Mad Upregulation and Id2 Repression Accompany Transforming Growth Factor (TGF)-β-mediated Epithelial Cell Growth Suppression*

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The growth inhibitory cytokine TGF-β enforces homeostasis of epithelia by activating processes such as cell cycle arrest and apoptosis. Id2 expression is often highest in proliferating epithelial cells and declines during differentiation. Recently, Id2 expression has been found to depend on Myc-Max transcriptional complexes. We observed that TGF-β signaling inhibits Id2 expression in human and mouse epithelial cell lines from different tissue origins. Furthermore, the observed Id2 down-regulation by TGF-β in mouse mammary epithelial cells occurs without a concurrent drop in c-Myc levels. However, sustained Id2 repression in these cells and in human keratinocytes coincides with induction of the Myc antagonistic repressors Mad2 and Mad4, decreased formation of Myc-Max heterodimers and the replacement of Myc-Max complexes with Mad-Max complexes on the Id2 promoter. These results argue that induction of Mad expression and Id2 down-regulation are important events during the TGF-β cytostatic program in epithelial cells.

Transforming growth factor β (TGF-β)1 plays a central role in maintaining epithelial tissue homeostasis under normal physiological conditions (1). Through anti-proliferative or apoptotic mechanisms, TGF-β can inhibit the growth of virtually all non-transformed epithelial cell types (2, 3). Disruption of TGF-β-mediated growth control occurs in many carcinomas (4). Furthermore, a selective loss of the antimetogenic effect in the cancerous state can reprogram the cellular response to TGF-β. The growth inhibitory cytokine TGF-β/H9252 plays a central role in maintaining epithelial tissue homeostasis under normal physiological conditions (1). Through anti-proliferative or apoptotic mechanisms, TGF-β can inhibit the growth of virtually all non-transformed epithelial cell types (2, 3). Disruption of TGF-β-mediated growth control occurs in many carcinomas (4). Furthermore, a selective loss of the antimetogenic effect in the cancerous state can reprogram the cellular response to TGF-β, which then becomes a stimulus for tumor cell invasion and metastasis (5). Identifying the TGF-β gene responses underlying the cytostatic effect in epithelial cells is of great interest.

TGF-β signals through a ligand-activated serine/threonine kinase complex that phosphorylates Smad transcription factors (6, 7). Upon translocation into the nucleus, the activated Smad proteins are rapidly directed to several hundred target genes by a host of Smad-binding cofactors (8, 9). In epithelial cells, a small group of TGF-β gene responses is devoted to arresting the cell cycle. These include induction of the G1 cyclin-dependent kinase (cdk) inhibitors, p15Ink4b, which directly inhibits cdk4 and cdk6 (10–12), and p21Cip1, which inhibits cdk2 (11, 13, 14). As a result of these cdk inhibitory events, cdk substrates such as pRb remain underphosphorylated and block cell cycle progression (15, 16).

Epithelial cells normally display another important set of TGF-β anti-proliferative gene responses involving repression of growth-promoting transcription factors. This group is best represented by c-Myc, a transcriptional regulator that can both activate or inhibit gene expression in favor of cell proliferation (17–19). In association with the protein Max, c-Myc binds to a-catenin and c-Myc is a transcriptional repressor in a variety of cell types (2). A c-Myc repressor complex consisting of Smad3, Smad4, E2F4/5 and the transcriptional repressor p107 mediates this effect (21). c-Myc down-regulation by TGF-β not only deprives the cell of growth promoting functions but also facilitates the induction of p15Ink4b and p21Cip1 (22–24). TGF-β-activated Smad complexes induce transcription when bound to specific sites within the p15Ink4b and p21Cip1 promoters, while TGF-β-mediated c-Myc repression removes this inhibitory influence from the same promoters (23). Thus, c-Myc down-regulation plays an integrative role in the TGF-β cytostatic program. However, not all cell types that are growth inhibited by TGF-β undergo c-myc down-regulation (25, 26), raising the question of whether a different but functionally equivalent mechanism is at play.

Id proteins function as negative regulators of basic helix-loop-helix (bHLH) transcription factors critical for cell differentiation (27, 28). Through interactions with Rb, Id proteins can also actively promote cell proliferation (29, 30) and recently, a role for Id2 as a survival and differentiation factor in the mammary gland during pregnancy has been demonstrated (31–33). TGF-β inhibits Id1, Id2, and Id3 expression in several cell types (34, 35). Transcriptomic analysis of different human epithelial cell lines has revealed that repression of these three Id genes, together with repression of c-myc and induction of p15Ink4b and p21Cip1, constitute a TGF-β cytostatic program shared by human epithelial cell lines of different tissue origins (36). Thus, in a manner reminiscent of c-Myc, the Id proteins...
are emerging as important targets of repression by TGF-β in the cytostatic program.

A link between c-Myc and Id2 expression was recently established by the demonstration that c-Myc binding to E-box motifs in the Id2 promoter supports Id2 expression (34). Consequently, c-Myc down-regulation by TGF-β may secondarily lead to Id2 inhibition (34). However, we observed a poor correlation between c-Myc levels and Id2 repression by TGF-β, particularly in mouse mammary epithelial cells. Therefore, we searched for a possible mechanism that might be functionally equivalent to c-Myc down-regulation. Here we show that in mouse mammary epithelial cells and human keratinocytes, sustained Id2 repression by TGF-β is paralleled by a strong induction of Mad4 and a replacement of Myc-Max complexes with Mad-Max complexes on the Id2 promoter.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The plasmid used as a template for the human β-actin riboprobe was generously provided by Mark Moasser (Memorial Sloan-Kettering Cancer Center, New York), and contains nucleotides 1005–1155 of human β-actin (GenBank™ accession number M10277) inserted as an EcoRI/HindIII fragment into pSP65. The human Id2 cDNA was inserted as a BamHI fragment into pBluescript II SK+ for use as a template for riboprobe synthesis.

Cell Culture and Growth Inhibition Assays—HaCaT keratinocytes were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). NMuMG cells were maintained in DMEM supplemented with 10% FBS, 2 mM glutamine, and 20 mM HEPES. Both HaCaT and NMuMG cells were obtained from the ATCC. In all cases, the culture media contained 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.5 μg/ml fungizone. Cells were cultured with 10 μM TGF-β for various lengths of time, ranging from 0–24 h. In some instances, NMuMG cells were preincubated with 10 μg/ml cycloheximide for 1 h prior to 6 h of TGF-β stimulation.

32P-Deoxyribonucleic incorporation assays were performed in 10% FBS as described previously (37). To generate an antisense riboprobe specific for human Id2, the template plasmid was linearized with XhoI and was used in an in vitro transcription reaction with T7 RNA polymerase. To synthesize the human β-actin antisense riboprobe, the template plasmid was digested with HindIII and transcribed with SP6 RNA polymerase.

Northern blot analysis was performed as previously described (38). To generate an antisense riboprobe specific for human Id2, the template plasmid was linearized with XhoI and was used in an in vitro transcription reaction with T7 RNA polymerase. To synthesize the human β-actin antisense riboprobe, the template plasmid was digested with HindIII and transcribed with SP6 RNA polymerase.

RESULTS

Sustained Inhibition of Id2 Expression by TGF-β without Concurrent c-Myc Down-regulation—We observed Id2 down-regulation within 3 h of TGF-β addition to epithelial cell lines from different tissue origins including NMuMG mouse mammary epithelial cells, HaCaT human skin keratinocytes, HPL1 human lung epithelial cells, MCF10A human mammary epithelial cells, and A549 human lung carcinoma cells (Fig. 1). To define the mechanism mediating this response we focused on NMuMG and HaCaT cells, which were efficiently growth arrested by TGF-β in a dose-dependent manner (Fig. 2A). We compared the kinetics of Id2 down-regulation in these cells with the kinetics of another important growth inhibitory gene response, namely, c-myc down-regulation (Fig. 2B). TGF-β caused a decrease in Id2 message, which dropped to undetectable levels by 6 h and remaining low at 24 h after TGF-β addition (Fig. 2B). Although two transcripts were evident by Id2 Northern blot analysis in HaCaT cells (Fig. 1, second panel; Fig. 2B, right panel), subsequent RNase protection analysis with an Id2 specific riboprobe revealed a rapid and sustained down-regulation of Id2 mRNA levels (Fig. 2C). This suggests that the lower transcript represents Id2 (Figs. 1 and 2B). The upper transcript has been detected by others (42), but its relationship to Id2 is unclear. Id2 protein levels were also markedly

AREAS OF IMPORTANT REPRESSION BY TGF-β IN THE CYTOSTATIC PROGRAM.

A LINK BETWEEN C-MYc AND ID2 EXPRESSION WAS RECENTLY ESTABLISHED BY THE DEMONSTRATION THAT C-MYc BINDING TO E-BOX MOTIFS IN THE ID2 PROMOTER SUPPORTS ID2 EXPRESSION (34). CONSEQUENTLY, C-MYc DOWN-REGULATION BY TGF-β MAY SECONDARILY LEAD TO ID2 INHIBITION (34). HOWEVER, WE OBSERVED A POOR CORRELATION BETWEEN C-MYc LEVELS AND ID2 REPRESSION BY TGF-β, PARTICULARLY IN MOUSE MAMMARY EPITHELIAL CELLS. THEREFORE, WE SEARCHED FOR A POSSIBLE MECHANISM THAT MIGHT BE FUNCTIONALLY EQUIVALENT TO C-MYc DOWN-REGULATION. HERE WE SHOW THAT IN MOUSE MAMMARY EPITHELIAL CELLS AND HUMAN KERATINOCYTES, SUSTAINED ID2 REPRESSION BY TGF-β IS PARALLELED BY A STRONG INDUCTION OF MAD4 AND A REPLACEMENT OF MYC-MAX COMPLEXES WITH MAD-MAX COMPLEXES ON THE ID2 PROMOTER.

EXPERIMENTAL PROCEDURES

PLOSMID CONSTRUCTION—THE PLASMID USED AS A TEMPLATE FOR THE HUMAN β-ACTIN RIBOPROBE WAS GENTLY PROVIDED BY MARK MOASSER (MEMORIAL SLOAN-KETTERING CANCER CENTER, NEW YORK), AND CONTAINS NUCLEOTIDES 1005–1155 OF HUMAN β-ACTIN (GENBANK™ ACCESSION NUMBER M10277) INSERTED AS AN ECORI/HINDIII FRAGMENT INTO PSP65. THE HUMAN ID2 CDNA WAS INSERTED AS A BAmHI FRAGMENT INTO PBLUESCRIPT II SK+ FOR USE AS A TEMPLATE FOR RIBOPROBE SYNTHESIS.

CELL CULTURE AND GROWTH INHIBITION ASSAYS—HACA T KERATINO CYTES WERE MAINTAINED IN DULBECCO’S MODIFIED EAGLE’S MEDIUM (DMEM) SUPPLEMENTED WITH 10% FETAL BOVINE SERUM (FBS). NMuMG CELLS WERE MAINTAINED IN DMEM SUPPLEMENTED WITH 10% FBS, 2 mM GLUTAMINE, AND 20 mM HEPES. BOTH HACA T AND NMuMG CELLS WERE OBTAINED FROM THE ATCC. IN ALL CASES, THE CULTURE MEDIA CONTAINED 100 UNITS/Ml PENICILLIN G, 100 μG/ML STRREPTOMYCN, AND 0.5 μG/ML FUNGIZONE. CELLS WERE CULTURED WITH 10 μM TGF-β FOR VARIOUS LENGTHS OF TIME, RANGING FROM 0–24 H. IN SOME INSTANCES, NMuMG CELLS WERE PREINCUBATED WITH 10 μG/ML CYCLOHEXIMIDE FOR 1 H PRIOR TO 6 H OF TGF-β STIMULATION.

32P-DEOXYRIBONUCLEIC INCORPORATION ASSAYS WERE PERFORMED IN 10% FBS AS DESCRIBED PREVIOUSLY (37). TO GENERATE AN ANTISENSE RIBOPROBE SPECIFIC FOR HUMAN ID2, THE TEMPLATE PLASMID WAS LINEARIZED WITH XHOI AND WAS USED IN AN IN VITRO TRANSCRIPTION REACTION WITH T7 RNA POLYMERASE. TO SYNTHESIZE THE HUMAN β-ACTIN ANTISENSE RIBOPROBE, THE TEMPLATE PLASMID WAS DIGESTED WITH HINDIII AND TRANSCRIBED WITH SP6 RNA POLYMERASE.

NORTHERN BLOT ANALYSIS WAS PERFORMED AS PREVIOUSLY DESCRIBED (38). TO GENERATE AN ANTISENSE RIBOPROBE SPECIFIC FOR HUMAN ID2, THE TEMPLATE PLASMID WAS LINEARIZED WITH XHOI AND WAS USED IN AN IN VITRO TRANSCRIPTION REACTION WITH T7 RNA POLYMERASE. TO SYNTHESIZE THE HUMAN β-ACTIN ANTISENSE RIBOPROBE, THE TEMPLATE PLASMID WAS DIGESTED WITH HINDIII AND TRANSCRIBED WITH SP6 RNA POLYMERASE.

RESULTS

SUSTAINED INHIBITION OF ID2 EXPRESSION BY TGF-β WITHOUT CONCURRENT C-MYC DOWN-REGULATION—WE OBSERVED ID2 DOWN-REGULATION WITHIN 3 H OF TGF-β ADDITION TO EPITHELIAL CELL LINES FROM DIFFERENT TISSUE ORIGINS INCLUDING NMuMG MOUSE MAMMARY EPITHELIAL CELLS, HACA T HUMAN SKIN KERATINO CYTES, HPL1 HUMAN LUNG EPITHELIAL CELLS, MCF10A HUMAN MAMMARY EPITHELIAL CELLS, AND A549 HUMAN LUNG CARCINOMA CELLS (FIG. 1). TO DEFINE THE MECHANISM MEDIATING THIS RESPONSE WE FOCUSED ON NMuMG AND HACA T CELLS, WHICH WERE EFFICIENTLY GROWTH ARRESTED BY TGF-β IN A DOSE-DEPENDENT MANNER (FIG. 2A). WE COMPARED THE KINETICS OF ID2 DOWN-REGULATION IN THESE CELLS WITH THE KINETICS OF ANOTHER IMPORTANT GROWTH INHIBITORY GENE RESPONSE, NAMELY, C-MYC DOWN-REGULATION (FIG. 2B). TGF-β CAUSED A DECREASE IN ID2 MESSAGE, WHICH DROPPED TO UNDETECTABLE LEVELS BY 6 H AND REMAINING LOW AT 24 H AFTER TGF-β ADDITION (FIG. 2B). ALTHOUGH TWO TRANSCRIPTS WERE EVIDENT BY ID2 NORTHERN BLOT ANALYSIS IN HACA T CELLS (FIG. 1, SECOND PANEL; FIG. 2B, RIGHT PANEL), SUBSEQUENT RNASE PROTECTION ANALYSIS WITH AN ID2 SPECIFIC RIBOPROBE REVEALED A RAPID AND SUSTAINED DOWN-REGULATION OF ID2 mRNA LEVELS (FIG. 2C). THIS SUGGESTS THAT THE LOWER TRANSCRIPT REPRESENTS ID2 (FIGS. 1 AND 2B). THE UPPER TRANSCRIPT HAS BEEN DETECTED BY OTHERS (42), BUT ITS RELATIONSHIP TO ID2 IS UNCLEAR. ID2 PROTEIN LEVELS WERE ALSO MARKEDLY
reduced in NMuMG cells over a similar time course, as determined by immunoblot analysis (Fig. 2D).

A sharp decline in c-myc message was observed at early time points in these cell lines (Fig. 2B). In NMuMG cells, this decrease was followed by a recovery of c-myc transcript levels that was noticeable by 3 h and complete by 12 h after TGF-β/H9252 stimulation (Fig. 2B). c-myc down-regulation was more sustained in HaCaT cells, which showed only a partial recovery of c-myc transcript levels by 24 h (Fig. 2B). The transient decrease in c-Myc expression in NMuMG cells was verified at the protein level by immunoblot analysis (Fig. 2D). The drop in c-Myc levels in these cells was more limited and transient than the drop in c-myc message, suggesting an additional attenuation of this response at the c-Myc translation and/or protein stability levels. This was in marked contrast to the effect of TGF-β/H9252 on Id2 mRNA and protein levels, which remained low out to 24 h post-stimulation (Fig. 2, B–D).

It has been suggested that TGF-β/H9252-mediated suppression of Id2 expression is downstream of c-Myc down-regulation by TGF-β given that c-Myc binding to E-box motifs in the Id2...
protein synthesis. Total RNA was isolated from NMuMG cells stimulated in the absence or presence of 100 μM TGF-β for 6 h and 10 μg/ml cycloheximide for 7 h, beginning 1 h prior to TGF-β addition, and subjected to Northern blot analysis. The membrane was sequentially probed for Id2 and GAPDH.

**Prolonged TGF-β Signaling Induces Max, Mad2, and Mad4 Expression in NMuMG and HaCaT Cells**—c-Myc has been shown to mediate induction of Id2 expression through E-box motifs located within the Id2 promoter (34). Using luciferase reporter assays we confirmed that the same E-box motifs were involved in Id2 repression by TGF-β signaling. In support of this possibility, we observed that the sustained repression of Id2 by TGF-β requires ongoing protein synthesis as the protein synthesis inhibitor cycloheximide blocked TGF-β-mediated suppression of Id2 expression in intact NMuMG cells (Fig. 3). Thus, long-term down-regulation of Id2 depends on a TGF-β-induced inhibitory activity.

**Reduced Myc/Max Heterodimer Formation in Response to TGF-β Stimulation**—The elevated levels of Mad2 and Mad4 observed in response to TGF-β suggests that the Mad proteins may antagonize c-Myc function by binding to Max and displacing c-Myc from the E-box element. To determine whether TGF-β stimulation can inhibit c-Myc/Max complex formation in the presence of appreciable c-Myc expression, we investigated the effect of TGF-β on the level of this complex. Immunoprecipitation experiments revealed a significant and specific increase in endogenous Max levels following TGF-β stimulation (Fig. 5). In the absence of TGF-β, c-Myc was efficiently associated with Max. However, treatment with TGF-β for 24 h dramatically reduced the amount of Myc that heterodimerized with Max despite the observed increase in endogenous Max levels (Fig. 5). This suggests that the formation of Myc/Max heterodimers is specifically reduced in response to TGF-β. The observed increase in Max levels therefore suggests that a significant amount of unbound Max is present to heterodimerize with members of the Mad family.

**Reduced c-Myc and Enhanced Mad Binding to the Id2 Promoter in Response to Sustained TGF-β Stimulation**—In order to directly determine the impact of these TGF-β-induced events on the protein complexes bound to the Id2 promoter, we decided to examine the levels of Max, c-Myc, Mxi-1/Mad2, and Mad4 that were bound to the Id2 promoter in intact cells, in the absence or presence of prolonged TGF-β treatment. Chromatin immunoprecipitation assays were performed using a region of the Id2 promoter containing the E-box elements. Consistent with the decreased formation of Myc/Max heterodimers, TGF-β stimulation caused a reduction in the level of c-Myc that was bound to the Id2 promoter (Fig. 6A). In contrast, a constant amount of Max was bound to the same region of the Id2 promoter in the absence or presence of TGF-β (Fig. 6A). The observed decreases in c-Myc, but not Max, binding to the Id2 promoter suggests that a shift from Myc/Max to Mad/Max complexes has occurred on the Id2 promoter in TGF-β-treated cells. Indeed, TGF-β increased the amount of endogenous Mad4, and to a lesser extent Mxi-1/Mad2, that was bound to the Id2 promoter (Fig. 6B). This suggests that the TGF-β-induced accumulation of Max-Mad complexes results in the binding of these transcriptional repressors to the Id2 promoter, contributing to the sustained repression of Id2.

**Discussion**

We have provided additional insights into the mechanism by which TGF-β mediates the cytostatic response in epithelial cell lineages. While c-Myc and Id2 are similarly downregulated during the acute phase of the TGF-β response in NMuMG and HaCaT cells, the sustained repression of Id2 may additionally involve c-Myc independent mechanisms since c-Myc levels may return to the basal state in the face of prolonged TGF-β signaling. This phenomenon is particularly evident in NMuMG cells, among the epithelial cell lines that we have investigated. Moreover, chronic Id2 repression is coincident with increased expression of Mxi-1/Mad2 and Mad4. We propose that TGF-β induced expression of Mxi-1/Mad2 and Mad4 causes a shift from Myc/Max to Mad/Max complexes that bind to the Id2 promoter, contributing to Id2 transcriptional repression (Fig. 7). The delayed kinetics of Mad induction by TGF-β suggest that Mxi-1/Mad2 and Mad4 function to help sustain, but not initiate, Id2 repression in response to TGF-β. The inability of Mxi-1/Mad2 or Mad4, when expressed exogenously, to repress Id2 expression indicates that additional TGF-β signals participate in this gene response (Fig. 7).

Given the observation that c-Myc can induce Id2 (34), and TGF-β suppresses c-Myc expression (1, 2), the ability of TGF-β to downregulate Id2 may represent a secondary event down-
stream of c-Myc repression. Indeed, the down-regulation of c-Myc has been shown to be an obligate event for Id2 repression in a mink lung epithelial cell line (34). However, our data from immortalized mouse mammary epithelial cells suggest that Id2 down-regulation can occur in the face of sustained c-Myc expression. The decrease in c-Myc expression observed at early time points in NMuMG cells following TGF-β/H9252 addition is subsequently erased by the re-emergence of c-Myc expression, while Id2 levels still remain low. Rather, our observations suggest a different mechanism that relies on the antagonism of c-Myc function. We show that TGF-β induces the expression of Mad family members, including a small increase in Mxi-1/Mad2 and a strong increase in Mad4 levels in NMuMG and HaCaT cells. Recently, it has been shown that c-Myc can repress Mad4 transcription through Miz-1 in undifferentiated mouse erythroleukemia cells, and that c-Myc down-regulation can lead to Mad4 induction upon differentiation of these cells (44). In the case of TGF-β-stimulated NMuMG cells, Mad4 expression is induced at time points when c-Myc levels are beginning to re-emerge, suggesting that c-Myc does not repress Mad4 in this context. We demonstrate that elevated levels of Mad proteins can influence the composition of transcriptional complexes on the Id2 promoter, resulting in a shift from Myc/Max complexes that occupy the E-box region in the absence of TGF-β, to Mad/Max complexes following TGF-β stimulation. Interestingly, individually expressing either Mxi-1/Mad2 or Mad4 was not sufficient to repress Id2 expression in NMuMG and HaCaT in the absence of TGF-β treatment. While these data do not exclude a role for Mxi-1/Mad2 or Mad4 in the down-regulation of Id2 expression, they indicate that additional TGF-β signals assist in mediating this gene response.

The mammary epithelium is a major physiological target of TGF-β (46) and its growth inhibitory action continually en-
forces homeostasis except during pregnancy (47–49). Id2 function has also been shown to be important for lobulo-alveolar development during pregnancy (31). Recently, studies have argued that Id2 protein is induced late during pregnancy (32) and functions as a differentiation factor in the mammary gland (33). Interestingly, enforced TGF-β signaling during late pregnancy in transgenic mice induces apoptosis that occurs at the same time that mammary gland apoptosis is observed in Id2-null mice (31, 50). Therefore, it is possible that loss of TGF-β signaling during mid to late pregnancy allows Id2 expression and mammary gland differentiation.

Expression of the mad gene family has often been correlated with differentiation, although certain members are easily detected in proliferating cells (18). However, Mad overexpression has not been shown to be an efficient inducer of differentiation in all cell lineages (51, 52). Instead, ectopic expression of Mad family members has been shown to impair proliferation in both non-transformed and tumor-derived cell lines, and can block transformation by multiple oncogenes (18). Although the loss of Mad1 or Mad3, by gene-targeting approaches, does not lead to developmental defects nor to tumor susceptibility (53, 54), hyperplasia in multiple tissues are observed in Mxi-1/Mad2-null animals (55). Together, these observations indicate that Mad proteins may function primarily to inhibit proliferation rather than promote differentiation.

The ability of TGF-β to induce Mad expression may antagonize c-Myc function in cell types that lack profound and sustained c-Myc down-regulation in response to this cytokine. Increased Mad levels, in concert with Max, compete with Myc/Max heterodimers for E-boxes within the Id2 promoter, providing a basis for sustained Id2 repression by TGF-β. In this regard we have identified a mechanism for Id2 down-regulation in response to chronic TGF-β signaling, that is distinct from rapid Id2 repression by this cytokine. This mechanism may contribute to the observed effects of extended TGF-β signaling in mammary epithelial cells. Given the tumor suppressive effects of TGF-β during mammary tumorigenesis (56), and the anti-proliferative effects ascribed to Mad family members (18), it will be interesting to determine if the tumor suppressive

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effects of TGF-β signaling in epithelial cell lineages relies on the induction of Mad expression.

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