF-β1 to both A375 and LOX cells caused increases

in binding of Smad3 and Smad4 to SBE #7AB as compared to the untreated counterparts, demonstrating that the seprase promoter is TGF-β-inducible in melanoma cells *in vivo*. In addition, c-Ski repressor protein levels were abolished in response to TGF-β1 stimulation and binding to the seprase promoter prevented in A375 cells accordingly. When taking into consideration all of the lines of evidence accumulated thus far, I felt comfortable in saying that the transcriptional regulation of the seprase gene was indeed taking place in these melanoma cell lines, and that this regulation was clearly dependent on TGF-β signaling. It seems extremely unlikely that the binding of Smads to the SBE #7AB DNA would in any way influence mRNA stability, but would most definitely be able to influence transcription given the role of the Smads as transcription factors. The same holds true for c-Ski binding to the SBE #7AB, I highly doubt that the binding of a transcriptional repressor protein to seprase promoter DNA would alter mRNA stability, but rather is much more likely to prevent active transcription off of the promoter. To examine whether or not c-Ski was actually capable of influencing seprase transcription I chose to overexpress c-Ski in both A375 and LOX melanoma cell lines.

Results indicated that the overexpression of c-Ski caused significant decreases in the level of endogenous seprase mRNA in both A375 and LOX cell lines as compared to vector-expressing control cell lines. In addition, c-Ski binding to SBE #7AB was enhanced, in both A375 and LOX cell lines demonstrating that the seprase promoter was directly targeted by this repressor to inhibit TGF-β-mediated gene transcription. The results suggested that c-Ski was predominantly repressing the seprase promoter through either the recruitment of additional co-repressors, blockade of transcriptional activators, or through the formation of inactive Smad complexes on the SBE (Akiyoshi et al., 1999; He et al., 2003; Luo et al., 1999; Suzuki et al., 2004). Although I did not absolutely prove this, I believe that if c-Ski prevented or interfered

with Smad complex formation (Ueki and Hayman, 2003; Wu et al., 2002) or prevented R-Smad phosphorylation (Prunier et al., 2003), we would expect a more diminished binding of Smad3/4 to the SBE as a result of the inability of the Smads to either enter the nucleus and/or bind to DNA which was not observed. A similar percentage of Smad binding was observed in both cell lines regardless if c-Ski was overexpressed or not. In addition, and similar to A375 cells, LOX cells were capable of TGF-β-induced ubiquitin-dependent proteasomal degradation of c-Ski, as addition of MG132 stabilized c-Ski protein significantly in LOX BP-Ski overexpressing cells as compared to LOX BP cells, and caused a more severe repression of seprase transcription as compared to BP/BP-Ski LOX cells in the untreated condition.

In addition to overexpressing the transcriptional repressor c-Ski in melanoma cell lines I wanted to validate by other additional approaches that blocking TGF-β signaling would result in a reduction in TGF-β-mediated seprase transcription. To this end, another genetic approach taken was to transiently express a dominant negative version of the TGF-β Type II receptor (TGF-β RII Δcyt) in both A375 and LOX cell lines. I observed decreases in seprase mRNA levels in both cell lines transfected with the dominant negative as compared to vector transfected controls as was expected. Although decreases were significant they were not profound, probably due to the fact that in the pooled transfectants not all cells were expressing the dominant negative, or to the same degree.

I also effectively blocked TGF-β-dependent seprase transcription in both melanoma cell lines by treatment of cells in culture with the specific TGF-β RI receptor chemical inhibitor SB-431542, or by administration of varying concentrations of a neutralizing antibody against TGF-β ligand. Taken together, these results indicate that targeting essentially any critical component of

the TGF-β signaling machinery will prevent TGF-β-dependent seprase transcription. Of particular interest were the experiments conducted with forced overexpression of c-Ski and treatment of cells with the neutralizing antibody. This is because the fundamental difference between the LOX and A375 cell line was a substantial difference in the amount of TGF-β1 produced by each cell line, and the differential expression/protein stability of c-Ski in these cell lines. Therefore, equalizing such differences, i.e. forced expression of c-Ski (in the presence of MG132 as it were for LOX cells to stabilize c-Ski), or neutralization of TGF-β1 production/autocrine signaling by administration of anti-TGF-β should have resulted in similar reductions in seprase mRNA production, and for the most part this is what I observed. LOX cells required a considerably higher concentration of neutralizing antibody but this is understandable given the amount of TGF-β1 that these cells are producing.

In conclusion, I find that seprase, which was cloned from the LOX human metastatic melanoma cell line (Goldstein et al., 1997; Pineiro-Sanchez et al., 1997) and associated with increased cellular invasiveness (Aoyama and Chen, 1990), is transcriptionally regulated in several human melanoma cell lines via the canonical TGF-β-Smad signaling pathway. I can conclude this upon the results of a combination of several different approaches, by no means any one definitive experiment or assay. Firstly, cell lines with differential levels of seprase mRNA also have similar differential seprase promoter activities, and in a similar ratio from cell line to cell line. Secondly, both endogenous seprase mRNA and seprase promoter activities are increased in A375 and LOX cells treated with TGF-β1. Thirdly, I located and validated the existence of a true Smad-binding element in the proximal seprase promoter which was both necessary and sufficient for TGF-β-dependent seprase transcription. This Smad-binding element was bound by Smad3 and Smad4 in a TGF-β-dependent manner indicating direct transcriptional

targeting, as was the presence of the c-Ski repressor in the cell line with lower expression of the gene. Finally, blockade of TGF-β signaling by genetic, chemical, or neutralizing antibody studies all reduced TGF-β-dependent seprase transcription. Future studies would be to further characterize additional binding sites and/or transcription factors involved in seprase transcription, and possibly in different cell or tumor types for example. Even more intriguing would be to assess the invasive or metastatic potential of A375 and LOX melanoma cell lines with altered TGF-β signaling (seprase transcription) through either stable genetic approaches (overexpression of ski, knock-down of Smads), or chemical inhibition of the TGF-β RI for example in xenograft spontaneous metastasis animal models.

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