Fetal Pancreas Transplants Are Dependent on Prolactin for Their Development and Prevent Type 1 Diabetes in Syngeneic but Not Allogeneic Mice

Gwladys Fourcade,[1] Bruno M. Colombo,[1,2] Sylvie Grégoire,[1] Audrey Baeyens,[1] Latif Rachdi,[3] Fanny Guez,[3] Vincent Goffin,[3] Raphael Scharfmann,[3] and Benoît L. Salomon[1]

Transplantation of adult pancreatic islets has been proposed to cure type 1 diabetes (T1D). However, it is rarely considered in the clinic because of its transient effect on disease, the paucity of donors, and the requirement for strong immunosuppressive treatment to prevent allogeneic graft rejection. Transplantation of fetal pancreases (FPs) may constitute an attractive alternative because of potential abundant donor sources, possible long-term effects due to the presence of stem cells maintaining tissue integrity, and their supposed low immunogenicity. In this work, we studied the capacity of early FPs from mouse embryos to develop into functional pancreatic islets producing insulin after transplantation in syngeneic and allogeneic recipients. We found that as few as two FPs were sufficient to control T1D in syngeneic mice. Surprisingly, their development into insulin-producing cells was significantly delayed in male compared with female recipients, which may be explained by lower levels of prolactin in males. Finally, allogeneic FPs were rapidly rejected, even in the context of minor histocompatibility disparities, with massive graft infiltration with T and myeloid cells. This work suggests that FP transplantation as a therapeutic option of T1D needs to be further assessed and would require immunosuppressive treatment. *Diabetes* 62:1646–1655, 2013

The conventional treatment of type 1 diabetes (T1D), consisting of the injection of insulin several times a day, alters the quality of life and prevents only imperfectly severe hypoglycemia and vascular complications. It is thus essential to develop alternative treatments. Transplantation of adult pancreatic islets is a simple procedure requiring minor surgery (islets are injected intravenously) that raised clinicians and patients' expectations (1). However, it is rarely considered in the clinic because of the paucity of donors (three cadaveric donors are often required per recipient) and the requirement for long-term immunosuppressive treatments, which have major side effects. In addition, insulin independence is only transient since the transplants do not produce enough insulin after a few years (1). This could be due to either transplant rejection or the lack of embedded stem cells in adult islets, which may be required for long-term renewal of β-cells. The use of fetal pancreases (FPs) as an alternative source of β-cells may provide a solution to these issues.

Transplantation of FPs to replace the function of destroyed β-cells in T1D has been envisioned for decades. Compared with adult pancreatic islets, fetal tissue appears to present several advantages: *1*) after transplantation, FPs continue their development, which is characterized by high proliferative capacity and differentiation into large numbers of mature insulin-producing pancreatic islets. Additionally, the presence of islet precursor cells in the ducts and possibly in immature islets may promote the long-term differentiation of insulin-producing cells (2–4). *2*) Various sources of FPs may be available for transplantation, even though some of them may raise ethical or technological issues at the moment. Human FPs could be obtained from fetuses after voluntary terminations of pregnancy, similar to human fetal cells that have already been used in clinical trials for the treatment of Parkinson or Huntington diseases (5). Xenotransplantation of pig FP could be another interesting option since pig insulin is perfectly functional in humans. Alternatively, insulin-producing cells may be generated from in vitro differentiation of embryonic or adult stem cells (6). *3*) Finally, several studies have shown that FPs at an early stage of development are less immunogenic than FPs from later stages of development or adult pancreases when transplanted across a xenogeneic barrier. Pig FPs from day 28 embryos (E28), just after the initial budding of the pancreas, were accepted in rats or macaques without host immunosuppression, whereas pancreas from E >35 embryos or adult islets were rejected in rats (7,8). Chick FPs from E15 but not from E18 embryos were accepted in rats (9). Human FPs from the first but not the second trimester of pregnancy were accepted in humanized mice (10). Surprisingly, the immunogenicity of FPs across an allogeneic barrier has been studied less extensively and only with transplants from late-stage fetal development. As expected from xenotransplantation studies, these transplants were rejected in a manner similar to adult pancreatic islets. For instance, allogeneic FPs derived from E17 to E18 rat or mouse embryos were rejected within 2 weeks (11–13). Based on previous reports of xenogeneic FP transplantation, we thus hypothesized that FPs from earlier stage (E12) rodent embryos may not be rejected in allogeneic recipients.

In this work, we first determined the minimal number of grafted E12 FPs required to control T1D in syngeneic mice. Contrary to previous studies in which diabetes was chemically induced before transplantation, we addressed this question in an inducible diabetes model, allowing for proper development of FPs into functional islets under

See accompanying commentary, p. 1382.

euglycemic conditions, followed by a selective destruction of endogenous islet β-cells. This is critical because hyperglycemia may impact on FP development (14). We found that as few as two FPs were sufficient to control glucose levels in diabetic mice in a syngeneic context. Interestingly, development of FP into insulin-producing cells was significantly delayed in male compared with female recipients. Finally, we observed that FPs from E12 mouse embryos were fully rejected in various allogeneic contexts.

RESEARCH DESIGN AND METHODS

Mice. C3H/HeNRJ (H-2^k), BALB/c × C3H/HeNRJ F1 (H-2^{dxk}), BALB/c × C57Bl/6 (B6) F1 (H-2^{dxb}), DBA/2 (H-2^d), BALB/c (H-2^d), B6, and BALB/c nude mice (pregnant or not) were obtained from Janvier Laboratories (Le Genest St. Isle, France). Ins-HA BALB/c transgenic mice expressing hemagglutinin (HA) under the control of the insulin promoter (15), TCR-HA BALB/c transgenic mice expressing a T-cell receptor specific for the HA$_{111}$ epitope presented by I-E^d (15) and neurogenin3-deficient mice (Ngn3$^{-/-}$) (16) (a gift from Dr. Ravassard, CNRS U975, Paris, France) were maintained under specific pathogen-free conditions in accordance with current European legislation. The Regional Ethics Committee approved all protocols.

Transplantation. FPs from E11 to E12 embryos were dissected using a microscope, as previously described (17). Some FPs were cultured overnight at 37°C in RPMI medium supplemented with 10% FCS. Adult pancreatic islets were purified as described previously (18). Recipient mice, anesthetized with a xylazine/ketamine mix, were transplanted with 1–10 FPs or 400 pancreatic islets under the left kidney capsule. Some FPs were grafted within a ring device placed under the kidney capsule as previously described (19).

Diabetes induction. Diabetes was induced in the ins-HA mouse model as previously described (20).

Bromocriptine treatment. Mice were implanted subcutaneously with pellets releasing 5 mg of bromocriptine per day for 21 days (Innovative Research of America) to inhibit prolactin production, as previously described (21). Sham control mice received placebo pellets.

Insulin quantification. The graft was manually homogenized, and proteins were extracted on ice in an acid/ethanol solution (49 parts 95% ethanol, 1 part concentrated hypochlorous acid) and then sonicated. Insulin was quantified by ELISA (Mercordia).

FP culture. FPs from E11 B6 embryos were cultured for 8 days, as previously described (22). When indicated, human prolactin (1 µg/mL; produced in our laboratory) and BrdU (10 µg/mL; Sigma-Aldrich) were added to the culture. Images were digitized and BrdU/insulin-positive cells were quantified blindly using Image J software (National Institutes of Health), as previously described (17).

Histology. Graft-bearing kidneys and FPs were either fixed in 4% or 10% formalin, embedded in paraffin, and cut in 4- to 5-µm sections, or they were frozen in optimal cutting temperature (OCT) compound, stored at −80°C, and cut in 8-µm sections. Paraffin sections were stained with hematoxylin-eosin. Paraffin (Figs. 1 and 3) or frozen sections (Fig. 5) were stained with anti-insulin (Sigma-Aldrich or DakoCytomation), antiglucagon (Immunostar), or anti-BrdU (Amersham Biosciences) antibodies, followed by fluorescent secondary antibodies (Jackson ImmunoResearch Laboratories or Beckman Coulter). Frozen sections were stained with phycoerythrin-, fluorescein isothiocyanate–, or allophycocyanin-labeled antibodies against CD4 (RM4-5), CD8 (53-6.7), CD11b (M1-70.15), CD11c (HL3), Gr-1 (RB6-8C5), and Foxp3 (FJK16s) (BD Biosciences). Nuclei were stained with Hoechst 33342 (Invitrogen). Tissues were observed by fluorescence microscopy (Leica Microsystems). The Metaview software was used for image analysis.

Statistical analyses. Statistical significance was calculated using a one-tailed Mann–Whitney nonparametric test or an unpaired Student t test.

RESULTS

Upon transplantation, two FPs are sufficient to normalize glucose levels in diabetic syngeneic recipients. We first determined the minimal number of FPs required for diabetes control. Because of the toxic impact of hyperglycemia on FP development (14), we set up a model in which we could specifically kill endogenous β-cells at any given time without affecting newly differentiated β-cells in the transplant. This was achieved by

transplanting FPs from wild-type (WT) BALB/c mice into transgenic BALB/c mice expressing the HA model antigen specifically in β-cells. Without transplantation, we previously showed that diabetes was induced within 10 days in these ins-HA transgenic mice by injecting HA-specific effector T cells (Teffs) that killed only HA-expressing β-cells (20). In this setting, any β-cell derived from the WT FP graft would be spared. In a first set of experiments, we used FPs that were maintained overnight in culture medium and then transplanted under the kidney capsule within a ring device in order to more easily localize the graft for histological analysis. Two to three weeks later, which is the time needed for FPs to differentiate into insulin-producing cells (2,3), mice were injected with HA-specific Teffs. Nongrafted controls became diabetic within 5–10 days, whereas female recipients transplanted with five to seven E12 FPs, but not with less than five E12 FPs, were fully protected from diabetes induced by Teff injection (Fig. 1A and Supplementary Fig. 1). After removal of the graft-containing kidney 6 to 7 weeks after transplantation, mice became hyperglycemic within 24 h, confirming that euglycemia was maintained solely by insulin produced by the transplant. Typical structures of dense pancreatic islets, rich in insulin- and glucagon-producing cells, were observed within the graft by immunohistology (Fig. 1B). Surprisingly, when we performed the same experiment in male ins-HA recipients, five to six E12 FPs regulated glycemia in only one of nine mice (Fig. 1C). Five weeks after transplantation, the graft was much smaller in male compared with female recipients, with only small numbers of pancreatic islets (Fig. 1D).

We then modified the protocol in order to increase the efficiency of FP development. Moreover, we implemented quantitative measurements of the graft insulin content by ELISA 3 weeks after transplantation of one to five E12 FPs in syngeneic WT BALB/c females. Using fresh FPs and in the absence of a ring, each FP produced an average of 11.3 µg of insulin as compared with only 2.4 µg using the initial protocol. Both the overnight culture and the ring device altered the FP development into insulin-producing cells (Supplementary Fig. 2). Except where noted, we preferentially used this new protocol (fresh FPs and no ring) for the remainder of the study. In this setting, we observed that as few as two E12 FPs were sufficient to regulate T1D in ins-HA females, which were made diabetic by injection of HA-Teffs 3 to 4 weeks earlier (Fig. 1E). As expected, mice became hyperglycemic hours after ablation of the grafted kidney. We confirmed the sex difference using this protocol since up to three FPs were not able to control T1D in ins-HA males (Fig. 1F). Taken together, our results show that as few as two FPs were sufficient to control T1D in female recipients, provided that the right transplantation protocol was used.

Delayed development of FP in male compared with female syngeneic recipients. To address whether FP development was impaired or just delayed in males, we measured the quantity of insulin produced by the transplant at various times after transplantation of one FP in WT BALB/c mice. In female recipients, the content of insulin per transplant was hardly detectable after 1 week, averaged 3.8 µg after 2 weeks, and reached a plateau of 8–15 µg after 3 weeks of development. Compared with females, insulin production was significantly lower in males after 2 and 3 weeks, but interestingly attained similar levels (~9 µg) after 6 weeks of development (Fig. 2A). We then performed functional studies in the ins-HA
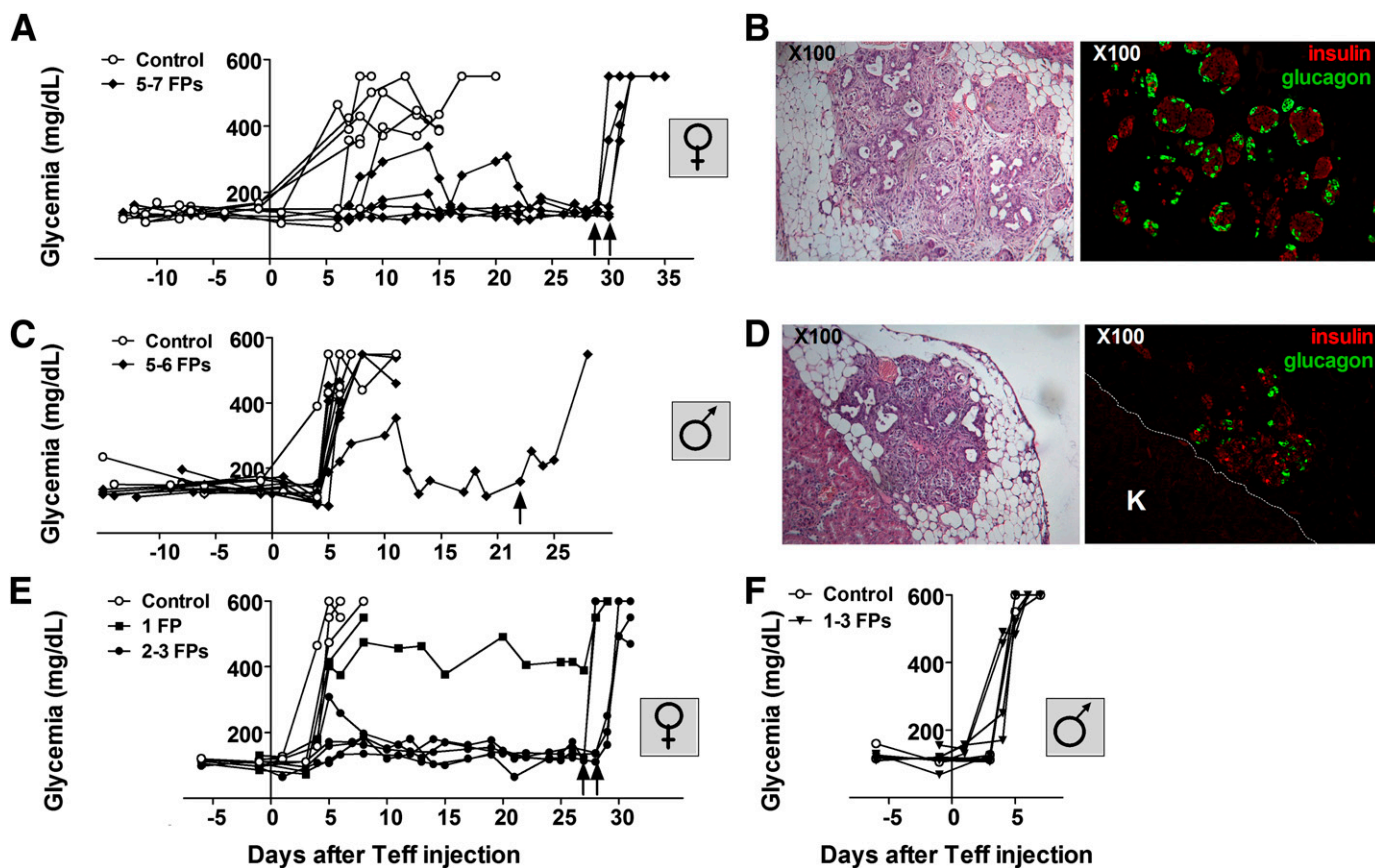
FIG. 1. Two FPs were sufficient to regulate diabetes in syngeneic mice. Female (*A*, *B*, and *E*) and male (*C*, *D*, and *F*) ins-HA mice were transplanted with five to seven overnight-cultured FPs in the presence of a ring device (*A–D*) or with one to three fresh FPs (*E* and *F*). Specific destruction of the recipient pancreatic β-cells was induced by injection of HA-specific Teffs 2 to 3 (*A–D*) or 3 to 4 (*E* and *F*) weeks later. Blood glucose levels of individual mice are shown in nontransplanted controls (*n* = 6, 5, 3, and 2 in *A*, *C*, *E*, and *F*, respectively) and FP-transplanted mice (*n* = 5, 9, 7, and 8 in *A*, *C*, *E*, and *F*, respectively). Arrows indicate the time of removal of the graft-containing kidney. *B* and *D*: Graft-bearing kidneys were analyzed by histology to visualize developed islets by hematoxylin-eosin staining (*left*) and by the presence of insulin- (red) and glucagon-expressing (green) cells (*right*). Representative sections are shown.

diabetes model. Male ins-HA mice were grafted with two to five E12 FPs and injected with HA-specific Teffs 6 weeks later to take into account the delayed development of FPs in males compared with females. All mice were protected from diabetes induction, and the protection was due to the transplant since these mice became rapidly diabetic following ablation of the grafted kidney (Fig. 2*B*). Thus, FP development was delayed rather than impaired in male compared with female recipients.

Role of prolactin and the immune system in the sex-related difference in FP development. The difference in the kinetics of FP development between males and females may be due to sex hormones since they appear to have an effect on proliferation, survival, and insulin production of β-cells (23). To address this question, we castrated male and female BALB/c mice before transplantation of one FP, eliminating hormones produced by gonads, and quantified insulin produced by the graft 3 weeks later. Interestingly, the difference in the insulin amount between males and females was maintained in castrated mice (Fig. 2*C*). The absence of a role for sex hormones on graft development was confirmed by the similar amount of insulin produced by the graft when comparing castrated mice and sham surgically operated controls in either males or females (Supplementary Fig. 3). Importantly, we could show that the sex of the

donor graft did not impact FP development (Supplementary Fig. 4).

We then studied the role of prolactin because this hormone is more abundant in females than males, and it may improve β-cell development and proliferation (24). To address this question, we treated mice with bromocriptine, an inhibitor of prolactin production (25,26). The higher level of insulin in transplants of female compared with male recipients at 3 weeks was confirmed again in control mice receiving the placebo. In contrast, insulin levels were decreased in females upon bromocriptine treatment, which reached levels observed in treated males. Thus, the sex difference was abolished in mice treated with bromocriptine (Fig. 3*A*)

To further support a role for prolactin in FP development, we first assessed expression of its receptor in E18 FPs. Expression was clearly detected in fetuses from WT mice but was severely reduced in fetuses from Ngn3$^{-/-}$ mice (Fig. 3*B*), which were genetically deficient for endocrine cell differentiation (16). This suggests that prolactin receptor was already expressed in β-cells during late fetal development. Then, we assessed the effect of prolactin in cultures of E11 FPs. The addition of prolactin after 7 days of culture increased the proportion of dividing β-cells (Fig. 3*C*), suggesting that this hormone increased β-cell proliferation during late fetal development.
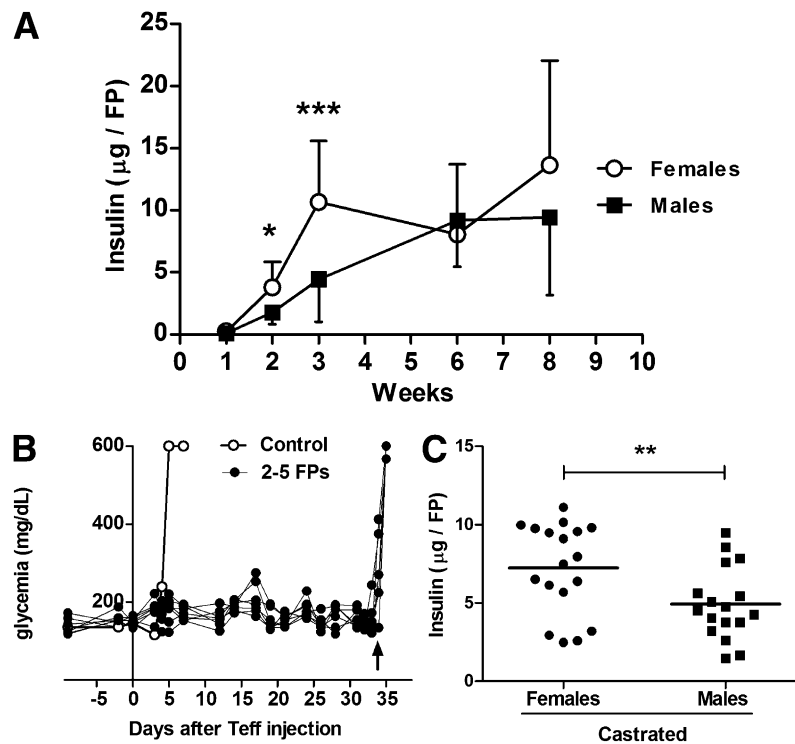
FIG. 2. Delayed FP development in male recipients in syngeneic condition. *A*: BALB/c males and females were grafted with one FP, and the insulin content of the graft was determined by ELISA at various times after transplantation. Means ± SEM are shown (*n* = 6, 7, 28, 5, and 13 at weeks 1, 2, 3, 6, and 8 in females, respectively, and *n* = 7, 8, 30, 8, and 13 at weeks 1, 2, 3, 6, and 8 in males, respectively). *B*: Ins-HA males were transplanted with two to five FPs followed by HA-specific Teff injection 6 weeks later to induce specific destruction of the recipient pancreatic β-cells at a later time point. Blood glucose levels of individual mice are shown in a nontransplanted control (*n* = 1) and FP-transplanted mice (*n* = 7). The arrow indicates the time of removal of the graft-containing kidney. *C*: Recipient BALB/c males and females were castrated under anesthesia 3 days to 12 weeks before transplantation (pool of four experiments that gave similar data). Insulin content of the graft was assessed in indicated groups 3 weeks after transplantation of one FP. Each symbol represents an individual mouse. *P < 0.05, **P < 0.005, ***P < 0.0005.

Since prolactin levels appeared lower in immunodeficient nude females than WT females (27), we studied FP development 3 weeks after transplantation in these mice. Interestingly, insulin levels were significantly decreased in nude females compared with control females but not in nude males compared with control males. As a result, the sex-related difference was abolished in nude recipients (Fig. 3*D*). In conclusion, in a syngeneic context, FP development was optimal and more rapid in females compared with males, which seemed to be linked to a higher prolactin level and a functional immune system.

Impaired FP development under hyperglycemic condition. Since T1D patients treated with insulin often have short periods of hyper- or hypoglycemia, it was important to evaluate FP development in a comparable context. Diabetes was first induced in ins-HA mice by HA-Teff injection, followed by insulin treatment at diabetes onset, transplantation of FP from ins-HA mice 5 to 6 days later, and quantification of graft insulin 14 days later (Supplementary Fig. 5*A*). Graft insulin was hardly detectable in mice that had repeated periods of hypo- (<60 mg/dL) and/or hyperglycemia (>300 mg/dL), whereas it was readily detected in the one mouse that had fewer peaks of moderate hyperglycemia (200–300 mg/dL) (Supplementary Fig. 5*B*). The lower graft insulin content in this latter mouse compared with values observed in BALB/c recipients (Fig. 2*A*) may be due to low-grade FP rejection by HA-Teffs. To further assess the impact of islet-specific autoreactive T cells on FP development, we performed an experiment in NOD mice. Two weeks after FP

transplantation in syngeneic 12-week-old NOD females, graft insulin contents were similar to the values obtained in the BALB/c model (Supplementary Fig. 5*C*). Thus, FP development appears to be impaired in mice with poor control of glycemia but not in the presence of islet-reactive T cells.

E12 FPs fail to control diabetes after transplantation in allogeneic hosts. We then studied the development of E12 FPs in allogeneic female recipients in order to test their immunogenicity. To address this question in the context of different degrees of alloreactivity, we grafted C3H or B6 FPs into BALB/c mice (full major histocompatibility complex [MHC] disparity), BALB/c × C3H F1 or BALB/c × B6 F1 FPs into BALB/c mice (semi-MHC disparity), and DBA/2 FPs into BALB/c mice (minor histocompatibility [mHC] disparity). These experiments were first performed in ins-HA BALB/c females using the initial protocol (overnight cultured FPs and ring device). In this setting, initial experiments showed that five or more syngeneic FPs were able to regulate T1D (Fig. 1*A*). Surprisingly, we observed that mice transplanted with 5–10 allogeneic FPs became diabetic 5–10 days after Teff injection regardless of the degree of disparity between donor and recipient (fully MHC, semi-MHC, and mHC) and as quickly as nontransplanted controls (Fig. 4*A*). Histological analyses showed that the grafts were highly infiltrated in all three allogeneic conditions (Fig. 4*B*) but not in syngeneic control recipients (Fig. 1*B*). Even in the context of mHC disparity, immune cell infiltration was already detectable at day 7, had increased at day 10, and was massive
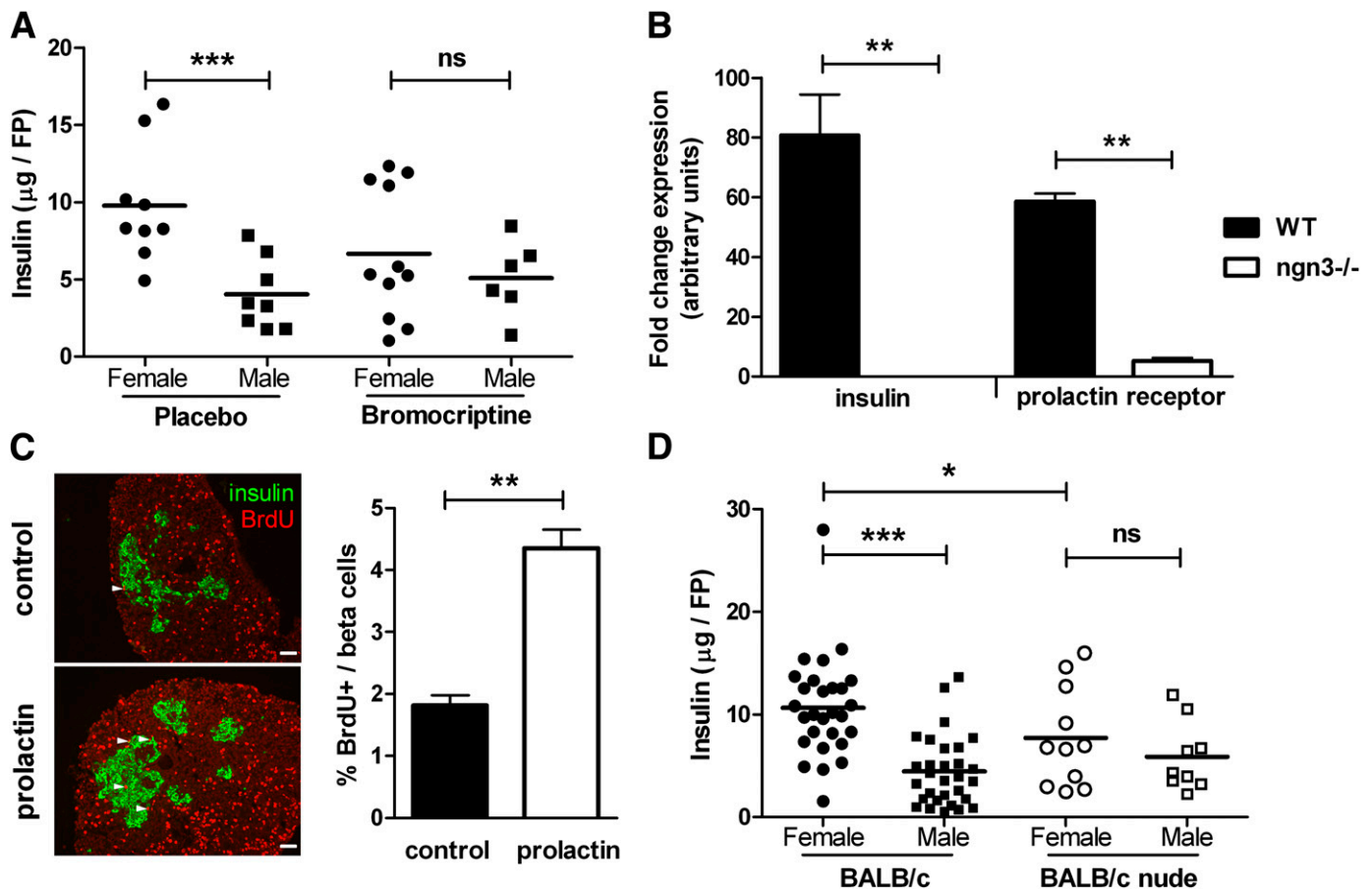
FIG. 3. Prolactin favored β-cell proliferation during FP development. *A*: Recipient BALB/c males and females were treated with a placebo or bromocriptine from the time of FP transplantation until euthanasia. Insulin content of the graft was assessed by ELISA in indicated groups 3 weeks after transplantation of one FP. Each symbol represents an individual mouse (pool of two experiments). *B*: RT-PCR quantification of insulin and prolactin receptor expression in E18 pancreases of WT or $Ngn3^{-/-}$ mice, as previously described (43). Cyclophilin A was used as the internal reference control. Means ± SEM of three independent experiments (pool of three FPs per experiment) are shown. *C*: Immunodetection and quantification of BrdU- (red) and insulin-positive (green) cells in pancreatic sections from 8-day culture of E11 FPs, which were treated with or without prolactin during the last 24 h and with BrdU during the last hour. Scale bar, 12.5 μm. Representative images (*left*) and means ± SEM of at least three independent experiments (four FPs per experiment) (*right*) are shown. *D*: Insulin levels of the graft in BALB/c (pool of seven to nine experiments) and BALB/c nude (pool of three experiments) female and male recipients transplanted 3 weeks earlier with one FP. Each symbol represents an individual mouse. *$P < 0.05$, **$P < 0.05$, ***$P < 0.0005$.

by day 15. Developing pancreatic islet structures could be observed at days 7 and 10 but were completely destroyed by day 15 (Supplementary Fig. 6). Thus, diabetes could not be prevented by FP transplantation in various allogeneic contexts, probably because of the rapid rejection of the transplanted FPs.

E12 FPs are fully rejected after allogeneic transplantation and infiltrated with T and myeloid cells. Since the presence of a ring device may increase inflammation and precipitate allograft rejection, we addressed the immunogenicity of E12 FPs using the improved FP transplantation protocol (fresh FP and no ring). We performed a kinetic analysis of insulin produced by the transplant in various allogeneic contexts, as a quantitative indicator of graft rejection. One week after transplantation, FP grafts produced only an average of 0.08–0.1 μg of insulin in the context of mHC or fully MHC disparities versus 0.25 μg in the syngeneic condition (Fig. 5*A*). The difference in insulin content was higher at week 2 (mean of 3.8 μg in syngeneic vs. 0.14–0.76 μg in the three allogeneic conditions) and even increased at week 3 (mean of 10.6 μg in syngeneic versus 0.03–0.9 μg in the three allogeneic conditions). In the context of mHC, semi-MHC, and

fully MHC disparities, the peak of insulin reached 0.9, 0.76, and 0.14 μg, respectively (Fig. 5*A*). In addition, the peak value was reached at later time points in the mHC condition as compared with the semi-MHC and fully MHC conditions. Thus, as expected, different intensities of graft rejection were observed depending on the allogeneic context, but even in the mHC context, rejection was strong enough to prevent proper and durable development of FPs. Immunohistology analyses confirmed these findings with only scarce and dispersed insulin- and glucagon-producing cells observed in the three allogeneic conditions at week 2 (Fig. 5*B* and *C*). At week 3, these cells were no longer detected, whereas they were abundant in the syngeneic condition (Fig. 5*B* and *C*). In conclusion, mouse E12 FPs are highly immunogenic and rapidly rejected in various allogeneic contexts, confirming the results obtained in the ins-HA model.

Previous studies have shown that during allograft rejection, the graft is infiltrated mostly by CD4 and CD8 T cells as well as myeloid cells (13,28). After validation of immunostaining on the spleen (Supplementary Fig. 7), we performed immunohistology of FP transplants to detect cells expressing CD4, CD8, Foxp3, CD11c, and Ly6C/G
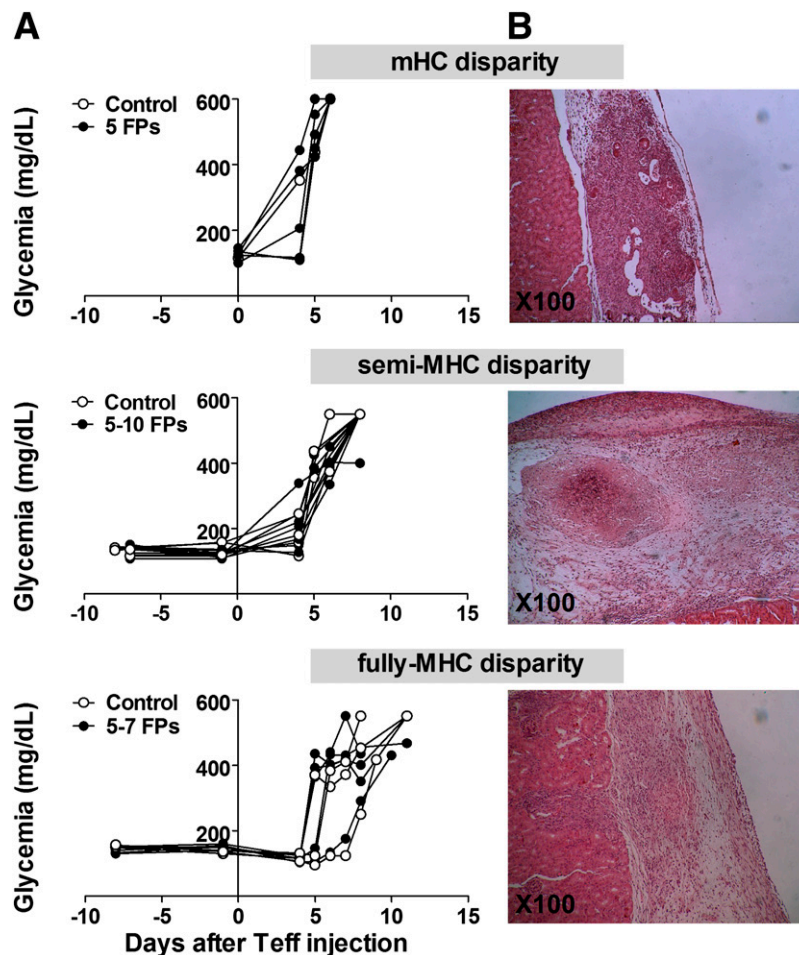
FIG. 4. Transplantation of FPs did not prevent diabetes in various allogeneic conditions. Ins-HA females were grafted with overnight-cultured 5–10 FPs in the presence of a ring device and injected with HA-specific Teffs 2 to 3 weeks later. Graphs show glycemia in individual recipients (*A*), and hematoxylin-eosin staining of a representative transplant is shown to illustrate cell infiltration (*B*). The experiment was performed in contexts of mHC disparity (DBA/2 donor, *n* = 5 and one nontransplanted control), semi-MHC disparity (C3H × BALB/c or B6 × BALB/c F1 donors, *n* = 8 and four nontransplanted controls), and fully MHC disparity (C3H donors, *n* = 5 and three nontransplanted controls).

(Gr1) at days 7, 14, and 21 after transplantation. In control mice transplanted with syngeneic grafts, we detected very few infiltrating immune cells (Fig. 6*A* and *B*). In contrast, in mice grafted with allogeneic FPs, we observed few T cells at day 7 but a high number of CD4$^+$, CD8$^+$, and Foxp3$^+$ T cells at days 14 and 21. Myeloid cells (CD11c$^+$ and Gr1$^+$ cells) were present in small to intermediate numbers in allogeneic conditions depending on the time point and individual mouse (Fig. 6*A* and *B*). No obvious difference in the type of infiltrating cells could be found between the various allogeneic contexts. As a control, we performed immunohistology of transplanted allogeneic adult pancreatic islets and observed similar T and myeloid cell infiltration (Fig. 6*A* and *B*). Thus, allogeneic E12 FPs were massively infiltrated with T and myeloid cells after transplantation.

DISCUSSION

When considering transplantation of FPs as a treatment for T1D, it is important to evaluate the minimal numbers of FPs required to control hyperglycemia. Previous studies addressing this question were performed in rats in which diabetes was induced by streptozotocin injection before transplantation. Since hyperglycemia was detrimental to the proper development of FPs (14), a finding confirmed in this study, recipient animals concomitantly received insulin administration. However, stable euglycemic condition could not be properly obtained in these animals because it would require repeated glycemia measurements and insulin injections. In this setting, at least six E17 to E18 FPs were required to control diabetes in syngeneic rats (2). Since normalization of glucose level is usually obtained in diabetic patients with only transient peaks of low hyperglycemia (200–300 mg/dL), it was essential to re-evaluate the question of the number of FPs required to control T1D in a context in which FPs develop in euglycemic animals. With this goal in mind, we developed a new mouse model allowing the exclusive destruction of the recipient's endogenous β-cells without affecting the transplanted FPs. Our data showed that as few as two E12 FPs were sufficient to control T1D in mice. It is likely that this number could be reduced to only one FP by further optimizing the experimental process. For instance, it has been shown that T1D was better controlled when FPs were preincubated with some growth factors before transplantation (7) or by shunting the venous drainage from the transplants to the liver (29). Additionally, it would be more appropriate to propose this transplantation in diabetic patients with well-controlled glycemia.

A

B

syngeneic

mHC disparity

semi-MHC disparity

fully-MHC disparity

weeks
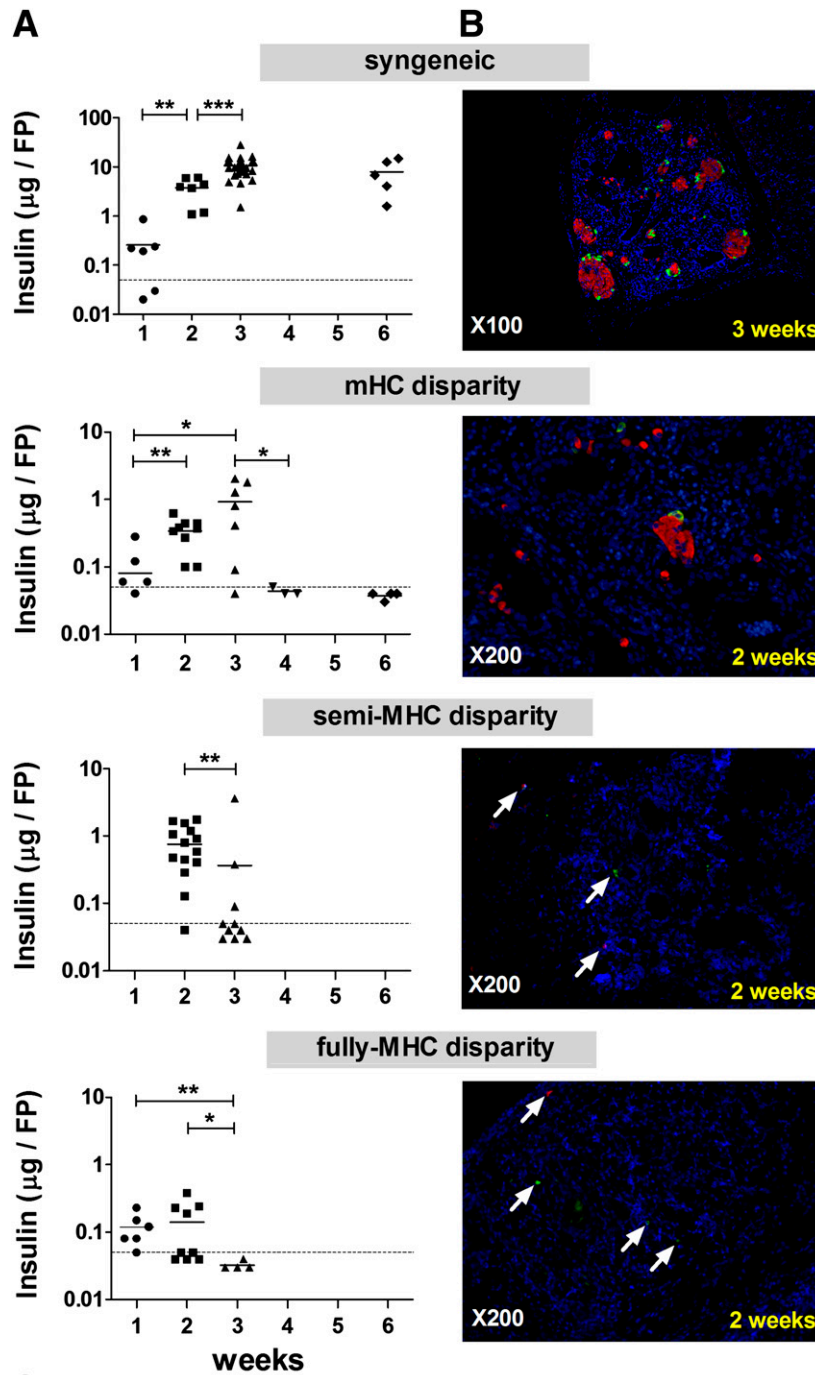
C

Week/ Graft	Insulin			Glucagon		
	1	2	3	1	2	3
Syngeneic			+++			+++
mHC	+	++	0	+	+	0
semi-MHC		+	0		+	0
fully-MHC	+	+ / 0	0	0	+	0

FIG. 5. Kinetics of insulin production by FP transplants depending on the degree of allogeneic disparities. BALB/c females were grafted with one fresh FP in syngeneic condition or in the context of mHC disparity (DBA/2 donor), semi-MHC disparity (B6 × BALB/c F1 donors), or fully MHC disparity (B6 donors). *A*: Insulin content of the graft was determined by ELISA at the indicated times after transplantation. Each symbol represents an individual mouse. The dotted line shows the limit of detection. In syngeneic conditions, the data are the same as the ones shown for females in Fig. 2*A*. *B*: Representative histology sections showing insulin (red)- and glucagon-expressing (green) cells 2 weeks after transplantation, except for the syngeneic context that was performed at week 3. Arrows indicate rare positive cells. *C*: Two to four mice per condition were scored for the presence of insulin- and glucagon-positive cells at different times after transplantation ($n = 1$ to 2 at week 1 and $n = 2$–4 at week 2 or 3). 0, no positive cells; +, scarce positive cells; ++, moderate cell numbers; +++, abundant positive cells; gray shading, not done. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.
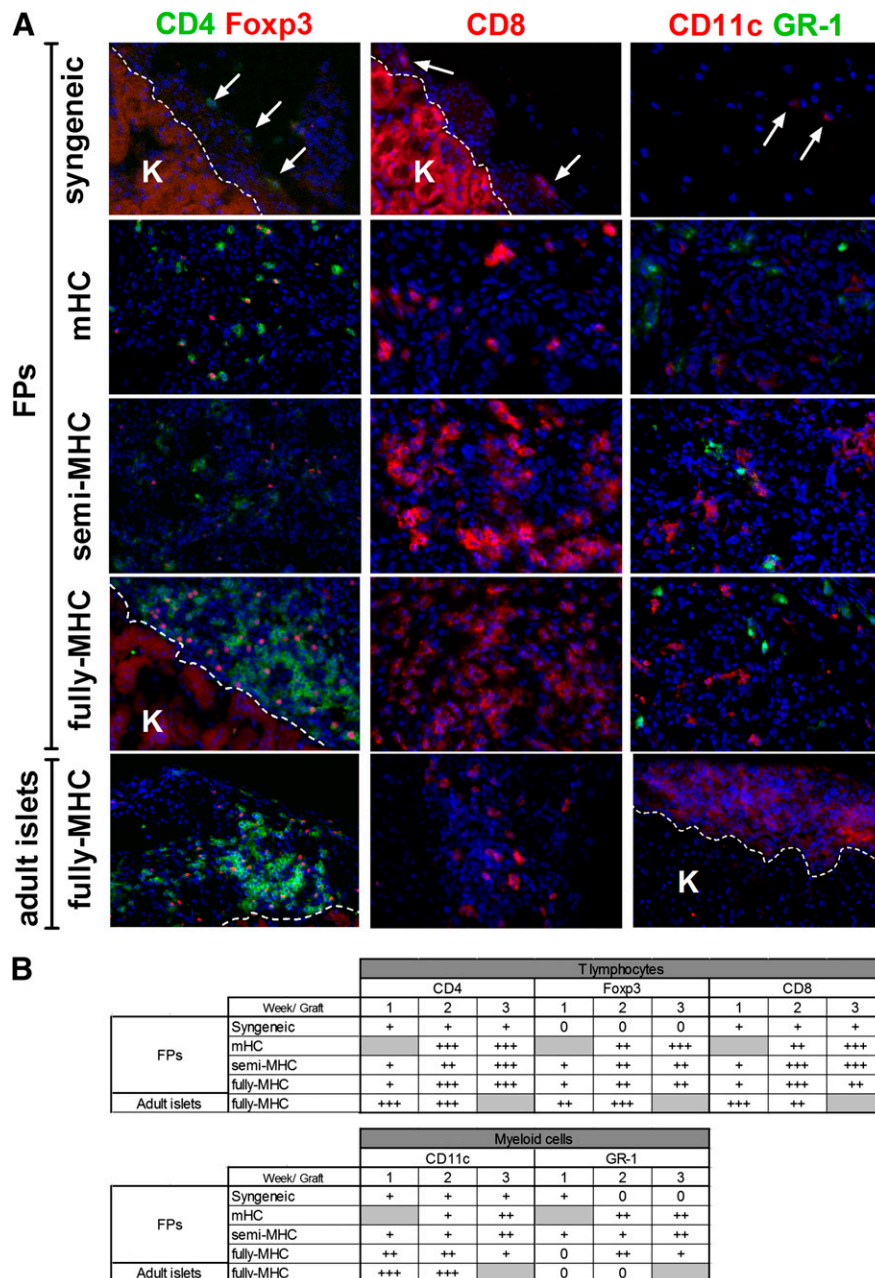
FIG. 6. T and myeloid cell infiltration of FP grafts after transplantation in various allogeneic conditions. *A*: Representative immunohistology sections of FP grafts labeled with the indicated markers (one fresh FP was grafted per mouse) 2 weeks after transplantation in syngeneic conditions (BALB/c→BALB/c, *n* = 2) or the context of mHC disparity (DBA/2→BALB/c, *n* = 2), semi-MHC disparity (B6 × BALB/c F1→BALB/c, *n* = 2 and B6 × BALB/c F1→B6, *n* = 2) or fully MHC disparity (B6→BALB/c, *n* = 3). A control mouse was grafted with adult pancreatic islets in a fully MHC disparity context (B6→BALB/c). *B*: Mice were scored for the presence of immune infiltrating cells at different times after transplantation (*n* = 1 to 2 at week 1 and *n* = 2–4 at week 2 or 3, except *n* = 1 for adult islets). 0, no cells; +, scarce cells; ++, moderate cell numbers; +++, massive infiltration throughout the graft; gray shading, not done.

Using this new model, we found a delay in the development of transplanted FPs in syngeneic males compared with females that was reproducibly observed in various settings (diabetes regulation or insulin quantification). To our knowledge, this sex discrepancy was not reported before despite the fact that many groups have performed syngeneic FP transplantation (14,28–32). This may be explained by the use of same-sex recipients, hyperglycemic recipients that do not present optimal conditions for FP development, or lymphopenic recipients in which we showed that the sex-related difference in graft development was abolished. Importantly, insulin production by transplanted islets also seems higher in women than men, suggesting that this sex-related difference might be specific to the endocrine pancreas. Alternatively, this difference might be also observed for other fetal tissues or tissues derived from embryonic or adult stem cells. This would be interesting to address since these different types of tissues may be transplanted in the future as part of the emerging field of regenerative medicine. Our study suggests that the environment and nature of the recipient have to be properly considered and may affect the function of the transplanted tissue. The normal physiological development of fetal tissues is taking place in

pregnant females, which undergo major hormonal modifications such as elevated levels of prolactin (33). One may speculate that the differentiation of adult stem cells or their derivatives, which usually occurs physiologically in nonpregnant animals, is less dependent on hormones that are increased during gestation.

We tested different parameters that could have accounted for the delayed development of FPs in males compared with females. Sex hormones were interesting candidates because they have an effect on proliferation, survival, and insulin production of β-cells (23), and prolactin levels were reduced after ovariectomy (34). However, our data showing that the difference in FP development between females and males was maintained after castration suggested that it was not due to differences in sex hormones. In contrast, prolactin, for which serum levels are twofold higher in females than males in the steady state (34), seems to be involved in the sex difference in FP development. Indeed, we showed that this difference was abolished upon administration of bromocriptine, an inhibitor of prolactin production (25), and that prolactin promoted β-cell proliferation during late FP development. Of note, this observation does not exclude the possibility that this hormone also has an effect on β-cell precursors to favor their differentiation. Our observation is compatible with several previous reports (23–25,35): *1*) overexpression of placental lactogen in β-cells, a hormone structurally related to prolactin that can bind the prolactin receptor and increases β-cell replication and insulin production; *2*) mice deficient in prolactin receptor have reduced β-cell mass and islet density; *3*) high levels of prolactin produced during gestation stimulate the proliferation of β-cells and increase insulin secretion in the maternal pancreas; and *4*) β-cells express high levels of the prolactin receptor during late gestation and lactation. Thus, prolactin appears to promote the proliferation of β-cells, not only in the mature pancreas, as previously shown (24), but also during late fetal development, and higher levels of prolactin in females than males may underlie the more rapid development of FPs in females.

Although it is still controversial (36–39), it has been suggested that FPs at their early stage of development (E28 pig FPs or E15 chick FPs) were well-tolerated in xenogeneic rat or monkey recipients. We were thus surprised when we found that E12 mouse FPs were rapidly rejected in allogeneic recipients even in a context of minor histocompatibility disparities. One possible explanation to reconcile our data with these previous studies is that FPs at an early stage of development would be less immunogenic across a xenogeneic than allogeneic barrier. An alternative explanation is that, compared with rat or monkey recipients, mice are more prone to reject FPs. Indeed, xenogeneic early developed FPs (i.e., from E28 pigs or E15 chick FPs) were accepted in rats or monkeys (7–9), whereas they were rejected in mice (31,38). Thus, it is difficult to speculate on the immunogenicity of allogeneic or xenogeneic FPs after transplantation in humans. If performed in T1D patients, additional complications could potentially arise from the possible reactivation of autoreactive T cells that have destroyed the patient's own β-cells, as has been observed after transplantation of allogeneic pancreatic islets (40). This question has been addressed previously by transplanting histocompatible E12 FPs in NOD mice that spontaneously develop autoimmune diabetes. The transplant developed into functional pancreatic islets when performed in prediabetic mice but

was rejected in diabetic mice (32), suggesting that severe autoimmunity may increase the FP rejection.

Depletion of CD4 and/or CD8 T cells prevents rejection of allogeneic adult islets or E17 to E18 FPs, showing their critical role in the rejection (13,41). T cell–mediated rejection was substantiated by histology of the rejected transplant showing massive infiltration with CD4$^+$ and CD8$^+$ T cells as well as myeloid cells (13,28,41). We observed a similar population of infiltrating cells after transplantation of mouse E12 FPs, suggesting that rejection was also T cell–mediated. Nevertheless, although common mechanisms may operate in rejection of adult and fetal tissues, several reports have shown that a mild immunosuppressive treatment was sufficient to prevent rejection of FPs across xenogeneic or allogeneic barriers (30,31,38,42). It would be important to reanalyze the efficacy of these treatments in male compared with female recipients. In conclusion, whereas only two FPs were sufficient to control T1D in syngeneic condition, allogeneic FP transplants were rapidly rejected, precluding their capacity to control T1D, even in the context of minor histocompatibility disparities. Thus, FP transplantation needs to be further studied in preclinical models before one may envisage its translation to the clinics.

REFERENCES

1. Shapiro AM, Ricordi C, Hering BJ, et al. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006;355:1318–1330
2. Brown J, Molnar IG, Clark W, Mullen Y. Control of experimental diabetes mellitus in rats by transplantation of fetal pancreases. Science 1974;184:1377–1379

3. Mandel TE. Fetal islet xenotransplantation in rodents and primates. J Mol Med (Berl) 1999;77:155–160

4. Castaing M, Péault B, Basmaciogullari A, Casal I, Czernichow P, Scharfmann R. Blood glucose normalization upon transplantation of human embryonic pancreas into beta-cell-deficient SCID mice. Diabetologia 2001;44:2066–2076

5. Peschanski M, Bachoud-Lévi A-C, Hantraye P. Integrating fetal neural transplants into a therapeutic strategy: the example of Huntington's disease. Brain 2004;127:1219–1228

6. Wu DC, Boyd AS, Wood KJ. Embryonic stem cells and their differentiated derivatives have a fragile immune privilege but still represent novel targets of immune attack. Stem Cells 2008;26:1939–1950

7. Rogers SA, Liapis H, Hammerman MR. Normalization of glucose post-transplantation of pig pancreatic anlagen into non-immunosuppressed diabetic rats depends on obtaining anlagen prior to embryonic day 35. Transpl Immunol 2005;14:67–75

8. Rogers SA, Chen F, Talcott MR, et al. Long-term engraftment following transplantation of pig pancreatic primordia into non-immunosuppressed diabetic rhesus macaques. Xenotransplantation 2007;14:591–602

9. Eloy R, Haffen K, Kedinger M, Grenier JF. Chick embryo pancreatic transplants reverse experimental diabetes of rats. J Clin Invest 1979;64: 361–373

10. Brands K, Colvin E, Williams LJ, Wang R, Lock RB, Tuch BE. Reduced immunogenicity of first-trimester human fetal pancreas. Diabetes 2008;57: 627–634

11. Lee MS, Wogensen L, Shizuru J, Oldstone MB, Sarvetnick N. Pancreatic islet production of murine interleukin-10 does not inhibit immune-mediated tissue destruction. J Clin Invest 1994;93:1332–1338

12. Muller CJ, Du Toit DF, Beyers AD, Page BJ, Muller N. Prolongation of rat fetal pancreas allograft survival using a nondepleting anti-CD4 monoclonal antibody W3/25. Transplant Proc 1998;30:4180–4183

13. Simeonovic CJ, Townsend MJ, Morris CF, et al. Immune mechanisms associated with the rejection of fetal murine proislet allografts and pig proislet xenografts: comparison of intragraft cytokine mRNA profiles. Transplantation 1999;67:963–971

14. Mullen YS, Clark WR, Molnar IG, Brown J. Complete reversal of experimental diabetes mellitus in rats by a single fetal pancreas. Science 1977; 195:68–70

15. Fisson S, Djelti F, Trenado A, et al. Therapeutic potential of self-antigen-specific CD4+ CD25+ regulatory T cells selected in vitro from a polyclonal repertoire. Eur J Immunol 2006;36:817–827

16. Gradwohl G, Dierich A, LeMeur M, Guillemot F. neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci USA 2000;97:1607–1611

17. Rachdi L, Aïello V, Duvillié B, Scharfmann R. L-leucine alters pancreatic β-cell differentiation and function via the mTor signaling pathway. Diabetes 2012;61:409–417

18. Valtat B, Dupuis C, Zenaty D, et al. Genetic evidence of the programming of beta cell mass and function by glucocorticoids in mice. Diabetologia 2011;54:350–359

19. Castaing M, Guerci A, Mallet J, Czernichow P, Ravassard P, Scharfmann R. Efficient restricted gene expression in beta cells by lentivirus-mediated gene transfer into pancreatic stem/progenitor cells. Diabetologia 2005;48: 709–719

20. Grinberg-Bleyer Y, Saadoun D, Baeyens A, et al. Pathogenic T cells have a paradoxical protective effect in murine autoimmune diabetes by boosting Tregs. J Clin Invest 2010;120:4558–4568

21. de Leeuw van Weenen JE, Parlevliet ET, Schröder-van der Elst JP, van den Berg SA, Willems van Dijk K, Romijn JA, Pijl H. Pharmacological modulation of dopamine receptor D2-mediated transmission alters the metabolic phenotype of diet induced obese and diet resistant C57Bl6 mice. Exp Diabetes Res 2011;2011:928523

22. Attali M, Stetsyuk V, Basmaciogullari A, et al. Control of beta-cell differentiation by the pancreatic mesenchyme. Diabetes 2007;56:1248–1258

23. Morimoto S, Morales A, Zambrano E, Fernandez-Mejia C. Sex steroids effects on the endocrine pancreas. J Steroid Biochem Mol Biol 2010;122: 107–113

24. Newbern D, Freemark M. Placental hormones and the control of maternal metabolism and fetal growth. Curr Opin Endocrinol Diabetes Obes 2011; 18:409–416

25. Freeman ME, Kanyicska B, Lerant A, Nagy G. Prolactin: structure, function, and regulation of secretion. Physiol Rev 2000;80:1523–1631

26. Durant S, Coulaud J, Homo-Delarche F. Bromocriptine-induced hyperglycemia in nonobese diabetic mice: kinetics and mechanisms of action. Rev Diabet Stud 2007;4:185–194

27. Pierpaoli W, Kopp HG, Bianchi E. Interdependence of thymic and neuroendocrine functions in ontogeny. Clin Exp Immunol 1976;24:501–506

28. Mandel TE, Kovarik J, Koulmanda M. A comparison of organ cultured fetal pancreas allo-, iso-, and xenografts (pig) in non-immunosuppressed nonobese diabetic mice. Am J Pathol 1995;147:834–844

29. Brown J, Mullen Y, Clark WR, Molnar IG, Heininger D. Importance of hepatic portal circulation for insulin action in streptozotocin-diabetic rats transplanted with fetal pancreases. J Clin Invest 1979;64:1688–1694

30. Millard PR, Garvey JF, Jeffery EL, Morris PJ. The grafted fetal rat pancreas: features of development and rejection. Am J Pathol 1980;100:209–224

31. Rogers SA, Liapis H, Hammerman MR. Intraperitoneal transplantation of pancreatic anlagen. ASAIO J 2003;49:527–532

32. Begum S, Chen W, Herold KC, Papaioannou VE. Remission of type 1 diabetes after anti-CD3 antibody treatment and transplantation of embryonic pancreatic precursors. Endocrinology 2009;150:4512–4520

33. Brown J, Heininger D, Kuret J, Mullen Y. Normal response to pregnancy in rats cured of streptozotocin diabetes by transplantation of one fetal pancreas. Diabetologia 1982;22:273–275

34. Sinha YN, Wickes MA, Salocks CB, Vanderlaan WP. Gonadal regulation of prolactin and growth hormone secretion in the mouse. Biol Reprod 1979; 21:473–481

35. Nielsen JH, Galsgaard ED, Møldrup A, et al. Regulation of beta-cell mass by hormones and growth factors. Diabetes 2001;50(Suppl. 1):S25–S29

36. Foglia RP, DiPreta J, Statter MB, Donahoe PK. Fetal allograft survival in immunocompetent recipients is age dependent and organ specific. Ann Surg 1986;204:402–410

37. Dekel B, Burakova T, Arditti FD, et al. Human and porcine early kidney precursors as a new source for transplantation. Nat Med 2003;9:53–60

38. Eventov-Friedman S, Tchorsh D, Katchman H, et al. Embryonic pig pancreatic tissue transplantation for the treatment of diabetes. PLoS Med 2006;3:e215

39. Hecht G, Eventov-Friedman S, Rosen C, et al. Embryonic pig pancreatic tissue for the treatment of diabetes in a nonhuman primate model. Proc Natl Acad Sci USA 2009;106:8659–8664

40. Vendrame F, Pileggi A, Laughlin E, et al. Recurrence of type 1 diabetes after simultaneous pancreas-kidney transplantation, despite immunosuppression, is associated with autoantibodies and pathogenic autoreactive CD4 T-cells. Diabetes 2010;59:947–957

41. Makhlouf L, Yamada A, Ito T, et al. Allorecognition and effector pathways of islet allograft rejection in normal versus nonobese diabetic mice. J Am Soc Nephrol 2003;14:2168–2175

42. Tchorsh-Yutsis D, Hecht G, Aronovich A, et al. Pig embryonic pancreatic tissue as a source for transplantation in diabetes: transient treatment with anti-LFA1, anti-CD48, and FTY720 enables long-term graft maintenance in mice with only mild ongoing immunosuppression. Diabetes 2009;58:1585–1594

43. Guillemain G, Filhoulaud G, Da Silva-Xavier G, Rutter GA, Scharfmann R. Glucose is necessary for embryonic pancreatic endocrine cell differentiation. J Biol Chem 2007;282:15228–15237