Letter to the Editor

p53 and its family members – reporter genes may not see the difference

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Dear Editor,

Luciferase-coupled reporter genes are widely used to investigate the activity of transcription factors. However, there is little awareness that the promoter sequences contained in these vectors are not always specific. We report here that both p63 and p73 activate Stratagene's p53 *PathDetect*[®] reporter plasmid, one of the most commonly used reporter genes. Thus data obtained using this system (or any similar one) should be interpreted with caution because they may not actually reflect p53 activity, but rather provide an integrated signal derived from different members of the p53 family.

To study effects caused by p53 mutations,¹ resulting in the loss of functional p53 activity^{2,3} and suppression of signalling from p53-related proteins such as p63 or p73,^{4,5} we generated a stable subline of LN-18 glioma cells where the expression of the endogenous mutant p53 (238 Cys→Ser)⁶ was sup-

pressed by more than 90% by pSUPER siRNA technology.⁷ RNA interference targeting p53 had no effects on the levels of p63 and p73 (Figure 1a). A transiently transfected pcDNA3-HA-p53 plasmid induced greater p53-Luc reporter gene activity in the p53-depleted cells than in the control transfectants, consistent with a dominant-negative effect of the endogenous p53 mutant (Figure 1b). We also noted that the mutant p53-depleted cells provided a significantly (Student's *t*-test, $P < 0.01$) increased p53-Luc signal (data not shown, see Figure 2e) even in the absence of wild-type p53 transfection which, by definition, could not be derived from wild-type p53 in this cell line.

Since we had previously demonstrated the expression of p73 in LN-18 cells,⁸ we hypothesized that this p53-like reporter gene activity was derived from the derepression of

Figure 1 Downregulation of mutant p53 in LN-18 glioma cells generates p53-like luciferase reporter gene activity. (a) LN-18 human malignant glioma cells²³ were stably transfected with pSUPERpuro control (left) or pSUPERpuro p53 plasmid (right)⁷ and analyzed for p53, p63, p73 or β -actin levels by immunoblot using murine anti-human p53 Bp53-12 (2 μ g/ml), goat anti-mammalian β -actin I-19 (1 μ g/ml) (both from Santa Cruz, Santa Cruz, CA, USA), mouse anti-mammalian p63 4A4¹⁰ or rabbit anti-human p73 antiserum (Sanofi, Paris, France).²⁴ (b) LN-18 pSUPER puro control (left) or p53-knockdown cells (right) were seeded into 96-well plates (6×10^3 cells/well) for 24 h and then cotransfected with 0.06 μ g of the PathDetect[®] p53 *cis*-reporter gene plasmid (Stratagene, La Jolla, CA, USA), 0.03 μ g of pcDNA3-HA-p53 and 0.01 μ g of pRL-CMV (Promega, Mannheim, Germany), using FuGene 6 (Roche, Mannheim, Germany). At 48 h after transfection, the cells were washed and lysed using Reporter Lysis Buffer (Promega). The respective activities of *Photinus pyralis* and *Renilla reniformis* luciferase were determined sequentially using the firelite dual luminescence reporter gene assay (Perkin-Elmer, Rodgau-Jügesheim, Germany). Background was subtracted from all values and the counts obtained from the measurement of firefly luciferase were normalized to pRL-CMV

Figure 2 p63 and p73 are strong activators of p53-Luc. (a) LN-308 glioma cells (left) and SV-FH45 non-neoplastic astrocytes, kindly provided by D Stanimirovic (Institute of Biological Sciences, National Research Council of Canada, Ontario, Ottawa, Canada) (right) were analyzed for p63 and β -actin levels by immunoblot using mouse anti-mammalian p63 4A4¹⁰ and goat anti-mammalian β -actin 1-19 (1 μ g/ml) (Santa Cruz). The size of the isoforms detected (corresponding to TAp63 β and Δ Np63 γ) is indicated. (b) LN-308 cells were cotransfected with p53-Luc, pRL-CMV and plasmids encoding p53 or the different isoforms of p73 (pcDNA3-HA-p53, pcDNA3-HA-p73 α , pcDNA3-HA-p73 β , pcDNA3-HA-p73 γ , pcDNA3-HA-p73 δ , pcDNA3-HA- Δ Np73 β).²⁵ *Photinus pyralis* and *Renilla reniformis* luciferase activities were determined as in Figure 1 and the values for p53-Luc/pRL-CMV are expressed relative to pcDNA3-HA-p53. (c) LN-308 cells were cotransfected with p53-Luc, pRL-CMV and plasmids encoding p53 or the different isoforms of p63 (pcDNA3myc-TAp63 α , pcDNA3myc-TAp63 β , pcDNA3myc-TAp63 γ , pcDNA3myc- Δ Np63 α , pcDNA3myc- Δ Np63 β , pcDNA3myc- Δ Np63 γ).¹⁰ (d) The cells were transfected as in (a) with the p73^{V156A} mutants (pcDNA3-HA-p73 α ^{V156V}, pcDNA3-HA-p73 β ^{V156V}, pcDNA3-HA-p73 γ ^{V156V} and pcDNA3-HA-p73 δ ^{V156V}) or the human p53^{V143A} mutant. At 24 h after transfection, the cells were shifted to 38.5°C (filled bars) or 32.5°C (open bars) and analyzed 24 h later. (e) LN-18 pSUPERpuro p53-transfected cells (filled bars) or control transfectants (open bars) were transiently cotransfected with p53-Luc, pRL-CMV and plasmids encoding the different isoforms of p63 and p73. Luciferase activities were determined as in (a) and are expressed relative to the activity induced by pcDNA-HA-p53 in LN-18 pSUPER puro p53 cells

p73 after the depletion of endogenous mutant p53. To assess whether the p53-Luc reporter responds to p73, we examined LN-308 cells, which express neither p53 nor p73^{6,8} nor p63 by immunoblot (Figure 2a). Cotransfection assays revealed that the transcriptionally most active p73 β isoform was even a stronger activator of the reporter plasmid than p53 itself. p73 δ was approximately as effective as p53. p73 α and p73 γ gave weaker, but still significant signals. Only the truncated p73 Δ N β remained transcriptionally silent (Figure 2b). Similar experiments performed with p63 showed that TAp63 β and TAp63 γ were also strong inducers of reporter gene activity, while TAp63 α gave only a weak signal, probably because of the rapid degradation of this isoform caused by the MDM-2-like activity of the α C-terminus.⁹ While the Δ Np63 α and Δ Np63 β isoforms remained transcriptionally silent, the Δ Np63 γ isoform, which lacks the p53-homologous transcrip-

tional activation domain at the N-terminus, induced strong reporter gene activity. This is consistent with this isoform being proapoptotic¹⁰ and may be due to an additional C-terminal activation domain,¹¹ which is repressed in the longer C-termini of other isoforms (Figure 2c). That we used p63 plasmids encoding the murine and not the human protein does not weaken our findings since p63 is more than 99% conserved between mice and men, with consensus sites being identical.

Temperature-sensitive mutants of p53 such as murine p53^{V135A} are widely used for the study of p53 function. A screening of a comprehensive p53 missense mutation library has revealed a huge number of temperature-sensitive mutations that could become valuable tools.¹² A set of similar mutants has been described for p73.¹³ Reporter gene analysis is ideally suited to assess their transcriptional activity

under various conditions. We found all p73^{V156A} mutants, the functional analogs to murine p53^{V135A} and human p53^{V143A}, to be highly temperature dependent. While they were almost transcriptionally silent at 38.5°C, transcriptional activity increased by more than 20-fold when the temperature was shifted to 32.5°C (Figure 2d).

Finally, to confirm the inhibition of p63- and p73-induced p53-like reporter gene activity by mutant p53, we transfected the various p63 and p73 isoforms in pSUPER puro p53-transfected LN-18 cells or control transfectants. The overexpression of transcriptionally active plasmids reliably induced luciferase activity, suggesting that p63 and p73 signalling can occur in the presence of mutant p53 variants and may mainly depend on the relative abundance of p53-related transcription factors in the cell.¹⁴ Yet, when mutant p53 expression was suppressed by pSUPER puro p53, the signals obtained were up to 10-fold stronger than in the respective control cells. It should be noted that some residual mutant p53 was still present in the knockdown cell line (Figure 1a). This may explain why p63 α as well as the p73 α and γ isoforms, which are weaker activators of the p53-Luc plasmid, are less well derepressed from p53-mediated suppression than the p73 β and p73 δ isoforms (Figure 2e), which induce stronger p53-like transcriptional activity (Figure 2b). Further, we found that the p53-like reporter gene activity observed in the p53 knockdown cell line was abrogated by the Δ Np63 α , Δ Np63 β and p73 Δ N β isoforms, confirming their dominant-negative action over the endogenous isoforms of p63 or p73.

These data demonstrate that the *PathDetect*[®] p53 *cis*-reporter gene plasmid, a typical reporter for the measurement of p53 transcriptional activity, is also activated by p63 and p73. This raises concerns about the specificity of p53-related data obtained by this and similar assays and suggests that often only integrated information on p53 and its family members is obtained. While several p53-dependent promoters including p21, bax, mdm2, gadd45 and cyclin G respond to p63¹⁵ and p73¹⁶ and thus resemble the artificial p53-responsive enhancer element (TGCCTGGACTTGCCTGG)₁₅ contained in the *PathDetect*[®] p53-Luc reporter, activation by all three family members is not a general characteristic of p53-dependent promoters. This is because only six of 36 p53-induced transcripts were upregulated by tetracycline-dependent induction of p73 α in DLD-1 colon cancer cells.¹⁷ Other promoters are activated by p63 and p73 only.¹⁸ Thus, the relative activity of p53, p63 and p73 isoforms appears to be promoter dependent, allowing for a finely differentiated

cellular response. Further fine-tuning may occur in a cell-type-specific way through the presence of cofactors like brn-3a, which show synergy with p53 at the p21, but antagonism at the bax promoter.¹⁹ Other cofactors like MDM-2,²⁰ ASPP/iASPP²¹ or PARC²² show different (MDM-2) or yet unexplored interactions with the various p53 family members, adding further complexity.

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