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Role of the mammalian target of rapamycin pathway and rapamycin in lentiviral vector gene transduction of hematopoietic stem cells

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Abstract

Purpose of review—A major goal in repopulating hematopoietic stem cell (HSC) gene therapies is achieving high-efficacy gene transfer, while maintaining robust HSC engraftment and differentiation *in vivo*. Recent studies have documented that rapamycin treatment of HSC during lentiviral vector transduction enhances gene transfer to human and mouse HSCs and maintains engraftment capacity. In this review, we place into context the role of mammalian target of rapamycin (mTOR) pathways in HSC quiescence and function, endocytic regulation, and lentiviral gene delivery.

Recent findings—Lentiviral vector transduction of human and mouse HSCs is considerably enhanced by rapamycin treatment. Furthermore, rapamycin preserves long-term engraftment of human and mouse HSCs. Investigations of cellular mechanisms that contribute to increased transduction in HSCs uncovered a role for mTOR inhibition-dependent activation of endocytosis.

Summary—Rapamycin enhances lentiviral vector transduction of HSCs through regulation of endocytic activity via mTOR inhibition. An important attribute of rapamycin treatment during transduction is the preservation of HSC function, allowing reconstitution of long-term hematopoiesis *in vivo* in murine models.

Keywords

gene therapy; hematopoietic stem cells; hematopoietic stem cell engraftment; mTOR; rapamycin

INTRODUCTION

A long sought-after holy grail in the field of hematopoietic stem cell (HSC) gene therapy is a method for highly efficient lentiviral vector transduction that does not impair in-vivo reconstitution potential of HSCs following human transplantation. Although modest lentiviral vector transduction efficiency of 15–25% has been reported in HSCs in the absence of cytokine activation, this level may not be sufficient for clinical use [1,2]. Various strategies have been demonstrated or proposed for improving transduction efficiency in HSCs, including small-molecule approaches such as proteasome inhibitors, PGE2, SR1, and

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pyrimidoindole derivatives, and genetic approaches such as knockdown of the p21 protein [3–7]. However, most of these methods are still in early-stage preclinical investigation, and none has yet been translated to clinical usage, due to treatment-associated cytotoxicity or cumbersome genetic manipulations of HSCs. Recently, we and others made the intriguing discovery that the clinically used drug rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR) kinase, greatly enhanced lentiviral transduction efficiency in human and mouse primitive HSCs, with very little detrimental, or even beneficial, effects on the engraftment and reconstitution potential of HSCs [8▪▪,9,10▪,11]. We uncovered the involvement of an unconventional and little-studied aspect of mTOR signaling – regulation of the endocytic pathway – in the enhancement of lentiviral transduction by rapamycin. Here, we review recent literature on the role of mTOR signaling in HSC function, mTOR regulation by rapamycin in hematopoietic cells, and the connection between mTOR signaling and the endocytic pathway that may inform the mechanistic basis of improved lentiviral vector transduction.

MAMMALIAN TARGET OF RAPAMYCIN SIGNALING PATHWAY

The mTOR kinase is a central molecule that coordinates cellular energy sensing pathways and metabolic flux. Studies in yeast, drosophila, and mammalian cell lines have allowed extensive mapping of the mTOR signaling pathway (reviewed in [12]). mTOR is regulated upstream by the phosphotidylinositol-3-kinase (PI3K) pathway, which plays a major role in promoting cell growth and proliferation. PI3K activity results in the phosphorylation and activation of protein kinase B (PKB or AKT). AKT in turn phosphorylates many cellular targets that are important in cell growth and survival, including the tuberous sclerosis complex (TSC). TSC is a negative regulator of mTOR; thus, inhibition of TSC by an active PI3K/AKT leads to activation of mTOR. The phosphatase and tensin homolog (PTEN) negates the action of PI3K and is a negative regulator of mTOR (Fig. 1).

Mammalian target of rapamycin is the active kinase subunit of two complexes, mTORC1 and mTORC2, which carry out nonredundant functions. The functions of mTORC1, which is conventionally thought to be rapamycin-sensitive, are much better mapped out. The mTORC1 complex is characterized by the presence of the protein Raptor. The most well studied downstream actions of mTORC1 include activation of protein translation through phosphorylation of ribosomal S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein (4E-BP1), and inhibition of autophagy. The mTORC2 complex is characterized by the presence of Rictor, and is traditionally thought to be rapamycin-insensitive. A well defined mTORC2-specific downstream effect is phosphorylation of AKT at Ser473 [13,14]. Thus, mTORC2 positively regulates signaling of the AKT/mTOR axis.

REGULATION OF MTORC1 AND MTORC2 BY RAPAMYCIN

Although rapamycin is extensively used as a canonical mTORC1 inhibitor, its use comes with a few important caveats. Firstly, rapamycin does not completely inhibit mTORC1, its canonical target, in mammalian cells, with a number of rapamycin-insensitive mTOR downstream processes such as 4E-BP1 phosphorylation and autophagy [15–18]. Rapamycin allosterically inhibits mTORC1 through binding to the adaptor protein FKBP12.

Rapamycin– FKBP12 does not bind mTORC2, which is traditionally believed to be insensitive to inhibition by rapamycin. However, since rapamycin–FKBP12 does bind free mTOR, prolonged rapamycin treatment (>24 h) impairs mTORC2 turnover by sequestering free mTOR [19]. In addition, different concentrations of rapamycin have been reported to differentially modulate mTORC1 and mTORC2, with high concentrations in the micromolar range inhibiting mTORC2 assembly [20]. Thus, the interpretations of studies reporting autophagy induction and function in hematopoietic cells in which micromolar concentrations of rapamycin were utilized may be confounded by the effects of mTORC2 induction [21,22].

For lentiviral vector transduction enhancement in HSCs, the inhibition of mTOR by rapamycin or other active site inhibitors is required, but it is unknown which mTOR complex is involved, or the downstream molecular events that regulate function following inhibition [8▪▪]. The effective rapamycin concentration for HSC transduction enhancement is in the micromolar range, concentrations at which mTORC2 inhibition may be invoked [20]. Supportive of a potential mTORC2-dependent mechanism, we found no evidence for the involvement of autophagy, an mTORC1-specific cytoprotective pathway. Although autophagy may contribute to certain aspects of lentiviral transduction of HSCs, such as the kinetics of intracellular trafficking of vector particles, it does not appear to affect the stable gain of transduction efficiency that results from rapamycin treatment.

ROLE OF MAMMALIAN TARGET OF RAPAMYCIN IN HEMATOPOIETIC STEM CELL QUIESCENCE

Mammalian target of rapamycin complexes play a central role in the regulation of cellular energy sensing and response pathways, which are particularly important for the function of primitive stem cells. Consistent with observations in other cell types, inhibition of mTOR exhibits a general effect in promoting HSC quiescence, and inappropriate activation leads to HSC proliferation, depletion, and oncogenic transformation, discussed in detail below.

EFFECT OF MAMMALIAN TARGET OF RAPAMYCIN ACTIVATION

The PI3K/AKT pathway, through regulation of mTOR, plays a key role in HSC quiescence. Over-activation of mTOR, either through increased AKT signaling [23], or depletion of negative regulators of mTOR, including PTEN, TSC, and PML, leads to HSC proliferation and depletion, as well as myeloproliferative disease [24–27]. Phenotypes associated with activation of AKT, depletion of PTEN, TSC, or PML, are reversed by rapamycin treatment, demonstrating involvement of mTOR [23,24,26,27]. Furthermore, HSC aging is associated with mTOR activation, and activating mTOR through TSC depletion in young mouse HSCs mimics an aged phenotype, which is corrected by rapamycin [28].

Mechanistically, mTOR activation may impact HSC quiescence by three potential mechanisms: increasing the amount of reactive oxygen species, as in the case of TSC depletion [26,29]; limiting fatty acid oxidation [30]; or deregulating the rate of protein synthesis, as in the case of PTEN depletion [31▪]; all three are detrimental for HSC maintenance (Fig. 1). Furthermore, the effect of mTOR activation on production of reactive

oxygen species may be mediated by repression of autophagy, as depleting Atg7 also leads to accumulation of mitochondria and generation of reactive oxygen species, and consequently proliferation and exhaustion of HSCs [32].

EFFECT OF MAMMALIAN TARGET OF RAPAMYCIN INHIBITION

The effect of mTOR inhibition on HSC function has been studied through pharmacological or genetic means. Rapamycin specifically reduced expansion of common myeloid progenitors (Lin^+CD34^+), but not primitive HSCs (Lin^-CD34^+) or lineage committed progenitors (Lin^+CD34^-) [33]. The different effects of rapamycin on primitive and progenitor cells may be due to different propensities for autophagy induction, as mouse HSCs are more prone to autophagy induction relative to myeloid progeny, due to the action of the transcription factor FOXO3A [34]. A similar mechanism may also underlie different rapamycin sensitivities of HSCs from different sources, as HSCs show augmented autophagy following G-CSF-mediated mobilization [35].

In recent years, generation of mouse models with conditional ablation of Raptor and Rictor has allowed specific examination of the functions of mTORC1 and mTORC2 complexes in hematopoiesis. Neither complex is essential for normal HSC maintenance under homeostatic conditions [36,37], but does affect differentiation of various downstream progenitors. While not essential in homeostatic hematopoiesis, mTORC1 is required for HSC regeneration following transplantation [36]. Inhibition of mTORC1 through Raptor depletion causes pancytopenia and skewing of hematopoiesis toward myelomonocytic lineages through impaired granulocyte and B-cell development, but does not affect proliferation of hematopoietic and myeloid progenitors [36,38,39]. Both mTORC1 and mTORC2 contribute to T-cell lymphopoiesis, but at different stages. Depletion of Raptor inhibits early T-cell development at the DN1 stage, whereas depletion of Rictor impairs development at the DN3 stage [39,40]. Interestingly, rapamycin blocks T-cell development at the DN3 stage, similar to the effect of Rictor depletion and distinct from the effect of Raptor depletion, suggesting that rapamycin may act to modulate mTORC2 [39]. The action of mTORC2 in hematopoiesis seems to specifically affect T-cell lymphopoiesis, as Rictor depletion does not affect the function of HSCs or development of B cell, erythroid, or myeloid lineages [37,40]. Depletion of either Raptor or Rictor protects from PTEN loss evoked leukemogenesis, indicating the involvement of both mTORC1 and mTORC2 in abnormal hematopoiesis in response to a hyperactive AKT pathway [36,37]. Of note, PTEN inhibits mTORC2 signaling in adult, but not neonatal HSCs, highlighting important differences in mTOR pathway regulation in HSCs of different origins that may be relevant to HSC transduction [37].

CONNECTION BETWEEN HEMATOPOIETIC STEM CELL QUIESCENCE AND TRANSDUCTION

Regulation of HSC quiescence is a key aspect in HSC transduction protocols, due to the need to maintain stem cell functions such as homing, engraftment, and reconstitution for subsequent transplantation following lentiviral vector transduction. However, in addition to its intrinsic importance, regulation of quiescence may be mechanistically linked to lentiviral

vector transduction efficiency. It has long been shown that the ability to transduce HSCs with lentiviral vectors is dependent on the cell activation state, as HSCs and progenitor cells need to exit G0 and progress to G1 to allow efficient transduction [41]. Accordingly, several strategies that improve lentiviral vector transduction in HSCs also affect cell cycling status. Rapamycin causes cell cycle delay in HSCs, consistent with previous studies [8▪▪]. Fibronectin, commonly used to enhance HSC lentiviral vector transduction, may also be implicated in HSC maintenance [42–44]. Silencing of the p21 protein leads to ex-vivo expansion of HSCs and decreases the fraction of HSCs in G0, and also increases lentiviral vector transduction efficiency [7,45]. However, we showed that rapamycin-mediated lentiviral vector transduction enhancement occurred via a rapidly dissipating effect on endocytosis of lentiviral vectors, which was likely distinct from rapamycin-mediated inhibition of cell cycle progression [8▪▪]. Nevertheless, cell activation status plays a key role in regulating HSC lentiviral vector transducibility through regulation of low-density lipoprotein receptor (LDLR) levels, the cellular receptor for vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped lentiviral vectors [46▪]. It is unclear whether mTOR is involved in the up-regulation of LDLR upon cellular activation; our findings indicated that rapamycin did not affect LDLR levels in quiescent or activated HSCs [8▪▪].

CONNECTION BETWEEN MAMMALIAN TARGET OF RAPAMYCIN AND ENDOCYTOSIS

Apart from its well studied role in regulating HSC quiescence, the mTOR pathway plays additional roles that are yet to be elucidated. Although we recently demonstrated a role for mTOR inhibition in enhancing endocytic entry of lentiviral vectors, resulting in increased transduction efficiency (Fig. 1), a relationship between mTOR and endocytosis has not been conclusively established [8▪▪]. It is evident that the endocytic machinery is involved in mTOR signaling. In yeast, TOR localizes to vesicular and endosomal membranes [47,48]. In mammalian cells, localization of mTOR to late endosomal and lysosomal compartments is involved in amino acid-induced mTORC1 signaling [49]. Furthermore, integrity of the late endosome is crucial for mTORC1 signaling, which is inhibited by blocking the conversion between early and late endosomes [50]. Rab5 GTPases, which regulate endosome formation and maturation, also mediate mTORC1 localization to the lysosomal compartment in both yeast and mammalian cells [51]. Therefore, several lines of evidence link mTOR signaling to functional endocytic machinery.

On the contrary, whether mTOR signaling in turn affects endocytic function has been more elusive. In drosophila, TOR interacts with clathrin-uncoating ATPase, and TOR signaling in the drosophila fat body differentially promotes bulk endocytosis while preventing endocytic degradation of amino acid transporter to increase nutrient availability. In this case, TOR is both regulated by endocytosis, as signaling is disrupted by disruption of endocytosis, and in turn regulates the endocytic process [52]. TOR may also have an indirect effect on protein endocytosis at the apical epithelium in drosophila pupal wing and mouse kidney proximal tubules, by inhibiting the transcription of the multiligand receptor Megalin [53].

While initially aiming to target postentry lentiviral restriction in HSCs, we instead showed that rapamycin-mediated transduction enhancement was solely attributed to enhanced vector

entry, demonstrating cytoplasmic entry to be a major restrictive step in lentiviral transduction of HSCs that can be regulated by mTOR (Fig. 1). The regulation of endocytic pathways in HSC by mTOR is a novel finding. Since VSV-G, which is commonly employed for lentiviral vector pseudotyping, mediates entry via the LDLR [54], this may be a byproduct of mTOR regulation of lipid metabolic pathways. Lipid metabolic pathways play a role in HSC function, as LDL promotes mouse HSC proliferation *in vitro* and *in vivo* [55]. Furthermore, mTOR functions in the regulation of lipid metabolic pathways and rapamycin have been reported to down-regulate the LDLR at both messenger and protein levels in hepatic cells [56,57]. However, we found in $CD34^+$ HSCs that rapamycin did not affect the cell surface levels of LDLR or bound lentiviral vectors, but rather increased the amount of vector cores in the cytoplasm. This suggests that rapamycin acts at the stages of vesicle internalization, trafficking, or lentiviral– endosomal fusion. The VSV-G pseudotype mediates cell entry in clathrin-coated vesicles that are dependent on actin for internalization [58]. Mechanistically, mTOR could potentially modulate lentiviral entry by acting on the cytoskeleton, as both yeast TOR2 and mammalian mTORC2 modulate organization of the actin cytoskeleton [59–62]. Actin cytoskeleton plays an important role in cargo sorting and maturation of early endosomes – the site of fusion for VSV-G pseudotyped vectors [63,64]. It is unclear whether mTOR inhibition affects other entry pathways in addition to clathrinmediated endocytosis, but there is evidence that drosophila TOR may be selective in promotion of clathrin vs. caveolin and raft-mediated endocytosis [52]. A genome-wide analysis of kinases in HeLa cells identified the mTOR pathway in the specific maintenance of clathrin-mediated endocytosis, as silencing of mTOR and signaling pathway members blocked clathrin-dependent endocytic events such as VSV infection, and transferrin internalization and trafficking [65]. This is in contrast to our finding that mTOR inhibition stimulates clathrin-dependent endocytosis of VSV-G pseudotyped lentiviral vectors in HSCs, and may reflect differences in cell type-specific metabolic programming.

CONCLUSION

The mTOR pathway is a key consideration in HSC gene therapy, both due to its well studied role in HSC maintenance, and our recent demonstration of its novel role in lentiviral vector endocytosis. The regulation of HSC endocytic machinery by mTOR reveals a novel aspect of mTOR signaling that is relevant to HSC gene therapy, and brings to light a number of further mechanistic and therapeutic questions. For example, the molecular events that bridge mTOR inhibition and endocytic enhancement need to be elucidated. Even though our findings suggest a non-mTORC1 mechanism in endocytic enhancement, certain aspects of mTORC1 signaling, such as autophagy, may be involved at additional stages of lentiviral vector entry. The contributions of mTORC1 and mTORC2 to the transduction process should be assessed, and may inform upon the choice of small molecule mTOR inhibitors with more specificity for therapeutic use. Additionally, it is unknown whether the regulation of endocytosis by mTOR exists as a physiological signaling pathway to bring in nonlentiviral vector ligands during metabolic stress, and whether this phenomenon is restricted to stem cells due to their particular metabolic requirements. Therapeutically, the efficacy of mTOR inhibitors in improving transduction by non-VSV-G-pseudotyped vectors that use alternative entry pathways remains to be evaluated. Finally, the dosage of mTOR

inhibitors needs to be optimized in animal and human studies to maximize both transduction efficiency and preservation of HSC function for clinical purposes.

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KEY POINTS

- Rapamycin significantly augments lentiviral gene delivery to HSCs while preserving engraftment potential.
- Rapamycin-mediated transduction in hematopoietic stem cells uncovered a role for mTOR inhibition-dependent activation of endocytosis.
- mTOR signaling regulates HSC quiescence and maintenance.

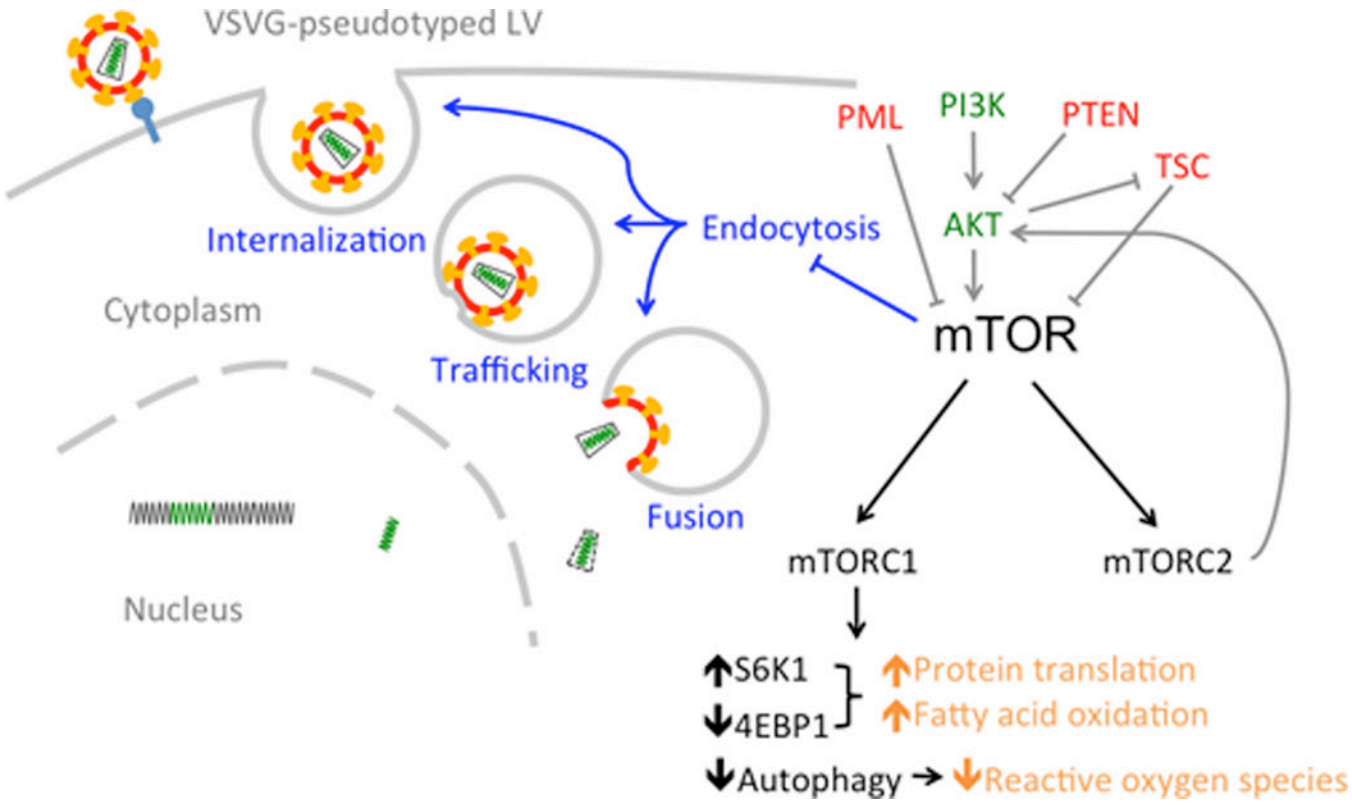

FIGURE 1.

The role of mTOR in HSC maintenance and transduction. Gray arrows denote upstream regulators of mTOR that are important for HSC maintenance (green: mTOR activators; red: mTOR inhibitors). Black arrows denote downstream effectors regulated by mTOR. mTOR is the active kinase subunit of two complexes: mTORC1 and mTORC2. mTORC1 regulates three processes implicated in HSC maintenance, shown in orange [26,29,30,31▪]. In addition to promoting HSC maintenance, mTOR inhibition also enhances lentiviral vector transduction of HSCs by enhancing one or more early steps of vector endocytosis, shown in blue, potentially via an mTORC2-mediated mechanism [8▪▪]. HSC, hematopoietic stem cell; mTOR, mammalian target of rapamycin.