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Xenotransplanted Embryonic Kidney Provides a Niche for Endogenous Mesenchymal Stem Cell Differentiation Into Erythropoietin-Producing Tissue

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ABSTRACT

Recent findings have demonstrated that stem cells can differentiate into mature tissue when supplied with a niche containing factors identical to those in the normal developmental program. A niche for the development of an organ can be provided by xenotransplantation of a similar developing organ. However, this process has many technical, safety, and ethical concerns. Here, we established xenotransplantation models that control endogenous mesenchymal stem cell (MSC) differentiation into mature erythropoietin (EPO)-producing tissue in a niche provided by a developing xenometanephros. Transplantation of rat metanephroi into mouse omentum, and similarly pig metanephroi into cat omentum, led to the recruitment of host cells and EPO production. EPO-expressing cells were not differentiated from integrating vessels because they did not coexpress endothelial markers (Tie-2 and VE-cadherin). Instead, EPO-expressing cells were shown to be derived from circulating host cells, as shown by enhanced green fluorescent protein (EGFP) expression in the grown transplants of chimeric mice bearing bone marrow from a transgenic mouse expressing EGFP under the control of the EPO promoter. These results suggest that donor cell recruitment and differentiation in a xenotransplanted developing organ may be consistent between species. The cells responsible for EPO expression were identified as MSCs by injecting human bone marrow-derived MSCs and endothelial progenitor cells into NOD/SCID mice. Furthermore, using metanephroi from transgenic ER-E2F1 suicide-inducible mice, the xenotissue component could be eliminated, leaving autologous EPO-producing tissue. Our findings may alleviate adverse effects due to long-lasting immunosuppression and help mitigate ethical concerns. STEM CELLS *2012;30:1228–1235*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Recently, pluripotent stem (PS) cells have been successfully isolated and established from various tissues, which has brought the possibility of using somatic stem cells for organ regeneration one step closer to realization. For stem cell use in the clinic, they must be differentiated into functionally mature forms, and many researchers have attempted to establish individual somatic cell types. However, only very few cell types, such as pancreatic β cells and cardiac myocytes, have been successfully established thus far [1, 2]. The major reason for this lack of success is because developmental programs that differentiate stem cells into mature cells consist of

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numerous factors, some of which are as yet unknown, and contribute to each other temporally and spatially in a tissue-specific manner. Elucidating the developmental program for each cell type one by one is very challenging. In this context, we previously found that bone marrow-derived mesenchymal stem cells (MSCs), but not embryonic stem cells (ESCs) or induced PS (iPS) cells, integrate into the kidney structure and acquire some renal functions when cultured with an immature metanephros in vitro [3]. Fetal organs, such as the metanephros have been suggested as less immunogenic and more feasible for transplantation because (a) antigen-presenting cells that mediate direct host recognition of alloantigens and xenoantigens would be absent [4], (b) donor antigens such as majorhistocompatibility complex class I and II may not be expressed by developing organs [5], and (c) the immune response to transplanted fetal tissue differs from that to adult tissue in terms of the elicitation of a T helper 2-biased response when the target organ is of fetal origin [6]. In fact, direct comparison of xenotransplantation clearly shows the immune advantage of developing precursor transplants over developed adult transplants in fully immunocompetent hosts [7]. Based on these observations, we speculated that we could use the developmental program of a developing organ by transplanting it into an ectopic site where it continued development in vivo. This procedure would facilitate the inward migration of autologous stem cells that would then be stimulated by the developmental program of the xeno-organ to mature into tissue-specific cells.

In addition, conventional xenotransplantation should require continuous and strong immunosuppression to avoid any humoral rejection that occurs across the xenogeneic barrier [8], which evokes various adverse effects including carcinogenicity and severe infection. In contrast, this in vivo programming system temporally uses xeno-organs as the source of the developmental program, and after establishment of the tissue of interest, the xenocomponent is no longer needed and can be discarded. Therefore, we introduced a cell fate-regulating system, in which a suicide gene is expressed on demand, and combined this system with the in vivo programming system.

In this study, we especially focused on the establishment of erythropoietin (EPO)-producing tissue. Although administration of recombinant human EPO is a widely used approach for treating and mitigating renal anemia in chronic kidney disease patients [9], such treatment is costly and comprises the highest annual drug sales worldwide [10]. In Japan, 83% of hemodialysis patients are prescribed recombinant EPO, and the mean EPO dose was 5,231 units per week in 2006, which extrapolates to more than $6,700 per person per year [11]. This amount has remained constant through the past decade [11]. In addition, the nonacquisition costs associated with anemia therapy in hemodialysis patients are considerable (more than $2,000 per person per year [12]). Furthermore, interruption of drug supply, which could occur at any time, such as during the disaster in Fukushima, Japan 2011, may be related to irremediable results. The ideal therapeutic focus appears to be the establishment of self-reliant tissue that secretes autologous proteins, because other than being possibly more cost effective, it would not suffer from an interruption to drug supply.

Here, we established a novel technique that uses the donor developmental program to differentiate endogenous stem cells into mature EPO-producing cells. In this system, immature metanephroi were transplanted into host omentum to provide a stem cell niche that recruits endogenous cells during its development, which stimulates them with a developmental program for differentiation into kidney residential cells. After establishment, the stem cell niche is eliminated by induction of a suicide gene with a regulating switching system, which may reduce adverse effects due to long-lasting immunosuppression, such as carcinogenesis and severe infection due to the immunocompromised state.

Materials and Methods

Animals

Wild-type Lewis rats and C57BL/6 and NOD/SCID mice were purchased from Sankyo Lab Services (Tokyo, Japan) and CLEA Japan (Tokyo, Japan, http://www.clea-japan.com), respectively. A breeding colony of VE-cadherin-Cre and CAG-Cre mice were obtained from the JCRB Laboratory Animal Resource Bank at the National Institute of Biomedical Innovation (NIBIO, Osaka, Japan, http://www.nibio.go.jp) and RIKEN BioResource Center (Tsukuba, Japan), respectively. Tie-2-Cre, EPO-BAC-GFP, and CAG-CAT-EGFP mice were established at the Laboratory Animal Center of The Jikei University School of Medicine (Tokyo, Japan) from breeding pairs that were kindly donated by Prof. Masashi Yanagisawa (The University of Texas Southwestern Medical Center at Dallas, TX), Prof. Masayuki Yamamoto (Tohoku University Graduate School of Medicine, Sendai, Japan), and Prof. Ryuichi Nishinakamura (Kumamoto University; Kumamoto Japan). Luciferase (Luc)/LacZ dual transgenic rats, EGFP transgenic rats, and ER-E2F1 mice were previously established by our laboratory [13]. Midday on the day when a vaginal plug was observed was designated as embryonic day (E) 0.5. Mice and rats were housed in a ventilated (positive airflow) rack and were bred and maintained under pathogen-free conditions. Eleven healthy specific pathogen-free (SPF) domestic cats weighing 3.7–5.7 kg (mean weight, 4.6 kg) were purchased from Nisseiken Co., Ltd. (Tokyo, Japan) and received humane care in compliance with the Care and Use of Laboratory Animals in the laboratory animal facility of Kitasato University.

Domestic hybrid pigs (Hypor) were maintained in a semi-windowless facility at a controlled temperature (15°C–30°C) and received a standard pig diet twice a day and water ad libitum. Pregnant sows were sacrificed under general anesthesia, and fetuses at E27-30 were recovered from the excised uterus. All experimental procedures were approved by The Committee for Animal Experiments and The Ethics Committees of The Jikei University, Meiji University, Jichi Medical University, and Kitasato University.

Metanephric Xenoimplantation

Rat metanephroi (E15), mouse metanephroi (E13-15), and pig metanephroi (E29-31) were dissected under a microscope and implanted in the omentum or epididymis of anesthetized host animals. In some experiments, rat fetal liver, brain, intestine, adrenal glands, pancreas, and lung were dissected from rat embryos (E15) and implanted in the omentum or epididymis of host rats. In the xenotransplantation between rats (donor) and mice (recipient), FK506 (2 mg/kg) was subcutaneously administered daily to the recipient from 1 day before transplantation until sacrifice. In the xenotransplantation between the pigs (donor) and cats (recipient), cyclosporine A was administered daily to the recipient starting on the day of transplantation. The amount of cyclosporine A was adjusted to serum trough levels of approximately 200 ng/ml. In the allotransplantation between B6 mice (donor) and NOD/SCID mice, an immunosuppressant was not administered.

Anemia Induction and Measurement of EPO Levels

To confirm increased levels of EPO protein in serum, both host kidneys were removed and mask native EPO production,

and then anemia was induced by a rapid withdrawal of blood from the vena cava at a volume of 2% vol/wt b.wt. Serum levels of EPO were measured using an radio immuno assay (RIA) kit (SRL, Tokyo, Japan) as described elsewhere [14].

Conventional and Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA was extracted from each tissue and subjected to RT-PCR as described elsewhere [14]. Primer sets, probes, and Polymerase Chain Reaction (PCR) conditions are shown in Supporting Information Table S1. In some experiments, the amount of EPO production was semiquantified using a TaqMan universal PCR master mix according to the manufacturer's instructions (Applied Biosystems Inc., Foster City, CA, http://www.appliedbiosystems.com). The amount of rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also measured using TaqMan Rodent GAPDH Control Reagents (Applied Biosystems), and the relative amount of rat-specific EPO cDNA was normalized to that of GAPDH cDNA.

Culture and Manipulation of Human and Rat MSCs and Human Endothelial Progenitor Cells

Human MSCs (hMSCs) were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, http://www.cambrex.com) and cultured using the manufacturer's recommended conditions. hMSCs were used within three passages to avoid phenotypic changes. The Luc/LacZ-dual-Tg rat was established by mating the Luc-rat and the LacZ-rat [15, 16]. Rat MSCs were established and maintained as described elsewhere [17]. Bone marrow mononuclear cells from a healthy volunteer were purchased from LONZA Walkersville Inc., http://www.lonza.com and human endothelial progenitor cells (EPCs) were established as described elsewhere [18]. It was confirmed that these cells contained an EPC-enriched population as assessed by the expression of CD31, VE-cadherin, vascular endothelial growth factor type 2 receptor, and von Willebrand factor. EPCs were used within five passages to avoid phenotypic changes. In some experiments, cells were labeled with LacZ using the MFG-LacZ retrovirus, as described elsewhere [19].

Bone Marrow Transplantation

Bone marrow transplantation (BMT) was performed as described elsewhere [20] with a few modifications. Bone marrow cells were flushed from the femur, tibia, and pelvis and washed with saline. Host mice were prepared for transplantation with a sublethal dose of irradiation (6.0 Gy) before 1×10^6 cells were injected into recipient mice via the tail vein.

In Vivo Imaging of Luc Expression

Rat MSCs (5×10^5 per mouse) from Luc/LacZ transgenic rats were injected into NOD/SCID mice via the tail vein every other day from day 3 after metanephros transplantation. Injected cells were traced by Luc expression measured 5 minutes after the injection of 20 mg/kg luciferin (Luc substrate) using an IVIS Imaging System 200 Series (Xenogen Corporation, Alameda, CA, http://www.xenogen.com).

X-Gal Assay

The X-Gal Assay was performed to assess the expression of the LacZ gene as described elsewhere [21].

Cell Fate Regulation Using ER-E2F1 Mouse

The ER-E2F1 mouse was generated by crossing CAG-ER-E2F1 mice, which express VENUS in the presence of Cre recombinase, with CAG-Cre mice. Metanephroi from these mice were implanted into rat omentum, which were then administered with FK506 (2 mg/kg per day) for 10 days. Tamoxifen (100 mg/kg per day) was also administered orally from day 4 to 10. After 10 days, the remaining kidney of the host rat was removed to avoid any effects of EPO from the native kidney. The next day, anemia was induced and 12 hours later, blood and mature metanephroi were collected for further analysis. Figure 1 shows the experimental protocol and flow diagram of the establishment of self-tissue. In some experiments, grown metanephroi were maintained for another 2 weeks in the omentum without immunosuppression, and then EPO production was measured as described above.

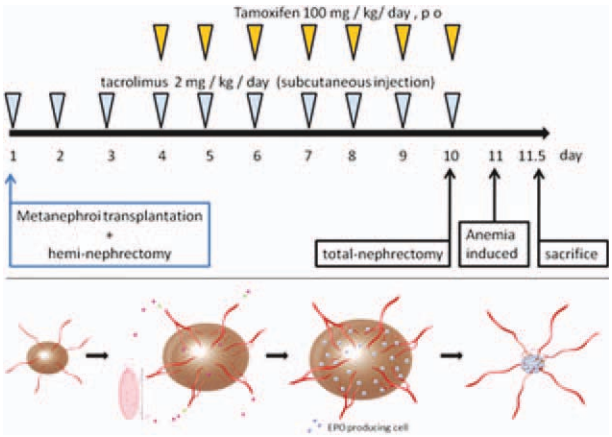

Figure 1. Experimental protocol and flow diagram of the establishment of self-tissue. The embryonic organ transplanted into the omentum recruits bone marrow mesenchymal stem cells, while it develops and provides a niche for them to differentiate into EPO-producing cells. The xenocomponent is then eliminated by a suicide gene contained in the metanephros under the control of an external drug. Abbreviation: EPO, erythropoietin.

Histochemical Analysis

Masson trichrome and periodic acid-schiff staining were performed as described elsewhere [22]. Immunohistochemical analysis was performed with the antibodies shown in Supporting Information Table S2, as described elsewhere [21] with some modification. Antigens were retrieved using a pressure chamber (Pascal; DAKO, Denmark, http://www.dako.com) according to the manufacturer's instructions.

Metanephric Organ Culture

Metanephroi were dissected and cultured on 12-mm diameter, 0.4 μm Nucleopore filters (Corning-Costar, Cambridge, MA, http://www.corning.com) at the air-fluid interface in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (FBS) (Gibco-BRL, Gaithersburg, MD, http://ja.invitrogen.com), 110 mg/l sodium pyruvate, and streptomycin and penicillin (50 U/ml each; Gibco). Medium was changed every 2 days. To assess the drug-inducible switch of E2F1 induction, metanephroi were cultured with 4-OH tamoxifen (300 nM; Sigma, http://www.sigmaaldrich.com) to induce apoptosis.

DNA Sequencing

Total RNA extracted from grown implants was subjected to cDNA synthesis. Rat and cat EPO expression was confirmed by PCR, and the downstream sequences (3′) of PCR products were identified using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

Establishment of Fluorescent Porcine MSCs

Kusabira-Orange Tg swine were established as described elsewhere [23]. Their MSCs were processed using the same

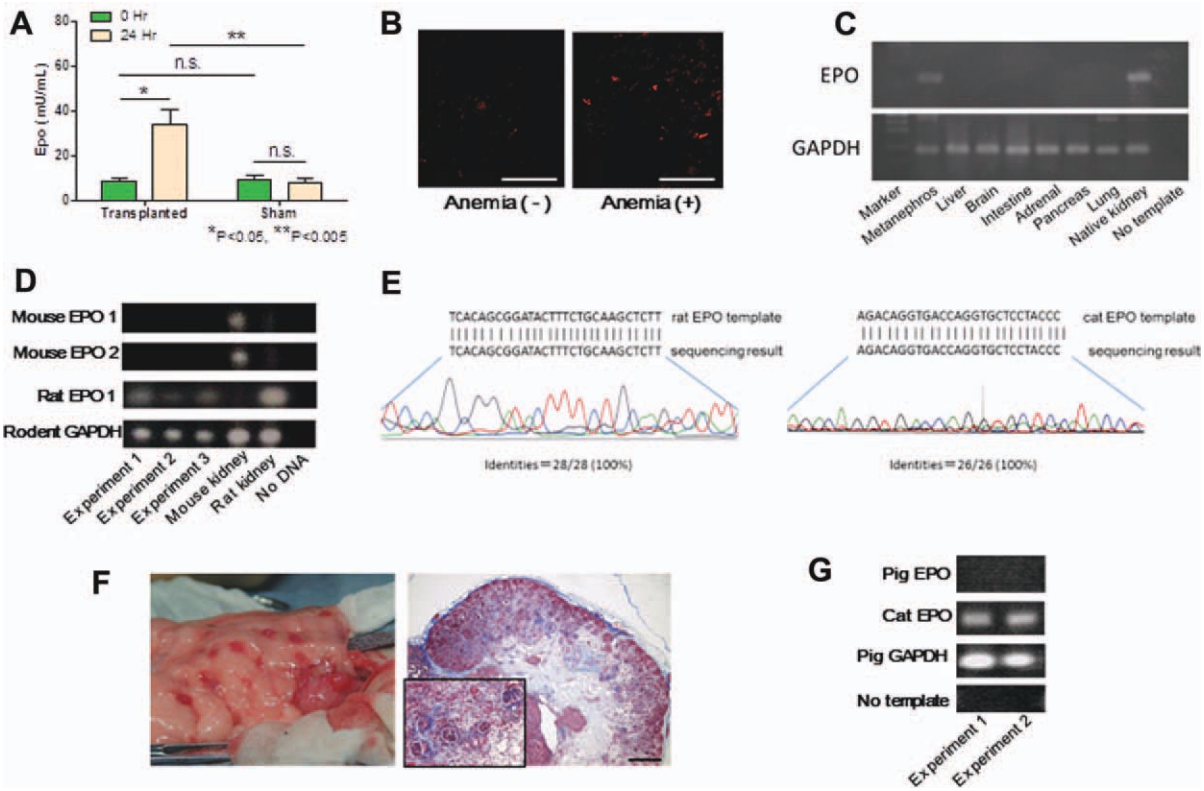

Figure 2. Host cells recruited to the transplant differentiate into EPO-producing cells. EPO expression by metanephroi grown in rat omentum as assessed by **(A)** RIA and **(B)** immunohistochemistry. **(C):** EPO expression by embryonic organs grown in the omentum and epididymis. Rat embryonic organs (E15) were transplanted into rat omentum and epididymis, and EPO expression was assessed by reverse transcription polymerase chain reaction (RT-PCR). As controls, EPO expression in native kidney and no template (negative control) was also assayed. **(D):** Mouse metanephroi transplanted into rat omentum and epididymis secreted rat EPO. To confirm the negative signal of mouse EPO, two differing pairs of species-specific primers were used to overcome the possibility of using an inappropriate primer pair. Results of three representative experiments are shown. **(E):** DNA sequences of the RT-PCR products of mouse metanephroi transplanted into rat omentum (left) and pig metanephroi transplanted into cat omentum (right) were analyzed to confirm that they contained unique sequences for rat and cat EPO DNA, respectively. **(F):** Appearance (left) and microscopic findings of pig metanephroi at 3 weeks after transplantation into cat omentum. **(G):** Pig metanephroi transplanted in cat omentum began to produce cat EPO. The results of two representative experiments are shown. Abbreviations: EPO, erythropoietin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; N.S., not significant.

protocol for MSC derivation from human adipose tissue [24] and cryopreserved in α-modified Eagle's medium (α-MEM; Gibco-BRL, Tokyo) supplemented with 10% FBS, 1% antibiotic-antimycotic (Gibco-BRL), and 10% dimethyl sulfoxide (DMSO; Merck, Tokyo). After thawing, cells were cultured in α-MEM supplemented with 10% FBS. At 70%–80% confluence, cells were harvested with 0.05% trypsin-EDTA (Gibco-BRL).

Metanephros Cryopreservation

Pig metanephroi were cryopreserved using the vitrification method described by Kagawa et al. [25] with slight modifications. Briefly, metanephroi were initially equilibrated in 7.5% ethylene glycol (EG) and 7.5% DMSO in handling medium (HM; 20 mM Hepes-buffered TCM199 + 20% [vol/vol] calf serum) for 25 minutes, followed by a second equilibration in vitrification solution (VS) consisting of 15% EG and 15% DMSO in HM for 20–50 minutes on ice. Metanephroi were then placed in a minimum volume of VS on a Cryotop device (Kitazato BioPharma, Fujinomiya, Japan, http://www.kitazato-biopharma.com) and then directly submerged into liquid nitrogen. For warming, the Cryotop was directly immersed in HM containing 1 M sucrose for 1 minute at 38.5°C. Then, metanephroi were transferred into HM with 0.5 M sucrose for 3 minutes at room temperature and then washed twice in HM for 5 minutes before transplantation.

Statistical Analysis

Data were expressed as the mean ± SE of the mean. Data from various groups were compared with the two-sample t test. In grouped analysis, a two-way analysis of variance and a Bonferroni post hoc test were used. $p < .05$ was considered significant.

Results

Initially, C57BL/6 (B6) mouse metanephroi (E13) were implanted in the omentum or epididymis of Lewis rats that were then administered FK506 daily for 10 days. Grown metanephroi expressed EPO as assessed by the serum EPO concentration and immunohistochemistry (Fig. 2A, 2B). This phenomenon was only found when the metanephros was transplanted, while other embryonic organs including the liver, brain, intestine, adrenal glands, pancreas, and lung showed no such ability (Fig. 2C). To examine the origin of EPO in grown metanephroi, PCR using species-specific primers and sequence analysis were performed. As shown in Figure 2D and 2E, EPO was of rat origin, suggesting that host cells migrated into the transplanted tissue and differentiated into EPO-producing cells. To confirm that this effect was common among species, pig metanephroi (E29-31) were implanted in the omentum of cats that received cyclosporine

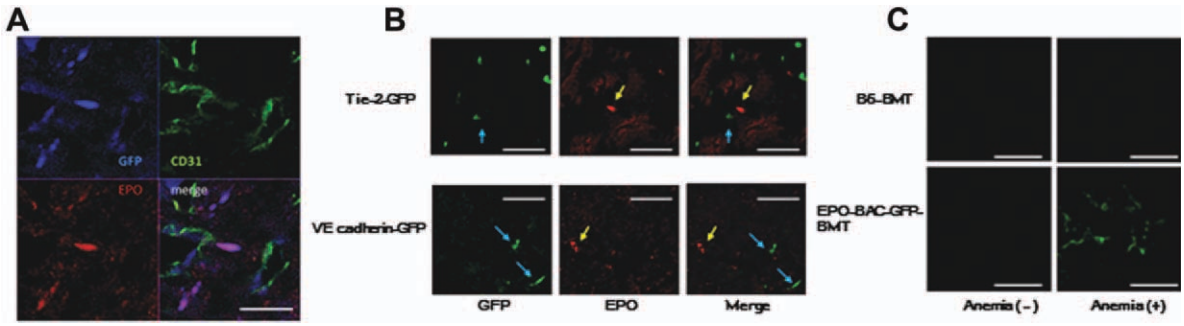

Figure 3. The origin of EPO-producing cells in transplanted metanephroi. **(A):** Host vessels that were integrated into metanephroi were not the origin of EPO-producing cells. B6 mouse metanephroi were implanted in the omentum of EGFP transgenic rats. CD31 (green); EPO (red); GFP (blue). **(B):** Host vessels that were integrated into metanephroi were not transdifferentiated into EPO-producing cells. B6 mouse metanephroi were implanted into the omentum of Tie-2-GFP and VE-cadherin-GFP mice GFP (blue); EPO (yellow). **(C):** Bone marrow-derived cells were demonstrated to be the origin of EPO-producing cells as follows. Bone marrow cells of EPO-BAC-GFP and control B6 mice were transplanted, and these chimeric mice were used as the host for metanephroi implantation, so that only EPO-producing cells derived from bone marrow cells expressed EGFP. Abbreviations: EPO, erythropoietin; GFP, green fluorescent protein.

daily. The volume of the transplant increased by more than 10^6-fold within 3 weeks (Supporting Information Movies S1, S2) without any sign of tumor formation (Fig. 1E), and the developing pig metanephroi were found to produce EPO of cat origin (Fig. 2D, 2F).

Immunohistochemical analysis revealed that EPO-producing cells in mouse metanephroi grown in the EGFP transgenic rat were positive for EGFP, but negative for CD31 (Fig. 3A), suggesting that integrated host endothelial cells were not producing EPO. Furthermore, to verify that EPO-producing cells were not transdifferentiated from the endothelial cells of integrated host vessels, two types of endothelial lineage reporter mice, Tie-2-Cre and VE-cadherin-Cre mice, were crossed with CAG-CAT-EGFP mice. After the cells of these crossed mice differentiated into an endothelial lineage, they were permanently labeled with EGFP; therefore, we used these mice as hosts for the metanephroi of wild-type mice. As shown in Figure 3B, EPO-producing cells never merged with the endothelial-lineage cells. This result indicated that metanephroi transplanted into the omentum provided a niche for host bone marrow cells to differentiate into EPO-producing tissue, and this effect did not occur via integrated host vessels. To clarify the origin of EPO production, bone marrow cells of a transgenic mouse expressing EGFP under the control of the EPO promoter (EPO-BAC-GFP mice) were transplanted into wild-type mice, thereby enabling the tracking of EPO-producing cells after transplantation. These chimeric mice were used as hosts, and syngeneic B6 mouse metanephroi were implanted into their omentum. EGFP-positive cells were detected in the grown metanephroi after induction of anemia, whereas no EGFP-positive cells were found even after anemia induction in the control group that were populated with syngeneic bone marrow of wild-type B6 mouse prior to metanephroi transplantation (Fig. 3C).

Next, we examined whether circulating bone marrow-derived cells (BMCs) had the ability to home exclusively to the developing metanephros and determine the fraction of BMCs responsible for such ability. We previously found that ESCs and iPS cells quickly form teratomas in the metanephros [3], suggesting that PS cells are not responsible for this phenomenon and that multipotent stem cells, which somehow commit to a mesenchymal lineage, in bone marrow may contribute to EPO production. Therefore, we focused on MSCs and EPCs, in which human MSCs and EPCs were retrovirally transfected with the LacZ gene and then intravenously injected into NOD/SCID mice hosting B6 metanephroi in their omentum. Immunohistochemical and RT-PCR analysis showed that the injected MSCs were recruited and began to express EPO, whereas EPCs had no such potential (Fig. 4A, 4B). BMT studies using Tie-2 and VE-cadherin mice also revealed that bone marrow-derived EPCs were not the source of EPO-producing cells (Fig. 4C). Injection of MSCs derived from the bone marrow of transgenic rats expressing both Luc and LacZ into chimeric NOD/SCID mice with B6 metanephroi in their omentum showed that the injected cells were recruited into metanephroi, but not into the native kidney, and that the recruited cells did indeed express EPO (Fig. 4D, 4E). LacZ-positive cells were scattered within the interstitium, although in a well-differentiated area in the metanephros, which resembled the structure observed in native kidney (Supporting Information Fig. S1). These results demonstrated that bone marrow-derived MSCs differentiated into EPO-producing cells only in the metanephros developing in the host animal. Therefore, host developmental signals must be important in this differentiation process.

Finally, we eliminated the xenocomponent using transgenic ER-E2F1 mice and inducible and targeted cellular suicide. E2F1 is a transcription factor that regulates cell proliferation, and its ectopic expression induces apoptosis in differentiated cells. Cells from the ER-E2F1 mouse can be eliminated on demand after tamoxifen administration. The efficacy of tamoxifen-induced apoptosis of these cells was confirmed in vitro, in which the metanephric scaffold from the ER-E2F1 mouse gradually disappeared while the injected MSCs continued to be maintained (Fig. 5A). Metanephroi from ER-E2F1 mice and B6 control mice were transplanted into Lewis rats that were daily administered with FK506 and tamoxifen (Fig. 1). Histological analysis showed that a mature kidney structure formed in transplants in the control B6 group but not in the ER-E2F1 group (Fig. 5B). The weight of grown transplants in ER-E2F1 mice was significantly lower at 1.3 ± 0.4 mg, as compared to 4.0 ± 0.4 mg in the control B6 group ($p < .001$; Fig. 5C). The number of cells of the grown transplants in ER-E2F1 mice was also lower at $4.4 \pm 1.7 \times 10^4$ cells per milliliter, as compared to $38.3 \pm 9.7 \times 10^4$ cells per milliliter from transplants grown in the control group ($p = .01$; Fig. 5D). However, quantitative PCR and RIA of serum EPO concentrations revealed that rat EPO was produced from metanephroi in both groups, with no significant differences in the amount of EPO produced (Fig. 5E, 5F). Thus, EPO-producing cells derived from host bone marrow maintained EPO production after elimination of the xenocomponent, that is, the metanephros. Finally, established tissues were maintained in the omentum with or without FK506 for another 2 weeks. EPO production was attenuated by transplants from wild-type metanephros even using FK506 due to parenchymal damage

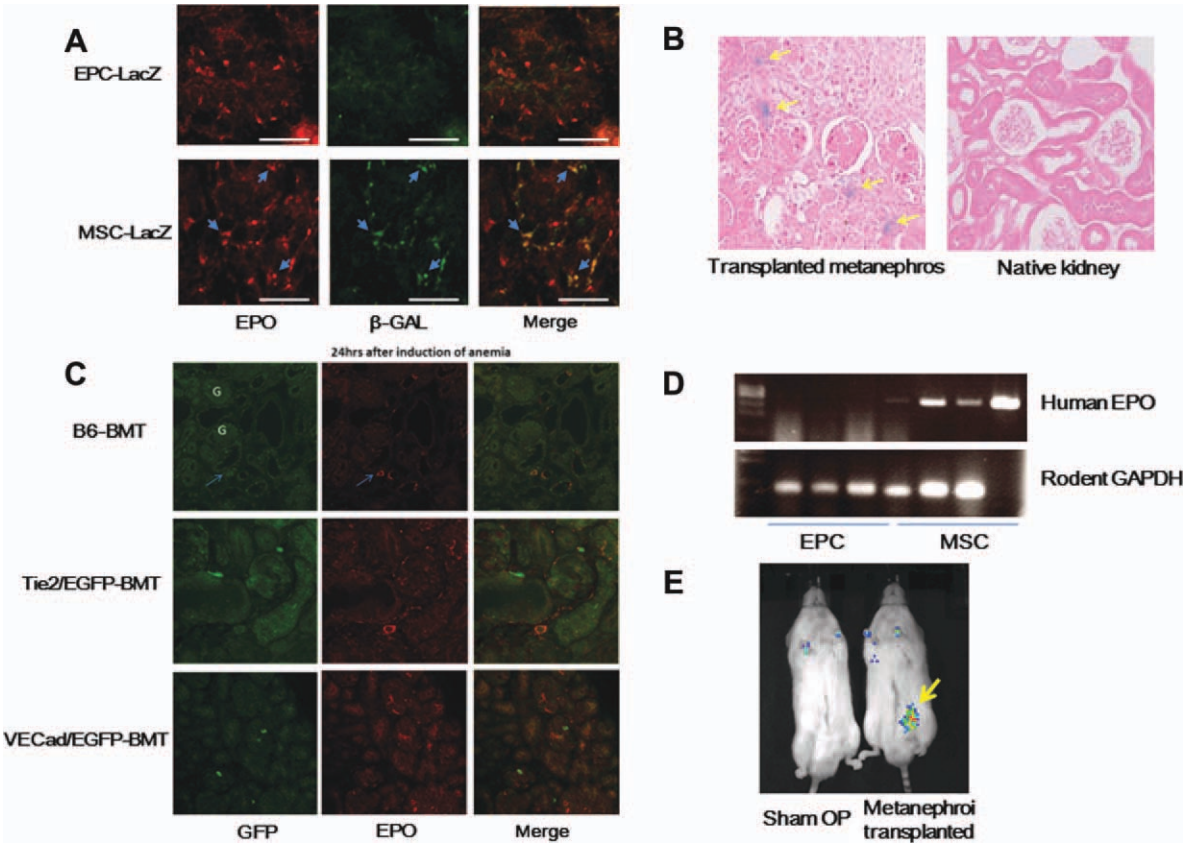

Figure 4. MSCs from bone marrow are the source of EPO-producing cells. **(A):** Human bone marrow-derived MSCs, but not EPCs, differentiate into EPO-producing cells in metanephroi transplanted into NOD/SCID mice. Human MSCs and EPCs bearing LacZ were injected via the tail vein, and the expression of EPO (red) and *β*-gal (green) in metanephroi was assessed by (A) two-color immunohistochemistry and **(B)** an X-gal assay. EPO-producing cells were localized in the interstitium of metanephroi. **(C):** To examine the contribution of endothelial-lineage cells to the development of EPO-producing cells, the bone marrow of Tie-2-EGFP and VE-cadherin mice was transplanted into wild-type B6 mice, and these chimeric mice were used as hosts for metanephric implantation. In these mice, bone marrow-derived cells (BMCs) that differentiated toward an endothelial lineage were permanently labeled with EGFP even if they subsequently transdifferentiated into other lineages. EGFP-positive cells did not merge with EPO-producing cells at any time point, indicating that EPO-producing cells were not derived from BMCs via the nonendothelial lineage pathway. G; glomerulus. **(D):** RT-PCR using human EPO-specific primers. **(E):** In vivo imaging of infused MSCs recruited to implanted metanephroi. Abbreviations: BMT, bone marrow transplantation; EGFP, enhanced green fluorescent protein; EPC, endothelial progenitor cell; EPO, erythropoietin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; MSC, mesenchymal stem cell.

by hydronephrosis [19]. However, transplants from E2F1 metanephros sustained EPO production even after 2 weeks without FK506 (Fig. 5G).

Conclusion

These data demonstrate that xenometanephroi can provide a niche for host BMCs to differentiate into EPO-producing tissues. Furthermore, the resultant EPO-producing cells can be manipulated to exclusively consist of host cell components using fate-controlled animals.

Discussion

One possible means of creating a niche for stem cells to differentiate into a mature tissue is to provide an artificial scaffold upon which stem cells can function. This strategy was recently used by Ott et al. [26] to successfully develop a beating heart using a cadaveric heart as the artificial scaffold. This technique was also applied to successfully regenerate liver [27] and lung [28], thereby generating functional hepatocytes and gas exchangers, respectively. However, in the case of kidneys, only morphologically similar but not functional kidney can be established [29], suggesting that three-dimensional architecture alone is not enough for a niche to differentiate complicated tissue, and additional signals are required. In this context, our system not only provides the scaffold but also provides the developmental program for endogenous MSCs to establish EPO-producing tissues. Most parts of the kidney are derived from mesoderm, and we previously found that MSCs differentiate into nephron and interstitial tissue if they are located at the site of nephrogenesis of developing embryo [22]. Accordingly, it seems natural that only endogenous MSC can differentiate into kidney lineage cells.

MSCs have been shown to be nonimmunogenic and have frequently been safely transplanted into allogeneic patients. In contrast, our system induces host bone marrow-derived MSCs to function naturally in a developmental niche, although the intermediate molecules that mobilize and attract MSCs have not been elucidated. Thus, our method appears to be safer because the xenocomponent only provides a scaffold for the niche and can be eliminated when it becomes unnecessary, thereby making it more suitable for clinical application (Supporting Information Fig. S2). To generate larger tissue, a

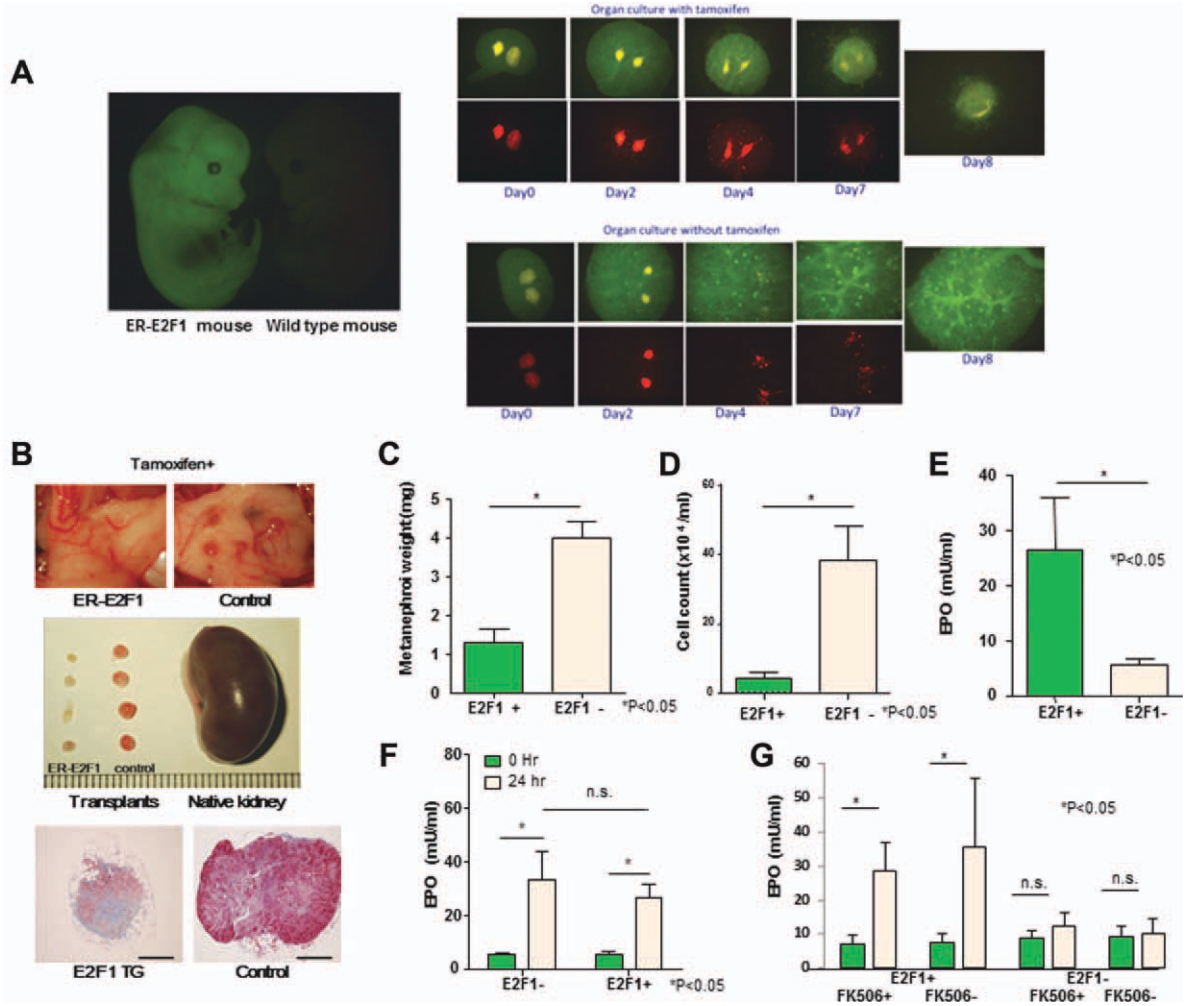

Figure 5. Elimination of the xenocomponent of metanephroi to generate completely self-EPO-producing tissue. **(A):** The ER-E2F1 mouse was generated by crossing CAG-ER-E2F1 mice, which express VENUS in the presence of Cre recombinase, with CAG-Cre mice. The resultant mice ubiquitously express EGFP (upper panel). Metanephroi were dissected from these embryos (E13) and the organs were cultured with injected mesenchymal stem cell (MSCs) from Kusabira-Orange transgenic pigs for 8 days in vitro with or without tamoxifen. EGFP-positive cells gradually disappeared in the presence of tamoxifen, while injected MSCs were maintained. **(B):** Appearance and histological findings of ER-E2F1 metanephroi that were transplanted into the omentum for 10 days. Controls were metanephroi from wild-type B6 mice, which were transplanted into the same rat with oral tamoxifen administration. Their weight and number of cells are shown in **(C)** and **(D)**, respectively. EPO-production was maintained even after elimination of the xenocomponent as shown by **(E)** quantitative PCR of EPO expression and **(F)** assessment of serum EPO concentrations. **(G):** Sustainability of EPO production by E2F1 metanephroi in rat omentum without immunosuppression. E2F1 metanephroi grown in the omentum with tamoxifen and FK506 administration were maintained for another 2 weeks without FK506. Abbreviations: EPO, erythropoietin; N.S., not significant.

bigger scaffold, such as a pig metanephros, would be needed and we have begun generation of a "suicide pig" for this purpose. However, providing pig metanephroi at the site of an operation would be highly inconvenient in a clinical setting because it requires special facilities to isolate the pig embryo. However, in this context, we previously confirmed that pig metanephroi can be cryopreserved and shipped when frozen using a particular cryopreservation technique [30] (Supporting Information Table S3).

It is currently unknown how many pig metanephroi would be required to enable a patient to recover from renal anemia and how long this xenotissue could be sustained for. Theoretically, it could be permanent since it is isolated from immune attack and integrated in its own body. Using a rat model, we previously found that transplantation of two metanephroi was enough to induce recovery from anemia [14], and our preliminary data using a cloned pig showed that one pig metanephros was enlarged by more than 30 g within 3 weeks. Pig metanephroi are approximately one-fifth the volume of a human kidney, which is theoretically enough to maintain EPO levels in the normal range. Therefore, we believe at least two to three pig metanephroi would be sufficient to sustain EPO therapy. The following is the estimated cost of the procedure in Japan. The pig and its maintenance fee in an SPF facility is approximately $6,000 (one pregnant sow yields approximately 10 embryos and one embryo has two metanephroi, therefore, $600 for each patient). Artificial fertilization costs approximately $150. Transplantation using an endoscopic procedure is approximately $2,000. If transplantation was required once a year, the total cost would be less than $3,000 per person per year excluding the initial investment. Therefore, even though transplantation may be required twice a year, consumptive costs may be cheaper when compared with recombinant EPO administration. Therefore, we believe that even if this technique would require renewal for unexpected reasons, it

would still be an effective alternative to the repeated injection of expensive recombinant EPO protein over the lifetime of chronic renal failure (CRF) patients. We are currently planning to launch a preclinical trial in a veterinary setting by treating pet cats, because more than 30% of pet cats die from CRF [31], and the provision of recombinant EPO administration is ineffective due to EPO alloantibody production [32]. In addition to conventional xenotransplantation, we characterized the use of a developing embryonic kidney as a temporary scaffold to provide MSCs with a niche to differentiate into mature kidney-specific cells. After their differentiation, the scaffold can be genetically eliminated so that long-term administration of an immunosuppressant is not required. This technique could be potentially used for the application of MSCs to other organs, such as the generation of insulin-producing tissue. After accumulating data on the safety and efficacy issues in a veterinary clinic, this method may be evaluated in a human clinical setting. This project (termed Project Yamaton-K) is expected to be intensively pursued, particularly in light of the 2011 Japanese earthquake, which revealed dialysis patients to be among the most vulnerable patient groups after such a disaster.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflict of interest.

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