A p38mapk-p53 cascade regulates mesodermal differentiation and neurogenesis of embryonic stem cells

Y Hadjal1,2, O Hadadeh1,4, CEI Yazidi1,2, E Barruet1,2 and B Binétruy*1,2

Embryonic stem cells (ESCs) differentiate in vivo and in vitro into all cell lineages, and they have been proposed as cellular therapy for human diseases. However, the molecular mechanisms controlling ESC commitment toward specific lineages need to be specified. We previously found that the p38 mitogen-activated protein kinase (p38MAPK) pathway inhibits neurogenesis and is necessary to mesodermal formation during the critical first 5 days of mouse ESC commitment. This period corresponds to the expression of specific master genes that direct ESC into each of the three embryonic layers. By both chemical and genetic approaches, we found now that, during this phase, the p38MAPK pathway stabilizes the p53 protein level and that interfering directly with p53 mimics the effects of p38MAPK inhibition on ESC differentiation. Anti-p53 siRNA transient transfections stimulate Bcl2 and Pax6 gene expressions, leading to increased ESC neurogenesis compared with control transfections. Conversely, p53 downregulation leads to a strong inhibition of the mesodermal master genes Brachyury and Mesp1 affecting cardiomyogenesis and skeletal myogenesis of ESCs. Similar results were found with p53−/− ESCs compared with their wild-type counterparts. In addition, knockout p53 ESCs show impaired smooth muscle cell and adipocyte formation. Use of anti-Nanog siRNAs demonstrates that certain of these regulations result partially to p53-dependent repression of Nanog gene expression. In addition to its well-known role in DNA-damage response, apoptosis, cell cycle control and tumor suppression, p53 has also been involved in vivo in embryonic development; our results show now that p53 mediates, at least for a large part, the p38MAPK control of the early commitment of ESCs toward mesodermal and neural lineages.

Received 31.5.13; revised 05.6.13; accepted 06.6.13; Edited by G Raschella

Subject Category: Experimental Medicine

Because of their pluripotency and unlimited proliferation properties, embryonic stem cells (ESCs) have been proposed as cellular therapy for human diseases. However, this potential is impaired by the poor efficiency of ESC differentiation into mature differentiated cells and by the persistence of undifferentiated cells with a tumorigenic capacity within differentiated cultures. Therefore, there is a crucial need for a better understanding of molecular mechanisms governing ESC commitment into specific lineages. Self-renewal of mouse ESCs is dependent on the intracellular pathways involving a complex interplay between specific epigenetic processes, miRNAs and transcription factors, such as Oct4, Nanog or Sox2 (see for reviews1,2). In vitro ESC differentiation can be induced by various experimental protocols resulting in the commitment into a variety of mature differentiated cell types (see for reviews3–5). This process can be modulated between the 2nd and 5th day of differentiation by the potent morphogen retinoic acid (RA) that induces differentiation into neurons and adipocytes6–8 and, conversely, inhibits cardiomyocyte and skeletal myotube formation.9,9,10 Although ESC differentiation experimental models recapitulate the successive steps from pluripotency to well-differentiated cells, the molecular mechanisms underlying these processes are poorly understood.

P38 Mitogen-activated protein kinase (p38MAPK) is activated by phosphorylation in response to osmotic stress, UV and various cytokines involved in inflammatory responses and has been proposed to regulate several cellular processes, such as proliferation, cell survival and differentiation.11,12 Deletion of the p38α gene leads to early embryonic lethality between 11.5 and 12.5 days,13,14 demonstrating an essential role of p38MAPK in development. We previously found that the p38MAPK pathway is necessary at two successive early steps during the differentiation process of ESC commitment into cardiomyocyte, endothelial cells, smooth muscle and skeletal muscle cell lineages, whereas it inhibits neurogenesis.10,15 Particularly, this p38MAPK control takes place during the critical first 5 days of ESC commitment, concomitantly with the expression of specific master genes that direct ESC into each of the three embryonic layers. To better understand the molecular mechanisms underlying these processes, we treated ESCs with a p38MAPK-specific

1INSERM U910, Faculté de Médecine, 27 Boulevard Jean Moulin, Marseille Cedex 5, France and 2Faculté de Médecine, Aix-Marseille Université, 27 Boulevard Jean Moulin, Marseille Cedex 5, France
*Corresponding author: B Binétruy, INSERM U910, Faculté de Médecine La Timone, 27 Boulevard Jean Moulin, Marseille Cedex 5 13385, France. Tel: 33 04 9132 4886; Fax: 33 04 9182 6958; E-mail: Bernard.Binetruy@univ-amu.fr

Keywords: mouse embryonic stem cells; p53; p38MAPK; neurogenesis; cardiomyogenesis; skeletal myogenesis

Abbreviations: ERK, extracellular signal-regulated kinase; EB, embryoid body; ESC, embryonic stem cell; MAPK, mitogen-activated protein kinase; MyH1, myosin heavy chain-1; RA, retinoic acid; RT-qPCR, reverse-transcriptase quantitative PCR; PBS, phosphate-buffered saline

Received 31.5.13; revised 05.6.13; accepted 06.6.13; Edited by G Raschella
inhibitor and performed microarray experiments on mRNAs extracted from treated versus untreated cells. Interestingly, one of the regulated targets is Bcl2, a well-known p53-inhibited gene.

The p53 protein is a short-lived, latent transcription factor, which is activated and stabilized in response to a wide range of cellular stresses; exacerbated p53 activation leads to dramatic cellular responses such as cell cycle arrest, apoptosis or senescence. Furthermore, p53 is a crucial tumor suppressor that is somatically mutated in about half of all the human cancers and has multiple roles in maintaining genomic stability in somatic cells (see for review 16). Its role in ESC biology seems paradoxical, indeed undifferentiated ESCs fail to undergo p53-dependent cell cycle arrest, apoptosis and senescence after DNA damage. By contrast, p53 can induce ESC differentiation by directly suppressing the expression of Nanog, one of the master transcriptional factor maintaining ESC self-renewal (Lin et al. 17 and see for review 18). However, its precise role in ESC commitment toward specific lineages is unknown.

We analyzed the role of p53 in ESC differentiation and found that p53 is an important mediator of p38MAPK-induced control of ESC commitment.

**Results**

Differential display experiments identify p53 as a potential mediator of the p38MAPK pathway. CGR8 mouse ESCs were induced to differentiate by forming embryoid bodies (EBs) and, at day 3, treated or not by the specific p38MAPK inhibitor PD169316, and mRNAs were extracted and analyzed by microarray experiments 48 h later. For each condition, three mRNA batches were hybridized on DNA chips spotted with 44 K mouse gene-specific sequences, and regulations of genes of interest were confirmed by reverse-transcriptase quantitative PCR (RT-qPCR) technique (data not shown). Among the identified targets, we focused our attention on one gene upregulated in our experiments, Bcl2, a well-known p53-inhibited gene.19,20 Interestingly, Bcl2 has already been described as a p38MAPK-inhibited gene and involved in ESC neurogenesis.21 Altogether, these data suggested that p53 could mediate the p38MAPK control on ESC differentiation and prompted us to verify this hypothesis.

Chemical inhibition of p53 mimics the effects of p38MAPK inhibition on target genes and ESC commitment. CGR8 ESCs induced to differentiate were treated by either the p38MAPK inhibitor or pifithrin-α, a specific p53 chemical inhibitor,22 and compared with untreated controls. As expected, p38MAPK inhibitor treatment induces Bcl2 gene expression and enhances neurogenesis, characterized by MAP2 gene expression; interestingly, p53 inhibition induces similar effects (Figures 1a and b). Conversely, cardiomyocyte formation was repressed by these treatments; as judged by Mesp1 expression (Figure 1c), a cardiac mesodermal master gene involved in ESC cardiomyogenesis23 and TroponinT2

**Figure 1** Chemical inhibition of p53 mimics the effects of p38MAPK inhibition on target genes and ESC commitment. EBs from wild-type CGR8 ESCs at day 0 were induced to differentiate and treated or not, between day 3 and 10, by either the PD169316 p38MAPK inhibitor or the pifithrin-α p53 inhibitor. mRNAs from EBs were extracted at day 10 (a and b) or at various time as indicated (c and d) and analyzed by real-time RT-PCR for the expression of Bcl2 (a), MAP2 (b), Mesp1 (c) and TroponinT2 (d) gene expression. Results are expressed in arbitrary units, with the values of untreated CGR8 control cells (Ct) taken as 1, and are the mean ± S.E.M. of at least three independent experiments. Significance to Ct values is given as: *P < 0.05 and **P < 0.01.
expression (Figure 1d), a cardiomyocyte-specific marker. These data suggest that the p38MAPK role in ESC differentiation could indeed be mediated by a p38MAPK-p53 cascade.

The p38 MAPK pathway regulates the stability of endogenous p53 proteins at day 3 of differentiation. In response to osmotic stress, p53 proteins have been shown to be directly phosphorylated by p38MAPK on several serine residues, and, in addition, p53 acetylation has been involved in human ESC differentiation. Therefore, we analyzed the consequences of p38MAPK inhibition on p53 protein expression and post-translational regulation. We could not find any significant effect of p38MAPK inhibition neither on phosphorylation of p53 serine residues 15 and 392 or on p53 acetylation (data not shown). As already shown, we found that the p53 protein level decreases rapidly as ESCs differentiated (Figure 2a); however, at day 3 of differentiation, a stronger decrease is systematically observed 24 h after p38MAPK inhibition (Figure 2b). Noteworthy, this effect is not detectable after 4 h of inhibition. This regulation is not at the p53 transcriptional level, neither at day 3 (Figure 2b, lower panel) or during the first 5 days of differentiation (Figure 2c), and is not associated to a control of the cytoplasmic/nucleic localization of p53 proteins (Figure 2d). Finally, we found that this effect is not mediated by a p38MAPK-dependent regulation of the proteasome activity. Indeed, although inhibition of the proteasome efficiently stabilizes p53 proteins, the destabilization of p53 proteins induced by p38MAPK inhibition is still present after treatment with the proteasome inhibitor MG132 (Figure 2e). Although additional works are necessary to understand the molecular mechanism by which the p38MAPK pathway augments p53 protein stability, the experiments shown suggest that p38MAPK inhibition leads to a shorter half-life of p53 protein. Furthermore, the fact that this regulation necessitates 24 h (Figure 2b) strongly suggests that it corresponds to an indirect mechanism involving intermediate partner(s).

To precise the role of p53 in ESC differentiation and to avoid potential unspecific effects of chemical compounds, we then

Figure 2: The p38MAPK regulates the stability of p53 proteins in CGR8 ESCs. (a) ESCs were induced to differentiate, and total cellular proteins were extracted at various time from day 0 to 5, as indicated, and analyzed by western blotting with either α-p53 antibodies or α-extracellular signal-regulated kinase (α-ERK) antibodies as loading control. (b) ESCs were induced to differentiate and, at day 3, treated or not, as indicated, by the p38MAPK inhibitor. Total cellular proteins or mRNAs were extracted 0, 4 or 24 h later and analyzed either by western blotting with α-p53 antibodies or α-ERK antibodies as loading control (upper panels) or by real-time RT-PCR for the expression of p53 gene (lower panel). (c) ESCs were induced to differentiate and treated or not, as indicated, by the p38MAPK inhibitor either from day 0 to 5 or from day 3 to 5. mRNAs were extracted at various time, as indicated, and analyzed by real-time RT-PCR for the expression of p53 gene. Results are expressed in arbitrary units, with the values of untreated CGR8 control cells at day 0 taken as 1, and are the means ± S.E.M. of at least three independent experiments. (d) ESCs were induced to differentiate and, at day 3, treated or not, as indicated, by the p38MAPK inhibitor. Cytoplasmic and nuclear cellular proteins were extracted 0, 4 or 24 h later and analyzed by western blotting with either α-p53 antibodies or α-ERK antibodies as loading control.
used two genetic approaches: transient transfections of anti-p53 siRNAs and analysis of differentiation capacities of ESC knockout for the p53 gene.27

siRNAs against p53 efficiently inhibit p53 expression and activity in ESCs. Anti-mouse-p53 siRNAs (siRNA SMART pool; Dharmaco, Waltham, MA, USA) were transiently transfected into undifferentiated CGR8 ESCs and p53 mRNA expression analyzed by RT-qPCR 24 and 48 h later. A strong decrease in expression is consistently observed compared with cells transfected with control siRNAs, with only 8.5–10% of p53 mRNA left (Figure 3a).

This p53 inhibition is also detected at the protein level, as shown by western blot experiment (Figure 3b). We then analyzed the p53 repression during differentiation; undifferentiated ESCs were transfected with control or anti-p53 siRNAs, the day after (day 0) ESCs were induced to differentiate, and p53 mRNAs were analyzed at days 0, 3, 4, 5, 7 and 12 of differentiation. A significant p53 repression persists until day 5 of the protocol, and is only lost at day 7 (Figure 3c).

Importantly, this inhibitory effect last long enough to cover the first 5 days of critical period for ESC commitment. We then investigated the effects of anti-p53 siRNA transfections on p53 activity. As expected, compared with control cells, transfections of anti-p53 siRNAs in ESCs lead to a 3–4-fold increase in Bcl2 expression (Figure 3d), whereas the expression of p21 gene, a positively regulated p53 target, is decreased by 70% at 24 h and 60% at 48 h (Figure 3e).

siRNA-mediated inhibition or homozygote gene knockout of p53 interfere with ESC differentiation. Differentiation capacities of CGR8 ESCs transfected by anti-p53 siRNAs compared with control siRNAs transfection and those of p53−/− ESCs compared with their wild-type counterparts were then analyzed. Anti-p53 siRNA-transfected CGR8 ESCs and p53−/− ESCs display, on the one hand, augmented neurogenesis (Figure 4a) and, on the other hand, a strong inhibition of cardiomyogenesis, illustrated by the decrease in gene expression of both the cardiac transcription factor Nkx2.5 (Supplementary Figure S1A) and the cardiac marker TroponinT2 (Figure 4b) compared with controls. This cardiac inhibition is also clearly detected by immunofluorescence experiments using α-troponinT2 antibodies (Figures 4c and d).

In addition to ESC neurogenesis and cardiomyogenesis, the p38MAPK pathway controls also endothelial cell, smooth and skeletal muscle cell10 and adipocyte28 lineage formation. Similarly to p38MAPK inhibition, we found that the two p53-inhibition approaches induce a strong inhibition of skeletal myogenesis compared with controls; as judged by the decrease in myosin heavy chain-1 (MyH1) mRNA (Figure 5a) and protein (Figure 5b) expression and by immunofluorescence experiments using α-MyH1 antibodies (Figures 5c and d).

By contrast, although p38MAPK inhibition leads to a decrease in endothelial and smooth muscle cell formation and to an increase in adipogenesis, these three cell lineages are not significantly affected by anti-p53 siRNA transfections. These lineages were characterized by gene expression of the following specific markers: Tie2 and Flk1 for endothelial differentiation (Supplementary Figures S1B and C, left parts); α-smooth muscle actin for smooth muscle cells (Figure 6a, left part); and peroxisome-proliferative-activated receptor gamma and adipocyte-specific lipid-binding protein for adipocytes (Figures 6b and c, left parts).

Similarly, endothelial differentiation is not modified in p53−/− ESCs compared with their wild-type counterparts (Supplementary Figures S1B and C, right parts). Surprisingly, smooth muscle differentiation 4, 5, 7 and 12 of differentiation. A significant p53 repression persists until day 5 of the protocol, and is only lost at day 7 (Figure 3c).
cell formation and adipogenesis are strongly inhibited in p53−/− ESCs (Figures 6a–c, right parts). These data reveal different biological effects of transient p53 inhibition by siRNAs versus p53 homozygote gene knockout, suggesting variable successive roles for this gene during ESC differentiation. Altogether, our results also suggest the existence of other mediators than p53 for the p38MAPK pathway to account for all its effects on ESC differentiation.

To precise the p53 role in ESC differentiation, we analyzed the effects of p53 inhibition on expression of master genes important during embryonic development and ESC commitment. We focused our analysis on master genes whose gene expression peaked during the day 0–5 period of ESC differentiation, and therefore susceptible to be regulated by siRNA transient transfection experiments. Consistent with neurogenesis regulation, the peak expression of the Pax6 ectodermal master gene is augmented by anti-p53 siRNA transfections (Figure 7a). We also investigated the expression of three master genes relevant to mesodermal lineages: Brachyury, Mesp1 and TBX1. Gene expressions of Brachyury and Mesp1, which peak at day 5, are almost completely abolished by p53 inhibition, either after anti-p53 siRNA transfections or in p53−/− cells (Figures 7b–e). These inhibitions could account for the p53 effects on mesodermal-derived differentiations. By contrast, although TBX1 is not significantly affected by anti-p53 siRNAs (Figure 7f), expression of this gene is strongly downregulated in knockout cells (Supplementary Figure S2A), suggesting for this gene a complex regulation by p53. Noteworthy, the peak of expression of TBX1 appears at day 7, later than Brachyury and Mesp1, which could explain the lack of regulation by anti-p53 siRNAs.

p53-dependent Nanog repression contributes partially to the p53 effects on ESC differentiation. ESC differentiation is accompanied by the rapid downregulation of Nanog gene expression, a crucial gene involved in ESC self-renewal. It has been shown that this regulation is mediated by p53, which, upon differentiation, gets activated and represses Nanog expression by direct binding to its promoter.17 We tested the hypothesis that this p53-dependent Nanog repression would contribute to p53 regulation of ESC differentiation. Consequently, Nanog expression should be augmented in p53−/− ESCs, and interfering with this expression should affect their differentiation capacities. Indeed, we found that a significant percentage of the level of Nanog expression in undifferentiated cells persists until day 12 of the differentiation of p53−/− ESCs, whereas this
expression is barely detected after day 5 into their wild-type counterparts (Figure 8a). This persistence of Nanog expression seems to profoundly perturb the commitment of p53/C0/ESCs. In addition to Nanog, the expression of Oct4, another key self-renewal transcription factor, is also persisting during differentiation instead of decreasing rapidly (Supplementary Figure S2B). This result is most likely due to the known capacity of these transcription factors to activate each other.1

To test the role of Nanog during p53/C0/ESC differentiation, we performed transient transfections of anti-Nanog siRNAs at day 0 and found that Nanog mRNA expression is inhibited by 70% (Figure 8b). This Nanog inhibition is sufficient to restore significant Brachyury and Mesp1 mRNA expressions, at days 5 (Figure 8c) and 7 (Figure 8d), respectively, of the differentiation process; by contrast, it has no effect on Pax6, Bcl2 (Supplementary Figures S3A and B) or TBX1 (data not shown) gene expressions. Subsequently, we observed the restoration of p53/C0/ESC differentiation capacities toward cardiomyocyte and smooth muscle cell formation (Figures 8e and f and Supplementary Figure S3C). However, it is worth to note that this restoration is partial, reaching only 30–50% of the level of their wild-type counterparts. Importantly, in these experiments, anti-Nanog siRNAs display no significant effect on neurogenesis (Figure 8g), which correlates with the lack of effect on Bcl2 and Pax6 gene expression. Likewise, although activating Brachyury expression, anti-Nanog siRNAs do not restore skeletal myogenesis (Figure 8h), showing that Brachyury regulation is not sufficient for this lineage. Altogether, our results suggest that p53-dependent Nanog repression is involved in p53 activation of cardiomyogenesis and smooth muscle formation, whereas neurogenesis inhibition and skeletal myogenesis activation are Nanog independent.

Discussion
We previously described a role for p38MAPK as an early switch of ESC commitment into mesodermal lineages (p38MAPK on) or neurons (p38MAPK off).10,15 This control takes place during the critical first 5 days of ESC commitment, concomitantly with the expression of specific master genes that direct ESC into each of the three embryonic layers. We found now that this effect is partially mediated by the transcription factor p53, unveiling an important p38MAPK-p53 cascade regulating the early commitment of ESCs. During the ESC differentiation process, p38MAPK induces a stabilization of p53 protein and increases its transcriptional activity. This cascade, regulating the expression of specific
master genes of ESC commitment, inhibits ectodermal and, conversely, activates several mesodermal lineages. Homozygous p53-knockout mice were found to survive to birth, but then to succumb rapidly to tumors, suggesting, at first, that the p53 gene is dispensable during the embryonic development but crucial as tumor-suppressor gene during the adult life. However, a high rate of developmental abnormalities is associated with p53 deficiency, which result to defects...
in neural tube closure, overgrowth of neural tissues and exencephaly, therefore suggesting a role for p53 in development.\textsuperscript{30,31} This apparent discrepancy could be due to compensation by the p53 family members p63 and p73, which are expressed in early mouse embryos, resulting in the incomplete penetrance of the developmental phenotype observed. Furthermore, in vitro, depending on the cellular model analyzed, p53 can either facilitate or suppress cell differentiation.\textsuperscript{32} In agreement with the in vivo negative role of p53 on neurogenesis, we found that this transcription factor is a potent early inhibitor of in vitro ESC neurogenesis and that this effect can be explained, at least partially, by the transcriptional repression of two genes Bcl2 and Pax6. Indeed, Bcl2 is known to be targeted by p38MAPK and is critical for neuronal commitment of ESCs,\textsuperscript{21} and Pax6 is a transcription factor long known to be essential for cortical development. Interestingly, it has been shown recently that Pax6\textsuperscript{\textasciitilde} ESCs displayed impaired neurogenesis.\textsuperscript{33}

Therefore, we hypothesize that, in our experiments, p53 inhibition leads to Bcl2 and Pax6 overexpressions, which in turn augment ESC neurogenesis. These regulations seem independent of the p53-induced repression of Nanog gene expression observed during differentiation, suggesting that the p53 effect is mediated by another mechanism.

p53 is necessary for myotube formation in C2C12 myoblasts\textsuperscript{34} and mesenchymal stem cell\textsuperscript{35} cellular models. Here we found a p53 requirement in skeletal myogenesis early on during ESC commitment, demonstrating a p53 control all along this process. This effect is associated to the control of the mesodermal master gene Brachyury, and we found that the p53-dependent repression of Nanog participates in this regulation. However, even if anti-Nanog siRNA transfections are capable to restore Brachyury expression, they do not restore skeletal myogenesis of p53\textsuperscript{\textasciitilde} ESCs. This result could be due to a partial Nanog downregulation or, alternatively, the involvement of additional molecular mechanisms corresponding to later roles of p53.

Not much was known about p53 and in vitro cardiomyocyte formation; however, it has been shown recently that the pharmacological compound icariin augments ESC cardiomyogenesis via activation of both p53\textsuperscript{36} and p38MAPK.\textsuperscript{37} Our results show that the p38MAPK-p53 cascade is required for cardiomyocyte formation from mouse ESCs. Furthermore, inhibition of Nanog gene expression in differentiated p53\textsuperscript{\textasciitilde} ESCs restore both a cardiac phenotype and the expression of two master genes important for cardiac development, Brachyury and Mesp1.\textsuperscript{38,39}

In conclusion, our results identify a p38MAPK-p53-Nanog cascade important for the ESC early commitment toward several lineages. However, Nanog repression does not account for all the effects of p53, and, likewise, the p53 activation is not sufficient to account for all the effects of the
p38MAPK signal transduction pathway on ESC differentiation, suggesting the existence of additional molecular mechanisms for these processes.

Materials and Methods

**Determination of ESCs.** Mouse ESCs CGR8 and p53−/− and their wild-type counterpart ESCs were grown on gelatin-coated plates and were induced to differentiate as described previously. Briefly, 1 × 10^5 ESCs were aggregated without leukemia-inhibitory factor to form EBs at day 0, cultivated in suspension until day 7, treated (for neurogenesis and adipogenesis) or not (for cardiomyogenesis, endothelial differentiation, smooth and skeletal myogenesis) with 10−7 M all-trans RA (Sigma, Saint-Quentin-Fallavier, France) daily between day 3 and 5, plated at day 7 on gelatin-coated six-well plate and treated with 85 nM insulin (Sigma) and 2 nM 3,3′,5′-triiodothyronine (Sigma) to induce terminal differentiation. Fresh medium containing the inducers was applied every 2 days. Neurons, cardiomyocytes, endothelial and smooth muscle cells were analyzed at day 12, and skeletal myotubes and adipocytes were analyzed at day 26.

During EB differentiation, 10 μmol/l of the p38 inhibitor PD169316 (Sigma) or 10 μmol/l of pifithrin-α (Sigma) were added to the culture media as indicated.

**RNA analysis.** Total RNA was prepared using trizol reagent (Invitrogen, Life Technologies, Saint-Aubin, France). Real-time RT-PCR was performed with the ABI Prism7300 (Applied Biosystems, Life Technologies) and Taqman or Mesa Green MasterMix (Eurogentec, Angers, France). The relative amounts of the different mRNAs were quantified by using the comparative CT method (2−(ΔΔCT)). 36B4 was used as housekeeper transcripts, and gene expressions were performed using Lipofectamine2000 (Invitrogen) according to the manufacturer’s pool (Dharmacon) or control siRNAs (Dharmacon). Transfection of siRNAs was indicated.

**Differentiation of embryonic stem cells into adipocytes in vitro.** 10^5 ESCs were separated by SDS-polyacrylamide gel electrophoresis on a 10% gel (Invitrogen) and transferred onto polyvinylidene difluoride membranes (Millipore, Molsheim, France). Membranes were incubated with antibodies against either mouse p53 (Cell Signaling, Ozyme, Saint-Quentin en Yvelines, France) or by quantitative real-time RT-PCR.

**Cell death and Disease.** 2009; 18: 1257–1265.

**ES differentiation control by a p38MAPK-p53 cascade**

Y Hadjil et al

Acknowledgements. We thank Dr. Blattner (Karlsruhe, Germany) for the gift of p53 wild-type and knockout ESC lines.

1. Young RA. Control of the embryonic stem cell state. Cell 2011; 144: 940–954.
2. Kashyap V, Rezende N, Scotland K, Shaffer S, Pennson J, Gudas L et al. Regulation of stem cell pluripotency and differentiation involves a mutual regulatory circuit of the NANGO, OCT4, and SOX2 pluripotency transcription factors with polycomb repressive complexes and stem cell microRNAs. Stem Cell Dev 2009; 18: 1093–1108.
3. Wobus AM. Potential of embryonic stem cells. Mol Aspects Med 2001; 22: 149–164.
4. Wobus AM, Boehler KR. Embryonic stem cells: prospects for developmental biology and cell therapy. Physiol Rev 2000; 85: 635–678.
5. Wobus AM, Guan K, Yang HT, Boehler KR. Embryonic stem cells as a model to study cardiac, skeletal muscle, and vascular smooth muscle cell differentiation. Methods Mol Biol 2002; 185: 127–156.
6. Rohwedel J, Guan K, Wobus AM. Induction of cellular differentiation by retinoic acid in vitro. Cells Tissues Organs 1999; 165: 190–202.
7. Ying Q-L, Stavridis M, Griftis D, Li M, Smith A. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoloc. Nat Biotechnol 2003; 21: 183–186.
8. Dani C, Smith AG, Desolin D, Leroy P, Bacciotti L, Villageois P et al. Differentiation of embryonic stem cells into adipocytes in vitro. J Cell Sci 1997; 110 (Pt 11): 1279–1285.
9. Hescheler J, Fleischmann BK, Wartenberg M, Bloch W, Koliosov E, Ji G et al. Establishment of ionic channels and signalling cascades in the embryonic stem cell-derived primitive endoderm and cardiovascular system. Cells Tissues Organs 1999; 165: 1–13.
10. Barret E, Hadadeh O, Peiretti F, Renault VM, Hadjal Y, Bernot D et al. p38 Mitogen activated protein kinase controls two successive-steps during the early mesodermal commitment of embryonic stem cells. Stem Cells Dev 2011; 20: 1233–1246.
11. Cuenda A, Cohen P. Stress-activated protein kinase iib/2) and a rafamycin-sensitive pathway are required for JCB12 myogenesis. J Cell Biol 1999; 274: 4341–4346.
12. Cuenda A, Rousselle S. p38 MAP-Kinasies pathway regulation, function and role in human diseases. Biochim Biophys Acta 2007; 1773: 1358–1375.
13. Tamura K, Sudo T, Senttifeben U, Dadam AM, Johnson R, Karm M et al. Requirement for p38i alpha in erythropoietin expression: a role for stress kinases in erythropoiesis. Curr Biol 2000; 10: 209–213.
14. Adams RH, Porras A, Alonso G, Jones M, Vintersten K, Panelli S et al. Essential role of p38 MAP kinase in placental but not embryonic cardiac developmental. Mol Cell 2000; 6: 109–116.
15. Aouadi M, Bost F, Caron L, Laurent K, Le Marchand Brutel Y, Bineatr Y et al. p38 Mitogen-activated protein kinase activity commits embryonic stem cells to either neurogenesis or cardiomyogenesis. Stem Cells 2006; 24: 1399–1406.
16. Vouwen KD, Blinder L, guided by the blind: the growing complexity of p35. Cell 2009; 137: 413–431.
17. Lin T, Choi C, Saito S, Mazur SJ, Murphy ME, Appella E et al. p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. Nat Cell Biol 2006; 7: 165–171.
18. Zhao T, Xu Y, p35 and stem cells: new developments and new concerns. Trends Cell Biol 2010; 20: 170–175.
19. Miyashita T, Harigai M, Hanada M, Reed JC. Identification of a p35-dependent negative response element in the bcl-2 gene. J Biol Chem 1999; 274: 3131–3135.
20. Wu Y, Meheu JW, Heckman CA, Aronis MA, Bexer LM. Negative regulation of bcl-2 expression by p35 in hematopoietic cells. Oncogene 2001; 20: 240–251.
21. Trouillas M, Saucourt C, Duval D, Gauthereau X, Thibault C, Dembele D et al. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science 1999; 285: 1733–1737.
22. Bondu A, Lapouge G, Paulissen C, Semerano C, Iacovino M, Kyba M et al. MesP1 acts as a master regulator of multipotent cardiovascular progenitor specification. Cell Stem Cell 2008; 3: 69–84.
23. Kishi H, Nakagawa K, Matsumoto M, Sugra M, Anda M, Taya Y et al. Osmotic shock induces G1 arrest through p53 phosphorylation at Ser33 by activated p38MAPK without phosphorylation at Ser15 and Ser28. J Biol Chem 2001; 276: 39115–39122.
24. Huang C, Ma WY, Maxner A, Sun Y, Dong Z. p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 392. J Biol Chem 1999; 274: 12229–12235.
25. Jain AK, Alton K, Iacovino M, Mahen E, Mliczarek R, Zwaal TP et al. p35 regulates cell cycle and microRNAs to promote differentiation of human embryonic stem cells. PLoS Biol 2012; 10: e1001286.
26. Solozobova V, Rolletscheck A, Blattner C. Nuclear accumulation and activation of p53 in embryonic stem cells after DNA damage. BMC Cell Biology 2009; 10: 46.
27. Aouadi M, Laurent K, Prot M, Le Marchand-Brustel Y, Binetruy B, Bost F. Inhibition of p38MAPK increases apoptosis from embryonic to adult stages. Diabetes 55: 281–289.

Conflict of Interest

The authors declare no conflict of interest.
29. Donahower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr., Butel JS et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 1992; 356: 215–221.
30. Armstrong JF, Kaufman MH, Harrison DJ, Clarke AR. High-frequency developmental abnormalities in p53-deficient mice. Curr Biol 1995; 5: 931–938.
31. Sah VP, Attardi LD, Mulligan GJ, Williams BO, Bronson RT, Jacks T. A subset of p53-deficient embryos exhibit exencephaly. Nat Genet 1995; 10: 175–180.
32. Almog N, Rotter V. Involvement of p53 in cell differentiation and development. Biochim Biophys Acta 1997; 1333: F1–27.
33. Nikoletopoulou V, Plachta N, Allen ND, Pinto L, Gotz M, Barde YA. Neurtrophin receptor-mediated death of misspecified neurons generated from embryonic stem cells lacking Pax6. Cell Stem Cell 2007; 1: 529–540.
34. Porrello A, Cerone MA, Coen S, Guthner A, Fontemaggi G, Cimino L et al. p53 regulates myogenesis by triggering the differentiation activity of pRB. J Cell Biol 2000; 151: 1295–1304.
35. Molchadsky A, Shats I, Goldfinger N, Pevsner-Fischer M, Olson M, Rimon A et al. p53 plays a role in mesenchymal differentiation programs, in a cell fate dependent manner. PLoS One 2008; 3: e3707.
36. Zhu D, Ou L, Zhang X, Lou Y. Icarin-mediated modulation of cell cycle and p53 during cardiomyocyte differentiation in embryonic stem cells. Eur J Pharmacol 2005; 514: 99–110.
37. Ding L, Liang XG, Hu Y, Zhu DY, Lou YJ. Involvement of p38MAPK and reactive oxygen species in icarin-induced cardiomyocyte differentiation of murine embryonic stem cells in vitro. Stem Cells Dev 2008; 17: 751–760.
38. Bost F, Caron L, Marchetti I, Dani C, Le Marchand-Brustel Y, Binetruy B. Retinoid acid activation of the ERK pathway is required for embryonic stem cell commitment into the adipocyte lineage. Biochem J 2002; 361(Pt 3): 821–827.

Cell Death and Disease is an open-access journal published by Nature Publishing Group. This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/3.0/