Please cite this article in press as: Tan et al., MFG-E8 Is Critical for Embryonic Stem Cell-Mediated T Cell Immunomodulation, Stem Cell Reports (2015), http://dx.doi.org/10.1016/j.stemcr.2015.09.005

Stem Cell Reports
Article

ISSCR

OPEN ACCESS

MFG-E8 Is Critical for Embryonic Stem Cell-Mediated T Cell Immunomodulation

Yuan Tan,[1] Bodour AlKhamees,[1] Deyong Jia,[1] Li Li,[1] Jean-François Couture,[1,2] Daniel Figeys,[1,2] Masahisa Jinushi,[3,*] and Lisheng Wang[1,2,4,*]

[1]Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada
[2]Ottawa Institute of Systems Biology, University of Ottawa, Ottawa, ON K1H 8M5, Canada
[3]Institute for Advanced Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjiku-ku, Tokyo 160-8582, Japan
[4]Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, ON K1H 8L6, Canada
*Correspondence: jinushi@z7.keio.jp (M.J.), lwang@uottawa.ca (L.W.)
http://dx.doi.org/10.1016/j.stemcr.2015.09.005

SUMMARY

The molecules and mechanisms pertinent to the low immunogenicity of undifferentiated embryonic stem cells (ESCs) remain poorly understood. Here, we provide evidence that milk fat globule epidermal growth factor 8 (MFG-E8) is a vital mediator in this phenomenon and directly suppresses T cell immune responses. MFG-E8 is enriched in undifferentiated ESCs but diminished in differentiated ESCs. Upregulation of MFG-E8 in ESCs increases the successful engraftment of both undifferentiated and differentiated ESCs across major histocompatibility complex barriers. MFG-E8 suppresses T cell activation/proliferation and inhibits Th1, Th2, and Th17 subpopulations while increasing regulatory T cell subsets. Neutralizing MFG-E8 substantially abrogates these effects, whereas addition of recombinant MFG-E8 to differentiated ESCs restores immunosuppression. Furthermore, we provide the evidence that MFG-E8 suppresses T cell activation and regulates T cell polarization by inhibiting PKCθ phosphorylation through the α3/5βV integrin receptor. Our findings offer an approach to facilitate transplantation acceptance.

INTRODUCTION

Understanding the molecular mechanisms governing transplantation acceptance is essential for successful stem cell-based therapies. Undifferentiated embryonic stem cells (ESCs) have been considered "immune privileged" tissues, but they become much more immunogenic upon in vivo differentiation (Robertson et al., 2007; Zhao et al., 2011). The low immunogenicity of undifferentiated ESCs has been associated with the low level of major histocompatibility complex (MHC) molecules and the absence of co-stimulatory molecules that would promote activation of allo-specific T cells (Boyd and Wood, 2009; Drukker et al., 2002, 2006). However, mechanisms underlying the lack of immunogenicity seem to be more complicated than originally speculated. Koch et al. have demonstrated that ESCs exert local immunosuppression through the production of soluble anti-inflammatory cytokines (Koch et al., 2008). We also observed that undifferentiated ESC-derived soluble factors (i.e., the water-soluble fraction of sonicated ESCs) modulate T cell functions (Mohib et al., 2012). However, the defined molecules and underlying mechanisms of their actions have not been revealed.

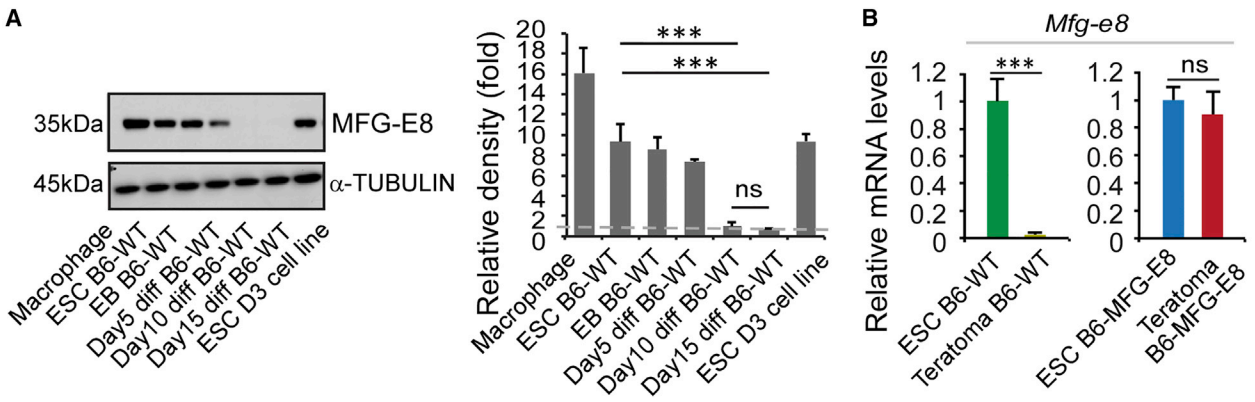
Here, we report that milk fat globule epidermal growth factor 8 (MFG-E8)/lactadherin is a key determinant. MFG-E8, a secreted glycoprotein, was initially identified in mammary gland epithelial cells. Further studies have revealed its anti-inflammatory functions in macrophages, where it assists in the phagocytosis of apoptotic cells through $\alpha_V\beta_3$/$\alpha_V\beta_5$ integrins (Brissette et al., 2012; Jinushi et al., 2007; Yang et al., 2011). However, molecular mechanisms pertinent to MFG-E8 expression in health and diseased conditions remain largely unknown. In addition, the functions of MFG-E8 on T cells and ESCs have not yet been reported. In this study, we demonstrate that MFG-E8 is essential in sustaining the "immune privileged" status of undifferentiated ESCs and in facilitating the acceptance of ESC-derived tissues across allogeneic MHC barriers and thus enabling the suppression of T cell-mediated immune responses through the PKCθ pathway.

RESULTS

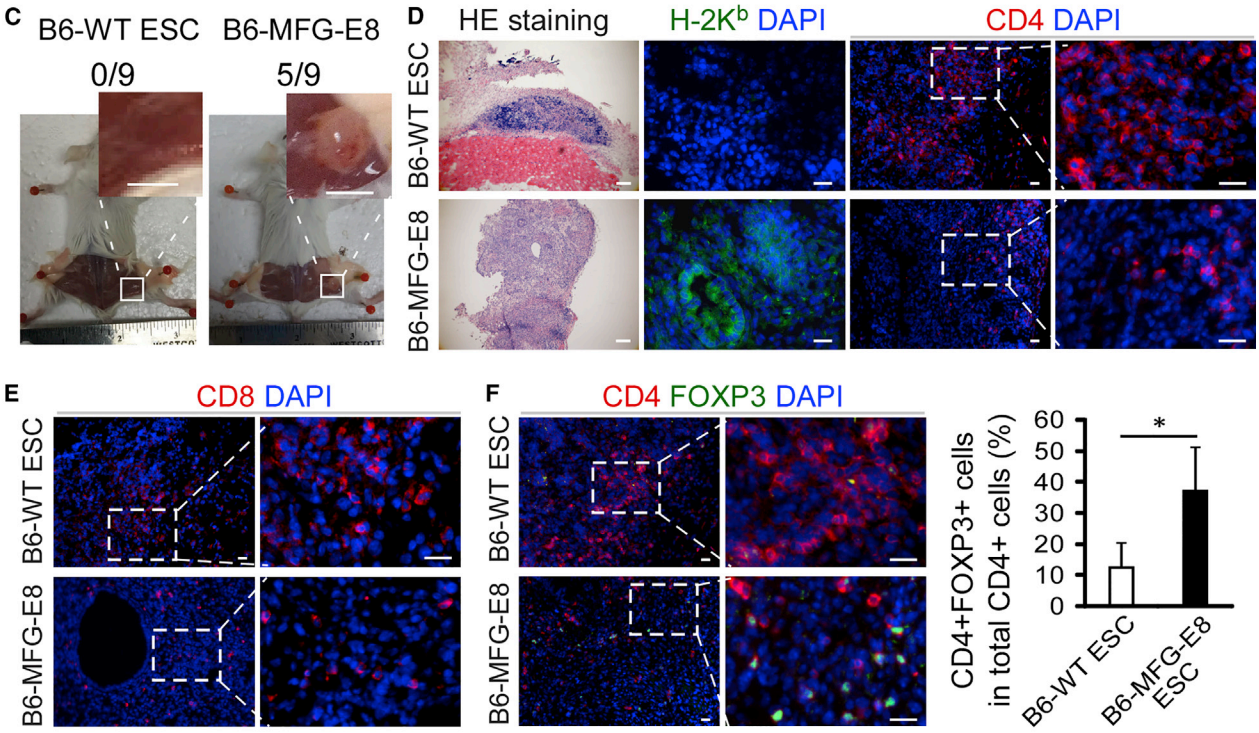
Undifferentiated, but Not Differentiated, ESCs Express High Levels of MFG-E8, and Upregulation of MFG-E8 Enables Survival and Engraftment of Undifferentiated and Differentiated Allogeneic ESCs

To define molecules with immunomodulatory potential in ESCs, we searched literature and proteomics databases (Graumann et al., 2008; Sarkar et al., 2012; Segura et al., 2005; Van Hoof et al., 2006) and selected the proteins that were closely associated with immune regulation and also highly expressed in and secreted by undifferentiated ESCs while diminished upon ESC differentiation. We found that, except for MFG-E8, other proteins originally selected were inefficient in the inhibition of T cell function in preliminary experiments and so were excluded from

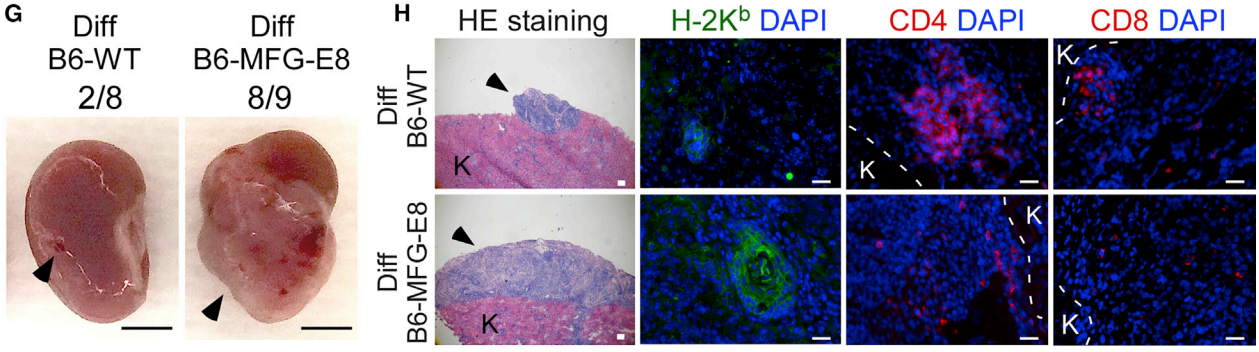
Please cite this article in press as: Tan et al., MFG-E8 Is Critical for Embryonic Stem Cell-Mediated T Cell Immunomodulation, Stem Cell Reports (2015), http://dx.doi.org/10.1016/j.stemcr.2015.09.005



Allogeneic transplantation: B6-WT or B6-MFG-E8 ESCs to BALB/c Recipients



Differentiated B6-WT or B6-MFG-E8 ESCs to BALB/c Recipients



(legend on next page)

Please cite this article in press as: Tan et al., MFG-E8 Is Critical for Embryonic Stem Cell-Mediated T Cell Immunomodulation, Stem Cell Reports (2015), http://dx.doi.org/10.1016/j.stemcr.2015.09.005

further investigation. As shown in Figures 1A and 2A, the gene and protein expression of MFG-E8 was diminished in a timely manner after spontaneous in vitro differentiation of embryoid body (EB) (i.e., formation of cellular aggregates). In addition, *Mfg-e8* gene was also downregulated after in vivo ESC differentiation (i.e., teratoma formation; Figure 1B). To determine whether MFG-E8 has an immunoregulatory function in ESCs, we transduced *Mfg-e8* gene into B6 wild-type ESCs (B6-WT, derived from C57BL/6 H-2^b mice) with a lentiviral vector (B6-MFG-E8 ESCs; Figure 2A) and implanted B6 B6-WT and B6-MFG-E8 ESCs into syngeneic C57BL/6 recipients. As expected, both B6-WT and B6-MFG-E8 ESCs formed teratomas containing significantly different levels of MFG-E8 proteins in the recipients (Figure 2B). In both instances, teratomas displayed the same differentiated structures representing three germ layers and showed a similar level of the MHC class I determinant H-$2K^b$ expression 4 weeks after injections (Figures 2C, 2D, and 3B). In addition, differentiated B6-WT ESCs showed a significant decrease in gene and protein expression of MFG-E8 in comparison to their undifferentiated counterparts. In contrast, teratomas generated from B6-MFG-E8 ESCs sustained a high level of MFG-E8 expression (Figures 1B and 2B). These results suggest that the differentiation capacity and MHC-I expression in B6 ESCs are not altered after MFG-E8 overexpression.

To determine whether MFG-E8 in ESCs was capable of facilitating graft acceptance across an MHC barrier, we subcutaneously transplanted B6-MFG-E8 ESCs into allogeneic BALB/c (H-2^d) mice. In allogeneic settings, B6-WT ESCs failed to form any detectable teratomas, whereas B6-MFG-E8 ESCs survived and generated five teratomas in nine implantations (Figure 1C). Immunofluorescent staining and histological analysis demonstrated that allogeneic B6-WT ESCs elicited severe graft infiltration of $CD4^+$ and $CD8^+$ T cells in the transplanted remnant (Figures 1D and 1E), suggesting that allogeneic B6-WT ESCs and their derivatives were destroyed by host alloimmune responses. Conversely, allogeneic B6-MFG-E8 ESCs formed typical teratomas with unnoticeable tissue necrosis but detectable MHC-I (H-$2K^b$) expression (Figure 1D). Importantly, in contrast to B6-WT controls, B6-MFG-E8 teratomas displayed limited numbers of infiltrating T cells while containing a high percentage of $CD4^+Foxp3^+$ regulatory T cells (Tregs; Figure 1F). Semiquantitative immunohistological analyses of syngeneic and allogeneic teratomas are summarized in Table S1.

To further determine whether MFG-E8 also facilitates engraftment of in vitro differentiated allogeneic ESCs, we derived EBs from B6-WT and B6-MFG-E8 ESCs by formation of cellular aggregates and cultured them in the absence of leukemia inhibitory factor for 7 days. This differentiation procedure has been used commonly in the field and represents a turning point for the early development of specialized tissues (Lumelsky et al., 2001; Mizuseki et al., 2003; Nat et al., 2012; Van Vliet et al., 2012). The differentiated EBs showed low levels of pluripotency genes (Figure 3E) but increased levels of MHC-I (H-$2K^b$) prior to transplantation under the kidney capsule of syngeneic or allogeneic recipients (Figure 3A). As expected, all

Figure 1. Undifferentiated, but Not Differentiated, ESCs Express High Levels of MFG-E8, and Upregulation of MFG-E8 Enables Survival and Engraftment of Undifferentiated and Differentiated Allogeneic ESCs

(A) Western blot analysis of MFG-E8 protein expression in undifferentiated ESCs and their differentiated derivatives. Results are representative of three independent experiments. Densitometry results represent means ± SD of cells obtained from three separate unpooled cultures with α-tubulin as a loading control. Abbreviation: ESC B6-WT, B6 ESC line derived from C57BL/6 H-2^b wild-type mouse; EB, embryoid body; Day5 diff B6-WT, day5 differentiated B6-wild-type ESCs; ESC D3 cell line, D3 ESC line derived from 129S2/SvPas mouse blastocysts.

(B) qPCR analysis of *Mfg-e8* mRNA levels in undifferentiated B6 ESCs and their differentiated derivatives in teratomas developing 4 weeks after injection into C57BL/6 mice. Data represent means ± SD of unpooled samples from three different mice.

(C) No detectable teratomas developed after transplantation of B6-WT ESCs into nine allogenic BALB/c mice, and five teratomas were generated after injection of B6-MFG-E8 ESCs into nine BALB/c mice ($p < 0.05$). Scale bars, 0.5 cm in insets.

(D–F) H&E staining of sections from B6-WT ESC injection sites (without teratoma formation) or B6-MFG-E8 ESC teratomas. Immunofluorescent staining of sections corresponding to each H&E staining shows expression of MHC-I (H-$2K^b$, green) and infiltrations of $CD4^+$ (red), $CD8^+$ (red), and $CD4^+/Foxp3^+$ (red/green with image analysis results; mean ± SD from three mice per group, three sections per mouse). Nuclei were counterstained with DAPI (blue). Scale bars, 200 μm (H&E) and 20 μm (immunofluorescent staining).

(G) Only two of eight allogeneic mice transplanted with B6-WT EBs show small implants, whereas eight of nine allogeneic mice transplanted with B6-MFG-E8 EBs are engrafted with larger implants. Arrowhead: implants. For a comparison, the same images are shown again in Figure S1A.

(H) Histological analysis of serial sections after 15-day transplantation of EBs differentiated from B6-WT and B6-MFG-E8 ESCs. Immunofluorescent staining of sections corresponding to each H&E staining shows expression of MHC-I (H-$2K^b$, green) and infiltrations of $CD4^+$ (red) and $CD8^+$ (red). Nuclei were counterstained with DAPI (blue). K, kidney; scale bars, 200 μm (H&E) and 20 μm (immunofluorescent staining).

See also Figure S1 for $CD4^+/Foxp3^+$ Treg results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Please cite this article in press as: Tan et al., MFG-E8 Is Critical for Embryonic Stem Cell-Mediated T Cell Immunomodulation, Stem Cell Reports (2015), http://dx.doi.org/10.1016/j.stemcr.2015.09.005

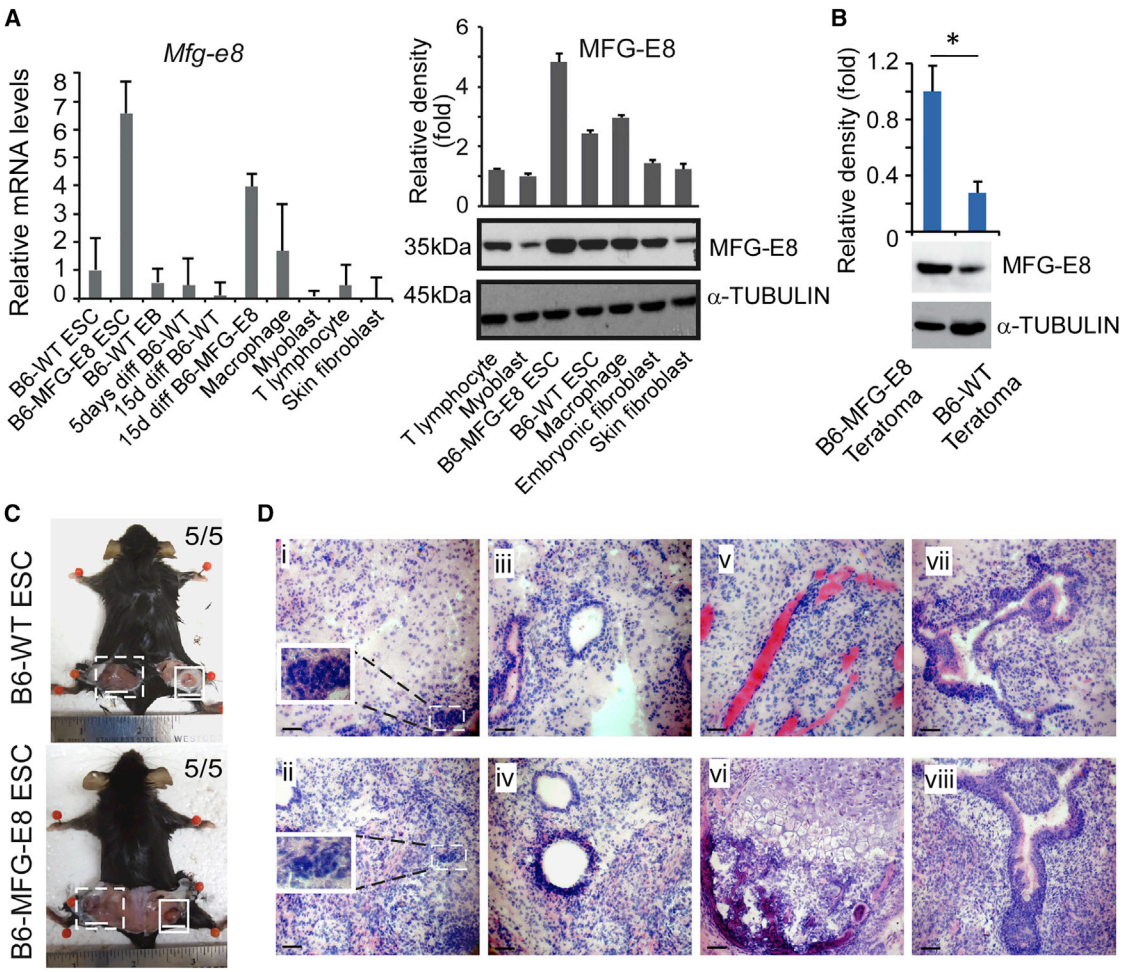


Figure 2. Teratoma Formation of B6-WT and B6-MEG-E8 ESCs in Syngeneic C57BL/6 Mice

(A) B6 wild-type ESCs (B6-WT) were transduced with a lentiviral vector encoding MFG-E8 and GFP, followed by drug selection to establish a stable ESC line with upregulated MFG-E8 expression (B6-MFG-E8 ESCs). qPCR and western blot were performed to assess MFG-E8 expression as indicated. *β-Actin* was used as a normalization control for qPCR and α-tubulin for western blot (which was quantified by densitometry from three independent experiments).

(B) The expression levels of MFG-E8 protein in teratomas after transplantation of B6-MFG-E8 and B6-WT ESC cells into syngeneic mice. n = 3, *p < 0.05.

(C) Both B6-WT and B6-MFG-E8 ESCs formed teratomas in syngeneic C57BL/6 mice after 4 weeks of transplantation into two flank regions (n = 5 mice). Scale bars, 1 cm.

(D) Teratomas generated from both B6-WT and B6-MFG-E8 ESCs were composed of tissues representing three germ layers, including ectoderm (i, ii: neural rosettes and glial tissue; iii, iv: cystic epithelium), mesoderm (v: striated muscle; vi: cartilage and osteoid island), and endoderm (vii, viii: glandular epithelium). Scale bars, 50 μm.

Abbreviations: Diff ESC, differentiated ESCs; Macrophage, RAW macrophage line as a positive control; Myoblast, C2C12 myoblast cells as a negative control.

implanted EBs were accepted by syngeneic mice and grew well (Figures 3F and 3G). However, in allogeneic BALB/c recipients, the majority of B6-WT EBs failed to grow and the implants showed severe infiltration of T cells with a low proportion of $CD4^+Foxp3^+$ Tregs. In contrast, eight of nine allogeneic mice transplanted with B6-MFG-E8 EBs were engrafted, and the implants showed limited T cell infiltration with a higher proportion of $CD4^+Foxp3^+$ Tregs (Figures 1G, 1H, and S1).

It is evident that overexpression of MFG-E8 in both undifferentiated and differentiated ESCs is sufficient to reduce allogeneic rejection (Figure 1), despite a significant increase in the expression of MHC-I (Figures 1 and 3) and co-stimulatory molecules that made differentiated ESCs more

Please cite this article in press as: Tan et al., MFG-E8 Is Critical for Embryonic Stem Cell-Mediated T Cell Immunomodulation, Stem Cell Reports (2015), http://dx.doi.org/10.1016/j.stemcr.2015.09.005

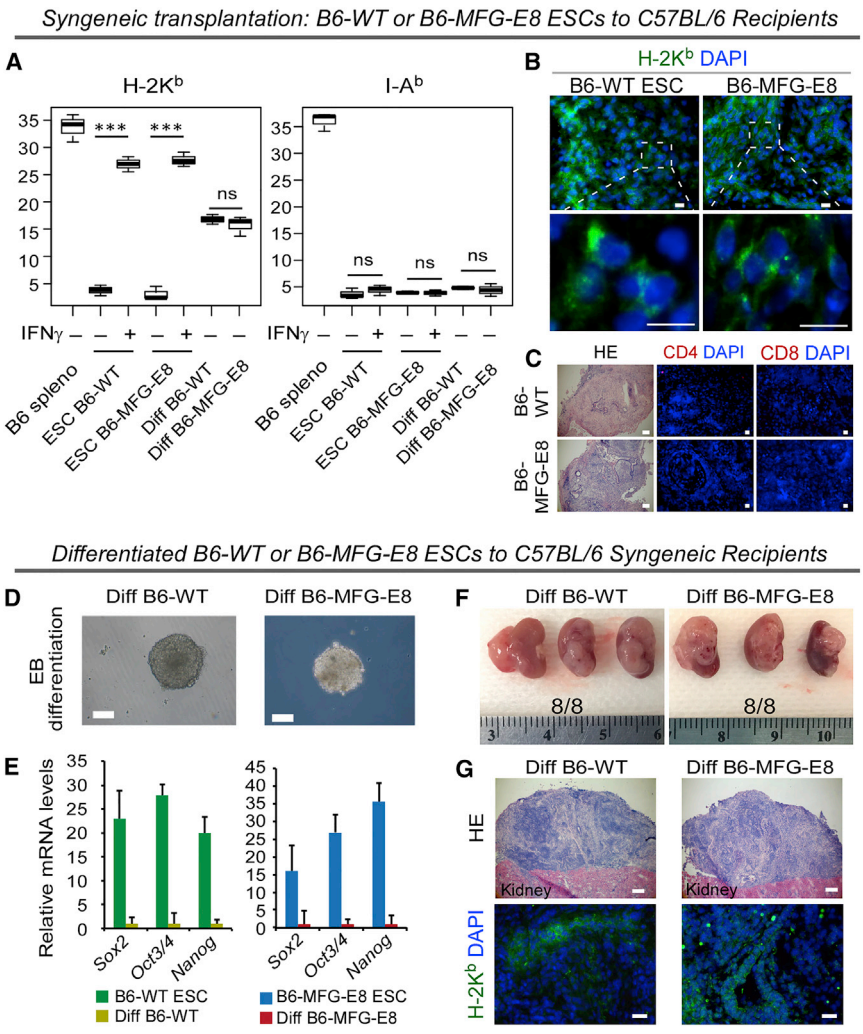


Figure 3. Immunogenicity of Tissues Derived from Undifferentiated and Differentiated B6-WT and B6-MFG-E8 ESCs in Syngeneic Recipients

(A) Boxplot of mean fluorescent intensity of MHC-I (H-2K^b) and MHC-II (I-A^b) in undifferentiated and differentiated (diff) B6-WT and B6-MFG-E8 ESCs. Data represent median ± interquartile range (P25–P75) based on flow cytometric analyses using three unpooled different cell passages and cultures for each group. ***p < 0.005; ns, not significant.

(B and C) The expression of H-2K^b (green), CD4 (red), and CD8 (red) in tumors generated from B6-WT and B6-MFG-E8 ESCs after implanted into syngeneic hosts (n = 5 mice in each group). Nuclei were counterstained with DAPI (blue). Scale bars, 10 μm (B), 200 μm (C, H&E), and 20 μm (C, CD4 and CD8).

(D and E) EBs derived from B6-WT and B6-MFG-E8 ESCs were differentiated for 7 days, showing a significant decrease in pluripotency genes (qPCR). Data represent mean ± SD of at least three independent experiments.

(F and G) The above EBs were transplanted into syngeneic mice C57BL/6 under kidney capsules and engrafted (eight out of eight for B6-WT and eight out of eight for B6-MFG-E8). Histological analysis of serial sections of syngeneic B6-WT and B6-MFG-E8 EBs 12 days after transplantation. The expression of H-2K^b (green) was detected. Scale bars, 100 μm, top panels (H&E); 20 μm, low panels (H-2K^b expression, green). Nuclei were counterstained with DAPI (blue).

immunogenic (Fairchild et al., 2007). Since overexpression of MFG-E8 does not inhibit MHC-I and II expression (Figure 1 and Figure 3), the immunoregulation of MFG-E8 is not through blocking MHC. Given that sustained MFG-E8 levels in the grafts were inversely associated with the presence of T cell infiltration (Figure 1; Table S1), MFG-E8 might contribute to graft acceptance by suppressing T cell responses. Although involvements of other innate inflammatory cells cannot be fully excluded, a study by Swijnenburg et al. has suggested that innate responses can be initiated by surgical procedures and are not completely specific to ESC implants (Swijnenburg et al., 2005). Accordingly, we focused on the direct role of MFG-E8 in T cell regulation, a question that has not been addressed.

MFG-E8 in ESCs Suppresses T Cell Activation and Proliferation

Based on the above in vivo observations and previous findings that ESC-derived soluble factors inhibited T cell activation (Mohib et al., 2012), we wanted to make a direct link between ESC-produced MFG-E8 and T cell activation and proliferation. ESC-derived soluble factors suppressed expression of activation markers (CD25, CD69, and CD44) in both CD4$^+$ and CD8$^+$ effector T cells stimulated in vitro, and neutralization of MFG-E8 with a specific antibody markedly mitigated the suppressive effects of ESC-derived soluble factors (Figures 4A and S2A), suggesting that MFG-E8 is responsible for ESC-mediated T cell inhibition.

To further assess the immunomodulatory functions of MFG-E8 on T cells, we stimulated the cultured mouse splenic T cells in the absence or presence of recombinant mouse MFG-E8 (rmMFG-E8). Treatment with rmMFG-E8 significantly suppressed both CD4$^+$ and CD8$^+$ effector T cell activation, resulting in a 2-fold decrease in CD25, CD44, and CD69 expression (Figure 4B). Conversely, introduction of MFG-E8 blocking antibody, but not an isotype control antibody, abrogated the suppression of activation

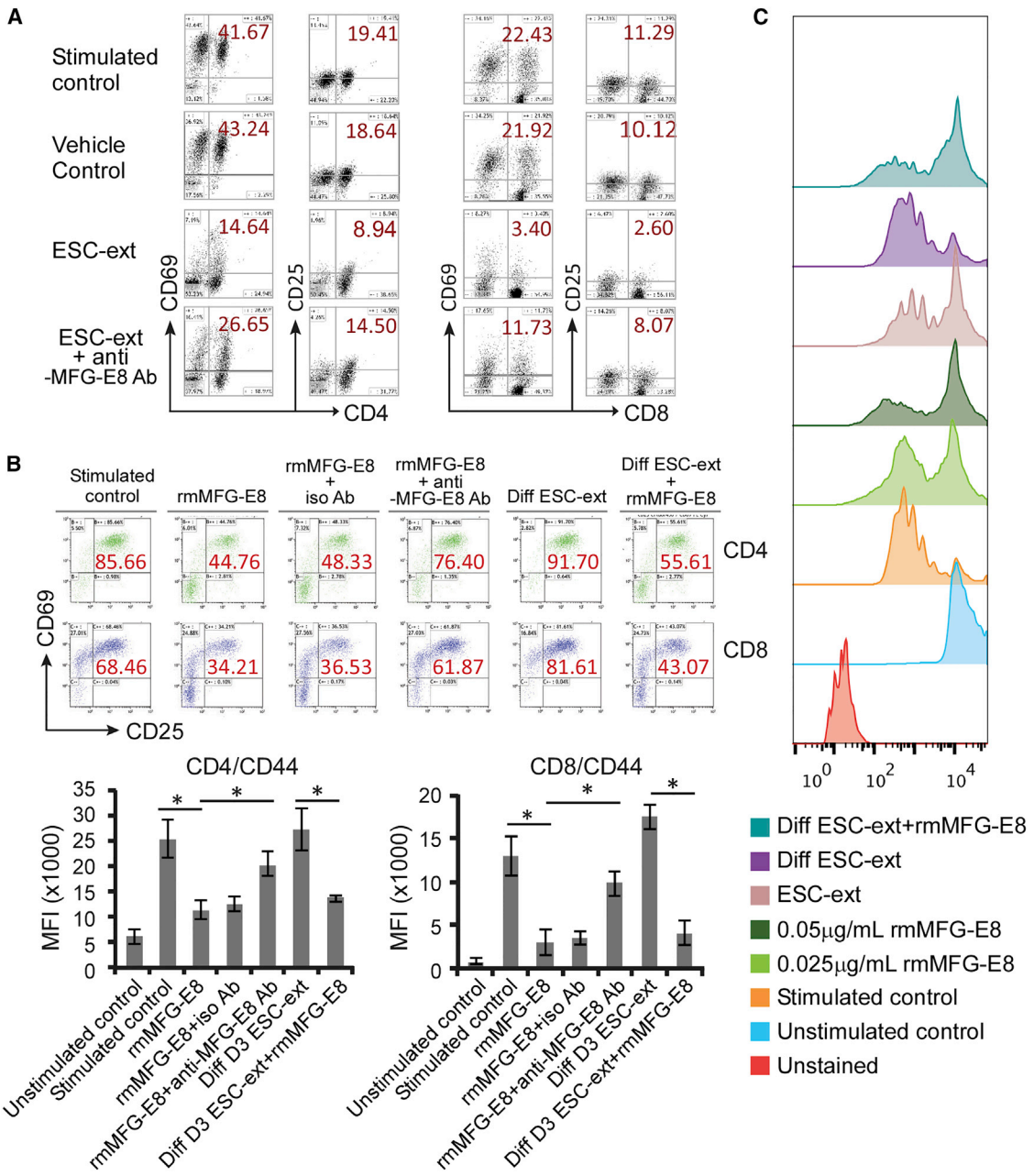


Figure 4. MFG-E8 in ESCs Suppresses T Cell Activation and Proliferation

Negatively selected C57BL/6 $CD3^+$ T cells were incubated with different treatment reagents, followed by stimulation with plate-bound anti-CD3/CD28 antibodies.

(A) After stimulation, T cells were either untreated (stimulated) or treated with vehicle control (Ct), ESC-derived soluble factors alone (ESC-ext prepared from D3 ESCs derived from 129S2/SvPas mouse blastocysts, 0.23 mg/ml), or in combination with anti-MFG-E8 antibody (2.5 μg/ml) for 24 hr. The cells were then stained for CD4, CD8, and activation markers CD25 and CD69 and subsequently analyzed by flow cytometry.

(B) The surface expression of CD25, CD44, and CD69 on $CD4^+$ and $CD8^+$ T cells was analyzed by flow cytometry in untreated control (Ct), stimulated control, recombinant mouse MFG-E8 (rmMFG-E8) alone, rmMFG-E8 with isotype antibodies (iso Ab), rmMFG-E8 with anti-MFG-E8 antibody, differentiated ESC-derived soluble factors (Diff ESC-ext from D3 ESCs), or Diff ESC-ext with rmMFG-E8. The dot plots are all from one experiment in which cells were stained for CD25 and CD69 markers, and three experiments were performed with similar results.

(legend continued on next page)

(Figure 4B). Moreover, $CD4^+$ and $CD8^+$ T cell activation was not affected after treatment with soluble factors derived from differentiated ESCs expressing low level of MFG-E8, with the majority of T cells showing upregulated activation markers after anti-CD3/CD28 stimulation. In contrast, reconstitution of differentiated ESC-derived soluble factors with rmMFG-E8 restored suppression, as indicated by a reduction of all three activation markers in T cells (Figure 4B). These results demonstrate that MFG-E8 is indeed a key component in ESC-mediated T cell inactivation.

We further tested whether MFG-E8 was capable of suppressing T cell proliferation using a carboxyfluorescein succinimidyl ester (CFSE) dilution assay with CFSE-labeled $CD3^+$ T cells. In the presence of rmMFG-E8, reduced cell divisions in activated T cells were observed, similar to those treated with undifferentiated ESC-derived soluble factors (Figure 4C). Conversely, differentiated ESC-derived soluble factors did not inhibit T cell proliferation, whereas MFG-E8 supplementation almost fully restored the suppressive effect on T cell proliferation (Figure 4C).

Together, the aforementioned results demonstrate that MFG-E8 is a key component in ESC-derived soluble factors that suppresses T cell activation and proliferation, providing the evidence that MFG-E8 produced by undifferentiated ESCs is directly involved in repressing T cell immune responses in addition to its conventional role in facilitating phagocytosis of apoptotic cells. Consequently, loss of MFG-E8 expression following ESC differentiation restores normal T cell responses to reject allogeneic implants; conversely, sustained MFG-E8 expression in differentiated cells inhibits T cell proliferation and activation, promoting graft acceptance (Figures 1 and 4).

Since cells deficient in MHC-I expression (e.g., ESCs) could be recognized by natural killer (NK) cells, we examined whether MFG-E8 also suppresses NK cell function as measured by interferon-γ (IFN-γ) production (Foley et al., 2014). As shown in Figure S2B, IFNγ production in fractionated NK cells was inhibited after treatment of the cells with ESC-derived soluble factors or rmMFG-E8. It is possible that NK cells participate in the early recognition of transplanted ESCs that express low MHC-I based on "missing-self" theory (Shifrin et al., 2014). Because differentiated B6-MFG-E8 EBs expressing MHC-I ($H\text{-}2K^b$) prior to transplantation are engrafted (Figure 3), it appears that T cells play a major role in successful engraftment, with the possible contribution of NK cells, warranting further investigation.

MFG-E8 in ESCs Suppresses Th1, Th2, and Th17 Subpopulations while Increasing Regulatory T Cells

Our results showed that B6-MFG-E8, but not B6-WT, implants displayed limited T cell infiltration with a high percentage of $CD4^+Foxp3^+$ Tregs (Figures 1 and S1). We therefore tested whether MFG-E8 in undifferentiated ESCs modulates T cell polarization induced by stimulation with anti-CD3/CD28 or phorbol myristate acetate (PMA). qPCR results examining the expression of lineage-specific transcription factors indicated a significant decrease in expression of transcriptional factors *Gata3* (Th2) and *Rorγt* (Th17) in T cells after treatments with rmMFG-E8 or undifferentiated ESC-derived soluble factors. Conversely, MFG-E8 neutralization restored *Gata3* and *Rorγt* expression (Figure 5A). Interestingly, *T-bet* (Th1) gene expression was unaffected in all treatment conditions (Figure 5A) despite a marked decrease in gene expression of the Th1-specific cytokine *Ifnγ* (Figure S3). In an attempt to further assess the changes in function of each T effector cell subset attributed to MFG-E8, T cells were intracellularly stained with IFN-γ (Th1), IL-4 (Th2), and IL-17 (Th17) antibodies. Consistent with changes in *Ifnγ* gene expression (Figure S3), T cells treated with rmMFG-E8 exhibited a notable decrease in IFN-γ-producing effector cells (Figure 5B). Likewise, in accordance with downregulated *Gata3* and *Rorγt* gene expression, the numbers of IL-4- and IL-17-producing T cells were significantly decreased (Figures 5C and 5D). Neutralization of MFG-E8 effectively ameliorated the suppressive effects of rmMFG-E8 or ESC-derived soluble factors, restoring the numbers of IFN-γ-, IL-4-, and IL-17-producing T cells (Figures 5B–5D). These data suggest that ESC-produced MFG-E8 inhibits the development of Th1, Th2, and Th17 subpopulations.

Furthermore, consistent with upregulated *Foxp3* and *Tgfβ* gene expression (genes associated with regulatory T cells; Figures 5A and S3) and increased proportions of $CD4^+Foxp3^+$ Tregs in the allografts after transplantation of B6-MFG-E8 ESCs and EBs (Figures 1F and S1B), the $CD4^+CD25^+Foxp3^+$ Treg subpopulation was markedly increased after treatment with rmMFG-E8 (Figure 5E). Of note, the elevated Treg subpopulation induced by ESC-derived soluble factors was almost completely blocked following the neutralization of MFG-E8 with an antibody (Figure 5E).

Collectively, our results provide direct evidence that ESC-produced MFG-E8 enhances polarization of the Treg subpopulation, suppresses Th1 function, and prevents the development of Th2 and Th17 T cells. Previous studies

Histograms represent the mean fluorescent intensity (MFI) of either $CD4^+$ or $CD8^+$ T cells with CD44 expression for each treatment group. Error bars indicate mean ± SD from three independent experiments; *$p < 0.05$ (ANOVA and Tukey HSD).

(C) Proliferation assays were performed on CFSE-labeled C57BL/6 $CD3^+$ splenic lymphocytes. Results are representative of three independent experiments.

See also Figure S2.

Please cite this article in press as: Tan et al., MFG-E8 Is Critical for Embryonic Stem Cell-Mediated T Cell Immunomodulation, Stem Cell Reports (2015), http://dx.doi.org/10.1016/j.stemcr.2015.09.005

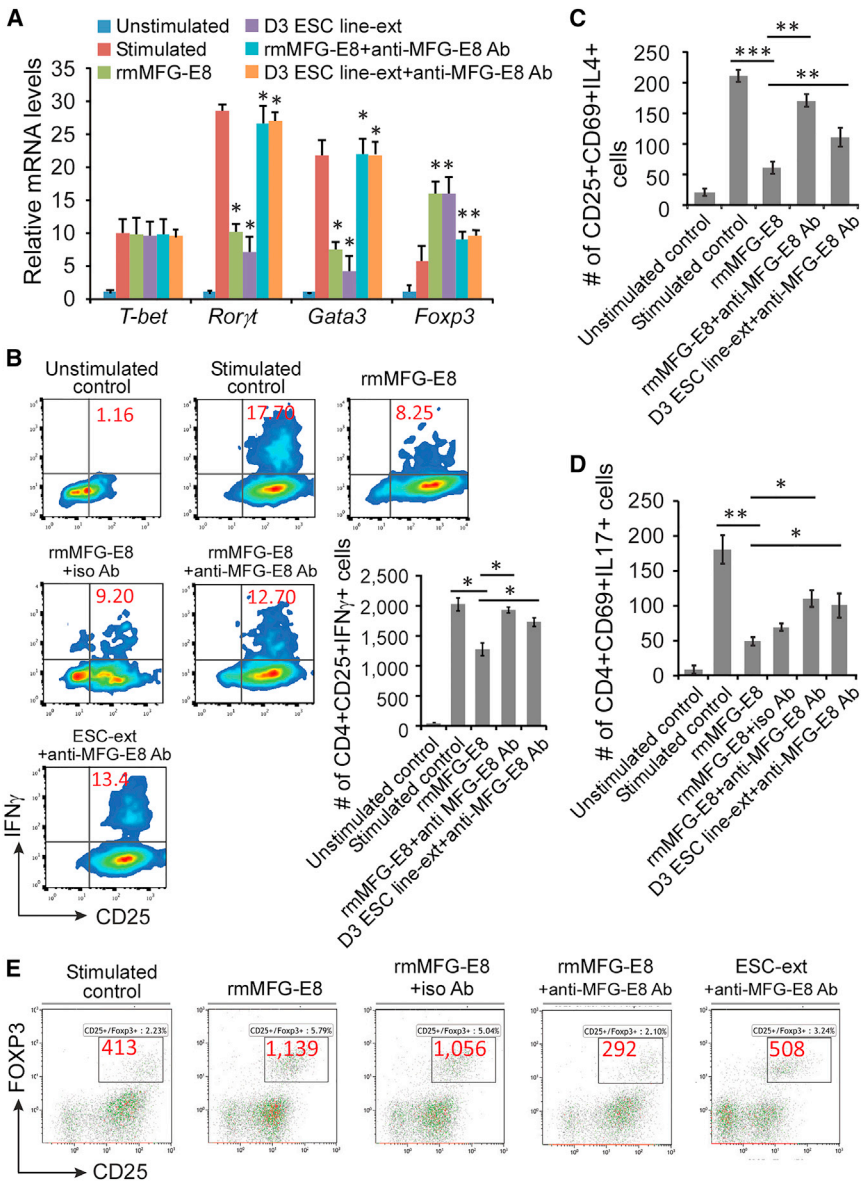


Figure 5. MFG-E8 in ESCs Suppresses Th1, Th2, and Th17 Subsets while Enhancing the Treg Subpopulation

(A) Gene expression of transcriptional factors representing Th1, Th2, and Th17 (qPCR). Purified $CD3^+$ splenic T cells were primed with anti-CD3/CD28 for 3 days and then treated with either D3 ESC-derived soluble factors (D3 ESC-ext, D3 ESC line derived from 129S2/SvPas mouse blastocysts) or rmMFG-E8 in the presence of absence of MFG-E8 blocking antibody, followed by PMA stimulation. Expression levels of mRNA of transcriptional factors (*T-bet*, *Rorγt*, *Gata3*, and *Foxp3*) were determined by qPCR. Data represent mean ± SD of at least three independent experiments; $^*p < 0.05$.

(B–D) rmMFG-E8 is capable of suppressing IFN-γ, IL-4, and IL-17 production. The cells were stained with fluorescent-labeled antibodies against CD4, CD25, or CD69 for surface markers and IFN-γ, IL-4, and IL-17 for intracellular cytokines, followed by flow cytometric analysis. Histograms are shown as mean ± SD of the absolute number of cells expressing each cytokine from three independent experiments ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$).

(E) Expression of FOXP3. T cells treated and stimulated as described above were stained for CD4 and CD25 surface markers and then analyzed for intracellular FOXP3 expression by flow cytometry to assess the Treg subpopulation. The dot plots are all from one experiment, and three experiments were performed with similar results.

See also Figure S3.

have shown that increased Treg infiltration in tumor tissues was associated with elevated macrophage MFG-E8 expression (Jinushi et al., 2009) and development of autoimmunity was correlated with downregulated MFG-E8 levels (Peng and Elkon, 2011; Yamaguchi et al., 2008). Our study provides a direct link between MFG-E8 and Th1, Th2, Th17, and Treg T cells, demonstrating that undifferentiated ESCs are capable of skewing immune response toward an anti-inflammatory or immune tolerant status via MFG-E8.

MFG-E8 in Undifferentiated ESCs Suppresses T Cell Activation via Inhibition of PKCθ Phosphorylation

Given an indispensable role of PKCθ in T cell responses (Healy et al., 2006) and our previous observation that ESC-derived soluble factors inhibit PKCθ phosphorylation (Mohib et al., 2012), we speculated that MFG-E8 might modulate T cells through PKCθ. Indeed, both undifferentiated ESC-derived soluble factors and rmMFG-E8 significantly suppressed PKCθ phosphorylation of activated T cells, and anti-MFG-E8 antibody abrogated the inhibitory effects (Figures 6A and 6B). Additionally, differentiated ESC- or myoblast C2C12-derived soluble factors containing little MFG-E8 did not significantly affect PKCθ phosphorylation in T cells. Conversely, supplement of differentiated ESC-derived soluble factors with rmMFG-E8 restored inhibition (Figure 6C).

It is known that T cells selectively upregulate integrin $\alpha_V\beta_3/\alpha_V\beta_5$ on their surface when encountering a specific

Please cite this article in press as: Tan et al., MFG-E8 Is Critical for Embryonic Stem Cell-Mediated T Cell Immunomodulation, Stem Cell Reports (2015), http://dx.doi.org/10.1016/j.stemcr.2015.09.005

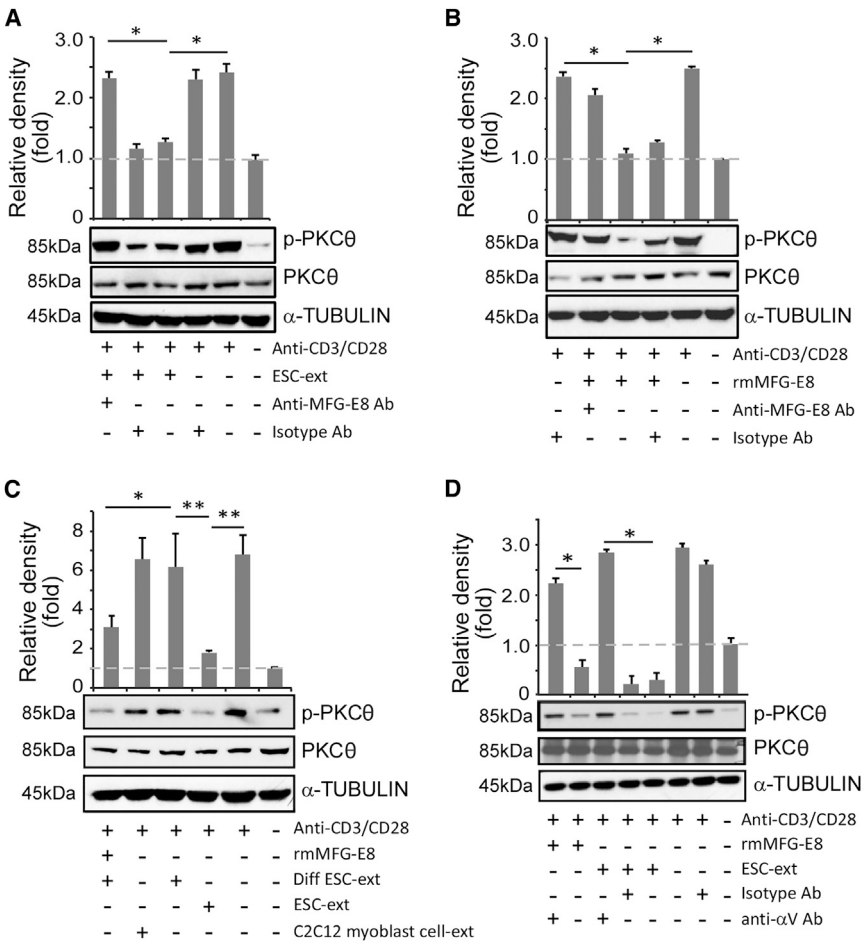


Figure 6. MFG-E8 in ESCs Inhibits PKCθ Activation

(A) Negatively selected C57BL/6 CD3$^+$ T cells were treated with undifferentiated ESC-derived soluble factors (ESC-ext, prepared from D3 ESCs derived from 129S2/SvPas mouse blastocysts) alone or in combination with either control isotype antibody or anti-MFG-E8 antibody, and stimulated with anti-CD3/CD28 antibodies. For western blot analysis, cell lysates were probed with antibodies to phosphorylated-PKCθ (Ther538), total PKCθ, and α-tubulin (as a loading control).

(B) Cells were treated with recombinant mouse MFG-E8 (rmMFG-E8) in the absence or presence of control isotype antibody or anti-MFG-E8 antibody and analyzed by western blot.

(C) Cells were treated with soluble factors derived from undifferentiated D3 ESCs (ESC-ext), differentiated D3 ESCs (Diff ESC-ext), or C2C12 myoblast cell line (C2C12 myoblast cell-ext) alone or in combination with rmMFG-E8 and then analyzed by western blot.

(D) The αV integrin-blocking antibody (anti-αV Ab) was introduced to restrain the binding of MFG-E8 to its receptor. Cell lysates were then analyzed for levels of PKCθ phosphorylation by densitometry with α-tubulin as a loading control. Bar graphs represent mean ± SD from at least three independent experiments.

See also Figure S4.

PKCθ activator, PMA (Huang et al., 1995; Luzina et al., 2009; Nohara et al., 2005). We reasoned that engagement of MFG-E8 to its integrin receptor on activated T cells might transduce a signal via PKCθ in ESC-mediated immunomodulation to control the level of T cell responses. As anticipated, western blot revealed that a blockade of $\alpha_V\beta_3/\alpha_V\beta$ receptor activity with an anti-CD51 (anti-α_V) antibody alleviated the inhibitory effect of ESC-derived soluble factors or rmMFG-E8 on PKCθ phosphorylation (Figure 6D).

Additionally, to further confirm that inhibition of PKCθ would suppress T cell function, we transfected purified splenic T cells with scrambled RNA or PKCθ small interfering RNA (siRNA) prior to stimulation with antiCD3/CD28 (Figure S4A). With PKCθ knockdown, T cell activation was impaired with a robust decrease in CD25$^+$/CD69$^+$ T cells in both CD4 and CD8 subpopulations (Figure S4B).

Furthermore, MFG-E8 specifically abolished PMA-induced PKCθ activation (Figure S4C). Since PMA is a well-known molecule capable of upregulating PKCθ activity, but not PKC-α, β, δ, ε, and δ82 (Isakov and Altman, 1987; Touraine et al., 1977; Tsutsumi et al., 1993), PKCθ is a key player involved in MFG-E8-mediated T cell regulation. A role for PKCθ in mouse cardiac rejection has been reported. PKCθ knockout mice exhibit an impaired alloimmune response and are susceptible to tolerance induction. While a subtherapeutic dose of anti-CD154 antibody or CTLA4-Ig failed to delay cardiac allograft rejection in wild-type mice, it could prevent cardiac allograft rejection in PKCθ knockout mice (Manicassamy et al., 2008; Wang et al., 2009). In this report, we made a connection between MFG-E8, PKCθ, and immune acceptance of differentiated ESC implants.

DISCUSSION

Molecules and mechanisms pertinent to the low immunogenicity of undifferentiated ESCs remain poorly understood. In this study, we demonstrated that MFG-E8 is enriched in undifferentiated but diminished in

differentiated ESCs (Figures 1 and 2); upregulation of MFG-E8 promotes successful engraftment of non-self ESCs and their derivatives across the MHC barrier (Figures 1, 2, 3, and S1; Table S1).

Apparently, MFG-E8 is a key determinant in ESC-induced T cell suppression in a PKCθ-dependent manner. By suppressing PKCθ phosphorylation (Figures 6 and S4), ESC-produced MFG-E8 inhibits Th1/Th2/Th17 subpopulations while increasing regulatory T cells (Figures 4, 5, and S1–S3). A study regarding MFG-E8-mediated tissue regeneration has shown that MFG-E8 transduced its downstream signaling for mucosal healing by activating intracellular PKCε in epithelial cells (Bu et al., 2007). Given the premise of similar structures shared between PKCθ and PKCε (Mellor and Parker, 1998), our findings regarding an MFG-E8 mechanistic function on PKCθ in T cells suggest diversified modes of MFG-E8 signaling in different cell types.

Our observations that MFG-E8 did not affect *T-bet* gene expression while suppressing Th1 function (i.e., IFN-γ production) seem conflicting, which might pertain uniquely to PKCθ. Studies have illustrated that synergistic inhibition of two pathways instead of PKCθ alone is crucial for complete suppression of Th1 differentiation (Lee et al., 2010; Smeets et al., 2012). The requirement of collaborative pathways for fully controlling Th1 differentiation may provide one possible explanation regarding our *T-bet* and IFN-γ results (Ma et al., 2012; Marsland and Kopf, 2008; Marsland et al., 2005).

In summary, we elucidated an immunomodulatory mechanism by which ESCs downregulate effector T cells while upregulating Tregs via MFG-E8. Importantly, sustaining a high level of MFG-E8 expression in differentiated ESCs overcomes T cell-mediated immune rejection and facilitates transplant acceptance. This work provides insights into a potential approach for stem cell-based therapies and autoimmune diseases.

EXPERIMENTAL PROCEDURES

Cell Lines, Animals, and Reagents

Mouse ESC lines C57BL/6 (B6) and D3 (derived from 129S2/SvPas mouse blastocysts) were obtained from ATCC. Jurkat T cells were maintained in RPMI supplemented with 10% fetal bovine serum. C57BL/6 (H-2^b) and BALB/c (H-2^d) mice (8–12 weeks old) were purchased from Charles River Laboratories and maintained at the University of Ottawa according to the Canadian Council on Animal Care guidelines under the protocol approved by the Animal Use Subcommittee (permit BMI-2025). Recombinant mouse MFG-E8 (rmMFG-E8) was purchased from R&D Systems, and MFG-E8 antibody was prepared as described previously (Jinushi et al., 2009). Other antibodies used were described below or in Supplemental Experimental Procedures. See Supplemental Experimental Procedures for detailed procedures.

Teratoma Formation, Transplantation of EBs under Kidney Capsules, and Histological and Immunofluorescent Analyses

B6-WT and B6-MFG-E8 ESCs were harvested, and one to three million cells were injected subcutaneously into the flank region of syngeneic C57BL/6 (n = 10) or allogeneic BALB/c (n = 18) mice to generate teratomas. Differentiated EBs were transplanted under the kidney capsules (syngeneic transplantation: n = 16; allogeneic transplantation: n = 17). Frozen tumor sections were evaluated after H&E staining or immunostaining. All of the specimens were coded so that the measurements were performed blindly for final analysis. See Supplemental Experimental Procedures for more details.

Lentiviral Production, MFG-E8 Transduction, and Characterization of B6-MFG-E8 ESC

293 T cells were transfected with MFG-E8 lentiviral vector encoding full-length MFG-E8 and a GFP marker (Addgene plasmid #46847), and two packing plasmids psPAX2 and pMD2.G (Addgene plasmids 12259 and 12260) using Lipofectamine 2000 (Invitrogen). Lentivirus was harvested and concentrated using Lenti-X concentrator (Clontech) according to the manufacturer's instruction. B6 wild-type (B6-WT) ESCs were infected with lentivirus in the presence of 10 μg/ml polybrene (Sigma). Puromycin selection (2 μg/ml) started 48 hr after lentiviral transduction for 2 weeks with daily medium change. After establishing a stable cell line with upregulated MFG-E8, B6-MFG-E8 ESCs were cultured in ESC growth medium containing 1 μg/ml of puromycin. See Supplemental Experimental Procedures for more details.

ESC Differentiation

ESCs were differentiated by formation of EBs and removal of LIF. See Supplemental Experimental Procedures for more details.

Preparation of ESC- and Other Cell-Derived Soluble Factors, qPCR, and Western Blot

This experimental method has been described previously (Mohib et al., 2010). See Supplemental Experimental Procedures for more information.

Fractionation of Mouse Splenic CD3$^+$ T Cells and NK Cells

Mouse spleens were removed aseptically and homogenized gently and lysed using ACK red blood cell lysis buffer (Cedarlane Laboratories, catalog no. A1049201). After two washes, splenocytes were re-suspended, and CD3$^+$ T cells (>92% purity) or NK cells (>90% purity) were fractionated by negative selection using a magnetic labeling kit according to manufacturer instructions (STEMCELL Technologies, catalog no. 19851). See Supplemental Experimental Procedures for more information.

Flow Cytometry and Intracellular Staining

Fractionated CD3$^+$ T cells were treated with different reagents as described in Results, followed by stimulation with plate-coated anti-CD3 and anti-CD28 (eBioscience, catalog no. 16-0031-85 and 16-0281-85) for 48 hr. Cells were then stained with antibodies

against CD4, CD8, and surface activation markers (CD25, CD44, and CD69) (eBioscience, catalog no. 15-0041-82, 11-0081-81, 48-0253-82, 25-0691-81, and 47-0441-82, respectively). Appropriate fluorochrome-conjugated isotype matched antibodies were used as negative controls. Analysis was carried out in a Beckman Coulter Cyan cytometer, and data were analyzed using Kaluza. See Supplemental Experimental Procedures for detailed procedures.

Statistical Analysis

Results are expressed as mean ± SD. Statistical significance was determined using a Student's t test, ANOVA and Tukey HSD or chi-square test wherever applicable. Results were analyzed using R studio, and considered significant with a p value < 0.05.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.09.005.

AUTHOR CONTRIBUTIONS

Y.T.: conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing; B.A., D.J., and L.L.: collection and assembly of data and data analysis and interpretation; J.F.C. and D.F.: provision of study materials; M.J., conception and design, provision of study materials, and data interpretation; L.W.: conception and design, data interpretation, manuscript writing, and financial support.

ACKNOWLEDGMENTS

We thank Drs. Kathryn Wright, Sylvain Lanouette, and Haggag Salah Zein Mostafa for their partial involvement in this study. This work is supported by operating grants from the Canadian Institutes of Health Research (CIHR) MOP-111224, the Office of the Assistant Secretary of Defense for Health Affairs and the Defense Health Agency, Research, Development, and Acquisition Directorate through the USAMRAA under award number W81XWH-10-BMFRP-EHDA, the Heart and Stroke Foundation of Ontario NA7186, and the Canadian Breast Cancer Foundation – Ontario Region.

Received: February 3, 2015
Revised: September 8, 2015
Accepted: September 9, 2015
Published: October 8, 2015

REFERENCES

Boyd, A.S., and Wood, K.J. (2009). Variation in MHC expression between undifferentiated mouse ES cells and ES cell-derived insulin-producing cell clusters. Transplantation *87*, 1300–1304.

Brissette, M.-J., Lepage, S., Lamonde, A.-S., Sirois, I., Groleau, J., Laurin, L.-P., and Cailhier, J.-F. (2012). MFG-E8 released by apoptotic endothelial cells triggers anti-inflammatory macrophage reprogramming. PLoS ONE *7*, e36368.

Bu, H.-F., Zuo, X.-L., Wang, X., Ensslin, M.A., Koti, V., Hsueh, W., Raymond, A.S., Shur, B.D., and Tan, X.-D. (2007). Milk fat globule-EGF factor 8/lactadherin plays a crucial role in maintenance and repair of murine intestinal epithelium. J. Clin. Invest. *117*, 3673–3683.

Drukker, M., Katz, G., Urbach, A., Schuldiner, M., Markel, G., Itskovitz-Eldor, J., Reubinoff, B., Mandelboim, O., and Benvenisty, N. (2002). Characterization of the expression of MHC proteins in human embryonic stem cells. Proc. Natl. Acad. Sci. USA *99*, 9864–9869.

Drukker, M., Katchman, H., Katz, G., Even-Tov Friedman, S., Shezen, E., Hornstein, E., Mandelboim, O., Reisner, Y., and Benvenisty, N. (2006). Human embryonic stem cells and their differentiated derivatives are less susceptible to immune rejection than adult cells. Stem Cells *24*, 221–229.

Fairchild, P.J., Robertson, N.J., Minger, S.L., and Waldmann, H. (2007). Embryonic stem cells: protecting pluripotency from alloreactivity. Curr. Opin. Immunol. *19*, 596–602.

Foley, B., Felices, M., Cichocki, F., Cooley, S., Verneris, M.R., and Miller, J.S. (2014). The biology of NK cells and their receptors affects clinical outcomes after hematopoietic cell transplantation (HCT). Immunol. Rev. *258*, 45–63.

Graumann, J., Hubner, N.C., Kim, J.B., Ko, K., Moser, M., Kumar, C., Cox, J., Schöler, H., and Mann, M. (2008). Stable isotope labeling by amino acids in cell culture (SILAC) and proteome quantitation of mouse embryonic stem cells to a depth of 5,111 proteins. Mol. Cell. Proteomics *7*, 672–683.

Healy, A.M., Izmailova, E., Fitzgerald, M., Walker, R., Hattersley, M., Silva, M., Siebert, E., Terkelsen, J., Picarella, D., Pickard, M.D., et al. (2006). PKC-theta-deficient mice are protected from Th1-dependent antigen-induced arthritis. J. Immunol. *177*, 1886–1893.

Huang, S., Endo, R.I., and Nemerow, G.R. (1995). Upregulation of integrins alpha v beta 3 and alpha v beta 5 on human monocytes and T lymphocytes facilitates adenovirus-mediated gene delivery. J. Virol. *69*, 2257–2263.

Isakov, N., and Altman, A. (1987). Human T lymphocyte activation by tumor promoters: role of protein kinase C. J. Immunol. *138*, 3100–3107.

Jinushi, M., Nakazaki, Y., Dougan, M., Carrasco, D.R., Mihm, M., and Dranoff, G. (2007). MFG-E8-mediated uptake of apoptotic cells by APCs links the pro- and antiinflammatory activities of GM-CSF. J. Clin. Invest. *117*, 1902–1913.

Jinushi, M., Sato, M., Kanamoto, A., Itoh, A., Nagai, S., Koyasu, S., Dranoff, G., and Tahara, H. (2009). Milk fat globule epidermal growth factor-8 blockade triggers tumor destruction through coordinated cell-autonomous and immune-mediated mechanisms. J. Exp. Med. *206*, 1317–1326.

Koch, C.A., Geraldes, P., and Platt, J.L. (2008). Immunosuppression by embryonic stem cells. Stem Cells *26*, 89–98.

Lee, K., Gudapati, P., Dragovic, S., Spencer, C., Joyce, S., Killeen, N., Magnuson, M.A., and Boothby, M. (2010). Mammalian target of rapamycin protein complex 2 regulates differentiation of Th1 and Th2 cell subsets via distinct signaling pathways. Immunity *32*, 743–753.

Lumelsky, N., Blondel, O., Laeng, P., Velasco, I., Ravin, R., and McKay, R. (2001). Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science *292*, 1389–1394.

Luzina, I.G., Todd, N.W., Nacu, N., Lockatell, V., Choi, J., Hummers, L.K., and Atamas, S.P. (2009). Regulation of pulmonary inflammation and fibrosis through expression of integrins alphaVbeta3 and alphaVbeta5 on pulmonary T lymphocytes. Arthritis Rheum. *60*, 1530–1539.

Ma, J., Ding, Y., Fang, X., Wang, R., and Sun, Z. (2012). Protein kinase C-θ inhibits inducible regulatory T cell differentiation via an AKT-Foxo1/3a-dependent pathway. J. Immunol. *188*, 5337–5347.

Manicassamy, S., Yin, D., Zhang, Z., Molinero, L.L., Alegre, M.L., and Sun, Z. (2008). A critical role for protein kinase C-theta-mediated T cell survival in cardiac allograft rejection. J. Immunol. *181*, 513–520.

Marsland, B.J., and Kopf, M. (2008). T-cell fate and function: PKC-θ and beyond. Trends Immunol. *29*, 179–185.

Marsland, B.J., Nembrini, C., Schmitz, N., Abel, B., Krautwald, S., Bachmann, M.F., and Kopf, M. (2005). Innate signals compensate for the absence of PKC-theta during in vivo CD8(+) T cell effector and memory responses. Proc. Natl. Acad. Sci. USA *102*, 14374–14379.

Mellor, H., and Parker, P.J. (1998). The extended protein kinase C superfamily. Biochem. J. *332*, 281–292.

Mizuseki, K., Sakamoto, T., Watanabe, K., Muguruma, K., Ikeya, M., Nishiyama, A., Arakawa, A., Suemori, H., Nakatsuji, N., Kawasaki, H., et al. (2003). Generation of neural crest-derived peripheral neurons and floor plate cells from mouse and primate embryonic stem cells. Proc. Natl. Acad. Sci. USA *100*, 5828–5833.

Mohib, K., Allan, D., and Wang, L. (2010). Human embryonic stem cell-extracts inhibit the differentiation and function of monocyte-derived dendritic cells. Stem Cell Rev. *6*, 611–621.

Mohib, K., AlKhamees, B., Zein, H.S., Allan, D., and Wang, L. (2012). Embryonic stem cell-derived factors inhibit T effector activation and induce T regulatory cells by suppressing PKC-θ activation. PLoS ONE *7*, e32420.

Nat, R., Salti, A., Suciu, L., Ström, S., and Dechant, G. (2012). Pharmacological modulation of the Hedgehog pathway differentially affects dorsal/ventral patterning in mouse and human embryonic stem cell models of telencephalic development. Stem Cells Dev. *21*, 1016–1046.

Nohara, K., Pan, X., Tsukumo, S., Hida, A., Ito, T., Nagai, H., Inouye, K., Motohashi, H., Yamamoto, M., Fujii-Kuriyama, Y., and Tohyama, C. (2005). Constitutively active aryl hydrocarbon receptor expressed specifically in T-lineage cells causes thymus involution and suppresses the immunization-induced increase in splenocytes. J. Immunol. *174*, 2770–2777.

Peng, Y., and Elkon, K.B. (2011). Autoimmunity in MFG-E8-deficient mice is associated with altered trafficking and enhanced cross-presentation of apoptotic cell antigens. J. Clin. Invest. *121*, 2221–2241.

Robertson, N.J., Brook, F.A., Gardner, R.L., Cobbold, S.P., Waldmann, H., and Fairchild, P.J. (2007). Embryonic stem cell-derived tissues are immunogenic but their inherent immune privilege promotes the induction of tolerance. Proc. Natl. Acad. Sci. USA *104*, 20920–20925.

Sarkar, P., Collier, T.S., Randall, S.M., Muddiman, D.C., and Rao, B.M. (2012). The subcellular proteome of undifferentiated human embryonic stem cells. Proteomics *12*, 421–430.

Segura, E., Amigorena, S., and Théry, C. (2005). Mature dendritic cells secrete exosomes with strong ability to induce antigen-specific effector immune responses. Blood Cells Mol. Dis. *35*, 89–93.

Shifrin, N., Raulet, D.H., and Ardolino, M. (2014). NK cell self tolerance, responsiveness and missing self recognition. Semin. Immunol. *26*, 138–144.

Smeets, R.L., Fleuren, W.W.M., He, X., Vink, P.M., Wijnands, F., Gorecka, M., Klop, H., Bauerschmidt, S., Garritsen, A., Koenen, H.J.P.M., et al. (2012). Molecular pathway profiling of T lymphocyte signal transduction pathways; Th1 and Th2 genomic fingerprints are defined by TCR and CD28-mediated signaling. BMC Immunol. *13*, 12.

Swijnenburg, R.-J., Tanaka, M., Vogel, H., Baker, J., Kofidis, T., Gunawan, F., Lebl, D.R., Caffarelli, A.D., de Bruin, J.L., Fedoseyeva, E.V., and Robbins, R.C. (2005). Embryonic stem cell immunogenicity increases upon differentiation after transplantation into ischemic myocardium. Circulation *112* (9, Suppl), I166–I172.

Touraine, J.L., Hadden, J.W., Touraine, F., Hadden, E.M., Estensen, R., and Good, R.A. (1977). Phorbol myristate acetate: a mitogen selective for a T-lymphocyte subpopulation. J. Exp. Med. *145*, 460–465.

Tsutsumi, A., Kubo, M., Fujii, H., Freire-Moar, J., Turck, C.W., and Ransom, J.T. (1993). Regulation of protein kinase C isoform proteins in phorbol ester-stimulated Jurkat T lymphoma cells. J. Immunol. *150*, 1746–1754.

Van Hoof, D., Passier, R., Ward-Van Oostwaard, D., Pinkse, M.W., Heck, A.J., Mummery, C.L., and Krijgsveld, J. (2006). A quest for human and mouse embryonic stem cell-specific proteins. Mol. Cell. Proteomics *5*, 1261–1273.

Van Vliet, P., Wu, S.M., Zaffran, S., and Pucéat, M. (2012). Early cardiac development: a view from stem cells to embryos. Cardiovasc. Res. *96*, 352–362.

Wang, L., Xiang, Z., Ma, L.L., Chen, Z., Gao, X., Sun, Z., Williams, P., Chari, R.S., and Yin, D.P. (2009). Deficiency of protein kinase C-theta facilitates tolerance induction. Transplantation *87*, 507–516.

Yamaguchi, H., Takagi, J., Miyamae, T., Yokota, S., Fujimoto, T., Nakamura, S., Ohshima, S., Naka, T., and Nagata, S. (2008). Milk fat globule EGF factor 8 in the serum of human patients of systemic lupus erythematosus. J. Leukoc. Biol. *83*, 1300–1307.

Yang, C., Hayashida, T., Forster, N., Li, C., Shen, D., Maheswaran, S., Chen, L., Anderson, K.S., Ellisen, L.W., Sgroi, D., and Schmidt, E.V. (2011). The integrin alpha(v)beta(3-5) ligand MFG-E8 is a p63/p73 target gene in triple-negative breast cancers but exhibits suppressive functions in ER(+) and erbB2(+) breast cancers. Cancer Res. *71*, 937–945.

Zhao, T., Zhang, Z.-N., Rong, Z., and Xu, Y. (2011). Immunogenicity of induced pluripotent stem cells. Nature *474*, 212–215.