FKBP12, the 12-kDa FK506-binding protein, is a physiologic regulator of the cell cycle

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FKBP12, the 12-kDa FK506-binding protein, is a ubiquitous abundant protein that acts as a receptor for the immunosuppressant drug FK506, binds tightly to intracellular calcium release channels and to the transforming growth factor β (TGF-β) type I receptor. We now demonstrate that cells from FKBP12-deficient (FKBP12$^{-/-}$) mice manifest cell cycle arrest in G_1 phase and that these cells can be rescued by FKBP12 transfection. This arrest is mediated by marked augmentation of p21(WAF1/CIP1) levels, which cannot be further augmented by TGF-β1. The p21 up-regulation and cell cycle arrest derive from the overactivity of TGF-β receptor signaling, which is normally inhibited by FKBP12. Cell cycle arrest is prevented by transfection with a dominant-negative TGF-β receptor construct. TGF-β receptor signaling to gene expression can be mediated by SMAD, p38, and ERK/MAP kinase (extracellular signal-regulated kinase/mitogen-activated protein kinase) pathways. SMAD signaling is down-regulated in FKBP12$^{-/-}$ cells. Inhibition of ERK/MAP kinase fails to affect p21 up-regulation. By contrast, activated phosphorylated p38 is markedly augmented in FKBP12$^{-/-}$ cells and the p21 up-regulation is prevented by an inhibitor of p38. Thus, FKBP12 is a physiologic regulator of cell cycle acting by normally down-regulating TGF-β receptor signaling.

FKBP12, the 12-kDa FK506-binding protein, was discovered as a receptor for the immunosuppressant drug FK506, which elicits its effects by binding to and inhibiting the calcium-activated phosphatase calcineurin (1, 2). Although first identified in immune cells, FKBP12 occurs in all tissues, with particularly high densities in the brain, and appears to have diverse functions, which have been only partially characterized (3). FKBP12 is a subunit of two intracellular calcium release channels, the inositol 1,4,5-trisphosphate receptor and the ryanodine receptor (4, 5), and is also a subunit of the TGF-β type I receptor (6, 7). FKBP12 inhibits basal signaling of these three receptors (4, 5, 8, 9) and also regulates the P-glycoprotein multidrug transporter (10, 11). FKBP12 is critical for life, because mice with targeted deletion of FKBP12 typically die during embryonic life, manifesting multiple abnormalities, including cardiac hypertrophy and augmented calcium release through the ryanodine receptor (12). FKBP12 occurs in very high concentrations in all cells and so might be expected to regulate fundamental aspects of cell biology. Accordingly, we examined several properties of FKBP12$^{-/-}$ fibroblasts and discovered that they grow much more slowly than wild-type cells. Our examination of relevant mechanisms led us to demonstrate that FKBP12 is a physiologic regulator of the cell cycle. Cells from FKBP12-deficient (FKBP12$^{-/-}$) mice are arrested in G_1 phase of the cell cycle. The arrest is mediated by enhanced TGF-β receptor signaling leading to overactivation of p21(WAF1/CIP1), a physiologic inhibitor of the cell cycle.

Methods

Immunocytochemistry. Fixation, permeabilization, blocking, and PBS washes were all performed as described (13).

Primary Cultures. Primary cultures of fibroblasts were established from mouse embryos at embryonic day 12.5 from FKBP12$^{-/-}$ mice and their wild-type littermates by the Genetics Resource Core Facility at The Johns Hopkins University School of Medicine, according to established protocols. A breeding pair of FKBP12$^{+/-}$ mice (12) was provided by M. M. Matzuk and W. Shou. Cells were maintained in standard cell-culture medium (DMEM/10% FCS/100 units/ml, penicillin/100 μg/ml, streptomycin/2 mM glutamine). All experiments were performed during passages 3–5. Genotyping was performed as reported (12), and Western blot analysis with anti-FKBP12 was used to confirm the results.

Transient Transfection. Transfection was performed according to the manufacturer's protocol for Lipofectamine/PLUS (GIBCO/BRL).

Phosphate Labeling and Immunoprecipitation. For [^{32}P]phosphate labeling, fibroblasts (50–60% confluency), plated in 10-cm dishes, were washed and preincubated with phosphate-free medium containing 0.2% dialyzed FCS. The cells were then incubated with medium containing [^{32}P]phosphate at 0.5 mCi/ml (1 Ci = 37 GBq) for 2 h at 37°C. Some cells were then treated 30 min with TGF-β1 (Calbiochem) at 10 ng/ml followed by lysis with 1 ml of lysis buffer A [50 mM Hepes, pH 7.4/50 mM NaCl/1 mM EDTA/0.5% Triton X-100/1 mM Na_3VO_4/50 mM NaF/10 mM sodium β-glycerophosphate/1 mM PMSF/aprotinin (5 mg/ml)/leupeptin (1 mg/ml)/pepstatin A (1 mg/ml)], and centrifuged at 14,000 × *g* for 10 min at 4°C. After normalizing the protein concentration, 2 μg of anti-Smad2/3 (Santa Cruz Biotechnology) and 40 μl of 50% slurry protein A&G agarose Plus (Calbiochem) were added to the supernatant and incubated with rotation at 4°C for 2 h. The agarose pellet was washed four times with 1 ml of lysis buffer and resuspended in 30 μl of sample buffer, and proteins were separated by SDS/PAGE. The gel was Coomassie-stained, dried, and exposed to film to detect ^{32}P radioactivity.

Western Blot and Cell Cycle Markers. Fibroblasts were plated in six-well dishes at 50% confluency incubated with TGF-β1 at 10 ng/ml or basic fibroblast growth factor (bFGF; Calbiochem) at 25 ng/ml for 18–24 h. Cells were then lysed with 200 μl of lysis buffer B [50 mM Hepes, pH 7.4/50 mM NaCl/1 mM EDTA/0.5% Triton X-100/1 mM PMSF/aprotinin (5 mg/ml)/leupeptin (1 mg/ml)/pepstatin A (1 mg/ml)] and centrifuged

Abbreviations: bFGF, basic fibroblast growth factor; ERK, extracellular signal-regulated kinase; FKBP12, 12-kDa FK506-binding protein; MAP kinase, mitogen-activated protein kinase; PCNA, proliferating-cell nuclear antigen; RT-PCR, reverse transcription–PCR, TGF-β, transforming growth factor β; PAI-1, plasminogen activator inhibitor-1.

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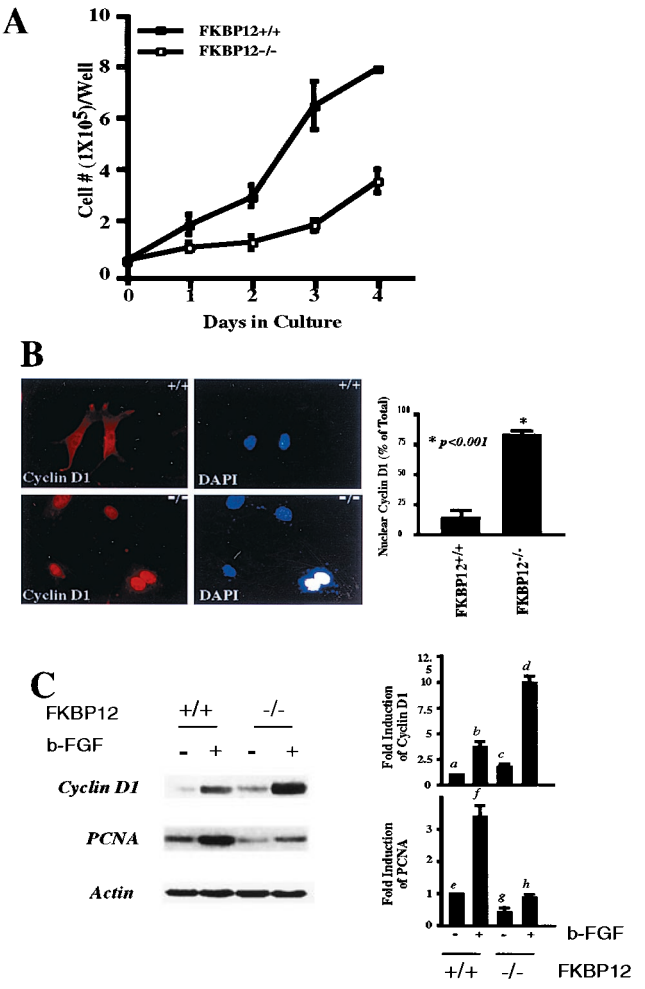

Fig. 1. FKBP12$^{-/-}$ fibroblasts are arrested in G_1 phase of the cell cycle. (*A*) Cell growth. For FKBP12$^{+/+}$ or FKBP12$^{-/-}$ cells, 5×10^4 cells were plated in six-well dishes and counted in 24-h intervals by hematocytometry. Trypan blue dye was used to count viable cells. Data points are the mean ± SEM from three experiments. (*B*) Cyclin D1 staining. (*Left*) Nuclear localization of cyclin D1 was observed in more than 80% of FKBP12$^{-/-}$ cells. (*Right*) The bar graph presents the mean ± SEM from three experiments, with at least 200 cells counted in 10 fields for each experiment (*, $P < 0.001$). (*C*) Cell cycle progression in FKBP12$^{-/-}$ cells stimulated with bFGF (25 ng/ml) was monitored by using cyclin D1 (G_1 phase) and PCNA (S phase) as markers. In FKBP12$^{+/+}$ cells, a 24-h bFGF treatment stimulates expression of cyclin D1 4-fold (bar b is different from bar a, $P < 0.001$) and expression of PCNA 3.5-fold (bar d is different from bar c, $P < 0.001$), indicating an increase in cells in S phase. In FKBP12$^{-/-}$ cells, cyclin D1 is stimulated 5-fold (bar d different from bar c, $P < 0.001$) with only a modest increase in PCNA, indicating G_1-phase blockade and failure to progress to S phase. Values are the mean ± SEM of five determinations.

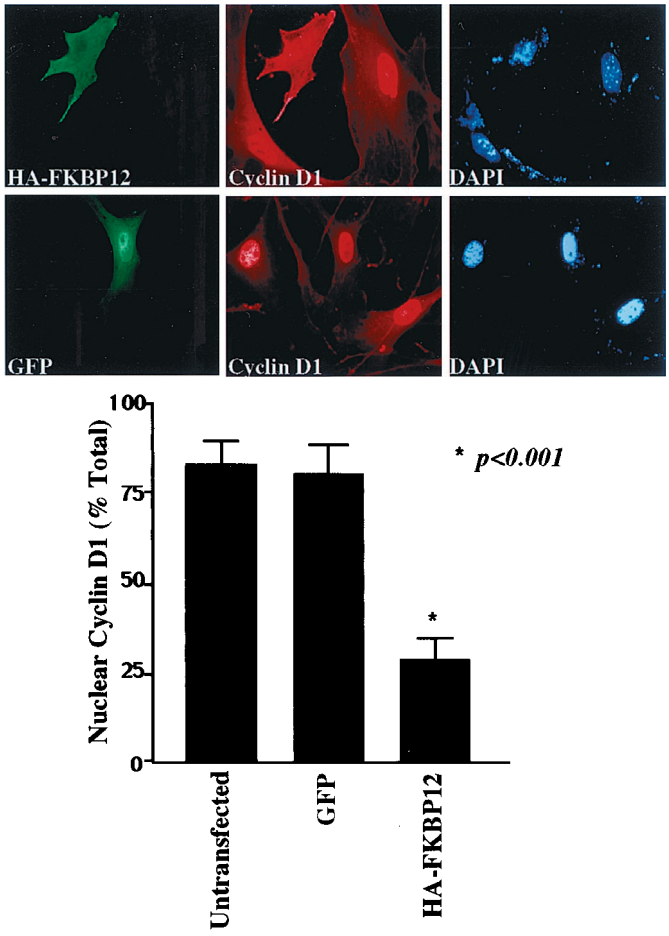

Fig. 2. FKBP12 transfection reverses the G_1 arrest of FKBP12$^{-/-}$ fibroblasts. Hemagglutinin (HA)-tagged FKBP12 was transfected into FKBP12$^{-/-}$ cells and nuclear localization of cyclin D1 was monitored. At least 200 transfected or untransfected cells in 10 fields were counted in three experiments with mean ± SEM reported in the bar graph. As a control green fluorescent protein (GFP) was transfected in FKBP12$^{-/-}$ cells and the transfected GFP cells were also counted.

at 14,000 × *g* for 10 min at 4°C. Protein (50 μg) was loaded in each well, SDS/PAGE was performed, and, after transfer to nitrocellulose paper, Western blots were performed at 1:500 dilution with antiserum to cyclin D1, p21, proliferating-cell nuclear antigen (PCNA), or actin (Santa Cruz Biotechnology) and a 1:2,000 dilution with anti-mouse horseradish peroxidase-coupled secondary antibody (DAKO). For phosphorylated p38 blots, cells were treated with TGF-β1 at 10 ng/ml for 30 min and lysed with lysis buffer A. Rabbit anti-phospho-p38 and anti-rabbit p38 were used at a 1:1,000 dilution with anti-rabbit horseradish peroxidase-coupled secondary antibody (DAKO).

Quantitative PCR. Fibroblasts were plated and, at 50% confluency, were treated with TGF-β1 at 10 ng/ml for 3 h. RNA was extracted with Trizol (Sigma) according to the manufacturer's protocol. Reverse transcription–PCR (RT-PCR) was performed on 1 μg of total RNA with the RT-PCR kit/Superscript II from (GIBCO/BRL) according to the manufacturer's protocol, using a dT_{12-18} primer. Real-time PCR was performed according to the Light Cycler instruction (DNA Master SYBR GreenI, Roche Diagnostics) with the master mixes supplied in the kit and 0.5 μM primers p21 (5′-CGGTCCCGTGGACAGTGAGCAG-3′, 5′-GTCAGGCTGGTCTGCCTCCG-3′), SMAD7 (5′-GCATTCCTCGGAAGTCAAGAGG-3′, 5′-CGTGTGGGTTTACAACCGCA-3′), or PAI-1 (5′-CCCACTTCTTCAAGCTCTTCCAG-3′, 5′-CTCATCCTGCCTAAGTTCTCTCTGG-3′). The actin primers (5′-CTCTTCCAGCCTTCCTTCCTGG-3′, 5′-CTTGCTGATCCACATCTGCTGG-3′) were used for normalization for normalization. With 10 ng of DNA per capillary and annealing at 56°C, 35 PCR cycles were monitored. Conventional curve analyses were done according to the Light Cycler instruction kit. For detection on agarose gels, the reaction was stopped at the logarithmic phase of product amplification. The product was electrophoresed on a 1% agarose gel and detected with ethidium bromide.

Results

Cell Cycle Is Arrested in G_1 Phase in FKBP12$^{-/-}$ Fibroblasts. We monitored cell growth of primary fibroblasts from wild-type

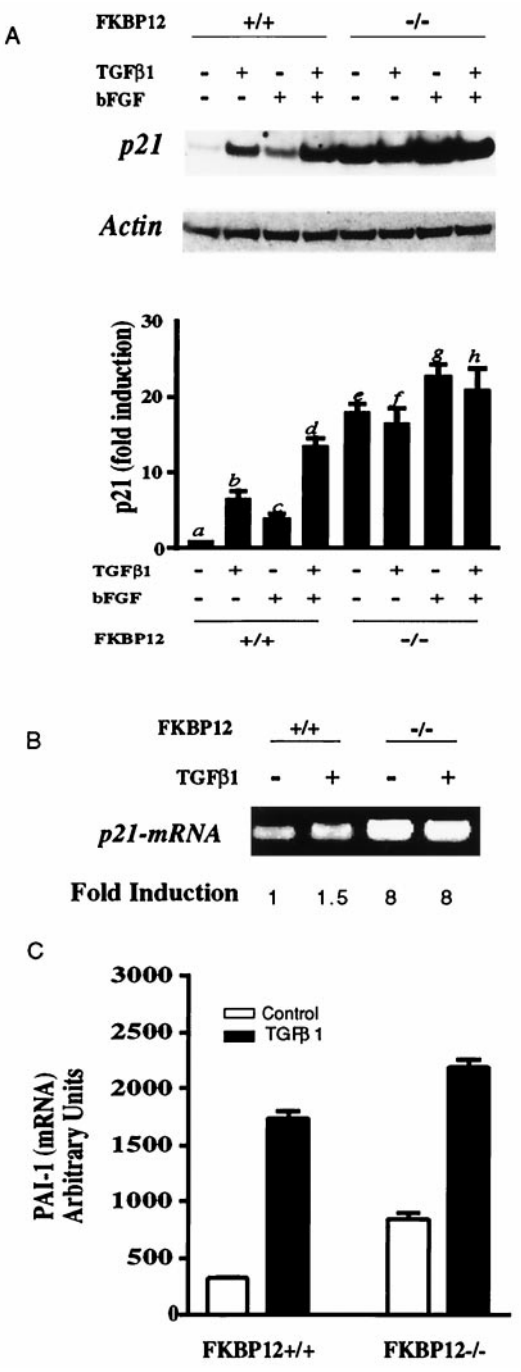

Fig. 3. Increased p21(WAF1/CIP1) expression in FKBP12$^{-/-}$ cells. (*A*) TGF-β1 and bFGF treatments for 24 h induce p21 protein in FKBP12$^{+/+}$ cells (bars b–d are different from bar a, $P < 0.01$) and their effects are additive (bar d is different from bars b and c, $P < 0.01$). In FKBP12$^{-/-}$ cells, p21 protein is greatly augmented (bar e is different from bar a, $P < 0.001$), and TGF-β1 does not elicit any further increase although bFGF does augment p21 (bar g is different from bar e, $P < 0.05$). Data are the mean ± SEM of five experiments. (*B*) p21 mRNA is augmented in FKBP12$^{-/-}$ fibroblasts. p21 mRNA was monitored by real-time PCR and RT-PCR; RNA was extracted from FKBP12$^{+/+}$ and FKBP12$^{-/-}$ cells. p21 mRNA was 8-fold higher in FKBP12$^{-/-}$ cells. TGF-β1 treatment (3 h) induced p21 in FKBP12$^{+/+}$ cells but not in FKBP12$^{-/-}$ cells. Data are representative of data from three experiments, whose results varied less than 15%. (*C*) PAI-1 mRNA is augmented in FKBP12$^{-/-}$ fibroblasts. PAI-1 mRNA was monitored by real-time PCR and RT-PCR; RNA was extracted from FKBP12$^{+/+}$ and FKBP12$^{-/-}$ cells. PAI-1 mRNA was 2.5-fold higher in FKBP12$^{-/-}$ cells than in FKBP12$^{+/+}$ cells. However, consistent with previous reports (12, 23), TGF-β1 treatment (3 h) induced PAI-1 in both FKBP12$^{+/+}$ cells and FKBP12$^{-/-}$ cells by 5- and 3-fold, respectively. Data are representative of data from three experiments, whose results varied less than 15%.

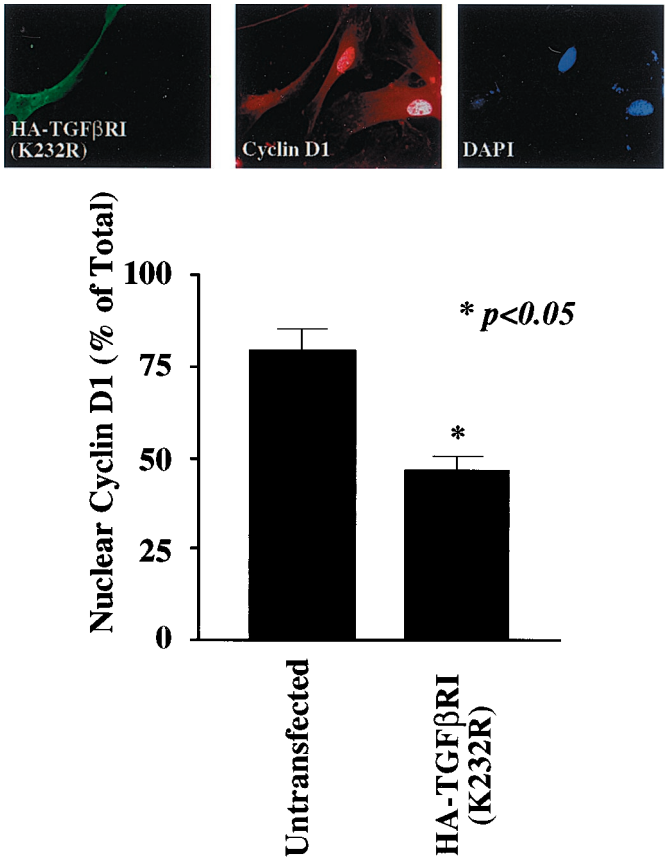

Fig. 4. Perturbation of TGF-β signaling pathway rescues cells from G_1-phase arrest. FKBP12$^{-/-}$ cells were transfected with hemagglutinin (HA)-tagged kinase-deficient (K232R) TGF-β RI, which decreased cells in G_1 phase as monitored by nuclear cyclin D1. Values in the bar graph are the mean ± SEM for three experiments, each conducted with at least 200 cells counted in 10 fields.

and FKBP12$^{-/-}$ mice (Fig 1*A*). Cell growth was markedly reduced in the FKBP12$^{-/-}$ fibroblasts whose cell number at 3 days was only 30% of wild-type values. To ascertain cell cycle progression, we stained cells for cyclin D1, which translocates from cytoplasm to nucleus in the G_1 phase (Fig. 1*B*). Nuclear cyclin D1 levels are augmented 7- to 8-fold in mutant fibroblasts, reflecting G_1-phase arrest (14). We examined the effects of bFGF (15), a known stimulator of cell cycle progression, on levels of cyclin D1 and PCNA, an S-phase marker (Fig. 1*C*). In wild-type fibroblasts, bFGF augmented levels of both cyclin D1 and PCNA. However, in mutant fibroblasts, it stimulated a much greater increase in cyclin D1 with substantially lower levels of PCNA, confirming a cell cycle blockade from G_1 to S phase.

We wondered whether the G_1 arrest is a direct consequence of FKBP12 loss or reflects alterations several steps removed from the early embryonic deletion of FKBP12. We overexpressed FKBP12 in the FKBP12$^{-/-}$ fibroblasts and observed much lower levels of nuclear cyclin D1 (Fig. 2).

p21 Enhancement in FKBP12$^{-/-}$ Cells. What molecular associations of FKBP12 might account for the G_1 arrest? FKBP12 is a subunit of the TGF-β1 receptor and inhibits basal signaling. TGF-β1 administration arrests the cell cycle in the G_1 phase by augmenting formation of p21 (16, 17), which induces G_1 arrest by inhibiting cyclin D-, E-, and A-dependent kinases (18, 19). Levels of p21 were negligible in untreated wild-type fibroblasts (Fig. 3*A*) but greatly augmented in mutant fibro-

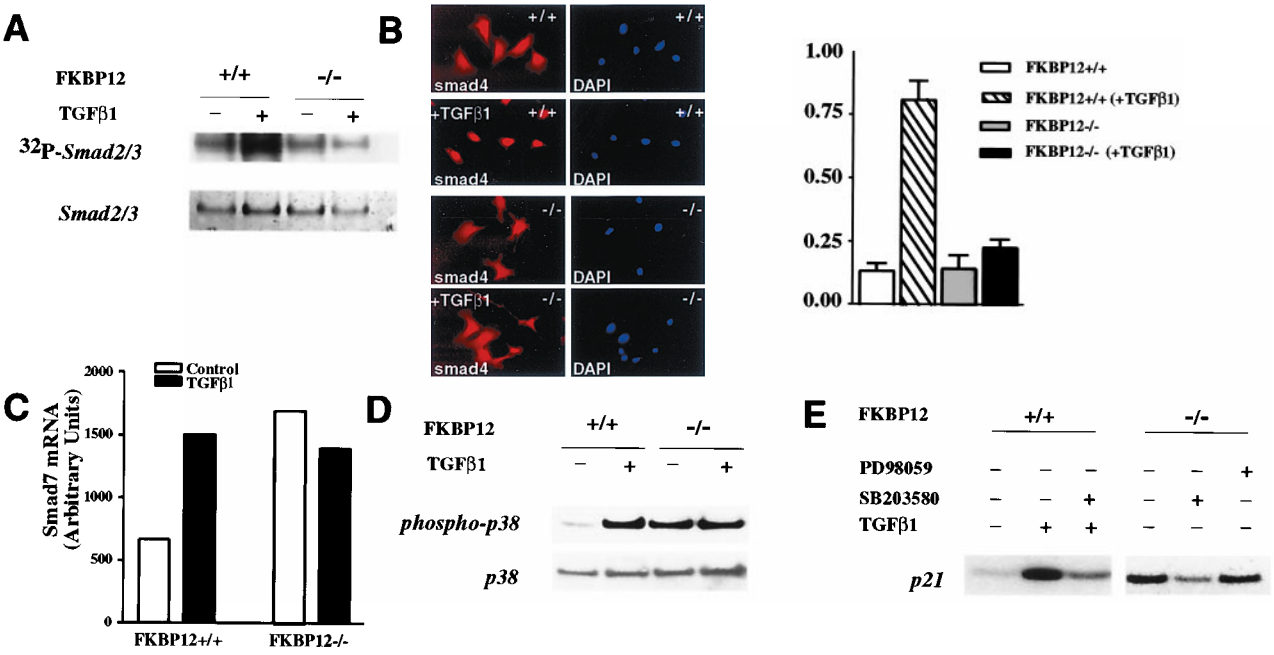

Fig. 5. Overactivation of TGF-β signaling is due to increased p38 MAP kinase signaling. (*A*) TGF-β1 treatment increases the phosphorylation of SMAD2/3 in FKBP12$^{+/+}$ but not FKBP12$^{-/-}$ cells. FKBP12$^{+/+}$ and FKBP12$^{-/-}$ cells were incubated with [^{32}P]orthophosphate. Immunoprecipitation with anti-SMAD2/3 was used to determine the amount of ^{32}P incorporated into SMAD2/3. There was no significant difference in basal SMAD2/3 phosphorylation in the FKBP12$^{+/+}$ or FKBP12$^{-/-}$ cells. TGF-β1 treatment (30 min) significantly increased SMAD2/3 phosphorylation in FKBP12$^{+/+}$ but not in FKBP12$^{-/-}$ cells. (*B*) TGF-β1 treatment increases nuclear localization of SMAD4 in FKBP12$^{+/+}$ but not in FKBP12$^{-/-}$ cells. After TGF-β1 treatment, nuclear localization of SMAD4 was observed in more than 80% of FKBP12$^{+/+}$ cells but no significant increase was seen in FKBP12$^{-/-}$ cells. (*C*) SMAD7 mRNA is significantly higher in FKBP12$^{-/-}$ than FKBP12$^{+/+}$ cells. TGF-β1 treatment increased SMAD7 mRNA in FKBP12$^{+/+}$ cells almost to the levels of the FKBP12$^{-/-}$ cells but did not augment the SMAD7 mRNA in the FKBP12$^{-/-}$ cells. (*D*) p38 MAP kinase is constitutively phosphorylated in FKBP12$^{-/-}$ fibroblasts. TGF-β1 treatment (30 min) increased phosphorylation of p38 in FKBP12$^{+/+}$ cells; however, p38, in FKBP12$^{-/-}$ cells, was already extensively phosphorylated and TGF-β1 treatment did not increase phosphorylation. No change in total p38 expression was observed relative to actin or total protein. The experiment was replicated three times. (*E*) Inhibition of p38 MAP kinase blocks p21 induction in FKBP12$^{-/-}$ fibroblasts. SB203580 (20 μM, 24 h), an inhibitor of p38 MAP kinase, blocked the TGF-β1 induction of p21 in FKBP12$^{+/+}$ cells and reduced p21 in the FKBP12$^{-/-}$ cells. PD98059 (20 μM, 24 h), an inhibitor working upstream of MAP kinase/Erk kinase, did not affect p21 in FKBP12$^{-/-}$ cells.

blasts. TGF-β stimulated p21 expression in wild-type cells. If the augmented basal levels of p21 in the mutant fibroblasts reflect enhanced TGF-β signaling, then one would not expect exogenous TGF-β to produce further increases. Indeed, TGF-β failed to enhance p21 levels of mutant fibroblasts. bFGF, on the other hand, acts as a growth factor predominantly through the p42/44 mitogen-activated protein kinase (MAP kinase, also known as Erk1/2, where Erk is extracellular-signal-regulated kinase) pathway, distinct from TGF-β signaling (15, 20–22). bFGF augmented further the already elevated levels of p21 in the mutant fibroblasts, whereas the combination of TGF-β and bFGF was no greater than that produced by bFGF alone.

To ascertain whether the augmented p21 protein in mutant fibroblasts reflected new synthesis or stabilization of the protein, we analyzed p21 mRNA by semiquantitative real-time PCR (Fig. 3*B*). Mutant fibroblasts displayed an 8-fold increase in p21 mRNA compared with wild-type controls, and TGF-β1 produced no further increase. As reported by others (12, 23), TGF-β1 still induced plasminogen activator inhibitor-1 (PAI-1) (Fig. 3*C*) in the FKBP12$^{-/-}$ cells, although there was an increase in basal PAI mRNA.

These findings strongly imply that enhanced TGF-β signaling in FKBP12$^{-/-}$ fibroblasts accounts for the cell cycle arrest. If this hypothesis is correct, then blockade of TGF-β signaling should reverse the cell cycle arrest. Overexpression of a kinase-dead TGF-β receptor in the mutant fibroblasts does markedly reduce nuclear cyclin D1, confirming that augmented TGF-β signaling mediates the cell cycle arrest (Fig. 4).

p38 MAP Kinase Augmentation Mediates p21 Up-Regulation in FKBP12$^{-/-}$ Cells. TGF-β signals through several cascades, including the SMADs, p42/44 MAP kinase, and p38 MAP kinase (20, 21, 24–26). Which of these is responsible for the augmented TGF-β signaling that causes cell cycle arrest? Phosphorylation of SMAD2/3 in mutant fibroblasts was reduced compared with that in wild-type fibroblasts, and TGF-β failed to stimulate SMAD phosphorylation (Fig. 5*A*). Phosphorylation of SMAD2/3 leads to translocation of SMAD4 into the nucleus (20, 21). TGF-β failed to stimulate SMAD4 nuclear translocation in mutant fibroblasts, consistent with its failure to augment SMAD2/3 phosphorylation (Fig. 5*B*). The SMAD signaling pathway is subject to feedback regulation in which translocation of SMAD7 from the nucleus to the cytoplasm down-regulates SMAD2/3 phosphorylation (27, 28). In mutant fibroblasts, we observed increased cytoplasmic SMAD7, which can explain the failure of TGF-β to augment SMAD2/3 phosphorylation and nuclear translocation of SMAD4 (Fig. 5*C*). These findings indicate that augmented SMAD signaling probably does not account for cell cycle arrest in mutant fibroblasts.

p38 MAP kinase is activated by phosphorylation (29, 30). Phosphorylated p38 levels are substantially greater in mutant than wild-type fibroblasts (Fig. 5*D*). If the augmented levels of phosphorylated p38 reflect enhanced basal TGF-β signaling, then exogenous TGF-β should not further increases these levels. Indeed, we observed no further increase in mutant fibroblasts after TGF-β treatment, whereas such treatment of wild-type fibroblasts augmented phosphorylation of p38.

If the cell cycle arrest of mutant fibroblasts is attributable to augmented p38 signaling, then inhibition of p38 should relieve

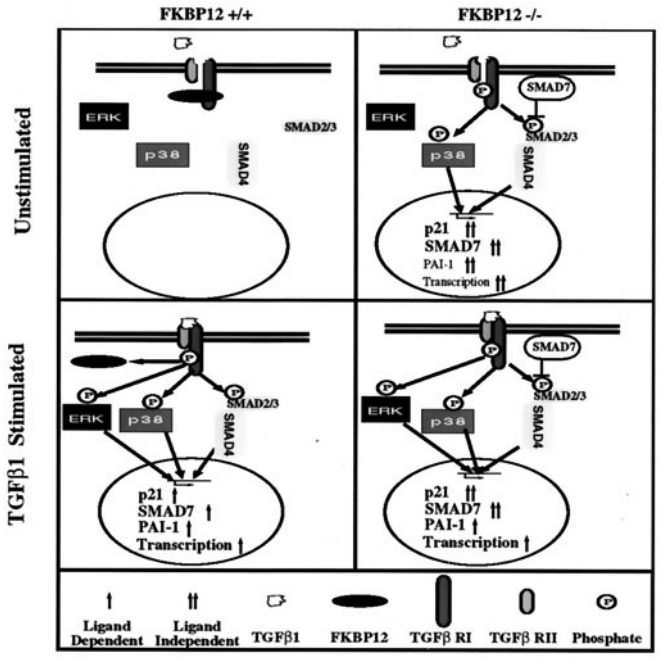

Fig. 6. Model depicting FKBP12 regulation of type I TGF-β receptor signaling. (*Left*) In FKBP12$^{+/+}$ cells, TGF-β1 induces gene transcription by SMAD2/3, p38, and ERK MAP kinase leading to induction of p21, SMAD7, PAI-1, and other genes under the influence of TGF-β receptor signaling. (*Upper Right*) FKBP12$^{-/-}$ cells are partially activated in a ligand-independent manner leading to augmentation of p21, SMAD7, and p38. (*Lower Right*) TGF-β1 treatment of the FKBP12$^{-/-}$ cells cannot further induce or activate these pathways due to feedback influenced by SMAD7. PAI-1 is basally activated in FKBP12$^{-/-}$ cells and can be further activated by TGF-β1.

the arrest. SB203580, a potent and selective inhibitor of p38 catalytic activity (31), substantially reduced the elevated p21 levels of mutant and wild-type fibroblasts (Fig. 5*E*). However, PD98059, a potent and selective inhibitor of MAP kinase/Erk kinase, failed to alter p21 levels.

Discussion

In the present study, we have discovered that FKBP12$^{-/-}$ cells manifest cell cycle arrest in the G_1 phase. This is a direct consequence of a loss of FKBP12, because the effect is rescued by FKBP12. The arrest stems from an up-regulation of p21, which arrests cell growth (19). The p21 stimulation arises because of overactivity of the TGF-β receptor in the absence of its ligand TGF-β1. The up-regulated p21 is unresponsive to TGF-β1 but can respond to bFGF, indicating that p21 in FKBP12$^{-/-}$ cells is not maximally activated but simply no longer responds to TGF-β1 because the TGF-β receptor system is already maximally active. TGF-β signals primarily through three pathways SMAD, p38 kinase, and MAP kinase. We could rule out the SMAD pathway as being responsible for p21 up-regulation, because levels of phosphorylated SMAD2/3 and translocation of SMAD4 into the nucleus are down-regulated in FKBP12$^{-/-}$ cells. The down-regulation of the SMAD system appears due to an up-regulation of SMAD7, which emerges from the nucleus to down-regulate SMAD2/3 phosphorylation in response to TGF-β1. We could rule out ERK/MAP kinase because p21 up-regulation is not blocked by the MAP kinase inhibitor PD98059. SB203580, an inhibitor of p38 kinase, however, does prevent p21 up-regulation. Because SB203580 can affect other kinases (32), we sought additional evidence for a role of p38. We found that the activated phosphorylated form of p38 is markedly up-regulated in FKBP12$^{-/-}$ cells and that this up-regulation is not affected by TGF-β1 treatment.

These findings are consistent with the following model (Fig. 6). The TGF-β receptor normally has two subunits, TGF-β receptor I (TGF-β RI) and TGF-β receptor II (TGF-β RII). FKBP12 is tightly bound to TGF-β RI and essentially appears to be a subunit of the receptor. Binding of TGF-β1 to the complex of TGF-β RI and TGF-β RII leads to phosphorylation of TGF-β RI by TGF-β RII causing FKBP12 to dissociate from TGF-β RI, which initiates receptor signaling.

TGF-β RI then signals through three distinct pathways (1). The ERK/MAP kinase pathway, acting through Ras leads to phosphorylation of MAP kinase/Erk kinase, which in turn phosphorylates MAP kinase, which translocates to the nucleus leading to the phosphorylation of transcription factor CREB (2). The p38 pathway is activated via interactions of TAK1 binding protein (TAB1) with TGFβ-activated kinase 1 (TAK1) leading to the phosphorylation and activation of p38, which can translocate to the nucleus and phosphorylate multiple targets both in the cytosol and nucleus (3). The SMAD pathway is initiated by TGF-β RI binding to and phosphorylating SMAD2/3, which then binds to SMAD4 leading to translocation of the SMAD complex into the nucleus. The SMAD complex acts as a transcription factor that augments the cell cycle and also leads to synthesis of SMAD7 in the nucleus. SMAD7 migrates back to the cytosol and directly prevents the association of SMAD2/3 with TGF-β RI.

In FKBP12$^{-/-}$ cells the basal inhibition of TGF-β receptor signaling by FKBP12 is lost leading to overactive signaling that, via p38 overactivation, leads to up-regulation of p21 (Fig. 6). p21 arrests the cell cycle in G_1 phase by preventing cyclin-dependent kinases 4 and 6 from phosphorylating the retinoblastoma gene product pRb. Without inhibition by p21, hyperphosphorylated pRb would release E2F, basally bound to Rb, enabling it to stimulate DNA synthesis and allow cell cycle progression (19).

Regulation of the cell cycle has been a target for the therapy of cancer and neurodegenerative diseases. For instance, nearly all pancreatic and colon cancers contain mutations that disable TGF-β signaling pathways (33). Inactivation mutations occur in third of ovarian cancers (33). We suggest FKBP12 as a therapeutic target. For instance, drugs that bind to FKBP12 to prevent its interaction with the TGF-β receptor and calcineurin would lead to cell cycle arrest but would not be immunosuppressant in the way that immunosuppressants such as FK506 exert their actions. Inhibition of the cell cycle is one component of the events involved in neurite extension in response to neurotrophic proteins such as nerve growth factor (34). The immunosuppressants FK506 and cyclosporin do stimulate neurite outgrowth by mechanisms distinct from their immunosuppressant effects. Thus, derivatives of cyclosporin A and FK506 that bind, respectively, to cyclophilin and FKBP12 but that fail to promote interactions with calcineurin are neurotrophic (35). Molecules much smaller than FK506 that retain affinity for FKBP12 and do not promote interactions with calcineurin display extremely potent neurotrophic and neuroprotective actions (36). The mechanism of their neural actions has not been established but might conceivably involve TGF-β receptor signaling.

One striking feature of FKBP12$^{-/-}$ mice is their embryonic lethality, with the majority of mutant mice dying at embryonic day 18.5 or earlier (12). The exact mechanism of lethality has not been clear. The mutant mice do manifest cardiac hypertrophy, which appears to be associated with increased calcium flux through ryanodine receptors (12). The augmented p38 kinase signaling that we observe in FKBP12$^{-/-}$ cells could play a role in the cardiac hypertrophy of the mutant mice, because augmented p38 signaling is associated with cardiac failure and development (37). If cardiac hypertrophy was the sole lethal

stimulus, then one might expect death to occur within a narrowly circumscribed time. However, the mutant mice die over a wide range of ages from embryonic day 12 to birth, which is more consistent with multiple causes, one of which might be the cell cycle arrest that we have identified. Cell cycle arrest might also explain the very small size of the mutant mice (12).

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1. Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I. & Schreiber, S. L. (1991) *Cell* **66,** 807–815.
2. Friedman, J. & Weissman, I. (1991) *Cell* **66,** 799–806.
3. Snyder, S. H., Lai, M. M. & Burnett, P. E. (1998) *Neuron* **21,** 283–294.
4. Cameron, A. M., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronnett, G. V. & Snyder, S. H. (1995) *Cell* **83,** 463–472.
5. Brillantes, A. B., Ondrias, K., Scott, A., Kobrinsky, E., Ondriasova, E., Moschella, M. C., Jayaraman, T., Landers, M., Ehrlich, B. E. & Marks, A. R. (1994) *Cell* **77,** 513–523.
6. Wang, T., Donahoe, P. K. & Zervos, A. S. (1994) *Science* **265,** 674–676.
7. Okadome, T., Oeda, E., Saitoh, M., Ichijo, H., Moses, H. L., Miyazono, K. & Kawabata, M. (1996) *J. Biol. Chem.* **271,** 21687–21690.
8. Chen, Y. G., Liu, F. & Massague, J. (1997) *EMBO J.* **16,** 3866–3876.
9. Wang, T., Li, B. Y., Danielson, P. D., Shah, P. C., Rockwell, S., Lechleider, R. J., Martin, J., Manganaro, T. & Donahoe, P. K. (1996) *Cell* **86,** 435–444.
10. Hemenway, C. S. & Heitman, J. (1996) *J. Biol. Chem.* **271,** 18527–18534.
11. Mealey, K. L., Barhoumi, R., Burghardt, R. C., McIntyre, B. S., Sylvester, P. W., Hosick, H. L. & Kochevar, D. T. (1999) *Cancer Chemother. Pharmacol.* **44,** 152–158.
12. Shou, W., Aghdasi, B., Armstrong, D. L., Guo, Q., Bao, S., Charng, M. J., Mathews, L. M., Schneider, M. D., Hamilton, S. L. & Matzuk, M. M. (1998) *Nature (London)* **391,** 489–492.
13. Ye, K., Compton, D. A., Lai, M. M., Walensky, L. D. & Snyder, S. H. (1999) *J. Neurosci.* **19,** 10747–10756.
14. Baldin, V., Lukas, J., Marcote, M. J., Pagano, M. & Draetta, G. (1993) *Genes Dev.* **7,** 812–821.
15. Johnson, D. E. & Williams, L. T. (1993) *Adv. Cancer Res.* **60,** 1–41.
16. Li, C. Y., Suardet, L. & Little, J. B. (1995) *J. Biol. Chem.* **270,** 4971–4974.
17. Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y. & Wang, X. F. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 5545–5549.
18. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. & Elledge, S. J. (1993) *Cell* **75,** 805–816.
19. Sherr, C. J. (1996) *Science* **274,** 1672–1677.
20. Massague, J. (1998) *Annu. Rev. Biochem.* **67,** 753–791.
21. ten Dijke, P., Miyazono, K. & Heldin, C. H. (2000) *Trends Biochem. Sci.* **25,** 64–70.
22. Frater-Schroder, M., Muller, G., Birchmeier, W. & Bohlen, P. (1986) *Schweiz. Med. Wochenschr.* **116,** 1160–1162.
23. Bassing, C. H., Shou, W., Muir, S., Heitman, J., Matzuk, M. M. & Wang, X. F. (1998) *Cell Growth Differ.* **9,** 223–228.
24. Derynck, R., Zhang, Y. & Feng, X. H. (1998) *Cell* **95,** 737–740.
25. Hartsough, M. T. & Mulder, K. M. (1995) *J. Biol. Chem.* **270,** 7117–7124.
26. Hanafusa, H., Ninomiya-Tsuji, J., Masuyama, N., Nishita, M., Fujisawa, J., Shibuya, H., Matsumoto, K. & Nishida, E. (1999) *J. Biol. Chem.* **274,** 27161–27167.
27. Hayashi, H., Abdollah, S., Qiu, Y., Cai, J., Xu, Y. Y., Grinnell, B. W., Richardson, M. A., Topper, J. N., Gimbrone, M. A., Jr., Wrana, J. L. *et al.* (1997) *Cell* **89,** 1165–1173.
28. Itoh, S., Landstrom, M., Hermansson, A., Itoh, F., Heldin, C. H., Heldin, N. E. & ten Dijke, P. (1998) *J. Biol. Chem.* **273,** 29195–29201.
29. Han, J., Lee, J. D., Bibbs, L. & Ulevitch, R. J. (1994) *Science* **265,** 808–811.
30. Han, J., Richter, B., Li, Z., Kravchenko, V. & Ulevitch, R. J. (1995) *Biochim. Biophys. Acta* **1265,** 224–227.
31. Young, P. R., McLaughlin, M. M., Kumar, S., Kassis, S., Doyle, M. L., McNulty, D., Gallagher, T. F., Fisher, S., McDonnell, P. C., Carr, S. A., *et al.* (1997) *J. Biol. Chem.* **272,** 12116–12121.
32. Lali, F. V., Hunt, A. E., Turner, S. J. & Foxwell, B. M. (2000) *J. Biol. Chem.* **275,** 7395–7402.
33. Massague, J., Blain, S. W. & Lo, R. S. (2000) *Cell* **103,** 295–309.
34. Mark, M. D. & Storm, D. R. (1997) *J. Biol. Chem.* **272,** 17238–17244.
35. Steiner, J. P., Connolly, M. A., Valentine, H. L., Hamilton, G. S., Dawson, T. M., Hester, L. & Snyder, S. H. (1997) *Nat. Med.* **3,** 421–428.
36. Snyder, S. H., Sabatini, D. M., Lai, M. M., Steiner, J. P., Hamilton, G. S. & Suzdak, P. D. (1998) *Trends Pharmacol. Sci.* **19,** 21–26.
37. Zhang, D., Gaussin, V., Taffet, G. E., Belaguli, N. S., Yamada, M., Schwartz, R. J., Michael, L. H., Overbeek, P. A. & Schneider, M. D. (2000) *Nat. Med.* **6,** 556–563.