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Stage-Specific Regulation of Reprogramming to Induced Pluripotent Stem Cells by Wnt Signaling and T Cell Factor Proteins

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SUMMARY

Wnt signaling is intrinsic to mouse embryonic stem cell self-renewal. Therefore, it is surprising that reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) is not strongly enhanced by Wnt signaling. Here, we demonstrate that active Wnt signaling inhibits the early stage of reprogramming to iPSCs, whereas it is required and even stimulating during the late stage. Mechanistically, this biphasic effect of Wnt signaling is accompanied by a change in the requirement of all four of its transcriptional effectors: T cell factor 1 (Tcf1), Lef1, Tcf3, and Tcf4. For example, Tcf3 and Tcf4 are stimulatory early but inhibitory late in the reprogramming process. Accordingly, ectopic expression of Tcf3 early in reprogramming combined with its loss of function late enables efficient reprogramming in the absence of ectopic Sox2. Together, our data indicate that the stepwise process of reprogramming to iPSCs is critically dependent on the stage-specific control and action of all four Tcfs and Wnt signaling.

INTRODUCTION

The generation of induced pluripotent stem cells (iPSCs) from fibroblasts by ectopic expression of Oct4, Sox2, cMyc, and Klf4 established a major landmark in the field of stem cell biology, as it allowed the generation of patient-specific pluripotent cells (Maherali et al., 2007; Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). The reprogramming process is quite robust, in that ectopic expression of the reprogramming factors works on a wide range of differentiated cells to produce iPSCs (Stadtfeld and Hochedlinger, 2010). However, reprogramming to iPSCs is inefficient, in that only a few somatic cells of the starting population transition to pluripotency after a latency period of around 2 weeks (Papp and Plath, 2013). Thus, events that are currently largely unknown need to occur to achieve reprogramming to the pluripotent state. Indeed, the starting cell type, the reprogramming factor combination used, the method of overexpression, and the culture conditions all have major effects on the activation of the endogenous pluripotency gene regulatory network and even the epigenetic state of the reprogrammed cells (Papp and Plath, 2013). In this study, we focus on the role of Wnt signaling in reprogramming to iPSCs.

The Wnt/β-catenin signaling pathway is intricately linked to the pluripotent state (Clevers and Nusse, 2012). For instance, mouse embryonic stem cells (ESCs) secrete active Wnt ligands and autocrine Wnt activity is required to prevent their differentiation (ten Berge et al., 2011), indicating that Wnt signaling is both necessary and sufficient for the self-renewal of these cells. Mouse ESCs can even self-renew efficiently in the absence of serum and extrinsic signals as long as Wnt/β-catenin signaling is stimulated and ERK kinases are inhibited ("2i" culture condition) (Ying et al., 2008).

Canonical Wnt signaling is classically described as functioning in two states. In the absence of a Wnt ligand, a complex of proteins, including Axin, Apc, Ck1, and Gsk3, stimulates the ubiquitin-mediated destruction of β-catenin (Clevers and Nusse, 2012). In the absence of stable β-catenin, T cell factor (Tcf) proteins (Tcf1, Lef1, Tcf3, and Tcf4 in mammals) transcriptionally repress Wnt target genes by interacting with corepressor proteins, such as Groucho or the C-terminal binding protein (Ctbp) and recruiting them to their DNA recognition sites through their high-mobility group (HMG) domain, which is nearly identical in all Tcfs (Clevers and Nusse, 2012). When a Wnt ligand activates the pathway, the β-catenin destruction complex is inhibited, enabling β-catenin to translocate to the nucleus, where it can bind to a conserved domain present near the amino terminus of all Tcfs (Clevers and Nusse, 2012). Upon binding to a Tcf, β-catenin can switch the activity of Tcfs from transcriptional repression to activation by recruiting coactivators, such as CBP (Takemaru and Moon, 2000). Although Tcfs share homologous HMG and β-catenin interaction domains, differences among individual Tcfs cause them to function uniquely within the Wnt pathway. For example, the effect of β-catenin binding can differ, either inducing the classic conversion from a repressor to transactivator for Tcf1 and Lef1 or only inactivating the repressor activity of Tcf3 (B.J.M., unpublished data). Thus,

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individual Tcfs can cause overlapping or diverse effects, depending on how their conserved and unique elements are regulated.

An important understanding of how Wnt signaling affects ESCs has been attained through the appreciation of the diverse effects of Tcfs. Together with core pluripotency transcription factors, Oct4, Sox2, and Nanog, Tcf3 co-occupies many pluripotency genes, including *Nanog* and *Esrrb* (Cole et al., 2008; Marson et al., 2008a; Martello et al., 2012; Tam et al., 2008; Yi et al., 2008). Ablation of Tcf3 stimulates *Nanog* and *Esrrb* expression, similar to the activation of Wnt/β-catenin signaling (Cole et al., 2008; Martello et al., 2012; Pereira et al., 2006; Yi et al., 2011), and allows self-renewal of ESCs in serum-free conditions without Wnt pathway stimulation (Yi et al., 2011). It is therefore thought that Tcf3 acts exclusively as a transcriptional repressor in ESCs, even in the presence of stable β-catenin. Tcf4 mainly displays similar transcriptional repressor activity as Tcf3, but it is expressed at low levels in ESCs (Pereira et al., 2006; Yi et al., 2011). By contrast, Tcf1 and Lef1 display β-catenin-dependent transcriptional activator activity in ESCs, and endogenous Tcf1 activity counteracts some, but not all, transcriptional repression by Tcf3 (Yi et al., 2011).

The central importance of Wnt signaling and inhibition of Tcf3 for self-renewal of mouse ESC has stimulated investigations into the effects of Wnt signaling on reprogramming to pluripotency. In experiments where somatic cell nuclei are reprogrammed to the pluripotent state upon fusion with ESCs, treating ESCs with exogenous Wnt3a, stabilized β-catenin, or downregulation of Tcf3 each stimulates the efficiency by which somatic cells are reprogrammed (Han et al., 2010; Lluis et al., 2008, 2011). The effects of Wnt3a or Tcf3 ablation on fusion-mediated reprogramming are substantial, increasing reprogramming efficiency up to 1,000-fold (Lluis et al., 2008, 2011). By contrast, Wnt/β-catenin stimulation or Tcf3 depletion cause only a weak enhancement of reprogramming to iPSCs (Lluis et al., 2011; Marson et al., 2008b). In addition, β-catenin was among the original 24 factors screened by Takahashi and Yamanaka, and its overexpression was found to have no significant effect on iPSC formation (Takahashi and Yamanaka, 2006). Currently, it remains unclear how Wnt signaling has such a substantial impact on the self-renewal of mouse ESCs and reprogramming by fusion with ESCs, yet causes relatively minor effects on the outcome of iPSC-based reprogramming experiments.

To elucidate how Wnt/β-catenin signaling affects reprogramming to iPSCs, we determined the effects of inhibiting or stimulating Wnt signaling and the requirement for the four Tcfs during different stages of the reprogramming process. Our results demonstrate that early events in reprogramming are stimulated by inhibiting Wnt signaling, whereas late events are stimulated by activating the pathway. These effects are mediated by differential activities of the four Tcfs, and dynamic manipulation of Tcf3 levels allows for the efficient formation of iPSCs without exogenous Sox2. Our findings showcase that the poor efficiency of reprogramming is at least partially caused by changing molecular requirements in the process, where events promoting one phase are inhibitory for a subsequent phase, calling for further optimization of the iPSC technology.

RESULTS

Wnt Signaling Is Essential for Late Stages of Reprogramming to iPSCs

We began elucidating the role of Wnt signaling in reprogramming to iPSCs by determining whether endogenous Wnt signaling is necessary for the process. In these experiments, we employed mouse embryonic fibroblasts (MEFs) carrying a single tetracycline-inducible polycistronic cassette encoding *Oct4*, *Sox2*, *cMyc*, and *Klf4* (inducible OSCK [iOSCK]) and a reverse tetracycline transactivator (*M2rtTA*) transgene (Figure S1A); induced reprogramming by addition of doxycycline; and assessed reprogramming efficiency under various treatments by quantifying colonies positive for expression of the pluripotency factor Nanog. To inhibit endogenous Wnt signaling, iOSCK MEFs were transduced with a retrovirus expressing Dickkopf1 (Dkk1), a secreted ligand and natural antagonist to the Wnt coreceptor LRP5/LRP6 (Mao et al., 2001). Reduction of transcript levels of the Wnt target gene *Axin2* and of TOPflash luciferase reporter activity confirmed that ectopic Dkk1 expression efficiently inhibited Wnt signaling (Biechele et al., 2009; Figures S1B and S1C). Notably, Dkk1 expression greatly reduced the numbers of Nanog-positive colonies (Figure 1A), suggesting that endogenous Wnt signaling is essential for the formation of iPSCs. To confirm that the reduction of Nanog-positive colonies was due to effects on Wnt signaling, IWP2, a potent small-molecule inhibitor of Porcupine, which is necessary for the processing and secretion of all Wnt ligands (Chen et al., 2009), was continuously added throughout the reprogramming process. This independent method of inhibiting endogenous Wnt signaling strongly blocked the formation of Nanog-positive colonies (Figure 1B), demonstrating that the production of active Wnt ligands is essential for reprogramming.

The substantial negative effects of inhibiting endogenous Wnt signaling (Figures 1A and 1B) contrasted with the minimal positive effects of exogenously stimulating Wnt reported previously, and there was even no effect when cMyc was included in the reprogramming cocktail with Oct4, Sox2, and Klf4 (Marson et al., 2008b). To determine if an effect of exogenous Wnt signaling could be observed with our experimental set-up, Nanog-expressing colonies were measured in reprogramming experiments continuously treated with purified recombinant Wnt3a protein, which strongly activated a TOPflash luciferase reporter (Figure S1C). Consistent with previous reports, we observed no increase in the reprogramming of iOSCK MEFs to Nanog-expressing colonies upon Wnt3a treatment (Figure 1C). Moreover, recombinant Wnt3a reduced the number of Nanog-expressing colonies by half, indicating that constitutive exogenous Wnt signaling is even inhibitory for the induction of Nanog when reprogramming is performed with our inducible OSCK polycistronic expression cassette.

Because both stimulating and blocking Wnt signaling resulted in an inhibition of iOSCK reprogramming, albeit to different extents, we examined the possibility of different responses to Wnt signaling during the progression to pluripotency. To test this, we divided the reprogramming process into early, mid, and late stages and observed the effects of Wnt inhibition and stimulation on reprogramming (Figure 1D). Early treatment with

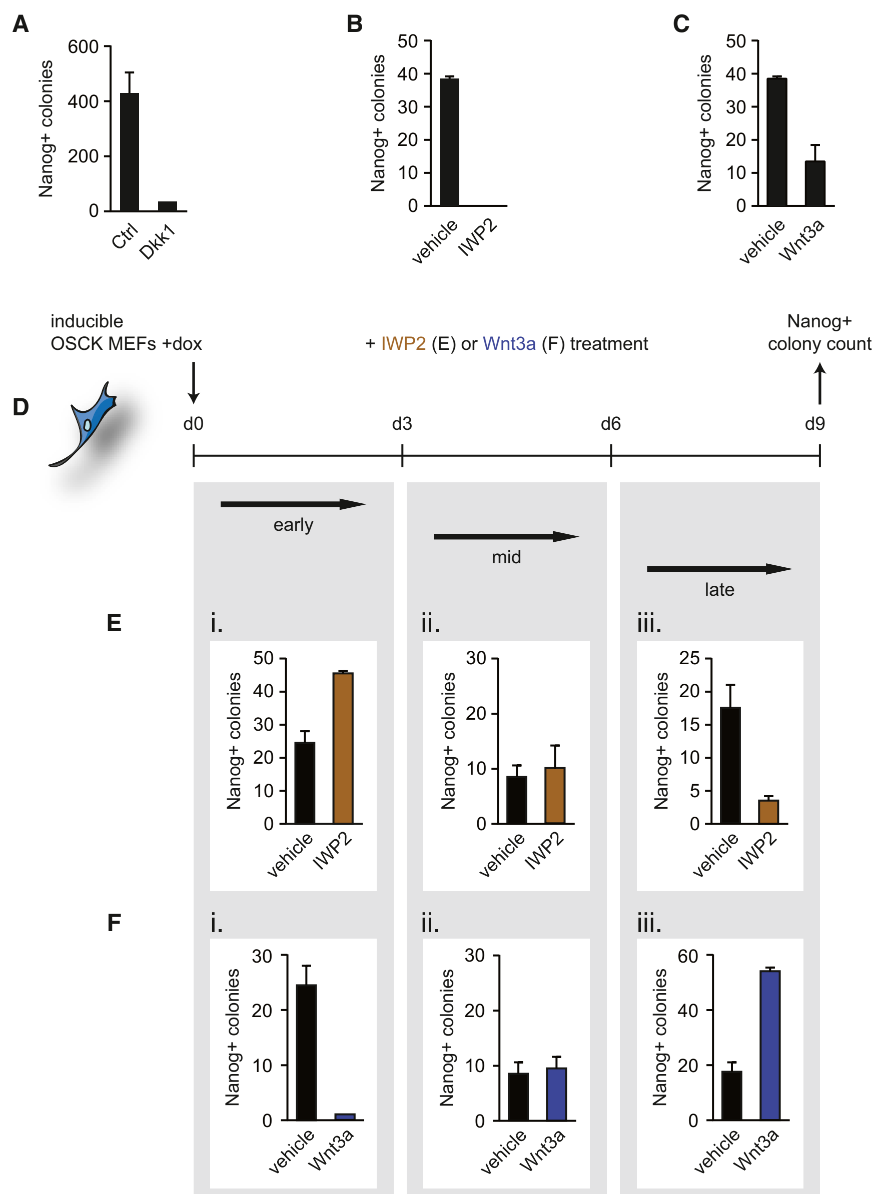

Figure 1. Biphasic Role of Wnt Signaling in Reprogramming to iPSCs

(A) iOSCK MEFs were transduced with a *Tomato* (Ctrl) and *Dkk1*-encoding retrovirus, respectively, and treated with dox to express the reprogramming factors, and Nanog-positive colonies were quantified at day 13.

(B) iOSCK MEFs were treated with dox and IWP2 or vehicle, respectively, continuously throughout reprogramming, and Nanog positive colonies were counted at day 9.

(C) As in (B), except that Wnt3a was added continuously throughout reprogramming.

(D) Schematic of the reprogramming experiments to determine the effect of Wnt3a or IWP2 during different stages of reprogramming.

(E) Nanog colony count for IWP2-treated reprogramming cultures as described in (D).

(F) Nanog colony count for Wnt3a-treated reprogramming cultures as described in (D). All reprogramming counts represent the average of two representative experiments, and error bars depict standard deviation.

See Figure S1 for additional information.

IWP2 yielded a 2-fold increase of Nanog-positive colonies, whereas late treatment strongly reduced reprogramming (Figure 1E). Conversely, Wnt3a addition early resulted in a dramatic inhibition of the formation of Nanog-positive colonies, whereas treatment late increased the number of Nanog-positive colonies over 2-fold (Figure 1F). The efficiency of reprogramming was not affected when either IWP2 or Wnt3a were added in the middle phase of the process (Figures 1E and 1F). Together, these data demonstrate a biphasic response of the iPSC reprogramming process to Wnt signaling.

The possibility that this biphasic response could be caused by different responses to graded levels of Wnt activity, as previously described by Cosma and colleagues for cell fusion experiments (Lluis et al., 2008), was ruled out by the dose-dependent manner by which IWP2 affected Nanog-positive colony formation (Figures S1D–S1F). Moreover, effects on levels of well-established Wnt targets, such as *Axin2* and *Tcf1*, confirmed that Wnt3a and IWP2 stimulated and inhibited Wnt signaling, respectively, as expected (Figures S1E and S1F).

We conclude that our experimental approach resolves reprogramming effects that were previously overlooked by continuous treatment of reprogramming cultures with Wnt pathway effectors. Two distinct phases of Wnt response affect two stages of reprogramming, which can be temporally defined as early and late stages. The conclusion that the stimulation of Wnt signaling establishes a strong barrier for early reprogramming events is further supported by the findings that preincubation of MEFs with Wnt3a before the start of reprogramming dramatically impaired reprogramming and preincubation with IWP2 enhanced reprogramming (Figure S1G). In contrast, the late phase not only depends on endogenously active Wnt signaling but also benefits from the ectopic stimulation of the pathway.

Tcf1/Lef1 and Tcf3/Tcf4 Have Opposing Roles in the Biphasic Response to Wnt Signaling during Reprogramming

Wnt ligands can elicit multiple downstream effects, some of which are independent of the canonical Tcf-β-catenin regulation of target genes. To determine whether the biphasic effects of Wnt signaling on reprogramming to iPSCs were mediated by Tcfs, we depleted each Tcf (*Tcf1, Lef1, Tcf3,* and *Tcf4*) during reprogramming by small interfering RNA (siRNA)-mediated

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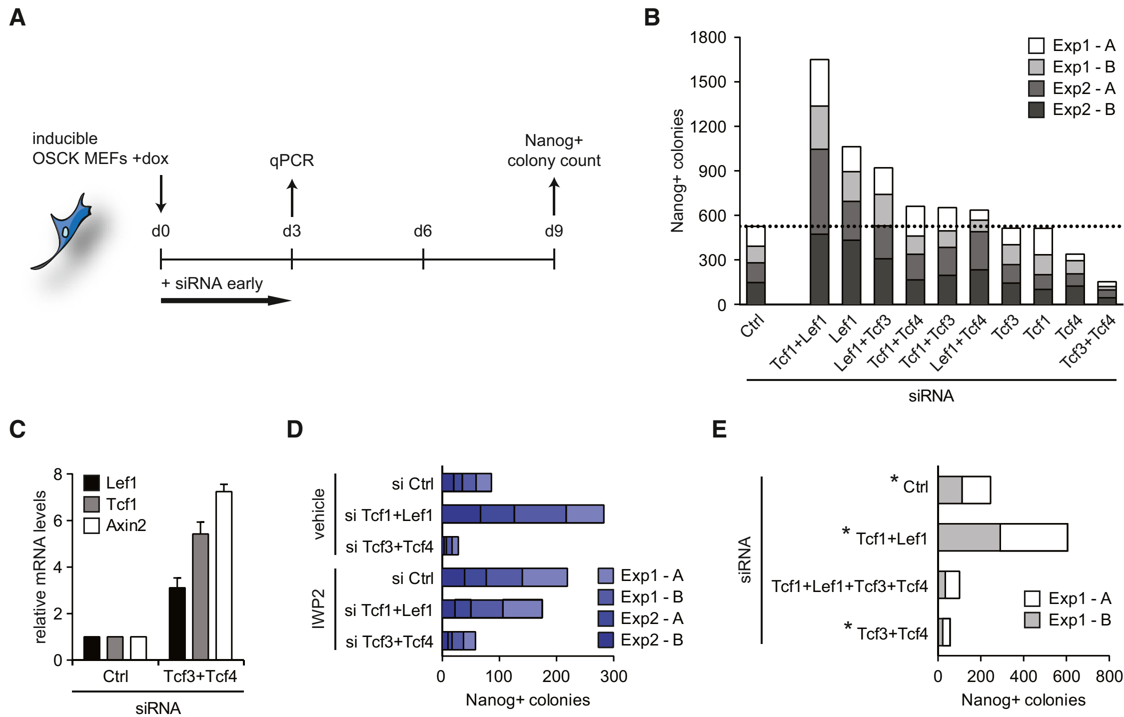

Figure 2. Tcf1 and Lef1 Inhibit whereas Tcf3 and Tcf4 Promote the Early Phase of Reprogramming

(A) Schematic of the reprogramming experiment testing the requirement of Tcfs in the early phase of reprogramming. iOSCK MEFs were transfected with siRNAs targeting Tcfs individually, in combination or with siCtrl twice: first 12 hr prior to the induction of OSCK factors and again together with dox addition. Knockdown was confirmed on day 3 of reprogramming, and Nanog-positive colonies were counted at day 10 (experiment 1) or day 9 (experiment 2).

(B) Nanog-positive colony count from two independent experiments (Exp1 and Exp2), each with two technical replicates (A and B).

(C) qPCR for *Lef1*, *Tcf1*, and *Axin2* transcript levels relative to siCtrl upon *Tcf3/Tcf4* double knockdown at day 3 of reprogramming. Values represent the average of duplicate sampling, and error bars represent standard deviation.

(D) As in (B), except that reprogramming cultures were treated with IWP2 or vehicle from day 0 to day 3 in addition to indicated siRNAs. Nanog-positive colony count from two independent experiments (Exp1 and Exp2), each with two technical replicates (A and B) is given.

(E) Number of Nanog-positive colonies upon simultaneous knockdown of all four Tcfs early in reprogramming. Knockdown was performed as part of experiment 1 shown in (B) and experimental conditions shared with (B) are indicated by asterisks.

See Figure S2 for additional information.

knockdown. We also tested the effect of depleting all possible pairs of Tcfs to address the potential for redundancy between family members. The requirement for the Tcfs was first examined during the early stage of reprogramming (Figures 2A and S2A–S2D). The strongest effects due to loss-of-function of a single Tcf early in the process were seen for *Lef1* and *Tcf4*, respectively (Figure 2B). Their knockdown had opposing effects on the induction Nanog-positive colonies; depletion of *Lef1* increased and *Tcf4* knockdown decreased colony numbers. These effects were magnified when the knockdown of *Lef1* was combined with that of *Tcf1* or when *Tcf4* was depleted together with *Tcf3* (Figure 2B). These findings reveal (1) redundancies among Tcf family members and (2) an antagonistic effect between two groups of Tcfs early in reprogramming: endogenous Tcf1 and Lef1 are inhibitors, whereas Tcf3 and Tcf4 are enhancers in this phase of reprogramming.

Based on the effects of Wnt signaling on the early reprogramming phase, one would predict that Tcf1 and Lef1 mediate Wnt effects, whereas Tcf3 and Tcf4 counteract Wnt effects during this stage. Consistent with this hypothesis, the transcript levels of Wnt target genes *Tcf1, Lef1,* and *Axin2* were significantly increased upon *Tcf3* and *Tcf4* knockdown early in reprogramming (Figure 2C). To test this hypothesis further, we combined IWP2 treatment with knockdown of *Tcf1/Lef1* or *Tcf3/Tcf4* early in reprogramming (Figures 2D, S2E, and S2F). The combined knockdown of *Tcf1/Lef1* increased the number of Nanog-positive colonies in the absence of IWP2 but failed to further increase colony numbers when endogenous Wnt signaling was blocked by IWP2 (Figure 2D). Thus, the effects of Tcf1 and Lef1 early in reprogramming overlap with those seen by inhibiting endogenous Wnt signaling, which is consistent with Wnt/β-catenin-dependent transcriptional activator activities for Tcf1 and Lef1 during this reprogramming phase. Conversely, knockdown of *Tcf3/Tcf4* inhibited reprogramming, regardless of the presence or absence of IWP2 (Figure 2D). The Wnt target genes *Tcf1*, *Lef1*, and *Axin2* were upregulated upon *Tcf3/Tcf4* knockdown, even in the presence of IWP2 (i.e., without active Wnt signaling) (Figure S2G). Therefore, the inhibitory effect of *Tcf3/Tcf4* depletion on early reprogramming does not require active Wnt signaling, which is most consistent with transcriptional repressor

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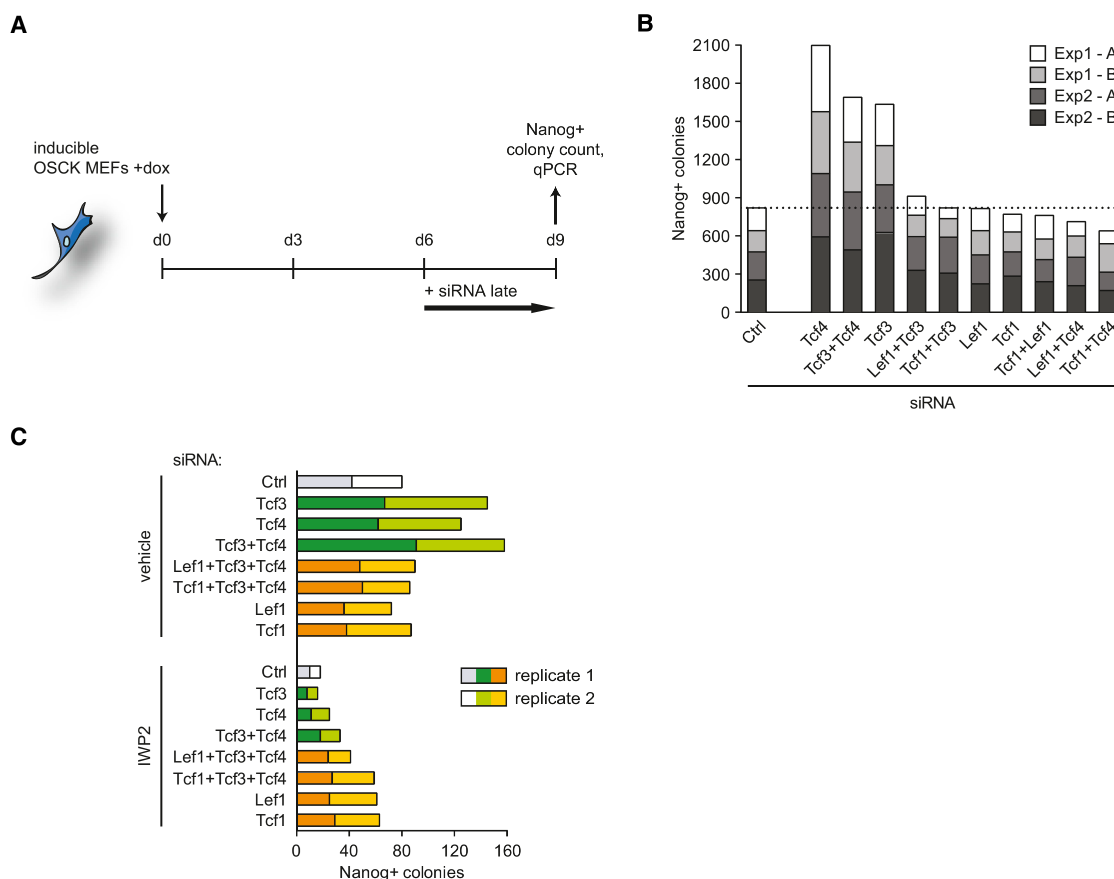

Figure 3. *Tcf3/Tcf4* Depletion Enhances the Late Phase of Reprogramming in a *Tcf1/Lef1*-Dependent Manner

(A) Schematic of the reprogramming experiment testing the requirement of Tcfs in the late phase of reprogramming. iOSCK MEFs were transfected with siRNAs targeting Tcfs individually or in combination with siCtrl once at day 6 postinduction of OSCK. Transcript levels and Nanog-positive colonies were quantified on day 10 (experiment 1) or day 9 (experiment 2).

(B) Nanog-positive colony count from two independent experiments (Exp1 and Exp2), each with two technical replicates (A and B).

(C) As in (B), except that the cultures were treated with IWP2 or vehicle from day 6 to day 9 in addition to indicated siRNAs. Nanog-positive colony count from technical replicates of a representative experiment is given.

See Figure S3 for additional information.

activities for Tcf3 and Tcf4. Notably, the simultaneous knockdown of all four Tcfs reduced the number of Nanog-positive colonies compared to control (Figures 2E, S2H, and S2I). This result indicates that the mediators of active Wnt signaling, *Tcf1* and *Lef1,* are not the critical targets of Tcf3 and Tcf4 repression during the early stage of reprogramming, as reducing the aberrant *Tcf1/Lef1* upregulation observed upon *Tcf3/Tcf4* depletion did not rescue the reprogramming efficiency.

To test the role of Tcfs during the late phase of reprogramming, we transfected siRNAs once on day 6 of reprogramming and assessed the formation of Nanog-positive colonies 3 days later (Figures 3A and S3A–S3D). Among all siRNA treatments, only the knockdown of *Tcf3* and *Tcf4*, individually or together, consistently enhanced reprogramming (Figures 3B and S3E), indicating that Tcf3 and Tcf4 inhibit late reprogramming events. Because the effect of the *Tcf3* and *Tcf4* double knockdown was not additive compared to their respective single knockdowns, Tcf3 and Tcf4 likely act in the same pathway. Importantly, the reprogramming enhancement due to *Tcf3* or *Tcf4* knockdown was nullified when *Tcf1* or *Lef1* were concurrently depleted (Figures 3B and S3F). Because these data demonstrated that the loss of Tcf3 or Tcf4 requires Tcf1 of Lef1 for a positive effect late in reprogramming, which are typically the mediators of active Wnt signaling, we next tested the requirement of Wnt signaling in this context more directly by combining the *Tcf3/Tcf4* knockdown with IWP2 treatment. Our results show that IWP2 prevented the enhancing effect of *Tcf3* or *Tcf4* depletion late in reprogramming (Figure 3C, green bars). This effect does not appear to be due to a dramatic change in *Tcf1* and *Lef1* levels (Figure S3G). Together, these findings indicate that depletion of *Tcf3* and *Tcf4* promotes the late stage of reprogramming through a mechanism that requires Tcf1 or Lef1 as mediators of active Wnt signaling.

Although depletion of *Tcf1* and/or *Lef1* during the late phase did not inhibit reprogramming (Figure 3B), their depletion mitigated the inhibitory effect of Wnt inhibition (i.e., IWP2 treatment)

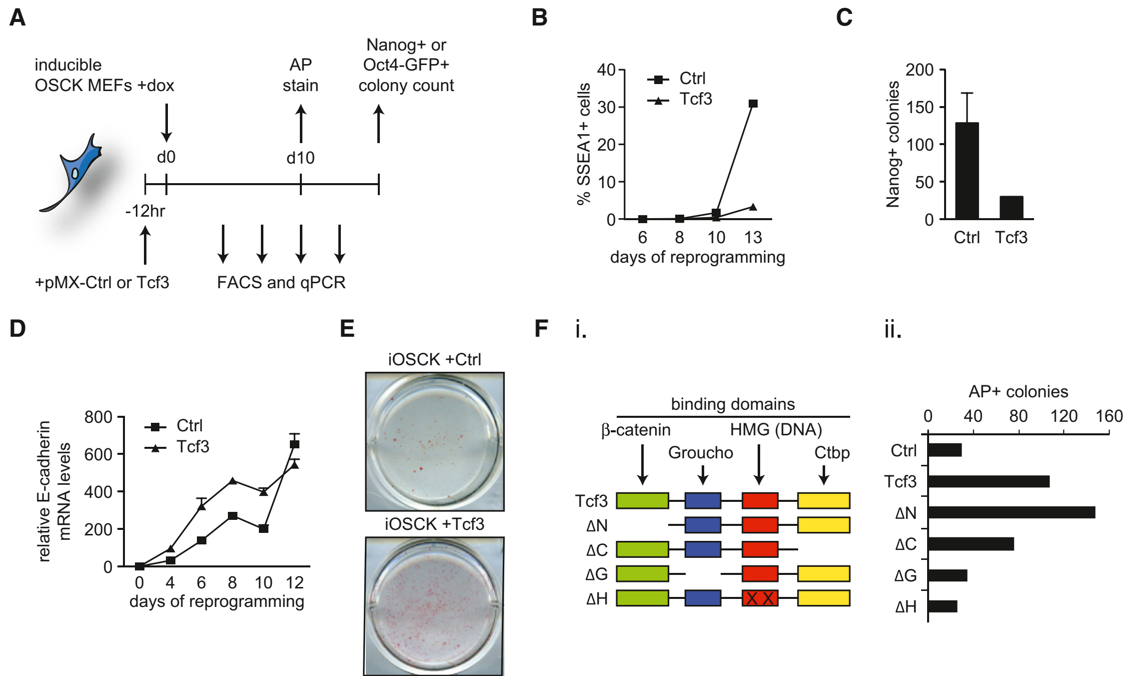

Figure 4. Ectopic Expression of Tcf3 Promotes Early Reprogramming Events but Inhibits the Induction of Pluripotency

(A) Experimental design for continuous Tcf3 overexpression from the pMX-retroviral vector during reprogramming of iOSCK MEFs. Tomato expression served as control overexpression (Ctrl).

(B) Fluorescence-activated cell sorting (FACS) analysis showing the percentage of SSEA1 positive cells at indicated times of a representative reprogramming experiment.

(C) Nanog colony count representing the average of two independent experiments, each with two technical replicates: one counted on day 11 and one on day 15. Error bars indicate standard deviation of the averaged values.

(D) Transcript levels of *E-cadherin* during reprogramming relative to MEFs. Values represent the average of duplicate sampling, and error bars represent standard deviation.

(E) Staining for AP activity at day 10 of reprogramming.

(F) Schematic of the Tcf3 domain structure and Tcf3 mutants used in this reprogramming experiment (i). Reprogramming was performed as in (A), with Tcf3 variants retrovirally expressed throughout reprogramming. AP colony count of a representative reprogramming experiment at day 11 is given (ii).

See Figure S4 for additional information.

during the late phase of reprogramming, even when combined with depletion of *Tcf3* and *Tcf4* (Figure 3C, orange bars). These data are most consistent with the interpretation that the activity of endogenous Wnt signaling during the late phase is necessary to prevent Tcf1 and Lef1 from becoming potent inhibitors of reprogramming. We speculate that, in the absence of active Wnt signaling late in reprogramming, Tcf1/Lef1 are transcriptional repressors at target genes that are essential for the induction of pluripotency. Depleting *Tcf1*/*Lef1* under ‘‘no Wnt’’ conditions would relieve the repressive effect and allow other, alternative pathways to activate these critical genes. Such alternative pathways may also explain why depletion of *Tcf1* and *Lef1* alone did not inhibit reprogramming as seen in Figure 3B. Although we favor this explanation, it is also possible that residual activity of Tcf1 or Lef1 after siRNA knockdown is enough to fulfill a critical function, which could be addressed in the future by using genetic knockout models.

Taken together, our data uncover different requirements of the Tcfs early and late in reprogramming, which is consistent with the changing role of Wnt signaling between the early and late stages. Early in reprogramming, Tcf3 and Tcf4 stimulate reprogramming and are inhibited by active Wnt signaling mediated by Tcf1 and Lef1. Late in reprogramming, Tcf3 and Tcf4 are inhibitory and regulate the activity of the Wnt signaling pathway. We posit that the distinct activities of individual Tcf factors are responsible for the biphasic effects of Wnt signaling on iPSC reprogramming.

Biphasic Effects of Tcf3 Affect the Requirement for Exogenous Reprogramming Factors

Given the fundamental role of Tcf3 in regulating pluripotency in ESCs, we reasoned that further elucidating how Tcf3 contributes to the biphasic Wnt signaling effect during reprogramming to iPSCs would provide the greatest insights into mechanisms of the process. First, we determined if overexpression of Tcf3 would affect the dynamics of reprogramming (Figure 4A). Constitutive Tcf3 expression throughout reprogramming reduced the number of cells positive for the surface marker SSEA1, which marks late reprogramming intermediates (Stadtfeld et al., 2008), and also decreased the formation of Nanog and *Oct4*-GFP-positive colonies in a dose-dependent manner (Figures 4B, 4C, S4A, and S4B). Proliferation of the reprogramming

culture was not affected by Tcf3 expression (data not shown), and quantitative PCR (qPCR) confirmed the reduction of *Nanog* and *Esrrb* transcripts in Tcf3-expressing reprogramming cultures (Figures S4C–S4E), confirming that Tcf3 overexpression is incompatible with late stages of iPSC formation. However, the expression of an early marker of reprogramming, E-cadherin (Cdh1), which marks the mesenchymal-to-epithelial transition (Samavarchi-Tehrani et al., 2010), was increased when Tcf3 was overexpressed (Figure 4D). Similarly, Tcf3 overexpression resulted in a dramatic increase in alkaline phosphatase (AP)-positive colonies, which normally arise at a midpoint of reprogramming (Figure 4E). Together, these data suggest that Tcf3 overexpression stimulates early reprogramming events and colony formation but inhibits later events, including pluripotency gene induction. These data are in agreement with the observation that depletion of endogenous *Tcf3*/*Tcf4* early in reprogramming is inhibitory (Figure 2), whereas depletion late promotes reprogramming (Figure 3).

Tcf3 has been described to function in mice exclusively as a transcriptional repressor, whereas the other Tcfs have been shown to be able to switch between repressor and activator states (Wu et al., 2012; B.J.M., unpublished data). The effects of Tcf3 overexpression on reprogramming enabled mutational analysis of the domains of Tcf3 required for stimulation of AP-positive colony formation using previously characterized mutants. Tcf3 mutants that lack the domain responsible for the interaction with β-catenin (ΔN) or Ctbp (ΔC) repress Tcf-β-catenin target genes similarly to wild-type Tcf3. Those that lack the groucho-interaction region (ΔG) or carry point mutations in the HMG DNA-binding domain (ΔH) do not repress Tcf-β-catenin target genes (Merrill et al., 2001; Figure 4Fi). During reprogramming, all Tcf3 mutants were expressed at similar levels and localized to the nucleus, ruling out the possibility that differences between mutants could be due to lack of expression or different subcellular localization (Figures S4F and S4G). Similar to wild-type Tcf3, expression of ΔC and ΔN mutants increased the number of AP-positive colonies (Figure 4Fii). By contrast, the ΔG and ΔH mutants that lacked repressor activity also lacked the ability to stimulate AP colony formation (Figure 4Fii). Therefore, direct binding of Tcf3 to DNA and Tcf3's repressor activity are important for stimulating the early phase of reprogramming.

To determine whether the effects of endogenous Tcf3 were modulated by the reprogramming factors, iPSC reprogramming was examined using all possible combinations of reprogramming factors. For these experiments, we established the genetic ablation of *Tcf3* during reprogramming by employing MEFs homozygous for a conditional *Tcf3* allele (Merrill et al., 2004) that also carry an estrogen receptor-tagged *Cre* recombinase transgene. These MEFs were initially transduced with separate retroviruses to express the reprogramming factors Oct4, Sox2, cMyc, and Klf4, and after splitting, half of the reprogramming culture was treated with tamoxifen (Tam) to induce *Tcf3* ablation. Upon 24 hr of exposure to Tam, excision of the loxp-flanked cassette (Figure 5A) and elimination of Tcf3 protein occurred efficiently (Figure 5B). Deletion of *Tcf3* increased the number of Nanog-positive colonies consistently but less than 2-fold without enhancing the kinetics of the process (Figure 5Ci). A similar effect due to *Tcf3* deletion was also observed when cMyc was omitted from the reprogramming factor cocktail (Figures 5Cii, 5D, and S5A). The enhancement of OSCK and OSK reprogramming by *Tcf3* loss was observed in media containing fetal bovine serum or knockout serum replacement, which is known to enhance reprogramming (Esteban et al., 2010; Figure S5B) and was not simply a consequence of an increased proliferation rate (Figures S5C and S5D). Deletion of *Tcf3* at the very beginning of the reprogramming process reduced the enhancing effect and yielded only a few more Nanog-positive colonies than control (Figure S5E), indicating that constitutive ablation of *Tcf3* throughout the entire reprogramming process causes only a minor increase in the overall efficiency. These data are consistent with our findings that the timing of Tcf3 activity is critical, due to the biphasic nature of Wnt effects on iPSC reprogramming.

Of all the possible combinations of reprogramming factors, ablation of *Tcf3* caused the strongest effect on OCK reprogramming. Previous studies have reported that reprogramming in the absence of ectopic Sox2 results in the generation of partially reprogrammed ESC-like colonies, in which the pluripotency network is not activated (Takahashi and Yamanaka, 2006). Initially, we found that a very small number of these ESC-like colonies obtained upon OCK-induced reprogramming expressed Nanog in the complete absence of *Tcf3* (Figures 5D and S5F), indicating that constitutive *Tcf3* deletion enabled OCK reprogramming but at an extremely low rate and with dramatically delayed kinetics compared to OSK or OSCK reprogramming. However, passaging-dependent mechanisms magnified this effect. Specifically, we observed that ESC-like colonies isolated and expanded from a $Tcf3^{-/-}$ OCK reprogramming culture at day 30 induced Nanog expression with high efficiency, whereas Nanog remained largely undetectable when clones from a parallel $Tcf3^{+/+}$ OCK reprogramming culture were expanded (Figure S5G). Similarly, splitting $Tcf3^{-/-}$ OCK reprogramming cultures resulted in the induction of Nanog expression in many colonies (Figures 6C and 6D).

These Nanog-positive $Tcf3^{-/-}$ OCK-reprogrammed cell lines displayed silencing of retroviral reprogramming factor expression and lacked *Tcf3* and retroviral *Sox2* integration (Figures S5H and S5I). Hierarchical clustering and Pearson correlation of genome-wide gene expression data showed that OSK and OCK $Tcf3^{-/-}$ iPSC lines were similar to wild-type ESCs and iPSCs and clearly different from MEFs and a line of partially reprogrammed OCK pre-iPSCs (Figures 5E and S5J; Tables S1 and S2). $Tcf3^{-/-}$ reprogrammed lines also produced teratomas with three embryonic germ layers (Figure S5K) and upregulated markers of each germ layer during embryoid body differentiation, albeit with delayed kinetics relative to wild-type iPSCs (Figure 5F), which is a characteristic of $Tcf3^{-/-}$ ESCs compared to wild-type ESCs (Yi et al., 2008). Furthermore, $Tcf3^{-/-}$ iPSC lines bear similar expression differences as $Tcf3^{-/-}$ ESCs when compared to their wild-type counterparts (Figure S5L), further indicating that they closely resemble $Tcf3^{-/-}$ ESCs. Together, these data demonstrate that reprogramming in the absence of Tcf3 and ectopic Sox2 yields bona fide iPSCs.

Given that *Tcf3* deletion is advantageous for the late stage of OSCK reprogramming and enabled completion of OCK reprogramming, we tested whether partially reprogrammed colonies that normally are the end-product of OCK reprogramming

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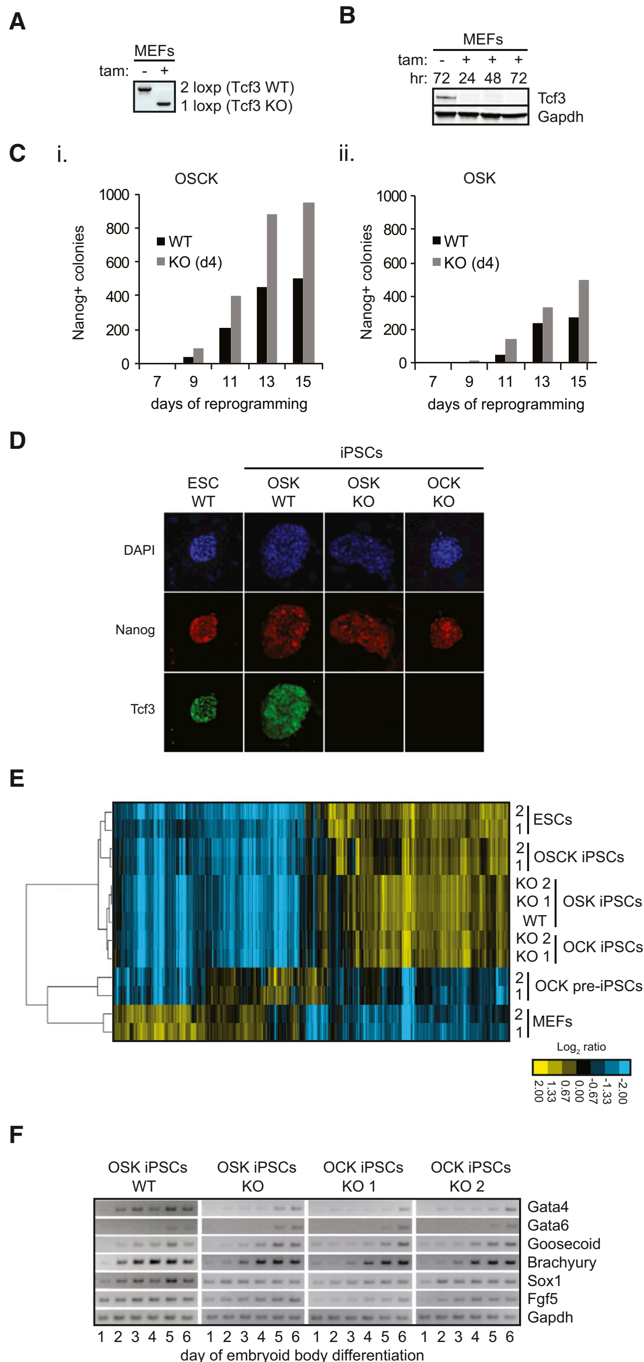

Figure 5. *Tcf3* Ablation Allows Reprogramming in the Absence of Sox2

(A) PCR genotyping of $Tcf3^{2loxp/2loxp}$;Tg(*UBC-Cre-ERT2*) MEFs after treatment with 1 μM tamoxifen (tam) or vehicle for 24 hr. Cells with homozygous 1loxp alleles are referred to as KO or *Tcf3*−/−, and cells with homozygous 2loxP alleles as WT or *Tcf3*+/+.

(B) Western blot for Tcf3 on MEFs described in (A) 24–72 hr after tam addition. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a loading control.

(C) $Tcf3^{2loxp/2loxp}$;Tg(*UBC-Cre-ERT2*) MEFs were transduced with separate retroviruses encoding OSCK (i) or OSK (ii) and treated with and without tamoxifen, respectively, at day 4 to delete *Tcf3*. Nanog-positive colonies were quantified at the indicated days of reprogramming by fixing parallel reprogramming wells and immunostaining for Nanog.

(D) Immunostaining for Nanog and Tcf3 in iPSC lines isolated from OSK or OCK reprogramming cultures in which *Tcf3* was deleted at day 4. DAPI staining marks nuclei. ESCs and OSK WT iPSCs serve as controls.

(E) Hierarchical clustering of log_2 expression ratios of indicated cell lines relative to the average intensity of each probe across all arrays. Only probes 2-fold differentially expressed between ESCs and MEFs were included.

(F) *Tcf3* WT and *Tcf3* KO OSK and OCK iPSC lines were differentiated by embryoid bodies. RNA was harvested at indicated time points and analyzed by semiquantitative RT-PCR for expression of representative genes of each of the three embryonic germ layers. GAPDH serves as a loading control.

See Figure S5 and Tables S1 and S2 for additional information.

(OCK pre-iPSCs), characterized by an ESC-like morphology and lack of pluripotency network expression (Sridharan et al., 2009; Takahashi and Yamanaka, 2006), are blocked from reaching pluripotency by Tcf repressor activity. Notably, knockdown of *Tcf3* and/or *Tcf4* yielded a large number of *Nanog*-GFP-positive colonies as early as 72 hr post-siRNA transduction, whereas *Tcf1* knockdown did not induce *Nanog*-GFP expression (Figures 6A, 6B, and S6A–S6C). *Tcf3* and *Tcf4* knockdown in OCK pre-iPSCs induced the Wnt signaling target genes *Lef1*, *Tcf1*, and *Axin2* (Figure S6D), and the concurrent knockdown of *Tcf1* dramatically inhibited the appearance of *Nanog*-GFP-positive colonies without affecting overall colony morphology or cell number (Figures 6B, S6E, and S6F). *Lef1* siRNA knockdown did not affect the OCK pre-iPSC to iPSC transition (data not shown). Together, these data indicate that *Tcf3* and *Tcf4* knockdown can rapidly trigger induction of pluripotency in OCK pre-iPSCs. Furthermore, the transition from OCK pre-iPSCs to pluripotency appears to require a similar mechanism as the late stage of OSCK reprogramming (i.e., a Tcf1/Lef1-dependent pathway, likely requiring active Wnt signaling).

Expression Changes due to Tcf3 Ablation Differ Early and Late in Reprogramming

To determine downstream genes mediating the effects of Tcf3, we analyzed the gene expression changes in OCK reprogramming cultures in the presence and absence of *Tcf3*. Parallel $Tcf3^{+/+}$ and $Tcf3^{-/-}$ OCK reprogramming cultures were split at day 21 to enhance the Tcf3-mediated reprogramming effect, and RNA samples were collected at several time points throughout the reprogramming experiment (Figures 6C and 6D). qPCR confirmed the decrease of *Tcf3* messenger RNA (mRNA) levels upon activation of *Cre* and the increase in *Nanog* transcript levels in the $Tcf3^{-/-}$ OCK reprogramming culture at late time points (Figure S6G). None of the tested endogenous Sox family members were precociously upregulated in the absence of *Tcf3* (Figure S6G), thereby discounting a simple mechanism by which *Tcf3* ablation could enable the induction of pluripotency in the absence of ectopic Sox2 (Nakagawa et al., 2008).

We next combined our genome-wide gene expression data with unsupervised short time-series expression miner (STEM) analysis (Ernst and Bar-Joseph, 2006) to capture expression differences and groups of coregulated genes between the $Tcf3^{+/+}$ and $Tcf3^{-/-}$ OCK reprogramming cultures (Tables S1, S2, and S3). The three most significant groups of coregulated genes are depicted in Figure 6E (Table S4). Group 1 genes are more

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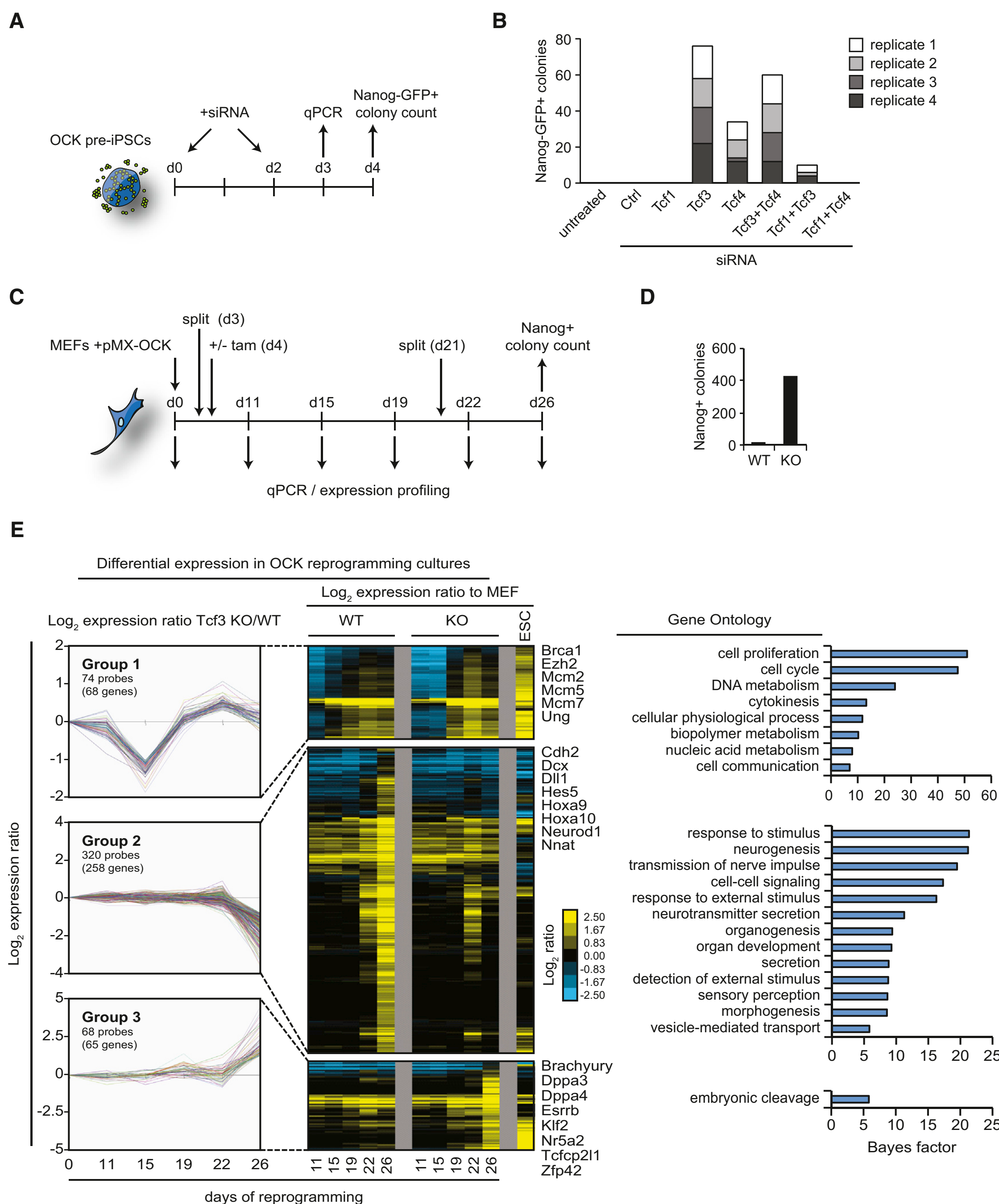

Figure 6. Regulation of OCK Reprogramming by Tcf3

(A) Schematic of the OCK pre-iPSC reprogramming experiment. Different siRNAs were transfected twice into wild-type OCK pre-iPSCs containing the *Nanog*-GFP reporter. Knockdown efficiency was assessed by qPCR 3 days after the initial transfection, and GFP-positive colonies were quantified 4 days after the initial siRNA transfection.

(B) *Nanog*-GFP positive colony count for a representative experiment with four technical replicates per condition is shown.

(C) Schematic of the OCK reprogramming experiment used for gene expression analysis. *Tcf3*$^{2loxp/2loxp}$;Tg(*UBC-Cre-ERT2*) MEFs were infected retrovirally with OCK and split on day 3. Half of the culture was treated with tamoxifen (tam) at day 4 to generate the Tcf3 KO condition, and the other half was exposed to vehicle control (WT). Both WT and KO reprogramming cultures were split again on day 21 to enhance the effect of *Tcf3* deletion on the induction of pluripotency. RNA from KO and WT reprogramming cultures was harvested at indicated time points from parallel reprogramming wells and analyzed for gene expression.

(D) Quantification of Nanog-positive colonies at day 26 of the OCK reprogramming experiment described in (C).

(E) STEM analysis for all transcripts that are at least 2-fold differentially expressed between *Tcf3* KO and WT OCK reprogramming cultures at any of the profiled time points during OCK reprogramming. The top three groups with significant patterns of coregulated gene expression changes are shown, and the number of

(legend continued on next page)

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highly expressed in ESCs than MEFs, initially (at day 15) expressed at lower levels in the *Tcf3*$^{-/-}$ reprogramming culture compared to the *Tcf3*$^{+/+}$ culture but slightly surpassed the levels of the *Tcf3*$^{+/+}$ culture by day 22. Based on gene ontology (GO) analysis, these genes function in the regulation of cell proliferation (Figure 6E). Group 2 genes are strongly induced in the *Tcf3*$^{+/+}$ reprogramming culture but not in the *Tcf3*$^{-/-}$ culture at day 26 and are implicated in morphogenesis and neuronal differentiation. Group 3 genes are more highly expressed in the *Tcf3*$^{-/-}$ reprogramming culture at day 26 and include several pluripotency-related genes, such as *Zfp42*, *Dppa3, Esrrb,* and *Tcfcp2l1*, consistent with the induction of faithful reprogramming specifically in the absence of *Tcf3*. These data indicate that OCK-transduced MEFs progress faster into an intermediate reprogramming state in the presence of *Tcf3* but then upregulate various lineage regulators later in the reprogramming process. Given that the expression of developmental genes has been suggested to be a barrier to reprogramming (Mikkelsen et al., 2008), these genes could block the entry into pluripotency. In the absence of *Tcf3*, the upregulation of a large number of developmental genes appears to be efficiently suppressed, which could overcome the pluripotency blockade.

To confirm that the suppression of developmental genes late in reprogramming is not simply a consequence of expression changes that occurred earlier in the process due to continuous *Tcf3* deletion, we determined direct expression changes upon *Tcf3* depletion in a late reprogramming stage. Depletion of *Tcf3* in OCK-pre-iPSCs led to the downregulation of a similar group of developmental genes as defined by group 2 (Figure S6H; Table S5). Interestingly, active Wnt signaling is known as a negative regulator of neural genes (Aubert et al., 2002; Yoshikawa et al., 1997). Because Wnt target genes, such as *Tcf1*, *Lef1*, *Cdx1*, and *Brachyury*, were upregulated both in late *Tcf3*$^{-/-}$ reprogramming cultures and *Tcf3*-depleted OCK-pre-iPSCs (Figures S6D and S6I) and active Wnt signaling is required for the enhancing effects of *Tcf3* deletion late in reprogramming (Figures 3 and 6B), the induction of Wnt signaling upon *Tcf3* deletion may therefore be directly responsible for the suppression of neural genes late in reprogramming.

Taken together, these data demonstrate that Tcf3 has different targets in the early and late stages of the process, which is consistent with the biphasic role of Wnt signaling during reprogramming.

Stage-Specific Modulation of Tcf3 Levels Enables Efficient OCK Reprogramming

The biphasic response to Wnt signaling and stage-specific effects of Tcfs indicate that, to arrive at the pluripotent state, individual cells progress through a Wnt "low" (or Tcf3 high) state followed by progression through a Wnt "high" (or Tcf3 low) activity state. To test this idea directly, we established a system that allowed us to manipulate *Tcf3* levels in a stage-dependent manner, where each cell expressed high *Tcf3* levels at the early stage and reduced *Tcf3* levels at the late stage of reprogramming (Figure 7A). Based on our data, we reasoned that elevated *Tcf3* should promote early reprogramming events and subsequent depletion of *Tcf3* would then promote late events. This hypothesis was tested in the context of OCK reprogramming, the best system to observe reprogramming enhancement in a *Tcf3*-dependent manner. We expressed *Tcf3* at a range of levels early in OCK reprogramming, from day 1 to day 8, taking advantage of a doxycycline-inducible expression system (Figure 7A). At 0 and 0.002 μg/ml of dox, representing MEF and ESC-like mRNA levels of *Tcf3*, respectively (Figure 7B), OCK reprogramming cultures appeared similar at day 8 of reprogramming, displaying nascent colonies (Figure S7A). At much higher *Tcf3* levels induced by 0.02 μg/ml dox (Figure 7B), more and bigger colonies emerged (Figure S7A). On day 8, dox was withdrawn to stop *Tcf3* overexpression and siRNAs targeting *Tcf3* were added to ensure the reduction of *Tcf3* in the late phase (Figure 7A). Reprogramming cultures were monitored daily for *Oct4*-GFP-positive colonies, prompting the following conclusions (Figure 7C): (1) *Tcf3* overexpression early in OCK reprogramming is not sufficient for the induction of reprogrammed cells. (2) *Tcf3* knockdown late, without prior overexpression of *Tcf3,* only yielded rare *Oct4*-GFP-positive colonies similar to our findings described in Figures 5 and S5. (3) Induction of ESC-like transcript levels of *Tcf3* early (0.002 μg/ml dox) followed by *Tcf3* knockdown late resulted in large numbers of *Oct4*-GFP-positive colonies. (4) Very high levels of *Tcf3* (0.02 μg/ml dox) early in reprogramming eventually gave rise to some *Oct4*-GFP-positive colonies when combined with *Tcf3* knockdown late, albeit with lower efficiency, even though this condition resulted in the most promising induction of ESC-like colonies at day 8, indicating that the exact levels of *Tcf3* early in reprogramming are critical.

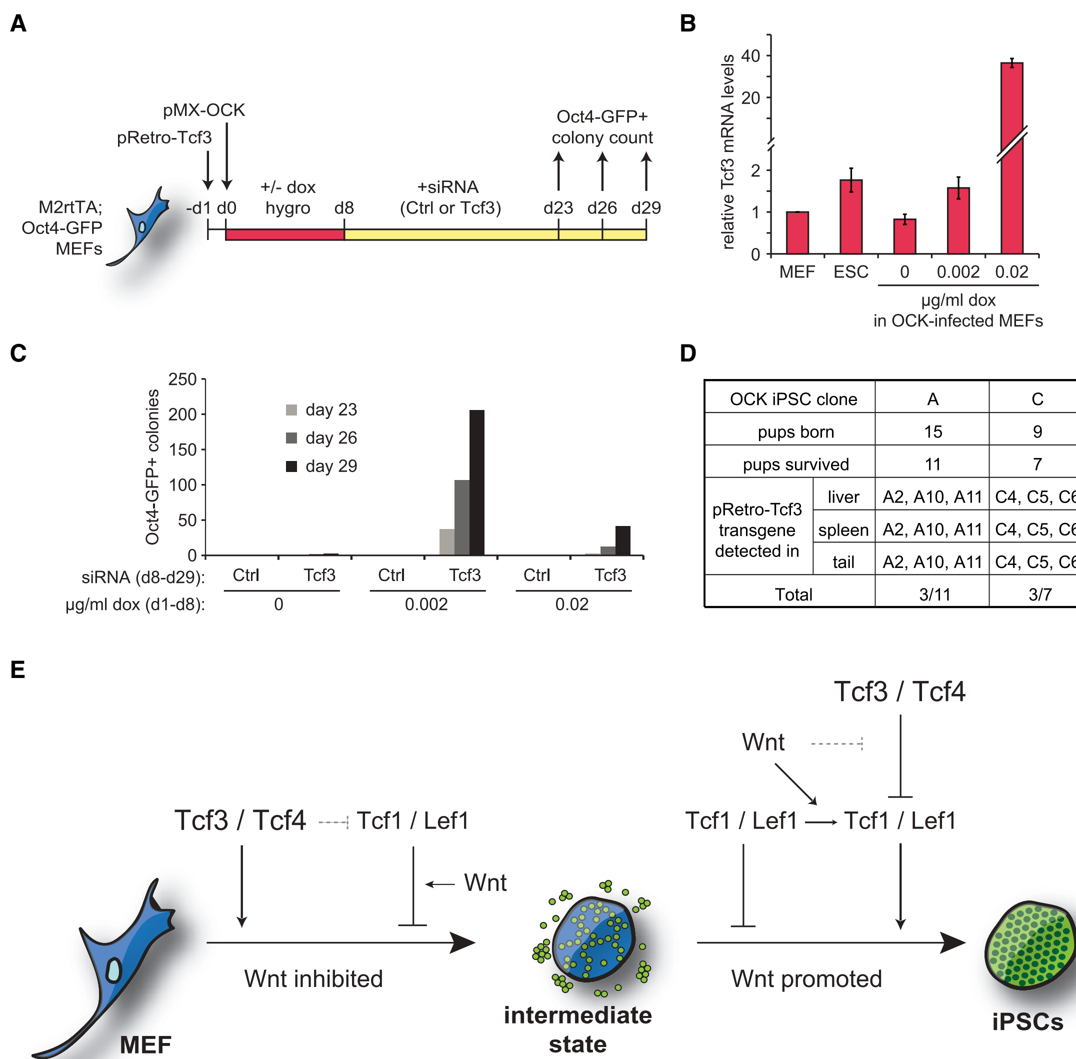
Three *Oct4*-GFP-positive OCK colonies, treated with 0.002 μg/ml dox, and subsequent *Tcf3* siRNA knockdown were stably expanded from this experiment and confirmed to lack the *Sox2* reprogramming vector (Figure S7B). These cell lines exhibited typical characteristics of pluripotent stem cells; in addition to their ESC-like morphology, they have silenced the retroviral expression of the reprogramming factors (Figure S7C), expressed the endogenous pluripotency genes *Sox2* and *Nanog,* and displayed ESC-like *Tcf3* transcript levels (Figures S7D and S7E). Upon blastocyst injection of two clones, we received pups with contribution of iPSCs to various tissues, as tested by PCR for the retroviral *Tcf3* transgene (Figures 7D and S7F).

Taken together, this experiment provides the proof of principle that, during reprogramming, cells transition through stages in which the activity of the Wnt/Tcf machinery dramatically differs and where precise levels of *Tcf3* are critical for achieving successful reprogramming.

DISCUSSION

Somatic cells en route to the pluripotent state undergo specific events, starting with the loss of somatic cell identity and

probes and genes belonging to each group is indicated. Left: $\log_2$ expression ratio between *Tcf3* KO and WT cultures for the probes in each group; middle: heat maps displaying the $\log_2$ expression ratios of probes belonging to these groups for both WT and KO reprogramming cultures, respectively, and of ESCs, all relative to MEFs; and right: significantly enriched GO terms for each group. Example genes from each group are also given.
See Figure S6 and Tables S1, S3, S4, and S5 for additional information.



OCK iPSC clone		A	C
pups born		15	9
pups survived		11	7
pRetro-Tcf3 transgene detected in	liver	A2, A10, A11	C4, C5, C6
	spleen	A2, A10, A11	C4, C5, C6
	tail	A2, A10, A11	C4, C5, C6
Total		3/11	3/7

Figure 7. Stepwise Modulation of *Tcf3* Levels Enables Efficient Reprogramming in the Absence of Sox2

(A) Scheme for the OCK reprogramming experiment with *Tcf3* level modulation. MEFs carrying the *M2rtTA* and *Oct4-GFP* transgenes were infected with a dox-inducible retrovirus (pRetro) encoding *Tcf3* and subsequently with constitutive retroviruses (pMX) encoding *OCK*. Dox was added during the first 8 days of reprogramming at different concentrations, and subsequently, *Tcf3* was depleted by repeated siRNA transfection every 3 days beginning on day 8 until day 29. *Oct4*-GFP-positive colonies were quantified at indicated days. Hygromycin was added from d0–d8 of reprogramming to ensure that all cells carry the pRetro-Hygro-*Tcf3*-expressing vector.

(B) Titration of *Tcf3* transcript levels is achieved by varying dox concentrations in OCK reprogramming. *Tcf3* transcript levels were measured in the reprogramming culture at day 4 and are presented relative to MEF levels. Note that 0.002 μg/ml dox induces ESC-like levels of *Tcf3*. Values represent the average of triplicate sampling, and error bars represent standard deviation. Expression of Tcf3 in virtually all cells was confirmed by immunostaining (data not shown).

(C) Quantification of *Oct4*-GFP-positive colonies at indicated days of reprogramming. *Tcf3* was induced at different levels using different amounts of dox, and siRNA was added as indicated.

(D) Characterization of mice obtained upon blastocyst injection of OCK iPSC clones A and C, which were expanded from reprogramming cultures treated with 0.002 μg/ml dox and subsequently depleted for *Tcf3*, as described in (C). Result of PCRs with primers specifically amplifying proviral integrations of pRetro-*Tcf3* on genomic DNA extracted from liver, spleen, and tails are summarized.

(E) Model summarizing the stage-specific roles of Tcfs and Wnt signaling in reprogramming to iPSCs, where the switch in Wnt response is associated with changing requirements for Tcfs and two pairs of Tcf family members, Tcf1/Lef1 and Tcf3/Tcf4, have opposing functions early and late in reprogramming.

See Figure S7 for additional information.

culminating in the expression of the full pluripotency network (Papp and Plath, 2013). In this study, we performed a comprehensive analysis of the role of Wnt signaling and the requirement of its transcriptional effectors Tcf1, Lef1, Tcf3, and Tcf4 in this process. Our work shows that reprogramming of mouse fibroblasts is biphasic with respect to its dependence on endogenous

Wnt signaling, Tcf proteins, and the consequences of ectopic Wnt stimulation (summarized in Figure 7E).

Two phases of Wnt signaling could be temporally separated into early and late stages of reprogramming, which enabled us to study the molecular roles for Wnt and Tcfs during each phase. In the early stage, the activation of Wnt signaling leads to a reprogramming block via Tcf1 and Lef1, likely due to induction of Wnt target genes that interfere with reprogramming events. In contrast, Tcf3 and Tcf4 promote early reprogramming events by repressing Wnt pathway target genes, including *Tcf1* and *Lef1*, and likely other targets not stimulated by Tcf1/Lef1 and active Wnt signaling. The targets of Tcf3/Tcf4 repression interfere with efficient reprogramming when expressed during the early stage. In the late stage, Wnt signaling promotes reprogramming. Interestingly, Tcf1/Lef1 and Tcf3/Tcf4 have opposing roles, as they did in the early stage; however, the relationship between Tcf1/Lef1 and Tcf3/Tcf4 is different compared to the early stage. Our data suggest that Tcf3 and Tcf4 repress the expression of Tcf1 and Lef1 late in reprogramming, thereby limiting the activity of Wnt signaling. Accordingly, deletion of *Tcf3/Tcf4* late in reprogramming enhances iPSC formation through a mechanism that requires Tcf1 or Lef1 and active Wnt signaling. Thus, Tcf1 and Lef1 appear to be critical target genes of Tcf3 and Tcf4 late in reprogramming. We propose that Wnt3a addition stimulates the late stage of reprogramming primarily by making Tcf1/Lef1 strong activators of key target genes and preventing Tcf1/Lef1 from acting as transcriptional repressors. Although we do not exclude a direct effect of Wnt3a on Tcf3 or Tcf4 activity or levels, our results suggest that Wnt stimulation acts upstream of Tcf1/Lef1 to enhance the late reprogramming phase. The late stage of reprogramming is likely unaffected by *Lef1* or *Tcf1* depletion, because alternative pathways are active that can act on a similar set of target genes. One such pathway may be the leukemia inhibitory factor (Lif)/Jak/Stat signaling pathway. Notably, in the presence of Lif, there is no consequence on ESC self-renewal upon *Tcf1* depletion (Yi et al., 2011). However, the ability of Wnt3a to sustain ESC self-renewal upon Lif withdrawal is stimulated by Tcf1 (Yi et al., 2011), indicating a redundancy between distinct signaling pathways in maintaining the pluripotent state, which may extend to a redundancy in acquiring pluripotency.

Throughout reprogramming, we suggest that the grouping of Tcf1/Lef1 versus Tcf3/Tcf4 reflects predominant Wnt-dependent activator functions of Tcf1/Lef1 and repressor functions of Tcf3/Tcf4. The observation that the four Tcfs fall into two distinct groups for their effect on reprogramming to iPSCs provides further insight into the roles of the factors as mediators of Wnt signaling. The grouping of the factors supports the diversification of the Tcf family into isoforms with specialized and distinct activities (Cadigan and Waterman, 2012). This contrasts the switch model pertaining to invertebrates, where a single Tcf gene product performs both activation and repression. The activator effect attributed to Tcf1/Lef1 during reprogramming is consistent with analysis of *Lef1*$^{-/-}$;*Tcf1*$^{-/-}$ double mutant mice, which display a *Wnt3a*$^{-/-}$ like phenotype (Galceran et al., 1999). The repressor activity of Tcf3/Tcf4 is consistent with the β-catenin independent effects caused by conditional *Tcf3* ablation in the skin of *Tcf4*$^{-/-}$ mice (Nguyen et al., 2009).

We made the striking discovery that solely manipulating the levels of Tcf3, from slight overexpression early to depletion late in the process, allows efficient and faithful reprogramming in the absence of ectopic Sox2. On a molecular level, this finding highlights a function of Sox2 that can be complemented by regulators of the Wnt pathway. The recently described competition between Tcf3 and Sox2 for binding at Oct-Sox DNA sites provides a possible mechanistic explanation for the effects of *Tcf3* ablation during late reprogramming (Zhang et al., 2013). Notably, our data highlight that the degree to which Wnt signaling activation and inhibition affect the early and late stages of reprogramming is dependent on the reprogramming factor combination used.

The duality of effects of Wnt during reprogramming provides a strong example of a factor being necessary at one step but being a barrier at a different step of the long reprogramming process. A priori, it is likely that many factors could cause similar biphasic or context-specific effects during reprogramming. Reprogramming methods that account for dynamic changes in signaling requirements, perhaps in other pathways, will more efficiently guide somatic cells into the desired pluripotent state. Moving forward, determining the reprogramming stage-specific target genes of Tcf1/Lef1 and Tcf3/Tcf4 under Wnt "on" and "off" conditions, along with different reprogramming factor combinations, will be a key question to answer to further understand the biphasic action of Wnt signaling in reprogramming to iPSCs.

EXPERIMENTAL PROCEDURES

Expression Constructs, Cell Lines, and Reprogramming Experiments

For reprogramming with retroviral factors, Oct4, Sox2, Klf4, and cMyc were expressed from pMX retroviruses, as previously described (Maherali et al., 2007). For overexpression, the complementary DNAs (cDNAs) encoding full-length *Tcf3* or its domain mutants (Merrill et al., 2001), *Dkk1*, or *Tomato* fluorescent protein (used as control) were also cloned into the pMX vector. For inducible *Tcf3* overexpression experiments, the *Tcf3* cDNA was cloned into the pRetroX-Tight-Hyg vector, allowing doxycycline-inducible expression in MEFs carrying the *M2rtTA* transgene in the *Rosa26* (*R26*) locus. For reprogramming experiments utilizing tet-iOSCK reprogramming factors, MEFs harboring the *R26-M2rtTA* and a single, doxycycline-inducible, polycistronic cassette coding for OSCK (Sommer et al., 2009) in the *Col1A* locus were generated from mice similarly to a published report (Stadtfeld et al., 2010). Some of the MEFs used for reprogramming carried the *Oct4-GFP* transgene (Szabó et al., 2002) or the *GFP* knockin in the endogenous *Nanog* locus (Maherali et al., 2007), as indicated. Reprogramming experiments, various treatments of reprogramming cultures with siRNAs or biologicals, and the characterization of generated iPSC lines were performed as described in the Extended Experimental Procedures.

Genome-wide Expression Profiling

RNA expression profiling was performed on the Affymetrix Gene Chip Mouse Genome 430 2.0 arrays at the UCLA microarray core facility, and a list of all data sets used in this study is given in Table S1. See Extended Experimental Procedures for the expression analysis performed.

RT- and Genotyping PCRs

Total RNA was isolated from cells using the RNeasy kit (QIAGEN) and cDNA generated with Superscript III (Invitrogen). qPCR values were generated using the ddCT method normalized to U6, unless otherwise indicated. Primers used for detecting expression of pMX transgenes and in the embryoid body assays have been previously described (Maherali et al., 2007; Yi et al., 2008). Primer sequences used to measure expression by qPCR and for PCR genotyping are listed in Table S6.

Western Blotting, Immunostaining, AP Detection, Cytometry, and Luciferase Assay

See Extended Experimental Procedures for details and antibodies used.

ACCESSION NUMBERS

Data are available in the Gene Expression Omnibus under accession number GSE46532.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.05.015.

LICENSING INFORMATION

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