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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

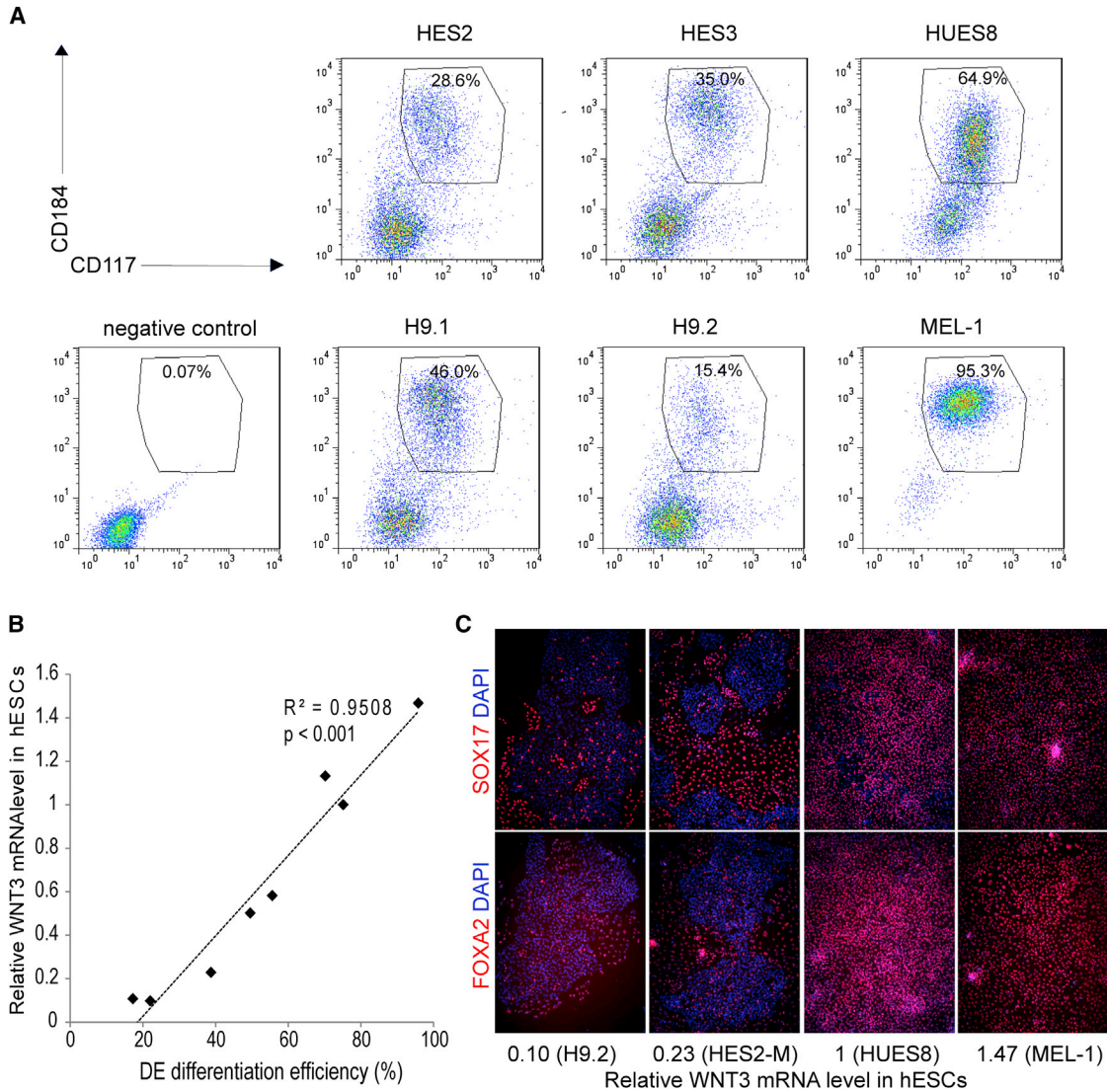


Figure 1. The WNT3 Level in hESCs Correlates with the DE Differentiation Potential

(A) Different hESC lines have different capacities for DE differentiation. The efficiency of DE differentiation was measured by the percentage of CD184 and CD117 double-positive cells after 4 days of induction.

(B) WNT3 messenger RNA (mRNA) levels in undifferentiated hESCs correlate with their DE differentiation efficiency after 4 days of induction. HES3, H9.2, MEL-1 (MG3) cultured with feeder, HUES8 cultured with Matrigel-mTeSR1, and H9.1 and HES2 cultured under both conditions were used in the differentiation assays. Shown is one representative result of three independent experiments.

(C) Immunofluorescent staining images show a correlation between WNT3 levels in hESCs and SOX17 or FOXA2 expression after 4 days of DE differentiation.

See also Figure S1.

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



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

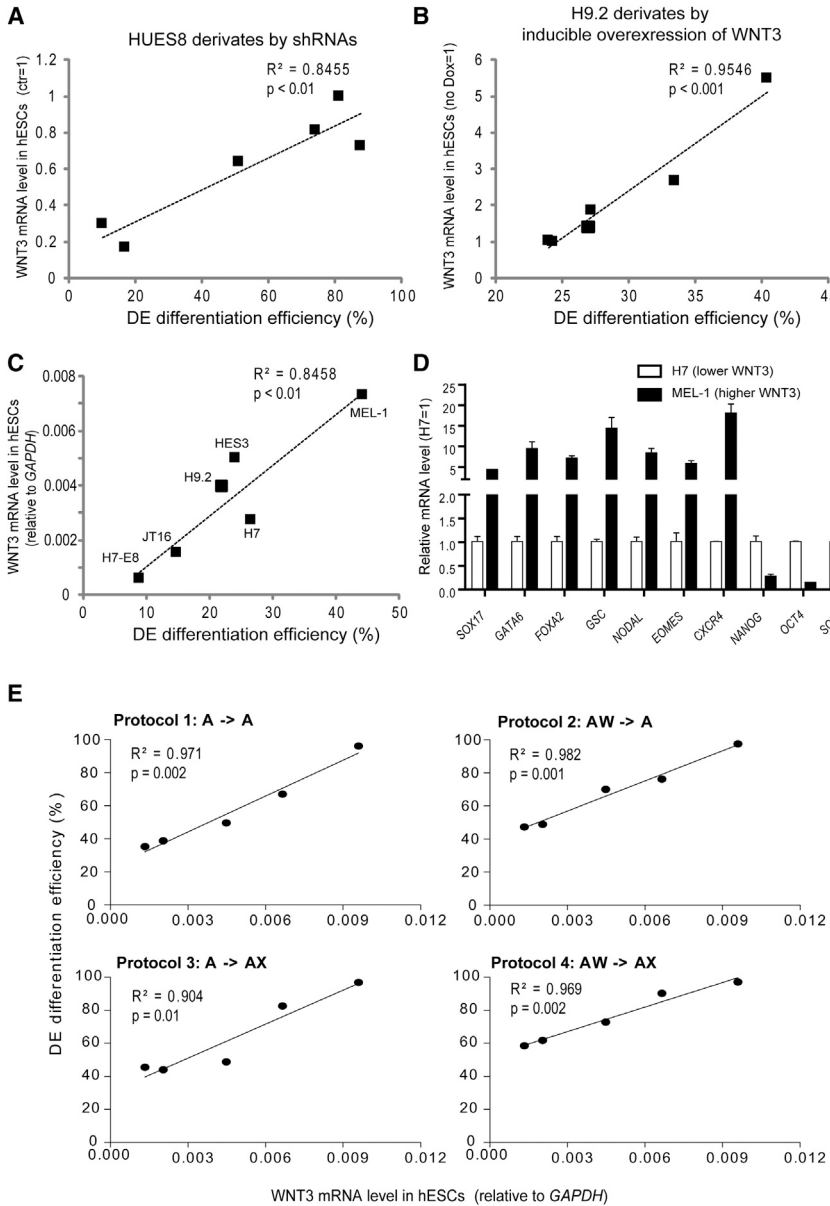


Figure 2. WNT3 Is a Functional Biomarker for Predicting the DE Differentiation Potential

(A) WNT3 levels in control and various knockdown HUES8 sublines correlate with their DE differentiation efficiency. Five shRNAs and one control shRNA were used to generate HUES8 sublines.

(B) Overexpression of WNT3 in the H9.2 hESC line in the undifferentiated state can improve the DE differentiation efficiency. The improvement correlates with the level of WNT3 expression.

(C) Independent verification of the predictive potential of WNT3 using different hPSC lines maintained in a different lab. hiPSC line JT16 and hESC lines HES3 (NKX2-5-GFP), H9, MEL-1 (NKX2-5-GFP), and H7 cultured on feeder or defined E8 system were used in this assay.

(D) qRT-PCR analysis of the expression of endoderm lineage genes and pluripotent genes after DE differentiation. Two cell lines, H7 and MEL-1, were chosen for this analysis based on their endogenous WNT3 expression levels in the undifferentiated state. Shown is one representative result (with SD from three technical replicates) of two independent experiments.

(E) Four previously described protocols (Jiang et al., 2013) were used to differentiate hESCs into DE, and the correlations between the WNT3 expression level of undifferentiated hESCs and the DE differentiation efficiency were analyzed. The four protocols included the following treatments in a serum-free medium: (1) A -> A (Activin A only for 4 days); (2) AW -> A (Activin A and Wnt3a for 2 days followed by Activin A only for another 2 days); (3) A -> AX (Activin A only for 2 days followed by Activin A and XAV 939 for another 2 days); and (4) AW -> AX (Activin A and Wnt3a for 2 days followed by Activin A and XAV 939 for another 2 days).
 See also Figure S2.

(Figure S2C). 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 knock-

down in hESCs resulted in impaired activation of mesoderm marker gene expression after a 1.5 day differentiation treatment (Figure S2E). Moreover, variable cardiomyocyte (mesoderm derivate) 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

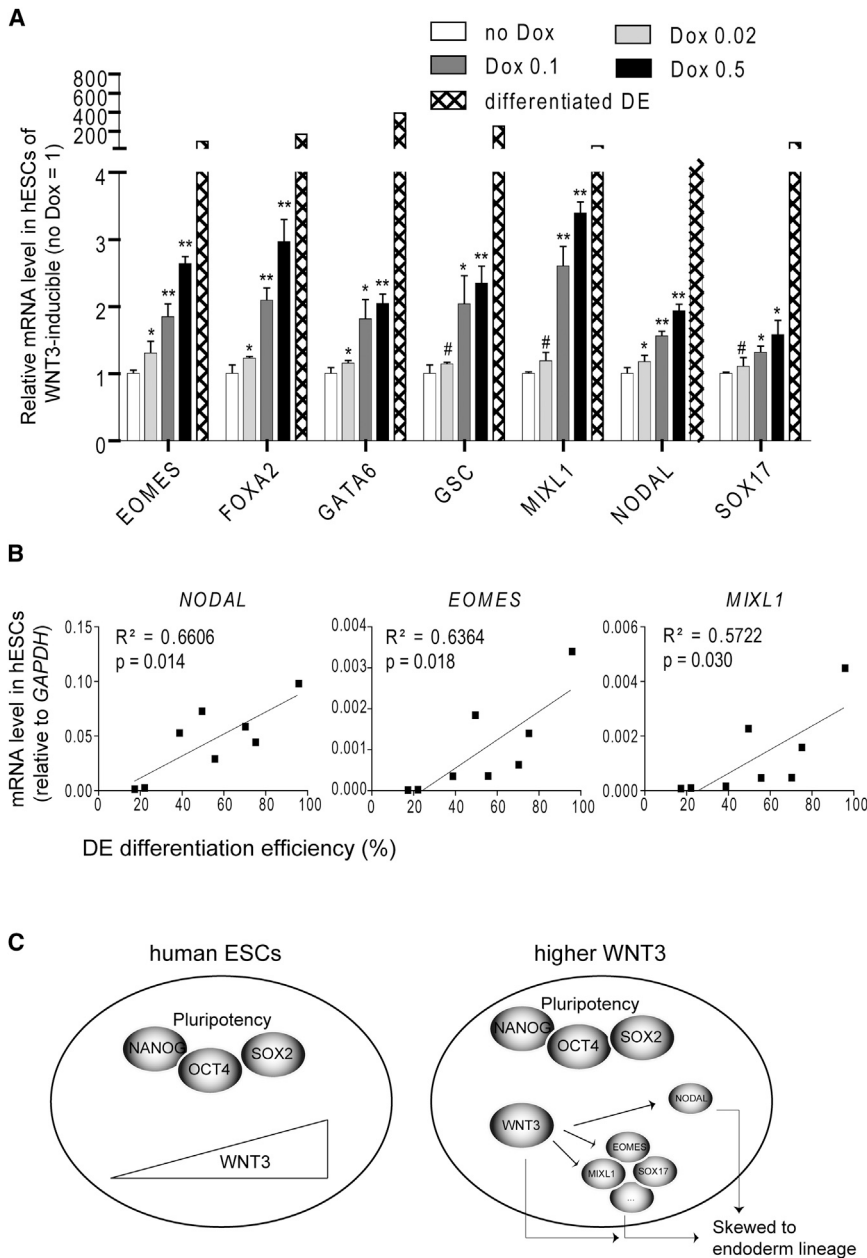


Figure 3. Dox-Induced *WNT3* Expression in hESCs Correlates Positively with the Expression Level of a Panel of Factors Important for Endoderm Differentiation

(A) qRT-PCR analysis demonstrates that Dox-induced *WNT3* expression in hESCs correlates with the expression of a panel of factors important for DE differentiation. However, the increased levels of the endoderm factors are not sufficient to drive DE differentiation. Data are shown with the SD from three biological replicates. The p value by Student's t test compared with "no dox" is shown as # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$. Differentiated DE samples serve as control. (B) Statistically significant correlations between the mRNA levels of the endoderm maker genes *NODAL*, *MIXL1*, and *EOMES* in hESCs and the DE differentiation efficiency of the various hESC lines.

(C) Hypothetic model explaining how the *WNT3* level in hESCs might be able to predict the DE differentiation potential. Left panel: The pluripotency of hESCs is independent of *WNT3*, and variable *WNT3* expression levels exist in hESC lines. Right panel: hESCs with higher *WNT3* expression. Key endoderm lineage factors are upregulated, which may render the hESCs poised for DE differentiation pending downregulation of the pluripotent factors by other differentiation signals.

See also Figure S3.

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 (Figures 3B and S3A). *NODAL*, *MIXL1*, 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 R^2 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 hESC lines. *WNT3* appears to be a functional marker, because the DE differentiation potential can be modulated by altering *WNT3* levels in hESCs. Our study establishes a simple method for predicting the DE differentiation poten-

tial 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 μ M β -mercaptoethanol supplemented with 8 ng/ml FGF2 (PeproTech)). Three H9 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 NKX2-5-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 multiwell 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 μ M 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 μ M]) 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 R^2 and p values are presented. Briefly, the coefficient of determination, R^2 , 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>.

LICENSING INFORMATION

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