Islet Regeneration During the Reversal of Autoimmune Diabetes in NOD Mice

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Nonobese diabetic (NOD) mice are a model for type 1 diabetes in humans. Treatment of NOD mice with end-stage disease by injection of donor splenocytes and complete Freund's adjuvant eliminates autoimmunity and permanently restores normoglycemia. The return of endogenous insulin secretion is accompanied by the reappearance of pancreatic β cells. We now show that live donor male or labeled splenocytes administered to diabetic NOD females contain cells that rapidly differentiate into islet and ductal epithelial cells within the pancreas. Treatment with irradiated splenocytes is also followed by islet regeneration, but at a slower rate. The islets generated in both instances are persistent, functional, and apparent in all NOD hosts with permanent disease reversal.

The NOD mouse exhibits spontaneous autoimmunity that causes diabetes through destruction of insulin-secreting pancreatic islets. A lymphoid cell–specific proteasome defect in these mice interrupts the presentation of self antigens by major histocompatibility complex (MHC) class I molecules that is required for negative selection of autoreactive naïve T cells (*1, 2*). The proteasome defect also impairs activation of the transcription factor nuclear factor–κB in pathogenic memory T cells, increasing their susceptibility to apoptosis induced by tumor necrosis factor–α (TNF-α) (*3–5*). Reselection of peripheral autoimmune naïve T cells is possible by the introduction of matched MHC class I–self peptide complexes, whereas self-directed autoimmune memory T cells can be reselected by treatment with TNF-α or by the induction of the endogenous TNF-α with complete Freund's adjuvant (CFA) (*5, 6*). Simultaneous treatment of severely diabetic NOD mice with both TNF-α and normal splenocytes partially or fully matched for MHC class I antigens thus restores self-tolerance and eliminates T cells directed against islets, resulting in permanent reversal of established diabetes (*7*). This "cure" is accompanied by the reappearance of insulin-secreting islets in the pancreas which can control blood glucose concentration in an apparently normal manner.

The new pancreatic islets in such treated NOD mice might arise from several sources, either endogenous or donor-derived sources. Donor nonlymphoid cells administered to mice or humans can undergo rare transdifferentiation events (*8–25*), although these findings remain controversial (*26, 27*). Alternatively, the regenerated islet cells in NOD mice might be the products of fusion between donor and host cells, in a mouse model of liver damage (*28, 29*). Such fusion events generate cells with marked chromosomal abnormalities (*30, 31*).

To investigate the origin of the new pancreatic islet cells in NOD mice, we examined the relative abilities of live versus irradiated donor splenocytes to restore normoglycemia (*32*). We injected CFA and either live or irradiated male $CByB6F_1$ mouse splenocytes into severely diabetic NOD females, which were used to ensure the absence of visible islets and insulitis that could obscure dead or dying islets (table S1). We controlled blood glucose concentration with a temporary (40-day) implant of syngeneic islets under the capsule of one kidney, which improved treatment efficacy. Similar to our previous data (*7*), six (67%) of the nine NOD mice that received live splenocytes remained normoglycemic after removal of the islet implant (Fig. 1A). In contrast, none of the eight animals that received irradiated splenocytes remained normoglycemic; they all rapidly developed severe hyperglycemia. (See supporting online text and fig. S1.) In another experiment, the islet transplant was maintained for 120 days before graft removal, to allow a longer period for islet regeneration. Of the 12 NOD mice that received live splenocytes, 11 (92%) remained normoglycemic for >26 weeks after disease onset or beyond 52 weeks of age. Moreover, 11 (85%) of the 13 animals that received irradiated splenocytes also remained normoglycemic for >27 weeks after

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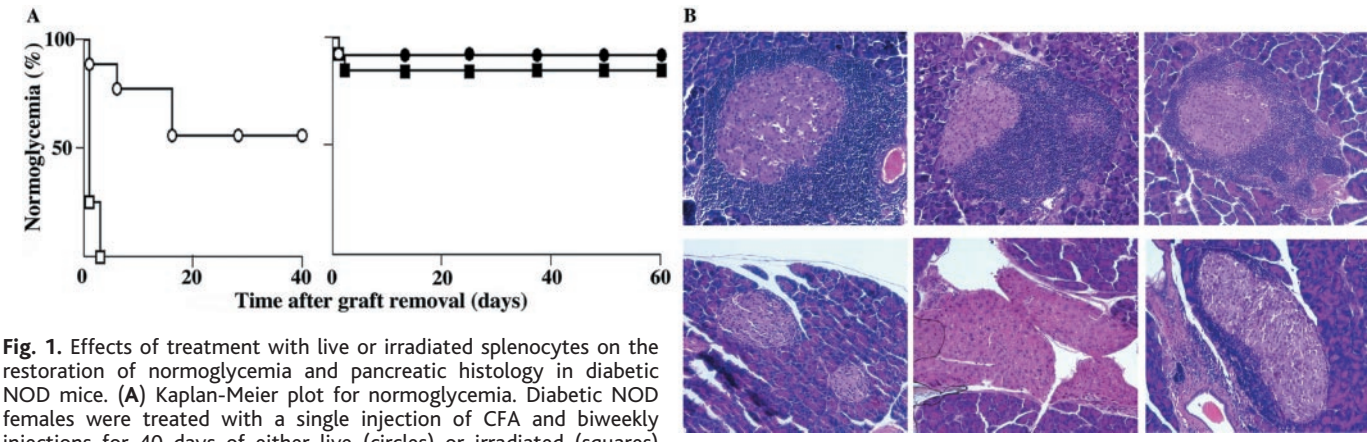


Fig. 1. Effects of treatment with live or irradiated splenocytes on the restoration of normoglycemia and pancreatic histology in diabetic NOD mice. (**A**) Kaplan-Meier plot for normoglycemia. Diabetic NOD females were treated with a single injection of CFA and biweekly injections for 40 days of either live (circles) or irradiated (squares) splenocytes from $CByB6F_1$ males. Syngeneic female islets transplanted subrenally at the onset of treatment were removed after either 40 days (left panel) or 120 days (right panel). Blood glucose concentration was monitored at the indicated times after islet graft removal, and the percentage of animals that remained normoglycemic was plotted. Data are from 9 and 8 (left panel) or from 12 and 13 (right panel) animals that received live or irradiated splenocytes, respectively; $P = 0.0002$ (left panel), $P = 0.68$ (right panel) for comparison between the two treatment groups. (**B**) Pancreatic histology. Three NOD mice successfully treated with either irradiated (top panels) or live (bottom panels) splenocytes were killed ~9 weeks after removal of the 120-day islet graft. Sections of each pancreas were stained with hematoxylin and eosin. Pronounced peri-insulitis was apparent only in the NOD mice treated with irradiated cells.

disease onset or beyond 48 weeks of age (Fig. 1A, table S2). Both live and irradiated splenocytes could thus effect permanent disease elimination, and with a longer period of imposed normoglycemia greatly increasing the frequency of functional islet recovery in both groups.

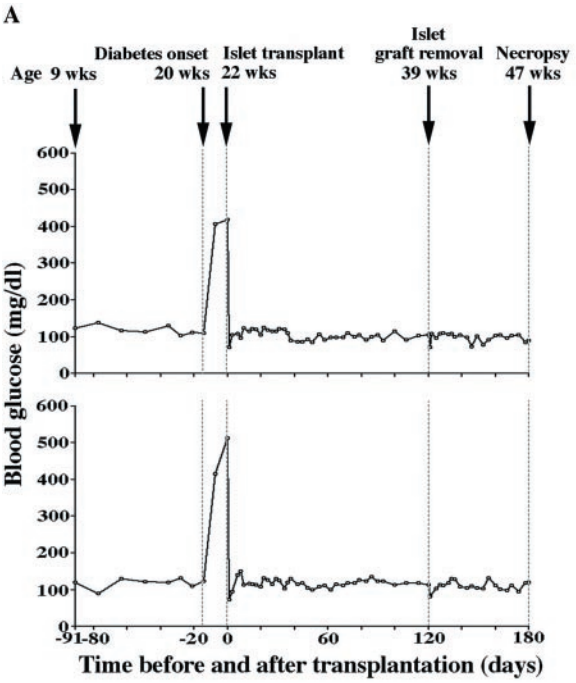
Mice treated with irradiated splenocytes that exhibited persistent normoglycemia for ~9 weeks after nephrectomy (table S2) exhibited the reappearance of pancreatic islets without invasive insulitis (autoreactive cells within the islets) but with pronounced peri-insulitis (circumferential lymphoid cells that do not progress to invasion) (Fig. 1B, table S3). In contrast, the pancreas of NOD mice that received live splenocytes exhibited the reappearance of pancreatic islets without invasive insulitis and with minimal or no peri-insulitis. The live splenocytes were thus necessary for reduction of peri-insulitis but not for the growth of new islets. Functionally, the restoration of long-term normoglycemia was indistinguishable between animals with disease reversal due to live or irradiated splenocytes.

We next tested mice that had been treated with live or irradiated splenocytes for the presence of live donor cells in blood, pancreas, and other tissues. Peripheral blood lymphocytes (PBLs) from NOD mice treated with irradiated $CByB6F_1$ splenocytes showed only background staining for H-2K^b (an indicator of live donor cells), indicating that no donor hematopoietic cells remained (Table 1; table S2 and fig. S2). In contrast, 4.4 to 12.6% of PBLs from NOD mice treated with live $CByB6F_1$ splenocytes were of donor origin. PBLs from an untreated NOD mouse contained only cells expressing H-2K^d, and those from a $CByB6F_1$ mouse contained exclusively cells coexpressing H-2K^b and H-2K^d. NOD mice treated with live splenocytes thus exhibited a persistent low level of blood chimerism with semiallogeneic cells that was achieved without continuous immunosuppression or lethal preconditioning.

Flow cytometry also revealed between 3.5 and 4.7% of cells positive for both H-2K^d and H-2K^b among splenocytes from five NOD mice successfully treated with live splenocytes; this confirmed the persistence of donor $CByB6F_1$ cells in all recipients (Table 1). Splenocytes from an untreated control NOD mouse showed a background level of 0.3% double-positive staining for both markers. $CByB6F_1$ donor splenocytes also contributed to T cells (CD3$^+$), monocytes (CD11b), and B cells (CD45R$^+$) (data not shown).

We then examined parenchymal tissues for chimerism by fluorescence in situ hybridization (FISH) analysis for detection of the Y chromosome of the male donor cells in two long-term normoglycemic NOD mice (Fig. 2A). Staining of serial pancreatic sections with antibodies to insulin revealed a homogeneous insulin content in the large islets (Fig. 2B; Table 1), consistent with the restored normoglycemia. Single-color FISH analysis revealed abundant nuclei positive for the Y chromosome within the islets (Fig. 2B; Table 1). In contrast, the exocrine portions of the pancreas were largely devoid of male cells. In these five animals, 29 to 79% of islet cells were of donor origin. No islets solely of host origin were detected.

Male donor cells also contributed to the epithelium of NOD female pancreatic ducts, although the distribution of male cells in this tissue was more heterogeneous than was that in islets (Fig. 2B, Table 1). Among the five treated NOD females studied in detail, 33 to 75% of the ducts contained genetic material of male origin. Ducts purely of host origin were never associated with an adjacent islet containing male cells. The proportion of male



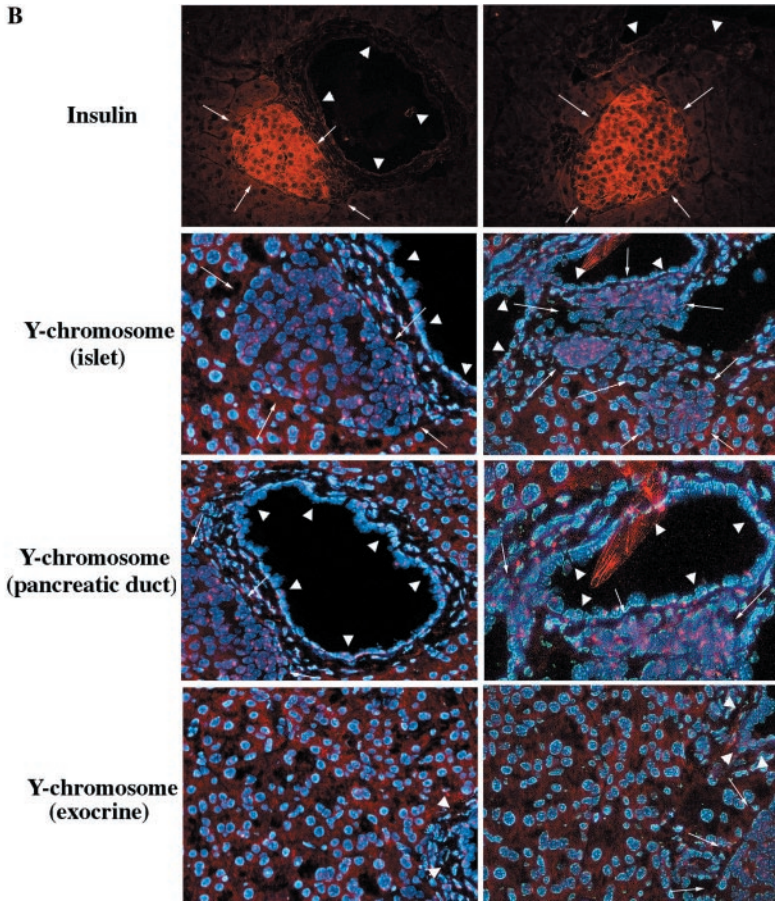


Fig. 2. Long-term restoration of normoglycemia and the direct contribution of live donor splenocytes to islet regeneration in successfully treated NOD female mice. (**A**) Blood glucose concentrations during the lifetime of two NOD females (top and bottom, nos. 789 and 790 in Table 1, respectively) successfully treated with CFA and $CByB6F_1$ male splenocytes, as well as with a temporary subrenal transplant of syngeneic islets. (**B**) Immunofluorescence and FISH analyses of serial pancreatic sections from the successfully treated NOD females 789 (left) and 790 (right). The two top panels show immunofluorescence staining of islets with antibodies to insulin (red); the three pairs of images below show FISH signals obtained with a Y chromosome–specific probe (pink dots) and nuclear staining with DAPI (blue) in sections containing islets (arrows), pancreatic ducts (arrowheads), and exocrine pancreas, respectively.

cells in the pancreatic ducts of the five NOD mice ranged from 9 to 41%. Single-color FISH analysis revealed abundant nuclei positive for the Y chromosome within both the exocrine and endocrine portions of the pancreas of control C57BL/6 male mice, whereas the pancreas of control C57BL/6 females was devoid of the Y chromosome (fig. S3A). We detected no evidence of engraftment, transdifferentiation, or fusion of male splenocytes in organs including the brain, liver, and kidney of treated NOD females (fig. S4B), which suggests that, in addition to the low level of hematopoietic chimerism observed, the marked incorporation of donor cells was selective for the diseased pancreas.

To examine whether the new islet cells arose by fusion of donor cells with endogenous islet cells, we evaluated >800 nuclei in β cells as well as >800 nuclei in adjacent exocrine tissue of the five treated NOD females studied in detail (fig. S3B and Table 2). At three scanning focal lengths, none of the regenerated cells within the islets was enlarged compared with the adjacent native exocrine cells. The β-cell nuclei were of normal size and did not contain multiple nucleoli. These observations suggest that the regenerated islet cells were not the products of fusion between donor splenocytes and endogenous dying or injured β cells, since hybrid cells contain markedly enlarged nuclei and multiple nucleoli and are tetraploid (*30, 31*). We cannot, however, exclude the possible occurrence of fusion followed by rapid and complete expulsion of host chromosomes.

We further examined the ploidy of the sex chromosomes of cells in the regenerated islets of successfully treated NOD mice by two-color FISH analysis with a Y chromosome–specific probe linked to fluorescein isothiocyanate (FITC) (green) and an X chromosome–specific probe conjugated with cyanine 3 (Cy3) (red). A NOD female treated with live male splenocytes exhibited only rare if any islet cells with an apparent XXY or XXXY genotype (Fig. 3). A normal complement of sex chromosomes was also observed in pancreatic duct epithelial cells. These results also indicate that the regenerated islet cells were not likely to be the result of fusion between donor male cells and host female cells. None of the islet cell nuclei examined in a NOD female treated with irradiated male splenocytes contained a detectable Y chromosome; rather, each nucleus yielded two red signals, corresponding to a genotype of XX (Fig. 3). Two-color FISH analysis of the

Table 1. Frequency and extent of donor engraftment in five NOD female mice with stable disease reversal after treatment with CFA and biweekly injections for 40 days with live $CByB6F_1$ male splenocytes. Subrenal islet transplants were removed by nephrectomy after 120 days. The donor cell composition of PBLs and splenocytes was determined by flow cytometry with antibodies specific for $H\text{-}2K^b$ or $H\text{-}2K^d$. That for pancreatic islets, exocrine cells, and ductal epithelial cells was determined by FISH with a Y chromosome–specific probe. The percentages of islets and pancreatic ducts containing donor cells are also shown.

Parameter	NOD recipient					Control NOD
	744	788	789	790	838	
Age (weeks)	57	46	47	47	39	38
Lymphoid system						
Donor PBLs (%)	4.4	5.8	12.6	8.3	10	0.3
Donor splenocytes (%)	3.5	4.7	4.0	3.5	3.9	0.3
Islets						
Having donor cells (%)	100	100	100	100	100	3
Percent donor cells (%)	29	41	79	37	46	2
Pancreas						
Donor pancreatic exocrine cells (%)	2	1	2	3	2	2
Having donor cells in ducts (%)	33	66	75	50	50	1
Percent donor cells in ducts (%)	9	15	41	35	11	1

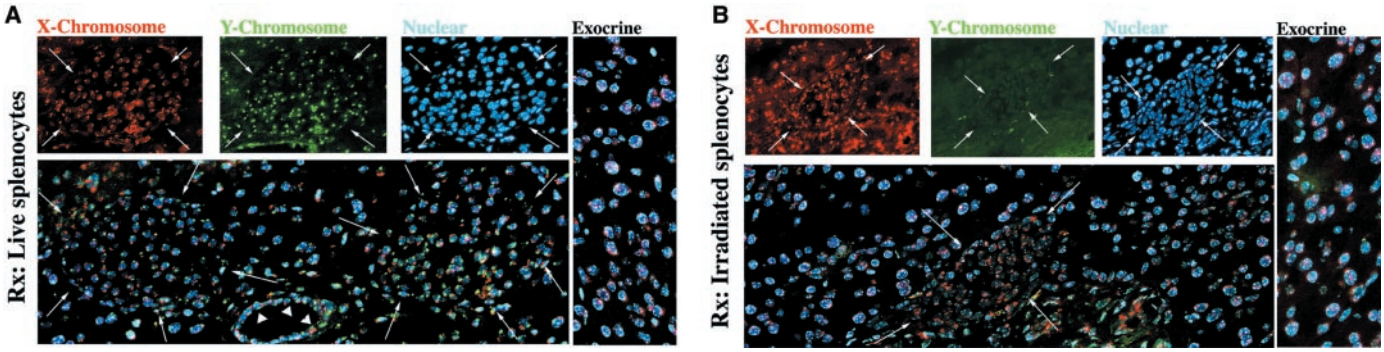


Fig. 3. Two-color FISH analysis of sex chromosomes in the pancreas of NOD female mice successfully treated with either live or irradiated male splenocytes. Pancreatic sections from NOD females treated with live (**A**) or irradiated (**B**) $CByB6F_1$ male splenocytes were subjected to nuclear staining with DAPI (blue) and to FISH analysis with a Cy3-conjugated X chromosome–specific probe (red dots) and an FITC-conjugated Y chromosome–specific probe (green dots). Purple represents overlap of Cy3 and DAPI signals. Arrows indicate outline of islets.

Table 2. Comparison of nuclear diameter between β cells and exocrine cells in a successfully treated NOD mouse (789 in Table 1). Data are means ± SD for the indicated number of nuclei examined. The *P* values for comparisons between islet and exocrine cells were obtained by Student's *t* test.

Scanning position	β cells (insulin positive)		Exocrine cells (insulin negative)		*P*
	No.	Nuclear diameter (pixels)	No.	Nuclear diameter (pixels)	
−3 μm	89	29.1 ± 4.0	91	36.0 ± 6.3	0.554
0 μm (standard)	85	33.0 ± 4.3	91	36.8 ± 6.5	0.054
+3 μm	112	32.0 ± 5.8	102	33.8 ± 7.0	0.147

pancreas of untreated female and male NOD mice revealed that, although this methodology can yield false-negative data (female nuclei with no red signal or only one red signal), it almost never yielded false-positive data (a green signal in the nucleus of a female cell or two green signals within an individual male nucleus) (fig. S4A).

Embryonic mesenchymal cells are able to differentiate into endothelial and endoderm cells, and they lack surface expression of CD45 (*33–37*). To examine whether nonlymphoid stem cells contribute to the regeneration of pancreatic islets in NOD mice, we injected 12-week-old NOD females with live $CD45^+$, $CD45^-$, or unfractionated $CByB6F_1$ splenocytes expressing enhanced green fluorescent protein (GFP). These experiments differed from our previous experiments: (i) The NOD females were prediabetic (with residual islet function but with active autoimmunity) at the start of treatment; (ii) they did not receive an islet graft; (iii) the number of cells injected was reduced to 4×10^5 to 5×10^5 administered four times over 2 weeks; and (iv) the regrowth of islets was monitored by detection of GFP immunofluorescence. All of the NOD females that received $CD45^+$, $CD45^-$, or unfractionated splenocytes remained normoglycemic during the monitoring period, whereas all untreated NOD littermates ($n = 10$) became diabetic.

Immunoblot analysis of cytoplasmic extracts prepared from the pancreas of NOD mice revealed more GFP for those treated >120 days earlier with $CD45^-$ splenocytes than for those treated with $CD45^+$ splenocytes (Fig. 4A). In addition, the pancreas of the prediabetic NOD females treated with either $CD45^-$ or unfractionated splenocytes contained islets positive for the GFP marker (Fig. 4B). Furthermore, the newly generated islets lacked invasive lymphocytes and were associated with minimal or no peri-insulitis, as revealed by costaining for insulin and CD45 (Fig. 4C). The proportion of islets containing cells of donor origin was markedly smaller for prediabetic NOD hosts treated with $CD45^-$ or unfractionated splenocytes than for severely diabetic NOD females treated with unfractionated splenocytes. This was as expected because the pancreas of the prediabetic mice still contained endogenous islets affected by peri-insulitis. Treatment of prediabetic animals with precursor cells thus rescues damaged islets and also promotes de novo islet regeneration. The islets of prediabetic NOD females treated with $CD45^+$ splenocytes were negative for the expression of GFP (Fig. 4B). Moreover, similar to the islet regeneration observed in severely diabetic NOD mice treated with irradiated splenocytes, the newly appearing islets in prediabetic NOD females treated with $CD45^+$ splenocytes were devoid of invasive insulitis but exhibited pronounced periinsulitis (Fig. 4C). The donor $CD45^+$ splenocytes, although essential for disease reversal, do not include cells able to participate directly in islet generation.

Overall, our data indicate that live male splenocytes injected into female diabetic NOD mice can provide cells ($CD45^-$ mesenchymal precursor cells) for the reconstitution of functional islets. The donor splenocytes also contribute to reversal of autoimmunity, possibly by reeducating naïve T cells through presentation of matched MHC class I molecules and self antigens, yielding islets almost free of any signs of autoimmunity. In contrast, diabetic NOD mice treated with irradiated splenocytes exhibit long-term restoration of normoglycemia as a result of islet regeneration but with markedly slower kinetics than those apparent in NOD animals treated with live splenocytes. Thus, adult diabetic NOD mice contain endogenous precursor cells capable of giving rise to new islet structures after the underlying autoimmune disease is eliminated. These syngeneic islets appear to function normally but succumb to stable, nonprogressive peri-insulitis.

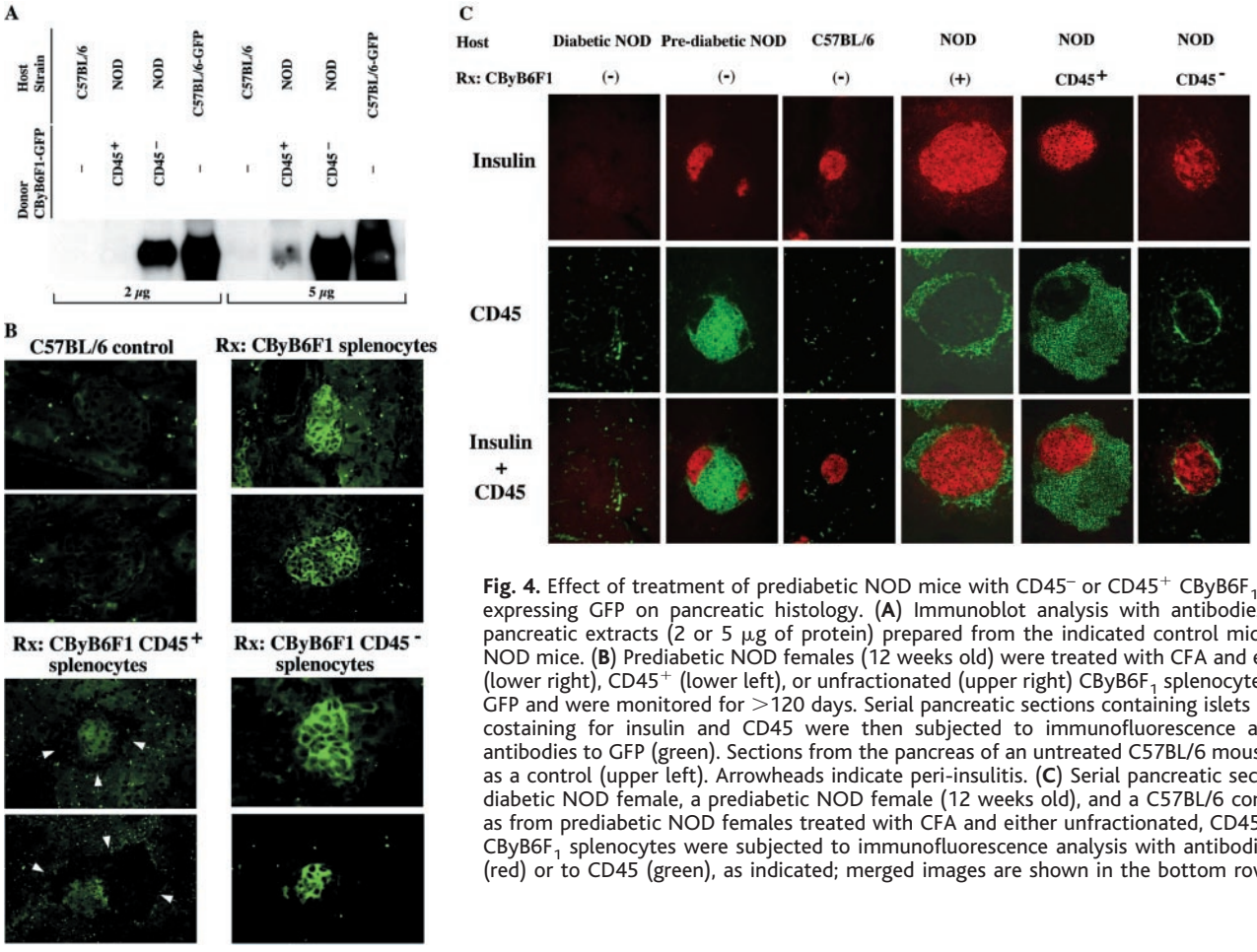


Fig. 4. Effect of treatment of prediabetic NOD mice with $CD45^-$ or $CD45^+$ $CByB6F_1$ splenocytes expressing GFP on pancreatic histology. (**A**) Immunoblot analysis with antibodies to GFP of pancreatic extracts (2 or 5 μg of protein) prepared from the indicated control mice or treated NOD mice. (**B**) Prediabetic NOD females (12 weeks old) were treated with CFA and either $CD45^-$ (lower right), $CD45^+$ (lower left), or unfractionated (upper right) $CByB6F_1$ splenocytes expressing GFP and were monitored for >120 days. Serial pancreatic sections containing islets identified by costaining for insulin and CD45 were then subjected to immunofluorescence analysis with antibodies to GFP (green). Sections from the pancreas of an untreated C57BL/6 mouse are shown as a control (upper left). Arrowheads indicate peri-insulitis. (**C**) Serial pancreatic sections from a diabetic NOD female, a prediabetic NOD female (12 weeks old), and a C57BL/6 control, as well as from prediabetic NOD females treated with CFA and either unfractionated, $CD45^+$, or $CD45^-$ $CByB6F_1$ splenocytes were subjected to immunofluorescence analysis with antibodies to insulin (red) or to CD45 (green), as indicated; merged images are shown in the bottom row.

Our findings with the NOD mouse may have implications for treatment of diabetes or other autoimmune diseases in humans. Both the ability of an exogenous population of adult spleen cells to correct established diabetes permanently and the presence of an endogenous population of NOD mouse stem cells able to give rise to new islets suggest that therapies to reverse autoimmune diabetes need not incorporate transplantation of exogenous adult islets. The use of fresh splenocytes eliminates the need for cell culture manipulations that transform stem cells of fetal or adult origin into malignant precursors or fusion hybrids with an abnormal DNA content. Because the cell donors and hosts are adults this system would preclude ethical issues associated with the use of embryonic stem cells, as well as concerns that the transdifferentiation of embryonic stem cells may be incomplete.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/302/5648/1223/DC1
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A Genetic Screen in *Drosophila* for Metastatic Behavior

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A genetic screen was designed in *Drosophila* to interrogate its genome for mutations sufficient to cause noninvasive tumors of the eye disc to invade neighboring or distant tissues. We found that cooperation between oncogenic Ras^{V12} expression and inactivation of any one of a number of genes affecting cell polarity leads to metastatic behavior, including basement membrane degradation, loss of E-cadherin expression, migration, invasion, and secondary tumor formation. Inactivation of these cell polarity genes cannot drive metastatic behavior alone or in combination with other tumor-initiating alterations. These findings suggest that the oncogenic background of tissues makes a distinct contribution toward metastatic development.

Most cancer fatalities are due to the ability of later stage tumors to metastasize, or form discontinuous secondary foci (*1*). Experiments in mammalian systems have indicated the presence of metastasis suppressor genes that can block some aspects of metastasis (*2*); however, relatively little is known about the genetic alterations sufficient to cause noninvasive tumors to become metastatic. *Drosophila melanogaster* is an important model system for studying cancer biology. Despite some important differences (i.e., the lack of a vascular circulatory system, and therefore of angiogenesis or vascular tumor cell transport), many biological processes related to tumorigenesis and metastasis are well conserved in flies (*3, 4*); hence *Drosophila* could be a model for the genetic basis of metastatic behavior. Using *Drosophila*, the majority of the genome can be interrogated for metastasis-promoting mutations, something vastly more difficult to accomplish in mammalian systems.

To develop a *Drosophila* metastasis model, we wished to (i) induce noninvasive tumors in a defined location, through either expression of an oncogene or inactivation of a tumor suppressor gene; (ii) genetically label these tumor cells with a visible marker such as green fluorescent protein (GFP); and (iii) explore whether additional genetic alterations in these cells could elicit metastatic behavior (i.e., the movement of GFP-labeled tumor cells into different tissues) (Fig. 1A). Our genetic scheme used *eyeless* promoter–driven FLP recombinase expression (*eyFLP*) (Fig. 1B) (*5, 6*). This allowed the introduction of multiple genetic alterations (either loss-of-function mutations or gene overexpression) into GFP-labeled cells specifically in the developing larval eye-antennal imaginal discs. Because tumor cells were genetically produced, mechanical disruption of cells and extracellular matrix was avoided. Also, tumor progression and metastatic behavior could be easily monitored and studied in living flies because GFP-labeled tumor cells could be observed in transparent larvae.

Clones of GFP-labeled wild-type cells were analyzed in the whole bodies of third-instar larvae, pupae, and adults (Fig. 2A; $n > 1000$) (*5*). GFP was observed in the larval eye-antennal imaginal discs as well as in the optic lobes of the brain, but was not detected in other adjacent tissues such as the ventral nerve cord (VNC, arrow in Fig. 2A) (*7*). GFP was also observed in other tissues and occurred in reproducible locations, depending on the particular *eyFLP* transgene used [mostly in the gonad (*5*)].

Alterations in the *Ras* oncogene or the *lats* tumor suppressor gene contribute to tumorigenesis in both flies and mammals (*8–11*). We generated flies with noninvasive tumors by inducing clones of cells either mutant for *lats* or expressing activated *Ras* (*UAS-Ras85D*^{G12V} or "Ras^{V12}", Figs. 1A and 2B; $n > 1000$ for each). In either case, the amount of GFP-marked tissue in the eye-antennal discs was noticeably increased, and most mutant flies died before adulthood. However, GFP-labeled mutant cells were always located in the same areas as in the wild type. Mutant cells were not seen outside of the eye-antennal disc/optic lobe region even after eye disc eversion, suggesting that tissue integrity was not compromised. Thus, this sys-

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