Original Research

Potentially immunogenic proteins expressed similarly in human embryonic stem cells and induced pluripotent stem cells

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Abstract

A major limitation of the use of cellular therapies is the loss of donor-derived cells because of immune incompatibility. While induced pluripotent stem (iPS) cells offer the potential for autologous transplant therapies, questions have been raised using a mouse model that specific antigens mediate the rejection of grafts after syngeneic transplants with iPS, but not embryonic stem (ES) cells. In this study, we examined whether the human homologs of these markers, HORMAD1, ZG16, and Cyp3A, are differentially expressed in human iPS versus ES cells, as well as undifferentiated and *in vitro*-differentiated cells. Both qRT-PCR and flow cytometric analyses demonstrated similar gene and protein expression profiles for iPS and ES cells regardless of differentiation state. Our data are consistent with a recent study in mice that showed no evidence of rejection of differentiated syngeneic iPS cells. Furthermore, our results suggest that expression of these gene products cannot predict differences in clinical outcomes between human iPS and ES-derived cells.

Keywords: Embryonic stem cells, induced pluripotent stem cells, tumor antigens, transplant therapy, immune rejection

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Introduction

The prospect of using induced pluripotent stem (iPS) cells in autologous cellular therapies has generated considerable interest for the treatment of numerous diseases. However, questions have been raised about the immunogenicity of iPS-based treatments. In particular, Zhao and coworkers[1] contended that in mice, that iPS cells are rejected in a syngeneic transplant setting because of expression of specific tumor antigens not found during normal embryonic development, or in transplanted embryonic stem (ES) cells. In contrast, other groups have demonstrated that there is little or no evidence that undifferentiated or differentiated iPS cells are immunogenic.[2,3] However, each of these studies examining the immunogenicity of iPS cells was conducted in mice, and fundamental differences exist between mouse and human iPS cells in their developmental state and pluripotency. Furthermore, the study demonstrating iPS cell rejection *in vivo* did not examine differentiated iPS cell products.

In this study, we examined whether antigens identified by Zhao and coworkers, HORMAD1, ZG16, and CYP3A, as mediators of immunogenicity of iPS cells are expressed in human iPS cells in comparison with human ES cells. Additionally, we compared expression of these markers in undifferentiated and differentiated human iPS and ES cell products. If undifferentiated iPS cells express high levels of immunogeneic proteins, as suggested by Zhao and coworkers, but differentiated iPS do not, these differences in immunogenicity can be exploited for the prevention of tumors in recipients of differentiated iPS cellular products.

Methods and materials

Cell culture and differentiation

iPS cell lines were cultured in DMEM/F12 medium supplemented with 20% knockout serum replacer, 0.1 mmol/L non-essential amino acids (NEAA), 1 mmol/L L-glutamine (all from Gibco/Life Technologies, Grand Island, NY), 0.1 mmol/L β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and 4 ng/mL basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, MN). ES cell lines were cultured in the same manner except Knockout DMEM (Gibco/Life Technologies) was used as the base medium. Cultures were maintained on irradiated mouse embryo fibroblasts and passaged enzymatically using 1 mg/mL collagenase IV (Invitrogen, Carlsbad, CA). Differentiation was achieved using embryoid body (EB) formation. Spontaneous differentiation was initiated by dissociating human iPS or ES cells using 1 mg/mL collagenase IV and

then culturing in six-well ultra-low attachment plates containing differentiation medium: DMEM (Gibco/Life Technologies) supplemented with 2% fetal bovine serum (Thermo Scientific/Hyclone, Rockford, IL), 1 mmol/L L-glutamine, 0.1 mmol/L NEAA, and 0.1 mmol/L β-mercaptoethanol.

RNA extraction and gene expression analysis

RNA was extracted from cell samples using the RNeasy Micro kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. cDNA was synthesized from 0.5 to 5 μg RNA using the SuperScript III First-Strand Synthesis system for RT-PCR kit (Invitrogen). Quantitative PCR (qPCR) was carried out with cDNA using SYBR Green (Applied Biosystems, Carlsbad, CA) on an Eppendorf Mastercycler (realplex^2). Primer sets were ordered from the University of Minnesota Genomics Center. The following primer sets were used: ZG16, left: GGTGGGAAT ACCCAGGAAGT, right: ATTCCATTCAAAGCCAATGC; HORMAD1, left: TGGACCACTCATGGACTTCA, right: GCTGGTAATCTGGGGGTGTA; Cyp3a4: left: CAAGAC CCCTTTGTGGAAAA, right: CGAGGCGACTTTCTT TCATC.

Flow cytometric analysis

Undifferentiated iPS and ES cells were collected by collagenase IV dissociation, and then dissociated with Accumax (Sigma-Aldrich). EBs were collected and dissociated with Accumax. Dissociated cells were stained using the following primary antibodies at 1:1000 dilution: rabbit anti-human CYP3A4/3A7 (BD Biosciences, San Jose, CA), mouse anti-human HORMAD1, clone 2E5 (Sigma-Aldrich), and mouse anti-human LOC124220 (Abnova, Taipei City, Taiwan). Rabbit IgG (Novus Biologicals, Littleton, CO), mouse IgG1 (R&D Systems), and mouse IgG (Invitrogen) were used as isotype controls. Secondary staining was performed using either goat anti-mouse Alexa Fluor 488 (Invitrogen) or goat anti-rabbit PE (R&D Systems). Surface staining was performed in PBS/1% FBS following blocking in 50 μg/mL goat IgG (R&D Systems). Intracellular staining was performed using the BD Cytofix/Cytoperm fixation/permeabilization solution kit (BD Biosciences). Samples were run on a BD FACSCalibur flow cytometry system and data analyzed using FlowJo (TreeStar, Ashland, OR).

Results

A recent report[1] indicated that differences in expression of tumor antigens were responsible for the rejection of grafts of mouse iPS-derived but not ES-derived cells in mice. In order to determine whether similar antigens are expressed in human-derived cells, gene expression of ZG16, HORMAD1, and CYP3A4 (human homolog to murine Cyp3a11) in the human H9 ES and L-1 iPS cell lines was assessed by qRT-PCR.

Gene expression profiles indicate no significant difference between ES and iPS cells

Results of qPCR analysis for 8 iPS and 2 ES cell lines are presented in Figure 1. Samples from 22 independent unstimulated differentiation experiments (iPS: 13 experiments, 8 individual lines; ES: 9 experiments, 2 individual lines) were analyzed. While higher ZG16 expression on average was observed in differentiated versus undifferentiated iPS cells ($P = 0.0005$), no significant difference was observed in the average expression levels between undifferentiated and differentiated iPS and ES cells.

Differences in gene expression were detected between cell lines and experimental repeats within the same cell line

In order to gain insight into specific sources of differences in marker expression that could be detected, two iPS and two ES cell lines were examined individually for several experimental repeats (Figure 2). A significant difference in HORMAD expression was observed between

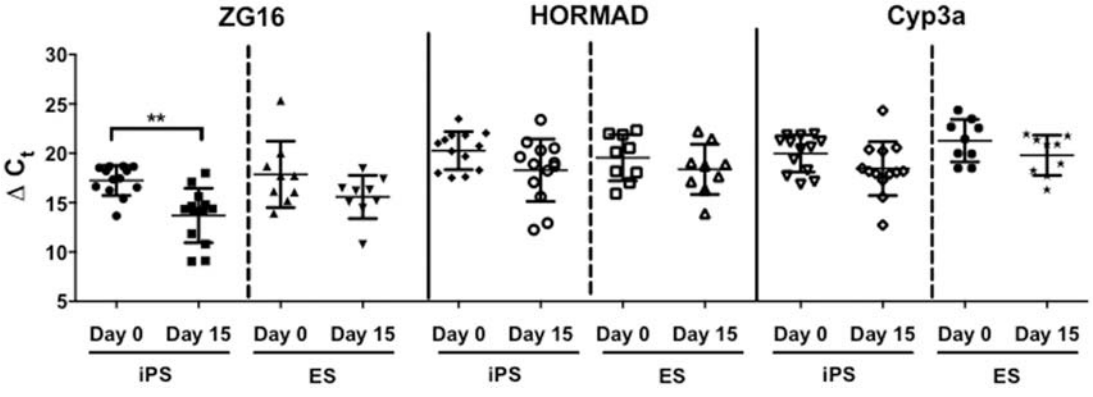

Figure 1 Similar gene RNA expression levels of ZG16, HORMAD1, and CYP3A were observed in embryonic stem (ES) and induced pluripotent stem (iPS) cells. ES and iPS cells were differentiated in embryoid body (EB) suspension culture for 15 days and samples collected for quantitative PCR (qPCR). Samples from 22 independent unstimulated differentiation experiments (iPS: 8 lines, 13 experiments; ES: 2 lines, 9 experiments) were analyzed. qPCR analysis demonstrated that ZG16, HORMAD1, and CYP3A were expressed in both ES and iPS lines and in both undifferentiated and day 15 differentiated cells. Expression levels were found to be not significantly different between ES and iPS cells by Mann–Whitney–Wilcoxon analysis for all timepoints. Expression levels for day 0 and day 15 differentiated samples were not significantly different, with the exception of ZG16 expression in iPS which was expressed at higher levels following differentiation ($P = 0.0005$)

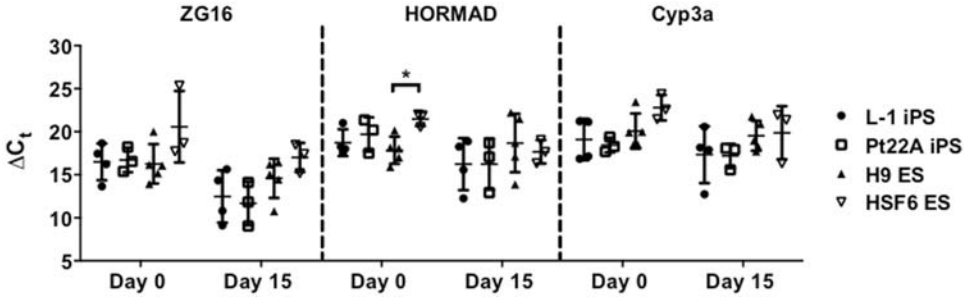

Figure 2 Variations in gene expression levels exist within individual induced pluripotent stem (iPS) and embryonic stem (ES) cell lines. Data from unstimulated differentiation experiments for two iPS and two ES lines were analyzed individually for several experimental repeats. Expression levels were found to be not significantly different between the individual undifferentiated and differentiated ES and iPS lines by Mann–Whitney–Wilcoxon analysis, except between undifferentiated H9 and HSF-6 ES cell lines ($P = 0.0357$). Average expression for each of the markers for the individual lines was not significantly different from the average expression for iPS and ES lines with the exception of HORMAD expression in undifferentiated H9 ES cells versus undifferentiated iPS cells ($P = 0.0338$)

undifferentiated H9 and HSF-6 ES cell lines ($P = 0.0357$), but not between differentiated cells from these lines. While there was a significant increase in ZG16 expression between the averaged results of undifferentiated and differentiated iPS cells, the expression in these two individual iPS cell lines was similar. This suggests that expression levels may vary among individual lines. However, when marker expression for each of the individual lines was compared with the average expression levels for undifferentiated and differentiated ES and iPS cells, the differences were not significant, suggesting that the average range was predictive of expression in individual lines, and that levels of each of these markers were consistent among ES and iPS lines. The exception to this pattern was HORMAD expression in undifferentiated H9 ES cells, which is higher than the average expression for undifferentiated iPS cells ($P = 0.0338$). These results demonstrate that expression of the potentially immunogenic markers varies among different lines and experiments, but no consistent variation in expression exists between iPS and ES cell lines. Overall, our findings suggest that there are no significant differences in gene expression of ZG16, HORMAD1, and CYP3A4 between undifferentiated and differentiated iPS and ES lines that can globally predict immunogeneic differences in cellular therapies. However, based on specific expression levels, individual cells lines could be chosen to reduce the risk of immune rejection from specific cell lines.

Variation in protein expression levels in individual ES and iPS lines support conclusions from gene expression analysis

In order to test the impact of expression levels on the presence of potentially immunogenic proteins in the cells, flow cytometric analysis was performed (Figure 3). The patterns of protein expression differ between each of the two iPS cell lines and the ES line analyzed. This supports the conclusion based on the gene expression data that differences in ZG16, HORMAD, and CYP3A4 expression patterns cannot be used to predict immunogenicity. Our findings indicate that a difference in expression between undifferentiated and differentiated iPS could not be exploited to promote immune clearing of residual tumor-forming pluripotent stem cells in cell products. Furthermore, these results suggest that it is unlikely that differences in immunogenicity of human iPS versus human ES cells will be observed as a consequence of differential expression of these markers.

Discussion

Zhao and coworkers identified a number of genes overexpressed in teratomas that were attributed to immunogenicity of syngeneic iPS cells in mice.[1] Three of these markers, ZG16, HORMAD1, and CYP3A11 are associated with tumors. In theory, expression of these markers could instigate an immune response against cells expressing them at increased levels. Pancreatic adenocarcinoma up-regulated factor (PAUF) is identical to common salivary protein 1/zymogen granule 16B (LOC124220) and is implicated in the progression of pancreatic cancer. PAUF also induces TLR/CXCR4 expression.[4–7] Alternatively, human ZG16 is down regulated in heptaocellular carcinoma.[8] HORMAD1 is identified as an immunogenic cancer/testis antigen[9] and an oncogene in basal subtype breast cancer.[10] HORMAD1 is also expressed in a substantial proportion of human epithelial ovarian cancers.[11] CYP3A4, human homolog to CYP3A11, encodes a member of cytochrome P450 monooxygenase family that is responsible for the oxidative metabolism of many drugs.[12] Polymorphisms in CYP3A4 have been associated with modest reduction in risk of breast cancer in younger patients[13] and with prostate cancer.[14] Others have demonstrated a correlation between higher expression of steroid and xenobiotic receptor (SXR)/CYP3A4 with higher survival rates for prostate cancer.[15] In addition to its association with cancer, CYP3A4 expression is correlated with the differentiation of a hepatocyte-like progenitor human cell line,[16] and the mouse homolog CYP3A11 with differentiation of mouse ES cells into mature hepatocytes.[17]

In this study, we observed differences in gene and protein expression of these markers between specific human ES and iPS lines, and between undifferentiated and differentiated cells. This demonstrates that human ES and iPS cells are more similar to each other than are mouse ES and iPS cells, based on expression of these markers. The differences correlate with those observed in the levels of development

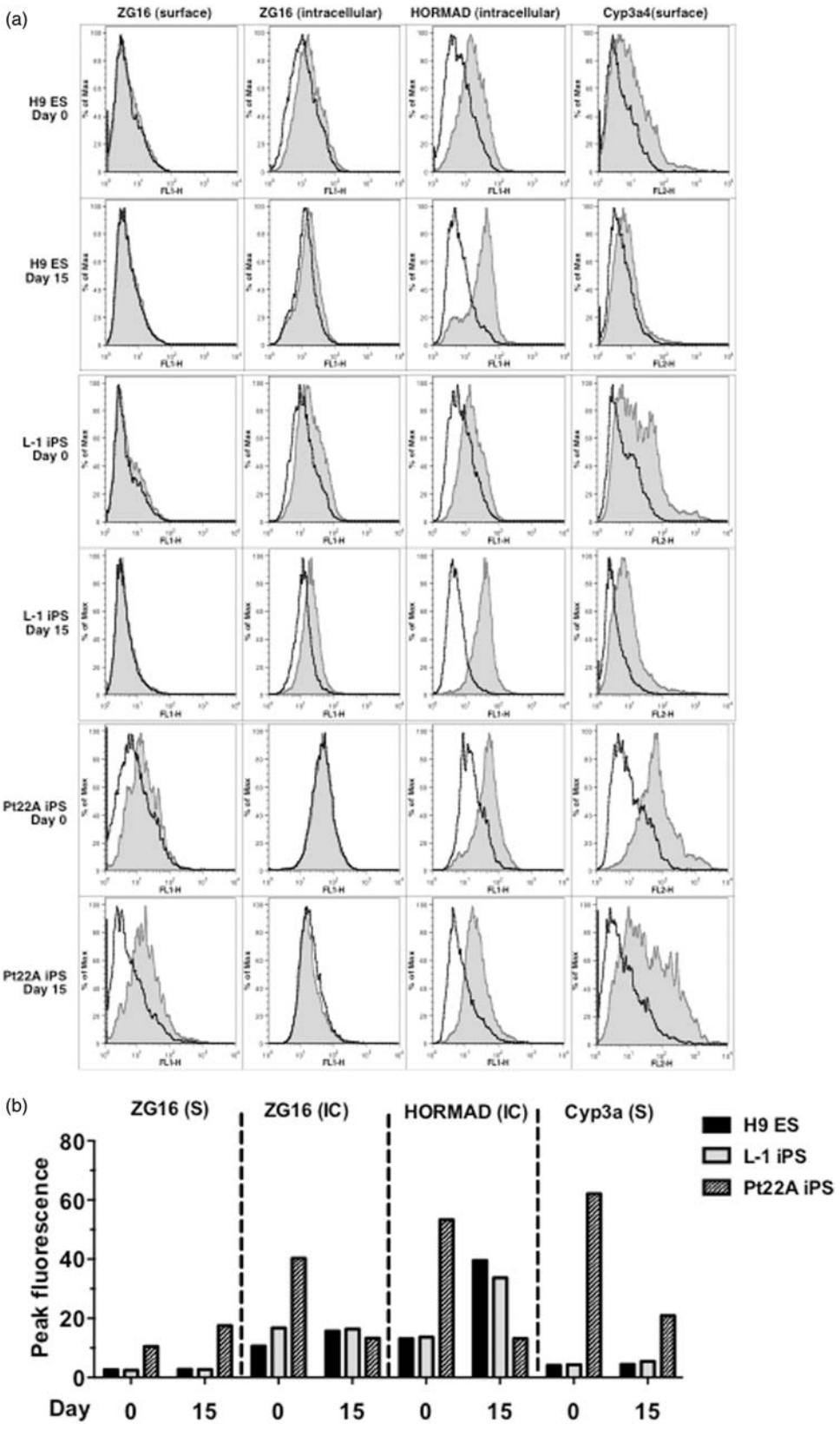

Figure 3 Embryonic stem (ES) and induced pluripotent stem (iPS) cells express varying levels of ZG16, HORMAD1, and CYP3A4 protein. (a) H9 ES, L-1 iPS, and Pt22A iPS cells were differentiated in embryoid body (EB) suspension culture for 15 days. Cells were dissociated and stained before and after differentiation with anti-human CYP3A4, ZG16, and HORMAD1 antibodies. Variation in protein expression levels was observed between differentiated versus undifferentiated cells and between the three different lines. Peak fluorescence values are summarized in (b)

represented by each stem cell population and species. Human ES and iPS are closer in developmental status than mouse ES and iPS, based on different states of pluripotency, which may account for these observed differences in gene/protein expression.[18–20]

The 2011 study by Zhao and coworkers examined the immunogenicity of teratomas generated by injection of undifferentiated ES and iPS.[1] More recently, Araki and coworkers saw no difference in immune responses between mouse ES and iPS. However, both of these studies relied on cells differentiated *in vivo*.[2] Cells differentiated *in vivo* would be subjected to different factors than those differentiated *in vitro*. Therefore, the observations made in these previous studies may not be relevant to clinical applications, as this approach would never be used for clinical production, and the transplanted cells unreliable for the conclusions about human therapies. Our data are in accord with more recent studies in which no difference in immunogenicity between mouse ES and mouse iPS differentiated in culture was observed.[3] Furthermore, since these gene products in mice do not mediate immune rejection in this model, the risk of rejection in human cellular therapies seems to be diminished over what was previously proposed. While these findings do not eliminate the possibility that fully differentiated iPS will be rejected,[21] it is not likely that expression of the markers examined herein can be used to predict immunogenicity.

Author contributions: KMM: design of experiments, collection and assembly of data, data analysis and interpretation, manuscript writing. UA and MC: collection and assembly of data, data analysis and interpretation. MTF: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

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