Hsp40. These data suggest a role for chaperones in pathologies involving  $\alpha$ synuclein in humans, such that Hsp70 may be a critical part of the neuronal arsenal that mitigates  $\alpha$ -synuclein toxicity. An alternative interpretation is that the presence of chaperones in aggregates results in their cellular depletion, due to sequestration, and this loss of chaperone function leads to degeneration.

We present data that implicates the molecular chaperone machinery in the pathogenesis of PD using a Drosophila model. Augmentation of Hsp70 activity in vivo suppresses a-synuclein neurotoxicity, whereas compromising chaperone function enhances a-synuclein-induced dopaminergic neuronal loss. Thus, chaperone machinery in flies helps to protect dopaminergic neurons against degeneration and attenuates the neurotoxic consequences of a-synuclein expression. Hsp70 may mitigate  $\alpha$ -synuclein toxicity by influencing the conformation of  $\alpha$ -synuclein in ways that are not revealed by the morphology of aggregates in Drosophila. Alternatively,  $\alpha$ -synuclein may be toxic because it interferes with chaperone activity, possibly by their sequestration, and it is this effect that is mitigated by added Hsp70. Our findings suggest a role for chaperones in human pathology, because human LBs and LNs in PD and other human synucleinopathies immunostain for Hsp70 and Hsp40. Chaperones may thus play a role in  $\alpha$ -synuclein toxicity, such that augmentation of chaperone stress pathways may be an effective approach in the treatment of several human neurodegenerative diseases including PD.

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24 1:100, Santa Cruz Biotechnology, Santa Cruz, CA), Drosophila Hsp70 [7FB (21), 1:500], ubiquitin (MAB 1510, 1:2000, Chemicon, Temecula, CA), and  $\alpha$ -synuclein [syn303 (26), 1:100 and 1:1000, at the latter dilution, syn303 selectively detects aggregated  $\alpha$ -synuclein; syn514 (26), 1:5, which only detects aggregated  $\alpha$ -synuclein]. Sections were incubated overnight with primary antibody at 4°C, followed by secondary antibody, Avidin-Biotin Complex incubation (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA), and developing with 3,3'-diaminobenzidine. For each data point, complete serial sections from three to five individual brains were examined. Similar results were seen in at least five independent experiments. No sex differences were noted. The extent of neuron loss was less than previously reported (7), even using the same transgenic lines. We did not see effects of a-synuclein on climbing behavior

- Supplementary data are available at Science Online at www.sciencemag.org/cgi/content/full/1067389/ DC1.
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Germline Transmission and Tissue-Specific Expression of Transgenes Delivered by Lentiviral Vectors

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Single-cell mouse embryos were infected in vitro with recombinant lentiviral vectors to generate transgenic mice carrying the green fluorescent protein (GFP) gene driven by a ubiquitously expressing promoter. Eighty percent of founder mice carried at least one copy of the transgene, and 90% of these expressed GFP at high levels. Progeny inherited the transgene(s) and displayed green fluorescence. Mice generated using lentiviral vectors with muscle-specific and T lymphocyte-specific promoters expressed high levels of GFP only in the appropriate cell types. We have also generated transgenic rats that express GFP at high levels, suggesting that this technique can be used to produce other transgenic animal species.

The ability to introduce and express exogenous genes of interest in animals has become an indispensable tool to modern biologists (1). Transgenic mice are currently generated by pronuclear injection; however, this technique is still relatively inefficient, technically demanding, costly, and impractical in most other animal species. Another approach to transgenesis is to use retroviruses as gene delivery vehicles because they are able to stably integrate into the genome of cells. However, the generation of transgenic animals with oncoretroviruses such as the Moloney murine leukemia virus (MoMLV) is impractical because silencing of the provirus during development results in low to undetectable levels of transgene expression (2, 3).

Lentiviruses are a class of retroviruses that cause chronic illnesses in the host organisms they infect. Among retroviruses, lentiviruses have the distinguishing property of being able to infect both dividing and nondividing cells, and this ability has led to their development as gene delivery vehicles (4).

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To determine whether, in contrast to oncoretroviruses, lentiviruses might be immune to developmental silencing, we used lentiviralbased vectors to generate transgenic mice and rats.

The lentiviral backbone used in these experiments is based on a self-inactivating vector described previously (5, 6) (Fig. 1, top). The vector was engineered to carry an internal promoter driving the GFP reporter gene. After testing several promoters, the human ubiquitin-C promoter was found to provide the most reliable expression across different cell types and was selected for subsequent experiments (7-11). To increase the level of transcription, the woodchuck hepatitis virus posttranscriptional regulatory element (WRE) was inserted downstream of GFP (12). To increase the titer of the virus, the human immunodeficiency virus-1 (HIV-1) flap element (13) was inserted between the 5' long terminal repeat (LTR) and the human ubiquitin-C internal promoter to generate the viral vector called FUGW (see Fig. 1, top). Viruses were pseudotyped with the vesicular stomatitis virus glycoprotein (VSVG) and concentrated by ultracentrifugation to approximately  $1 \times 10^6$  infectious units (I.U.)/µl.

Approximately 10 to 100 pl of concentrated virus was injected into the perivitelline space of single-cell mouse embryos (14-16). After 72 hours in culture, GFP expression was apparent in the blastula- or morula-stage embryos developing from the infected zygotes [see supplementary data (17)]. Embryos were implanted into pseudopregnant females and were carried to term (15). In an initial trial, Southern blot analysis showed that 14 (82%) of 17 founder animals carried at least one copy of the integrated transgene (18). GFP fluorescence, indicating expression from the transgene, was seen in the paws, tails, and face of 13 (76%) of these founder animals (18) (Fig. 1, A and B). In a second trial, 49 (87.5%) of 56 founder animals carried at least one copy of the transgene, and 45

**Table 1.** Embryo viability and rates of implantation, transgenesis, and expression. M, mouse; FUGW, ubiquitin C promoter-GFP; FMHGW, myogenin promoter-H2B-GFP; PV, perivitelline injection; CI, viral co-incubation. The range and

(80%) of the founders expressed GFP (Table 1). All GFP-positive animals carried an integrated provirus, and all animals with two or more copies of the provirus expressed the transgene at levels detectable by direct viewing of GFP fluorescence. The intensity of GFP fluorescence correlated positively with copy number, as estimated qualitatively (17). All major tissues and organs, including skin, bone, skeletal muscle, cardiac muscle, lung, liver, thymus, spleen, stomach, intestine, kidney, brain, retina, and gonads, were GFP-positive (see Fig. 1, C through F, for a representative data set).

The delivery of the virus by injection into the perivitelline space yielded transgenics with high efficiency; however, the number of integrated proviruses in the genome varied substantially from animal to animal, ranging from 0 to more than 20 (17). A likely source of this variability is difficulty in controlling the volume of virus delivered into the perivitelline space during the injection. As an



**Fig. 1.** GFP is expressed in embryos and the tissues of adult mice derived from the infection of zygotes with the FUGW lentiviral vector. (Top) Diagram of the lentiviral vector FUGW used to generate transgenic mice and rats. Only the relevant portions of the plasmid are shown. All vectors have the CMV enhancer substituted for the U3 region of the 5' LTR (pCL configuration) to maximize expression of viral RNA genomes during transient transfection (29).  $\Delta$ U3 denotes a deletion in the U3 region of the 3' LTR that renders the 5' LTR of the integrated provirus transcriptionally inactive (5). The positions of the restriction sites Pst I and Bam HI used for Southern blot analysis of proviral copy number are indicated. (Bottom) Expression of GFP in the (A) face, (B) paw, (C) brain, (D) heart, (E) liver, and (F) kidney of a transgenic founder. The animal shown here carried eight proviral insertions. The animal was killed at 6 weeks of age by an overdose of anesthesia, intracardially perfused with fixative, and viewed immediately under a fluorescent dissecting microscope. A wild-type animal, identically prepared and photographed, is included for comparison. BF, brightfield photograph; WT and TG, fluorescent images of wild-type control and transgenic animals, respectively.

average number of proviral insertions for each experimental group is determined only from those animals that are transgenic, that is, the animals that carry one or more copies of the transgene.

Animal	Viral construct	Method	Virus conc. ×10³ (I.U./µl)	No. embryos			No. animals			No. copies	
				Treated	Viable	Implanted	Born	Transgenic	Expressing	Average	Range
 M	FUGW	PV (trial 1)	10 <sup>3</sup>	117	81	78	17	14	13	6.2	1–12
М	FUGW	PV (trial 2)	10 <sup>3</sup>	153	150	119	56	49	45	9	1–21
м	FUGW	`cı ´	20	45	_*	29	5	5	5	7.2	2–12
М	FUGW	CI	4 (trial 1)	25	_*	18	7	5	5	3.8	2–7
М	FUGW	CI	4 (trial 2)	120	_*	59	11	8	7	2.6	1–5
М	FUGW	CI	0.8	75	_*	40	8	1	1	1	
М	FMHGW	PV	10 <sup>3</sup>	106	86	74	15†	11	3 of 7‡	4.8	2–15
Rat	FUGW	PV	10 <sup>3</sup>	233	210	130	22	13	9	3.3	1–7

\*See (16). †Six founder embryos were recovered at 11.5 dpc (see text). ‡GFP expression can be determined only after sacrificing these animals. Data is unavailable from animals kept for breeding.

alternative procedure, we removed the zona pellucidae and incubated the denuded embryos with the lentiviral suspension at 2  $\times$  10<sup>4</sup> I.U./µl, 4000 I.U./µl, and 800 I.U./µl (16). Embryos were cultured in vitro for approximately 3 days to the morula or blastocyst stage, when they were implanted into the uterus of timed pseudopregnant females (15). Denuded embryos were delayed in their development in vitro with respect to their untreated counterparts; furthermore, the rate of implantation was lower than that of virusinjected embryos implanted with intact zona pellucidae (18 versus 38%). All animals developing from embryos incubated with 2  $\times$ 10<sup>4</sup> I.U./µl carried at least six proviral integrations, two (28.6%) of seven animals developing from embryos incubated with 4000 I.U./µl carried one or two copies of the provirus, and one of eight animals derived from embryos incubated with 800 I.U./µl carried the transgene. A second trial with 4000 I.U./ µl gave comparable results (Table 1). Although there is still some nonlinearity and irreproducibility, this method of virus delivery allows for better control of the number of proviral integrations per genome. Furthermore, incubating embryos in a virus-containing solution is a process that requires no specialized equipment and may be easier for many laboratories that wish to use this technique.

Founders carrying transgene(s) transmitted most of them to a fraction of their progeny (18) (Fig. 2). The bands corresponding to the proviral insertions characteristic of the founder animals segregated among the progeny. In the Southern blots of the founder animals, we occasionally observed animals with bands of varying intensity, suggesting they were genetic mosaics (see arrow in Fig. 2). In contrast, the intensity of the bands corresponding to the insertions in the progeny was uniform. Furthermore, ubiquitous GFP expression similar to that of the founder animals was observed in transgenic F<sub>1</sub> progeny, indicating that the provirus was not inactivated through one round of gametogenesis and development (17). All animals carrying two or more insertions of the FUGW provirus expressed GFP at levels detectable by direct fluorescence. However, among transgenic lines carrying one proviral insert, approximately half expressed the transgene at levels detectable by direct fluorescence (Fig. 2). In one single-insertion line in which GFP expression was not observed by direct viewing, GFP was detectable by Western blot analysis in some tissues (brain, testes), but not in others (heart, lung, liver, kidney, spleen, skeletal muscle) (17, 18). This suggests that the specific genomic locus into which an individual provirus has integrated may affect the transcriptional activity of some transgenes delivered by this method.

To determine whether lentiviral vectors could be used to express genes in a tissuespecific manner, we engineered a viral vector, FMHGW, in which a histone 2B-GFP (H2B-GFP) fusion gene was driven by the myogenin promoter, the activity of which is specific to skeletal muscle (19). The H2B-GFP reporter was used to concentrate the fluorescence in the nuclei, making the signal more intense (20, 21). Transgenic animals were generated with the FMHGW viral vector by delivering the lentivirus into the perivitelline space of single-cell embryos, as described above (16). Two of the six embryos recovered at day 11.5 of pregnancy showed GFP fluorescence in the paraxial and cephalic somites, limb buds, and extraocular muscles in the pattern expected for the myogenin protein muscle (19) (Fig. 3). Immunofluorescence of frozen tissue sections with an antibody raised against GFP showed that expres-

Fig. 2. The F<sub>1</sub> progeny inherit the proviral transgene(s) from the transgenic founders. Southern blot analysis of proviral transgene insertions in the F1 progeny of three transgenic founders. Founder mouse 14 was generated by FUGW perivitelline injection, whereas founder mouse 18 was generated by FUGW viral co-incubation. Genomic DNA from each animal was digested with Pst I and hybridized with a GFP+WRE probe. The numbered lane in each group is the founder animal, and the lettered lanes represent the F1 progeny resulting from outcrossing that founder animal to a wild-type animal. The arrow indicates a band of lower intensity in founder mouse 14 that cor-

sion was limited to the nuclei of cells in the skeletal muscle lineage, whereas cells of the skin, cartilage, neural tube, heart, lung, and intestines were negative (18) (Fig. 3, C and D). Southern blot analysis of these embryos showed that although all six embryos were transgenic, only those animals carrying six or more copies of the proviral insert expressed at levels detectable by direct GFP fluorescence (17). Fifteen-day-old animals derived from FMHGW-infected zygotes showed GFP fluorescence in the nuclei of skeletal muscle in the tongue, limbs, chest, and jaw but not in cardiac or smooth muscle or other nonmuscle tissues examined (17), reflecting the known specificity of myogenin expression. F<sub>1</sub> progeny from three independent founders expressed H2B-GFP exclusively in the skeletal muscle lineage. Furthermore, progeny carrying as few as one FMHGW proviral integrant expressed H2B-



responds to a proviral insertion that was not transmitted to the progeny in this litter, likely due to low or no germline contribution (see text). Plus signs above each lane indicate GFP expression in that animal detectable by direct viewing of the live animal under a conventional fluorescent microscope.

Fig. 3. Lentiviral vectors carrying the myogenin promoter (FMHGW) direct expression of a histone-GFP fusion protein to the skeletal muscle lineage. (A) H2B-GFP is expressed in the somites and emerging muscles of the limb buds, eye, and jaw in an 11.5days post coitum (dpc) embryo derived from FMHGW-injected zygotes. (B) Higher magnification view of (A) showing the boundaries between somites. (C and D) Immunofluorescence against GFP in tissue sections of an 11.5 dpc FMHGW-derived animal shows specific staining for GFP in the somites (arrows). The signal from the rhodamine-conjugated secondary antibody is pseudocolored in yellow for clarity. Sections are counterstained with the



nuclear dye Hoechst 33342, and the images are overlayed. Image in (C) corresponds to a coronal section in the lumbar region showing the localization of GFP immunofluorescence to the somites and its exclusion from flanking skin and cartilage. Image in (D) is a frontal section through the cervical region showing the localization of GFP immunofluorescence to the somites on either side of the neural tube (nt).

GFP in the appropriate tissue types at high levels detectable by direct viewing with a fluorescent microscope (17).

In addition, a viral vector containing GFP driven by the T lymphocyte–specific proximal *lck* was also delivered by perivitelline injection to mouse embryos, resulting in mice expressing GFP exclusively in the thymus (*17*).

To determine whether exogenous genes introduced into single-cell embryos via lentiviral vectors could be expressed in mammals other than mice, the FUGW lentivirus was delivered to single-cell rat embryos by perivitelline injection, essentially as described above (16). Introduction of exogenous genes into the germ line of rats has, so far, been difficult and inefficient (22). Of the 22 founder pups born, 13 (59.1%) carried one or more proviral insertions as determined by Southern blot analysis (17), and 9 (40.9%) expressed GFP at levels detectable by direct viewing of the skin under a fluorescent microscope (Fig. 4 and Table 1). GFP-positive founders were crossed to wild-type animals, and F, progeny rats carrying as few as one copy of the provirus expressed GFP as determined by direct viewing with a fluorescent microscope (17), showing that a GFP-expressing transgene is not silenced by transmission through the germ line.

The technique of transgenesis by lentiviral gene delivery to one-cell embryos overcomes many of the limitations of pronuclear injection (23). It is more efficient (Table 1), less invasive to the embryos, more cost-effective, and technically less demanding. Delivering lentiviruses by coincubation with denuded embryos obviates the need for micromanipulation and may be an easier option for many laboratories wanting to make transgenic animals. Furthermore, because the lentiviral delivery technique does not require visualization of the pronucleus, it has the potential to be extended to other animal species. For example, using this technique we have generated transgenic rats, a species into which the introduction of exogenous genes has so far been difficult and inefficient (22). Because the VSVG protein that mediates viral entry finds receptors on the cells of all vertebrates, including primates, we believe lentiviral transgenesis will be easily extended to all mammals. Furthermore, it may allow for the germline introduction of exogenous genes into birds, a class of animals for which no satisfactory method exists for creating transgenics (24). The combination of a high efficiency of transgenesis, low cost, and scalability for high throughput allows for other applications of the lentiviral transgenesis technique such as finescale mutagenesis of regulatory sequences, large-scale insertional mutagenesis screens, or gene trapping.

The method of lentiviral transgenesis has some limitations. First, as with other retroviral vectors, use of these lentiviruses for purposes of transgenesis may be limited either by sequences that can decrease viral titers. such as splicing or polyadenylation signals in the transgene, or by insertion of transgenes larger than 10 kilobases between the LTRs. Second, in cases where multiple proviral insertions are necessary for high levels of expression, the establishment of pure breeding transgenic lines might be complicated by the independent segregation of the proviruses. However, as we have shown for the ubiquitin and myogenin promoters, one copy can be sufficient for high-level GFP expression detected by direct viewing with a fluorescent microscope. We expect that the method of lentiviral transgenesis will complement pronuclear injection, as it will be preferred for some applications and inappropriate for others. For instance, if an experiment requires the introduction of large pieces of DNA or the use of a promoter that is known to require many copies for expression, pronuclear injection would be the preferred method for the





**Fig. 4.** Ubiquitous GFP expression in rats derived from the delivery of lentiviruses to single-cell embryos in vitro. Brightfield and fluorescent images of the paws of newborn rats derived from FUGW-injected embryos. The transgenic pup, carrying four copies of the proviral insert, expresses GFP in the paw, as well as all tissues and organs examined. A littermate carrying no transgene is included for comparison.

generation of such transgenic mice (23).

Our results revealed that, in contrast to oncoretroviruses, lentiviral vectors are not developmentally silenced. One possible explanation is that the transcriptionally inactive state of the lentiviral LTRs, a consequence of the self-inactivating deletion in the U3 region of the 3' LTR of the vector (5), might allow the provirus to escape epigenetic mechanisms of silencing. Alternatively, oncoretroviralbased and lentiviral vectors might have different intrinsic susceptibilities to silencing, perhaps as a consequence of their contrasting lifestyles. Whereas oncoretroviruses rely on germline transmission as one form of spreading, lentiviruses rely exclusively on horizontal and non-germline vertical transfer (25). Thus, organisms might have evolved mechanisms to suppress the activity of endogenous oncoretroviruses that would otherwise lead to their parasitic expansion in the genome (26). In contrast, such mechanisms might not target lentiviral sequences because endogenous lentiviruses have not been found in any mammalian genome (25).

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- Several constitutive promoters—the human cytomegalovirus (CMV) enhancer/promoter, human CMV enhancer/chicken β-actin promoter, mouse pgk promoter, and the human ubiquitin-C promoter—were tested in vitro on 293T human fibroblasts, 3T3 murine fibroblasts, mouse primary lymphocytes, and rat primary glial cells (8–11).
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lentiviruses were delivered to them on the same day of collection.

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- 17. Supplementary material is available at *Science* Online at www.sciencemag.org/cgi/content/full/1067081/ DC1.
- 18. Animals were analyzed for the presence of the transgene and the number of copies of the transgene by Southern blot analysis with a probe that hybridizes to both the GFP and WRE sequences. Genomic DNA used for Southern blot analysis was always extracted from the tails of the animals. Expression of GFP was determined by viewing the skin of the animal directly under an inverted fluorescence microscope or a fluorescence dissecting microscope. For higher sensitivity, immunofluorescence and Western blot analysis were performed with a polyclonal antibody raised against GFP (gift of H. Wong, UCLA, Los Angeles, CA).
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