Title
Induction of Pluripotent Stem Cells Via Wnt Signaling Pathway.

Author
Ng, Damian Tse Chun

Publication Date
2013

Induction of Pluripotent Stem Cells Via Wnt Signaling Pathway

Damian Tse Chun Ng
Independent Study Project
University of California, San Diego

Abstract

Human somatic cells are capable of being reprogrammed to induced pluripotent stem (iPS) cells through the introduction of several transcription factors (Oct4, Sox2 and either Klf4, and c-Myc or Lin28 and Nanog) [1-2]. While iPS cells hold therapeutic potential for the future of regenerative medicine, current methods of induction and maintenance of the pluripotent state are extremely inefficient (with reported efficiencies of <0.01%) and pose significant oncogenic risk [3]. Several studies have demonstrated that the efficiency of reprogramming can be significantly enhanced by modifying culture conditions through the addition of small molecules or growth factors [4]. The goal of this project is to study the impact of growth factors encoded by the Wnt gene family on the reprogramming and the regulation of the induced pluripotent state. This work will address the utility of Wnt proteins as reprogramming agents and potentially establish a safer method to generate iPS cells than is currently available.

Here we propose a set of experiments to determine whether Wnt proteins, a class of signaling molecules with potent stem cell activities, and their signaling pathways regulate the acquisition of the pluripotent state. WT83 human foreskin fibroblasts were infected with retroviruses containing Oct-4, Sox-2, Klf-4 and c-Myc (Yamanaka factors), and assessed for iPS reprogramming efficiency while activating or inhibiting Wnt signaling. Wnt signaling was activated by adding either canonical Wnt3a or non-canonical Wnt5a proteins, or concurrently transducing lentiviruses encoding β-catenin. Wnt signaling was inhibited by adding small

molecule antagonists of Wnt signaling IWP and IWR, or concurrently transducing with lentiviruses encoding Axin.

11 days after gene transduction, fibroblasts were assessed for iPS cell reprogramming efficiency by staining for alkaline phosphatase, a marker for pluripotency, and quantifying the number of AP-positive colonies. In comparison to untreated cells, we observed a significant increase in AP-positive colonies when we activated Wnt signaling with Wnt3a or β-catenin overexpression. Conversely, treatment with the small molecule Wnt antagonists IWP and IWR or transduction of an Axin transgene led to a reduction in AP-positive colonies. Furthermore, treatment with Wnt5a, which antagonizes Wnt/b-catenin signaling, significantly impaired reprogramming efficiencies. We performed Wnt-promoter driven luciferase reporter gene assays to confirm and validate the effects of Wnt proteins, Wnt antagonists, and β-catenin and Axin overexpression on Wnt signaling.

These studies suggest that Wnt/β-catenin signaling is indeed a crucial component mediating the reprogramming of induced pluripotent stem cells, and holds potential as a method of generating iPS cells without the oncogenic risks of viral transduction.

Background

Induced pluripotent stem (iPS) cells are created by reprogramming somatic cells with transduction of four transcription factors Oct4, Sox2, Kfl4, and c-Myc [1] or Oct4, Sox2, Nanog and Lin28 [2]. These iPS cells are thought to hold therapeutic potential, as somatic cells from an individual could be reprogrammed and utilized to generate patient-specific cell types suitable for transplantation [5]. For instance, it has been postulated that iPS cell technology could be used to

produce tissue for patients with retinal degeneration [6], neurodegenerative disorders such as Parkinson's disease or familial dysautonomia [7], or blood disorders such as sickle cell anemia [8]. However, most current methods of reprogramming employ retroviruses, which stably integrate into the host genome thereby potentially altering gene regulation and expression. Such genomic alterations can have dangerous consequences as observed in early gene therapy trials [9]. Thus, alternative methods of generating iPS cells, which do not involve genomic integration of potential oncogenes, need to be established in order to improve the prospect of therapeutic applications of iPS cells.

One potential alternative method to achieve the iPS cell state is through the manipulation of the culture conditions, or the extracellular microenvironment. Many developmental studies have demonstrated that differentiation and regenerative processes are regulated by multiple developmental signaling pathways. One such developmental signaling pathway is regulated by Wnt genes. Currently, 19 distinct WNT genes have been identified that code for secreted lipid-modified glycoproteins [10]. Mutations in WNT genes or disruptions in the Wnt signaling pathway can catastrophically alter human embryonic development [11] and can contribute to disease, most notably cancer [12-14]. For example loss of WNT3 function has been shown to lead to tetra-amelia, a rare and fatal genetic defect [15]. Additionally, the Wnt signaling cascade has been demonstrated to exert significant effects on adult stem cells of the intestine [16], skin [17-18], brain [19-22], and blood [23-25].

More recent studies also suggest that Wnt signaling influences the efficiency of induction of the pluripotent state, even in the absence of c-Myc [4]. This raises the possibility of manipulating Wnt signaling as a means of inducing pluripotency without the use of viral transduction of transcription factor genes, which carry the risk of oncogenic mutations.

The goal of this project is to study the influence of Wnt signaling on the regulation of induced pluripotency. We hypothesize that Wnt signaling is a necessary component of the reprogramming process capable of inducing and maintaining the pluripotent state. The results of this project should provide significant insight into the possibility of utilizing Wnt proteins as reagents to generate and maintain iPS cells.

Research Design

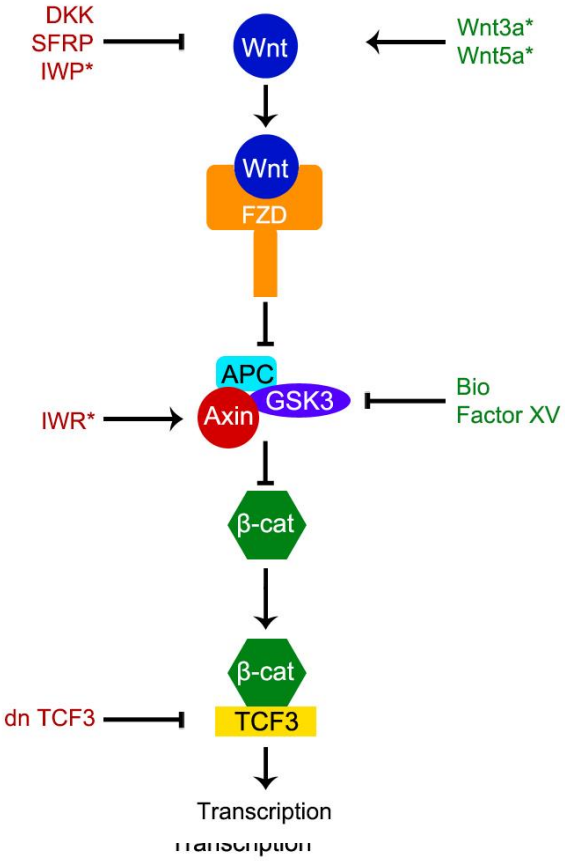
To investigate the role of Wnt signaling in reprogramming, we performed standard reprogramming methods [1] while activating or inhibiting Wnt signaling. Two to three weeks post-transduction, we counted iPS cell colonies to obtain a relative measure of the efficiency of iPS cell generation.

1) Reprogramming while activating Wnt signaling: We transduced WT83 human foreskin fibroblasts with the four known reprogramming factors OCT4, SOX3, KLF4 and MYC. At the same time, we transduced β-catenin, a downstream component of Wnt signaling, to constitutively activate Wnt signaling. In separate experiments, we activated Wnt signaling by adding either canonical Wnt3a or non-canonical Wnt5a proteins. These three approaches serve to ectopically activate Wnt signaling through distinct mechanisms.

2) Reprogramming while inhibiting Wnt signaling: We transduced WT83 human foreskin fibroblasts with the four known reprogramming factors OCT4, SOX3, KLF4 and MYC. To inhibit Wnt signaling, we transduced Axin at the same time. Axin overexpression acts to enhance β-catenin degradation. Finally, we inhibited Wnt signaling by treating cells undergoing reprogramming with the small molecule antagonist IWP [26]. IWP acts to

prevent secretion of Wnt protein, thereby inhibiting Wnt signaling. Together, these approaches serve to potently inhibit Wnt signaling by two different methods during the process of iPS cell generation.

A



B

	↓ Wnt Signaling	↑ Wnt Signaling
Gene Transduction	↑ Axin	↑ β-catenin
Soluble Protein (Growth Factor)		Wnt3a Wnt5a
Small Molecule	IWP IWR	

Figure 1: (a) Schematic of Wnt signaling pathway, displaying corresponding experimental approaches as described in (b). Reagents highlighted in green stimulate the Wnt signaling cascade, while reagents highlighted in red inhibit the pathway. Wnt3a acts through the canonical signaling pathway, binding to Wnt receptor FZD, which inhibits the "destruction complex" consisting of Axin, APC, and GSK3 from phosphorylating and destroying β-catenin. β-catenin is then free to bind TCF3 and initiate target gene transcription. Wnt5a binds to FZD and acts through a distinct and non-canonical signaling pathway. Our experimental approaches will target components along the Wnt signaling pathway to either increase or decrease the downstream effects of Wnt.

Materials and Methods

1) **Cell Culture:** We cultured HEK 293T human embryonic kidney and WT83 adult human foreskin cell lines in DMEM 1X (Cellgro) supplemented with 10% fetal bovine serum (Hygro) and 1% penicillin-streptomycin (Gibco). We seeded Hues9 human embryonic stem cells and iPS cells on a monolayer of irradiated mouse embryonic fibroblasts (MEF)

mouse embryo fibroblasts and cultured in DMEM/F12 1X (Cellgro) with 20 ng/ml human fibroblast growth factor 2 (FGF2, Millipore), 20% KnockOut Serum Replacement (Gibco), 55 μM 2-mercaptoethanol, and 2% NEAA (Invitrogen). 10 mM valproic acid was added to this culture media during iPS cell reprogramming.

2) **Lentiviral Production:** We cultured HEK 293T cells in DMEM 1X (Cellgro) supplemented with 10% fetal bovine serum (Hygro) to approximately 80% confluence. We then transfected these cells with lentiviral packaging vectors CMV-GP and VSVG, and either pMX-hc-Myc, pMX-hKLF4, pMX-hSox2, pMX-hOct4, Axin-HIV-IRE, or β-catenin-HIV-IRE lentiviral plasmid constructs using 1 μg/μL PEI. Cells were transfected for 5-6 hours, then washed and cultured in DMEM with 4% FBS for 72 hours. We filtered the culture media was then filtered through a 0.22 μM filter unit, and collected and concentrated lentivirus by ultracentrifugation at 60,000 x g and 4°C for 2 hours. After discarding of supernatant, pelleted lentivirus was resuspended in DMEM High Glucose (Cellgro) at 4°C overnight until ready for transduction.
3) **Induced Pluripotent Stem Cell Production:** We seeded WT83 human foreskin fibroblasts to 50% confluency in 6-well plates over a monolayer of MEFs for 24 hours. We then transduced these cells with Yamanaka lentiviral cocktail containing equimolar concentrations of Klf4, Sox2, Oct4, and c-Myc for 5-6 hours. Cells were then washed and grown in DMEM/F12 1X (Cellgro) with 20 ng/ml FGF2 (Millipore), 20% KnockOut Serum Replacement (Gibco), 55 μM 2-mercaptoethanol and 2% NEAA (Invitrogen) for 96 hours. At 96 hours of growth, we added 10 mM valproic acid to the culture in addition to Wnt3a, Wnt5a, IWP, or IWR small molecules as indicated. We also secondarily transduced these cells with lentiviruses encoding Axin or β-catenin as indicated. Cells

were incubated in this culture media at 37 °C and 5% CO_2 for a total of 11 days before being assessed for iPS cell production.

4) **Luciferase Reporter Assay:** We transfected HEK 293T cells with the Wnt reporter called TOP (TCF Optimal Promoter)-Flash (=luciferase) and subsequently transduced them with lentivirus encoding β-catenin or Axin, with or without Wnt3a. The cells were lysed in luciferase lysis buffer (25 mM Tris-HCl pH 7.8, 2mM CDTA, 2mM DTT, 1% Triton X-100, and 10% glycerol) and transferred to a 96-well white bottom plate. For each well, we added 50 μL luciferin substrate (BD Biosciences) and measured luminescence using a luminometer.
5) **Alkaline Phosphatase Staining:** To assess for iPS cell production, we used Alkaline Phosphatase Staining Kit (Stemgent) for in situ AP staining. Plates were fixed for 1-2 minutes in AP fixing solution (Stemgent), and quenched with 2.6M Glycine. Plates were then washed with PBST (1 X phosphate buffered saline + 10% Tween-20 or Triton X-100), and placed in AP staining solution (Stemgent) for 2 minutes.

Results

Induced pluripotent stem cell colony formation is observed within 11 days of reprogramming

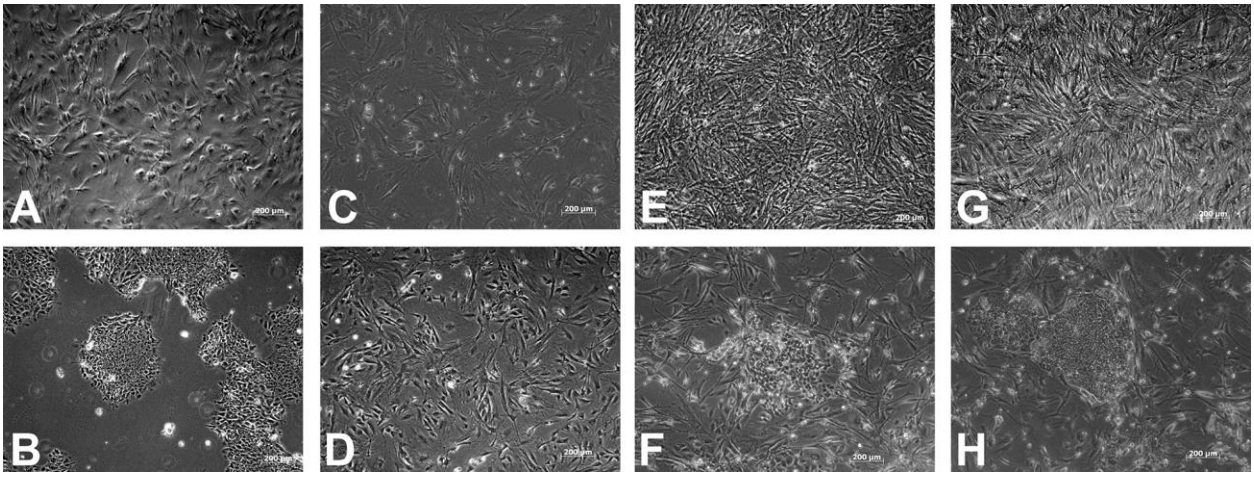


Figure 2: Morphology of WT83 undergoing iPS cell reprogramming. (a) WT83 human foreskin fibroblasts cultured in standard media. (b) Hues9 human embryonic stem cells cultured in standard media as positive control. (c-h) WT83 cells were transduced with lentiviruses containing Klf4, Sox2, Oct4 and c-Myc (Yamanaka factors) and observed for changes in cell morphology at day 4 (d), day 8 (f), and day 11 (h) post-transduction. As a negative control, WT83 cells were mock transduced with empty lentiviruses and cell morphology was observed at day 4 (c), day 8 (e), and day 11 (g) post-transduction.

We performed standard reprogramming methods following the Yamanaka protocol [1] to produce iPS cells from WT83 human foreskin fibroblasts. WT83 human foreskin fibroblasts were seeded onto PMEF mouse embryo fibroblasts, transduced with lentiviruses encoding Klf4, Sox2, Oct4, and c-Myc. To verify successful iPS cell reprogramming, we monitored these cells for growth and colony formation (Fig. 2). On day 8 post-transduction, we could observe changes in cell morphology. Cells were no longer growing in a monolayer, and signs of small hESC-like colonies could be observed (Fig. 2F). By day 11 post-transduction, we noted large colonies appearing similar in morphology to that of Hues9 human embryonic stem cells (Fig. 2H). From these observations, we determined that early signs of successful iPS cell reprogramming could be assessed 11 days post-transduction with the Yamanaka factors.

Confirming effect of β-catenin or Axin overexpression on Wnt signaling activity

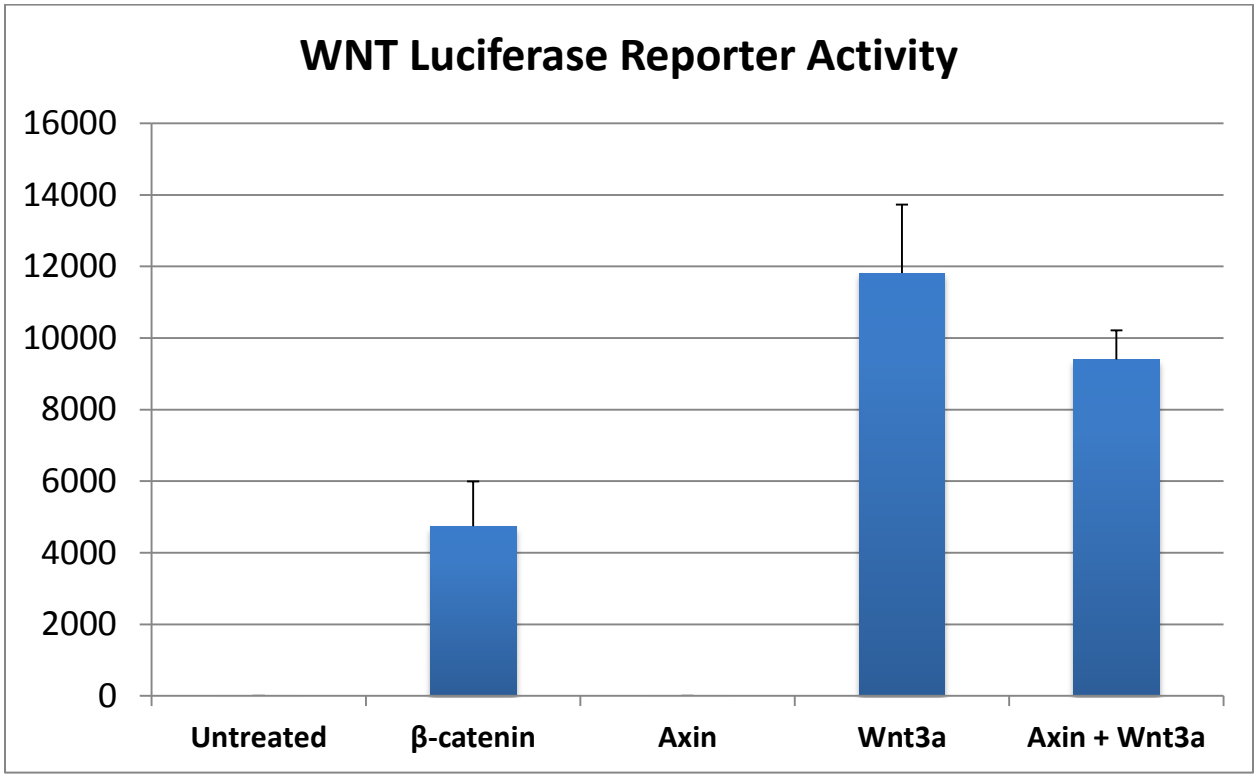


Figure 3: Assessing Wnt signaling activity via TCF reporter gene luciferase assay. HEK 293T cells were transfected with TOPFlash TCF luciferase reporter assay plasmid and transduced with lentivirus encoding β-catenin or Axin, with or without Wnt3a.

To verify that Wnt signaling can be modulated by β-catenin or Axin overexpression, we performed a TCF reporter gene luciferase assay. We transfected HEK 293T cells with the Wnt reporter called TOP (TCF Optimal Promoter)-Flash (=luciferase) and subsequently transduced with lentiviruses encoding β-catenin or Axin, with or without the addition of Wnt3a. Cells were incubated at 37 °C and 5% CO_2 for 24 hours prior to being assessed for luciferase activity (Fig. 3). We observed no baseline Wnt signaling activity in the untreated cells, as well as in cells transduced with the Axin overexpression vector. However, we noted a significant increase in Wnt reporter activity with β-catenin overexpression. We also observed increased Wnt reporter

activity with the addition of Wnt3a, an effect that was notably blunted with Axin overexpression. This result confirmed that β-catenin overexpression upregulates, while Axin overexpression downregulates Wnt signaling activity.

Effect of Wnt signaling modulation on iPS cell formation

We produced induced pluripotent stem cells as above via standard reprogramming methods following the Yamanaka protocol [1]. On day 4 of reprogramming, we secondarily transduced cells with lentivirus encoding Axin or β-catenin, or treated with small molecules Wnt3a, Wnt5a, IWP, or IWR. On day 11 of reprogramming, we stained cells for alkaline phosphatase, and counted AP-positive colonies to assess for iPS cell production (Fig. 4, 5). Compared to untreated samples (Fig. 4A), we noted a significant increase of AP-positive colonies in iPS cells treated with Wnt3a (Fig. 4B), but not Wnt5a (Fig. 4C). Conversely, we observed a significant reduction of AP-positive colonies in iPS cells treated with IWP (Fig. 4D), but not with IWR (Fig. 4E). We also observed a slight reduction of AP-positive colonies in iPS cells with Axin overexpression (Fig. 4F), and a slight increase of AP-positive colonies with β-catenin overexpression (Fig. 4G). Overall, these results suggest that Wnt3a and β-catenin overexpression increase the efficiency of iPS colony formation, while IWP and Axin overexpression decrease the efficiency of iPS colony formation.

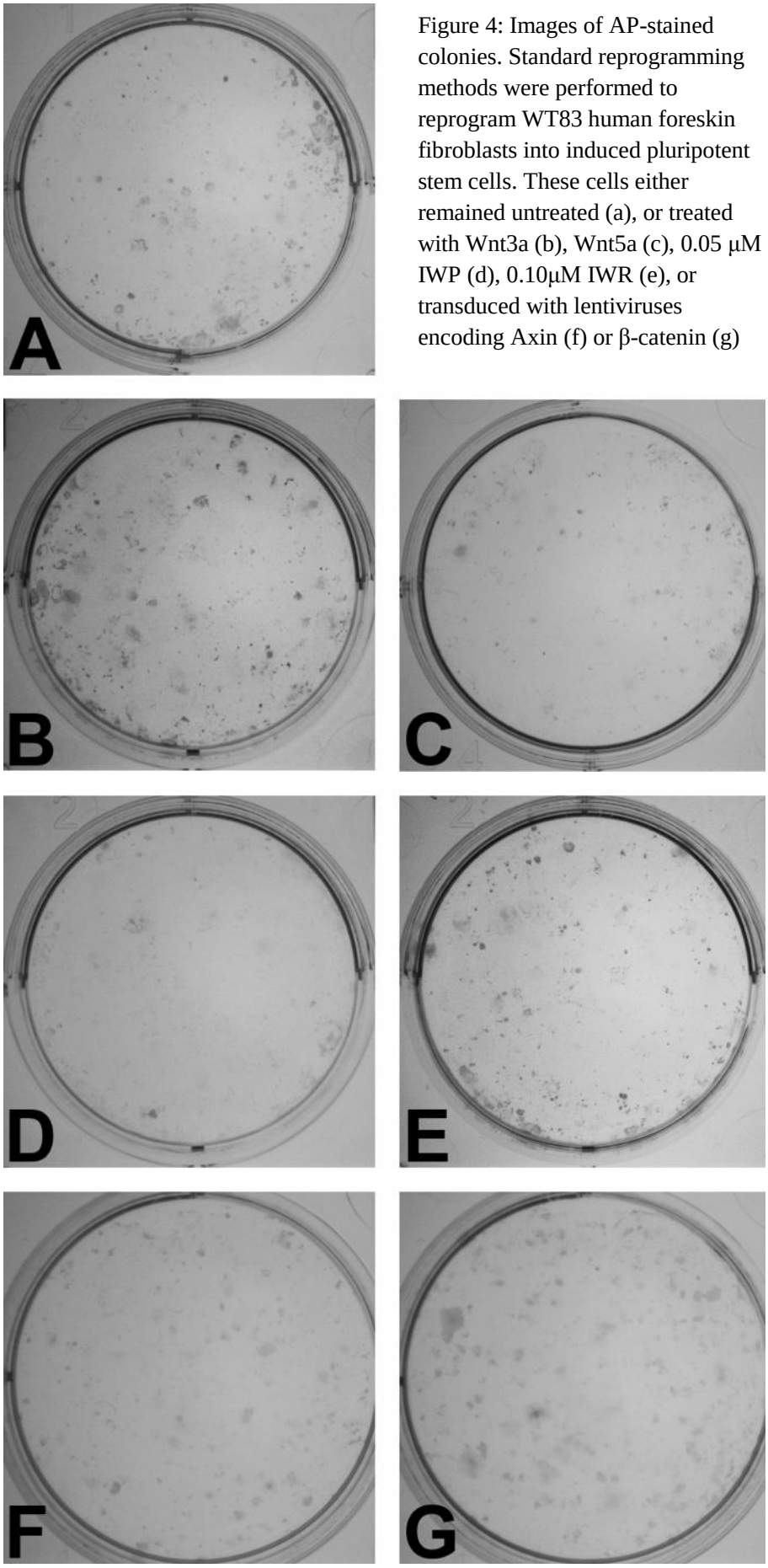


Figure 4: Images of AP-stained colonies. Standard reprogramming methods were performed to reprogram WT83 human foreskin fibroblasts into induced pluripotent stem cells. These cells either remained untreated (a), or treated with Wnt3a (b), Wnt5a (c), 0.05 μM IWP (d), 0.10μM IWR (e), or transduced with lentiviruses encoding Axin (f) or β-catenin (g)

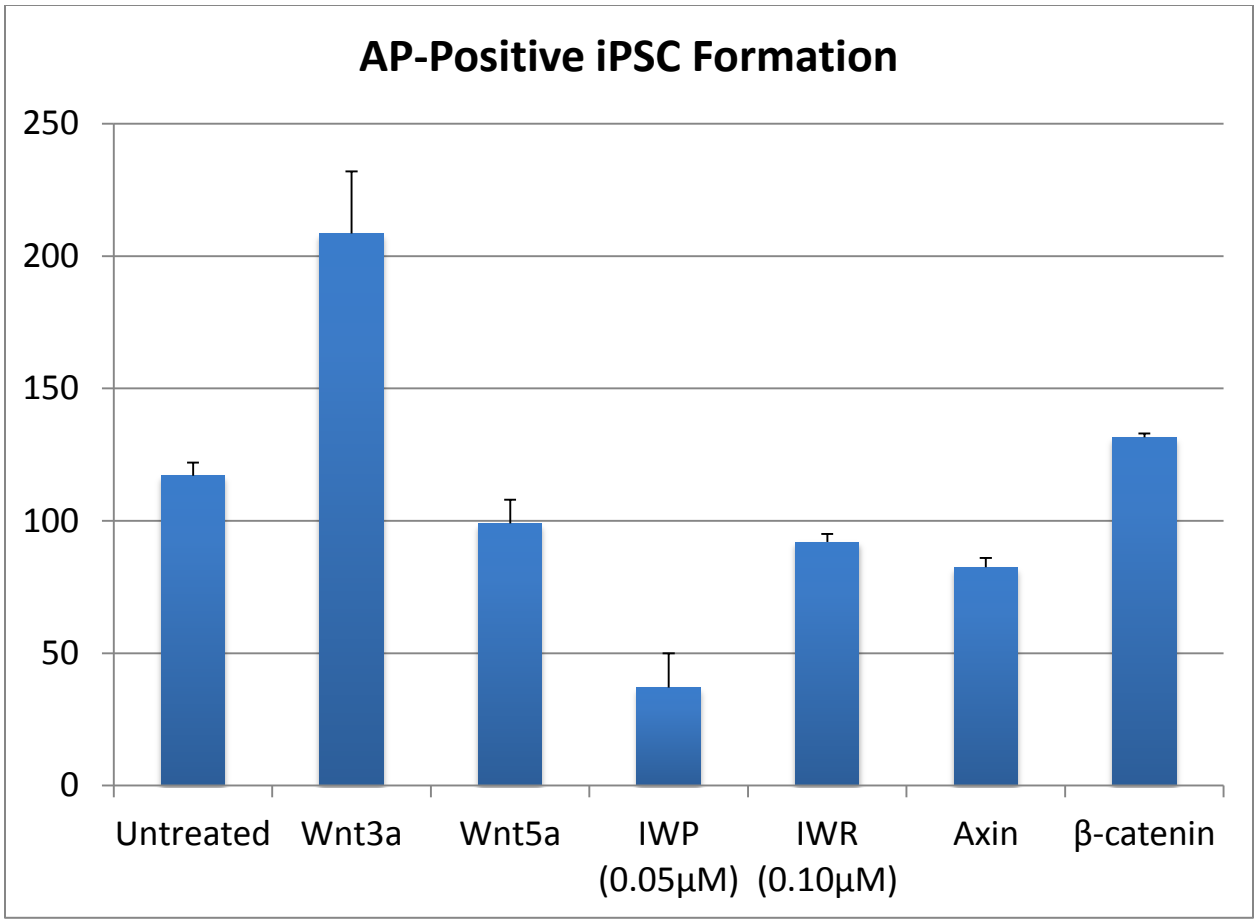


Figure 5: Quantification of alkaline phosphatase staining induced pluripotent stem cell colonies treated with Wnt3a, Wnt5a, 0.05 µM IWP, 0.10µM IWR, or transduced with lentiviruses encoding Axin or β-catenin compared to untreated negative control.

Discussion

In this study, we successfully reprogrammed WT83 human foreskin fibroblasts into induced pluripotent stem cells via lentiviral transduction of OCT4, SOX2, and KLF4 (Fig. 2). We also established a method of quantifying iPS cell formation and reprogramming efficiency by staining for alkaline phosphatase, a marker of pluripotent stem cells, and quantifying the number of AP-positive iPS cell colonies 11 days post-Yamanaka factor transduction. Using these

methods, we were able to detect changes in iPS cell reprogramming efficiency via alterations in the Wnt signaling pathway.

The present findings suggest that Wnt signaling influences the efficiency of induced pluripotent stem cell generation. In comparison to untreated negative control (Fig. 4A), we observed a significant increase of AP-positive colonies in iPS cells treated with Wnt3a (Fig. 4B). Consistent with this finding was the fact that a reduction in AP-positive colonies was observed in iPS cells treated with 0.05 μM IWP (Fig. 4D), a small molecule antagonist that prevents Wnt secretion. Furthermore, in preliminary experiments, the Willert lab found that inhibition of reprogramming with IWP could be rescued by the addition of exogenously added Wnt3a (data not shown, K.Willert and J. Ross). Together, these findings strongly suggest that an endogenous Wnt signaling loop is required during reprogramming.

Furthermore, we assessed whether regulating Wnt signaling activity via overexpression of Axin or β-catenin is sufficient to affect iPS cell reprogramming efficiency. Upon confirming that Axin or β-catenin overexpression modulates Wnt signaling activity via TCF reporter gene luciferase assay (Fig. 3), we proceeded to reprogram iPS cells secondarily transduced with lentivirus encoding either Axin or β-catenin. Indeed, we observed a reduction of AP-positive colonies in iPS cells overexpressing Axin (Fig. 4F), and an increase of AP-positive colonies in iPS cells overexpressing β-catenin (Fig. 4G).

Interestingly, we observed a slight reduction in AP-positive colony formation with the addition of Wnt5a (Fig. 4C). It is important to note that unlike Wnt3a, Wnt5a acts through a distinct and non-canonical signaling pathway. In fact, it has been reported that Wnt5a antagonizes the Wnt/β-catenin signaling pathway [27-28]. Our findings are consistent with Wnt5a's

role as an antagonist in this pathway, reducing the efficiency of iPS cell reprogramming. Additionally, iPS cell reprogramming did not appear to be affected by the addition of small molecule inhibitor IWR, as there was no significant difference in AP-positive colony formation observed (Fig. 4E). Subsequent experiments in the Willert lab did find a reduction in iPS reprogramming with the addition of IWR, consistent with all other data (data not shown, K.Willert and J. Ross). It is possible that the concentration of IWR that we treated the cells with was inadequate to produce a significant effect. This could have been better elucidated through additional dose-response studies with TCF reporter gene luciferase assays using multiple doses of IWR.

Although the quantification of alkaline phosphatase-positive colonies was sufficient in our studies to detect statistically significant differences in iPS cell formation in the setting of modifying the Wnt signaling pathway, colony counting remains an imprecise method of quantification. Other methods, such as immunocytological staining for markers for pluripotency and flow cytometry may yield more accurate and precise values for comparing iPS cell reprogramming efficiency. Unfortunately, attempts thus far in our lab at flow cytometry with immunocytostaining for pluripotency markers such as Tra-1-81 or SSEA-3 have been unsuccessful in detecting significant differences between embryonic stem cells, induced pluripotent and somatic cells. This issue may be resolved in the future by establishing a line of WT83 cells stably transfected with GFP driven by promoters for pluripotency markers, such as OCT4.

Our finding that modulating the Wnt signaling pathway, especially Wnt3a, can affect iPS cell reprogramming efficiency provides several insights. First, the fact that Wnt3a increases the rate at which iPS cell are generated raises the possibility of using Wnt3a or other small molecule

agonists as a means of inducing pluripotency. This would eliminate the need for lentiviral transduction of transcription factor genes, which carry the risk of oncogenic mutations. Second, our findings strongly suggest that canonical Wnt signaling is required during the process of reprogramming a somatic cell to an induced pluripotent stem cell. Specifically, the small molecule IWP inhibits endogenous Wnt protein processing and secretion. IWP treated fibroblasts do not yield iPS cells unless we add exogenous Wnt3a protein, thereby overcoming the IWP-imposed block on Wnt secretion.

Based on these findings, Dr. Jason Ross in the Willert lab has been pursuing additional strategies to disrupt Wnt processing and secretion. One such strategy is through the disruption of Porcupine (PORCN), the downstream target inhibited by IWP. PORCN is an ER membrane-bound *O*-acyltransferase essential for all Wnt secretion and signaling [29]. Germ line mutations in PORCN result in focal dermal hypoplasia (FDH or Goltz syndrome), an X-linked dominant disorder that manifests primarily in heterozygous females and is associated with defects in tissues derived from ecto-mesoderm, such as superficial skin striation and atrophy, fat herniation, mucous membrane papillomas, or even limb shortening and syndactyly [30]. More specifically, Proffitt et al. demonstrated that PORCN-null cells lack the ability to secrete Wnt3a, and that secretion was rescued with the addition of wild type PORCN overexpression [30]. Similarly, recent studies in the Willert lab have been directed at reprogramming fibroblasts obtained from skin biopsies of patients with FDH. Preliminary studies show that FDH-fibroblasts harboring mutations in PORCN fail to reprogram, and that this defect can be rescued with the addition of Wnt3a (data not shown, K. Willert and J. Ross). This observation is consistent with our finding that IWP inhibit iPS cell reprogramming and can be reversed by the addition of Wnt3a. Thus, we speculate that Wnt signaling not only increases the efficiency of iPS cell reprogramming, but

also is an essential component required for reprogramming. We also speculate that disorders such as FDH can be corrected by circumventing PORCN and restoring the canonical Wnt signaling pathway using Wnt3a or other small molecule agonists.

In summary, the goal of this study was to determine if Wnt proteins and Wnt signaling pathways regulate the acquisition of the pluripotent state. Indeed, we have discovered that alterations in Wnt signaling are capable of modulating the efficiency of induced pluripotent stem cell reprogramming. Further inquiry into the role of Wnt3a in iPS cell formation may provide insight into the process by which pluripotency is achieved, and also to investigate methods for correcting disorders of Wnt signaling.

References

1. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-676 (2006).
2. Yu, J., *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917-1920 (2007).
3. Okita, K., Ichisaka, T. & Yamanaka, S. Generation of germline-competent induced pluripotent stem cells. *Nature* **448**, 313-317 (2007).
4. Marson, A., *et al.* Wnt signaling promotes reprogramming of somatic cells to pluripotency. *Cell Stem Cell* **3**, 132-135 (2008).
5. Nishikawa, S., Goldstein, R.A. & Nierras, C.R. The promise of human induced pluripotent stem cells for research and therapy. *Nat Rev Mol Cell Biol* **9**, 725-729 (2008).
6. Comyn, O., Lee, E. & MacLaren, R.E. Induced pluripotent stem cell therapies for retinal disease. *Curr Opin Neurol* **23**, 4-9 (2010).
7. Hacein-Bey-Abina, S., *et al*. LMO2-associated clonal T-cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**, 415-419 (2003).
8. Robinton, D.A., *et al.* The promise of induced pluripotent stem cells in research and therapy. *Nature* **481**, 295-305 (2012).
9. Hanna, J. *et al*. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science* **318**, 1920-1923 (2007).

10. Nuss, R. Human Wnt Genes. Vol. 2010 (2009).
11. Logan, C.Y. & Nusse, R. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* **20**, 781-810 (2004).
12. Clevers, H. Wnt/beta-catenin signaling in development and disease. *Cell* **127**, 469-480 (2006).
13. Kinzler, K.W. & Vogelstein, B. Lessons from hereditary colorectal cancer. *Cell* **87**, 159-170 (1996).
14. Klaus, A. & Birchmeier, W. Wnt signalling and its impact on development and cancer. *Nat Rev Cancer* **8**, 387-398 (2008).
15. Niemann, S., *et al.* Homozygous WNT3 mutation causes tetra-amelia in a large consanguineous family. *Am J Hum Genet* **74**, 558-563 (2004).
16. Gregorieff, A. & Clevers, H. Wnt signaling in the intestinal epithelium: from endoderm to cancer. *Genes Dev* **19**, 877-890 (2005).
17. Gat, U., DasGupta, R., Degenstein, L. & Fuchs, E. De Novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. *Cell* **95**, 605-614 (1998).
18. Ito, M., *et al.* Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding. *Nature* **447**, 316-320 (2007).
19. Chenn, A. & Walsh, C.A. Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science* **297**, 365-369 (2002).
20. Kalani, M.Y., *et al.* Wnt-mediated self-renewal of neural stem/progenitor cells. *Proc Natl Acad Sci U S A* **105**, 16970-16975 (2008).
21. Lie, D.C., *et al.* Wnt signalling regulates adult hippocampal neurogenesis. *Nature* **437**, 1370-1375 (2005).
22. Soen, Y., Mori, A., Palmer, T.D. & Brown, P.O. Exploring the regulation of human neural precursor cell differentiation using arrays of signaling microenvironments. *Mol Syst Biol* **2**, 37 (2006).
23. Reya, T., *et al.* A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature* **423**, 409-414 (2003).
24. Willert, K., *et al.* Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **423**, 448-452 (2003).
25. Luis, T.C., *et al.* Wnt3a deficiency irreversibly impairs hematopoietic stem cell self-renewal and leads to defects in progenitor cell differentiation. *Blood* **113**, 546-554 (2009).
26. Chen, B., *et al.* Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat Chem Biol* **5**, 100-107 (2009).
27. Topol, L., *et al.* Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent beta-catenin degradation. *J Cell Bio* **162**, 899-908 (2003).
28. Li, J., *et al.* WNT5A antagonizes WNT/β-catenin signaling and is frequently silenced by promoter CpG methylation in esophageal squamous cell carcinoma. *Cancer Biol Ther.* **10**, 617-624 (2010).
29. Covey, T.M., *et al.* PORCN Moonlights in a Wnt-Independent Pathway That Regulates Cancer Cell Proliferation. *PLoS ONE* **7**, e34532 (2012).
30. Proffitt, K.D., *et al.* Precise Regulation of Porcupine Activity Is Required for Physiological Wnt Signaling. *J Biol Chem* **287**, 34167-34178 (2012).