Comparable Generation of Activin-Induced Definitive Endoderm via Additive Wnt or BMP Signaling in Absence of Serum

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http://dx.doi.org/10.1016/j.stemcr.2014.05.007

SUMMARY

There is considerable interest in differentiating human pluripotent stem cells (hPSCs) into definitive endoderm (DE) and pancreatic cells for in vitro disease modeling and cell replacement therapy. Numerous protocols use fetal bovine serum, which contains poorly defined factors to induce DE formation. Here, we compared Wnt and BMP in their ability to cooperate with Activin signaling to promote DE formation in a chemically defined medium. Varying concentrations of WNT3A, glycogen synthase kinase (GSK)-3 inhibitors CHIR99021 and 6-bromoindirubin-3'-oxime (BIO), and BMP4 could independently co-operate with Activin to effectively induce DE formation even in the absence of serum. Overall, CHIR99021 is favored due to its cost effectiveness. Surprisingly, WNT3A was ineffective in suppressing E-CADHERIN/CDH1 and pluripotency factor gene expression unlike GSK-3 inhibitors or BMP4. Our findings indicate that both Wnt and BMP effectively synergize with Activin signaling to generate DE from hPSCs, although WNT3A requires additional factors to suppress the pluripotency program inherent in hPSCs.

INTRODUCTION

Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), collectively known as human pluripotent stem cells (hPSCs), can potentially be differentiated into clinically useful cell types for in vitro disease modeling, drug screening, and cell replacement therapy. Given the explosion in diabetes and its complications worldwide, the directed differentiation of hPSCs into definitive endoderm (DE) and subsequently pancreatic cells is of immense interest (Teo et al., 2013a). In 2005, Novocell (now ViaCyte) reported the ability to derive > 80% of DE from hESCs with the use of 100 ng/ml Activin A (hereafter referred to as "Activin") in the presence of 0.2%–2% fetal bovine serum (FBS; D'Amour et al., 2005). To complement Activin/Nodal signaling in inducing DE formation, Wnt and BMP signaling activators were then introduced (Table S1 available online). Developmentally, this mimics the complex *Nodal*, *Wnt* (*Wnt3*, *Wnt3a* and *Wnt5a*) and *Bmp* (*Bmp4*) signaling, which operate during gastrulation, primitive streak, and early DE formation in the mouse embryo (Arnold and Robertson, 2009; Lawson et al., 1999; Liu et al., 1999; Ohinata et al., 2009; Teo et al., 2011; Yamaguchi et al., 1999).

The coactivation of Wnt and Activin/Nodal signaling (albeit in the presence of FBS) is commonly used as described by D'Amour et al. (2006). They reported that Activin and WNT3A (specifically 100 ng/ml Activin, 25 ng/ml WNT3A, and 0.2% FBS) can induce more than 80% of DE cells (D'Amour et al., 2006). Alternatively, activation of Wnt signaling via the inhibition of glycogen synthase kinase (GSK)-3 (specifically 100 ng/ml Activin or 500 nM IDE1, 3 μM CHIR99021 [GSK-3 inhibitor], and 2% FBS) has also been recently reported to induce 70%–80% of DE cells (Illing et al., 2013; Kunisada et al., 2012). In contrast, the work of Teo, Dunn (Phillips et al., 2007; Teo et al., 2012), and Vallier (Vallier et al., 2009) independently demonstrated that 10–50 ng/ml of BMP4 can synergistically act with Activin (in a defined medium without FBS) to induce more than 80% of DE cells. However, it is still uncertain how Wnt compares with BMP in cooperating with Activin/Nodal signaling in a chemically defined medium to induce DE formation.

We sought to clarify and define the Activin-Wnt-BMP signaling relationship using hiPSCs that we derived (Teo et al., 2013b). Here, we report a head-to-head comparison of Wnt versus BMP in combination with Activin signaling in a chemically defined medium without FBS to induce DE formation. Unlike previous reports (25 ng/ml WNT3A plus FBS; Table S1), we observed that a high dose of WNT3A (100 ng/ml, without FBS) is required to maximally induce DE in the presence of 100 ng/ml Activin. The activation of Wnt signaling with the use of two independent GSK-3 inhibitors (CHIR99021 or 6-bromoindirubin-3'-oxime [BIO]; Sato et al., 2004) or BMP signaling with BMP4 can also maximally induce DE cells. Thus, both Wnt and BMP signaling can effectively cooperate with Activin signaling to induce DE formation.

Effective differentiation of hPSCs into DE is coupled with the suppression of E-CADHERIN/CDH1 and pluripotency. Whereas BMP signaling has been reported to suppress E-CADHERIN/CDH1 and pluripotency (Teo et al., 2011, 2012), we observed that WNT3A is not as effective as
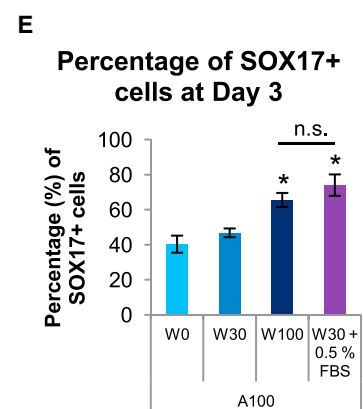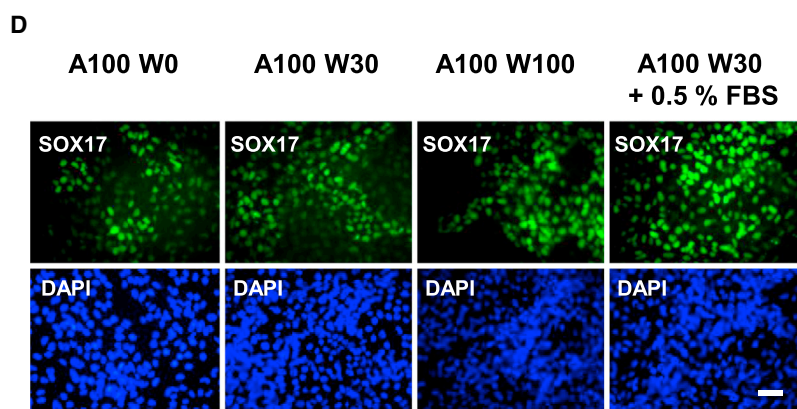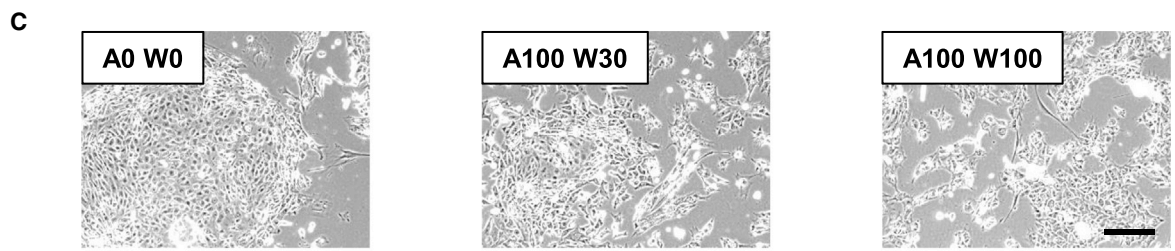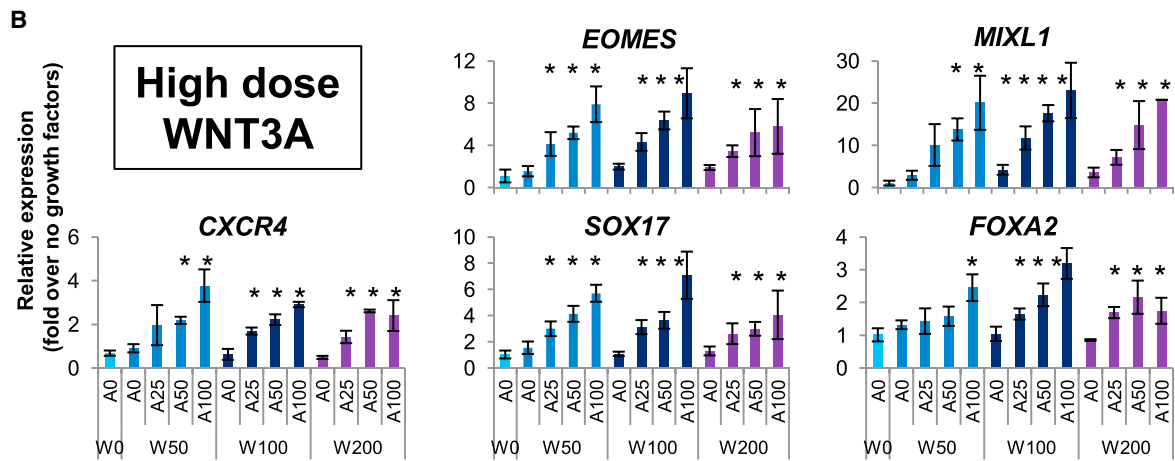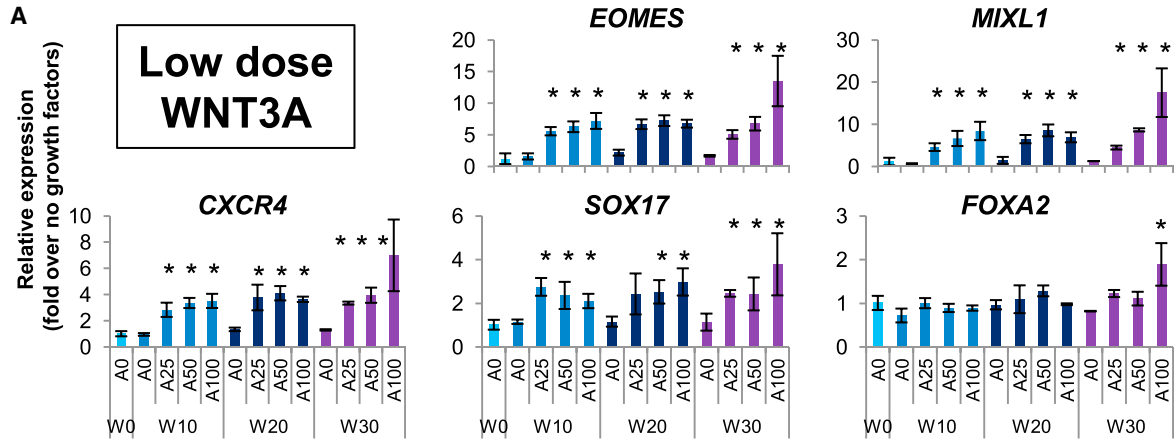
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GSK-3 inhibitors in suppressing both E-CADHERIN/CDH1 and the pluripotency program. This could partly explain the continued requirement for FBS when deriving DE with WNT3A. Therefore, our study identifies the unprecedented need for an additional compound to suppress pluripotency when combining Activin and WNT3A to generate DE cells without serum.

RESULTS

High Dose of WNT3A Is Sufficient to Cooperate with Activin to Induce DE without Serum

Many previous studies have reported reliance upon Activin, WNT3A, and FBS to differentiate hPSCs into DE (Table S1). To determine whether WNT3A can cooperate with Activin in a chemically defined medium in the absence of serum (RPMI 2% B27) to induce DE from hiPSCs, we used 10–30 ng/ml WNT3A with 0–100 ng/ml Activin in optimization experiments. Dismally, 30 ng/ml WNT3A and 100 ng/ml Activin only marginally increase the expression of DE markers *SOX17* and *FOXA2* (Figures 1A and 1C–1E). Unlike previous reports, a low dose of WNT3A is insufficient to effectively promote DE formation in serum-free conditions. We subsequently increased the dose of WNT3A and determined that 100 ng/ml WNT3A can effectively result in maximal DE marker gene expression (Figures 1B–1E) without a dose-responsive increase in mesodermal markers *BRACHYURY* and *TBX6*, or extraembryonic marker *SOX7* (Figure S1A). Morphologically, cells induced with 100 ng/ml Activin and 30 or 100 ng/ml WNT3A looked indistinguishable but different from no growth factor condition (Figure 1C). However, immunostaining and quantitative analyses for SOX17 DE marker clearly demonstrated that 100 ng/ml Activin + 100 ng/ml WNT3A gave rise to DE cells with a comparable efficiency as that of 100 ng/ml Activin + 30 ng/ml WNT3A + 0.5% FBS, as opposed to 100 ng/ml Activin + 30 ng/ml WNT3A (Figures 1D and 1E). Together, these findings suggest that FBS is necessary for synergistic activity with 100 ng/ml Activin

and 25 ng/ml WNT3A to efficiently induce DE formation. Thus, FBS + 25 ng/ml WNT3A can be replaced with a high dose of WNT3A (100 ng/ml) to induce DE.

GSK-3 Inhibitors, CHIR99021 and BIO, Cooperate with Activin to Induce DE without Serum

To further define the synergism between Wnt and Activin signaling in inducing DE without serum, we adopted complementary approaches to activate Wnt signaling by inhibiting the downstream signaling molecule GSK-3, using two independent GSK-3 inhibitors, CHIR99021 or BIO, which block the degradation of β-catenin, thereby allowing its nuclear translocation and activation of downstream target genes. Kunisada et al. (2012) and Illing et al. (2013) used a GSK-3 inhibitor to demonstrate that 3 μM CHIR99021 with 100 ng/ml Activin or 500 nM IDE1 and 2% FBS induces 70%–80% of DE cells.

Initially, we used a range of doses of CHIR99021 (0.5, 3, or 9 μM) together with Activin to prompt DE differentiation (Figure 2A). Interestingly, 9 μM CHIR99021 induces maximal *CXCR4* gene expression independent of the requirement for Activin. However, cardinal DE markers *EOMES*, *SOX17*, and *FOXA2* are suppressed at such a dose, suggesting that excessive Wnt signaling activation is refractory to DE formation (Figure 2A). Thus, we lowered the doses of CHIR99021 to 1, 3, and 5 μM. Morphologically, cells induced with 100 ng/ml Activin and 1 or 3 μM CHIR99021 were indistinguishable but different from no growth factor condition. We finally confirmed that 3 μM CHIR99021 (together with 100 ng/ml Activin) maximally induces DE differentiation in our chemically defined medium (Figures 2B and 2C) without a dose-responsive increase in mesodermal markers *BRACHYURY* and *TBX6*, or extraembryonic marker *SOX7* (Figure S1B). The increasing dose of CHIR99021 increases mesodermal marker gene expression independent of the dose of Activin (Figure S1B), indicating a fine balance in its use for DE (1–3 μM) versus mesoderm (>3 μM) formation.

To complement the use of CHIR99021 in deriving DE cells, we used another GSK-3 inhibitor, BIO (0.5, 2, or 5 μM), to

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Figure 1. A High Dose of WNT3A Is Required for Activin-Induced DE Formation in Absence of Serum

(A and B) Expression of mesendoderm (*EOMES*, *MIXL1*) and DE (*CXCR4*, *SOX17*, *FOXA2*) markers in hiPSCs differentiated for 3 days in the presence of Activin (0, 25, 50, 100 ng/ml) and (A) low dose (10, 20, 30 ng/ml) or (B) high dose (50, 100, 200 ng/ml) of WNT3A. Asterisk (*) indicates p < 0.05 compared to no growth factor (A0 W0).

(C) Morphology of hiPSCs differentiated for 3 days in the presence of no growth factors (A0 W0; left) or 100 ng/ml Activin (A100) plus either 30 ng/ml (W30; middle) or 100 ng/ml (W100; right) WNT3A. Scale bar represents 200 μm.

(D) Immunostaining for the SOX17 DE marker on hiPSCs differentiated for 3 days in the presence of A100 and WNT3A (W0, W30, W100) ± 0.5% fetal bovine serum (FBS). Scale bar represents 50 μm.

(E) The percentage of SOX17+ cells after differentiation of hiPSCs for 3 days in the presence of A100, A100 W30, A100 W100, or A100 W30 + 0.5% FBS. Asterisk (*) indicates p < 0.05 compared to A100 W0. All error bars represent SD of three biological replicates. A, Activin; W, WNT3A; and FBS, fetal bovine serum. DAPI is the nuclear stain.

See also Figure S1.
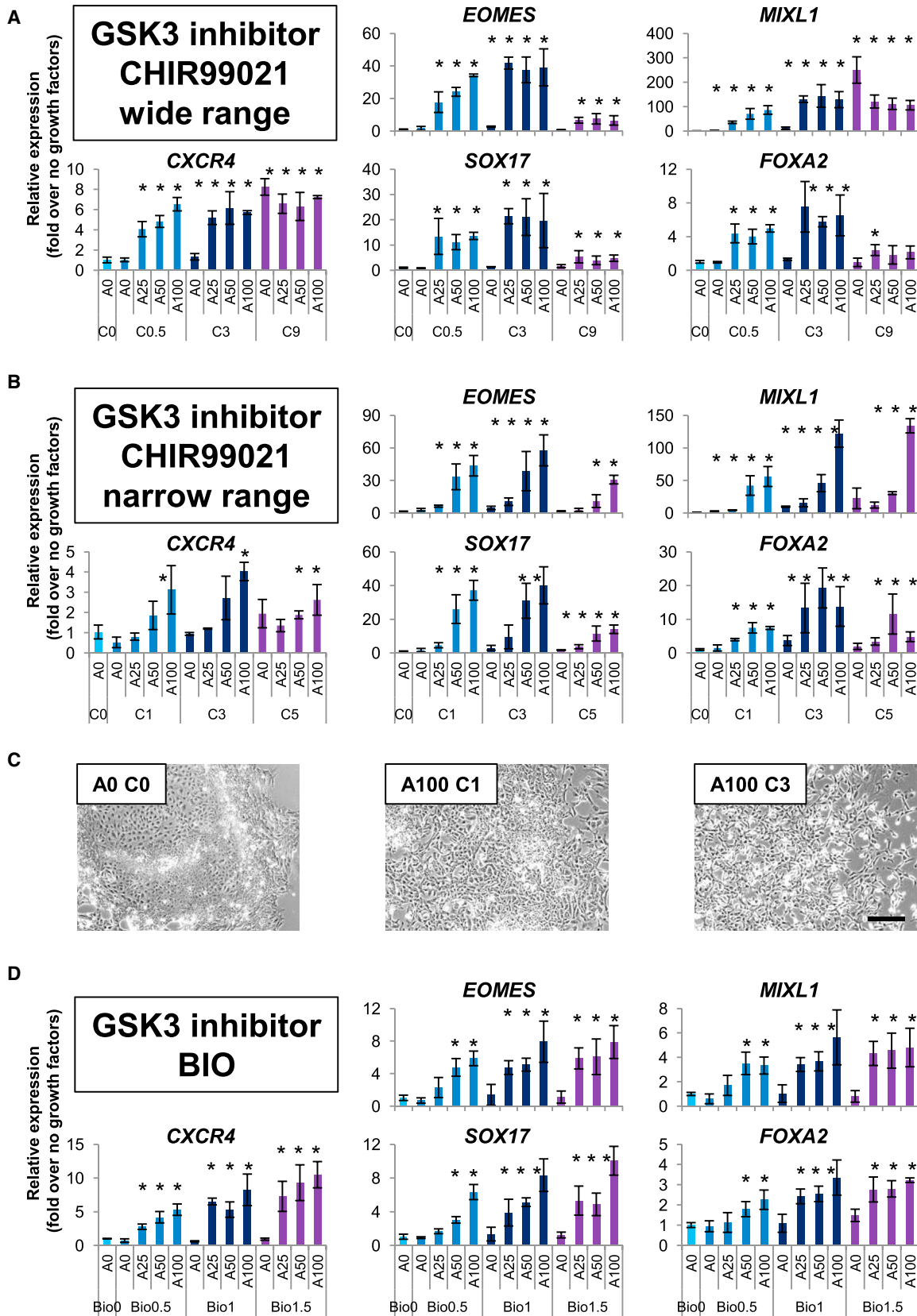
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activate Wnt signaling and observed that ≥2 μM resulted in excessive cell death (data not shown). Subsequently we determined that 1.5 μM BIO together with 100 ng/ml Activin is optimal for generating DE cells from hPSCs without serum (Figure 2D), again without a dose-responsive increase in mesodermal markers *BRACHYURY* and *TBX6*, or extraembryonic marker *SOX7* (Figure S1C).

Wnt and BMP Signaling Can Enhance Activin-Induced DE with Comparable Efficiencies without Serum Supplementation

Next, to confirm that BMP4 and Activin can induce DE from hPSCs without serum, we performed similar dose-response experiments. These experiments ascertained that 100 ng/ml Activin and 25–50 ng/ml BMP4 can elicit maximal expression of DE markers *CXCR4*, *SOX17*, and *FOXA2* (Figure 3A), without a dose-responsive increase in mesodermal markers *BRACHYURY* and *TBX6*, or extraembryonic marker *SOX7* (Figure S1D). Because both Wnt and BMP can cooperate with Activin signaling to generate DE cells in our chemically defined differentiation condition, we then compared the various optimal DE-inducing conditions in the same experiment. Time course analyses indicated that Wnt and BMP signaling-based conditions induced a peak expression of mesendodermal marker *EOMES* between days 2 and 3 and DE markers *CXCR4* and *SOX17* on day 3 in one hiPSC and one hESC line (Figures S2A–S2C). Thus, we compared these DE-inducing conditions on day 3 of differentiation.

Interestingly, the combination of 100 ng/ml Activin and 3 μM CHIR99021 resulted in the highest expression of mesendoderm (*EOMES*, *MIXL1*) and DE (*CXCR4*, *SOX17*, *FOXA2*) marker gene expression (Figure 3B), although the percentage of SOX17+ DE cells (as determined by cell count) is comparable with the other DE-inducing conditions (Figures S3B and S3C). Fluorescence-activated cell sorting (FACS) analyses performed on two different hPSC lines further confirmed the observation that the various optimal DE-inducing conditions generated comparable percentages of CXCR4+SOX17+ DE cells (Figures 3C and S3E). Reassuringly, cells differentiated in the optimal DE-inducing conditions morphologically resembled each other but were different from no growth factor condition

(Figure S3D). Among the optimal DE-inducing conditions, 3 μM CHIR99021 induced mesendodermal marker *BRACHYURY* and a marginal increase in mesodermal marker *TBX6* but none of them induced extraembryonic tissue marker *SOX7* dramatically (Figure S3A).

To further ascertain that the various optimal DE-inducing conditions were comparable with published methods, we undertook a head-to-head comparison with protocols outlined in Kroon et al. (2008) and Touboul et al. (2010), using the same differentiation medium (RPMI 2% B27). Because Touboul et al. (2010) utilized 20 ng/ml FGF2 (F20) and 10 μM LY294002 (LY10) in addition to Activin and BMP4, we applied the same amount of FGF2 and LY294002 to all our DE-inducing conditions because the aim was to determine if the various Wnt and BMP signaling-induced DE (in the presence of 100 ng/ml Activin) were comparable with published methods. In our comparison performed on two different hPSC lines, we found that our various optimal DE-inducing conditions were comparable with Touboul et al. (2010) and at times superior to Kroon et al. (2008) in terms of the induction of DE (*CXCR4*, *SOX17*, *FOXA2*) marker gene expression (Figure S4A).

Next, we investigated whether our DE-inducing conditions generated DE derivatives comparably. To this end, we differentiated these DE cells into pancreatic progenitors using a published protocol (Teo et al., 2012) with some modifications (A.K.K.T. et al., unpublished data). Our DE-inducing conditions gave rise to pancreatic progenitor markers *SOX9* and *PDX1* comparably with Kroon et al. (2008) in two different hPSC lines (Figure S4B), demonstrating equivalence in terms of their differentiation potential.

Wnt Signaling via WNT3A Is Not as Effective as BMP4 or the GSK-3 Inhibitors, CHIR99021 and BIO, in Suppressing E-CADHERIN and Pluripotency of hPSCs during DE Induction

During DE differentiation, hPSCs undergo epithelial to mesenchymal transition evidenced by morphological changes (Figures 1C, 2C, and S3D) and a decrease in epithelial marker E-CADHERIN/CDH1 (D'Amour et al., 2005). In addition, pluripotency program is suppressed, with rapid plummeting of pluripotency factor SOX2 expression


Figure 2. GSK-3 Inhibitors CHIR99021 and BIO Can Promote DE Formation

(A and B) Expression of mesendoderm (*EOMES*, *MIXL1*) and DE (*CXCR4*, *SOX17*, *FOXA2*) markers in hiPSCs differentiated for 3 days in the presence of Activin (0, 25, 50, 100 ng/ml) and (A) wide range (0.5, 3, 9 μM) or (B) narrow range (1, 3, 5 μM) of CHIR99021.

(C) Morphology of hiPSCs differentiated for 3 days in the presence of no growth factors (A0 C0; left) or 100 ng/ml Activin (A100) plus either 1 μM (C1; middle) or 3 μM (C3; right) CHIR99021. Scale bar represents 200 μm.

(D) Expression of mesendoderm and DE markers in hiPSCs differentiated for 3 days in the presence of Activin (0, 25, 50, 100 ng/ml) and BIO (0.5, 1, 1.5 μM). Asterisk (*) indicates $p < 0.05$ compared to no growth factor (A0 C0, A0 Bio0). All error bars represent SD of three biological replicates. A, Activin; C, CHIR99021.

See also Figure S1.
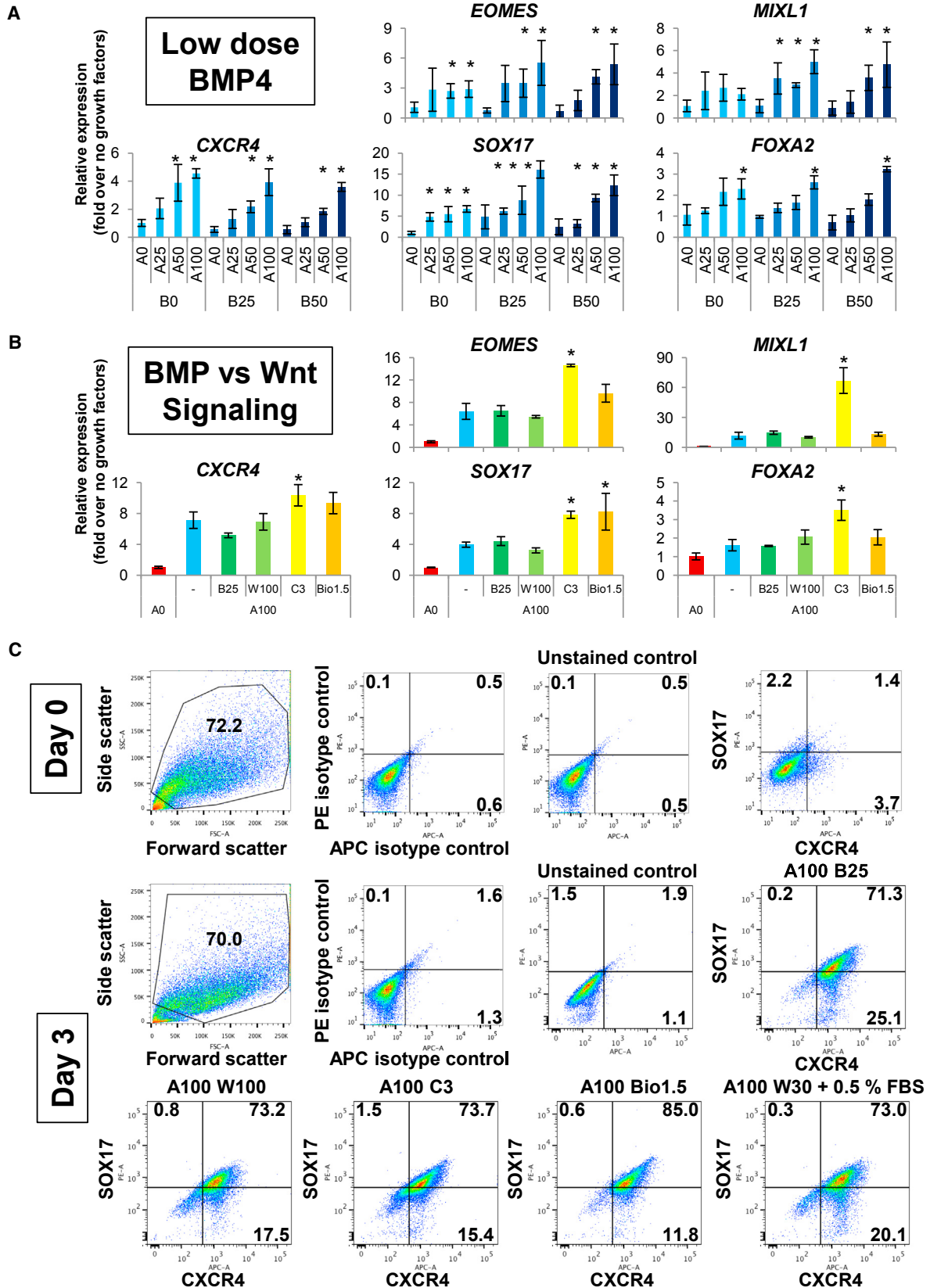
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followed by NANOG and lastly OCT4 (Teo et al., 2011, 2012). To ascertain that both Wnt and BMP signaling-induced DE cells are committed toward DE cell fate, we investigated the impact of varying doses of Wnt and BMP signaling activators on epithelial marker E-CADHERIN/CDH1 and pluripotency factor gene expression. Despite a high dose of WNT3A being able to significantly induce DE marker gene expression (with 100 ng/ml Activin), the expression level of E-CADHERIN/CDH1 did not decrease with increasing doses of WNT3A (Figure 4A). Surprisingly, pluripotency factors SOX2 and NANOG were only marginally affected by increasing doses of WNT3A with a background of 100 ng/ml Activin (Figures 4A–4C). In contrast, increasing doses of BMP4 and GSK-3 inhibitors, CHIR99021 and BIO, resulted in significant suppression of E-CADHERIN/CDH1 and pluripotency factors SOX2 and NANOG (Figures 4A–4C). Collectively, these data suggest that additional factors are required to work with WNT3A to suppress E-CADHERIN/CDH1 and pluripotency factors during differentiation toward DE. Therefore, CHIR99021, BMP4, and BIO may be preferred for DE differentiation because they also aid in the suppression of pluripotency (Figures 4A–4C).

DISCUSSION

We report the use of a chemically defined differentiation medium to compare DE-inducing effects by Wnt or BMP signaling in the presence of Activin signaling. This direct comparison demonstrates that both Wnt and BMP can independently synergize with Activin signaling to comparably and effectively induce DE cells from hPSCs in the absence of FBS. Numerous reports have established that 25 ng/ml WNT3A is optimal for inducing DE in the presence of Activin and FBS (Table S1). However, 10–30 ng/ml WNT3A is insufficient to synergize with 100 ng/ml Activin to effectively generate DE cells in our serum-free system. The need for 100 ng/ml WNT3A for efficient DE formation in a chemically defined system suggests that FBS (which exhibits batch-to-batch variability) introduces additional differentiation-promoting factors to supplement 25 ng/ml WNT3A in inducing DE cells.

Although Kunisada et al. (2012) and Illing et al. (2013) used CHIR99021 to induce DE differentiation from hPSCs, they also included FBS in their protocol. We now provide several alternative methods for effectively inducing DE cells, all via the activation of Wnt signaling pathway (in addition to the indispensable Activin signaling pathway). These include 100 ng/ml WNT3A, 3 μM CHIR99021, and 1.5 μM BIO, all of which exhibit similar effects in DE induction. This three-pronged approach confirms the ability of Wnt to synergize with Activin signaling to induce DE in a chemically defined medium. Our comparison with Kroon et al. (2008) (WNT3A-based) and Touboul et al. (2010) (BMP4-based) further demonstrates that these three Wnt signaling-based optimized conditions induce DE comparably and efficiently, and give rise to DE derivatives with equivalent potential.

However, it is surprising to note that WNT3A is not as effective in downregulating E-CADHERIN/CDH1 and pluripotency factors as compared to GSK-3 inhibitors (or even BMP4) in a serum-free system. It is known from early mammalian development that the expression of *Oct4*, and in particular *Sox2* and *Nanog* (Avilion et al., 2003; Hart et al., 2004) coincides with the in situ expression of *Wnt3*, *Wnt3a* and *Wnt5a* but not that of *Bmp4* (Arnold and Robertson, 2009; Liu et al., 1999; Tam and Loebel, 2007; Teo et al., 2011; Yamaguchi et al., 1999). Thus, given the overlapping expression of *Wnt* and pluripotency genes but not that of *Bmp*, it is conceivable that Wnt does not play a major role in suppressing pluripotency in contrast to BMP. In this study, we reveal fundamental mechanistic differences between the two developmental signaling pathways: (1) BMP signaling suppresses pluripotency and simultaneously promotes DE formation, whereas (2) Wnt signaling mostly serves to synergistically promote DE formation and may require additional factors to suppress pluripotency. FBS, which is commonly added in presence of WNT3A, could be promoting both differentiation and suppressing pluripotency.
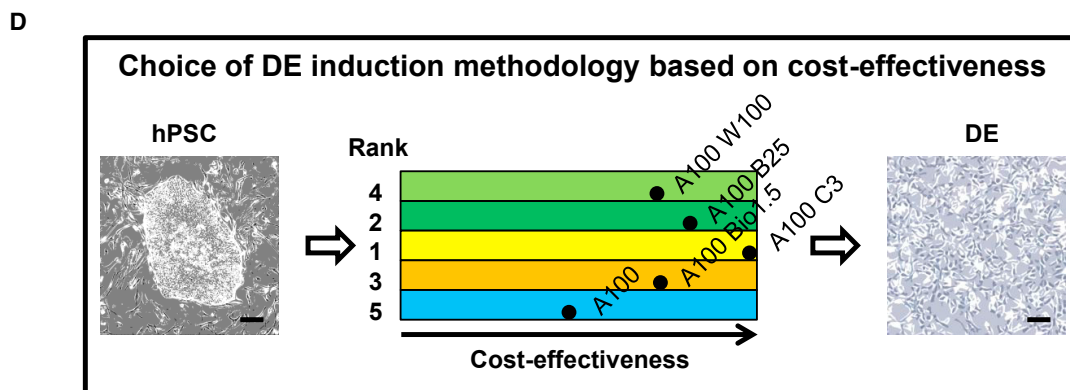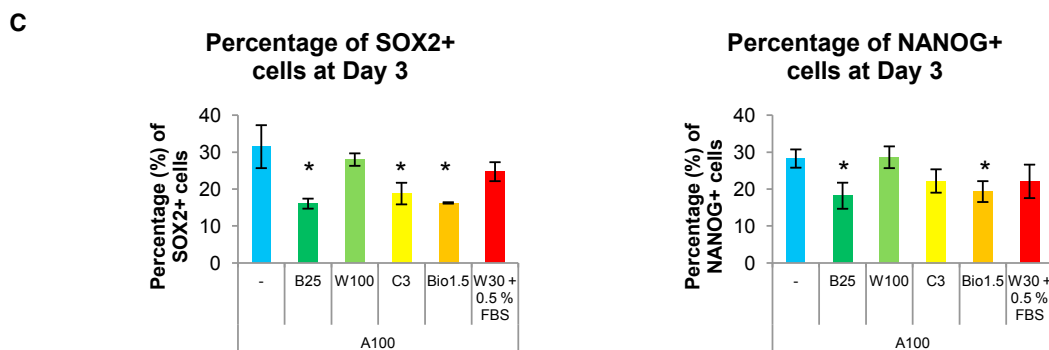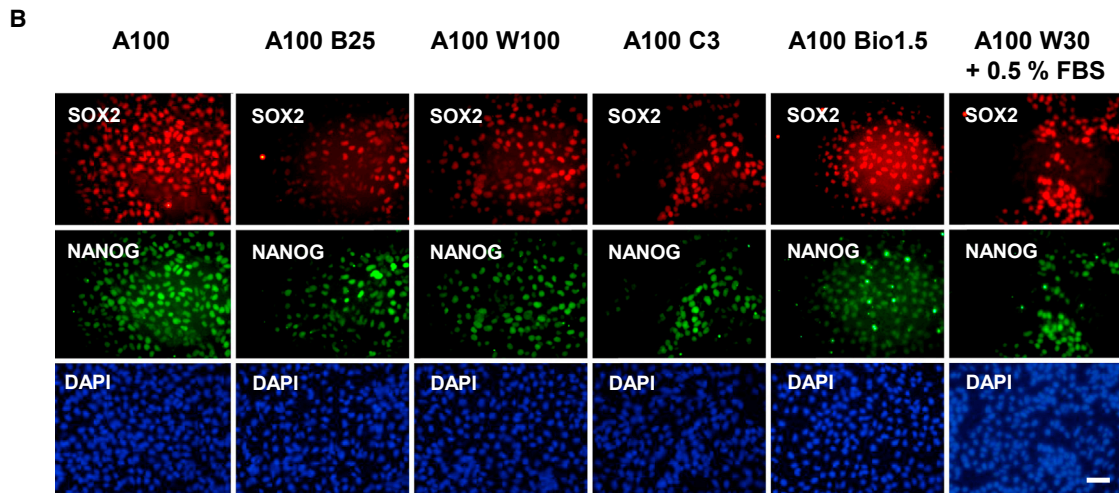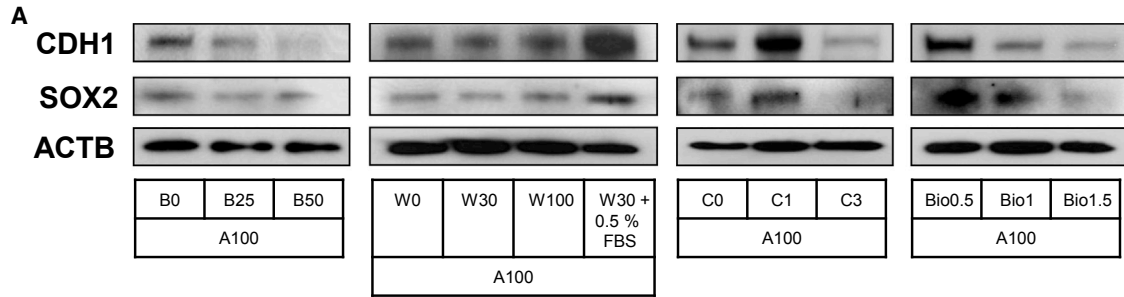
Although the use of GSK-3 inhibitors results in a downregulation of E-CADHERIN/CDH1 and pluripotency factors SOX2 and NANOG, and GSK-3 inhibition activates downstream Wnt signaling, the inhibition of GSK-3 could potentially affect other signaling pathways in which GSK-3 is active, such as cellular proliferation and migration (Doble

Figure 3. Activators of Wnt and BMP Signaling Can Enhance Activin-Induced DE Formation with Comparable Efficiencies
(A and B) Expression of mesendoderm (*EOMES*, *MIXL1*) and DE (*CXCR4*, *SOX17*, *FOXA2*) markers in hiPSCs differentiated for 3 days in the presence of (A) Activin (0, 25, 50, 100 ng/ml) and BMP4 (0, 25, 50 ng/ml) or (B) no growth factor (A0), 100 ng/ml Activin alone (A100), A100 plus either 25 ng/ml BMP4 (B25), 100 ng/ml WNT3A (W100), 3 μM CHIR99021 (C3), or 1.5 μM BIO (Bio1.5). Asterisk (*) indicates p < 0.05 compared to no growth factor (A0 B0, A0). All error bars represent SD of three biological replicates.
(C) The percentage of CXCR4+SOX17+ DE cells determined by FACS analyses in undifferentiated hiPSCs or after differentiation of hiPSCs for 3 days in the presence of A100 B25, A100 W100, A100 C3, A100 Bio1.5, or A100 W30 + 0.5% FBS. A, Activin; B, BMP4; W, WNT3A; C, CHIR99021; FBS, fetal bovine serum.
See also Figures S1–S4 and Table S1.

A

B

C

Percentage of SOX2+ cells at Day 3

Percentage of NANOG+ cells at Day 3

D

Choice of DE induction methodology based on cost-effectiveness

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and Woodgett, 2003). This is in contrast to the specific WNT3A-induced Wnt signaling. In our experiments, we also observed that WNT3A (up to 200 ng/ml) was not as effective as the GSK-3 inhibitors in elevating β-catenin transcript expression during DE differentiation (data not shown). This could imply that WNT3A-induced Wnt signaling events occurred solely at the protein level. Further, this suggests that GSK-3 inhibitors potentiate the increased presence of β-catenin, thereby strengthening Wnt signaling (besides inhibiting the GSK-3-containing destruction complex). Therefore, the inhibition of multifunctional GSK-3 coupled with the increased β-catenin expression could partly account for the differences observed between WNT3A-induced and GSK-3 inhibition-induced Wnt signaling. Specific to CHIR99021 (a potent and highly selective GSK-3 inhibitor as compared to BIO), the strong inductive effects on mesendodermal (*EOMES*, *MIXL1*, *BRACHYURY*) marker gene expression (Kubo et al., 2004; Tada et al., 2005) and a marginal increase in mesodermal marker *TBX6* indicate that excessive Wnt signaling via CHIR99021 (>3 μM) is likely to promote mesoderm while suppressing DE formation.

Taken together, CHIR99021 appears to be the preferred choice in combination with Activin (rank 1, Figure 4D) in generating DE cells based on its superior ability to induce mesendoderm as well as its cost effectiveness (~2 cents/ml of media). BMP4 would be favored if not for its ~20-fold greater cost (rank 2, Figure 4D), whereas BIO, although less expensive, exhibits cytotoxic effects (rank 3, Figure 4D) and WNT3A is both expensive and requires a high dose to be effective (rank 4, Figure 4D).

In summary, we demonstrate that both Wnt and BMP can cooperate with Activin signaling to induce DE with comparable efficiencies in a chemically defined serum-free medium. DE generated from both Wnt+Activin and BMP+Activin signaling can give rise to derivatives such as pancreatic progenitor cells (Kunisada et al., 2012; Teo et al., 2012). Thus, DE made via these two differing signaling pathways is conceptually consistent and physio-logically relevant. Future genome-wide comparisons could reveal interesting mechanistic insights specific to Wnt and BMP signaling that are relevant for DE specification.

EXPERIMENTAL PROCEDURES

Cell Culture

hiPSCs derived from AG16102 skin fibroblasts (Coriell Institute) or hESCs (CHB8) were cultured in conditions as described previously (Teo et al., 2013b). To initiate DE differentiation, hiPSCs were treated with Collagenase IV and Dispase at 1:1 ratio for 3–5 min before being manually passaged into small clumps and placed in a 70 μm cell strainer to deplete the feeders (Teo et al., 2012). hiPSCs were then differentiated 2 days later in RPMI 2% B27 (no vitamin A; serum-free chemically defined medium; Teo et al., 2012) supplemented with the appropriate growth factors and chemical compounds. Cells were harvested after 3 days of differentiation. To generate pancreatic progenitors, DE was further differentiated with 50 ng/ml Activin for 2 days, 50 ng/ml fibroblast growth factor-2 (FGF2), 3 μM all-*trans* retinoic acid (RA), and 10 mM nicotinamide (NIC) for 5 days; 50 ng/ml FGF2, 3 μM RA, 10 mM NIC, and 20 μM DAPT for 4 days; and 50 ng/ml FGF2, 10 mM NIC, and 20 μM DAPT for 3 days.

qRT-PCR, Immunostaining, Western Blot, and FACS Analyses

Methods for qRT-PCR, immunostaining, western blot, and FACS analyses have been described previously (Teo et al., 2012, 2013b). The percentage of DE and pluripotent cells was obtained by dividing the number of SOX17+, SOX2+, and NANOG+ cells over the total number of DAPI+ cells. The average of three separate images was used for cell counting analyses. A p value <0.05 indicates statistical significance by Student's t test. All error bars represent SD of three biological replicates in at least two independent experiments. Primers and antibodies are provided in Table S2.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.05.007.

Figure 4. BMP4, CHIR99021 and BIO Are More Effective at Suppressing E-CADHERIN and Pluripotency as Compared to WNT3A
(A) Western blot analyses for CDH1, SOX2, and ACTB protein levels in hiPSCs differentiated for 3 days in 100 ng/ml Activin A (A100) plus increasing doses of BMP4 (B), WNT3A (W), CHIR99021 (C), or BIO (Bio).
(B) Immunostaining for SOX2 and NANOG pluripotency factors on hiPSCs differentiated for 3 days in the presence of A100 or A100 plus either 25 ng/ml BMP4 (B25), 100 ng/ml WNT3A (W100), 3 μM CHIR99021 (C3), 1.5 μM BIO (Bio1.5), or 30 ng/ml WNT3A (W30) + 0.5% fetal bovine serum (FBS). Scale bar represents 50 μm.
(C) The percentage of SOX2+ and NANOG+ cells after differentiation of hiPSCs for 3 days in the presence of A100, A100 B25, A100 W100, A100 C3, A100 Bio1.5, or A100 W30 + 0.5% FBS. Asterisk (*) indicates p < 0.05 compared to A100. All error bars represent SD of three biological replicates.
(D) Summary of growth factor combination choice for DE induction based on cost effectiveness. A100 C3 (rank 1) is preferred followed by A100 B25 (rank 2) and then A100 Bio1.5 (rank 3) or A100 W100 (rank 4). A100 is least preferred (rank 5). For every 1 ml of media, CHIR99021 costs ~2 cents, BMP4 costs ~48 cents, BIO costs ~1 cent, and WNT3A costs ~$2.30. BIO is less preferred than BMP4 due to its cytotoxic effects. Scale bar represents 200 μm. A, Activin; B, BMP4; W, WNT3A; C, CHIR99021; FBS, fetal bovine serum; DE, definitive endoderm; and hPSC, human pluripotent stem cell. DAPI is the nuclear stain.

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