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# S6K in geroconversion

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**Markers of cellular senescence depend in part on the MTOR (mechanistic target of rapamycin) pathway. MTOR participates in geroconversion, a conversion from reversible cell cycle arrest to irreversible senescence. Recently we demonstrated that hyper-induction of cyclin D1 during geroconversion was mostly dependent on MEK, whereas rapamycin only partially inhibited cyclin D1 accumulation. Here we show that, while not affecting cyclin D1, siRNA for p70S6K partially prevented loss of RP (replicative/regenerative potential) during p21-induced cell cycle arrest. Similarly, an inhibitor of p70 S6 kinase (PF-4708671) partially inhibited phosphorylation of S6 and preserved RP, while only marginally prevented cyclin D1 induction. Thus S6K and MEK play different roles in geroconversion.**

Cell cycle arrest is not yet senescence.[1] To induce senescence in cell culture, cells first need to be arrested by different means such as telomere shortening, DNA damage, cytotoxic stresses, as well as strong oncogenic stimulation (Ras and Raf), which induce cell cycle arrest by induction of CDK inhibitors such as p21 and p16.[2-14] Importantly, cells become arrested in growth-promoting conditions (in the presence of serum, nutrients, and oxygen, which all activate MTOR, like in proliferating cells). At first, arrested cells are not senescent. Yet, still active MTOR initiates the conversion to senescence, named gerogenic conversion or geroconversion.[1] Under the pressure of MTOR, cells acquire markers of senescence: hypertrophy (a large, flat cell morphology), cellular hyper-functions, including hyper secretion of cytokines, hyper-elevated levels of cyclins D1 and E and loss of regenerative/replicative potential (RP), i.e., the ability to resume proliferation when cell cycle is released (**Fig. 1A**). The process of geroconversion is a proper target for suppression of senescence without abrogating cell cycle arrest. Inhibition of MTOR by rapamycin, p53, hypoxia, and MEK inhibitors suppresses geroconversion, preserving RP (**Fig. 1B**) and preventing other markers of senescence (in cell type-dependent manner, in the case of hypoxia, p53, and MEK inhibitors).[15-25] Recently, we demonstrated that MEK inhibitors completely prevented induction of cyclin D1, even when MTOR remained fully activated.[26] In contrast, the effect of rapamycin on cyclin D1 was modest compared with the complete elimination of cyclin D1 by MEK inhibition. The MTOR pathway was mostly responsible for loss of RP and hypertrophy.[26] p70 S6 kinase 1 (S6K1) is a crucial substrate of MTOR given that knockdown of S6K1 extends lifespan in mice.[27] Here we compared consequences of inhibition of MEK[26] with inhibition of S6K1, using RNAi technology (**Fig. 2**).

siRNA for S6K1 decreased level of phosphorylated p70S6K1 (**Fig. 2A**). Both siRNA for MEK1 and S6K1 decreased acidification of cell culture medium as evident by reddish color compared with yellow medium in control cells (**Fig. 2B**), reflecting inhibition of lactic acid production.[28] siRNA for MEK1 and S6K1 also decreased cell size, as was measured by the amount of protein per cell (**Fig. 2B**). This effect was especially prominent with siRNA for S6K1 (**Fig. 2B**). Finally, both siRNAs for MEK and S6K1 preserved RP in HT–p21 cells treated with IPTG (**Fig. 2C**).

We next used small-molecule kinase inhibitors (**Fig. 3**). As expected, both

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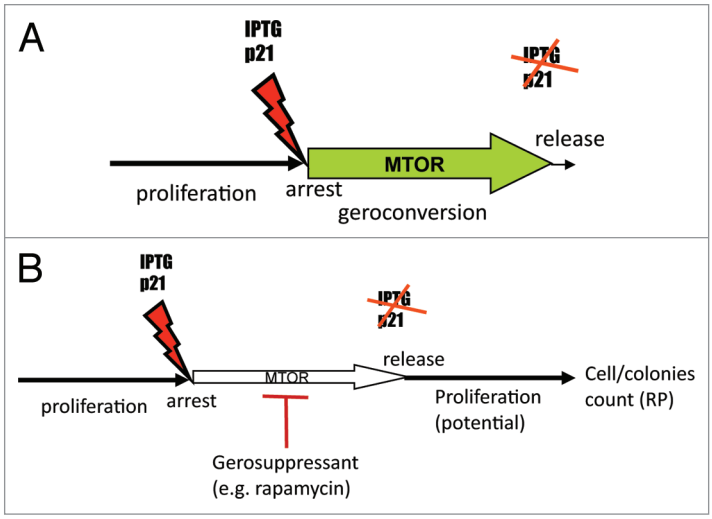


**Figure 1.** How to measure geroconversion and gerosuppression. (**A**) Geroconversion (conversion from arrest to senescence). In proliferating cells, the MTOR pathway is active (especially in malignant cells used as a model). When the cell cycle is arrested, MTOR drives geroconversion (during 3–5 days in cell culture conditions). Senescent cells cannot proliferate after abrogation of cell cycle arrest (release). As a particular example, cells expressing ectopic IPTG-inducible p21 can be arrested by addition of IPTG.57 When IPTG is removed, then the cells are released. (**B**) Gerosuppression. Inhibition of the MTOR pathway suppresses geroconversion. Cells resume proliferation, when cell cycle is released. A number of colonies or cells may serve as a quantification of gerosuppression (determined by dividing a number of colonies [or cells] in (**B**) by respective numbers in panel (**A**). B/A = regenerative or replicative potential (RP).

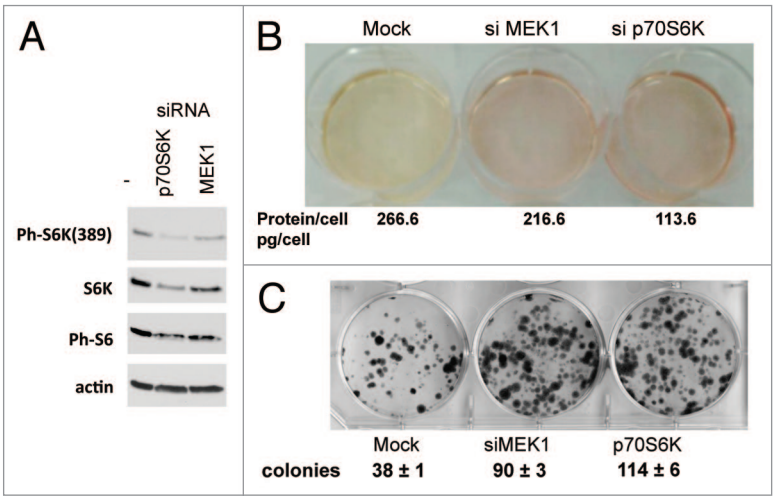


**Figure 2.** Effects of siRNA for MEK and S6K1 on senescence. (**A**) HT–p21cells transfected with siRNA for MEK1 or p70S6K1 or with lipofectamine alone were lysed 4 days after transfection and immunoblotted with the indicated antibodies. (**B**) HT–p21 cells were transfected with siRNA for MEK1 or S6K1 or with lipofectamine alone (Mock). Next day cells were trypsinized and plated at low density. After 6 days in culture, wells were photographed for the color of the media, trypsinized, and counted, then lysed. Protein amount per cell was determined (shown as pg/cell). (**C**) Regenerative/replicative potential (RP). HT–p21 cells, transfected with siRNA for MEK1 or S6K1 or with lipofectamine alone (Mock), were split 4 days after transfection and treated with IPTG for 3 days. (Note: IPTG causes cell cycle arrest in HT–p21 cells by inducing ectopic p2157). Then IPTG was washed out, and colonies were grown for 10 days and stained with Crystal Violet. A number of colonies is presented as mean ± SD.

rapamycin and everolimus prevented loss of RP in HT–p21 cells (**Fig. 3A**). This potent gerossupressive effect can explain life-extending and anti-aging effects of rapamycin in diverse species,[29] including yeast,[30] worm,[31] flies,[32-34] and mice.[35-45] Treatment with inhibitors of S6K (PF-478671)[46] and MEK (PD184352), especially at concentration 10 μM, preserved RP of IPTG-treated HT–p21 cells (**Fig. 3A**). These results were consistent with the effects of siRNAs for S6K1 and MEK (**Fig. 2C**). In addition, we tested inhibitors of several related pathways: p90/RSK (SL 0101-1 and BRD7389), phospholipase D2 or PLD2 (halopemide), and JNK (SP600125). PLD2 is known to activate the MTOR/S6K pathway.[47-50] P90/RSK was chosen as a target, because it phosphorylates S6 independently from the MTOR pathway. Lastly, JNK is involved in aging and age-related pathology in *Drosophila*.[51-54] However, effects of these inhibitors on gerosuppression were insignificant (data not shown).

In agreement with our recent work,[28] MEK inhibitors (PD184352 and U0126) eliminated cyclin D1, whereas effect of rapamycin on cyclin D1 accumulation was incomplete, and cyclin D1 was still visible on the longer exposured blot (**Fig. 3B**). Inhibitor of S6K (PF478671) only slightly decreased levels of cyclin D1, indicating that effects of rapamycin on cyclin D1 accumulation may involve different pathways. In fact, it was shown that MTOR increases cyclin D1 through inactivation of 4EBP1.[55,56] We also investigated 2 inhibitors of p90 RSK (SL 0101-1 and BRD7389). These inhibitors did not affect MTOR pathway and just slightly decreased cyclin D1 (**Fig. 3B**). Compared with SL 0101–1, BRD7389 exerted a stronger effect on cyclin D1, which could be due to its toxicity at concentration 10 μM. Halopemide affected neither phosphorylation of S6 nor cyclin D1 levels. Thus, we identified p70 S6K as a target for gerosuppression, yet an inhibitor of p70 S6K did not decrease cyclin D1 levels. In contrast, MEK inhibitor was extremely effective in prevention of cyclin D1 accumulation, confirming our conclusion that markers of senescence can be dissociated. At standard concentration of 10 μM, inhibitors of p90 ribosomal S6 kinase or RSK (SL 0101-1 and BRD7389), Jun N-terminal kinase (SP600125), and phospholipase D2 (halopemide) failed to suppress geroconversion in this preliminary assessment. It is possible that, while ineffective as

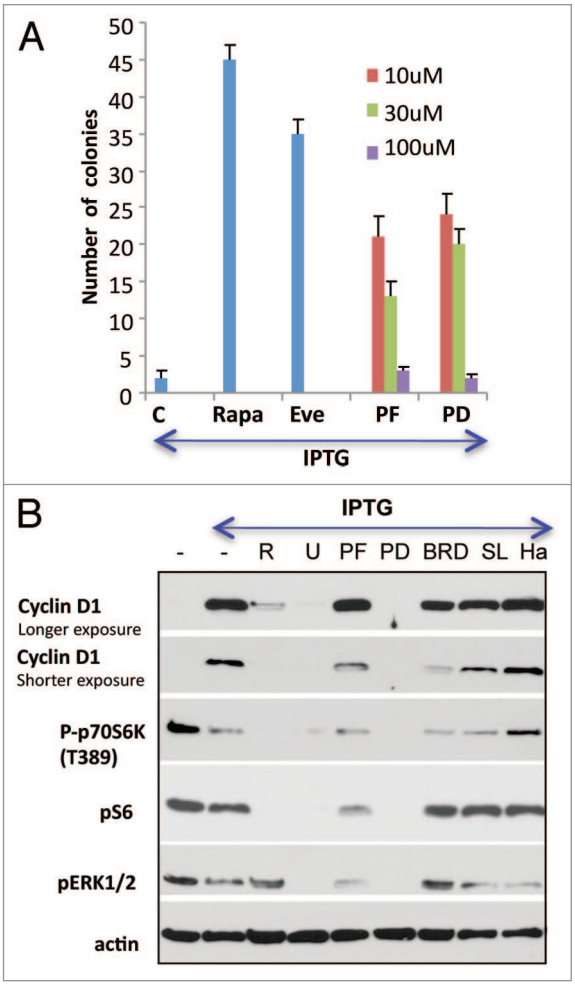


**Figure 3.** Effects of kinase inhibitors on senescence. (**A**) RP. HT–p21 cells were treated with IPTG and with indicated drugs. Rapa, rapamycin 500 nM; Eve, Everolimus 500 nM; PF, PF4708671; PD, PD184352: 10; 30; 100 μM. After 3 days, drugs were washed out and cells were allowed to grow. A number of colonies represents RP. (**B**) Immunoblot. HT–p21 cells were treated as indicated for 24 h and then lysed. Immunoblotting was performed with the indicated antibodies. R – rapamycin 500 nM; All other drugs were used at 10 μM. U, U0126; PF, PF4708671; PD, PD184352; BRD, BRD7389; SL, SL 0101-1; Ha, Halopemide.

single drugs in this cell line, SL 0101-1, and SP600125 might potentiate effects of other gerosupressants and be effective in drug combinations as a cocktail of inhibitors at low doses. Finally, the effects of gerosuppressants may be cell type-specific and detailed study is under way.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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