

Supplemental Digital Content 1

(Items 1-10)

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1. MHC-inbred miniature swine are unique translational animals that bridge between rodents and primates: A number of small animal models have successfully demonstrated allogeneic tolerance induction, however, many attempts to extrapolate such protocols to large animal models or to the clinic have been unsuccessful. Although non-human primates (NHPs) are phylogenetically close to humans, the economic cost, lack of defined major histocompatibility complex (MHC) genetics and time-to-maturity make primate models inappropriate for such studies. For gestational, anatomic, economic reasons and defined genetic background, MHC-inbred swine are the preferred experimental animals for the translation of small animal tolerance data to primates, however very few of these models exist.

2. CLAWN-miniature swine: CLAWN-miniature swine, aged less than 12 months, were obtained from the Japan Farm CLAWN Institute (Kagoshima, Japan). CLAWN-miniature swine are genetically-typed, MHC-inbred miniature swine that were established by Nakanishi and his colleagues at Kagoshima University (1,2). In this study, c2-type swine were used as donors of fully MHC-mismatched renal allografts to c1-type recipients.

3. Additional information of HGF: HGF, which is a potent stimulator of hepatocyte proliferation, was obtained from the purified plasma of a patient with fulminant hepatic failure (3,4). HGF is a multifunctional, pleiotropic protein with mitogenic, motogenic, and morphogenic effects in a wide variety of cells, including endothelial cells and renal proximal tubular epithelial cells (5). It is an important regulator of kidney function and a potent renoprotective agent (6), preserving normal structure and function and accelerating recovery in experimental models of acute renal failure and ischemic injury (7,8). HGF also ameliorates chronic renal injury in a variety of models (9,10), that have been ascribed to its anti-apoptotic, anti-fibrotic (11), and anti-inflammatory effects in myriad pathologies (12,13).

4. c-Met analysis: c-Met, the HGF receptor, is a trans-membrane protein that has tyrosine-kinase activity and is involved in embryonic development, cell proliferation, and wound healing (14). c-Met is normally expressed by cells of epithelial origin, while expression of HGF is restricted to cells of mesenchymal origin. Upon HGF stimulation, c-Met induces several biologic responses that collectively give rise to a complex biological response referred to as the invasive growth program (3,6). In our study, immunohistochemistry demonstrated that the

renal-allograft tubular epithelium and interstitium expressed higher levels of c-Met during rejection (Fig. 5b-d). Enhanced expression of c-Met in this case may have been due to acute inflammation, likely as a result of rejection. This is consistent with previous reports that showed c-Met activation in injured organs, but not in intact tissues (15). On the other hand, the improved expression of c-Met may represent positive feedback due to higher, local levels of HGF.

5. Confirmation of full-MHC mismatch by MLR and CML assays:

MLR and CML assay responses between genetically-typed c1 and c2 CLAWN-miniature swine confirmed that these animals were fully mismatched at the MHC locus. MLR responses, as assessed by CSFE, between donor-recipient pairs showed an average stimulation index of 7 ± 1.3 , confirming that these animals were mismatched at Class II. The percent specific lysis (PSL) observed in PKH-26 CML assays in which naïve recipient PBMCs were stimulated with donor PBMCs was $24.4\% \pm 1.7$ at an effector-to-target ratio of 100:1; In contrast, self-MHC matched donor PBMCs lead to a PSL of 0-5%, indicating that recipient-donor pairs were also mismatched at Class I.

6. Kidney transplantation: Following bilateral nephrectomies, heterotopic renal transplantation to the right iliac space was performed via a paramedian incision. The graft renal artery was anastomosed end-to-end to the recipient's external iliac artery. The donor renal vein was anastomosed end-to-side to the recipient's iliac vein. There were no complications due to the ligation of the distal side of the external iliac artery. Total cold ischemic time was kept to less than 60 minutes.

7. Insertion of the catheter to infuse HGF selectively to renal allografts

A previous report by Ido et al. demonstrated that recombinant human HGF administered intravenously or via the portal vein accumulated in livers and spleen but not in kidneys (16). In order to maintain a high concentration of HGF in renal allografts, we developed a new method for HGF delivery allowing HGF to accumulate in the graft selectively. A silicone-rubber catheter (Clinical Supply Co., LTD. Japan) was introduced through the flank abdomen and inserted into a branch of the iliac artery on the day of transplantation. The catheter tip was directed toward the renal graft artery. An infusion pump was used to continuously administer either hHGF (cloned by Dr. Tsubouchi).

8. Cellular assays: PBMCs were obtained by gradient centrifugation using HISTOPAQUE (Sigma, St Louis, MO) as previously reported (17). Allogeneic proliferative responses were assessed by carboxyfluorescein diacetate succinimidyl ester-mixed lymphocyte reaction (CFSE-MLR) assays as previously described (17). Stimulation indices were calculated by dividing the proliferative index of allogeneic combinations by that of the self-control combination. Cell mediated T cell cytotoxic killing was assessed by PKH-26 cytotoxicity assay (CML) assays as previously described (Reference 37 in the main text).

9. Assessment of FoxP3+ CD4+CD25+ lymphocytes: The percentage of

FoxP3+CD4+CD25+ positive cells in PBMCs was assessed by FACS analysis using FITC conjugated anti-CD4 (VMRD, Inc., Washington, USA), Bio conjugated anti-CD25 (Fitzgerald industries, MA, USA), and PE conjugated FoxP3 (eBioscience Inc., San Diego, CA). Cells stained with anti-CD4 and CD25 mAbs were washed, resuspended in 1 ml of freshly prepared Fixation/Permeabilization working solution (FoxP3 staining buffer, eBioscience, Inc.), and incubated at 4°C for 60 minutes. Cells were washed once in Permeabilization Buffer and stained with fluorochrome FJK-16s (anti-mouse/rat FoxP3 PE, eBioscience).

10. Measurement of the concentration of human recombinant HGF (hHGF):

The concentration of hHGF in serum and tissue extracts was determined using a commercially available ELISA kit (Otsuka Pharmaceutical Co., Tokushima, Japan). Fresh tissues were homogenized in cold Ca²⁺-, Mg²⁺-free phosphate-buffered saline (PBS) containing 0.4% EDTA-2Na and 500 units/ml of aprotinin. The homogenates were centrifuged at 9000×g for 20 min at 4°C, and then at 105,000×g for 1 h at 4°C. HGF levels were measured from the supernatants.

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