Regulated Delivery of Therapeutic Proteins After in Vivo Somatic Cell Gene Transfer

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Stable delivery of a therapeutic protein under pharmacologic control was achieved through in vivo somatic gene transfer. This system was based on the expression of two chimeric, human-derived proteins that were reconstituted by rapamycin into a transcription factor complex. A mixture of two adeno-associated virus vectors, one expressing the transcription factor chimeras and one containing erythropoietin (Epo) under the control of a promoter responsive to the transcription factor, was injected into skeletal muscle of immune-competent mice. Administration of rapamycin resulted in 200-fold induction of plasma Epo. Stable engraftment of this humanized system in immune-competent mice was achieved for 6 months with similar results for at least 3 months in a rhesus monkey.

The biotechnology industry has spawned a new class of therapeutics based on the delivery of recombinant proteins. Delivery of these therapeutics requires repeated parenteral administration, usually through direct injection. Problems of delivery limit the broader use of these therapeutic proteins because of toxicity associated with unfavorable pharmacokinetics and poor patient compliance. Somatic gene therapy has been considered as an alternative means to delivery of therapeutic proteins through the stable transduction of cells capable of secreting proteins into the blood. Indeed, therapeutic levels of a number of proteins have been achieved with this approach (*1*). Vector systems have generally been based on constitutive promoters that result in stable but unregulated gene expression (*1*). In most clinical applications, however, it will be necessary to regulate the expression of the gene to maintain concentrations of the protein within the therapeutic window to maximize efficacy without toxicity and to respond to the evolving nature of the patient's disease.

A number of systems for achieving regulatable transgene expression are in development (*2*). Important properties of such a system include low baseline expression and high induction ratio as well as positive control by an orally bioavailable small-molecule drug. One approach for developing this kind of system is through the expression of heterologous transcription factors designed to selectively bind the transgene promoter and activate its transcription in response to a drug. We have explored the potential of a system for regulating genes based on the ability of the drug rapamycin to bind simultaneously to two human proteins FKBP12 and FRAP (*3*). Transcriptional activation is achieved in vivo through rapamycin-induced reconstitution of a transcription factor complex that is formed by the coupling of two independently expressed proteins: a unique DNA-binding domain called ZFHD1, genetically fused to FKBP, and the activation domain of the p65 subunit of nuclear factor kappa B (NFκB), fused with the rapamycin-binding domain of FRAP, termed FRB. Successful constitution of this system in vivo requires the presence of genes encoding both hybrid transcription factors together with the transgene driven by the inducible promoter. This system has demonstrated inducible expression in stably transfected tumor cells analyzed in vitro or after transplantation in vivo in immune-deficient mice (*4*).

The goal of this study was to evaluate the potential of the rapamycin-inducible system in the context of direct in vivo gene transfer in mice and nonhuman primates with adeno-associated virus (AAV) as a vector. Previous studies have demonstrated the utility of AAV for achieving efficient and stable transgene expression while avoiding destructive cellular immune responses to the transgene product (*5–7*). Packaging limits of AAV required that the three components of the system be incorporated into two vectors, one vector that expresses the transcription factors from a single transcriptional unit with an internal ribosome entry site and the second vector containing the therapeutic gene (in this study, murine and rhesus monkey Epo) driven by a promoter recognized by the ZFHD1 DNA-binding domain (*8*). Infection of the permissive human cell line 84-31 (*9*) with equal quantities of the two AAV vectors at high multiplicity of infection resulted in full reconstitution of the regulated system with at least a 100-fold induction after exposure to rapamycin (*10*).

Reconstitution of the regulated system in vivo requires transduction of the target cell with both vectors requiring a very efficient delivery system. We selected skeletal muscle as the target in part because its component cells, the muscle fibers, are long syncytia with extended nuclear domains. Reconstitution of the system could theoretically be achieved by independent transduction events targeting different nuclei within the same fiber. Furthermore, murine skeletal muscle is permissive for AAV transduction (*5, 6*).

Initial experiments were performed in immune-deficient mice injected with transcription factor and murine Epo target gene vectors into the tibialis anterior and quadriceps (Fig. 1). There was no increase in plasma Epo or change in hematocrit before challenge with rapamycin for a period of either 30 (Fig. 1A) or 71 (Fig. 1B) days after vector administration. Single administration of rapamycin (1 mg/kg) resulted in an immediate 100-fold rise in plasma Epo to 100 to 150 mU/ml that returned to baseline within 14 days (Fig. 1B). A more sustained increase of plasma Epo was achieved after repeated rapamycin administrations (1 mg/kg × 10 in Fig. 1A and 5 mg/kg × 6 in Fig. 1B). Induction of Epo resulted in an increase in hematocrit to 70 to 75%, which slowly returned to baseline over a 2- to 3-month period after the withdrawal of rapamycin, consistent with the half-life of erythrocytes in mice (Fig. 1A). The hematocrit returned to high levels when these animals were challenged with a second dose of rapamycin (1 mg/kg) 140 days after the initial vector administration (Fig. 1A).

Experiments were repeated with AAV vectors in three groups of immune-competent animals to more closely simulate clinical applications (Fig. 2). All groups responded initially to a single administration of rapamycin (10 mg/kg) with an identical rise in plasma Epo. The second and third doses in each group were single injections of different amounts of rapamycin. These studies clearly showed a direct relation between dose of rapamycin and peak plasma Epo in a range of rapamycin between 0.02 mg/kg (no induction) and 0.4 mg/kg (maximal induction). Maximum induction of Epo actually increased over the first 6 to 8 weeks because of a delay in fiber transduction that steadily increases over this time period (*5, 6*). A

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similar dose response was observed after repeated administration of rapamycin (three doses repeated every other day) in doses of 0.1 (Fig. 2A), 0.2 (Fig. 2B), and 0.3 mg/kg (Fig. 2C). There was no diminution of inducible Epo expression over time, with each group showing peak levels of inducible Epo equal to 700 mU/ml 6 months after vector injection. These data confirm the advantage of AAV in evading destructive cellular responses despite expression of neoantigens (*7*) and demonstrate the ability to titrate in vivo expression of the transgene with the dose of rapamycin.

Critical to the evaluation of this system for regulated expression of therapeutic proteins in humans is demonstration of success in nonhuman primates. These experiments have been difficult to perform because of limitations in the production of AAV. A method for producing AAV was developed on the basis of the use of a cell line stably expressing the AAV genes *rep* and *cap* and an adenovirus-AAV (Ad/AAV) hybrid vector. Advantages of this system include high production of vector per cell, simplicity in scaling up, and absence of replication-competent AAV in the final preparations (*11*).

An AAV vector was constructed containing the cDNA for rhesus monkey Epo driven from the constitutive promoter of the immediate early region of cytomegalovirus (CMV). Vector was produced with the traditional approach based on transfection and the method that uses the hybrid virus. Equal quantities of each vector preparation (5×10^{12} genomes per kilogram) were injected into the quadricep muscles of two adolescent rhesus monkeys (Fig. 3, A and B). A substantial increase in plasma Epo to 100 mU/ml was seen in each animal that has persisted for almost 1 year (the duration of the experiment in one animal). Both animals developed severe polycythemia, requiring at least weekly phlebotomies to maintain their hematocrits at <65%.

Regulated expression of Epo was attempted in a rhesus monkey by coinjecting into its quadricep muscle vectors expressing the transcription factors (4×10^{12} genomes per kilogram) and rhesus monkey Epo from the Z12 promoter (4×10^{13} genomes per kilogram). There was

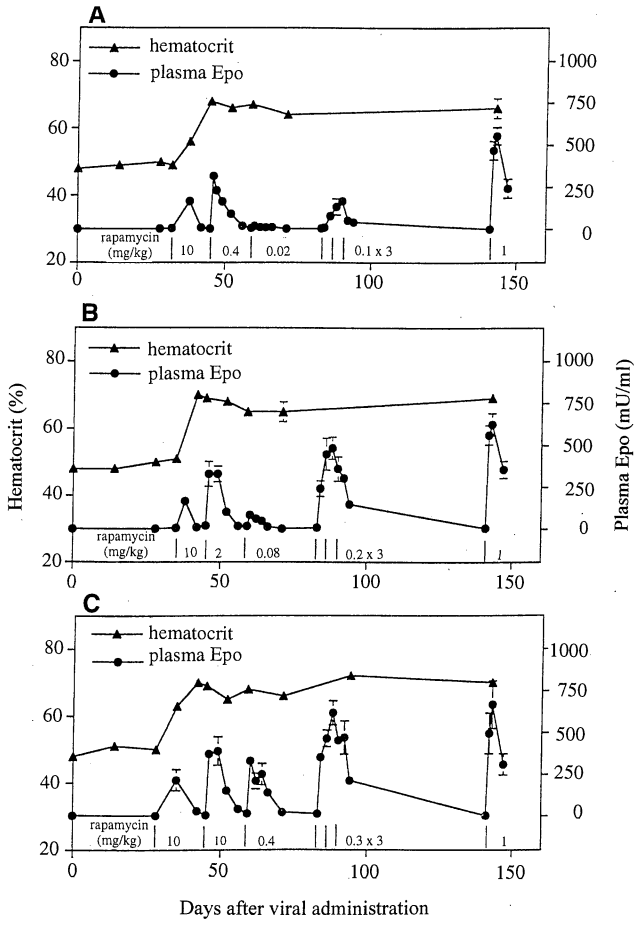
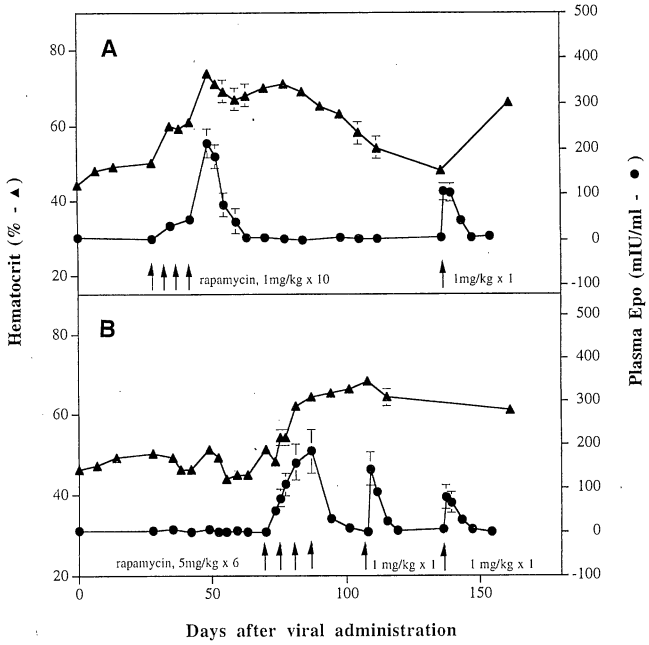


Fig. 2. Regulated Epo expression in immune-competent balb/c mice receiving intramuscular injection of inducible AAV vectors. Four- to 5-week-old male balb/c mice were injected intramuscularly with 2×10^{11} genome copies of AAV.CMVTF1 and AAV.Z12mEpo3S3 at a 1:1 ratio. Rapamycin was given intraperitoneally at the indicated time and dose. Blood samples were taken retroorbitally at various time points. Hematocrit counts were determined by an IEC microcapillary reader, and circulating plasma Epo concentrations were measured by ELISA (Quantikine IVD kit). Values are mean ± SEM for five mice per group. (**A** to **C**) Hematocrits and plasma Epo levels in mice receiving rapamycin with different dosing schedules.

Fig. 1. Regulated Epo expression in immune-deficient balb/c nude mice receiving intramuscular injection of inducible AAV vectors. Four- to five-week-old male balb/c nude mice were injected with 2×10^{11} genome copies of recombinant AAV. The viruses, a 1:1 mixture of AAV.CMVTF1 and AAV.Z12mEpo3S3, were suspended in 100 μl of phosphate-buffered saline (PBS) and administered into four intramuscular sites (quadriceps and tibialis anterior on both sides, 25 μl per site). Rapamycin was initially dissolved in *N*,*N*-dimethylacetamide as a stock solution and then diluted to each specific concentration in a mixture with the final concentration of 4% *N*,*N*-dimethylacetamide, 10% polyoxyethylene glycol (average molecular weight of 400), and 1.7% polyoxyethylene sorbitan monooleate. The injection was given intraperitoneally in a total volume of 100 μl. Doses of rapamycin are indicated in the figure. Blood samples were taken retroorbitally at various time points. Hematocrit counts were determined by an IEC microcapillary reader, and circulating plasma Epo concentrations were measured by enzyme-linked immunosorbent assay (ELISA) (Quantikine IVD kit; R&D Systems, Minneapolis, Minnesota). Values are mean ± SEM for 10 mice per group. (**A**) Hematocrits and plasma Epo levels in mice receiving the first rapamycin challenge at day 30 after viral administration. The initial rapamycin dose, indicated by four arrows, was 1 mg/kg × 10 with a 1-day interval between injections; the second dose, indicated by one arrow, was 1 mg/kg × 1. (**B**) Hematocrits and plasma Epo levels in mice receiving the first rapamycin challenge at day 71 after viral administration. The initial rapamycin dose, indicated by four arrows, was 5 mg/kg × 6 with a 1-day interval between injections; the second and third doses, shown by single arrows, were 1 mg/kg × 1.



no increase in plasma Epo (Fig. 4B) or hematocrit (Fig. 4A) for 29 days after vector injection before rapamycin administration, confirming low or no basal expression in vivo from the inducible promoter. Administration of rapamycin (0.5 mg/kg) resulted in rapid induction of Epo from 2 (lower limit of detection) to 97 mU/ml that returned to baseline in about 14 days (Fig. 4B). This induction was associated with an increase in hematocrit from 42 to 60% (Fig. 4A). A similar initial induction of Epo and increase in hematocrit was observed in four other rhesus monkeys (*12*). Rechallenge with the same dose of rapamycin on day 50 resulted in an identical and reversible induction of Epo, demonstrating the stability and consistency of the system (Fig. 4B). The peak induction of recombinant Epo was decreased 20-fold at 3 months and became indistinguishable from endogenous Epo after 4 months. This could be due to immune response to the highly antigenic protein domains that were incorporated into the NH_2-terminus of both transcription factors (influenza virus hemagglutin and SV40 nuclear localization sequence) or a decrease in the level of transcription factors below a critical threshold (*13*).

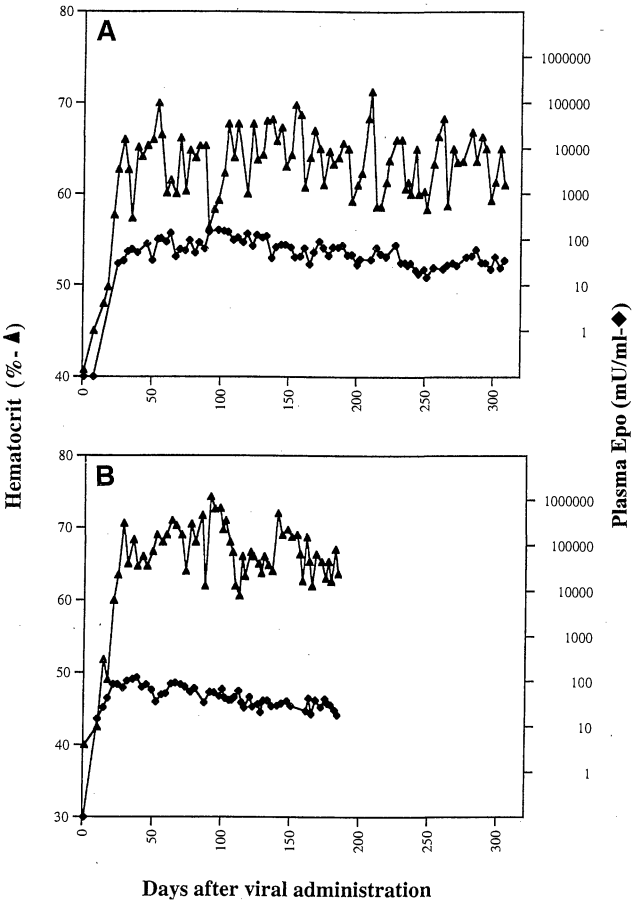


Fig. 3. Epo expression in nonhuman primates receiving intramuscular injection of AAV vector with a constitutive promoter. Two rhesus monkeys were injected intramuscularly with AAV.CMVrmEpo3 (5×10^{12} genomes per kilogram). The viruses were suspended in 10 ml of PBS and administered into ten intramuscular sites (vasta lateralis on both sides, five sites each side, 1 ml per site). Blood samples were taken by venopuncture of the saphenous vein at various time points. Hematocrit counts were determined by an IEC microcapillary reader, and circulating plasma Epo concentrations were measured by ELISA (Quantikine IVD kit). (**A**) Hematocrits and plasma Epo levels in the rhesus monkey receiving AAV produced by transfection method (RQ1582, female, 60 months old, 4.5 kg). (**B**) Hematocrits and plasma Epo levels in the rhesus monkey receiving AAV produced by the hybrid system (93B644, male, 60 months old, 5.5 kg).

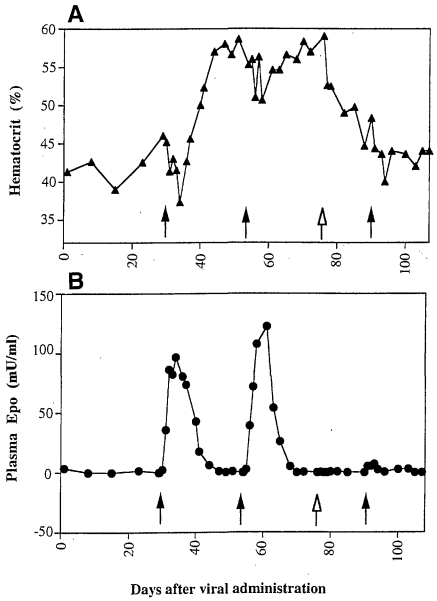


Fig. 4. Regulated Epo expression in nonhuman primates receiving intramuscular injection of inducible AAV vectors. One rhesus monkey (95C002, male, 30 months old, 5.1 kg) was injected intramuscularly with a mixture of AAV.CMVTF1 (4×10^{12} genomes per kilogram, vector made by the transfection method) and AAV.Z12rmEpo2S2 (4×10^{13} genomes per kilogram, vector made by the hybrid method). The viruses were suspended in 10 ml of PBS and administered into 10 intramuscular sites (vasta lateralis on both sides, five sites each side, 1 ml per site). Rapamycin was initially dissolved in *N,N*-dimethylacetamide as a stock solution and then diluted to each specific concentration in a mixture with the final concentration of 4% *N,N*-dimethylacetamide, 10% polyoxyethylene glycol (average molecular weight 400), and 1.7% polyoxyethylene sorbitan monooleate. Injection was given intravenously in a total volume of 2.5 ml (0.5 mg/kg), as indicated by the closed arrow heads. An analog of rapamycin with 10-fold decreased biological activity was administered at 0.5 mg/kg, as indicated by the open arrow heads. Blood samples were taken by venopuncture of the saphenous vein at various time points. Hematocrit counts were determined by an IEC microcapillary reader, and circulating plasma Epo concentrations were measured by ELISA (Quantikine IVD kit). (**A**) Hematocrit counts. (**B**) Plasma Epo concentrations.

In summary, we describe an approach for delivering therapeutic proteins based on somatic gene transfer. This system has advantages over tetracycline-based repression because basal expression in vivo is essentially zero and expression is extinguished by simply removing the drug (*14*). Furthermore, expression at peak induction is quite robust, approaching levels of a strong constitutive promoter. The use of muscle as a target may facilitate reconstitution of this system from two vectors by mixing the expressed transcription factors with the transgene in the multinucleated muscle fiber.

AAV is clearly the preferred vector in this application for a number of reasons. Gene expression, as measured from constitutive promoters, remains stable over an extended period of time (up to 12 months in our model) in part because of the fact that AAV appears to integrate into the chromosomal DNA of the recipient cell (*5, 6*). Successful application of this strategy may be achieved in immune-competent hosts because of the ability of AAV to avoid destructive immune responses to the transcription factors or transgene products in some settings (*7*). Delivery of therapeutic proteins with this system offers several potential advantages over the traditional method of repeated parenteral injections. The pharmacokinetics and pharmacodynamics of the therapeutic protein may be more favorable because of steady and titratable protein concentrations, which may be held within the therapeutic window while avoiding peaks that may be associated with toxicity. Patient compliance should be enhanced because of the convenience of a pill over an injection. Finally, manufacturing of individual proteins is replaced by a generic approach for manufacturing the drug and the vector.

References and Notes

1. R. O. Snyder *et al.*, *Hum. Gene Ther.* **8**, 1891 (1997); S. K. Tripathy *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 10876 (1996); N. Naffakh *et al.*, *Hum. Gene Ther.* **7**, 11 (1996); R. O. Snyder *et al.*, *Nature Genet.* **8**, 270 (1997); S.

Connelly, J. M. Gardner, R. M. Lyons, A. McClelland, M. Kaleko, *Blood* **87**, 4671 (1996); P. Moullier, D. Bohl, J. M. Heard, O. Danos, *Nature Genet.* **4**, 154 (1993).
2. D. Bohl and J. M. Heard, *Hum. Gene Ther.* **8**, 195 (1997); D. Bohl, N. Naffakh, J. M. Heard, *Nature Med.* **3**, 299 (1997); Y. Wang, J. Xu, T. Pierson, B. W. O'Malley, S. Y. Tsai, *Gene Ther.* **5**, 432 (1997); Y. Wang, B. W. O'Malley Jr., S. Y. Tsai, B. W. O'Malley, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8180 (1994); D. No, T. P. Yao, R. M. Evans, *ibid.* **93**, 3346 (1996).
3. E. J. Brown *et al.*, *Nature* **369**, 756 (1994); R. F. Standaert, A. Galat, G. L. Verdine, S. L. Schrieber, *ibid.* **346**, 671 (1990).
4. V. M. Rivera *et al.*, *Nature Med.* **2**, 1028 (1996); S. R. Magari, V. M. Rivera, J. D. Iuliucci, M. Gilman, F. Cerasoli Jr., *J. Clin. Invest.* **100**, 2865 (1997).
5. P. D. Kessler *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14082 (1996); X. Xiao, J. Li, R. J. Samulski, *J. Virol.* **70**, 8098 (1996).
6. K. J. Fisher *et al.*, *Nature Med.* **3**, 306 (1997).
7. K. Jooss, Y. Yang, K. J. Fisher, J. M. Wilson, *J. Virol.* **72**, 4212 (1998).
8. The transcription factor construct AAV.CMVTF1 contains a CMV promoter (*15*) driving expression of a bicistronic gene with the following components: an activation domain fusion (the FRB fragment of human FRAP in which the threonine at amino acid 2098 was mutated to phenylalanine fused to an activation domain derived from the p65 subunit of human NF-κB), the internal ribosome entry site derived from encephalomyocarditis virus, the DNA-binding domain fusion (the ZFHD1 DNA-binding domain and three tandemly repeated copies of human FKBP12), and an 830–base pair (bp) portion of the rabbit β-globin gene containing the final intron and a 3′ untranslated region (UTR). The FRB-p65 and ZFHD1-3xFKBP fusion proteins both contain an NH_2-terminal epitope tag from influenza virus hemagglutin and nuclear localization signal from SV40 large T antigen (*4, 16*). Reporter construct AAV.Z12mEpo3S3 contains the 12 ZFHD1-binding sites (*4*), a minimal interleukin-2 promoter (*4*), a chimeric intron from pCI vector (Promega), a murine Epo cDNA, an SV40 late gene 3′ UTR, and a 2.7-kb stuffer containing a 1367-bp internal portion of human placental alkaline phosphatase coding sequence followed by a 626-bp portion of the human growth hormone 3′ UTR and a 720-bp portion of the 3′ rabbit β-globin intron and PAS (*4*). Reporter construct AAV.Z12rmEpo2S2 is similar except that the Epo cDNA is derived from a rhesus monkey Epo and the vector lacks the chimeric intron and the 720-bp rabbit β-globin portion of the stuffer. Reporter construct AAV.CMVrmEpo3 contains a human CMV enhancer-promoter, a chimeric intron from pCI vector, a rhesus monkey Epo cDNA, and an SV40 late gene 3′ UTR. Recombinant AAV was generated by cloning CMVTF1, Z12mEpo3S3, Z12rmEpo2S2, and CMVrmEpo3 expression cassettes into Xba I–restricted pSub 201 backbone (*17*). The resulting plasmids were used to produce recombinant AAV by a triple transfection method (*18*) or by an Ad/AAV hybrid system (*11*).
9. K. J. Fisher *et al.*, *J. Virol.* **70**, 520 (1996).
10. X. Ye *et al.*, unpublished data.
11. G.-P. Gao *et al.*, *Hum. Gene Ther.* **9**, 2353 (1998).
12. An additional four rhesus monkeys were injected with a mixture of AAV vectors expressing Epo and the transcription factors as described in Fig. 4. Each responded to a dose of rapamycin (0.5 mg/kg) with a brisk and reversible induction of Epo with a concurrent increase in hematocrit. Group 1 had (transcription factor vector dose of 0.75×10^{13} genomes per kilogram and Epo vector dose of 1.0×10^{13} genomes per kilogram) peak plasma Epo levels of 220 and 420 mU/ml. Group 2 had (transcription factor vector dose of 0.75×10^{13} genomes per kilogram and Epo vector dose of 2.5×10^{13} genomes per kilogram) peak Epo levels of 560 and 120 mU/ml.
13. Antibodies to the influenza virus hemagglutinin tag were detected in serum of the nonhuman primate 3 months after gene transfer, although it is unclear whether their presence predicts functional cellular immune responses.
14. K. G. Rendahl *et al.*, *Nature Biotechnol.* **16**, 757 (1998).
15. X. Ye *et al.*, *J. Biol. Chem.* **271**, 3639 (1996).
16. V. M. Rivera, *Methods* **14**, 421 (1998).
17. R. J. Samulski, L.-S. Change, T. Shenk, *J. Virol.* **61**, 3096 (1987).
18. W. Xiao, S. C. Berta, M.-M. Lu, J. Tazelaar, J. M. Wilson, *ibid.* **72**, 10222 (1998).
19. Funding was provided by grants from the NIH [P30 DK47757-05 (J.M.W.) and P01 AR/NS43648-03 (H. L. Sweeney)], the Muscular Dystrophy Association, ARIAD Pharmaceuticals, and Genovo, a company J.M.W. founded and in which he holds equities. Technical support from N. Courage was greatly appreciated. W. Xiao provided plasmids for AAV production.

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Processing of the Notch Ligand Delta by the Metalloprotease Kuzbanian

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Signaling by the Notch surface receptor controls cell fate determination in a broad spectrum of tissues. This signaling is triggered by the interaction of the Notch protein with what, so far, have been thought to be transmembrane ligands expressed on adjacent cells. Here biochemical and genetic analyses show that the ligand Delta is cleaved on the surface, releasing an extracellular fragment capable of binding to Notch and acting as an agonist of Notch activity. The ADAM disintegrin metalloprotease Kuzbanian is required for this processing event. These observations raise the possibility that Notch signaling in vivo is modulated by soluble forms of the Notch ligands.

The Notch (N) signaling pathway defines an evolutionarily conserved cell interaction mechanism that controls cell fate by modulating the cell's response to developmental signals (*1, 2*). The N receptor is cleaved in the trans-Golgi network as it traffics toward the plasma membrane and eventually forms a ligand-competent heterodimeric molecule (*3*). Both known ligands, Delta (Dl) and Serrate (Ser), are thought to act as transmembrane proteins that interact via their extracellular domains with N receptors that are expressed on adjacent cells (*2, 4*). Given the similar phenotypes produced by loss of Notch signaling and loss-of-function mutations in the *kuzbanian* (*kuz*) gene [a gene encoding a putative member of the ADAM family of metalloproteases (*5*)], it has been suggested that Kuz may be involved in the cleavage of N (*6*). This hypothesis is not corroborated by recent biochemical studies, indicating that the functionally crucial cleavage of N in the trans-Golgi network is catalyzed by a furinlike convertase (*7*).

A genetic screen to identify modifiers of the phenotypes associated with the constitutive expression of a dominant negative transgene of *kuz* (*kuzDN*) in developing imaginal discs identified *Delta* as an interacting gene (*8*). Flies expressing this dominant negative *kuz* construct, despite carrying a wild-type complement of *kuz*, became semi-lethal when heterozygous for a loss-of-function *Delta* mutation (*8*). In contrast, *Delta* duplications rescued the phenotypes associated with *kuzDN* (Fig. 1). The *kuzDN* flies display extra vein material (especially deltas at the ends of the longitudinal veins), wing notching (observed with a low penetrance), extra bristles on the notum, and have small rough eyes (Fig. 1, A and E) (*6*). When *kuzDN* flies carried three, as opposed to the normal two, copies of wild-type *Notch*, the bristle and eye phenotypes were not affected (*8*), nor were the vein deltas altered (Fig. 1D). However, the *kuzDN* phenotypes were effectively suppressed by *Delta* duplications (Fig. 1, B and F), indicating that a higher copy number of Dl molecules is capable of overriding the effect of the *kuzDN* construct.

The interaction between *Delta* and *kuz* was further explored through their respective protein products. Dl antigen was expressed in a stably transfected S2 cell line and was examined with an extracellular domain–specific antibody (*9*) (Fig. 2A). A fragment migrating

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