Stem cell research

Potent activation of the Wnt pathway by a Wnt surrogate-Fc fusion protein

Introduction

The promise of stem cells as a tool to understand human development and disease progression and as a basis for cell therapy applications is predicated on the ability to direct stem cell differentiation into specific cell types [1,2]. Protocols for the *in vitro* differentiation of stem cells into a wide range of specialized cell types have been devised, and nearly all methods focus on manipulating cell signaling pathways using small molecules, recombinant growth factors, or both [1,2].

Wnt signaling is a prominent stem cell pathway that is involved in the differentiation of many cell types, including cardiomyocytes [3] and intestinal epithelial organoids [4]. Secreted Wnt growth factors signal through the family of Frizzled (FZD) receptors and LRP5/6 co-receptors to regulate gene expression and diverse cellular behaviors [5]. However, endogenous Wnt proteins are lipidated and hydrophobic, which makes the production of soluble recombinant Wnt ligands very challenging and typically results in poor bioactivity [6,7]. The Gibco™ Wnt surrogate-Fc fusion protein offers a novel soluble solution to this problem. The Wnt surrogate-Fc fusion protein (aka Wnt surrogate) is engineered to bridge the FZD and LRP5/6 receptors to activate Wnt signaling. Here we demonstrate the high potency of the Wnt surrogate in Wnt reporter assays and demonstrate its ability to induce the expression of direct Wnt target genes in human induced pluripotent stem cells (iPSCs). These results demonstrate that the Wnt surrogate performs comparably to common Wnt agonists such as human or mouse recombinant Wnt3a, Wnt-conditioned medium, and the GSK3β inhibitor CHIR99021, but at significantly lower concentrations. Furthermore, using next-generation sequencing we demonstrate that this mimetic protein closely recapitulates the recombinant human Wnt3a–dependent transcriptome in iPSCs, suggesting that the Wnt surrogate may be a useful alternative to recombinant Wnt ligands.

Results

Composition of Wnt surrogate

The Wnt surrogate-Fc fusion protein is an engineered Fc fusion protein composed of the LRP-binding domain of the extracellular Wnt modulator Dickkopf (DKK), linked to a designed ankyrin repeat protein (DARPin) that was chosen as a selective, high-affinity binder of FZD receptors [8]. More specifically, this engineered Wnt mimetic interacts with FZD1, FZD2, FZD5, FZD7, and FZD8 [8,9]. In this way, the Wnt surrogate can bridge FZD and LRP5/6 receptors to activate Wnt signaling (Figure 1).

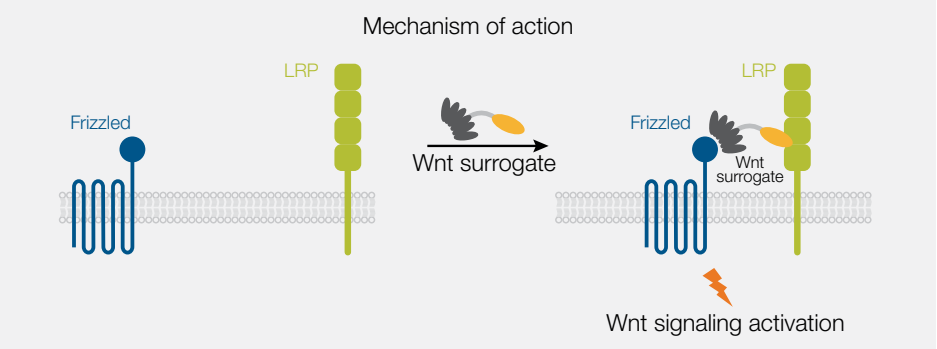
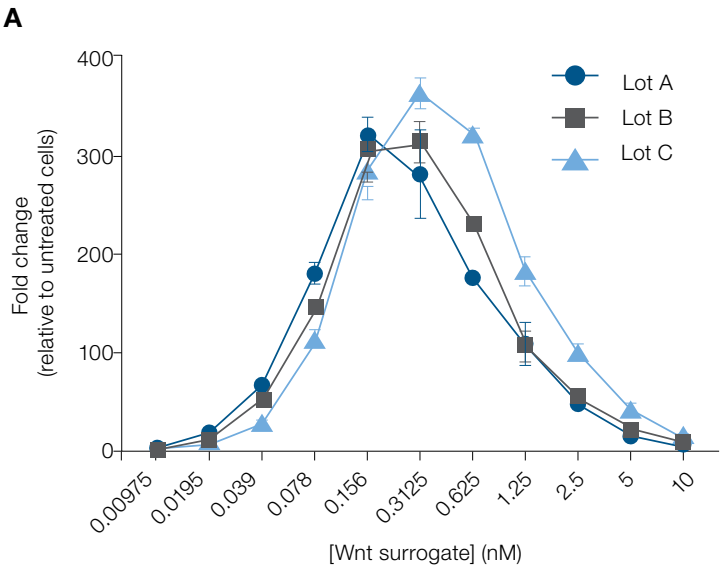


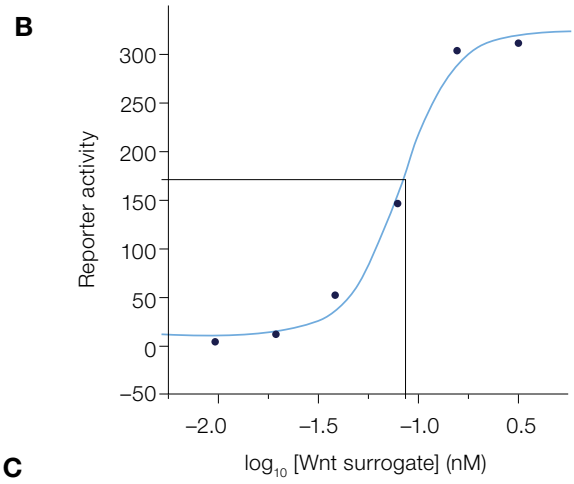
Figure 1. Mechanism by which the Wnt surrogate-Fc fusion protein activates the Wnt pathway. The Wnt surrogate-Fc fusion protein is composed of the LRP-binding domain of the extracellular Wnt modulator Dickkopf (DKK; orange) linked to a designed ankyrin repeat protein (DARPin; gray) that binds FZD receptors to activate the Wnt pathway.

Wnt surrogate potently activates a Wnt reporter assay

The Topflash assay is a well-established method for characterizing the activity of Wnt pathway modulators [10]. It is composed of Wnt-responsive DNA elements that control the expression of a reporter (i.e., luciferase) that can be measured to infer Wnt activity. We cultured a stable 293T cell line containing a Wnt luciferase reporter in DMEM basal medium supplemented with 20% fetal bovine serum (FBS) and antibiotics. To characterize the activity of the Wnt surrogate-Fc fusion protein, the 293T reporter cells were seeded into clear-bottom, white-walled 96-well plates and allowed to attach overnight. Cells were then treated with varying concentrations of the Wnt surrogate for 18–20 hr, followed by luciferase assays. Figure 2A shows that the Wnt surrogate had a dose-dependent effect across the picomolar (pM) to nanomolar (nM) range. At concentrations of ~0.156–0.625 nM, the Wnt surrogate-Fc fusion protein was a strong agonist, though this activation potential decreased at higher concentrations. This drop-off in activity at high doses may be specific to this reporter assay, because Wnt target genes are activated by higher concentrations of the Wnt surrogate in assays described later. The activity profile was consistent across multiple manufactured lots of the Wnt surrogate (Figure 2A). We further analyzed the 0–0.625 nM activity range to determine the half-maximal effective concentration (EC_{50}) range across 3 manufactured lots. As shown in Figure 2B and 2C, the EC_{50} range of the Wnt surrogate was 0.068–0.106 nM in this assay.

Next, we found that the Wnt surrogate activates Wnt signaling at lower concentrations compared to commonly used Wnt agonists, Wnt3a-conditioned medium and recombinant Wnt3a. Wnt3a conditioned medium was prepared using a stable cell line that secretes Wnt3a protein into the growth medium. This conditioned medium was blended with the growth medium for the luciferase reporter cell line at different volume/volume (v/v) ratios, mimicking the approach often reported in the literature [4]. In this comparison, the Wnt surrogate at ~100–5,000 pM activated the reporter more strongly than 6.25–50% v/v Wnt3a-conditioned medium (Figure 3A), thus offering a potent means of stimulating this pathway without the need for serum-containing conditioned medium. Likewise, the Wnt surrogate strongly activated the Wnt reporter cell line at picomolar concentrations, whereas recombinant mouse or human Wnt3a required nano- to micromolar concentrations to achieve similar levels of reporter activation (Figure 3B). Together, these data suggest the Wnt surrogate is a potent, defined, and cost-effective Wnt pathway agonist.





C

Sample	EC_{50} (log_{10} [Wnt surrogate] (nM))	Concentration (nM)
Lot A	–1.17	0.068
Lot B	–1.08	0.082
Lot C	–0.97	0.106

Figure 2. The Wnt surrogate activates Wnt signaling with low lot-to-lot variability. (A) Dose-dependent response of the Wnt reporter to the Wnt surrogate demonstrates strong and consistent activation in the sub-nanomolar concentration range. **(B, C)** EC_{50} analysis of the 0–0.625 nM Wnt surrogate activity range indicated a half-maximal response between 0.068 and 0.106 nM in the Topflash assay. The table shows the average of 4 experiments for each lot.

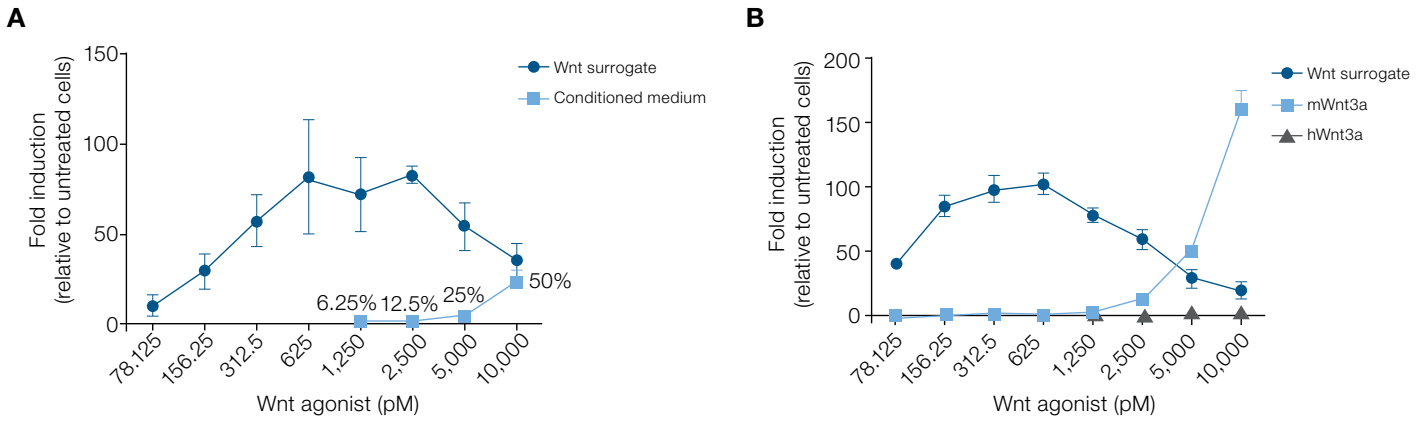


Figure 3. The Wnt surrogate strongly activates Wnt signaling at lower concentrations than conditioned medium and recombinant Wnt3a. **(A)** The Wnt surrogate induced higher levels of reporter activation than the Wnt3a-conditioned medium. Note that the actual concentration of Wnt3a in conditioned medium is unknown and varies from batch to batch. The activity profile of conditioned medium is overlaid on the Wnt surrogate's dose-response curve to visually compare the levels of reporter activation for these two Wnt agonists. **(B)** The Wnt surrogate strongly activates the Wnt reporter at lower concentrations than do the recombinant mouse and human Wnt3a (mWnt3a, hWnt3a, respectively). Each experiment was performed twice with four technical replicates at each concentration; representative data are shown here.

Activity of Wnt surrogate in human iPSCs

Wnt agonists are commonly used to direct the differentiation of pluripotent stem cells into mesoderm and other cell lineages. Given that the Wnt surrogate functions through a subset of Frizzled receptors, we first confirmed gene expression patterns of the Frizzled (FZD) family in undifferentiated human iPSCs. Similar FZD family expression profiles were measured in 2 different PSC culture media systems (Figure 4A). Robust expression of Wnt surrogate–specific FZD receptors (gray arrows) was observed, indicating these cells should be responsive to the Wnt surrogate. To analyze the Wnt surrogate's activation potential for Wnt target genes, we treated undifferentiated Gibco™ human iPSCs with the Wnt surrogate at a range of concentrations and then assayed several known Wnt target genes by qPCR using Applied Biosystems™ TaqMan® Assays. The Wnt surrogate potently induced the expression of the Wnt target genes and early mesoderm markers [11], *SP5* and *T* (aka *BRACHYURY*), in a dose-dependent manner (Figure 4B, C). Interestingly, the bell-shaped response observed in the Topflash assay (Figure 2A) was much less pronounced and exhibited a wider range of increasing dose-dependent Wnt activation when measuring endogenous target gene expression.

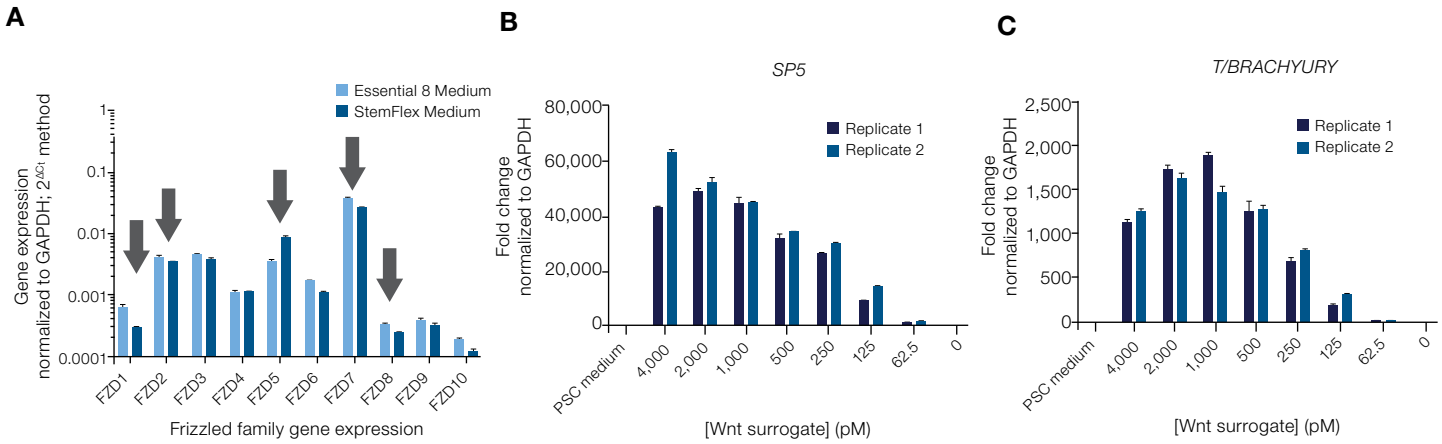


Figure 4. Wnt surrogate activates endogenous Wnt target genes in iPSCs to promote early germ layer differentiation. **(A)** iPSCs maintained in a variety of PSC culture media systems (Gibco™ Essential 8™ Medium and Gibco™ StemFlex™ Medium) showed consistent expression of all 10 Frizzled family members by qPCR using TaqMan Assays. Gray arrows indicate the detected levels of Wnt surrogate–specific FZD family genes. Relative expression was determined with the $2^{\Delta Ct}$ method. **(B, C)** iPSCs were exposed to Wnt surrogate diluted in DMEM/F-12 for 24 hr. Replicates 1 and 2 indicate independent experimental repeats. Relative fold activation was calculated using the $2^{-\Delta\Delta Ct}$ method relative to the untreated condition (i.e., 0 pM = DMEM/F-12 only).

Wnt surrogate recapitulates the Wnt3a global transcriptional response

As shown previously, the Wnt surrogate strongly activated endogenous Wnt target genes. However, given the composite nature of this mimetic protein (Figure 1), we next sought to characterize the Wnt surrogate–dependent transcriptome to determine any discordance with commonly used Wnt pathway agonists on a much broader scale. To this end, we first compared the activity of the Wnt surrogate with two commonly used recombinant Wnt ligands, human and mouse Wnt3a, and the small-molecule GSK3β inhibitor CHIR99021 (Figure 5). Human iPSCs were exposed to Wnt pathway agonists for ~22–24 hr, and the expression of *T* and *SP5* were assayed by qPCR using TaqMan Assays. These results demonstrated that 100–500 pM of the Wnt surrogate performed similarly to 2.4–2.5 nM (i.e., ~100 ng/mL) recombinant human or mouse Wnt3a, while the CHIR99021 chemical inhibitor exhibited the strongest activity, albeit at 3 µM; these are commonly published concentrations for these reagents. Together, these data indicate the potential to utilize the Wnt surrogate to activate key Wnt target genes in place of other Wnt pathway agonists or small molecules.

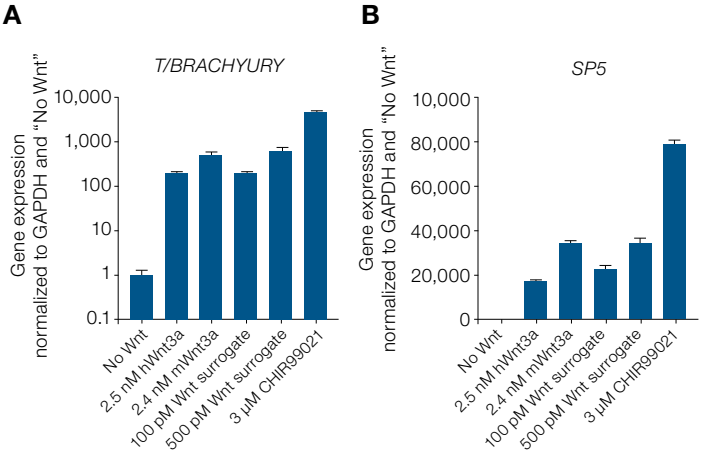


Figure 5. The Wnt surrogate has higher potency for Wnt target gene activation than recombinant Wnt3a protein. Human iPSCs were treated for ~24 hr with recombinant human (h) and mouse (m) Wnt3a, Wnt surrogate, or CHIR99021 diluted in DMEM/F-12. N = 3, representative experiment shown here.

We then extended this analysis using RNA sequencing (RNA-Seq) to characterize the Wnt surrogate and recombinant human Wnt3a global transcriptional responses in order to gain a broader understanding of their effect on cells. Human iPSCs were treated with 500 pM Wnt surrogate or 2.5 nM hWnt3a to generate their respective global transcriptomes. Linear regression analysis determined these datasets to be highly similar, with $R^2 = 0.9943$ (Figure 6A). Parsing these transcriptomes to identify all differentially expressed genes ($P < 0.05$, >2-fold gene expression change relative to untreated cells) led to the detection of 887 (Wnt surrogate) and 739 (hWnt3a) differentially expressed genes, highlighting the utility of conducting this type of broad transcriptional analysis. The 25 most highly activated genes after treatment with the Wnt surrogate include many common Wnt targets, such as *T* and *SP5*, whose levels were comparably activated by hWnt3a (Figure 6B). These data strongly suggest both Wnt agonists have similar activity profiles.

To give context to the role of the genes affected by the Wnt surrogate, we used gene ontology (GO) analysis; this is a means of analyzing large gene expression datasets to determine if well-known functions, such as cell signaling pathways or biological processes, are strongly enriched in a given dataset. GO analysis works by comparing a gene dataset, such as a list of differentially expressed genes, with lists of genes that are previously defined by functional attributes. GO pathway analysis of datasets of Wnt agonist–affected genes identified by our RNA sequencing experiments confirmed that the Wnt surrogate and recombinant human Wnt3a were strongly associated with the same signaling pathways. Figure 6C shows that the 5 pathways modulated by the Wnt surrogate with the most statistical significance were the same as those modulated by hWnt3a. Importantly, overrepresented genes modulated by the Wnt surrogate and hWnt3a were most strongly correlated with the Wnt signaling pathway (Figure 6C). Since the Wnt surrogate and hWnt3a act on similar genes and cell signaling pathways, these data suggest that the Wnt surrogate could be used to replace hWnt3a in existing protocols, potentially with some optimization of the concentration around 500 pM.

In addition to the Wnt surrogate and hWnt3a, we also identified the transcriptional response with recombinant mWnt3a and CHIR99021. We compared all 4 transcriptome datasets using a threshold criterion of $\log_2(\text{rpm}+1)$ and >2.5-fold change in gene expression levels across all samples, which identified 4,744 highly expressed genes. Hierarchical cluster analysis of the global transcriptional response was used to identify the relationships between these datasets. Heat map visualization of these gene expression patterns shows a remarkable amount of similarity between all datasets (Figure 7). The dendrogram further delineates the relationship between these datasets: the untreated and StemFlex Medium–cultured cells (clade 1) segregated from all the Wnt agonist–treated samples as the principal separation in this analysis; further, all 3 recombinant Wnt ligands clustered together (clade 2), distinct from the CHIR99021-treated cells (clade 3) (Figure 7). Importantly, the Wnt surrogate, hWnt3a, and mWnt3a experimental replicates were mixed further, suggesting a high degree of similarity between these gene expression profiles.

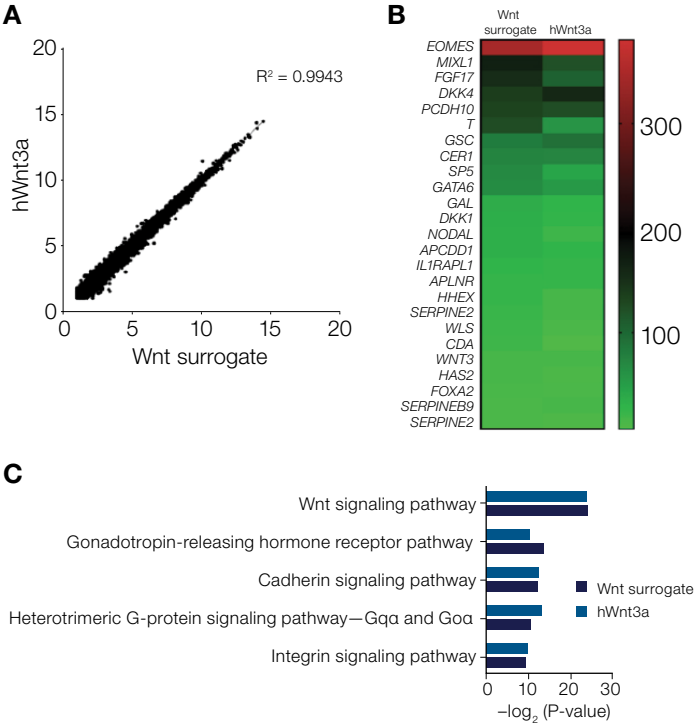


Figure 6. The Wnt surrogate and hWnt3a modulate the same gene pathways in human iPSCs. (A) Linear regression analysis of the global transcriptomes of cells treated with Wnt surrogate and hWnt3a (log_2 (rpm+1)). **(B)** Heat map of the 25 genes most significantly upregulated by Wnt surrogate and the relative fold change of these genes in the hWnt3a-upregulated gene set. Color scale bar is fold change relative to untreated cells. This represents the average of 3 independent sequencing experiments. **(C)** Gene ontology pathway analysis demonstrates that the native human Wnt3a sequence and the engineered Wnt surrogate have highly similarly activity profiles.

Lastly, principal component analysis (PCA) of these datasets using complete transcriptome datasets reinforces the hierarchical clustering patterns. PCA plots are used to simplify the comparison of multidimensional datasets with many variables, in this case genes. This statistical approach allows us to compare large datasets and illustrate the variance between them along the PCA plot axes. Initial analysis included RNA-Seq data from untreated A549 cells as a control to demonstrate the ability of this approach to distinguish unrelated datasets (Figure 8A). As expected, all RNA-Seq datasets derived from Gibco human iPSCs were easily distinguished from A549 cells along the PCA1 axis. To further visualize differences among the iPSC-derived datasets, we removed the A549 samples from the analysis, which subsequently recapitulated the cluster patterns seen in the hierarchical clustering analysis (Figure 7). This further demonstrated that the transcriptional responses to the Wnt surrogate and recombinant human and mouse Wnt3a were concordant and more distinguishable from untreated, StemFlex Medium, and CHIR99021-treated samples than from each other (Figure 8B). It should be noted that CHIR99021 is a GSK3β inhibitor, and GSK3β is a highly promiscuous kinase with more than 120 targets; thus, although the CHIR99021 chemical is a potent Wnt pathway activator, it is likely nonspecific [12], which may account for its distinct transcriptional profile compared to those of other Wnt agonists.

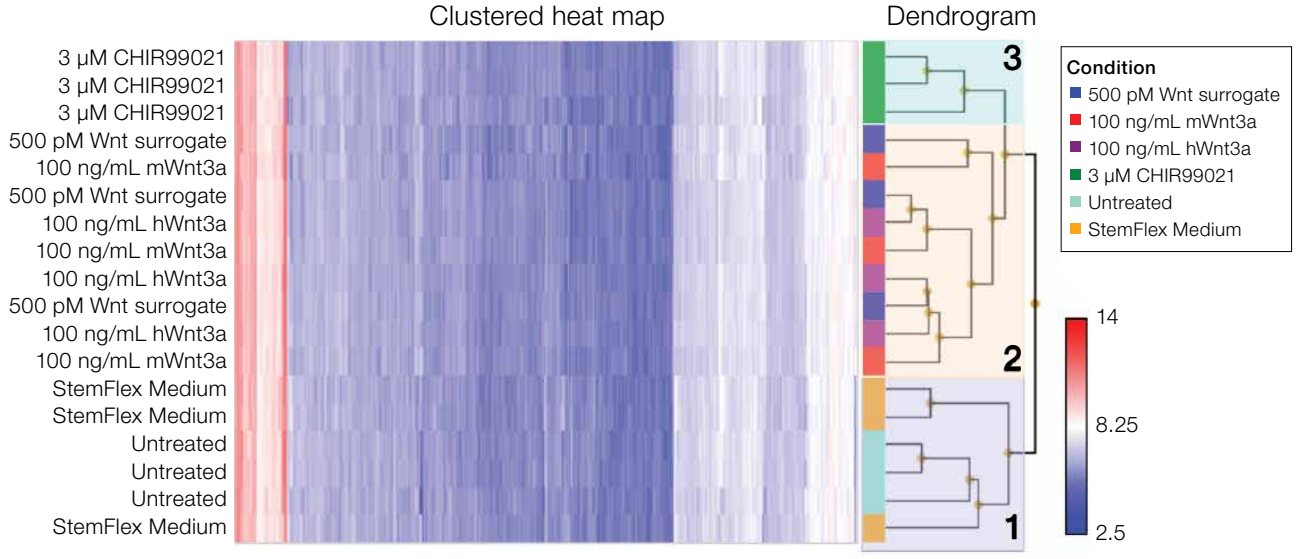


Figure 7. The Wnt surrogate transcriptome is highly similar to Wnt3a-regulated transcriptomes. Untreated cells were cultured in DMEM/F-12 in the absence of Wnt agonist; StemFlex Medium is the PSC culture medium. All conditions were assayed 3 times as indicated by the labels on the left of the heat map. The heat map contains 4,744 genes with log_2 (rpm+1) and >2.5-fold change in gene expression threshold; rpm = reads per million. Red–blue color scale bar represents fold change. Clades 1–3 of the dendrogram highlight the 3 clusters of most similar treatments. This figure was generated with Applied Biosystems™ Transcriptome Analysis Console (TAC) Software, which is limited to ≤5,000 genes for hierarchical cluster analysis.

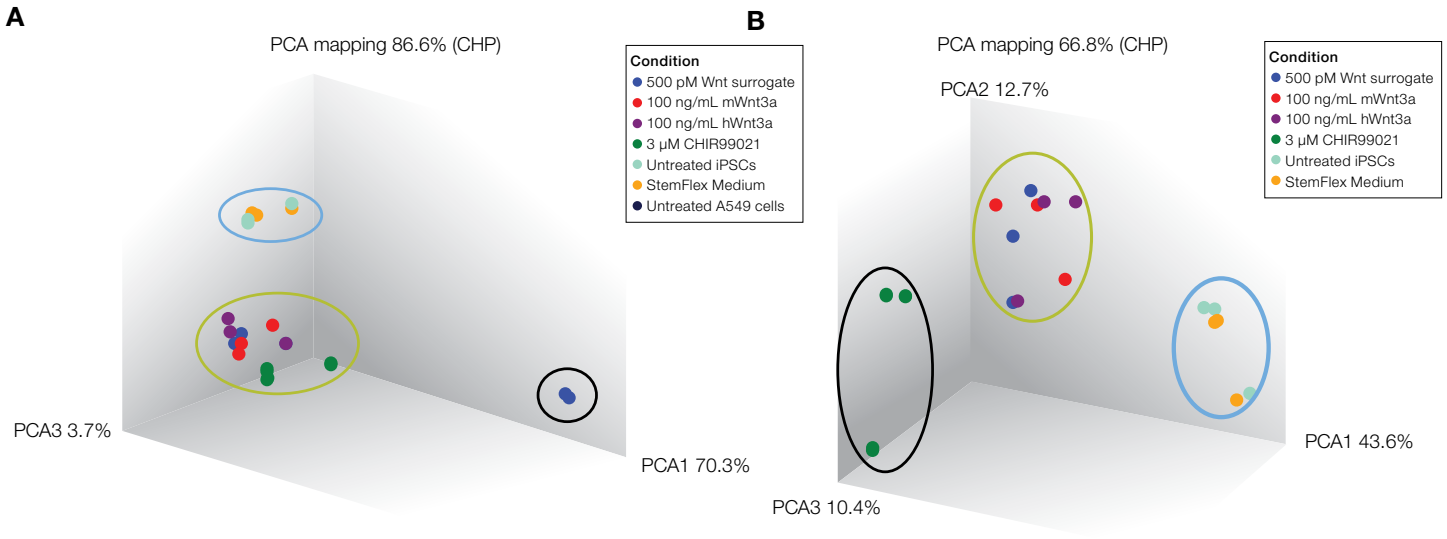


Figure 8. Principal component analysis (PCA) illustrates the comparable global transcriptional responses of the Wnt surrogate and recombinant Wnt3a in iPSCs. (A) Untreated A549 cells were included as a negative control to demonstrate the ability of this assay to distinguish between the datasets. **(B)** The PCA plot reveals that the untreated iPSCs and StemFlex Medium samples are significantly discordant from the Wnt agonist treatments. A small but notable difference between Wnt surrogate (blue) and CHIR99021 (green) treatments was also observed. Differences between dataset replicates likely reflect experimental variability. The ovals indicate the closest dataset groupings.

Conclusion

Wnt pathway activators are critical for numerous stem cell differentiation strategies and for the maintenance of epithelial stem cell–derived organoid models [13]. Here we have demonstrated that a novel Wnt pathway agonist, a Wnt surrogate-Fc fusion protein, is a highly potent activator of the Wnt pathway. We demonstrated similar response levels from picomolar concentrations of the Wnt surrogate and nanomolar concentrations of commercially available recombinant Wnt3a, as well as from commonly used protocols for Wnt-conditioned medium. Moreover, the Wnt surrogate induced a global transcriptional response in human iPSCs that strongly correlated with the other Wnt agonists and was most similar to that of wild type–based recombinant human Wnt3a, strongly suggesting that the Wnt surrogate is a true Wnt mimetic. In sum, the Wnt surrogate-Fc fusion protein is a highly potent and soluble Wnt pathway activator that overcomes known limitations of commercially available recombinant Wnt ligands and may be a useful alternative for studying the Wnt pathway in development and disease [9].

Table 1. Key reagents and equipment used in this study.

Product	Assay ID	Cat. No.
Wnt surrogate-Fc fusion protein, 25 µg	–	PHG0401
Wnt surrogate-Fc fusion protein, 100 µg	–	PHG0402
Wnt surrogate-Fc fusion protein, 500 µg	–	PHG0403
Human Episomal iPSC Line	–	A18945
Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	–	A1413302
Fetal Bovine Serum (FBS)	–	16140071
StemFlex Medium	–	A3349401
Essential 8 Medium	–	A1517001
DMEM/F-12	–	11320033
Ion Chef Instrument	–	4484177
Ion GeneStudio S5 System	–	A38194
Ion 540 Chip Kit	–	A27765
Ion AmpliSeq Transcriptome Human Gene Expression Panel	–	A31446
Varioskan LUX Multimode Microplate Reader	–	VLBLATGD2
QuantStudio 12K Flex Real-Time PCR System	–	thermofisher.com/quantstudio
TRIzol Reagent	–	15596026
TaqMan Fast Advanced Master Mix	–	4444557
High-Capacity cDNA Reverse Transcription Kit	–	4374966
SuperScript VILO cDNA Synthesis Kit	–	11754050
T/BRACHYURY TaqMan Assay	Hs00610080_m1	4331182
SP5 TaqMan Assay	Hs01370227_mH	4331182
FZD1 TaqMan Assay	Hs00268943_s1	4331182
FZD2 TaqMan Assay	Hs00361432_s1	4331182
FZD3 TaqMan Assay	Hs00907280_m1	4331182
FZD4 TaqMan Assay	Hs00201853_m1	4331182
FZD5 TaqMan Assay	Hs00361869_g1	4331182
FZD6 TaqMan Assay	Hs01095627_m1	4331182
FZD7 TaqMan Assay	Hs00275833_s1	4331182
FZD8 TaqMan Assay	Hs00259040_s1	4331182
FZD9 TaqMan Assay	Hs00268954_s1	4331182
FZD10 TaqMan Assay	Hs00273077_s1	4331182
GAPDH TaqMan Assay	–	4326317E

References

1. Rowe RG et al. (2019) Induced pluripotent stem cells in disease modelling and drug discovery. *Nat Rev Genet* 20:377–388.
2. Liu G et al. (2020) Advances in pluripotent stem cells: history, mechanisms, technologies, and applications. *Stem Cell Rev Rep* 16:3–32.
3. Zhao M et al. (2019) Deciphering role of Wnt signalling in cardiac mesoderm and cardiomyocyte differentiation from human iPSCs: four-dimensional control of Wnt pathway for hiPSC-CMs differentiation. *Sci Rep* 9:19389.
4. Dutta D et al. (2017) Disease modeling in stem cell–derived 3D organoid systems. *Trends Mol Med* 23:393–410.
5. Nusse R et al. (2017) Wnt/β-catenin signaling, disease, and emerging therapeutic modalities. *Cell* 169:985–999.
6. Willert K et al. (2003) Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 423:448–452.
7. Tüysüz N et al. (2017) Lipid-mediated Wnt protein stabilization enables serum-free culture of human organ stem cells. *Nat Commun* 8:14578.
8. Dang LT et al. (2019) Receptor subtype discrimination using extensive shape complementary designed interfaces. *Nat Struct Mol Biol* 26:407–414.
9. Miao Y et al. (2020) Next-generation surrogate Wnts support organoid growth and deconvolute frizzled pleiotropy *in vivo*. *Cell Stem Cell* 27:840–851.
10. Veeman MT et al. (2003) Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Curr Biol* 13:680–685.
11. Huggins IJ et al. (2017) The WNT target SP5 negatively regulates WNT transcriptional programs in human pluripotent stem cells. *Nat Commun* 8:1034.
12. Beurel E et al. (2015) Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. *Pharmacol Ther* 148:114–131.
13. Huch M et al. (2015) Modeling mouse and human development using organoid cultures. *Development* 142:3113–3125.