Suppression of Latent Transforming Growth Factor (TGF)-β1 Restores Growth Inhibitory TGF-β Signaling through microRNAs

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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-β1 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-β1 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-β1 processing factors, thrombospondin-1, and furin, respectively. Our data suggest a novel mechanism in which latent TGF-β1 binds a membrane-bound serine/threonine receptor complex (TβRI/TβRII), 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. 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 cystostatic 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-

The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2.
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-β1 in several cancer cell lines and observed apoptosis after 3 days. TGF-β1 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-β1 inhibits cytocstatic 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 cytocstatic TGF-β signaling at the earliest point in the pathway. Furthermore, it implies that in these instances pharmacological targeting of TGF-β1 mRNA may be of therapeutic value in pathological mechanisms caused by loss of the cytocstatic 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-β1 targeting human TGF-β1 (NM_000660) is CCAACUAUUGCUUCAGCUC (1712–1730); siTGF-β1(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-β1 (100-B), latent TGF-β1 (299-1T/F), and TGF-β neutralizing antibody (MAB1835; Clone 1D11) were from R&D Systems. SB431542 was from Santa Cruz Biotechnology (SC-7181; TSP1: SC-81755; FURIN: SC-20801). After washing membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1–2 h at room temperature. Chemiluminescence was measured in sealed plates in 1% FBS-containing media and transfected with siRNAs. Supernatants were acidified with 1M HCl and neutralized with 1.2 m NaOH/0.5 m HEPES prior to assay for total TGF-β1. The concentrations of active TGF-β1 were analyzed on non-acidified samples. TGF-β1 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 (R 0278; Sigma). Protein concentrations were determined using a BCA assay (Thermo Fisher Scientific 23225), 10–20 µg of protein was mixed with equal quantities of SDS loading buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, 0.2% bromphenol 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-β1: 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-β1 (SC119746) was from OriGene (Rockville, MD). cDNA of THBS1 (plba-hsps1) 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; Polypei) according to manufacturer’s protocol. SiRNAs were transfected after 24 h with indicated doses. After 24 h, supernatants were removed and 30 µl 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 µl 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-
measurements of cell-associated caspase 3/7 activity cells were lysed in PBS containing 1% Triton X-100 and 5 μl of lysates were mixed with equal volumes of substrate and otherwise assayed as above.

RESULTS

Inhibition of TGF-β1 mRNA Causes Apoptosis in Cervical Carcinoma Cells—Like most cervical carcinoma cell lines HeLa express high levels of TGF-β1 (36). They respond to exogenously-delivered, mature recombinant TGF-β1 but they do not undergo apoptosis (data not shown). Independent treatment of HeLa cells with two siRNAs (siTGFβ1 and siTGFβ1(s)) specific to the TGF-β1 sequence led to a dose-dependent down-regulation of TGF-β1 mRNA by greater than 80% at 15 nx by both siRNAs (Fig. 1A), though siTGFβ1 was more potent in most assays. Loss of TGF-β1 mRNA caused a rapid (24 h) reduction of intracellular un-nicked latent TGF-β1 protein, which was detected by a pan anti-TGF-β antibody (Fig. 1B). Reduction in total TGF-β1 protein in cell supernatants was quantified using an ELISA specific for the TGF-β1 isoform after treatment with acid (Fig. 1C), which converts the majority of latent nicked TGF-β1 to the mature form (37). Cells undergoing TGF-β1 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 siTGFβ1 into Caski and Siha cell lines (Fig. 1E). The results suggest that high levels of TGF-β1 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-β1 RNAi (Fig. 2A).

miRNAs Contribute to TGF-β1-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-β1 RNAi-mediated apoptosis, we measured the expression of 448 miRNAs in cells treated with a single dose of siTGFβ1 or oligofectamine control using stem-loop PCR. From the screen we selected 48 miRNAs for a repeat study using both TGF-β1 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-β1 RNAi-induced apoptosis, we measured caspase 3/7 activity after transfection of siTGFβ1 in combination with double-stranded RNA reagents. In experiments designed to control for unspecific effects of siRNA against TGF-β1, delivery of increasing doses of a plasmid expressing (latent) TGF-β1 mRNA rescued the cells from siTGFβ1-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-β1 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 siTGFβ1 (Fig. 2F). The anti-miRNA oligonucleotide of miR-34a (AMO-34a) inhibited siTGFβ1-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-β1 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 siTGFβ1-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-β1 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-β1 RNAi, we assayed for phospho-SMAD2 (P-SMAD2) activity after TGF-β1 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-β1 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-β1 mRNA had been reduced by ~50% (Fig. 3C).
port of this data, cells treated with siTGF$\beta$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-$\beta$ secreted by most cells is in a latent nicked form. Given the multi-step nature of TGF-$\beta$ processing, it seemed plausible that an increased TGF-$\beta$ signaling activity might arise from increased processing of extracellular latent TGF-$\beta$, despite declining levels of intracellular TGF-$\beta$ due to RNAi. We therefore examined siTGF$\beta$1-treated HeLa cells for indications of elevated TGF-$\beta$ processing. Concentrating cell supernatants facilitates detection of trace quantities of total extracellular TGF-$\beta$ by Western blot (45). However, our anti-TGF$\beta$ antibody does not distinguish between the three mature

FIGURE 1. Down-regulation of TGF-$\beta$1 by RNAi leads to apoptosis in cervical carcinoma cells. A, TGF-$\beta$1 mRNA after transfection of Hela cells with siRNAs targeting TGF-$\beta$1 and a control siRNA (siCon). Total RNA was isolated 48 h post-transfection. Relative expression of TGF-$\beta$1 mRNA is displayed (mean of PCR triplicates; single RNA samples ± S.D.). B, cell-associated latent un-nicked TGF-$\beta$1 after siRNA treatment. Proteins from Hela cells 24 h post-transfection were analyzed using a pan anti-TGF-$\beta$ antibody (left panel) and were quantified by densitometry (right panel). C, total secreted TGF-$\beta$1 after siRNA transfection. Supernatants from Hela cells grown in 1% FBS media were assayed by ELISA after acidification 72 h post-transfection. Total TGF-$\beta$1 protein is displayed (mean of triplicate transfections ± S.D.). D, caspase 3/7 activity in Hela cells was measured 48 h and 72 h post-transfection (mean of triplicate transfections ± S.D.). E, Caski and Siha cells were treated with increasing doses of siTGF$\beta$1. Caspase 3/7 activity was measured 72 h post-transfection (mean of triplicate transfections ± S.D.).
FIGURE 2. Changes in expression of selected mRNAs, miRNAs, and proteins upon TGF-β1 RNAi in Hela cells. A, selected mRNAs after transfection with TGF-β1 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 were displayed (mean of PCR triplicates; single RNA samples ± 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. D, attenuation of siTGFβ1-mediated caspase 3/7 induction after co-transfection with plasmid expressing TGF-β1 cDNA. Cells were first treated with increasing doses of TGF-β1 expressing plasmid, 24 h later transfected with an apoptosis-inducing dose of siTGFβ1 (30 nM). Caspase 3/7 activity was measured 72 h post-transfection (mean of triplicate transfections ± S.D.). E, attenuation of siTGFβ1-mediated caspase 3/7 induction after co-transfection with selected miRNAs or AMOs. Cells were treated simultaneously with an apoptosis-inducing dose of siTGFβ1 (15 nM) and increasing doses of indicated reagents. Caspase 3/7 activity was measured 72 h post-transfection (mean of triplicate transfections ± 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 siTGFβ1. Caspase 3/7 activity was measured 72 h post-transfection (mean of triplicate transfections ± S.D.).
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 siTGFβ1 (Fig. 3D). As RNAi lowered levels of the un-nicked precursor latent TGF-β1 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-β1 ELISA, which measures total TGF-β1 protein with excellent sensitivity after acid treatment of supernatants converts nicked latent TGF-β1 to the mature (12 kDa) form. We transfected cells with siTGFβ1 and then measured mature TGF-β1 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-β1 (Fig. 1C). Forgoing acid treatment, however, returned only background values, indicating undetectable amounts of mature TGF-β1 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-β1 without acidification, recombinant nicked latent TGF-β1 protein was added to cultures of siCon- and siTGFβ1-treated cells prior to ELISA at 72 h. siTGFβ1, but not siCon treatment now increased mature TGF-β1 up to 3-fold (Fig. 3E). We concluded that TGF-β1 RNAi mediates secretion of factors that are capable of processing exogenously-added latent TGF-β1 to mature TGF-β1. Extrapolating, we surmised that these same factors would also be capable of converting endogenously-derived latent TGF-β1 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 siTGFβ1 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-β1 mRNA indicating that the apoptosis in these cells did not result from RNAi caused by transfer of any siTGFβ1 from the primary transfection. C33-A is a non-HPV infected cervical carcinoma cell line which does not express TβRII. 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 TβRII. We transfected Hela cells with a fixed dose of siTGFβ1 in combination with increasing doses of either the blocking anti-TGF-β antibody, or a selective inhibitor of TβRI (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-β1 antibody or SB431542 upon supernatant transfer from the siTGFβ1-treated HeLa cells (Fig. 3H). All taken together, the results implied that apoptosis occurred, at least partially, through restoration of cytostatic TGF-β signaling via TβRI and/or TβRII.

**TGF-β1 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-β1 RNAi (Fig. 2A), however both proteins showed a strong induction on siTGFβ1 treatment more in accordance with a post-transcriptional activation (Fig. 3D). Transfection of cells with a cDNA expressing THBS1 (Fig. 4A) was associated with 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-β1 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

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**FIGURE 3. Activation of TGF-β1 signaling upon down-regulation of latent TGF-β1 in Hela cells.** A, time course of AKT-phosphorylation and p53 induction after treatment with siTGFβ1. Proteins were analyzed by Western blot. Bar graphs show densitometric evaluations. B, time course of SMAD2 phosphorylation. C, SMAD binding element lucerase 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 ± S.D.). Hela cells treated with siTGFβ1. Total RNA was collected after 7 h and TGF-β1 mRNA levels were measured (right panel). D, induction of TGF-β processing factors upon TGF-β1 RNAi. Cells cultured in media containing 1% FBS were treated with siTGFβ1. Lysates and concentrated supernatants were analyzed by Western blotting 24 h post-transfection. E, induction of processing activity by siTGFβ1 treatment. Cells grown in 1% FBS media were treated with siRNAs: 20 ng/ml recombinant latent TGF-β1 was added 0, 24, and 48 h post-transfection. TGF-β1 in supernatants was measured by ELISA without acidification 72 h post-transfection (mean of triplicate transfections ± S.D.). F, caspase 3/7 induction in indicator cell lines by conditioned media of siTGFβ1-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 ± 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 ± 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 ± S.D.).
FIGURE 4. Increased maturation of TGF-β upon down-regulation of latent TGF-β1 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). Caspase 3/7 activity was measured from supernatants of recipient HeLa cells 48 h post-transfer (right panel) (mean of triplicate transfections ± 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 ± 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-β1 and FURIN mRNAs are displayed (mean of PCR triplicates; single RNA samples ± S.D.). I, HeLa cells were simultaneously transfected with 15 nM siTGFβ1 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 we used luciferase reporter constructs bearing their full-length 3'-UTRs. In contrast to siCon, increasing doses of siTGF-β 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 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-β1 (Fig. 4G), whereas for miR-24, it was likely due to intracellular accumulation of the un-nicked latent TGF-β1 as FURIN was repressed. To confirm the functional importance of the repression of miR-18a and miR-24 during the siTGF-β1-mediated processing of latent TGF-β, we again co-transfected cells with each mimic in combination with a 15 nM dose of siTGF-β1. Similar to the TGF-β1 overexpression vector, increasing amounts of either miR-18a or miR-24 counteracted the effects of siTGF-β1: levels of latent TGF-β1 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. 4J).

In summary, our results demonstrate that TGF-β1 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-β1 the data also suggests that latent TGF-β1, 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-β1 RNAi Activates TGF-β Processing in LN-18 Glioblastoma Cells**—We next investigated whether TGF-β1 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-β1 (45). We confirmed that LN-18 cells do not undergo caspase 3/7 induction upon treatment with human recombinant TGF-β1. LN-18 cells transfected with increasing concentrations of siTGF-β1 yielded a dose-dependent down-regulation of TGF-β1 mRNA (Fig. 5A) and a reduction of total TGF-β1 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 HeLa. The miRNAs 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-β1 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-β1 to treated LN-18 cells to assay for changes in TGF-β1 processing. Increasing doses of siTGF-β1 led to increased amounts of mature TGF-β1 in comparison to siCon treated cells (Fig. 5F). We examined levels of selected miRNAs in treated cells. SiTGF-β1 reduced miR-18a levels by ~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-β1 (Fig. 5H).

The results in LN-18 cells partially replicate the results from HeLa cells. Inhibition of latent TGF-β1 by RNAi leads to increased TSP1 (but not FURIN), increased processing of latent TGF-β1, 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 growth inhibition in gastric cancer cells by blocking the synthesis of P21 and BIM (22). Despite a
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 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-β1 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-β1 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-β1 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 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-β1 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
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-β1, by distinct mechanisms and partially abrogated the apoptosis in a manner similar to a TGF-β1 overexpression construct. The data demonstrate important roles for latent TGF-β1, 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-β1 RNAi which, in turn, lowers levels of latent TGF-β1, 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-β1 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-β1 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-β1 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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