TGF- β 1 Regulates the Expression of Multiple Max-Interacting Transcription Factors in Balb/MK Cells: Implications for Understanding the Mechanism of Action of TGF- β 1

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ABSTRACT

Appropriate transforming growth factor- β 1 (TGF- β 1) signaling is required to preserve homeostasis of diverse tissues during development. At the cellular level, one function of TGF- β 1 that is critical for preserving homeostasis is the ability to arrest cell growth. TGF- β 1 arrests growth by blocking the function of the *c-myc* proto-oncogene. *c-myc* function is determined by the level of *c-myc* expression relative to other Max-interacting transcription factors, and TGF- β 1 has been shown to inhibit *c-myc* expression by inhibiting *c-myc* transcription. However, whether TGF- β 1 might also increase the expression of a Max-interacting factor that blocks *myc* function by competing with *myc* for Max binding is not known. Therefore, we determined the effect of TGF- β 1 on the expression of Max-interacting transcription factors in Balb/MK cells. We found unexpectedly that Balb/MK cells express both *N-myc* and *c-myc*. The pattern of *N-myc* expression during the cell cycle differs from that of *c-myc*, indicating that mRNA accumulation is controlled by mechanisms specific to each gene. TGF- β 1 rapidly inhibits *N-myc* mRNA expression; thus *N-myc* is a novel target of TGF- β 1 in Balb/MK cells. More importantly, we found that TGF- β 1 induces the expression of the putative tumor suppressor genes *Mad4* and

Mxi1 in both the Balb/MK and Mv1Lu cell lines. *Mad4* and *Mxi1* are novel targets of TGF- β 1, known to inhibit cell growth by antagonizing the interaction of Myc with Max. Thus, our results suggest that the induction of *Mad4* and *Mxi1* may function in tandem with the inhibition of *N-myc* and *c-myc* to mediate the growth inhibitory function of TGF- β 1. (*Pediatr Res* 50: 67–75, 2001)

Abbreviations

TGF- β 1, transforming growth factor- β 1
Balb/MK, mouse keratinocyte cell line
pRb, retinoblastoma protein
cdk, cyclin-dependent kinase
DRB, 5,6 dichloro-1-beta-D-ribofuranosylbenzimidazole
Mv1Lu, mink lung epithelial cell line
HDAC, histone deacetylase
EGF, epidermal growth factor
FBS, fetal bovine serum
FACS, fluorescence-activated cell sorter
TCE, TGF- β 1 control element

TGF- β 1 is the prototypical member of a large family of growth factors that preserve tissue homeostasis during development (1, 2). One function of TGF- β 1 that is critical for normal development is the ability to regulate cell proliferation. To illustrate, animals grow larger during development

principally by accumulating more cells through the process of cell division (3). Virtually every cell relies on signals from the extracellular environment to decide whether it is appropriate to continue to divide, or whether it should revert to the nondividing, so-called quiescent state. Deregulated signaling through the TGF- β 1 pathway results in a diverse array of developmental abnormalities depending on the tissue type and the component of the TGF- β 1 signaling pathway that is affected [reviewed in Raff (2)]. Accordingly, the study of TGF- β 1 function has revealed many of the components of signaling pathways that are critical for the appropriate regulation of cell division during development.

The direct effect of TGF- β 1 on the growth of many cell types *in vitro* and *in vivo* is to inhibit cell proliferation (4, 5). TGF- β 1 arrests cell growth by inhibiting key proto-oncogenes

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and activating key tumor suppressor genes that affect cell cycle progression. The first evidence of this came with the observation that the response of cells to the TGF- β 1 growth arrest signal is dependent on cell cycle position. In general, cells decide whether to divide based on extracellular growth signals during the G₁ phase of the cell cycle, before the onset of DNA synthesis (S-phase) [reviewed in Pardee (6)]. Accordingly, TGF- β 1 has been shown in many cell types to reversibly arrest cell cycle progression at a point in late G₁ when added before the G₁/S transition (7–10). A key event in the G₁ cell cycle arrest induced by TGF- β 1 is the ability of TGF- β 1 to convert the retinoblastoma tumor suppressor protein (pRb) to the underphosphorylated, growth-suppressive form that binds E2F (8, 11, 12). TGF- β 1 inhibits the phosphorylation of pRb by inhibiting the activation of Cdks during G₁. TGF- β 1 can inhibit both cyclin and Cdk expression [reviewed in Satterwhite *et al.* (10) and Ko *et al.* (13)], and also can induce specific inhibitors of Cdk activation (14, 15).

The *c-myc* proto-oncogene functions at least in part to mediate the activation of Cdks by extracellular growth-promoting signals [(7, 12, 16–21), also reviewed in Obaya *et al.* (22)]. Cells do not progress normally through G₁ when *c-myc* expression is perturbed, indicating that *c-myc* has an essential function in G₁ (23–25). Inhibition of *c-myc* transcription by TGF- β 1 treatment was first shown to be important in the TGF- β 1-induced arrest of mouse keratinocytes (26), and has been reported in numerous other TGF- β 1-responsive cell lines (27–32). Subsequently it has been shown that inhibition of *c-myc* by TGF- β 1 is required for inactivation of Cdk4 by induction of the p15^{Ink4b} Cdk inhibitor (33). Conversely, activation of a conditional Myc–estrogen receptor chimera in Balb/MK cells resulted in resistance to TGF- β 1-induced late G₁ arrest, indicating that c-Myc overactivity is sufficient to override the inhibitory effects of TGF- β 1 on the cell cycle machinery (34). Forced expression of *c-myc* in Mv1Lu cells prevented the inactivation of Cdk4 in response to TGF- β 1, principally by preventing induction of p15^{Ink4b} (33). Thus, blocking *myc* function results in a block of the phosphorylation of pRb by Cdks in late G₁. Blocking *myc* function is both necessary and sufficient to mediate the TGF- β 1-induced anti-proliferative signal.

c-Myc functions as a transcription factor by binding with the protein Max to form a heterodimer. It is known that c-Myc competes with other proteins for Max binding, and the relative abundance of each of these proteins is the primary force determining Max dimer composition. For example, signals that induce cell differentiation have been shown to induce expression of the Mad family of proteins including Mxi1 (35, 36). When present in abundance, Mad proteins bind Max. These Mad/Max heterodimers oppose the function of Myc/Max dimers on artificial Myc-responsive reporter constructs (35, 37). Mad/Max heterodimers repress transcription by recruiting HDAC to the DNA *via* the mSin3A corepressor [reviewed in Pazin and Kadonaga (38)]. This model describing the regulation of *myc* function continues to evolve as new members in this regulatory scheme are identified. Further, it is becoming clear that the various partners of Max confer very complex effects on wild-type promoters depending on sequence varia-

tions in the *myc* binding site or in the regions flanking the *myc* binding site (39–41). Although the inhibition of *c-myc* expression by TGF- β 1 has been studied extensively in the past (12, 26, 42), the possibility that TGF- β 1 might block *myc* function by inducing the expression of a Max-interacting transcription factor that opposes *myc* function has not been reported.

We performed studies to determine whether TGF- β 1 affects the expression of a group of Max-interacting transcription factors in Balb/MK. Balb/MK is a spontaneously immortalized cell line derived from primary mouse keratinocytes. Balb/MK cells are nontransformed and sensitive to TGF- β 1-induced growth arrest; therefore, they provide a useful model system in which to study gene expression in the control of nontumorigenic cell proliferation. We did not expect to find expression of *N-myc* or *L-myc* in Balb/MK cells, because the expression of multiple *myc* proto-oncogenes has been described only in transformed cells. However, because the *c-myc*, *N-myc*, and *L-myc* proteins are thought to be able to function interchangeably in promoting cell growth [reviewed in Marcu *et al.* (43)], we predicted that if *N-myc* or *L-myc* were expressed, TGF- β 1 would inhibit the expression. Conversely, several of the Max-interacting transcription factors that oppose *myc* function are known or putative tumor suppressor genes. Consistent with the idea that TGF- β 1 functions in part by activating tumor suppressor genes, we predicted that TGF- β 1 might increase the expression of one of the Max-interacting transcription factors previously known to oppose *myc* function.

METHODS

Cell culture and synchronization. Balb/MK cells (a gift from Stuart Aaronson) were grown in 175 cm² flasks (Falcon 3045, Franklin Lakes, NJ, U.S.A.) or 150 cm² flasks (Corning, Cambridge, MA, U.S.A.) in an incubator in 5–7% CO₂ at 37°C. Growth medium consisted of minimal essential medium with 5 mg/L calcium (MEM Lo Ca²⁺, JRH Biosciences, Lenexa, KS, U.S.A.), 8% dialyzed FBS (JRH Biosciences, Lenexa, KS, U.S.A. and GIBCO BRL, Rockville, MD, U.S.A.), and 4 ng/mL EGF (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Medium was replaced daily or every other day.

To obtain cell cycle synchronization, cells were cultured in synchronization medium (MEM Lo Ca²⁺, 0.1% FBS, with no added EGF) for 72 h. Balb/MK cells became arrested in the G₀ phase of the cell cycle at subconfluence by 72 h. G₀-arrested cells were restimulated to grow by replacing the synchronization medium with growth medium at time zero. Because the addition of growth medium functioned to stimulate the G₀-arrested cells to reenter the cell cycle, the addition of growth medium to G₀-arrested cells is referred to as mitogenic stimulation, and the growth medium as mitogen.

Mv1Lu cells (CCL-64, American Type Culture Collection, Rockville, MD, U.S.A.) were grown in 150-cm² flasks (Corning, Cambridge, MA, U.S.A.) in an incubator in 5% CO₂ at 37°C. Growth medium consisted of Dulbecco's modified Eagle medium (DMEM, GIBCO BRL, Rockville, MD, U.S.A.), 5% dialyzed FBS (GIBCO BRL, Rockville, MD, U.S.A.), and 4 ng/mL EGF (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

Rapidly growing, pooled primary mouse keratinocytes were isolated and cultured *in vitro* using a standard protocol (44). All studies involving animal subjects were approved as required by the respective Institutional Review Boards.

Porcine TGF- β 1 (R&D Systems, Minneapolis, MN, U.S.A.) was added to a final concentration of 10 ng/mL at the times indicated.

DNA synthesis was assessed in synchronized cultures by measuring [3 H]-thymidine incorporation. Forty microcuries of [3 H]-thymidine (Dupont/NEN, Boston, MA, U.S.A.) was added per 175-cm 2 flask, and labeling was allowed to proceed for 2 h. Cells were then washed, fixed in 10% trichloroacetic acid, and lysed in 0.2 N sodium hydroxide. [3 H]-thymidine incorporation (cpm) was measured in triplicate at time zero in quiescent cells and after 24 h of EGF-induced growth, both with and without the addition of TGF- β 1. Cell cycle-synchronized Balb/MK had approximately 100-fold higher [3 H]-thymidine incorporation in S-phase than in G $_0$ /G $_1$, and the addition of TGF- β 1 in mid-G $_1$ consistently inhibited S-phase entry by 60–70% in independent experiments (10).

Cell cycle distribution was determined using a standard protocol for FACS analysis (45).

RNA isolation, Northern analysis, and quantitation. Cells were harvested at the times indicated in each figure, and either total RNA (Figs. 1 and 2D) or polyadenylated RNA (Figs. 2A, 3A, and 4) was isolated using a standard protocol (46, 47). Whole cell lysates were pooled from one to two flasks for each time point when total RNA was isolated, and two to three flasks when polyadenylated RNA was isolated. RNA was resolved by electrophoresis on an agarose gel and transferred to Hybond, Zetaprobe, or Magnacharge membranes. Complete transfer was confirmed by ethidium bromide staining.

Total RNA from rapidly growing, pooled primary mouse keratinocytes was a gift from Dr. Anna Maria Garcia.

[32 P]dATP- and [32 P]dCTP- (Amersham, Piscataway, NJ, U.S.A. or Dupont/NEN, Boston, MA, U.S.A.) labeled probes were made using a random primer DNA labeling kit (Boehringer Mannheim, Indianapolis, IN, U.S.A.). The following cDNA templates were used for random primed labeling: the 0.7-kb *Bam*HI/*Pst*II fragment from plasmid SP65 1B15 containing the rat cyclophilin cDNA, kindly provided by Patria E. Danielson; the 850-bp *Hinc*II/*Pst*II fragment from the p277 construct containing the murine N-*myc* gene, kindly provided by Ron DePinho; the 1-kb *Xba*I/*Sca*I insert from pSV-*c-myc* containing murine *c-myc*, kindly provided by Robert Weinberg; the 1-kb *Eco*RI fragment from the human Mad1 cDNA (35); the 2.5-kb murine Mxi1 cDNA, kindly provided by Bunsiti Simizu; the 1.2-kb *Eco*RI fragment from the Mad3 cDNA, the 1.2-kb *Eco*RI fragment from the Mad4 cDNA, the 1.5-kb *Hind*III-*Xba*I fragment from pcDNA3/Hdac containing the histone deacetylase-1 cDNA, and the 1.7-kb *Hind*III fragment from pSPmSin3A containing the mSin3A cDNA, kindly provided by Don Ayer; the 550-bp *Eco*RI fragment from pVZ1 p21Max containing the human Max cDNA (48); the 800-bp *Sac*I fragment from mL2Gemb containing the second exon of the murine L-*myc* gene, kindly provided by Kari Alitalo; and the 550-bp *Hind*III fragment from SVpSP65-B-*myc* containing human B-*myc* (49). The N-*myc* and *c-myc* DNA fragments

used for probes were subcloned into pBluescript and sequenced to confirm their identities. Hybridizations were performed at 42°C using 1×10^6 cpm/mL of labeled DNA in hybridization buffer containing 50% formamide.

Images and quantification of signal intensity were obtained using a phosphorimager (Molecular Dynamics). Statistical comparisons were made using a two-tailed *t* test.

Inhibition of mRNA synthesis and mRNA half-life determination. The selective mRNA synthesis inhibitor DRB was used to rapidly block N-*myc* mRNA production, and the decay of N-*myc* message was calculated by Northern analysis of N-*myc* abundance. A final concentration of 100 μ M DRB was used; this was the lowest dose that maximally inhibited (85%) [3 H]-uridine incorporation measured for a 1-h period after addition of DRB to Balb/MK cells (data not shown). This dose of DRB did not affect the rate of DNA synthesis in Balb/MK cells as measured by [3 H]-thymidine incorporation during the first 2 h of DRB treatment (data not shown). Vehicle alone (DMSO at a final concentration of 0.2%) did not affect either [3 H]-uridine or [3 H]-thymidine incorporation (not shown). N-*myc* signal intensity was normalized to cyclophilin expression to control for loading differences among samples. This approach may yield a slight overestimation of the true length of the half-life, because cyclophilin levels also slowly decrease after addition of DRB. However, the half-life of cyclophilin was found to be >12 h long, and the cyclophilin mRNA expression seen by Northern analysis did not vary significantly from the intensity of the ribosomal bands, which apparently were not affected by DRB based on ethidium staining of the gel before blotting (not shown). As cyclophilin expression is not affected by TGF- β 1, this approach has the advantage of allowing very accurate comparison between TGF- β 1-treated and -untreated samples at any given time point after the addition of DRB in blots made with either total RNA or RNA enriched for polyadenylated message.

RESULTS

N-*myc* is rapidly inhibited by TGF- β 1. Because inhibition of *c-myc* mRNA expression has been shown to play an important role in mediating the effects of TGF- β 1 on cell cycle progression, we performed Northern analysis to determine whether TGF- β 1 might affect N-*myc* or L-*myc* mRNA expression. L-*myc* mRNA was not detected in Balb/MK cells (not shown). However, by Northern analysis we detected a single 2.9-kb band corresponding to the expected size of the N-*myc* mRNA using a 32 P-labeled fragment from the 3' untranslated region of the murine N-*myc* cDNA, a region of N-*myc* that has no homology to *c-myc*. The N-*myc* band was distinct from the 2.5-kb band detected using a probe from exon 2 of the murine *c-myc* gene, indicating that the probes did not cross-hybridize.

Because TGF- β 1 arrests cell cycle progression in the G $_1$ phase, we examined mRNA expression in Balb/MK cells that were stimulated to progress through the early stages of the cell cycle in a synchronized manner. Signal intensity for each sample was normalized to cyclophilin mRNA expression. We found that TGF- β 1 inhibited N-*myc* mRNA expression within 1 h after treatment during G $_1$ (Fig. 1, A and B). The inhibition

Figure 1. TGF- β 1 rapidly inhibits N-myc mRNA expression. *A*, response of N-myc expression to TGF- β 1 during the G₁ phase of the cell cycle determined by stimulating quiescent (G₀) Balb/MK cells to reenter the cell cycle by addition of mitogen (growth medium) at 0 h. (+) indicates TGF- β 1 added to cells 6 h after mitogen. Control cells began S-phase approximately 12 h after mitogen, whereas TGF- β 1-treated cells arrested in G₁. RNA was isolated for Northern blot analysis from cells harvested at the times indicated. Results shown from this single experiment are representative of multiple assays. Shown below is a diagram of experimental design indicating the predominant cell cycle phase at each time point. *B*, signal intensity in Fig. 2A was normalized to cyclophilin expression and represented graphically. Dashed line, N-myc mRNA expression in cells treated with TGF- β 1 6 h after mitogen. *C*, pattern of N-myc mRNA expression differs from that of c-myc during G₁. Black line, N-myc expression in control cells; gray line, c-myc expression in control cells (c-myc Northern not shown). *D*, cell cycle synchronization is not required for N-myc inhibition by TGF- β 1. N-myc mRNA expression was rapidly inhibited by TGF- β 1 in randomly cycling Balb/MK cells. (+) indicates TGF- β 1 added to asynchronous, rapidly proliferating cells at 0 h. RNA was isolated for Northern blot analysis from cells harvested at the times indicated. Cyclophilin expression was used as a control. The inhibition of N-myc is long-lasting, persisting for ≥ 24 h. Results shown from this single experiment are representative of multiple assays.

of N-myc occurred well in advance of the onset of DNA synthesis, which began approximately 12 h after mitogen, and the inhibition persisted for ≥ 10 h. Thus, the kinetics of the inhibition suggest that inhibition of N-myc is important in mediating the G₁ cell cycle arrest induced by TGF- β 1.

Interestingly, we found that the pattern of expression of N-myc differed from that of c-myc during the G₁ phase of the cell cycle (Fig. 1C). Expression of both N-myc and c-myc was low in nondividing, quiescent Balb/MK. c-myc was rapidly induced by mitogenic stimulation and continued to accumulate during early G₁, reaching maximum levels at 6 h. In contrast, N-myc had a very rapid spike of expression early in G₁. By 4 h, N-myc mRNA again began to accumulate, and N-myc expression increased through the middle and most of the second half of G₁, when c-myc levels were decreasing. The inhibition of c-myc expression by TGF- β 1 in Balb/MK has been published previously (26).

Likewise, the inhibition of N-myc expression was rapid and sustained in asynchronous, randomly cycling Balb/MK cells, persisting for ≥ 24 h after TGF- β 1 treatment (Fig. 1D). The inhibition of N-myc in randomly cycling cells indicates that the inhibition of N-myc is not dependent on cell cycle synchronization in G₁. Randomly cycling cells were used for subsequent analyses.

N-myc message stability is unaffected by TGF- β 1. The rapid inhibition of N-myc expression by TGF- β 1 suggests that the N-myc mRNA half-life is <1 h in TGF- β 1-treated Balb/MK cells, and therefore a potential target of regulation. To determine the N-myc mRNA half-life, and whether message stability might be affected by TGF- β 1, message abundance was measured by Northern analysis of RNA from randomly cycling Balb/MK cells after treatment with DRB, a specific inhibitor of RNA-polymerase II-dependent mRNA synthesis (50). In independent experiments, the N-myc half-life was found to be 21.3 ± 3.2 min (mean $\pm$ SD, $n = 3$). There was no effect of TGF- β 1 on the N-myc mRNA stability in any experiment, suggesting that TGF- β 1 inhibits N-myc mRNA synthesis (Fig. 2).

Mxi1 and Mad4 are induced by TGF- β 1. Because some members of the Max-interacting transcription factor family inhibit cell proliferation by antagonizing Myc activity, we performed northern analysis of mRNA expression in randomly cycling Balb/MK cells to determine whether TGF- β 1 might induce expression of a Myc antagonist (Fig. 3A). Cell cycle distribution was determined for each sample by FACS analysis (Fig. 3B, 0–20 h) or thymidine incorporation (Fig. 3B, 24 h). Balb/MK cells began to accumulate in the G₁ phase of the cell cycle between 4 and 8 h after TGF- β 1 treatment. A modest induction of Mxi1 and Mad4 expression was evident at 4 h, before cell cycle arrest. A marked induction of Mxi1 and Mad4 expression was seen by 8 h, the point when G₁ arrest was first detected, and expression continued to increase for 24 h (Fig. 3, A and C). The inhibition of N-myc and c-myc by TGF- β 1 is shown for comparison.

Mad3 signal was not consistently detected; however, a slight Mad3 signal was induced by TGF- β 1 in one experiment (not shown). The low reproducibility of Mad3 signal detection did not allow us to conclude whether expression of Mad3 is regulated by TGF- β 1. The mRNA expression levels of Max,

Mad1, *B-myc*, *HDAC*, and *mSin3A* were not affected by TGF- β 1 (not shown).

To determine whether the induction of *Mxi1* and *Mad4* by TGF- β 1 is restricted to the Balb/MK cell line, we examined expression in Mv1Lu cells. Mv1Lu is a polyclonal, nontransformed cell line that is commonly used as a model system for studying mechanisms of TGF- β 1 growth arrest. Neither *N-myc* nor *L-myc* expression was detected in Mv1Lu cells (not shown), and *c-myc* mRNA expression was inhibited by TGF- β 1 as previously reported (Fig. 4A) (32). *Mxi1* and *Mad4* expression was significantly elevated in Mv1Lu cells treated with TGF- β 1 for 24 h (Fig. 4). We conclude that the induction of *Mxi1* and *Mad4* by TGF- β 1 is associated with cell cycle arrest in both Balb/MK and Mv1Lu cells. The mRNA expression of *Max*, *Mad1*, *B-myc*, *HDAC*, and *mSin3A* was not affected by TGF- β 1 in Mv1Lu cells, and expression of *Mad3* was not detected (not shown).

DISCUSSION

Balb/MK express *N-myc* and *c-myc*. The finding that Balb/MK cells express both *N-myc* and *c-myc* was quite unexpected, because previously the expression of multiple *myc* proto-oncogenes in epithelial cell lines has been described only

Figure 2. *N-myc* mRNA stability is not affected by TGF- β 1. *A*, mRNA synthesis inhibitor, DRB, was added to randomly cycling Balb/MK cells at 0 h. TGF- β 1 added to cells at 10 (+) or 30 (*) min after DRB. RNA was isolated for Northern blot analysis from cells harvested at the times indicated. *B*, *N-myc* signal intensity normalized to cyclophilin expression and represented graphically (semi-logarithmically). *N-myc* mRNA levels decreased immediately after the cessation of mRNA synthesis because of DRB treatment. The *N-myc* mRNA half-life in this experiment was 20 min, and message stability did not differ for the TGF- β 1-treated cells. Results shown from this single experiment are representative of multiple assays.

Figure 3. TGF- β 1 rapidly induces expression of the *myc* antagonists *Mxi1* and *Mad4* in Balb/MK cells. *A*, (+) indicates TGF- β 1 added to asynchronous, rapidly proliferating Balb/MK cells at 0 h. RNA was isolated for Northern blot analysis from samples harvested at the times indicated. *B*, analysis of cell cycle distribution by FACS (at times 0–20 h) or [3 H]-thymidine incorporation (at 24 h) indicated that TGF- β 1 induced G₁ arrest and inhibited thymidine incorporation. *C*, signal intensity normalized to cyclophilin expression and represented graphically (percent of control is equivalent to expression in normalized TGF- β 1-treated sample relative to expression in corresponding normalized untreated sample). TGF- β 1 treatment rapidly induced the mRNA expression of *Mxi1* and *Mad4* relative to control. The rapid inhibition of *N-myc* and *c-myc* mRNA expression is shown for comparison. The expression of *Max*, *Mad1*, *Mad3*, *B-myc*, *HDAC*, and *mSin3A* was not affected by TGF- β 1 (not shown). Results shown from this single experiment are representative of multiple assays.

Figure 4. *Mxi1* and *Mad4* mRNA expression is also induced by TGF- β 1 in randomly cycling Mv1Lu cells. *A*, (+) indicates TGF- β 1 added to asynchronous, rapidly proliferating cells at 0 h. RNA was isolated for Northern blot analysis from cells harvested at 24 h. Paired samples were run on the same Northern blot ($n = 3$ independent experiments). *B*, signal intensity normalized to cyclophilin expression, and change in expression after TGF- β 1 represented graphically. *Mxi1* and *Mad4* were induced to $430 \pm 28\%$ (mean $\pm$ SD) and $405 \pm 76\%$ of control, respectively ($p < 0.05$). *c-myc* was inhibited to $25 \pm 3\%$ of control ($p < 0.05$).

in transformed cells. Thus, we have identified the first non-transformed cell line that expresses both proto-oncogenes. Because growth of cells in culture can substantially alter the patterns of gene expression from that which is observed *in vivo*, we wondered whether N-*myc* expression in Balb/MK cells might be a characteristic reflective of the cell of origin. Although our experiments did not address this specifically, presumptive evidence supports the conclusion that the Balb/MK cell line was derived from a skin cell that normally expresses N-*myc*. Within the skin of newborn mice, N-*myc* is expressed in cells comprising the outer root sheath of the hair bulb (51, 52). We know that Balb/MK cells were derived from normal skin, have stringent growth factor requirements, are anchorage dependent, and do not form tumors in nude mice. As further support, we have examined N-*myc* expression in rapidly growing primary mouse keratinocytes isolated from newborn mice and grown in the same manner as those from which the Balb/MK cell line was derived (53). We found that N-*myc* is not induced in cultured primary mouse keratinocytes that do not normally express N-*myc* *in vivo* (data not shown). Taken together, these observations indicate that N-*myc* expression in Balb/MK cells is not associated with a transforming event, and suggest that the Balb/MK cell line originated from a progenitor cell from the outer root sheath of the hair bulb that normally expresses N-*myc* *in vivo* (51, 52). Thus, Balb/MK is a previ-

ously well-characterized, nontransformed cell line that we have shown to be useful to study mechanisms that regulate N-*myc* expression. As such, the Balb/MK cell line may reveal important information about the regulation of N-*myc* during development, as well as the mechanisms that might regulate the proliferation of cells involved in hair growth.

TGF- β 1 inhibits myc. We found that TGF- β 1 inhibited N-*myc* expression in Balb/MK cells, and the inhibition occurred well in advance of detectable changes in cell cycle distribution. The functions of N-*myc* as a regulator of cell growth suggest that the inhibition of N-*myc* is an important mediator of the TGF- β 1 growth inhibition signal. N-*myc* has growth-promoting activities similar to c-*myc* and L-*myc* *in vivo* and *in vitro*, and N-*myc* overexpression has been implicated in a variety of malignancies [reviewed in Marcu *et al.* (43)]. Although the activity of N-*myc* in regulating cell growth is thought to be similar to c-*myc*, the normal expression patterns of these genes during development differs markedly, and each has essential functions that are not compensated for by other *myc* proteins (51, 54–56). Our observation that N-*myc* and c-*myc* have different patterns of expression during the G₁ phase of the cell cycle in Balb/MK cells suggests that N-*myc* and c-*myc* are subject to dissimilar regulatory forces, and their proteins serve highly individualized, nonoverlapping functions in Balb/MK cells. c-*myc* appears to be necessary for cell proliferation, and the inhibition of c-*myc* expression in Balb/MK cells is sufficient to inhibit cell growth (57). Similarly, N-*myc* is essential for the proliferation of cells in which it is expressed. Antisense N-*myc* expression in neuroblastoma cell lines inhibited soft agar growth, indicating that N-*myc* plays an essential role in the proliferation of these cancer cells (58–60). Furthermore, homozygous disruption in the N-*myc* gene in mice resulted in embryonic lethality with multiple defects thought to be caused by impaired cell proliferation in epithelial tissues that normally express N-*myc* (56, 61–63). Recently, activation of a conditional c-Myc–estrogen receptor chimera in Balb/MK cells resulted in resistance to TGF- β 1-induced late G₁ arrest, indicating that c-Myc overactivity is sufficient to override the inhibitory effects of TGF- β 1 on cell cycle progression (34). Taken together, these data support the conclusion that inhibition of c-*myc*, and probably also N-*myc*, is necessary for TGF- β 1-mediated growth inhibition of Balb/MK cells. The fact that N-*myc* has similar growth-promoting properties to c-*myc* suggests that deregulated N-*myc* expression is a mechanism that allows cancer cells to escape TGF- β 1-induced growth arrest.

Evidence that TGF- β 1 may regulate essential functions of N-*myc* during development comes from studies of normal epithelial development in mice. Using a gene knockout approach, mice expressing subnormal levels of N-*myc* were generated by the fortuitous insertion of a neomycin cassette into the first intron of the N-*myc* gene (64). The cassette, intended to disrupt the gene, was occasionally spliced from the mRNA resulting in low levels of a normal N-*myc* transcript. Mice homozygous for this mutation died of respiratory distress shortly after birth and were found to have a marked inhibition in pulmonary airway branching (64). Subsequently it was shown that the TGF- β 1-mediated inhibition of airway branch-

ing morphogenesis of 11.5- to 14.5-d murine lung explant cultures was associated with an inhibition of N-*myc* mRNA expression in the distal airway epithelium (65). Inasmuch as TGF- β 1 expression is normally found in the airway epithelium (66), these data suggest that TGF- β 1 plays an important role in the modulation of N-*myc* expression in the lung. On the basis of colocalization, TGF- β 1 might regulate N-*myc* expression in the hair bulb (52, 67), just as it might regulate N-*myc* expression in multiple developing epithelial tissues where both are expressed. The inhibition of N-*myc* by TGF- β 1 appears to require the presence of pRb, as N-*myc* inhibition was not seen in TGF- β 1-treated lung bud explants from Rb-gene knockout mice (68). Beyond this, the mechanism of TGF- β 1 inhibition of N-*myc* expression in normal cells has remained largely unknown.

Our data suggest that TGF- β 1 inhibits N-*myc* mRNA synthesis in Balb/MK cells, because TGF- β 1 treatment did not affect mRNA stability. Because TGF- β 1 has been shown to inhibit transcription of the c-*myc* promoter through a sequence called the TGF- β 1 control element (TCE), we have examined the N-*myc* promoter for regions similar to the consensus TCE (42). The sequence for the consensus TCE (GNGTNGGNGA) was proposed on the basis of comparison of the c-*myc* TCE with the TGF- β 1 inhibitory element (TIE) in the transin/stromelysin gene (42). The mouse N-*myc* promoter contains several regions of homology to the consensus TCE. Regions differing by only one base from the consensus are found at -1021, -224, -162, -137, and -74. The region from -169 to -112 contains seven tandem or overlapping repeats that differ by only one or two bases from the consensus TCE. The identification of regions in the N-*myc* promoter that are similar to the consensus TCE suggests that the inhibition of N-*myc* and c-*myc* may be mediated through the same mechanism.

TGF- β 1 induces Mxi1 and Mad4. Our results provide the first evidence that TGF- β 1 induces the expression of genes, *Mxi1* and *Mad4*, that directly antagonize *myc* function. By antagonizing Myc activity, Mxi1 and Mad4 appear to function as tumor suppressors (69, 70). One function of TGF- β 1 that is critical for preserving tissue homeostasis is the ability of TGF- β 1 to suppress tumor formation (34, 71). The tumor suppressor functions of TGF- β 1 appear to be predominantly related to the growth arrest signal, whereby TGF- β 1 inhibits proto-oncogenes and activates tumor suppressor genes. The ability of TGF- β 1 to inhibit cell growth is commonly lost as normal cells transform into cancer, and many of the alterations in gene expression that lead to cancer also appear to allow cells to escape TGF- β 1-induced growth arrest. Thus, one of the hallmark features of TGF- β 1 is its ability to activate the functions of tumor suppressor genes, and our observation that TGF- β 1 induces the expression of *Mxi1* and *Mad4* is consistent with this paradigm.

Interestingly, *Mxi1* and *Mad4* are thought to have essential roles in the process of cell differentiation during development (70), yet the G₁ arrest of both Balb/MK and Mv1Lu cells in response to TGF- β 1 is completely reversible, and permanent phenotypic changes have not been described. Our results suggest that the role of *Mxi1* and *Mad4* induction by TGF- β 1 may be limited to inhibiting cell growth, rather than initiating cell

differentiation. In this regard, our findings are similar to the reports indicating that Myc antagonists appear to mediate cell cycle arrest in the absence of differentiation in response to interferon (72) and serum withdrawal (73). Because induction of a *myc* antagonist such as *Mxi1* or *Mad4* has been shown uniformly to be sufficient to arrest cell growth (36, 69, 74, 75), it appears that induction of *Mxi1* or *Mad4* by TGF- β 1 might be sufficient to mediate cell cycle arrest in the rare circumstance in which c-*myc* does not appear to be inhibited by TGF- β 1. In other words, the induction of *Mxi1* and *Mad4* expression by TGF- β 1 alters the threshold so that the cell subsequently requires higher levels of *myc* expression to sustain cell growth.

In summary, the novel finding that TGF- β 1 regulates the expression of N-*myc*, *Mxi1*, and *Mad4* in Balb/MK cells supports the idea that these transcription factors mediate essential functions of TGF- β 1. Until now, TGF- β 1 was thought to block *myc* function exclusively by inhibiting *myc* expression. However, when viewed as a larger regulatory scheme, the induction of *Mxi1* and *Mad4* mRNA expression by TGF- β 1 suggests that the ratios of these proteins shift to form a relative predominance of Mxi1-Max and Mad4-Max dimers. Earlier reports have firmly established that a predominance of Mxi1-Max or Mad4-Max dimers will oppose the effect of Myc-Max dimers on the transcription of key growth regulatory genes, and thereby induce cell cycle arrest. Thus, our results suggest that the induction of *Mad4* and *Mxi1* may function in tandem with the inhibition of N-*myc* and c-*myc* to mediate the growth inhibitory and tumor suppressor functions of TGF- β 1. Although we have demonstrated that TGF- β 1 regulates the expression of multiple Max-interacting factors in two cell lines commonly used as model systems for studying TGF- β 1-induced growth arrest, it remains to be determined whether TGF- β 1 might target Max-interacting transcription factors in a cell type-specific manner. For example, it is possible that TGF- β 1 might affect the expression of Max-interacting factors other than N-*myc*, c-*myc*, *Mxi1*, and *Mad4* in certain cell types. Further studies are needed to determine the degree to which the induction of *Mxi1* and *Mad4* may mediate the effects of TGF- β 1 signaling on cell growth or differentiation during development. In addition, the relative importance of the distinct mRNA expression patterns of these genes in Balb/MK cells awaits a more comprehensive understanding of the relative abundance and transcriptional targets of the Max-containing protein dimers during the cell cycle.

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