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**WNT3 Is a Biomarker Capable of Predicting the Definitive Endoderm Differentiation Potential of hESCs**

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Generation of functional cells from human pluripotent stem cells (PSCs) through in vitro differentiation is a promising approach for drug screening and cell therapy. However, the observed large and unavoidable variation in the differentiation potential of different human embryonic stem cell (hESC)/induced PSC (iPSC) lines makes the selection of an appropriate cell line for the differentiation of a particular cell lineage difficult. Here, we report identification of WNT3 as a biomarker capable of predicting definitive endoderm (DE) differentiation potential of hESCs. We show that the mRNA level of WNT3 in hESCs correlates with their DE differentiation efficiency. In addition, manipulations of hESCs through WNT3 knockdown or overexpression can respectively inhibit or promote DE differentiation in a WNT3 level-dependent manner. Finally, analysis of several hESC lines based on their WNT3 expression levels allowed accurate prediction of their DE differentiation potential. Collectively, our study supports the notion that WNT3 can serve as a biomarker for predicting DE differentiation potential of hESCs.

**INTRODUCTION**

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) are promising cell sources for cell therapy due to their capacity for unlimited self-renewal as well as pluripotency (Robinton and Daley, 2012). Currently, various in vitro differentiation protocols have been developed for the generation of different functional cell types. However, each of these differentiation protocols is typically developed based on one or a limited number of hESC lines and may not be applicable to other cell lines (Cohen and Melton, 2011). Indeed, different hESC and iPSC lines exhibit substantial variation in their capacity to differentiate into different cell lineages (Bock et al., 2011; Osafune et al., 2008). A recent genome-wide gene-expression analysis of a large collection of hESC and iPSC lines generated a scorecard based on the expression level of ~500 lineage-specific genes (Bock et al., 2011). However, this method is technically complicated and not economical for routine laboratory studies. Thus, a simple and economical experimental approach for accurately predicting hESC and iPSC differentiation potentials is needed. In addition, a recent study demonstrated that expression of the miR-371-3 cluster can predict the neural differentiation propensity of human PSCs (hPSCs; Kim et al., 2011), which suggests that the lineage-specific differentiation potential can be predicted with the use of simple biomarkers. Here, we utilized a well-established definitive endoderm (DE) differentiation system (D’Amour et al., 2005; Jiang et al., 2007) that has also been demonstrated to work very well in our lab (Jiang et al., 2013), and established WNT3 as a biomarker capable of predicting the DE differentiation potential of hESCs.

**RESULTS AND DISCUSSION**

To identify a biomarker capable of predicting the hESC differentiation potential, we collected all of the hESC lines in the Zhang lab (HES2, HES3, HUES8, H9.1, H9.2, and MEL-1) and subjected them to a commonly used DE differentiation protocol (D’Amour et al., 2005; Jiang et al., 2013). We previously confirmed that CD184 and CD117 double-positive cells could mark DE cells via an activin-A-based hESC differentiation protocol (Jiang et al., 2013), which is consistent with other reports (Nostro et al., 2011; Pan et al., 2011). Hence, we measured the DE differentiation efficiency by quantifying the percentage of CD184 and CD117 double-positive cell populations using flow cytometry. Consistent with previous reports (Bock et al., 2011; Osafune et al., 2008), we observed a variable efficiency for DE differentiation ranging from 15.4% for H9.2 to 95.3% for MEL-1 (Figure 1A). Further analysis of the differentiation potential of the H9 cell lines maintained in different labs using the same differentiation protocol revealed a variable differentiation efficiency (Figure S1A available online). In addition, we also observed a variable
differentiation efficiency of each cell line when it was maintained under different culturing conditions (Figure S1B). Collectively, these results suggest that the differentiation potential of hESCs is affected not only by their genetic background but also by other factors, including accumulated genetic and epigenetic changes during long-term culturing.

To identify a molecular marker that can serve as an indicator of the differentiation potential of the cell lines tested, we analyzed the expression levels of a group of genes in hESCs that include pluripotent transcription factors, modulators of various signaling pathways known to be involved in DE differentiation (Mfopou et al., 2010), and epigenetic factors implicated in DE differentiation (Jiang et al., 2013). Among the genes analyzed, the expression level of WNT3 in hESCs has the best correlation with the DE differentiation potential (Figures 1B, 1C, and 4).
S1C), suggesting that the WNT3 expression level in hESCs is a potential biomarker for predicting the DE differentiation potential.

If the WNT3 expression level in hESCs is a true predictor of the potential for DE differentiation, we anticipated that manipulation of the WNT3 level in hESCs should affect their DE differentiation capacity. To test for this possibility, we designed five different small hairpin RNAs (shRNAs) that target different regions of WNT3 and generated five stable WNT3 knockdown HUES8 sublines. We first confirmed that knockdown of WNT3 does not affect hESC maintenance, as the expression of the pluripotent factors was maintained in these WNT3 knockdown HUES8 sublines (Figure S2A). We then subjected the parental HUES8 line and derived sublines to the same differentiation protocol and determined their DE differentiation efficiency, which ranged from 10% to 80%, by quantifying the percentage of CD184 and CD117 double-positive cells. Interestingly, when the differentiation efficiency of these HUES8 sublines was plotted against the WNT3 levels in the undifferentiated cell state, we observed an excellent correlation (Figure 2A).

Next, we asked whether the differentiation potential of a “poor” hESC line with a low DE differentiation potential could be improved by enforced expression of WNT3. To this end, we constructed a doxycycline-inducible WNT3 overexpression lentiviral vector and transfected it into H9.2, one of the hESC lines with a low endogenous WNT3 level and low DE differentiation potential (Figure 1). We first confirmed that doxycycline is able to induce WNT3 expression in this modified H9.2 hESC line in a doxycycline dosage-dependent manner (Figure S2B). We then confirmed that doxycycline treatment does not alter the expression of pluripotent genes (OCT4, NANOG, and SOX2) or AXIN2, a marker of canonical WNT/beta-catenin activity (Figure S2B). This indicates that transient overexpression of WNT3 alone does not affect hESC maintenance, and that WNT3 is functionally different from WNT signaling pathway activation, which could induce hESCs to differentiate (Davidson et al., 2012). After 4 days of culturing under different concentrations of doxycycline, these cells were subjected to DE differentiation (without doxycycline). We found that doxycycline-induced WNT3 expression in undifferentiated H9.2 cells improved their differentiation efficiency, and, more importantly, the improvement occurred in a WNT3 dosage-dependent manner (Figure 2B). Collectively, the above results support our observation that the DE differentiation potential correlates with WNT3 expression levels in hESCs.

To evaluate the predictive potential of WNT3, we performed the same differentiation experiment at Duke University using all of the hESC/hiPSC lines available in the Bursac lab (including hESC lines HES3 [NKX2-5-GFP], H7, and H9 and MEL-1 [NKX2-5-GFP], and hiPSC line JT16). We first analyzed the WNT3 expression level in these cell lines by quantitative PCR (qRT-PCR), which allowed us to rank order these cell lines based on their WNT3 expression levels. We then subjected these cell lines to differentiation and quantified their differentiation efficiencies. In almost perfect agreement with our prediction, we found that the differentiation efficiency is highly correlated with the WNT3 levels of these hESC/hiPSC lines (Figure 2C), confirming the predictive capacity of WNT3. To further support our cell-surface-marker-based fluorescence-activated cell sorting (FACS) results, we analyzed the expression of endoderm lineage markers and pluripotent genes after DE differentiation. We chose two hESC lines (H7 [low WNT3] and MEL-1 [high WNT3]) based on their endogenous WNT3 expression levels. The results shown in Figure 2D clearly demonstrate that the expression levels of a panel of DE marker genes are higher in MEL-1 compared with the H7 cell line, suggesting a correlation between their DE differentiation potential and the endoderm marker gene-expression levels in hESCs.

Activin/Nodal-SMAD2/3 signaling is necessary and sufficient for human endoderm induction (Brown et al., 2011; D’Amour et al., 2005), but many other signaling pathways, particularly the WNT signaling pathway (Bone et al., 2011; Hay et al., 2008), also modulate DE differentiation (Mfopou et al., 2010). We recently reported that WNT signaling exhibited a biphasic role in modulating DE differentiation of hESCs, as treatment with either the WNT agonist Wnt3a at the beginning of differentiation or the WNT antagonist XAV 939 at a later stage of differentiation, or both, could increase DE differentiation (jiang et al., 2013). To test whether the capacity of the WNT3 expression level in hESCs to predict the DE differentiation potential would still be applicable under these differentiation conditions, we assayed four differentiation conditions with five different hESC lines (HUES8, HES2, HES3, H9.1, and MEL-1). The results shown in Figure 2E demonstrate that the WNT3 expression levels in hESCs were still correlated with their DE differentiation efficiency under the different differentiation conditions (Figure 2E). These results indicate that although different differentiation protocols might result in variable differentiation efficiency, the predictive capacity of the WNT3 expression level in hESCs is not affected by differentiation conditions.

Thus far, we have demonstrated an excellent correlation between WNT3 levels in hESCs and their DE differentiation potential. However, it is not clear whether the variable WNT3 levels in hESC lines can also serve as a predictor when the hESCs are subjected to differentiation for other lineages. To address this question, we further characterized the WNT3-depleted HUES8 line. First, qRT-PCR analysis demonstrated that WNT3 knockdown upregulated neuroectoderm lineage marker gene expression when treated under the neuroectoderm differentiation condition.
Second, immunostaining revealed that WNT3 knockdown increased the PAX6- or NESTIN-positive neural lineage cell population when the cells were subjected to further neural differentiation (Figure S2D). These data suggest that WNT3 knockdown in hESCs can increase their neuroectoderm differentiation potential. We then evaluated the effect of WNT3 knockdown in hESCs on their potential for differentiation toward the mesoderm lineage, the common precursor for mesoderm and DE. qRT-PCR analysis demonstrated that WNT3 knockdown in hESCs resulted in impaired activation of mesoderm marker gene expression after a 1.5 day differentiation treatment (Figure S2E). Moreover, variable cardiomyocyte (mesoderm derive) differentiation efficiency was observed among the different hESC lines, with MEL-1 (highest WNT3 level) generating more beating cells than H9 and H7 when they were subjected to the same differentiation procedure (data not shown). This result is consistent with the observation that MEL-1 exhibited the highest DE differentiation potential (Figure 2C). Collectively, these
results raised the possibility that WNT3 levels in hESCs may predict not only the DE differentiation potential but also the mesoderm (positive correlation) and neuroectoderm (negative correlation) differentiation potentials. Further studies are needed to quantitatively evaluate the correlation between the WNT3 expression level in hESCs and their differentiation potential for mesoderm and neuroectoderm lineages.

To begin to understand why the WNT3 levels in hESCs can serve as a biomarker for predicting the DE differentiation potential, we asked whether higher levels of WNT3 in hESCs can alter the expression levels of key DE differentiation genes. To this end, we induced the expression of WNT3 at different levels using the inducible WNT3-overexpression hESC line (Figure S2B). Although WNT3 overexpression did not significantly alter the expression levels of pluripotent genes (Figure S2B), it upregulated a panel of key endoderm lineage genes, including EOMES, FOXA2, GATA6, GSC, MIXL1, NODAL and SOX17. However, the expression levels of these genes were still much lower compared with those in differentiated DE (Figure 3A). Interestingly, the expression levels of these endoderm genes in the various hESC lines generally correlated with their DE differentiation potential.
Potential (Figures 3B and S3A). NODAL, MIXLI, and EOMES exhibited a statistically significant correlation (p < 0.05; Figure 3B), although it was not as good as that of WNT3 based on the coefficient R² value (Figure 1B). Importantly, EOMES (EOMESODERMIN), which interacts with SMAD2/3 to initiate the transcriptional network governing endoderm formation, has been reported to mark the onset of endoderm specification (Teo et al., 2011). These data suggest that WNT3-mediated upregulation of key DE differentiation genes could predispose hESCs to DE differentiation. However, it is currently unclear how WNT3 upregulation leads to higher levels of endoderm marker gene expression in hESCs. The facts that the expression level of AXIN2, a canonical WNT signaling pathway marker, did not exhibit a significant change in WNT3-overexpression hESCs (Figure S2B), and that no significant correlation between AXIN2 and DE differentiation capacity was observed (Figure S1C) suggest that the canonical WNT signaling pathway may not be the key factor behind WNT3’s capacity to predict the DE differentiation potential. Consistent with this notion, treatment of hESCs with Wnt3a, a WNT agonist, did not significantly alter DE differentiation efficiency (Figures S3B and S3C). The use of recombinant WNT3 protein may help convert “poor” hESC lines with a low DE differentiation potential into “good” ones with a high DE differentiation potential.

Although the exact mechanism by which WNT3 levels in hESCs predict their differentiation potential is not known, we propose a working model that can explain all of our data so far (Figure 3C). It has been established that the identity of hESCs is mainly determined by the core pluripotency regulatory networks (Boyer et al., 2005). Variable levels of lineage factors, such as WNT3, can be tolerated in hESCs, which is supported by the fact that knockdown or overexpression of WNT3 in hESCs does not affect pluripotent gene expression (Figure S2). However, it is likely that higher levels of WNT3 could result in upregulation of multiple factors critical for DE differentiation, rendering the hESCs poised for DE differentiation. Upon receiving other differentiation signals that downregulate the pluripotency transcription network, hESCs could initiate differentiation toward DE. Consistent with this hypothesis, a recent study demonstrated that although constitutive overexpression of SOX17 in hESCs does not affect hESC maintenance, it can restrict hESCs to DE lineage differentiation (Séguin et al., 2008).

In summary, we have identified WNT3 as a biomarker capable of predicting the DE differentiation potential of hESCs, which should facilitate efforts to understand and generate endoderm cell lineages.

**EXPERIMENTAL PROCEDURES**

**hESC Maintenance and DE Differentiation**

hESC line HUES8 was cultured in Matrigel-coated dishes with mTeSR1 medium (STEMCELL Technologies) according to the manufacturer’s instructions. hESC line MEL-1 and HES3 with INS-GFP reporter (Micallef et al., 2012) were cultured on mitomycin C inactivated mouse embryonic fibroblast (iMEF) with regular hESC medium (Dulbecco’s modified Eagle's medium [DMEM]/F12, 20% [vol/vol] knockout serum replacement, 1% [vol/vol] nonessential amino acids, 1% [vol/vol] GlutaMax, 1% [vol/vol] penicillin-streptomycin, 55 μM β-mercaptoethanol supplemented with 8 ng/ml FGF2 (PeproTech)). Three hESC lines of different origin (H9.1 from the Zhang lab, H9.2 from the Bursac Lab, and H9.3 from the Deng lab (Peking University; Jiang et al., 2007) and the HES2 line were cultured either on iMEF with regular hESC medium or on Matrigel-coated dishes with mTeSR1 medium. Only those cells with undifferentiated morphology were used for the DE differentiation assay.

For Figures 2C and 2D, the hESC lines H7, H9, MEL-1, and HES3 with NRX2-5-GFP reporter (Elliott et al., 2011), and hiPSC line JT-16 were cultured on iMEF with regular hESC medium. H7 cultured on defined Essential 8 Medium (Invitrogen) was also used in one experiment. The experiments shown in Figure 2C and 2D were performed in the Bursac lab.

For DE differentiation, routinely cultured hESCs with undifferentiated morphology were replated into Matrigel-coated multi-well plates with ESC medium. To measure the correlation between gene expression and DE differentiation efficiency, we used half of the cells for gene-expression analysis and the other half for the subsequent DE differentiation assay. One day later, the cells were washed twice with DMEM/F12 and then cultured for 4 days in basal medium (DMEM/F12, 55 μM 2-mercaptoethanol, 1% [vol/vol] nonessential amino acids, 0.5% [vol/vol] B27 without vitamin A [Invitrogen], 0.25% [vol/vol] N2 [Invitrogen]) supplemented with 100 ng/ml recombinant human activin A (PeproTech) or other growth factors or chemicals (available upon request; murine Wnt3a [PeproTech, 50 ng/ml] and XAV 939 [Tocris, 1 μM]) were used for Figure 2E). The medium was changed every day.

**Statistical Analysis**

All data are representative of three or more experiments. Errors are the standard deviation (SD) of averaged results. Correlation analysis was performed with GraphPad Prism software, and R² and p values are presented. Briefly, the coefficient of determination, R², was calculated to determine how well a regression line fit the set of data. The significance (probability) of the correlation coefficient was determined from the t statistic, which indicates whether the observed correlation coefficient would have occurred by chance if the true correlation was zero.

More detailed information can be found in Supplemental Experimental Procedures, and details regarding the primers used in this work are provided in Table S1.
SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2013.03.003.

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