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Supplemental Information

WNT3A Promotes Hematopoietic or Mesenchymal Differentiation from hESCs Depending on the Time of Exposure

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Inventory of Supplemental Information

Supplemental data

The following Tables are provided as Excel files

Table S3. Average probe signals from the 51 probe sets differing more than 3 fold in day 4 WNT3A/BMP4 (WB) E^+G^+ vs. BMP4 (B) E^+G^+ EBs, related to Figure 3.

Table S4. Average probe signals of the 93 probe sets differing more than 3 fold in day 4 WNT3A/BMP4 (WB) E^-G^+ vs. BMP4 (B) E^-G^+ EBs, related to Figure 3.

Table S5. Average probe signals and fold change of the 689 probe sets upregulated ≥3-fold in both WNT3A and BIO mesospheres compared to undifferentiated hESCs, related to Figure 7.

Supplemental Experimental Procedures

SUPPLEMENTAL DATA

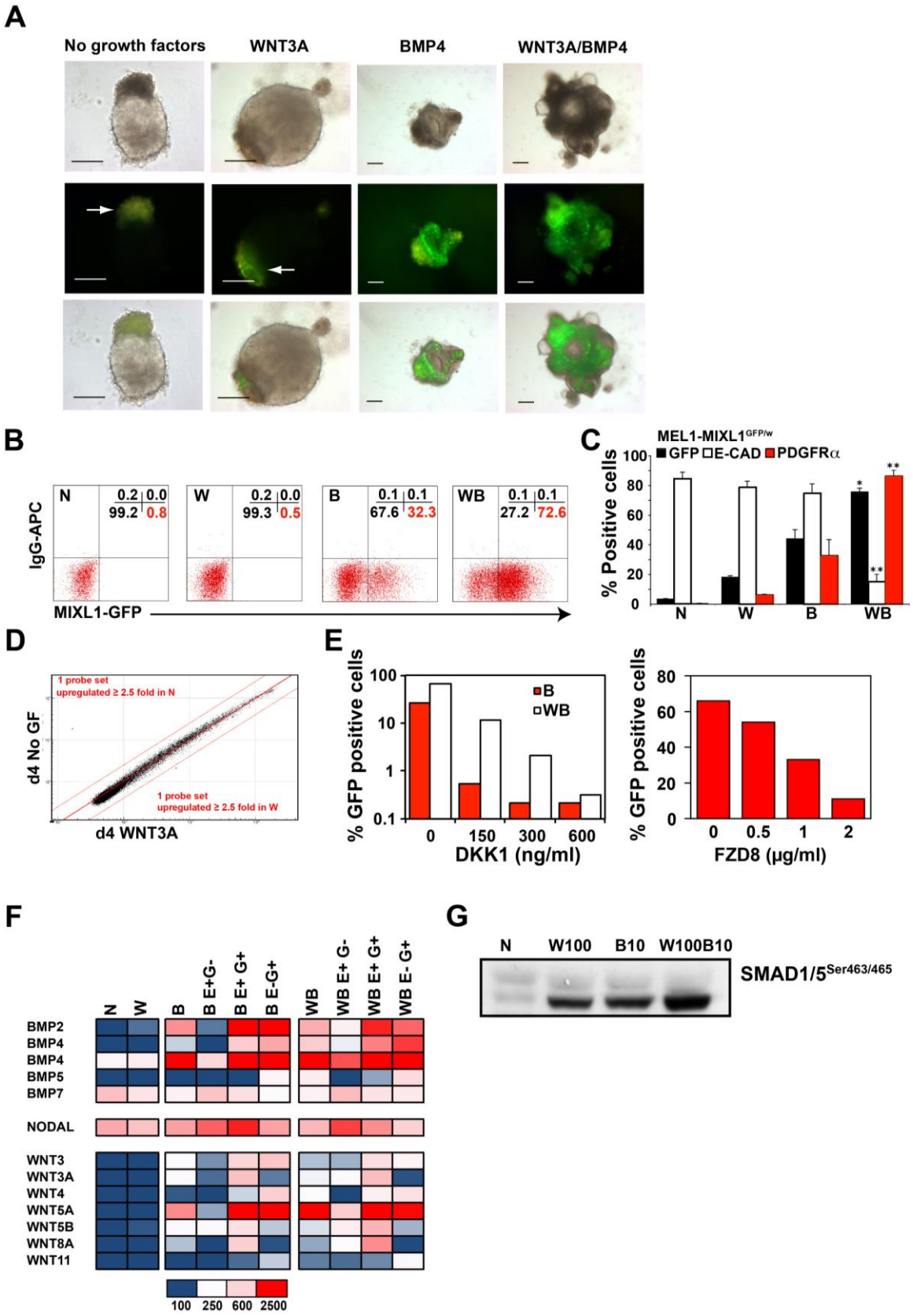

Figure S1. WNT3A synergises with BMP4 to induce mesoderm, related to Figure 1. (A) Examples of d4 EBs formed in the presence or absence of the indicated growth factors. Bright field, fluorescence and overlay images showing that WNT3A treated EBs were larger than those formed in no growth factors and that WNT3A/BMP4 treated EBs are larger than those formed in BMP4

alone. Significant yellow autofluorescence (not confirmed as GFP by flow cytometry) was observed in the no growth factor and the WNT3A stimulated EBs (arrows). Note different magnification for no growth factor and WNT3A EBs. Scalebars, 200 μm. (B) Flow cytometric analysis in which d4 EB cells were labelled with isotype control antibodies. These results were used to set the gates for analysis of E-CADHERIN and MIXL1-GFP expression shown in Figure 1B. EBs were formed in the factors indicated. Note that very little MIXL1-GFP expression was observed in EBs differentiated in WNT3A alone or in the absence of exogenous growth factors. N, No growth factors; W, 100ng/ml WNT3A; B, 10ng/ml BMP4; WB, WNT3A/BMP4. (C) Histogram representing the mean expression of MIXL1-GFP, E-CAD and PDGFRα assayed by flow cytometry at d4 in EBs formed using the MEL1-MIXL1$^{GFP/w}$ cell line in no growth factors (N), 40-100ng/ml WNT3A (W), 10-30ng/ml BMP4 (W) and 40-100ng WNT3A / 10-30ng/ml BMP4 (WB). Data represents the mean±SEM from 5 independent experiments. B and WB groups were compared using Student's t-test. Asterisks indicate $p<0.01$ (*) and $p<0.001$ (**). (D) Similarity of transcriptional profiles of d4 unsorted EBs differentiated in no growth factors or in WNT3A. (E) Histogram showing the percentage of MIXL1-GFP expressing cells in BMP4 treated cultures into which increasing concentrations of DKK1 or FZD8 FC were titrated. (F) Heat map showing mean signal intensity of BMPs, NODAL and WNTs in d4 EBs and their sorted fractions differentiated in the indicated growth factors. Note the similarity in patterns of expression between BMP4 and WNT3A/BMP4 analysed fractions. The scale in arbitrary units is shown. (G) Western blot of whole cell lysates examining the phosphorylation of SMAD1/5 in hESCs after 30 min treatment with the indicated growth factors. This is an independent repeat of the experiment shown in Figure 1F. Abbreviations: N, No growth factors; W, WNT3A; B, BMP4; WB, WNT3A/BMP4. The concentration of growth factors in ng/ml is indicated.

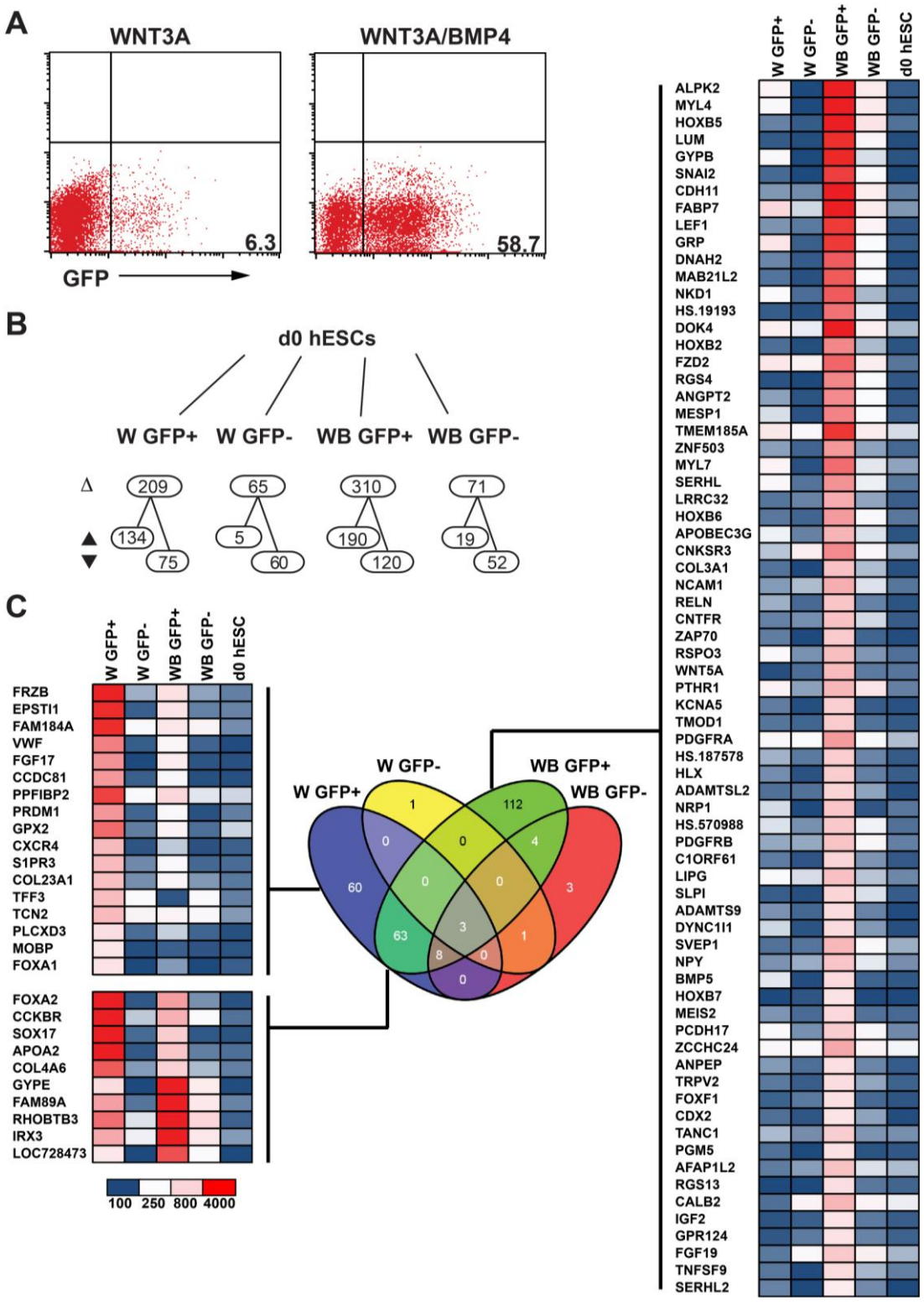

Figure S2. Comparison of GFP$^+$ cells from WNT3A and WNT3A/BMP4 differentiated EBs, related to Figure 1. (A) Flow cytometry profiles showing GFP$^+$ and GFP$^-$ fractions sorted from WNT3A (W) and WNT3A/BMP4 (WB) differentiated d4 EBs. (B) Number of genes in each fraction whose expression differed ≥ 5-fold from undifferentiated hESCs. (C) Venn diagram showing the numbers of upregulated genes in the analysed fractions and heat maps of selected genes from W GFP$^+$ and WB GFP$^+$ fractions. The heat maps represent the mean signal intensity for genes whose expression differed ≥ 5-fold from undifferentiated hESCs and ≥3-fold between W GFP$^+$ and WB GFP$^+$. See also Tables S1 and S2.

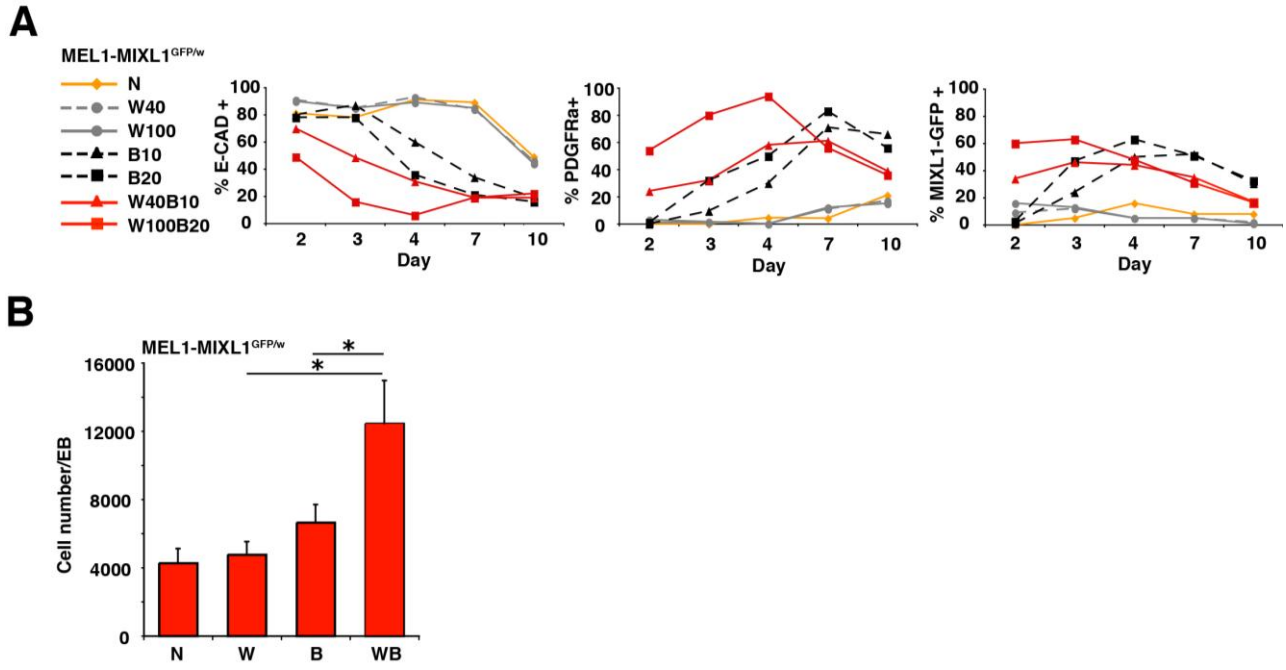

Figure S3. WNT3A accelerates BMP4 dependent mesoderm differentiation and enhances EB size, related to Figure 2. (A) Graphical representation of flow cytometry analysis from a representative time course experiment examining percentage of MIXL1-GFP-, E-CAD- and PDGFRα- expressing cells in EBs formed using the MEL1-MIXL1$^{GFP/w}$ cell line differentiated in APEL medium supplemented with the growth factors indicated. (B) Histogram representing the mean cell number per d4 EB formed using the MEL1-MIXL1$^{GFP/w}$ cell line in cultures induced with the growth factor combinations indicated. Data represents the mean±SEM from 5 independent experiments. B and WB groups were compared using Student's t-test. Asterisks indicate $p<0.05$ (*). Abbreviations: N, No growth factors; W, WNT3A; B, BMP4; WB, WNT3A/BMP4.

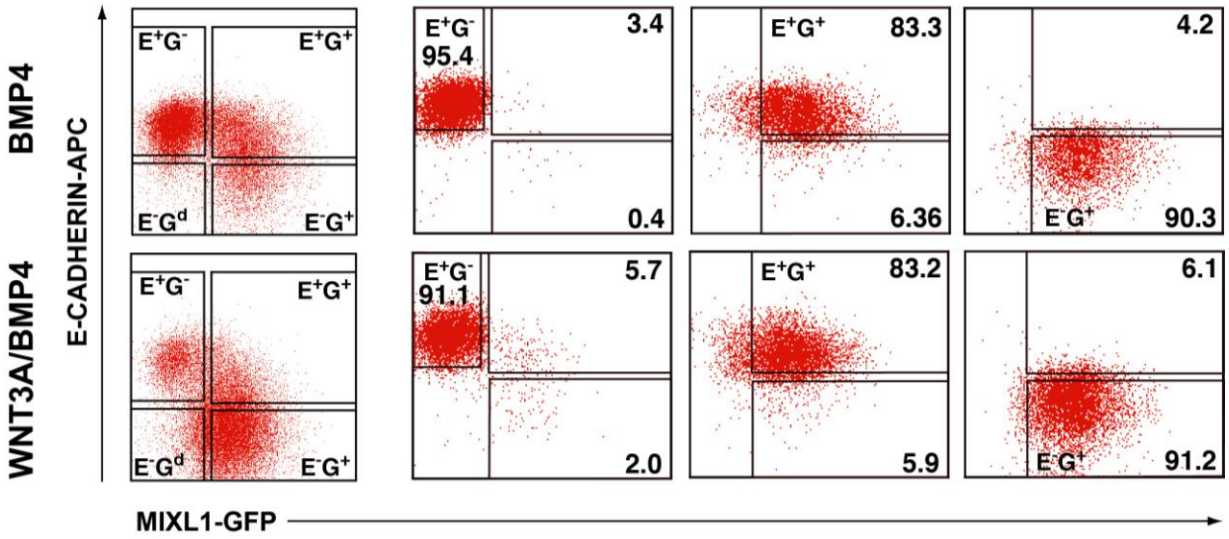

Figure S4. Reanalysis of flow sorted fractions from BMP4 and WNT3A/BMP4, related to Figure 3. Flow cytometric profiles documenting the reanalysis of sorted fractions from a representative experiment contributing to the data presented in Figures 3, 4 and S5. The $E^-G^{d\ (dim)}$ cell fraction was not reanalysed due to low cell numbers.

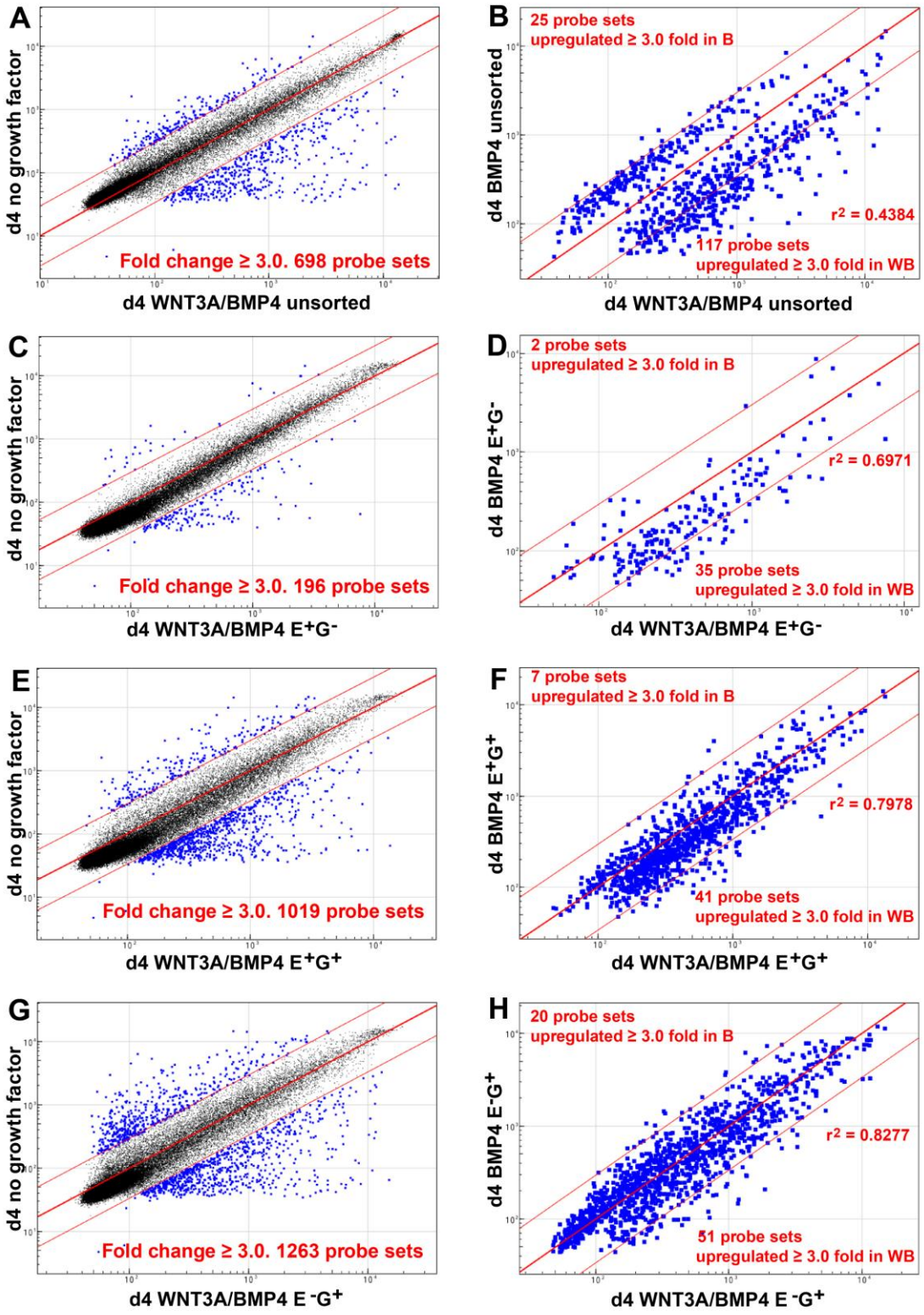

Figure S5. Similar transcriptional profiles of sorted cell populations from WNT3A/BMP4 and BMP4 differentiated EBs, related to Figure 3. (A, C, E, G) Scatter-plot comparison of transcriptional profiles of d4 EBs cultured in WNT3A/BMP4 and d4 EBs cultured in the absence of growth factors. A comparison between unsorted populations is shown in panel A, whilst data using sorted fractions is shown in panels C, E and G. Enhanced dots outside the parallel red lines indicate probe sets differing by ≥ 3-fold from the mean. (B, D, F, H) The levels of transcription of the probe sets enriched in WNT3A/BMP4 EBs (identified in panels A, C, E and F) were compared to the transcript levels for the same probe sets in equivalent sorted fractions from d4 BMP4 EBs. These

data show that i) the d4 WNT3A/BMP4 E^+G^- fraction, which is most ectodermal in character, displayed the greatest similarity to d4EBs differentiated in the absence of growth factors (panel C), ii) probe sets enriched in sorted fractions of WNT3A/BMP4 cultures were expressed at similar levels in the corresponding fractions in BMP4 induced EBs (panels D, F and H), highlighting the similarity of the cells in each fraction under BMP4 or WNT3A/BMP4 conditions. Differences in the relative size of each population explain the weaker correlation between the transcriptional profiles in unsorted populations of BMP4 and WNT3A/BMP4 cultures (panel B).

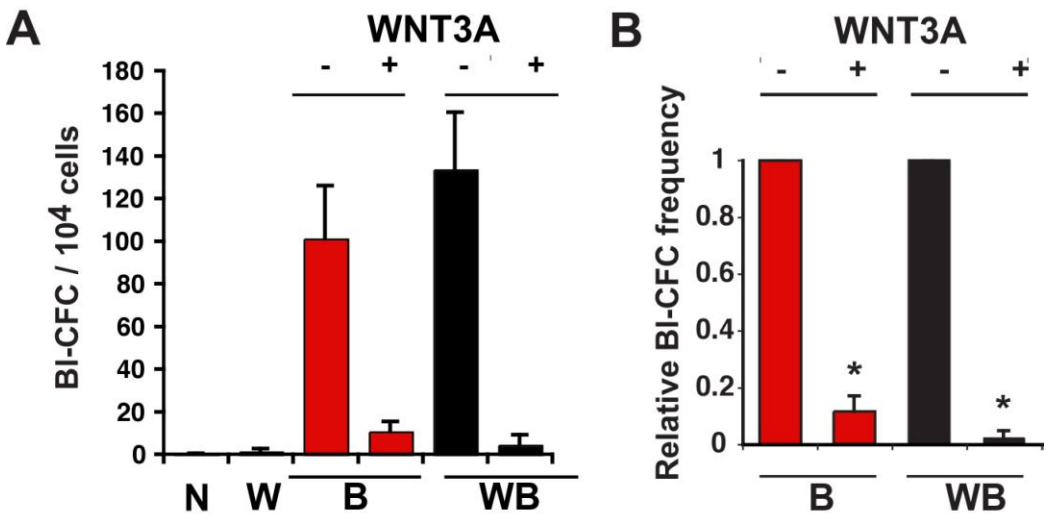

Figure S6. WNT3A addition to methylcellulose cultures suppresses hematopoiesis from d4 EBs, related to Figure 6. (A) Frequency of hematopoietic Bl-CFCs in methylcellulose cultures containing hematopoietic growth factors with (+) or without (-) additional WNT3A added to the methylcellulose. Cultures were seeded with cells from MEL-MIXL1$^{GFP/w}$ hESC derived EBs that had been initially differentiated for 4 days without added growth factors (N), or in the presence of WNT3A (W), BMP4 (B) or WNT3A/BMP4 (WB). (B) Colony frequency was normalised to methylcellulose cultures supplemented with hematopoietic growth factors alone, demonstrating the suppressive effect of WNT3A. Data in A and B represents the mean±SEM of 4 independent experiments. WNT3A (+) and (-) groups (panel B) were compared using Student's t-test. Asterisks indicate $p<0.001$ (*).

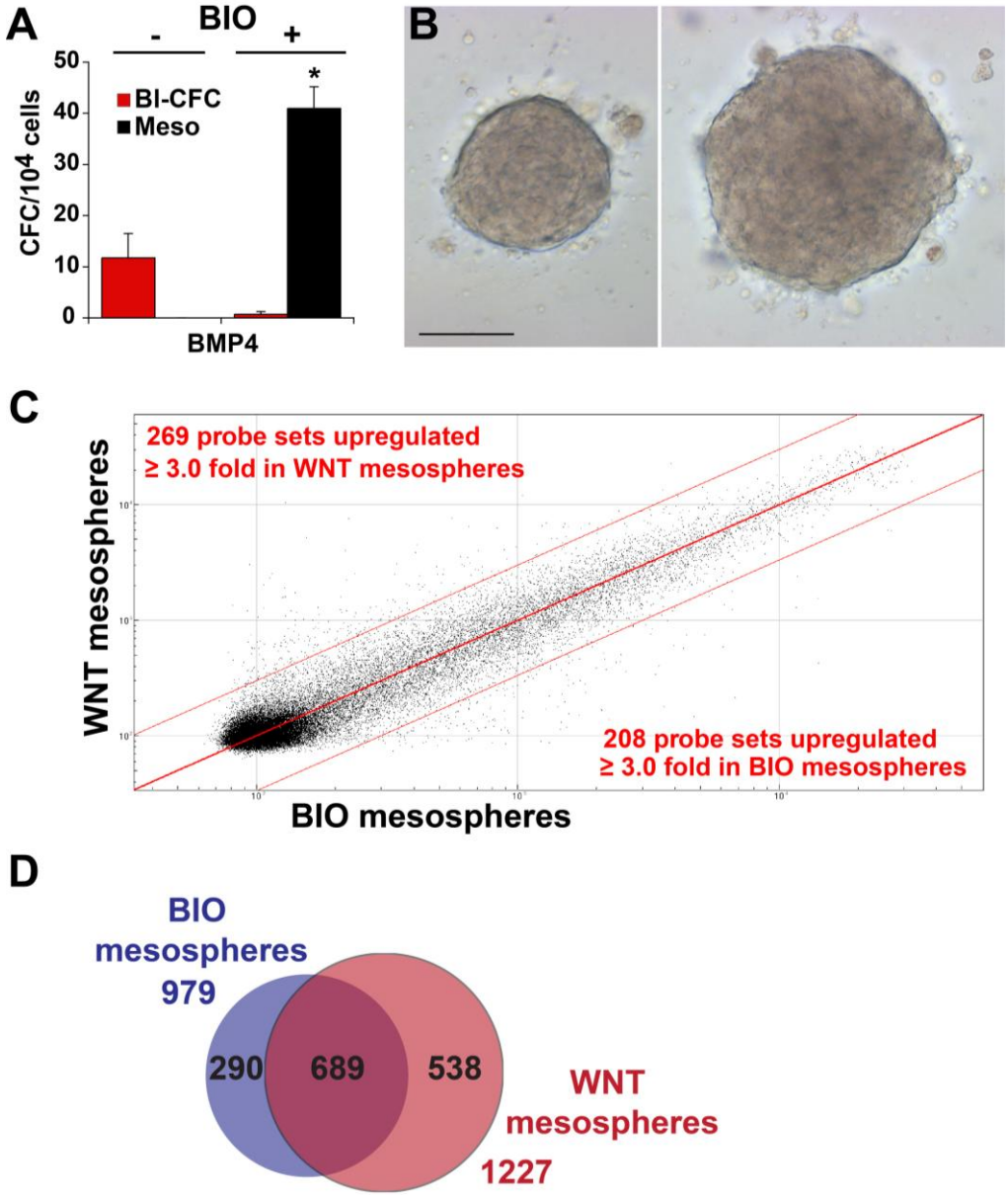

Figure S7. BIO addition to methylcellulose cultures suppresses hematopoiesis and promotes mesospheres, related to Figure 6. (A) Frequency of hematopoietic blast colonies (Bl-CFC) and non hematopoietic mesodermal colonies (Meso) from MEL-MIXL1$^{GFP/w}$ EBs grown in the presence of BMP4 (B) for 4 days that were subsequently disaggregated and cultured in methylcellulose containing hematopoietic growth factors with (+) or without (-) 5□M BIO addition to the methylcellulose. The low frequency of Bl-CFCs reflects the low concentration of BMP4 (2-10 ng/ml) used in these experiments. Data represents the mean ± SEM of 4 independent experiments. The generation of Mesospheres was statistically different between the treatment groups using Student's t-test (*, $p<0.05$). (B) Morphology of mesospheres, showing two examples, formed in methylcellulose cultures supplemented with BIO. Scalebar, 100μM. (C) Scatter-plot comparison of transcriptional profiles of the mesodermal colonies forming in methylcellulose cultures supplemented with either WNT3A (WNT mesospheres) or BIO (BIO mesospheres) in addition to hematopoietic growth factors. The numbers of probe sets differing by ≥ 3-fold from the mean are indicated. (D) Venn diagram demonstrating the similarity between probe sets differentially expressed in WNT and BIO generated mesospheres. Approximately 70% of probe sets upregulated in BIO mesospheres were also upregulated in WNT mesospheres. The probe sets in common between BIO and WNT mesospheres are listed in Table S5.

EXCEL FILES OF MICROARRAY DATA TABLES

Table S1. Average probe signals in 224 probe sets differing >5.0 fold in WNT3A (W) GFP+ vs d0 hESC, related to Figure 1.

Table S2. Average probe signals in 346 probe sets differing >5.0 fold in WNT3A/BMP4 (WB) GFP+ vs d0 hESC, related to Figure 1.

Table S3. Average probe signals from the 51 probe sets differing more than 3 fold in day 4 WNT3A/BMP4 (WB) $E^{+}G^{+}$ vs. BMP4 (B) $E^{+}G^{+}$ EBs, related to Figure 3.

Table S4. Average probe signals of the 93 probe sets differing more than 3 fold in day 4 WNT3A/BMP4 (WB) $E^{-}G^{+}$ vs. BMP4 (B) $E^{-}G^{+}$ EBs, related to Figure 3.

Table S5. Average probe signals and fold change of the 689 probe sets upregulated ≥3-fold in both WNT3A and BIO mesospheres compared to undifferentiated hESCs, related to Figure 7.

Supplemental Experimental Procedures

Cell culture, differentiation and flow cytometric analysis:
Growth factors and inhibitors used during embryoid body formation, hematopoietic differentiation and methylcellulose colony forming assays. Unless otherwise indicated, all growth factors were recombinant human proteins.

Application	Growth factor/ Inhibitor	Concentration	Supplier
EB formation	Wingless (WNT)3A (mouse)	40-100ng/ml	Millipore Corporation
	Bone Morphogenetic Protein (BMP)4	10-30ng/ml	PeproTech
	NOGGIN	300ng/ml	R & D Systems
	SB431542	4 μM	Sigma-Aldrich
	SB203580	10 μM	Sigma-Aldrich
	U0126	10 μM	Sigma-Aldrich
	FZD8 FC	0.5 - 2 μg/ml	R & D Systems
	DKK1	0.15 - 1 μg/ml	R & D Systems
	BIO	5 μM	Calbiochem
Hematopoietic differentiation cultures	BMP4	40ng/ml	PeproTech
	Stem cell factor (SCF)	50ng/ml	PeproTech
	Vascular endothelial growth factor) VEGF	30ng/ml	PeproTech
	Interleukin (IL)3	30ng/ml	PeproTech
	Erythropoietin (EPO)	3U/ml	PeproTech
Methylcellulose colony forming assays	SCF	100ng/ml	PeproTech
	VEGF	50ng/ml	PeproTech
	IL3	30ng/ml	PeproTech
	Interleukin (IL)6	30ng/ml	PeproTech
	Thrombopoietin (TPO)	30ng/ml	PeproTech
	Erythropoietin (EPO)	3U/ml	PeproTech
	FLT3 Ligand (FLT3L)	30ng/ml	PeproTech

Antibodies used for flow cytometry analysis and western blotting.

Antibody	Manufacturer and cat # or clone name
mouse anti-human E-CADHERIN	Zymed, cat #13-1700
mouse anti-human PDGFRα	BD Biosciences, cat #556001
mouse anti-human CD31	BD Biosciences, cat #555444
PE-conjugated mouse anti-human CD34	BD Biosciences, cat #348057
APC-conjugated mouse anti-human CD45	BD Biosciences, cat# 555483
PE-conjugated rat anti-human CXCR4	BD Biosciences cat#555974
PE-, APC-conjugated goat anti-mouse IgG	BD Biosciences, cat #550589 and #550826
PE-, APC-conjugated mouse IgG	BD Biosciences, cat# 555751 and #555749
PE-conjugated rat IgG	BD Biosciences, cat#555844
Mouse IgG	BD Biosciences, cat#557273 and # 554126
SMAD1/5 (Ser463/465)	Cell Signaling, 41D10
SMAD4	Santa Cruz, B8
β-ACTIN	MP Biomedicals, C4