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SHORT TAKE

Rapamycin and other longevity-promoting compounds enhance the generation of mouse induced pluripotent stem cells

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Summary

Reprogramming of somatic cells to a pluripotent state was first accomplished using retroviral vectors for transient expression of pluripotency-associated transcription factors. This seminal work was followed by numerous studies reporting alternative (noninsertional) reprogramming methods and various conditions to improve the efficiency of reprogramming. These studies have contributed little to an understanding of global mechanisms underlying reprogramming efficiency. Here we report that inhibition of the mammalian target of rapamycin (mTOR) pathway by rapamycin or PP242 enhances the efficiency of reprogramming to induced pluripotent stem cells (iPSCs). Inhibition of the insulin/IGF-1 signaling pathway, which like mTOR is involved in control of longevity, also enhances reprogramming efficiency. In addition, the small molecules used to inhibit these pathways also significantly improved longevity in Drosophila melanogaster. We further tested the potential effects of six other longevity-promoting compounds on iPSC induction, including two sirtuin activators (resveratrol and fisetin), an autophagy inducer (spermidine), a PI3K (phosphoinositide 3-kinase) inhibitor (LY294002), an antioxidant (curcumin), and an activating adenosine monophosphate-activated protein kinase activator (metformin). With the exception of metformin, all of these chemicals promoted somatic cell reprogramming, though to different extents. Our results show that the controllers of somatic cell reprogramming and organismal lifespan share some common regulatory pathways, which suggests a new approach for studying aging and longevity based on the regulation of cellular reprogramming.

Key words: longevity; drosophila; rapamycin; induced pluripotent stem cells.

Following the successful generation of induced pluripotent stem cells (iPSCs) using transiently expressed reprogramming factors in different combinations (Takahashi & Yamanaka, 2006; Meissner *et al.*, 2007; Wernig *et al.*, 2007; Yu *et al.*, 2007; Nakagawa *et al.*, 2008), a large number of groups reported studies on reprogramming efficiency as well as some molecular mechanisms underlying the reprogramming process (Feng *et al.*, 2009). So far, several chemicals have been shown to enhance the generation of iPSCs. The majority of reported chemicals modulate genome-wide chromatin structure and gene activities (Huangfu *et al.*, 2008a; Mikkelsen *et al.*, 2008; Shi *et al.*, 2008a; Liang *et al.*, 2010). An antioxidant, vitamin C (Vc), and inhibitors of the TGF-β, MEK, and GSK3 pathways also exhibit positive effects on iPSC generation (Shi *et al.*, 2008b; Silva *et al.*, 2008; Ichida *et al.*, 2009; Maherali & Hochedlinger, 2009; Esteban *et al.*, 2010; Li *et al.*, 2010), which suggests that manipulation of the signaling pathways that control cell growth and proliferation may contribute to efficient cell reprogramming.

Rapamycin is a clinically used immunosuppressant that inhibits the mammalian target of rapamycin (mTOR) pathway, involved in cell proliferation, motility, and survival. Rapamycin restores self-renewal in hematopoietic stem cells of aged mice (Chen *et al.*, 2009) and prevents epidermal stem cell exhaustion induced by Wnt-1 expression in mouse skin (Castilho *et al.*, 2009). In the current study, we tested the effects of rapamycin on somatic cell reprogramming. Following the retroviral transduction of four vectors that express Sox2, Klf4, Oct4, and c-Myc (SKOM), primary mouse embryonic fibroblasts (MEFs) from $OG2^{+/-}/ROSA26^{+/-}$ (OG2) mice (containing a transgenic Oct4-GFP reporter) were treated with rapamycin from days 1 to 3 after infection. On day 16 postinfection, we observed the appearance of morphological ES-like and GFP-positive colonies (Fig. 1A, left panel). Rapamycin treatment increased the number of GFP-positive colonies in a dose-dependent manner up to 0.3 nM (Fig. 1A, right panel). FACS analysis of SKOM-infected OG2 MEFs on day 12 postinfection displayed an over 5-fold increase in GFP-positive cells in samples treated with 0.3 nM rapamycin (Fig. 1B). Furthermore, we found that treatment of MEFs with rapamycin on days 1–3 postinfection was more effective in promoting reprogramming than treatment at later times after infection (Fig. 1C). These results suggest that rapamycin acts early in the reprogramming process.

To confirm the pluripotency of iPSCs generated after rapamycin treatment, we randomly picked colonies to establish multiple iPSC lines. All of these lines were morphologically indistinguishable from mouse ES cells, and they stained positive for alkaline phosphatase (Fig. 1D). The endogenous expression of *Oct4*, *Sox2* and *Nanog* as well as the silencing of exogenous retroviral factors expression was verified by real-time RT–PCR (Fig. 1E,F). A randomly chosen clone (iPS-Rapa#4) was then subjected to further analysis. These cells expressed high levels of the pluripotency

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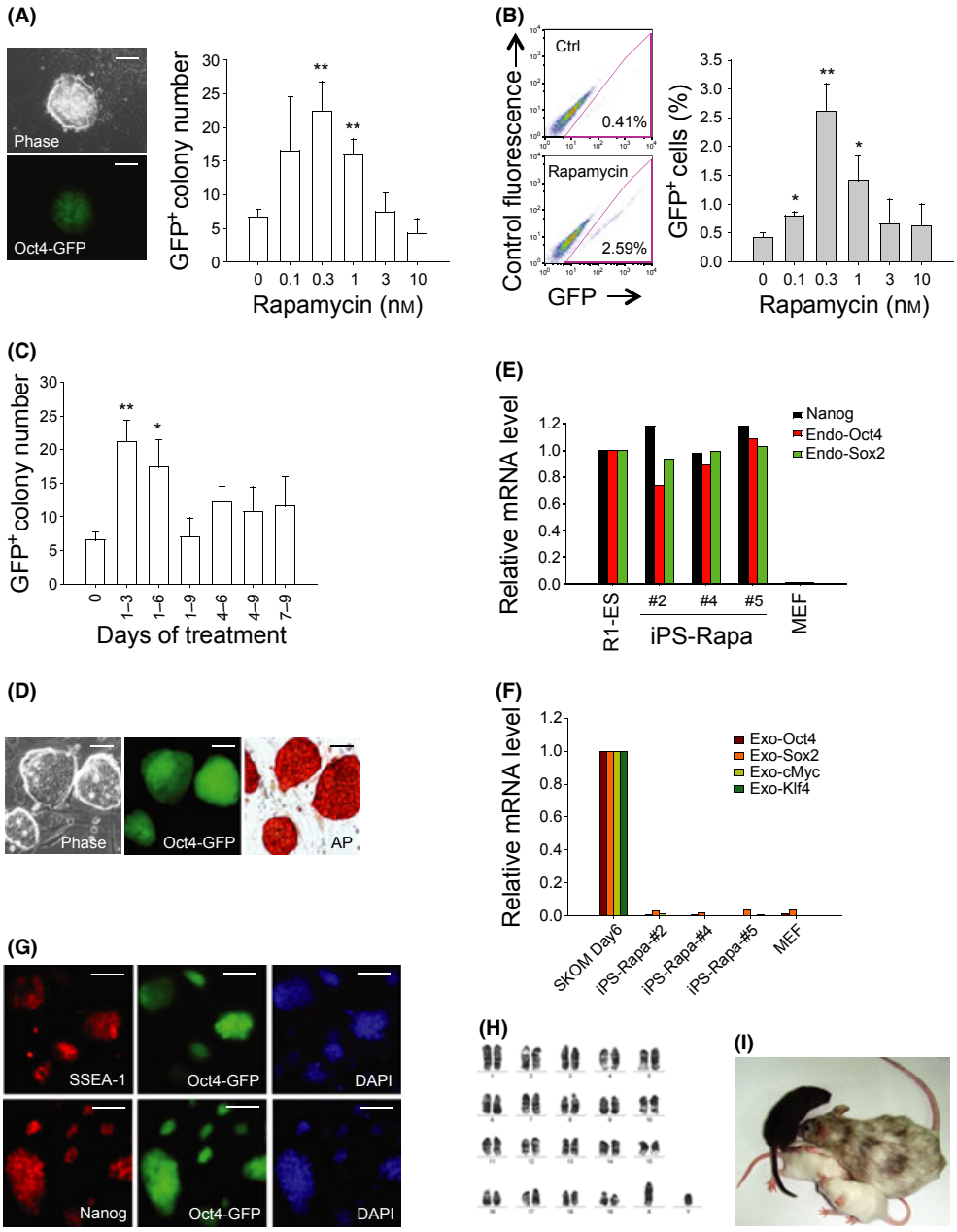
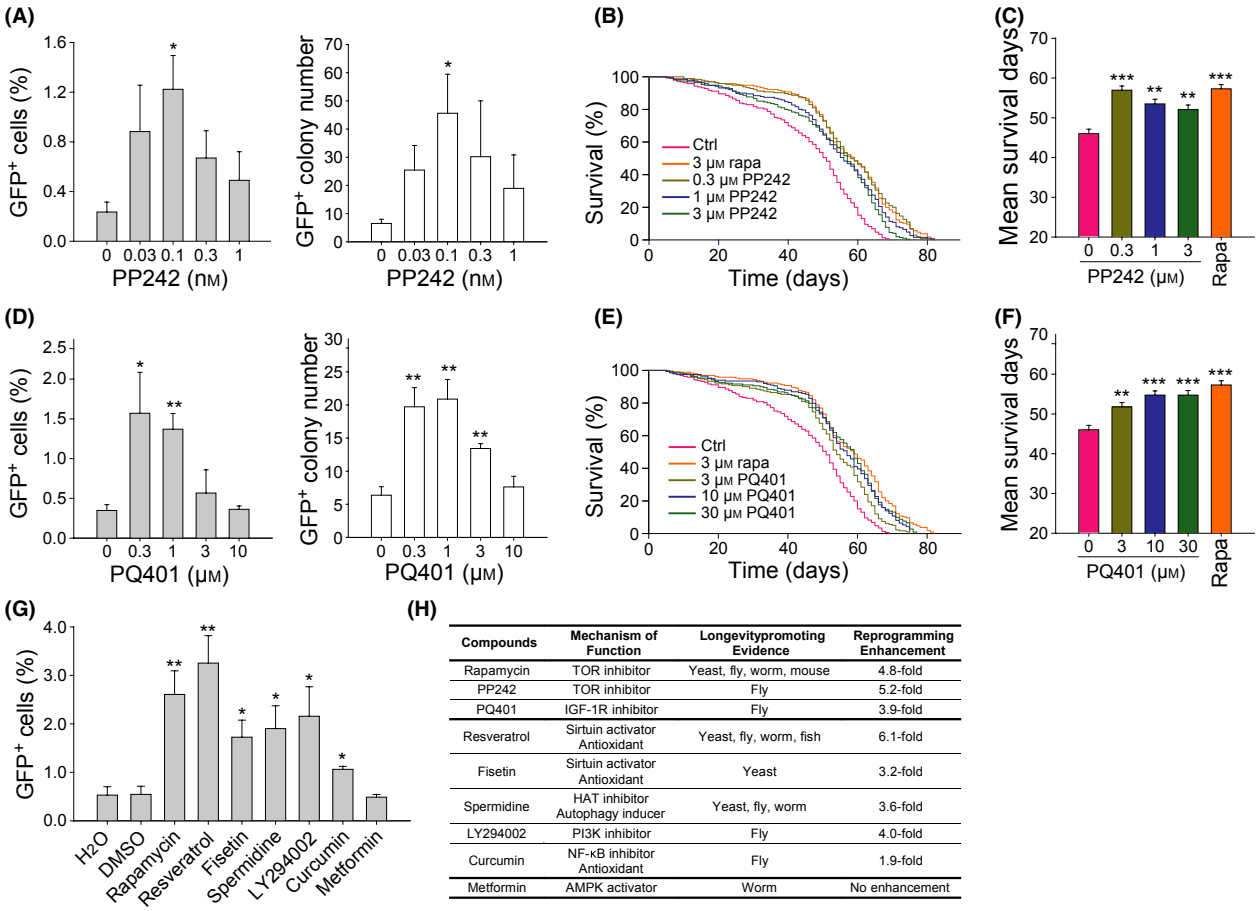


Fig. 1 Rapamycin promotes the generation of induced pluripotent stem cells (iPSCs). (A) Left: Representative pictures of GFP$^+$ colonies, which emerged on day 16 in SKOM-infected mouse embryonic fibroblasts (MEFs). Scale bars represent 100 μm. Right: The number of GFP$^+$ colonies, which were generated from rapamycin-treated SKOM-infected MEFs. Rapamycin at the indicated concentrations was added to the culture medium of SKOM (Sox2, Klf4, Oct4, and c-Myc)-infected MEFs for 3 days. GFP$^+$ colonies were counted on day 20 postinfection. Bars represent the number of GFP$^+$ colonies/40 000 cells initially plated. (B) The percentages of GFP$^+$ cells induced in SKOM-infected MEFs treated with rapamycin. The percentage of GFP$^+$ cells was determined by FACS analysis on day 12 postinfection. Left: Representative FACS data demonstrate an increase in the percentage of GFP$^+$ cells after treatment of 0.3 nM rapamycin. Right: The percentages of GFP$^+$ cells, which were induced in SKOM-infected MEFs that were treated with different concentrations of rapamycin, were determined. Data are represented as the mean ± SEM. At least 4 independent experiments were performed. $^{**}P < 0.01$, $^{*}P < 0.05$. (C) The GFP$^+$ colonies were generated from SKOM-infected MEFs treated with rapamycin (0.3 nM) at different times after infection. The number of GFP$^+$ colonies was determined as described in (A). (D) Representative pictures of the iPSCs generated in the presence of rapamycin. Left: phase contrast; middle: Oct4-GFP; right: alkaline phosphatase (AP) staining. Scale bars = 50 μm. (E) Relative gene expression (compared with ES cells) of endogenous pluripotency markers in iPSCs generated after rapamycin treatment. Endo = endogenous. (F) The silencing of ectopically expressed reprogramming factors in established iPSC lines generated in the presence of rapamycin. Exo = exogenous. (G) The immunostaining patterns for Nanog and SSEA-1 in the iPS-Rapa-#4 cells. Scale bars represent 50 μm. (H) Karyotype spread of iPS-Rapa-#4 cells. (I) The chimeric mouse and its offspring produced by the iPS-Rapa-#4 cells.

markers SSEA1 and Nanog (Fig. 1G), and were karyotypically normal (Fig. 1H). Most importantly, these cells were competent to generate germline chimeras (Fig. 1I). These results suggest that rapamycin treatment does not compromise iPSC pluripotency.

To test whether the reprogramming-enhancing effect of rapamycin was because of its inhibition of the mTOR pathway, we used PP242, another potent mTOR pathway inhibitor (Apsel *et al.*, 2008). PP242 functions as a selective inhibitor that targets the ATP-binding domain of



(H)

Compounds	Mechanism of Function	Longevitypromoting Evidence	Reprogramming Enhancement
Rapamycin	TOR inhibitor	Yeast, fly, worm, mouse	4.8-fold
PP242	TOR inhibitor	Fly	5.2-fold
PQ401	IGF-1R inhibitor	Fly	3.9-fold
Resveratrol	Sirtuin activator Antioxidant	Yeast, fly, worm, fish	6.1-fold
Fisetin	Sirtuin activator Antioxidant	Yeast	3.2-fold
Spermidine	HAT inhibitor Autophagy inducer	Yeast, fly, worm	3.6-fold
LY294002	PI3K inhibitor	Fly	4.0-fold
Curcumin	NF-κB inhibitor Antioxidant	Fly	1.9-fold
Metformin	AMPK activator	Worm	No enhancement

Fig. 2 Longevity-promoting compounds enhance the reprogramming of somatic cells. (A and D) Left panel: Percentages of GFP$^+$ cells induced in SKOM-infected mouse embryonic fibroblasts (MEFs) treated with the indicated compounds. Right panel: Number of GFP$^+$ colonies generated from SKOM-infected MEFs treated with the indicated compounds. Data are presented as mean ± SEM. At least four independent experiments were performed. $^{**}P < 0.01$, $^{*}P < 0.05$. (B, C, E and F) Survival curves of male w1118 flies fed with PP242 (B and C) or PQ401 (E and F). Data are presented as mean ± SEM. $^{***}P < 0.001$, $^{**}P < 0.01$. Rapa = Rapamycin. (G) Percentages of GFP$^+$ cells induced in SKOM-infected MEFs treated with the indicated compounds. Data are represented as mean ± SEM. At least four independent experiments were performed. $^{**}P < 0.01$, $^{*}P < 0.05$. (H) A summary of the compounds used in this study.

mTOR. Treatment with 0.1 nM PP242 led to a 5-fold increase in the reprogramming efficiency similar to rapamycin (Fig. 2A). These results indicate that inhibition of mTOR activity promotes somatic cell reprogramming.

Rapamycin extends the lifespan in various model organisms (Fontana *et al.*, 2010). Therefore, we tested whether inhibition of the mTOR pathway by PP242 had similar lifespan-extending effects to rapamycin in fruit flies (Fig. 2B,C). Treatment of PP242 significantly extended the lifespan of fruit flies similar to rapamycin (Fig. 2B,C). These results suggest that mTOR might be a common pathway that mediates both longevity and somatic cell reprogramming efficiency.

Similar to the mTOR pathway, the IIS pathway is ubiquitously expressed, and its inhibition is associated with increased longevity and the delayed onset of age-related disorders in diverse species (Fontana *et al.*, 2010). Therefore, we tested whether inhibition of the IIS pathway by PQ401, an inhibitor of IGF1 receptor, could also extend the lifespan and promote somatic cell reprogramming. Indeed, PQ401 treatment led to an almost 4-fold increase in the reprogramming efficiency of the SKOM-infected MEFs (Fig. 2D) and extended the lifespan of flies (Fig. 2E,F). These results further support a correlation between mechanisms regulating longevity and mechanisms regulating reprogramming efficiency.

We then tested the effects of six other longevity-promoting compounds on somatic cell reprogramming, including two sirtuin activators (resveratrol and fisetin), an autophagy inducer (spermidine), a PI3K inhibitor (LY294002), an antioxidant (curcumin), and an adenosine monophosphate-activated protein kinase (AMPK) inhibitor (metformin). We treated SKOM-infected OG2 MEFs with these compounds from day 1 to day 3 postinfection and subjected them to FACS analysis on day 12. Resveratrol, fisetin, spermidine, LY294002, and curcumin enhanced reprogramming to different extents (Fig. 2G and Fig. S1), but the AMPK activator, metformin, did not improve reprogramming efficiency.

Taken together, our results show a functional correlation between the regulation of cell reprogramming and that of organismal longevity. These compounds modulate organismal longevity by targeting different signaling pathways (Fig. 2H). Therefore, further investigation to unravel the molecular mechanisms of their reprogramming-promoting effects would be of importance to understand the functional correlation between the regulation of cell reprogramming and that of organismal longevity. Furthermore, many chemicals that enhance mouse somatic cell reprogramming also have similar positive effects on human somatic cell reprogramming (Huangfu *et al.*, 2008b; Feng *et al.*, 2009; Esteban *et al.*, 2010; Mali *et al.*, 2010; Zhu *et al.*, 2010). Therefore, our findings in

mouse cells may also be applicable to human cells, which is under current investigation. It is reported that the reprogramming process can be divided into three phases, namely initiation, maturation, and stabilization (Samavarchi-Tehrani *et al.*, 2010). Studies have shown that TGF-beta inhibitors function at the initiation stage of reprogramming (Li *et al.*, 2010; Samavarchi-Tehrani *et al.*, 2010), whereas MEK inhibitors function at the maturation and stabilization phase (Shi *et al.*, 2008b). Our results show that rapamycin and other longevity-related chemicals function at the initial stage of reprogramming. These chemicals may facilitate the bypass of certain reprogramming barriers, e.g., by preventing cell senescence that is induced by the reprogramming factors (Banito *et al.*, 2009; Li *et al.*, 2009) or by facilitating the mesenchymal-to-epithelial transition (Li *et al.*, 2010; Samavarchi-Tehrani *et al.*, 2010), or by other mechanisms.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Percentages of GFP^+ cells induced in SKOM-infected MEFs treated with indicated compounds.

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