

Suppression of Latent Transforming Growth Factor (TGF)- β 1 Restores Growth Inhibitory TGF- β Signaling through microRNAs⁵

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Cancer cells secreting excess latent TGF- β are often resistant to TGF- β induced growth inhibition. We observed that RNAi against TGF- β 1 led to apoptotic death in such cell lines with features that were, paradoxically, reminiscent of TGF- β signaling activity and that included transiently enhanced SMAD2 and AKT phosphorylation. A comprehensive search in HeLa cells for potential microRNA drivers of this mechanism revealed that RNAi against TGF- β 1 led to induction of pro-apoptotic miR-34a and to a globally decreased oncomir expression. The reduced levels of the oncomirs miR-18a and miR-24 accounted for the observed derepression of two TGF- β 1 processing factors, thrombospondin-1, and furin, respectively. Our data suggest a novel mechanism in which latent TGF- β 1, thrombospondin 1, and furin form a microRNA-mediated regulatory feedback loop. For cells with high levels of latent TGF- β , this provides a potentially widespread mechanism of escape from TGF- β -mediated growth arrest at the earliest point in the signaling pathway, TGF- β processing.

Transforming growth factor- β (TGF- β)² polypeptides are cytokines from a large family of ligands and receptors, which regulate epithelial, neuronal, and immune cells by controlling proliferation, differentiation, and survival processes with an intricate complexity. Cells synthesize the three isoforms of TGF- β in excess and their rate-limiting, complex mechanisms of activation are regulated in a cell type and stimulation-specific fashion (1). TGF- β 1 is expressed as a pro-peptide precursor comprising the mature form and a latency-associated peptide (LAP). The pro-peptide dimerizes and is nicked before secretion by furin-like proteases but remains self-associated. Secretion is promoted after conjugation of a latent TGF- β -binding protein (LTBP) to the LAP forming a large latent complex (LLC), which associates with the extracellular matrix. Mature TGF- β can be released from the LLC *in vitro* by heating or acid but *in vivo* release requires cell surface furin proteases as well as extracellular matrix proteins such as thrombospondin-1 (THBS1; TSP1) and integrins that induce conformational changes in the complex to promote dissociation of mature

ligand. The mechanisms of activation, and also the type of intracellular signaling, may depend on the TGF- β -containing complex bound to the cell surface. Consequently, cells may respond differently to TGF- β from autocrine or exogenous origin by activating, for example, alternative growth inhibitory pathways (2). By maintaining a source of latent TGF- β close to its site of action, cells can initiate rapid signaling without the need for new protein synthesis (3). Once activated, TGF- β binds a membrane-bound serine/threonine receptor complex (T β RI/T β RII), which phosphorylates various substrates. These include transcription factors SMAD2 and SMAD3, which accumulate in nuclear complexes with co-activators and co-repressors, or molecules from numerous non-Smad pathways (4). The cellular response to TGF- β is thus a balanced activation of Smad and/or non-Smad signaling pathways determined by cellular “context” (5, 6), as well as signaling thresholds and signaling duration (7, 8).

TGF- β signaling via SMADs causes growth inhibition of epithelial cells by transcriptional induction of cyclin-dependent kinase inhibitors *P21* and *P15*, and the repression of transcription factors *MYC*, *ID1*, and *ID2* (9, 5). TGF- β is often found up-regulated in tumors and tumor cell lines (10, 11) but resistance to growth inhibitory TGF- β signaling is an important and common event in tumorigenesis (12). Whereas some tumors acquire somatic changes in TGF- β signaling components *e.g.* mutations in SMADs or TGF- β receptors, others become resistant to the antiproliferative response while maintaining the ability to signal. Here, TGF- β becomes an oncogenic factor inducing proliferation, angiogenesis, and metastasis. Several mechanisms of resistance have been documented, mostly involving downstream pathway components (13). In particular, the PI3K-AKT survival pathway plays a prominent role in cancers which are resistant to cytostatic TGF- β signaling (14, 15), for example by blocking induction of *P21* in glioblastoma cells (16). Thus, inhibition of PI3K-AKT signaling restored TGF- β growth arrest in glioblastoma (16) and in colon carcinoma cell lines (15).

MicroRNAs (miRNAs) are single-stranded RNAs of ~22 nucleotides which regulate the expression of a large fraction of genes. MiRNAs bind with partial sequence complementarity to sites, usually in the 3'-UTRs of mRNAs, and inhibit protein translation or induce mRNA degradation. An increasingly recognized feature of miRNA function is the parallel targeting of regulatory pathway members by multiple miRNAs (17). Therefore, even relatively modest changes in the expression levels of individual miRNAs and their protein targets often have signif-

⁵ The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables S1 and S2.

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² The abbreviations used are: TGF, transforming growth factor; LAP, latency-associated peptide; LLC, large latent complex; miRNA, microRNA.

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icant phenotypic consequences. Signaling pathways use miRNAs to switch the gene expression programs of cells (18, 19) and not surprisingly, the TGF- β pathway has many documented examples of miRNA activity including: *miR-15/16* (20), *miR-224* (21), *miR-106b-25* (22), *miR-200* family (23–26), *miR-155* (27), *miR-181b/d* (28), *miR-21* (29), *miR-17~92* (30, 31), and *miR-24* (32–35).

In a search for miRNAs which play central roles in disease-associated mechanisms we selected TGF- β signaling for investigation. We conducted RNAi against TGF- β 1 in several cancer cell lines and observed apoptosis after 3 days. TGF- β 1 depletion in HeLa and LN-18 cells was associated with induction of TGF- β processing factors and transient TGF- β signaling prior to apoptosis. In HeLa cells apoptosis was preceded by repression of *miR-18a* and *miR-24*, which target *THBS1* and *FURIN*, respectively. The data suggest that in some cell lines latent TGF- β 1 inhibits cytosolic TGF- β signaling through post-transcriptional repression of its own processing factors. For tumor cells which secrete high levels of latent TGF- β this represents potentially an important mechanism of escape from cytosolic TGF- β signaling at the earliest point in the pathway. Furthermore, it implies that in these instances pharmacological targeting of TGF- β 1 mRNA may be of therapeutic value in pathological mechanisms caused by loss of the cytosolic response to TGF- β signaling.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—Hela (ATCC, CCL-2) and LN-18 (ATCC, CRL-2610) cells from LGC (Molsheim, FR) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma). siTGF β 1 targeting human TGF- β 1 (NM_000660) is CCAACUAUUGCUUCAGCUC (1712–1730); siTGF β 1(s) is CGUGGAGCUGUACCAGAAA (1368–1386). siRNAs, miRNA mimics and inhibitors were from Dharmacon, siCon was from Ambion (AM4640). RNAs were transfected using Oligofectamine (12252-011, Invitrogen) according to manufacturer's instructions. Recombinant human mature TGF- β 1 (100-B), latent TGF- β 1 (299-LT/CF), and TGF- β neutralizing antibody (MAB1835; Clone 1D11) were from R&D Systems. SB431542 was from Santa Cruz Biotechnology (SC-204265).

RNA and miRNA Q-PCR—Total RNA was extracted using mirVanaTM miRNA Isolation kit (AM1560; Ambion). For mRNA analysis, 1 μ g of total RNA was reverse transcribed using the M-MLV reverse transcriptase kit (28025-013, Invitrogen) according to manufacturer's instructions. Expression levels were assayed using Power SYBR[®] Green PCR Master Mix (4367659; Applied Biosystems). PCR cycling conditions were 95 °C/10 min and 40 cycles of (95 °C/15 s; 60 °C/1 min). Values were normalized using GAPDH or average of all measured mRNAs. RT-PCR primers sequences are shown in [supplemental Table S1](#).

For miRNA analyses, the TaqMan[®] Human MicroRNA Assay set v1.0 (4383443; Applied Biosystems) was used. Total RNA was reverse transcribed with miRNA-specific RT primers and amplified with TaqMan miRNA-specific primers. cDNA was synthesized from 10–20 ng of total RNA in a 15 μ l volume

using TaqMan MicroRNA Reverse Transcription kit (4366597; Applied Biosystems), according to manufacturer's instructions. All samples were measured in triplicates; the threshold cycle (CT) values were transformed to relative quantities.

ELISA—TGF- β 1 in cultured cells was quantified with human TGF- β 1 DuoSet ELISA (DY240; R&D Systems), according to the manufacturer's instructions. Cells were grown in 96-well plates in 1% FBS-containing media and transfected with siRNAs. Supernatants were acidified with 1 M HCl and neutralized with 1.2 M NaOH/0.5 M HEPES prior to assay for total TGF- β 1. The concentrations of active TGF- β 1 were analyzed on non-acidified samples. TGF- β 1 in the FBS was initially 180 pg/ml for the 1% FBS concentration used in these experiments.

Protein Analysis—Cells were lysed with RIPA lysis buffer (R0278; Sigma). Protein concentrations were determined using a BCA assay (Thermo Fisher Scientific 23225), 10–20 μ g of protein was mixed with equal quantities of SDS loading buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, 0.2% bromophenol blue). Samples were heated at 99 °C for 5 min, separated on SDS gels and transferred to polyvinylidene difluoride membranes. Non-specific membrane binding was blocked for 1 h at room temperature with 5% BSA (or milk) in phosphate-buffered saline containing 0.05% Tween 20. Membranes were incubated overnight at 4 °C with primary antibodies from Cell Signaling (TGF- β 1: 3711; P-SMAD2: 3108; SMAD2: 3122; P-AKT: 4060; AKT 9272) and from Santa Cruz Biotechnology (p53: SC-126; P21: SC-71811; TSP1: SC-81755; FURIN: SC-20801). After washing membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1–2 h at room temperature in blocking buffer. Signals generated by the chemiluminescent substrate (ECL(+); Amersham Biosciences) were captured by a cooled CCD camera (Bio-Rad). Protein bands were quantified by densitometry using the analysis software ImageJ.

Plasmid DNA Transfections and Luciferase Assays—Firefly luciferase reporter genes containing full-length human 3'-UTRs from *THBS1* (S211182) and *FURIN* (S209837) were from SwitchGear Genomics (Menlo Park, CA). cDNA of TGF- β 1 (SC119746) was from OriGene (Rockville, MD). cDNA of *THBS1* (pbla-htsp1) was from InvivoGen (San Diego, CA). SBE-Luc reporter construct was a gift from Dr. B. Vogelstein and contains four SMAD2/3 binding elements. For luciferase assays, HeLa cells were grown in white 96-well plates, 20 ng of plasmid DNAs were transfected per well using jetPEI (101-10; Polyplus) according to manufacturer's protocol. siRNAs were transfected after 24 h with indicated doses. After 24 h, supernatants were removed and 30 μ l of Bright-Glo Luciferase substrate (E2610; Promega) was added to each well. Luminescence signals were measured on a microtiter plate reader (Mithras LB940, Berthold Technologies).

Apoptosis Assay—Caspase-3/7 activity was measured in supernatants and lysates of transfected cells using a chemiluminescent substrate (Caspase-Glo 3/7 substrate, G8090, Promega). Cells were grown in 96-well plates, and transfected with siRNAs. For time-course experiments, 5 μ l of supernatants were transferred from the same wells at 24, 48 and 72 h time points to white 384-well plates and mixed with equal volumes of substrate. Chemiluminescence was measured in sealed plates after 30 min at room temperature in a plate reader. For mea-

surements of cell-associated caspase 3/7 activity cells were lysed in PBS containing 1% Triton X-100 and 5 μ l of lysates were mixed with equal volumes of substrate and otherwise assayed as above.

RESULTS

Inhibition of TGF- β 1 mRNA Causes Apoptosis in Cervical Carcinoma Cells—Like most cervical carcinoma cell lines HeLa express high levels of TGF- β 1 (36). They respond to exogenously-delivered, mature recombinant TGF- β 1 but they do not undergo apoptosis (data not shown). Independent treatment of HeLa cells with two siRNAs (siTGF β 1 and siTGF β 1(s)) specific to the TGF- β 1 sequence led to a dose-dependent down-regulation of TGF- β 1 mRNA by greater than 80% at 15 nM by both siRNAs (Fig. 1A), though siTGF β 1 was more potent in most assays. Loss of TGF- β 1 mRNA caused a rapid (24 h) reduction of intracellular un-nicked latent TGF- β 1 protein, which was detected by a pan anti-TGF- β antibody (Fig. 1B). Reduction in total TGF- β 1 protein in cell supernatants was quantified using an ELISA specific for the TGF- β 1 isoform after treatment with acid (Fig. 1C), which converts the majority of latent nicked TGF- β 1 to the mature form (37). Cells undergoing TGF- β 1 RNAi showed signs of increasing caspase 3/7 activity on day 2 post-transfection, which rose dramatically on day 3 (Fig. 1D) and was associated with massive cell death. Similar results were obtained after the transfection of siTGF β 1 into Caski and Siha cell lines (Fig. 1E). The results suggest that high levels of TGF- β 1 are essential for the survival of cervical carcinoma cells. In order to ascertain the mechanism of cell death, RNA from treated cells was analyzed for the expression of cell death genes using real-time PCR (Q-PCR). Several p53 response genes including *CDKN1A* (P21), *BAX*, *TNFR10B*, *FAS*, and *BTG2* were strongly induced at 48h (Fig. 2A). Accordingly, analysis of protein samples from treated cells showed dose-dependent up-regulation of p53 and P21 proteins (Fig. 2B). HeLa cells express wild-type p53 and RB proteins which are inactivated by HPV E6 and E7, respectively. Inhibition of viral protein expression reactivates these tumor suppressor proteins (38) and indeed, HPV E6 mRNA was repressed as a result of TGF- β 1 RNAi (Fig. 2A).

miRNAs Contribute to TGF- β 1-RNAi-mediated Apoptosis in HeLa Cells—Most miRNA-mRNA interactions are fine-tuning contributions to the robustness of systems (17). In a small number of cases, however, a single miRNA-mRNA interaction is capable of switching a gene expression program in cells. The dysregulation of such interactions may contribute to disease-causing mechanisms and therefore represent potential drug targets. In a first step to identify miRNAs which influence TGF- β 1 RNAi-mediated apoptosis, we measured the expression of 448 miRNAs in cells treated with a single dose of siTGF β 1 or oligofectamine control using stem-loop PCR. From the screen we selected 48 miRNAs for a repeat study using both TGF- β 1 siRNAs at three doses. We set stringent standards for the analysis of miRNA data because, compared with treatment-induced changes in mRNA expression, fold changes in individual miRNAs are typically lower. We called "absent" any miRNAs with cycle threshold (CT) values higher than 29. Only miRNAs which were regulated in the same direction by both

siRNAs were considered of interest. A large fraction of miRNAs which passed these filters has been previously associated with cancer and/or elements of TGF- β signaling (Fig. 2C). For example, the large induction of *miR-34*, which is capable of inducing apoptosis alone by targeting genes involved in cell cycle and apoptosis, was consistent with the induction of p53 (39, 40). On the other hand, the modest down-regulation of oncomirs *miR-18a*, *miR-20b*, *miR-93*, *miR-24*, *miR-181b/d*, and *miR-155* was consistent with a network of antiproliferative miRNA activity. Furthermore, many of these miRNAs have been previously reported to be repressed during TGF- β signaling including *miR-24*, *miR-181b/d*, *miR-224*, *miR-155*, and *miR-17~92* cluster.

To identify miRNAs whose regulation contributed to the TGF- β 1 RNAi-induced apoptosis, we measured caspase 3/7 activity after transfection of siTGF β 1 in combination with double-stranded RNA reagents. In experiments designed to control for unspecific effects of siRNA against TGF- β 1, delivery of increasing doses of a plasmid expressing (latent) TGF- β 1 mRNA rescued the cells from siTGF β 1-mediated caspase 3/7 induction (Fig. 2D). Interestingly, a clear reproducible reduction in background caspase 3/7 activity was also apparent at the highest dose of plasmid treatment alone, and correlated with the highest levels of TGF- β 1 mRNA and protein (data not shown). A siRNA to *TP53* (siP53) (41) also partially reversed the apoptosis and served as an additional positive control (Fig. 2E). The negative control siCon showed a small effect whereas *miR-34* showed no effect in combination with siTGF β 1 (Fig. 2F). The anti-miRNA oligonucleotide of *miR-34a* (AMO-34a) inhibited siTGF β 1-induced apoptosis almost to background levels (Fig. 2E). Mimics of *miR-18*, *miR-24*, and *miR-181* were able to reduce partially caspase 3/7 induction, consistent with a shared role in the apoptotic phenotype.

Repression of Latent TGF- β 1 Leads to Increased TGF- β Processing—The relationship between AKT cell survival and p53 cell death pathways has been described as a balance (4, 42). Analysis of protein from siTGF β 1-treated cells showed a dose-dependent rise in phospho-AKT (P-AKT) on day 1, followed by dose-dependent decreases on days 2 and 3. This rise appeared to precede p53 induction, and the onset of apoptosis (Fig. 3A). A transient induction of AKT activity prior to apoptosis was also described during an investigation of TGF- β processing/signaling in mink lung epithelial cells (43). Taking together this as well as other literature reports (15, 44) with our observations led us to suspect that paradoxically we were observing a restoration of growth-inhibitory TGF- β signaling activity during TGF- β 1 RNAi. It therefore also implied that the high levels of latent TGF- β produced by cells contributed to their resistance to TGF- β induced growth inhibition, possibly through elevated AKT signaling (15). Mindful of the difficulties of measuring TGF- β activity in cells undergoing TGF- β 1 RNAi, we assayed for phospho-SMAD2 (P-SMAD2) activity after TGF- β 1 RNAi at 7, 24, and 48 h (Fig. 3B). After 24 and 48 h levels of P-SMAD2 decreased, as expected for a potent, time-dependent down-regulation of TGF- β 1 ligand. However, a transient induction of P-SMAD2 was observed at 7 h post-siRNA transfection in comparison to slightly elevated levels of total SMAD2. At this point TGF- β 1 mRNA had been reduced by ~50% (Fig. 3C). In sup-

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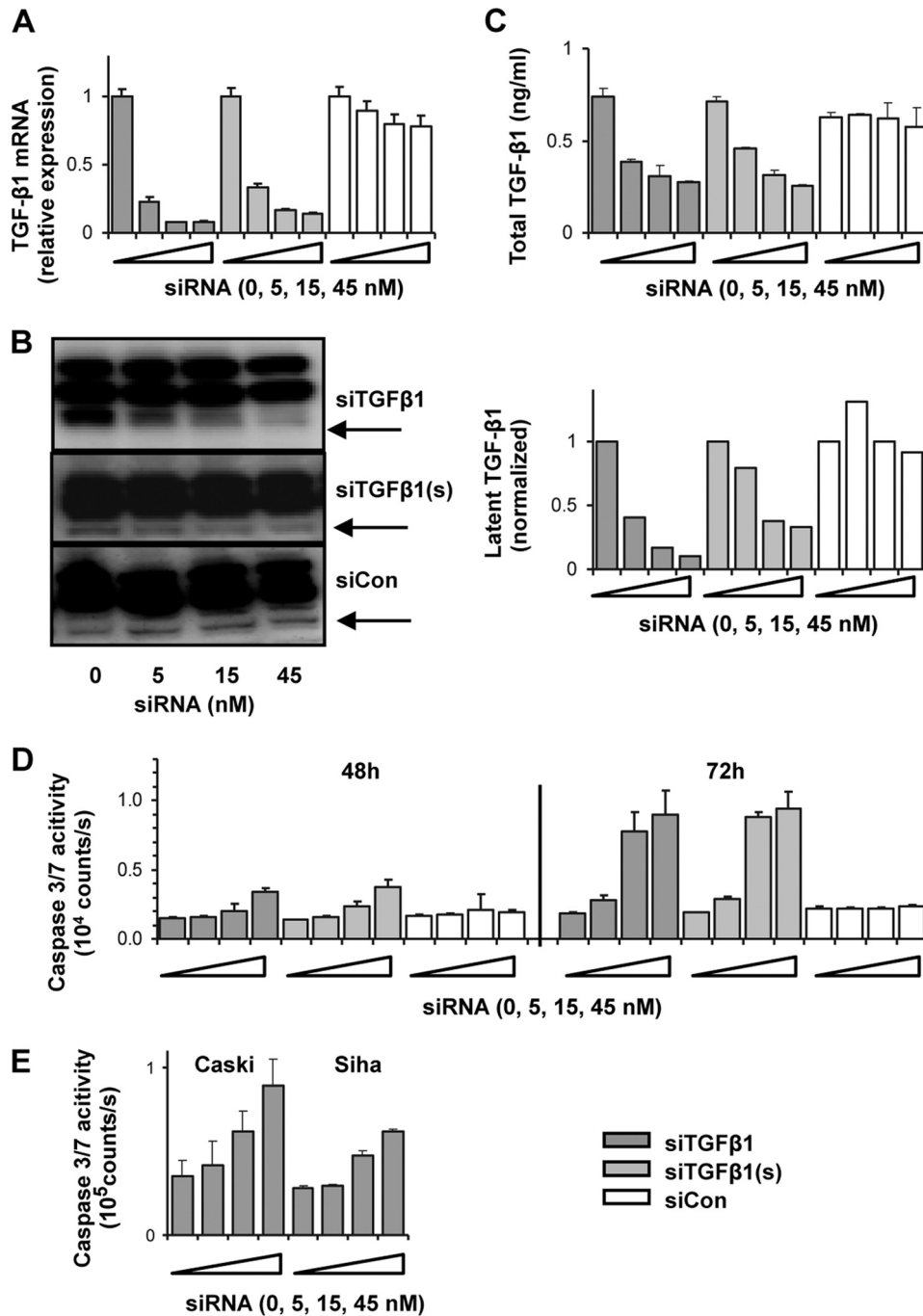


FIGURE 1. Down-regulation of TGF- β 1 by RNAi leads to apoptosis in cervical carcinoma cells. *A*, TGF- β 1 mRNA after transfection of HeLa cells with siRNAs targeting TGF- β 1 and a control siRNA (siCon). Total RNA was isolated 48 h post-transfection. Relative expression of TGF- β 1 mRNA is displayed (mean of PCR triplicates; single RNA samples \pm S.D.). *B*, cell-associated latent un-nicked TGF- β 1 after siRNA treatment. Proteins from HeLa cells 24 h post-transfection were analyzed using a pan anti-TGF- β antibody (left panel) and were quantified by densitometry (right panel). *C*, total secreted TGF- β 1 after siRNA transfection. Supernatants from HeLa cells grown in 1% FBS media were assayed by ELISA after acidification 72 h post-transfection. Total TGF- β 1 protein is displayed (mean of triplicate transfections \pm S.D.). *D*, caspase 3/7 activity in HeLa cells was measured 48 h and 72 h post-transfection (mean of triplicate transfections \pm S.D.). *E*, Caski and SiHa cells were treated with increasing doses of siTGF β 1. Caspase 3/7 activity was measured 72 h post-transfection (mean of triplicate transfections \pm S.D.).

port of this data, cells treated with siTGF β 1 and a luciferase reporter gene construct bearing four SMAD binding elements (SBE) in its promoter yielded an induction of luciferase activity at both 8 and 24 h (Fig. 3C).

The majority of TGF- β secreted by most cells is in a latent nicked form. Given the multi-step nature of TGF- β processing, it seemed plausible that an increased TGF- β signaling activity

might arise from increased processing of extracellular latent TGF- β , despite declining levels of intracellular TGF- β due to RNAi. We therefore examined siTGF β 1-treated HeLa cells for indications of elevated TGF- β processing. Concentrating cell supernatants facilitates detection of trace quantities of total extracellular TGF- β by Western blot (45). However, our anti-TGF β antibody does not distinguish between the three mature

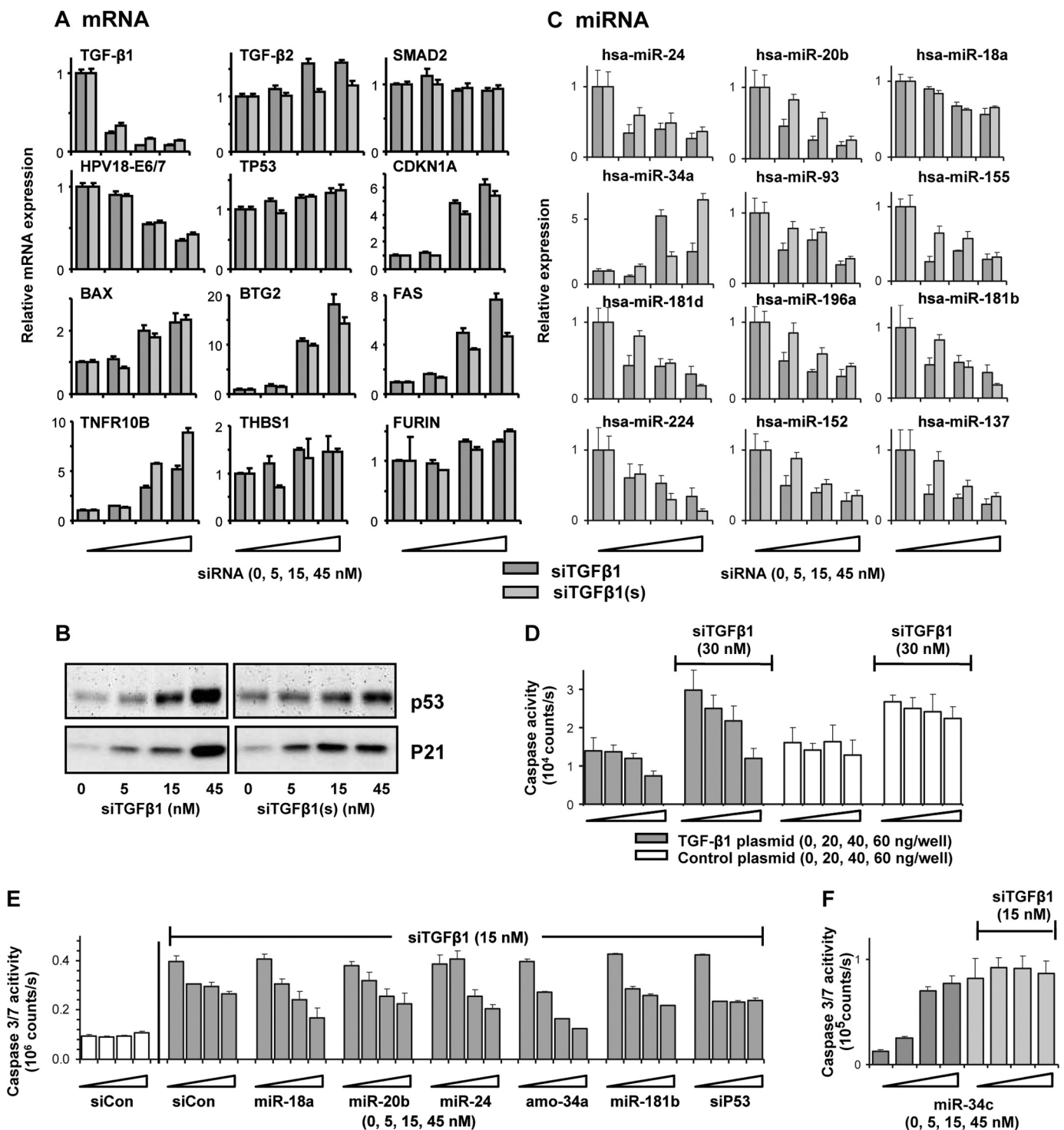
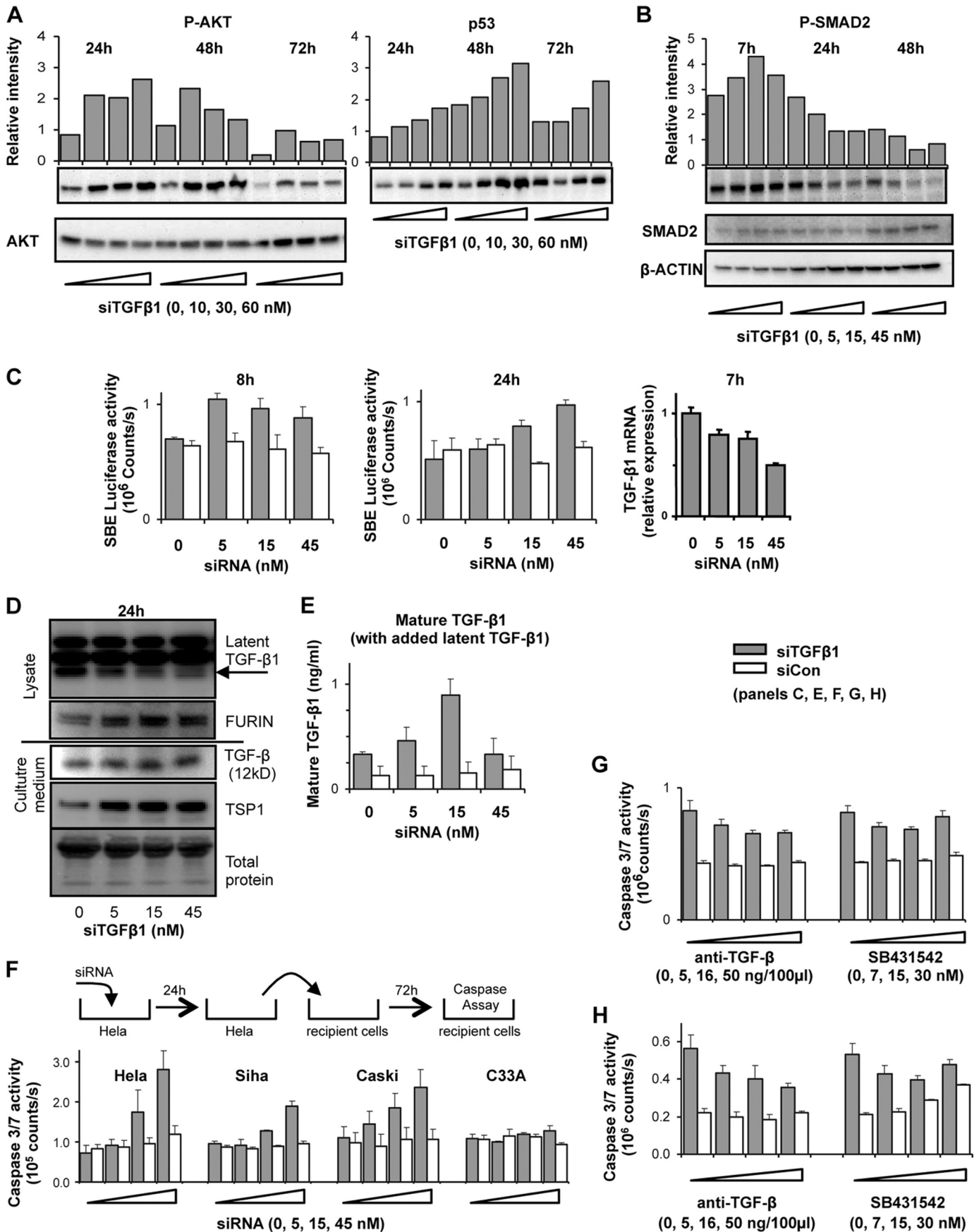


FIGURE 2. Changes in expression of selected mRNAs, miRNAs, and proteins upon TGF- β 1 RNAi in HeLa cells. *A*, selected mRNAs after transfection with TGF- β 1 siRNAs. Total RNA was subjected to Q-PCR analysis 48 h post-transfection. Data were normalized to average of CT values of all assayed mRNAs. Relative expressions of mRNAs are displayed (mean of PCR triplicates; single RNA samples \pm S.D.). *B*, p53 and P21 proteins after siRNA treatment. Cell lysates were analyzed with antibodies 48 h post-transfection. *C*, miRNA expression after siRNA treatment. Total RNA was isolated 48 h post-transfection and subjected to miRNA stem-loop Q-PCR. Data were normalized to hsa-miR-30c. Relative miRNA expression levels are displayed. Numerical data including *p* values are shown in [supplemental Table S2](#) (mean of PCR triplicates; single RNA samples \pm S.E.). *D*, attenuation of siTGF β 1-mediated caspase 3/7 induction after co-transfection with plasmid expressing TGF- β 1 cDNA. Cells were first treated with increasing doses of TGF- β 1 expressing plasmid, 24 h later transfected with an apoptosis-inducing dose of siTGF β 1 (30 nM). Caspase 3/7 activity was measured 72 h post-transfection (mean of triplicate transfections \pm S.D.). *E*, attenuation of siTGF β 1-mediated caspase 3/7 induction after co-transfection with selected miRNAs or AMOs. Cells were treated simultaneously with an apoptosis-inducing dose of siTGF β 1 (15 nM) and increasing doses of indicated reagents. Caspase 3/7 activity was measured 72 h post-transfection (mean of triplicate transfections \pm S.D.). *F*, HeLa cells were treated with increasing doses of hsa-miR-34c alone or in combination with an apoptosis-inducing 15 nM dose of siTGF β 1. Caspase 3/7 activity was measured 72 h post-transfection (mean of triplicate transfections \pm S.D.).

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TGF- β isoforms, nor small amounts of TGF- β present from the outset in the serum needed for cell proliferation (see “Experimental Procedures”). We examined latent and total TGF- β protein in cellular lysates and concentrated cell media, respectively with increasing doses of siTGF β 1 (Fig. 3D). As RNAi lowered levels of the un-nicked precursor latent TGF- β 1 in lysates, so levels of total TGF- β cytokine appeared to increase, though we could not be certain of the source nor the isoform. We therefore turned to the TGF- β 1 ELISA, which measures total TGF- β 1 protein with excellent sensitivity after acid treatment of supernatants converts nicked latent TGF- β 1 to the mature (12 kDa) form. We transfected cells with siTGF β 1 and then measured mature TGF- β 1 after 72 h, with and without acidic work-up. ELISA after 1 M HCl acid treatment revealed a strong dose-dependent reduction of total TGF- β 1 (Fig. 1C). Forgoing acid treatment, however, returned only background values, indicating undetectable amounts of mature TGF- β 1 in the supernatants. As an alternative, we therefore modified the protocol in effort to measure changes in processing activity. To achieve levels of measurable mature TGF- β 1 without acidification, recombinant nicked latent TGF- β 1 protein was added to cultures of siCon- and siTGF β 1-treated cells prior to ELISA at 72 h. SiTGF β 1, but not siCon treatment now increased mature TGF- β 1 up to 3-fold (Fig. 3E). We concluded that TGF- β 1 RNAi mediates secretion of factors that are capable of processing exogenously-added latent TGF- β 1 to mature TGF- β 1. Extrapolating, we surmised that these same factors would also be capable of converting endogenously-derived latent TGF- β to mature TGF- β , and that this was likely the source of transient signaling through SMAD2 prior to apoptosis.

To determine whether increased extracellular TGF- β -processing activity contributed to apoptosis during TGF- β RNAi, we used un-treated “indicator” recipient cells, as previously described (43). Supernatants from HeLa cells treated with escalating doses of siTGF β 1 or siCon were transferred after 24 h to four cervical carcinoma cell cultures (HeLa, Caski, Siha, C33-A) and caspase 3/7 activity was assayed after a further 72 h (Fig. 3F). The transferred supernatants contained no detectable caspase 3/7 activity prior to transfer to indicator cultures (see Fig. 1D). Dose-dependent induction of caspase 3/7 activity was observed in HeLa, Caski, and Siha indicator cells. RNA isolated from the HeLa indicator culture at 24 h post-transfer showed no down-regulation of TGF- β 1 mRNA indicating that the apoptosis in these cells did not result from RNAi caused by transfer of any siTGF β 1 from the primary transfection. C33-A is a non-

HPV infected cervical carcinoma cell line which does not express T β R1. No caspase 3/7 activation was observed after media transfer to C33-A recipient cultures, suggesting that apoptosis in HeLa, Caski, and Siha indicator cells and also, presumably, in the primary transfected HeLa cells required functional T β R1. We transfected HeLa cells with a fixed dose of siTGF β 1 in combination with increasing doses of either the blocking anti-TGF- β antibody, or a selective inhibitor of T β R1 (SB431542). Inductions of caspase activity were partially attenuated (Fig. 3G). However, a greater attenuation was obtained by treating the indicator HeLa cells with either the anti-TGF- β antibody or SB431542 upon supernatant transfer from the siTGF β 1-treated HeLa cells (Fig. 3H). All taken together, the results implied that apoptosis occurred, at least partially, through restoration of cytosolic TGF- β signaling via T β R1 and/or T β R2.

TGF- β 1 RNAi Up-regulates TSP1 and FURIN—We searched next for the source of elevated TGF- β processing activity. TGF- β stimulates transcription of many of its own activators including FURIN and THBS1, a member of the secreted thrombospondin family (46). Soluble TSP1 binds to and activates latent TGF- β in cell supernatants as well as in a cell-free system (47). FURIN is a protease, which nicks latent TGF- β primarily in the cell but there are also reports of its secretion (48). The mRNA levels of THBS1 and FURIN were slightly elevated after TGF- β 1 RNAi (Fig. 2A), however both proteins showed a strong induction on siTGF β 1 treatment more in accordance with a post-transcriptional activation (Fig. 3D). Transfection of cells with a cDNA expressing THBS1 (Fig. 4A) was associated with a 3-fold increase in caspase 3/7 activity 2–3 days post-transfection, and a 5–6 fold increase in recipient HeLa cells on media transfer (Fig. 4B). Recently, THBS1 was shown to be targeted by miRNAs of the miR-17~92 cluster (49), which is itself transcriptionally repressed by p53 (50). In one account miR-18a was unveiled as a major regulator of tumor angiogenesis via its interaction with THBS1 (18). Recently, miR-18a was shown to regulate SMAD2 (31). The relatively long 3'-UTR of FURIN shows conserved predicted binding sites for miR-17~92, miR-137, and miR-24 (www.targetscan.org; V5.1), all of which were repressed by TGF- β 1 RNAi (Fig. 2C). MiR-24 is one of the most highly expressed miRNAs in HeLa cells and is reportedly suppressed by Smad signaling (35). Furthermore, expression of miR-24 was reported to be altered by TGF- β in hepatocellular carcinoma cells (51), although functions of miR-24 in TGF- β signaling are likely cell-type specific (32). To determine

FIGURE 3. Activation of TGF- β 1 signaling upon down-regulation of latent TGF- β 1 in HeLa cells. A, time course of AKT-phosphorylation and p53 induction after treatment with siTGF β 1. Proteins were analyzed by Western blot. Bar graphs show densitometric evaluations. B, time course of SMAD2 phosphorylation. C, SMAD binding element luciferase reporter assay. Cells were transfected with SBE4 plasmid and were treated with siRNAs. Luciferase activity was measured 8 h (left panel) and 24 h (center panel) post-siRNA transfection. Relative luciferase activity is displayed (mean of triplicate transfections \pm S.D.). HeLa cells treated with siTGF β 1. Total RNA was collected after 7 h and TGF- β 1 mRNA levels were measured (right panel). D, induction of TGF- β processing factors upon TGF- β 1 RNAi. Cells cultured in media containing 1% FBS were treated with siTGF β 1. Lysates and concentrated supernatants were analyzed by Western blotting 24 h post-transfection. E, induction of processing activity by siTGF β 1 treatment. Cells grown in 1% FBS media were treated with siRNAs: 20 ng/ml recombinant latent TGF- β 1 was added 0, 24, and 48 h post-transfection. TGF- β 1 in supernatants was measured by ELISA without acidification 72 h post-transfection (mean of triplicate transfections \pm S.D.). F, caspase 3/7 induction in indicator cell lines by conditioned media of siTGF β 1-treated HeLa cells. Cells were treated with siRNAs for 24 h. The conditioned media were transferred to recipient cells and caspase 3/7 activity was measured 72 h later (mean of triplicate transfections \pm S.D.). G, suppression of caspase 3/7 activation in transfected HeLa cells by TGF- β inhibitors. HeLa cells were treated with 15 nM siRNAs in combination with increasing doses of either the blocking anti-TGF- β antibody, or SB431542. Caspase 3/7 activity was measured 72 h post-transfection (mean of triplicate transfections \pm S.D.). H, suppression of media transfer-induced caspase 3/7 activation in indicator cells by TGF- β inhibitors. HeLa cells were treated with 15 nM siRNAs and after 24 h the conditioned media were transferred to non-transfected recipient HeLa cells. TGF- β blocking antibody or SB431542 were added to the recipient cells and caspase 3/7 activation was measured after 72 h (mean of triplicate transfections \pm S.D.).

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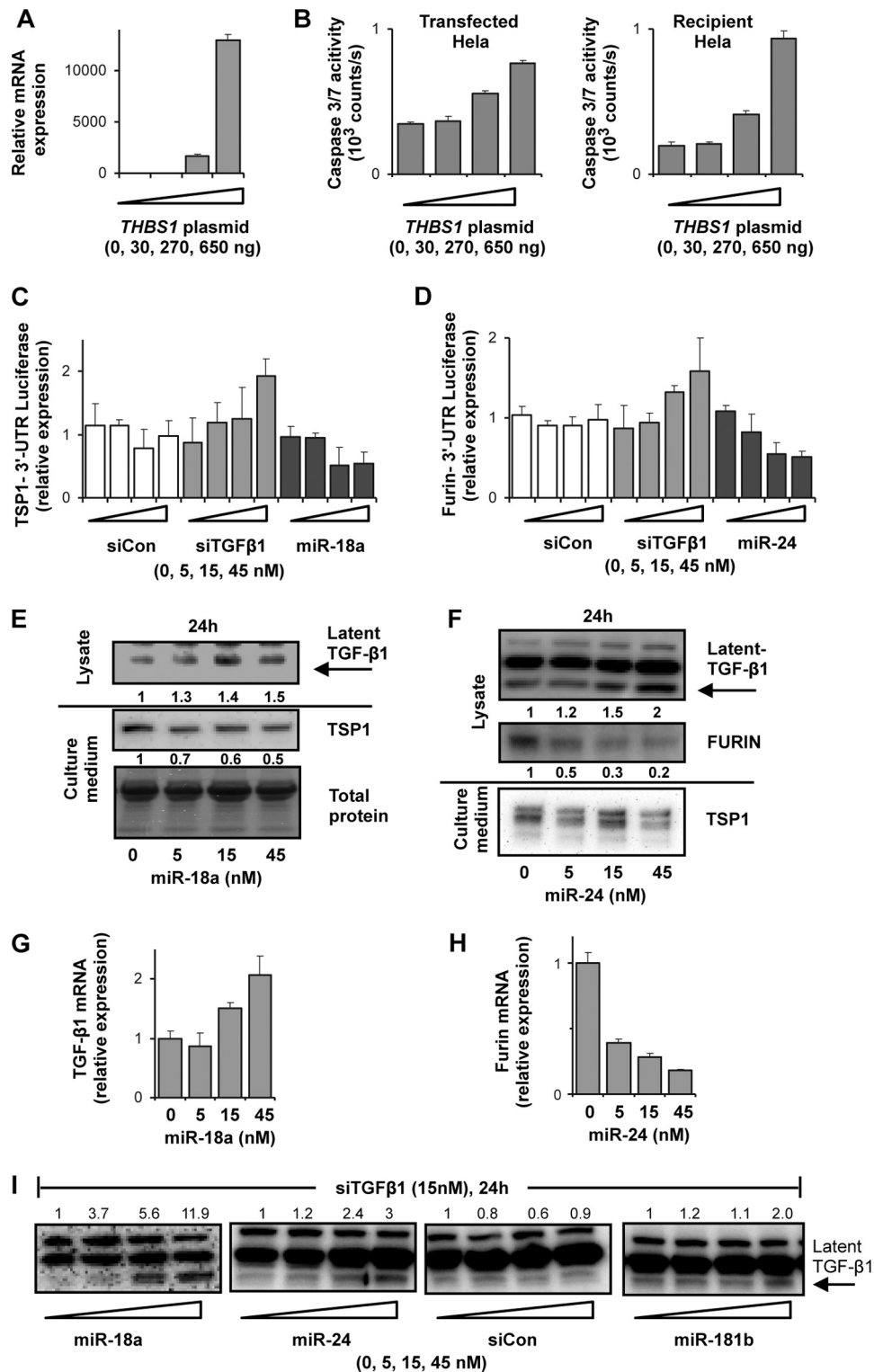


FIGURE 4. Increased maturation of TGF- β upon down-regulation of latent TGF- β in HeLa cells involves miRNAs. *A*, HeLa cells transfected with *THBS1* plasmid. Total RNA was isolated 24 h post-transfection and subjected to Q-PCR using *THBS1* specific primers. *B*, HeLa cells transfected with *THBS1* plasmid. Caspase 3/7 activity was measured from supernatants 24 h post-transfection (*left panel*) (mean of triplicate transfections \pm S.D.). Caspase 3/7 activity was measured from supernatants of recipient HeLa cells 48 h post-transfer (*right panel*) (mean of triplicate transfections \pm S.D.). *C* and *D*, cells transfected with *THBS1* and *FURIN* 3'-UTR reporter plasmids were treated after 24 h with siRNAs or miRNAs. Luciferase activity was measured 48 h after plasmid transfections. Relative luciferase activity is displayed (mean of triplicate transfections \pm S.D.). *E* and *F*, cells grown in media containing 1% FBS were treated with miRNAs. Western blots of proteins from lysates, and supernatants are displayed. *G* and *H*, HeLa cells were transfected with miR-18a and miR-24. Total RNA was isolated 72 h post-transfection and Q-PCR analysis was performed. Relative expressions of TGF- β 1 and *FURIN* mRNAs are displayed (mean of PCR triplicates; single RNA samples \pm S.D.). *I*, HeLa cells were simultaneously transfected with 15 nM siTGF β 1 and increasing doses of miR-18a, miR-24, siCon, and miR-181b. Western blot analyses were performed 24 h post-transfection.

whether *THBS1* or *FURIN* were post-transcriptionally up-regulated by siTGF β 1 we used luciferase reporter constructs bearing their full-length 3'-UTRs. In contrast to siCon, increasing doses of siTGF β 1 elevated luciferase activity suggesting that the induction of these factors during TGF- β RNAi derived to a significant degree from derepression of their UTR (Fig. 4, C and D). To establish whether *miR-18a* and *miR-24* contributed to the regulation of TSP1 and FURIN during TGF- β RNAi, we co-transfected miRNA mimics and their respective reporter constructs into cells. *miR-18a* and *miR-24* dose-dependently inhibited luciferase-*THBS1* and luciferase-*FURIN* by up to 50% (Fig. 4, C and D). To confirm that *miR-18a* and *miR-24* are able to regulate endogenous TSP1 and FURIN, respectively, we isolated protein from cells treated independently with miRNA mimics. A strong reduction in TSP1 was observed 24 h after treatment with *miR-18a* (Fig. 4E), whereas no inhibition was obtained from *miR-24* (Fig. 4F). In contrast, *FURIN* was very strongly repressed by *miR-24*, at both mRNA (Fig. 4H) and protein levels (Fig. 4F). Moreover, basal levels of latent un-nicked TGF- β 1 in cell lysates rose as levels of extracellular TSP1 and cellular FURIN dropped on addition of *miR-18a* and *miR-24*, respectively, but not on treatment with *miR-20b* (data not shown). In the case of *miR-18a* mimic, this resulted at least partly from increased transcription of TGF- β 1 (Fig. 4G), whereas for *miR-24*, it was likely due to intracellular accumulation of the un-nicked latent TGF- β 1 as FURIN was repressed. To confirm the functional importance of the repression of *miR-18a* and *miR-24* during the siTGF- β 1-mediated processing of latent TGF- β , we again co-transfected cells with each mimic in combination with a 15 nM dose of siTGF- β 1. Similar to the TGF- β 1 overexpression vector, increasing amounts of either *miR-18a* or *miR-24* countered the effects of siTGF- β 1: levels of latent TGF- β 1 protein were raised (Fig. 4I) and caspase 3/7 activity was attenuated (Fig. 2, D and E). No such effects were obtained from siCon and only a minor effect was observed at the highest dose from addition of *miR-181b* (Fig. 4I).

In summary, our results demonstrate that TGF- β 1 RNAi activates TGF- β -processing factors TSP1 and FURIN in part by attenuating their post-transcriptional repression by *miR-18a* and *miR-24*, respectively. Furthermore, as *miR-18a* and *miR-24* accumulate latent un-nicked TGF- β 1 the data also suggests that latent TGF- β 1, *miR-18a*, *miR-24*, TSP1, and FURIN are members of a regulatory feedback loop controlling, at least in part, the cytostatic response to TGF- β in HeLa cells. Extrapolating, the experiments suggest a new mechanism in which cancer cells inhibit processing of TGF- β to its active mature form to achieve resistance to growth inhibitory signaling. As recombinant mature TGF- β artificially added to cells is also not growth inhibitory, an alternative mechanism may be responsible here, consistent with accounts of clear differences in the pathways by which exogenously added mature TGF- β and autocrine TGF- β inhibit cell growth in some cell lines (2).

TGF- β 1 RNAi Activates TGF- β Processing in LN-18 Glioblastoma Cells—We next investigated whether TGF- β 1 RNAi activates TGF- β processing in cell types other than HeLa. TGF- β plays an important role in malignant glioblastoma. LN-18 cells, derived from a malignant glioma (52), carry a non-functional (heterozygous) *TP53* gene (53) and express high levels of

TGF- β 1 (45). We confirmed that LN-18 cells do not undergo caspase 3/7 induction upon treatment with human recombinant TGF- β 1. LN-18 cells transfected with increasing concentrations of siTGF β 1 yielded a dose-dependent down-regulation of TGF- β 1 mRNA (Fig. 5A) and a reduction of total TGF- β 1 protein present in supernatants (Fig. 5B). Interestingly, levels of *THBS1* mRNA increased by 2-fold (Fig. 5A), indicating perhaps a stronger transcriptional regulation of the gene in these cells compared with in HeLa. The mRNAs of *FURIN*, *SMAD2*, *CDKN1A*, and *TP53* remained constant. After 3 days, LN-18 cells underwent apoptosis as shown by induction of caspase 3/7 activity (Fig. 5C). Western blots from cell lysates showed reduction of latent TGF- β 1 and a corresponding dose-dependent increase in P-SMAD2 and p53, but no major change in FURIN (Fig. 5D). Isolation of protein from concentrated media enabled probing for the regulation of TSP1 and total TGF- β . The former showed a strong dose-dependent up-regulation (Fig. 5E), however only traces of TGF- β could be observed. We turned therefore to the ELISA protocols with non-acid work-up. In contrast to HeLa cells, it was not necessary to add recombinant latent TGF- β 1 to treated LN-18 cells to assay for changes in TGF- β 1 processing. Increasing doses of siTGF β 1 led to increased amounts of mature TGF- β 1 in comparison to siCon treated cells (Fig. 5F). We examined levels of selected miRNAs in treated cells. SiTGF β 1 reduced *miR-18a* levels by \sim 30%, consistent with the induction of TSP1 protein (Fig. 5G). As in HeLa, transfection of LN-18 with miR-18 mimic alone led to accumulation of latent TGF- β 1 (Fig. 5H).

The results in LN-18 cells partially replicate the results from HeLa cells. Inhibition of latent TGF- β 1 by RNAi leads to increased TSP1 (but not FURIN), increased processing of latent TGF- β 1, activation of P-SMAD2 and caspase 3/7 induction. Elevated levels of TSP1 likely derive from both transcriptional and post-transcriptional regulation with *miR-18a* contributing to the latter. The induction of caspase 3/7 activity in this cell line was therefore also consistent with restoration of the TGF- β cytostatic effect. However, the apoptotic mechanism appears not to involve transcriptional activity of p53 or P21, in accordance with *P53* status and previous literature reports of unusual mechanisms of apoptosis in certain glioma cell lines (54).

DISCUSSION

Dysregulation of TGF- β signaling is at the heart of a variety of important diseases. In many cancers only the tumor suppressor function of TGF- β is inactivated and therefore TGF- β signaling not only fails to protect cells against uncontrolled proliferation, but it also drives invasion and metastasis. A detailed understanding of the mechanisms by which tumor cells lose only the TGF- β growth inhibitory response might lead to safe, new, and efficacious therapeutic strategies. Several mechanisms have been described but are mostly concentrated on the downstream effectors of cytostatic TGF- β signaling (13). They include two accounts concerning TGF- β -associated oncomirs. In one, the repression of *SMAD5* by *miR-155* was associated with the development of leukemia/lymphoma in transgenic mice (55). In the second, overexpression of the *miR-106b*~*25* cluster attenuated TGF- β 1 growth inhibition in gastric cancer cells by blocking the synthesis of P21 and BIM (22). Despite a

microRNA Control of TGF- β Processing

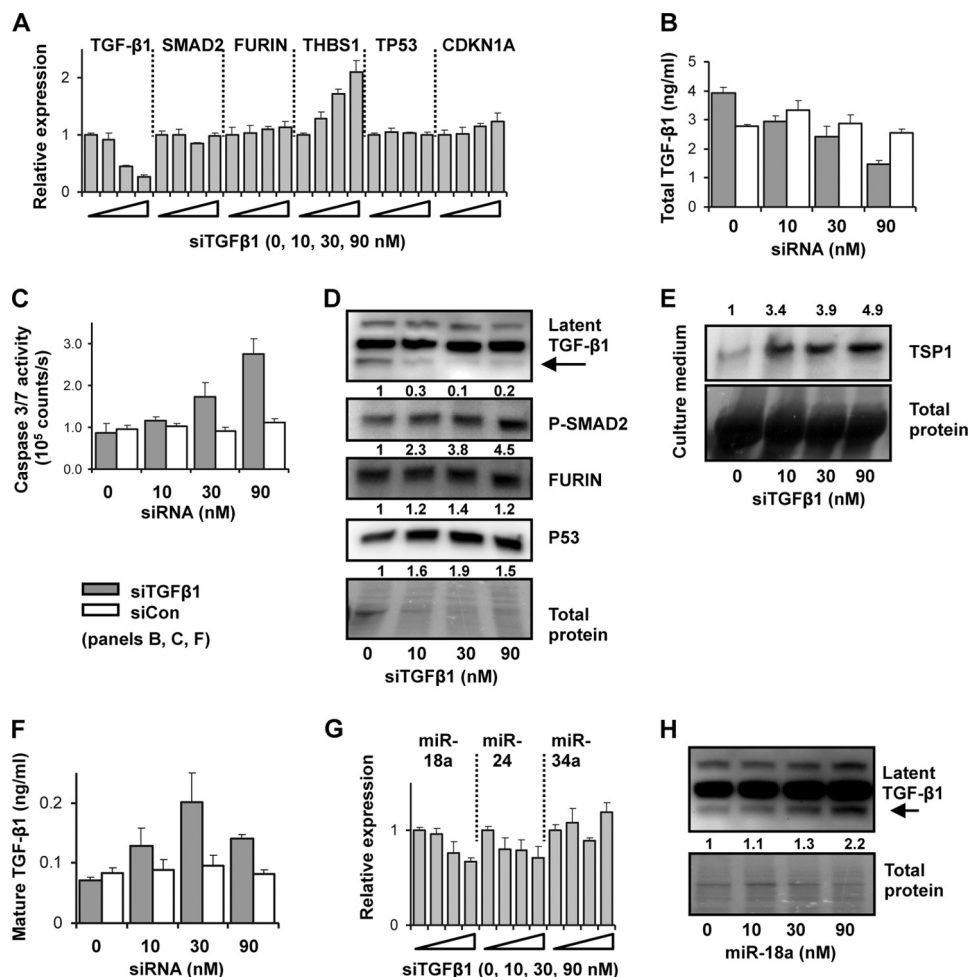


FIGURE 5. Reactivation of TGF- β signaling upon down-regulation of latent TGF- β by siTGF β 1 in LN-18 cells. *A*, cells were transfected with siTGF β 1, and Q-PCR analysis was performed 48 h post-transfection. Relative expression of mRNA is displayed (mean of PCR triplicates; single RNA samples \pm S.D.). *B*, cells grown in 5% FBS media were treated with siRNAs. Supernatants were collected 72 h post-transfection, and total TGF- β 1 was measured by ELISA after acidification. Relative expression of TGF- β 1 protein is displayed (mean of triplicate transfections \pm S.D.). *C*, cells grown in 5% FBS media were treated with siRNAs. Caspase 3/7 activity was measured from lysates 72 h post-transfection (mean of triplicate transfections \pm S.D.). *D* and *E*, cells grown in media containing 1% FBS were treated with siTGF β 1. Protein was isolated from lysates, and concentrated media after 24 h. Western blots of latent TGF- β 1, P-SMAD2, FURIN, p53 from lysates, and TSP1 from concentrated media are displayed. *F*, cells grown in 5% FBS media were treated with siRNAs. Cell supernatants were collected 72 h post-transfection and mature TGF- β 1 measured by ELISA without acidification. Mature TGF- β 1 protein is displayed (mean of triplicate transfections \pm S.D.). *G*, Q-PCR was performed on total RNA isolated after 48 h from cells transfected as in *F*. Relative miRNA expression is displayed (mean of PCR triplicates; single RNA samples \pm S.E.). *H*, cells grown in media containing 1% FBS were treated with miR-18a. Western blot analysis of latent TGF- β 1 was performed on lysates after 24 h.

vast literature on TGF- β , aspects of its maturation in the context of cancer are rarely reported. Nevertheless, it is well-known that cells synthesize excess TGF- β precursors, that the processing and activation of the ligand precursors are rate-limiting steps in their bioavailability and that high levels of TGF- β detected by ELISA are commonly found circulating in cancer patients. In general, also, cancer cell lines which have lost the cytostatic response to TGF- β 1 often express high levels of latent TGF- β and in cervical carcinoma cell lines specifically, levels of latent TGF- β correlate inversely with cytostatic response (36).

To our knowledge there are no reports which clarify a causal mechanistic link between high levels of latent TGF- β and the loss of the TGF- β cytostatic response; however, the failure to activate secreted latent TGF- β was proposed as a possible source of resistance long ago (56). Here we show that latent TGF- β 1 inhibits the TGF- β cytostatic response in some tumor

cell lines through a regulatory feedback loop involving miRNA control of latent TGF- β processing factors. We demonstrated that inhibition of TGF- β 1 by RNAi in TP53-positive HeLa and TP53-mutated LN-18 cell lines induces caspase 3/7 activity and cell death. Data from a variety of experiments in these two cell lines pointed to restoration of cytostatic TGF- β signaling as the source of the apoptosis. We discovered an increased latent TGF- β processing activity in HeLa and LN-18 supernatants undergoing TGF- β 1 RNAi derived from elevated levels of secreted TSP1 and leading, in turn, to induction of P-SMAD2. In HeLa cells this increased extracellular TGF- β 1 processing activity was reinforced by a strong up-regulation of a second TGF- β processing factor, intracellular FURIN. The results of a large miRNA expression profile after TGF- β 1 RNAi indicated potentially important post-transcriptional contributions to this mechanism. First, the relatively modest suppression of several oncomirs and the strong induction of miR-34a were suggestive

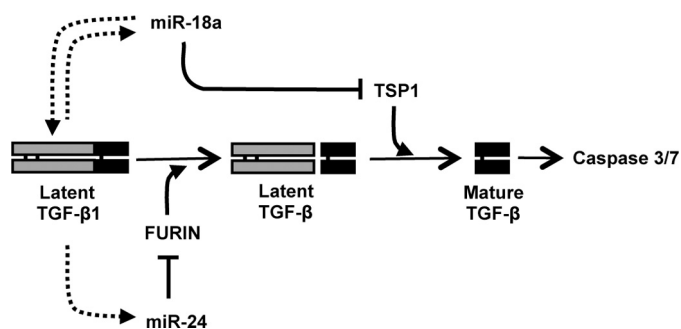


FIGURE 6. Latent TGF- β 1, TSP1, FURIN, miR-18a, and miR-24 are part of a regulatory feedback loop. High levels of latent TGF- β 1 indirectly maintain expression of miR-18a and miR-24 in HeLa cells, resulting in repression of TSP1 and FURIN, reduced processing of latent TGF- β precursors, increased TGF- β 1 transcription and accumulation of un-nicked latent TGF- β 1. Upon TGF- β 1 RNAi miR-18a and miR-24 are inhibited, TSP1 and FURIN are induced, thereby increasing latent TGF- β processing and leading to apoptosis. Dotted arrows represent indirect regulations, filled arrows represent direct interactions.

of a coordinated antiproliferative miRNA network in operation, somewhat akin to accounts of wide-scale miRNA reprogramming by VEGF signaling (18) or MYC-driven transcription (57). Second, rescue experiments using mimics of miR-18a and miR-24, which target TGF- β processing factors THBS1 and FURIN, respectively, increased levels of intracellular un-nicked latent TGF- β 1, by distinct mechanisms and partially abrogated the apoptosis in a manner similar to a TGF- β 1 overexpression construct. The data demonstrate important roles for latent TGF- β 1, its processing factors THBS1 and FURIN as well as miR-18a and miR-24 in a feedback loop that regulates at least in part the maturation and cytostatic activity of TGF- β (Fig. 6). We did not conduct a full transcriptomic or proteomic analysis and therefore we cannot exclude that other mediators may play significant roles in the mechanism. The feedback loop in HeLa cells is interrupted by TGF- β 1 RNAi which, in turn, lowers levels of latent TGF- β 1, miR-18a, and miR-24, thereby de-repressing THBS1, FURIN, and latent TGF- β processing. Two reports offer plausible explanations as to how high homeostatic levels of latent TGF- β 1 in HeLa maintain indirectly the expression of oncomirs miR-18a and miR-24. In one, p53 was shown to repress transcriptionally the miR-17-92 cluster containing miR-18a (50); in the second, miR-24 was shown to be inhibited via SMAD signaling sites in its promoter (35).

Our data suggest an additional mechanism for cancer cells which secrete high levels of (latent) TGF- β 1 to develop resistance to TGF- β -mediated growth inhibition. This mechanism stands apart from others because it takes place at the earliest point of TGF- β signaling, the processing stage. The relevance of these findings for cancer are potentially important because: 1) a high proportion of patient tumors which secrete large amounts of this cytokine are reported to be refractory to TGF- β growth inhibition and 2) restoration of TGF- β cytostatic response may be achievable pharmacologically through the use of antisense or double-stranded oligoribonucleotides directed to TGF- β 1 mRNA. Our findings add a new layer of complexity to the biology of TGF- β . They emphasize the importance of considering latent and mature TGF- β as distinct entities and the processing of latent TGF- β as an integral part of growth inhibitory TGF- β signaling.

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