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Hypoxia and gerosuppression

The mTOR saga continues

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Growth-promoting and nutrient/mitogen-sensing pathways such as mTOR convert p21- and p16-induced arrest into senescence (geroconversion). We have recently demonstrated that hypoxia, especially near-anoxia, suppresses geroconversion. This gerosuppressive effect of hypoxia correlated with inhibition of the mTOR/S6K pathway but not with modulation of the LKB1/AMPK/eEF2 pathway. Here we further show that mTOR inhibition is required for gerosuppression by hypoxia, at least in some cellular models, because depletion of TSC2 abolished mTOR inhibition and gerosupression by hypoxia. Also, in two cancer cell lines resistant to inhibition of mTOR by both p53 and hypoxia, hypoxia did not suppress geroconversion. Therefore, the effects of hypoxia on the oxygen-sensing mTOR pathway and geroconversion are cell type-specific. We also briefly discuss replicative senescence, organismal aging and free radical theory.

Hypoxia can cause cell cycle arrest. However, reversible cell cycle arrest is not yet irreversible senescence.[1] Indeed, hypoxia did not cause senescence in several cell lines tested by us, and we did not find well-documented reports of hypoxia-induced senescence. This may seem puzzling given that hypoxia activates hypoxia-inducible factor (HIF) and HIF-dependent secretion of VEGF, PAI, IGF-I and other cytokines. And hyper-secretory phenotype or senescence-associated secretory phenotype (SASP) is one of the hallmarks of cellular senescence.[2-5]

Possibly, while inducing some manifestations of senescence such as secretory phenotype, hypoxia suppresses the underlying senescence-driving (gerogenic) process. Thus, a senescent program (conversion from cell cycle arrest to senescence or geroconversion) depends in part on the nutrient-sensing and growth-promoting mTOR (target of rapamycin) pathway. Activation of the mTOR pathway is involved in secretion of numerous cytokines as a part of hypersecretory phenotype of senescent cells.[6-8] Importantly, the mTOR pathway is responsible for a large-cell morphology and irreversible loss of regenerative (replicative) potential. Rapamycin suppresses geroconversion during cell cycle arrest.[9-17] Hypoxia inhibits mTOR.[18-27] This may not only explain why hypoxia does not cause senescence, but also why it suppresses geroconversion caused by senescence-inducing agents. For example, induction of ectopic p21 by IPTG causes cell cycle arrest without inhibiting mTOR, thus leading to senescence in HT-p21 cells.[28] These cells acquired a large-flat morphology and lost regenerative (replicative) potential, becoming unable to resume proliferation after p21 is switched off. If p21 was induced under hypoxia, cells were arrested but did not become large and retained regenerative (replicative) potential, forming colonies upon IPTG removal.[28] Using several inducers of senescence, we demonstrated this phenomenon in a variety of cell lines. In all cases, suppression of geroconversion coincided with the inhibition of mTOR by hypoxia. It was independent from p53, HIF-1 and AMPK. Although hypoxia exerted multiple other effects, it seems that inhibition of mTOR was sufficient to

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suppress senescence, because rapamycin was even more effective than hypoxia as a gerosuppressor (in the same cell lines) and did not have additive effects with hypoxia.[28] Here we further showed that, at least in HT-p21 cells, the inhibition of mTOR was required for gerosuppression. We infected these cells with lentivirus expressing shRNA for TSC2 (shTSC2), which decreased levels of TSC2, a negative regulator of mTOR (**Fig. 1A**). TSC2 knockout prevented inhibition of mTOR by hypoxia, as evidenced by persistent phosphorylation of S6K and S6 (downstream targets of mTOR complex 1) and Akt (a downstream target of mTORC2) under hypoxia (**Fig. 1A**). Notably, both inhibition of pS6 phosphorylation[28] and Akt phosphorylation (**Fig. 2A**) were HIF-1 independent. In contrast, rapamycin increased Akt phosphorylation in the same cell line[10] (**Fig. 2B and C**). Hypoxia partially prevented loss of replicative/regenerative potential (RP), meaning that some cells could resume proliferation after IPTG was washed out (**Fig. 1B**). In contrast, hypoxia failed to prevent loss of RP in HT-p21 cells with depleted TSC2, indicating that inhibition of mTOR is required for gerosuppression by hypoxia at least in these cells.

Furthermore, we have previously identified cell lines in which hypoxia did not inhibit mTOR and geroconversion.[28] This is reminiscent of the effect of nongenotoxic induction of p53 by nutlin-3a. Nutlin-3a inhibited mTOR and suppressed geroconversion during p21-induced arrest in HT-p21 cells and in normal cells.[29,30] Yet, it did not inhibit mTOR in some cancer cell lines and MEFs.[31,30] Next, we chose cell lines (A549 and MCF-7) in which low concentrations of nutlin-3 did not inhibit mTOR (**Fig. 3**). These cells become senescent following treatment with nutlin-3a (**Figs. 4 and 5**). Unlike rapamycin, hypoxia did not inhibit mTOR in A549 and MCF-7 cells (**Fig. 3**). In agreement, hypoxia did not suppress morphological senescence caused by nutlin-3a, whereas rapamycin did (**Figs. 4 and 5**). Thus, inhibition of mTOR by hypoxia seems to be a prerequisite of gerosuppression by hypoxia.

Our studies can explain abrogation of replicative senescence by hypoxia in mouse

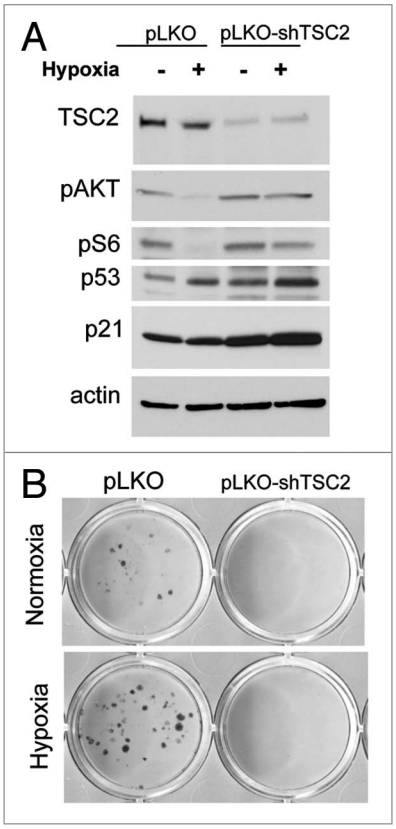

Figure 1. TSC2 is required for gerosuppression in HT-p21–9 cells. HT-p21–9 cells, infected with lentivirus vector pLKO or pLKO-shTSC2 (see ref. 31), were treated with IPTG in normoxia or 0.2% O_2 hypoxia. (**A**) After 24 h, cells were lysed, and immunoblotting was performed with the indicated antibodies as described previously.[28] (**B**) After 4 d, cells were washed and allowed to regrow. Colonies were stained with Crystal Violet as described previously.[28]

embryonic fibroblasts (MEFs) observed by Campisi and coworkers.[32] In fact, hypoxia inhibits mTOR in MEF cells.[20] Rapamycin can also suppress senescence in MEFs; however, its effect is limited by its cytostatic effect.[33] We can speculate that mild hypoxia slightly inhibited mTOR without inhibiting cell proliferation, thus creating a condition for avoidance of mTOR-dependent senescence. Since hypoxia is a normal physiological condition inside an organism, this may explain why geroconversion of normal cells may take decades in humans.

Our study has one startling implication. Thousands of experiments with oxygen and hypoxia were interpreted as the evidence for the free radical theory of aging. Yet, these data can have alternative

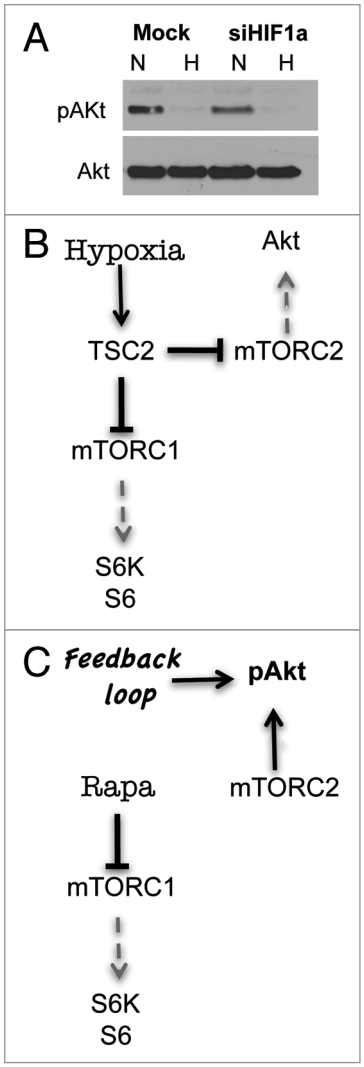

Figure 2. Hypoxia inhibits AKT phosphorylation in HIF-1-independent manner in HT-p21–9 cells. (**A**) HT-p21–9 cells, mock-transfected or transfected with siRNA for HIF-1α, were incubated under normoxia or hypoxia (0.2% O_2) as described in (see ref. 28). After 3 d, cells were lysed and immunoblotting was performed as described[28] using anti-pAkt (Ser 473) antibodies. The other proteins are shown in the PNAS paper (Fig. 2B in ref. 28). (**B**) Hypothetical schema of the effects of hypoxia on pS6 and pAkt in HT-p21–9 cells. In TSC2-dependent and HIF-1-independent manner, hypoxia inhibits mTORC1 and mTORC2 and thus inhibits phosphorylation of S6 and Akt. (**C**) Hypothetical schema of the effects of rapamycin on pS6 and pAkt in HT-p21–9 cells. Rapamycin inhibits mTORC1 and stimulates Akt phosphorylation via a feedback loop.

explanations. Instead of accumulation of random damage caused by free radicals, oxygen can activate oxygen-sensing pathways such as TOR (**Fig. 6**). Interestingly,

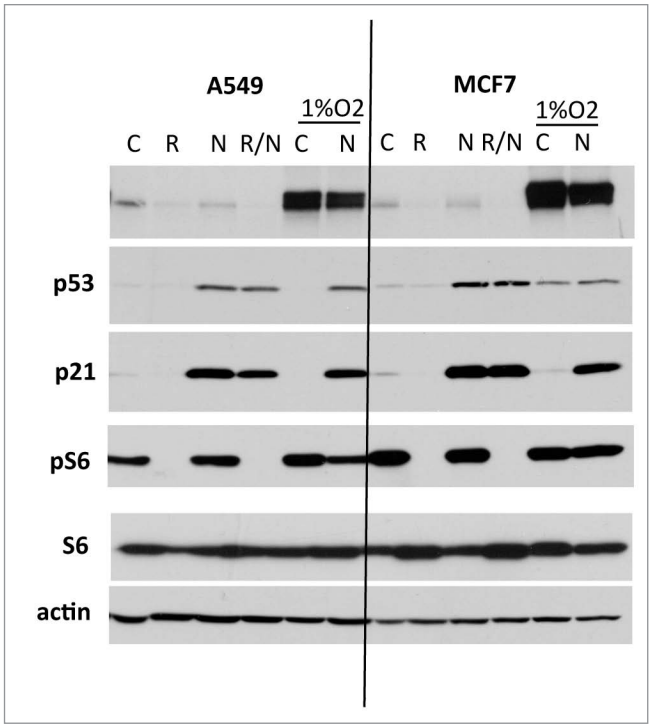

Figure 3. The effect of hypoxia and nutlin-3a on MCF-7 and A549. A549 and MCF7 cancer cells were treated with 5 uM of nutlin-3a (N) with or without 10 nM (A549) or 100 nM (MCF7) rapamycin (R) under normoxia or 1% O_2 hypoxia. C, control. After 24 h, cells were lysed, and immunoblotting was performed with the indicated antibodies (see ref. 28).

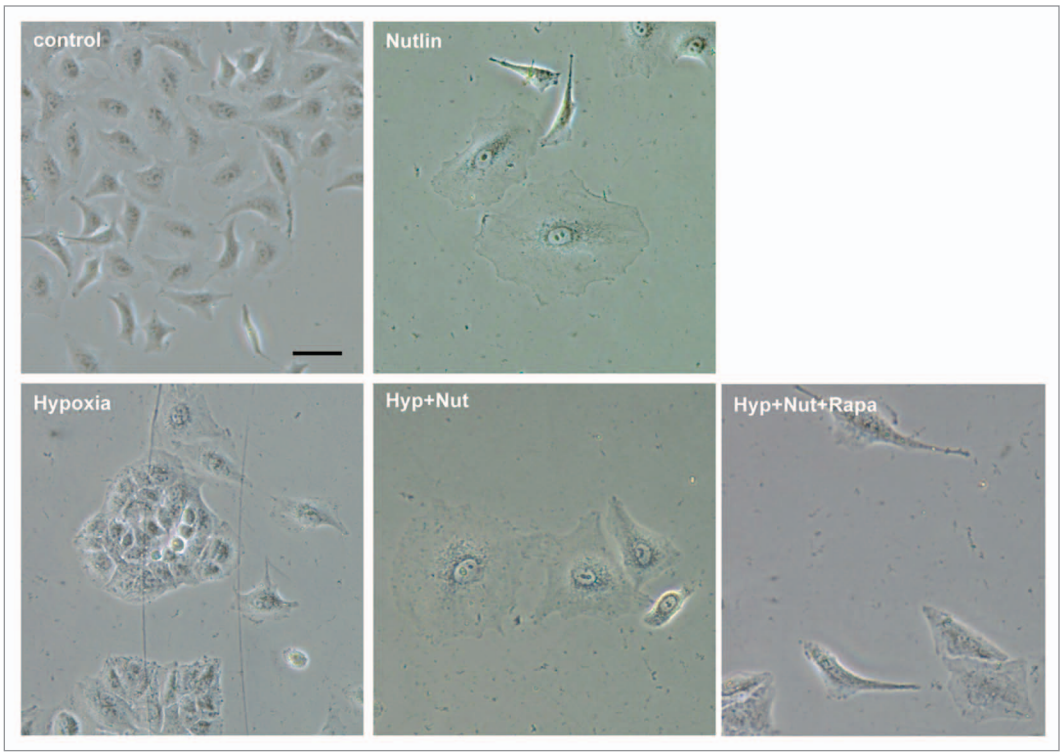

Figure 4. The effect of hypoxia and rapamycin on nutlin-3a-induced senescence in A549 cells. A549 cells were treated with 5 uM nutlin-3a (Nut) and 10 nM rapamycin (Rapa) under normoxia or 1% O_2 hypoxia (Hyp) for 4 d and stained for β-Gal. Bar = 100 um.

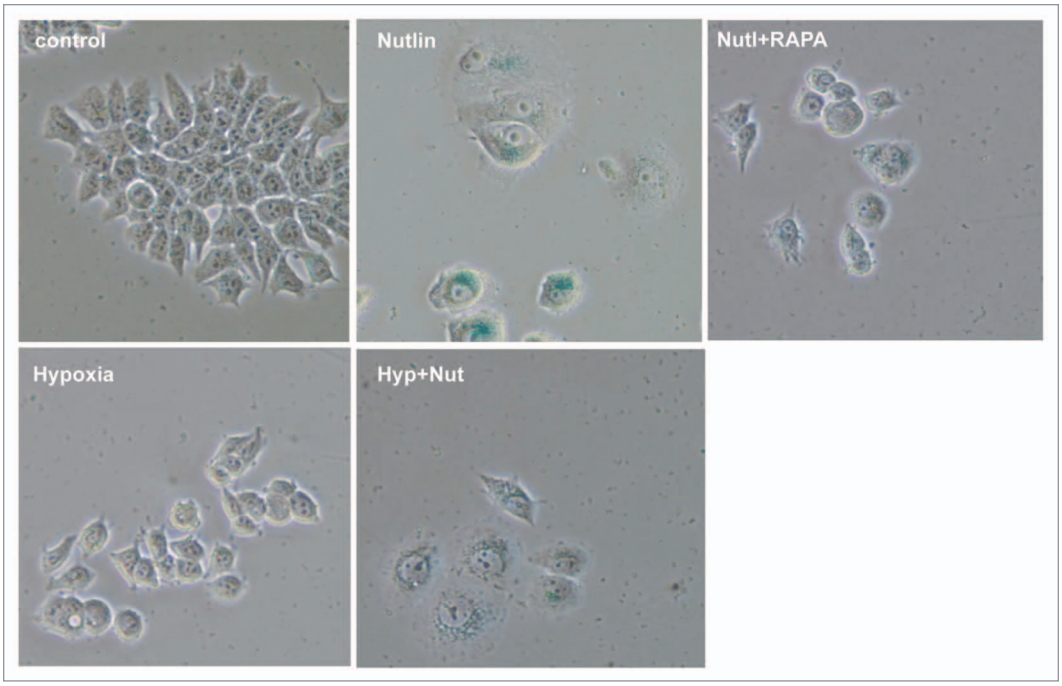

Figure 5. The effect of hypoxia and rapamycin on nutlin-3a-induced senescence in MCF-7 cells. MCF-7 cells were treated with 5 uM nutlin-3a (Nut) and 100 nM rapamycin (Rapa) under normoxia or 1% O_2 hypoxia (Hyp) for 4 d and stained for β-Gal. Bar = 100 um.

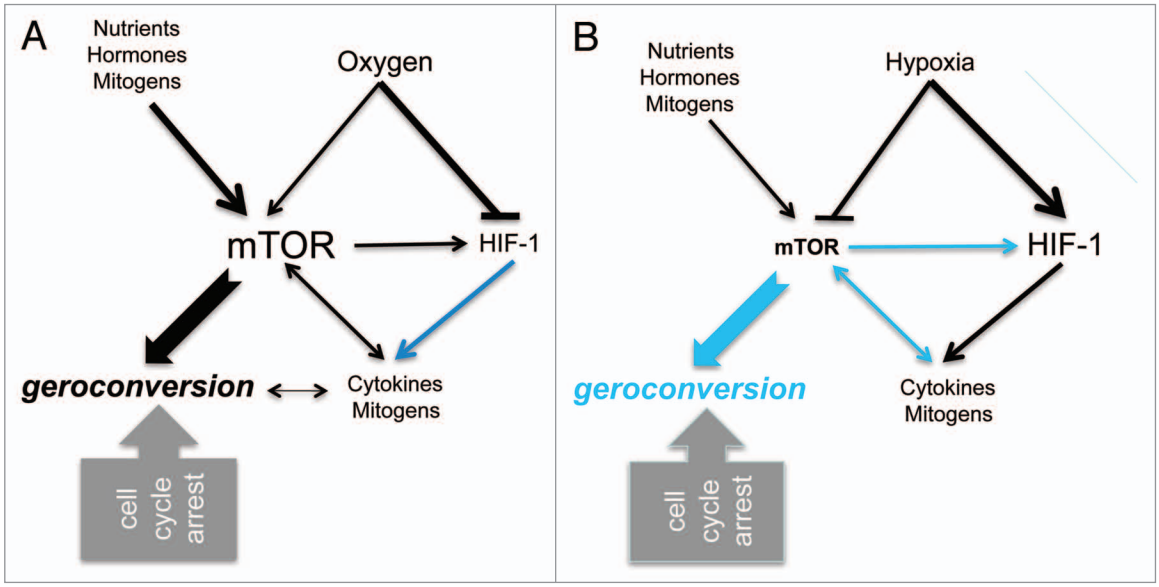

Figure 6. The relationships between hypoxia, HIF-1, mTOR and geroconversion. (**A**) Under normoxia, when the cell cycle is arrested, still active mTOR drives cellular senescence. mTOR is activated by nutrients, mitogens, cytokines and oxygen. As a part of geroconversion, mTOR stimulates cytokine secretion. Black lines: stimulatory and inhibitory effects. Blue lines: inactive under normoxia. (**B**) Under severe hypoxia, the mTOR pathway and geroconversion are partially inhibited. Hypoxia-inducible factor (HIF) is accumulated. HIF-1-dependent cytokine secretion may activate the mTOR pathway in neighboring oxygenated cells. Blue lines, inactive under hypoxia.

NAC (the most commonly used agent to decrease free radicals) turned out to inhibit the mTOR pathway in some cells too.[34] In our experiments, the gerosuppressive effect of hypoxia depended on whether it inhibited the mTOR pathway. Slight genetic alterations, differences between cell lines and levels of oxygen may determine the effect of oxygen on geroconversion. This is difficult to reconcile with the free radical theory. Also, free radical theory of aging does not fit observations in model organisms.[35-48] In agreement, inhibition of TOR prolongs lifespan in model organisms,[49-61] supporting the notion that mTOR-driven cellular hyper-functions (cellular aging) lead to age-related diseases and organismal death.[62-65]

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