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Combinatorial Modulation of Signaling Pathways Reveals Cell-Type-Specific Requirements for Highly Efficient and Synchronous iPSC Reprogramming

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SUMMARY

The differentiated state of somatic cells provides barriers for the derivation of induced pluripotent stem cells (iPSCs). To address why some cell types reprogram more readily than others, we studied the effect of combined modulation of cellular signaling pathways. Surprisingly, inhibition of transforming growth factor β (TGF-β) together with activation of Wnt signaling in the presence of ascorbic acid allows >80% of murine fibroblasts to acquire pluripotency after 1 week of reprogramming factor expression. In contrast, hepatic and blood progenitors predominantly required only TGF-β inhibition or canonical Wnt activation, respectively, to reprogram at efficiencies approaching 100%. Strikingly, blood progenitors reactivated endogenous pluripotency loci in a highly synchronous manner, and we demonstrate that expression of specific chromatin-modifying enzymes and reduced TGF-β/mitogen-activated protein (MAP) kinase activity are intrinsic properties associated with the unique reprogramming response of these cells. Our observations define cell-type-specific requirements for the rapid and synchronous reprogramming of somatic cells.

INTRODUCTION

Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by the enforced expression of transcription factor combinations such as *Oct4*, *Klf4*, *Sox2*, and *c-Myc* (OKSM) (Takahashi and Yamanaka, 2006), generating a unique platform to study developmental processes and model disease in cell culture (Cherry and Daley, 2013). An intriguing hallmark of induced pluripotency is the comparatively low efficiency at which stable pluripotent cell lines are established, which ranges between 0.1% and 10% for most somatic cell types (Stadtfeld and Hochedlinger, 2010). This is associated with the asynchronous reactivation of endogenous pluripotency loci including *Oct4* (also known as *Pou5f1*), *Nanog*, and *Utf1* and a lag phase of 2 or more weeks before a self-sufficient pluripotent state is established, which is only successful in a small subset of cells (Buganim et al., 2012; Polo et al., 2012). Studies in fibroblasts, the most commonly used cells for iPSC derivation, suggest that the gradual establishment of a chromatin environment permissive for OKSM activity may underlie these slow and stochastic reprogramming kinetics (Apostolou and Hochedlinger, 2013; Koche et al., 2011; Soufi et al., 2012). Intriguingly, some somatic cell types appear more amenable for the extensive epigenetic remodeling associated with acquisition of pluripotency. For example, within the hematopoietic system, immature progenitors form iPSCs more readily than differentiated cells (Eminli et al., 2009); however, the molecular reasons for this observation remain unknown.

In agreement with the importance of chromatin remodeling for iPSC formation, small molecule compounds that alter the activity of chromatin-modifying enzymes can facilitate fibroblast reprogramming (Li et al., 2013). An example is the antioxidant ascorbic acid (AA), which serves as a cofactor for α-ketoglutarate-dependent dioxygenases such as Tet proteins and JmjC domain-containing histone demethylases (Monfort and Wutz, 2013). Enhancement of iPSC formation has also been reported upon modulation of cellular signaling pathways. For example, activation of Wnt signaling by natural ligands (Marson et al., 2008) or chemical inhibition of glycogen synthase kinase 3 (GSK3) (Li and Ding, 2010), an antagonist of CTNNB1 (also known as β-catenin), has been shown to promote iPSC formation. However, evidence for an inhibitory role of the Wnt pathway early in reprogramming has also been provided by Aulicino et al. (2014) and Ho et al. (2013). Inhibition of transforming growth factor β (TGF-β) signaling supports both early (Li et al., 2010; Samavarchi-Tehrani et al., 2010) and late events (Ichida et al., 2009; Maherali and Hochedlinger, 2009) during the transition of fibroblasts to a pluripotent state.

Here, we have investigated possible synergisms between compounds that affect chromatin modulators and cellular signaling pathways during iPSC reprogramming. In addition to fibroblasts, we focused on somatic progenitor cells with the goal to identify cell-intrinsic features that facilitate iPSC formation. Taking this approach, we describe cell-type-specific requirements for the rapid and synchronous acquisition of pluripotency and provide molecular

insight into why specific somatic cells are particularly amenable to reprogramming.

RESULTS

Synergism between Small Molecule Compounds Allows Highly Efficient Reprogramming of Mouse Fibroblasts

To study synergisms between signaling pathways during iPSC formation, we focused on TGF-β and Wnt signaling because a role for these pathways in fibroblast reprogramming has been documented, and highly specific compounds targeting them are available. Specifically, we employed the TGF-β antagonist ALK5 inhibitor II ("iAlk5") (Maherali and Hochedlinger, 2009) and the GSK3β antagonist CHIR99021 ("CHIR") (Li et al., 2009), which activates Wnt signaling by stabilizing CTNNB1. In light of the importance of chromatin remodeling for iPSC formation, we included AA, an enzymatic cofactor that facilitates fibroblast reprogramming (Esteban et al., 2010).

We tested the aforementioned compounds using "reprogrammable MEFs" (mouse embryonic fibroblasts) (Stadtfeld et al., 2010) engineered to express a doxycycline (Dox)-inducible cassette encoding OKSM (Sommer et al., 2009) from a single defined genomic position and a GFP reporter from the endogenous *Oct4* locus, whose expression is a hallmark of the pluripotent state. We initially administered Dox and different combinations of compounds for 6 days to reprogrammable MEFs seeded in bulk. This was followed by 3 days of culture in unsupplemented mouse embryonic stem cell (ESC) media to select for fully reprogrammed cells (Figure 1A). In Dox alone, these conditions were insufficient for *Oct4* reactivation or to generate stable ESC-like colonies (Figures 1B and 1C; Figure S1A available online), but each of the three compounds individually allowed small numbers of Dox-independent colonies to emerge, with efficiencies ranging from 0.5% (CHIR) to about 2% (AA and iAlk5) of input cells (Figure 1B). The dual compound combinations of CHIR plus AA and CHIR plus iAlk5 slightly elevated colony numbers (5%), whereas the combination of iAlk5 and AA led to a more dramatic increase (21%) (Figure 1B). This synergistic effect was most pronounced when using all three chemicals together, frequently resulting in *Oct4* reactivation at day 6 (Figure S1A) and stable Dox-independent iPSC colonies at an average efficiency of 41% (Figures 1B and 1C). For simplicity, we will refer to the combination of AA, iAlk5, and CHIR as "3c."

Timed withdrawal experiments revealed that a minimum of 3 days of OKSM expression was sufficient for a small subset (~2%) of MEFs to enter a self-sustained pluripotent state in the presence of 3c. This was followed by a dramatic (~25-fold) increase in colony numbers between days 3 and 6 (Figure 1D), after which colony numbers plateaued. These observations indicate exceptionally rapid and synchronized acquisition of pluripotency, which is in stark contrast to the slow and sporadic reprogramming observed in Dox only (Figure 1D). To accurately determine reprogramming efficiencies, we conducted single-cell seeding experiments with reprogrammable MEFs that harbor a ubiquitously expressed red fluorescent dsRed transgene (Vintersten et al., 2004) (Figure S1B), thus excluding cells that fail to seed and reducing the risk of scoring satellite colonies (Smith et al., 2010). This stringent assay showed that 7 days in Dox plus 3c was sufficient for about 80% of MEFs to give rise to colonies that maintained ESC-like morphology in the absence of exogenous OKSM expression (Figure 1E). This correlated with the reactivation of *Oct4* in the comparatively short time window between day 4 and day 7 in most colonies in Dox plus 3c (Figure 1F). In contrast, less than 2% of MEFs acquired Dox independence after 12 days of OKSM expression in Dox only with *Oct4* reactivation occurring late and in rare colonies (Figures 1E and 1F).

At the molecular level, the synergistic effect exerted by 3c on iPSC formation was accompanied by the upregulation of large numbers of genes including ESC-associated transcripts such as *Tet1*, *Epcam*, *Eras*, and *Nanog* in day 4 reprogramming intermediates (Brambrink et al., 2008; Stadtfeld et al., 2008), which was not seen with individual compounds (Figures 1G and 1H). Gene Ontology (GO) analysis supported the conclusion that many stem cell-specific loci were only activated in the presence of all three compounds, whereas individual chemicals facilitated the silencing of specific aspects of the somatic program, such as collagen synthesis (by iAlk5) and mesodermal differentiation potential (by AA) (Table S1). Immunofluorescence confirmed accelerated reactivation of pluripotency loci such as *Nanog*, *Utf1*, *Lin28*, and *Sall4*, whose protein products were detectable in the majority of nascent colonies at day 6 in Dox plus 3c (Figures S1C and S1D). Together, these observations suggest a strong synergism between 3c compounds and reprogramming factors that allows reactivation of pluripotency loci that remain refractory in Dox-only conditions or in the presence of individual compounds. Colonies that emerged in Dox plus 3c and in Dox alone contained similar numbers of cells. An accelerated cell cycle therefore does not seem to be specifically associated with the dramatically increased reprogramming efficiency (Figure S1E).

Clonal iPSC lines derived in the presence of 3c yielded chimeras with germline transmission (Figure S1F; Table S2). This shows that the enhanced reprogramming kinetics does not come at the expense of impaired functional properties of derivative pluripotent cell lines.

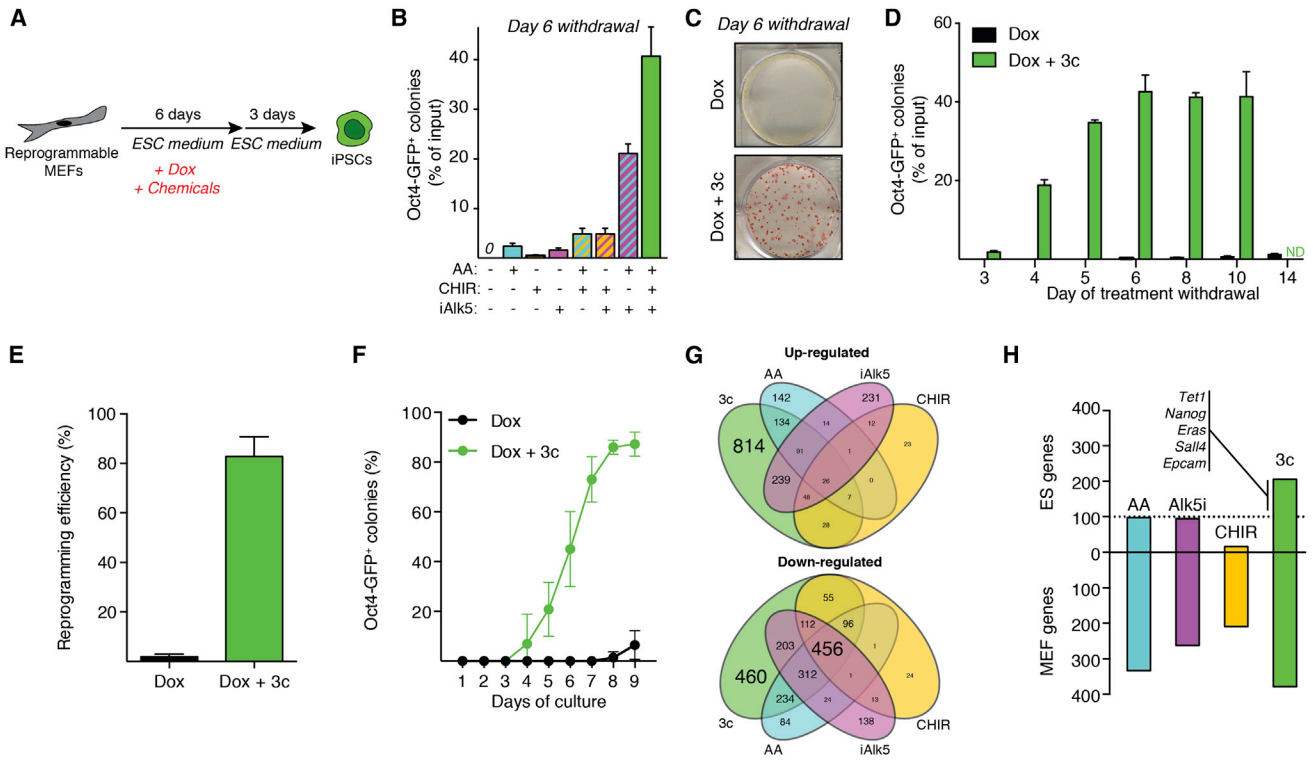

Figure 1. Effect of Combined TGF-β Inhibition, Wnt Activation, and AA on Fibroblast Reprogramming

(A) Approach to identify chemicals that allow MEF reprogramming in 6 days or less.

(B) Percentage of Dox-independent colonies that formed in the presence of indicated compounds after 6 days of exogenous OKSM expression. Shown are the mean and range of two independent experiments.

(C) Representative alkaline phosphatase (AP) staining of Dox-independent colonies that formed after 6 days of OKSM expression from 500 input MEFs.

(D) Percentage of Dox-independent colonies derived after withdrawal of compound on the indicated day (mean and range of two independent experiments). ND, not determined.

(E) Percentage of wells (96-well plate format) seeded with individual dsRed$^+$ reprogrammable MEFs that gave rise to Dox-independent Oct4-GFP$^+$ colonies after 12 days in Dox (n = 3 experiments) or 7 days in Dox + 3c (n = 5 experiments). Error bars show SDs.

(F) Percentage of nascent colonies that contained Oct4-GFP$^+$ cells at the indicated day of reprogramming, measured by fluorescence microscopy. Error bars indicate SDs of 3 independent experiments with at least 20 colonies analyzed.

(G) Venn diagrams of genes at least 1.5-fold up- or downregulated in reprogramming intermediates derived in the presence of indicated compounds compared to Dox alone.

(H) Quantification of the top ESC-specific and MEF-specific genes that showed significant expression changes in intermediates derived in the presence of indicated compounds compared to Dox alone. Select ESC-associated genes exclusively upregulated in Dox plus 3c are indicated.

See also Figure S1.

Synchronous Induction of Pluripotency in Somatic Progenitor Cell Populations

So far, our results show that combined Wnt activation and TGF-β inhibition in the presence of AA greatly facilitates the induction of pluripotency upon OKSM expression in MEFs. We next sought to investigate whether 3c compounds can also enhance the reprogramming of somatic progenitor cells. Upon bulk culture of purified granulocyte-macrophage progenitors (GMPs), an immature blood cell type previously reported to yield iPSCs at efficiencies up to 30% after 2 weeks of OKSM expression (Eminli et al., 2009), we obtained Dox-independent colonies at numbers corresponding to about 50% of input cells after 3 days in Dox plus 3c (Figure S2A). After 6 days—when we observed factor-independent colonies in Dox-only conditions at about 5% efficiency—colony numbers in the presence of 3c exceeded the number of input GMPs, suggesting a propensity to generate satellites.

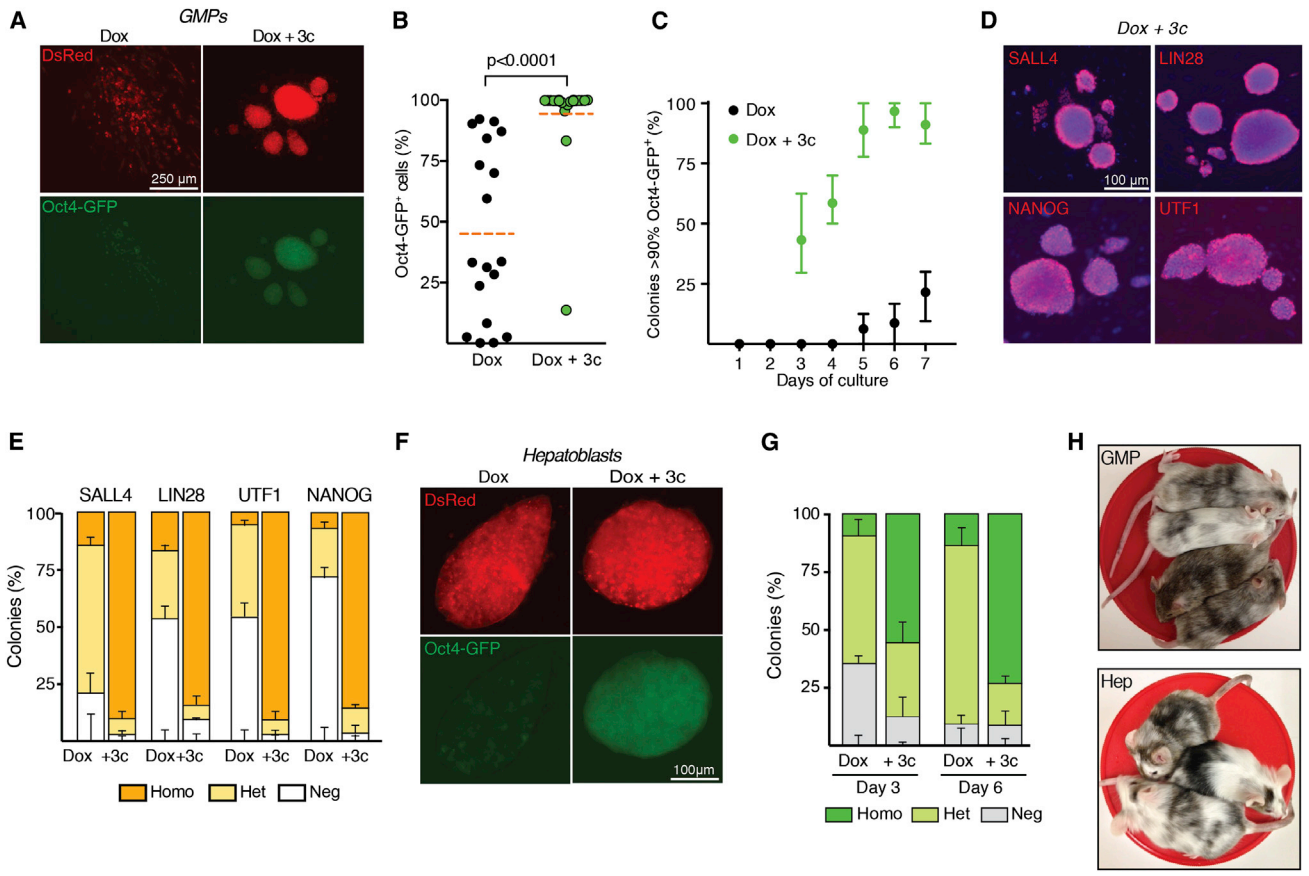

Figure 2. Reprogramming of Blood Progenitors and Hepatoblasts in 3c Conditions

(A) Representative images of *Oct4-GFP* and *dsRed* expression in colonies formed from individual GMPs after 6 days of culture. Note that most dsRed$^+$ cells are Oct4-GFP$^+$ in Dox + 3c, but not in Dox alone.

(B) Percentage of Oct4-GFP$^+$ cells in wells undergoing reprogramming after single-cell seeding of GMPs and culture for 6 days in Dox only (n = 18 clonal colonies) or Dox + 3c (n = 20 clonal colonies), measured by flow cytometry.

(C) Percentage of clonal GMP-derived iPSC colonies in which at least 90% of cells were Oct4-GFP$^+$ at the indicated time points. Shown are the mean and range of 2 or 3 independent experiments with at least 30 colonies analyzed.

(D) Representative superimposed images of GMP-derived colonies on day 4 of reprogramming in Dox + 3c after immunostaining against indicated pluripotency markers and counterstaining with DAPI.

(E) Quantification of GMP-derived colonies that express indicated pluripotency markers on day 4 of reprogramming in a homogeneous (Homo; >90% of positive cells) or heterogeneous (Het; 1%–90% of positive cells) fashion or are marker negative (Neg; <1% of positive cells). Shown are the mean and range of 2 independent experiments with 14 or more colonies scored each.

(F) Representative fluorescence microscopy images of hepatoblast-derived colonies after culture for 6 days.

(G) Quantification of *Oct4* expression in hepatoblast-derived colonies on days 3 and 6 of reprogramming as defined for (E). Shown are the mean and range of 2 independent experiments with 15 or more colonies scored each.

(H) Images of chimeric mice obtained after blastocyst injection of hepatoblast (Hep) iPSCs or GMP iPSCs derived in Dox plus 3c.

See also Figure S2.

Importantly, single-cell seeding of dsRed transgenic GMPs confirmed reprogramming efficiency of ~95% in Dox plus 3c. These experiments also revealed that the overall percentage of colonies containing Oct4-GFP$^+$ cells was similarly high with GMPs and MEFs in Dox plus 3c (Figure S2B). However, whereas almost all nascent GMP-iPSCs in Dox plus 3c expressed endogenous *Oct4* in a homogeneous manner, MEF-derived colonies in Dox plus 3c (as well as GMPs in Dox-only conditions) represented a heterogeneous mixture of Oct4-GFP$^+$ and Oct4-GFP$^-$ cells (Figures 2A and S2B). Quantification by flow cytometry confirmed this difference and documented an average GFP-labeling index of >95% in the presence of but only about 40% in the absence of 3c (Figures 2B and S2C). Time course analyses of clonal GMP cultures revealed that colonies with a GFP-labeling index of greater than 90%

appeared as early as day 3 in Dox plus 3c and frequently encompassed all nascent colonies by day 6 (Figure 2C). At this time, successfully reprogramming cultures initiated with single GMPs in the presence of compounds on average contained over 5,000 Oct4-GFP$^+$ cells and only negligible numbers of GMP-derived Oct4-GFP$^-$ cells (Figure S2D). The pluripotency markers NANOG, UTF1, LIN28, and SALL4 were also expressed homogeneously by the majority of colonies obtained after 4 days of culture in Dox plus 3c (Figures 2D and 2E). These results demonstrate that 3c conditions allow the vast majority of GMPs to acquire pluripotency in a highly synchronous manner upon OKSM expression. In contrast, the frequency of colonies with an Oct4-GFP-labeling index greater than 90% in Dox only remained below 10% (Figure 2C). Indeed, all GMP-derived iPSC colonies in Dox only contained cells that failed to reactivate *Oct4* (Figures 2B and S2D) and expressed NANOG and UTF1 in a heterogeneous manner (Figure S2E).

We next conducted timed Dox-withdrawal experiments with reprogrammable hepatoblasts, an endodermal progenitor cell unrelated to blood progenitors or MEFs that can be prospectively isolated by flow cytometry (Gailhouste, 2012). We observed rapid emergence of Dox-independent colonies upon culture of hepatoblasts in Dox plus 3c, but not Dox only (Figure S2F). Similar to GMPs, in the presence of 3c, the majority of cells within nascent hepatoblast-derived colonies reactivated *Oct4* and expressed LIN28 and NANOG (Figures 2F and S2G). Quantification after single-cell seeding showed *Oct4* expression in 95% of hepatoblast-derived colonies in Dox plus 3c, with about 80% of them—but only few colonies in the presence of Dox only—expressing this locus in a homogeneous manner (Figures 2G and S2H). Stable iPSC lines derived from GMPs and hepatoblasts yielded postnatal chimeras upon blastocyst injection (Figure 2H; Table S2). Together, these observations show that 3c reprogramming in both hepatoblasts and GMPs results in rapid and homogeneous reactivation of ESC-specific loci, which is strikingly different from the stochastic reprogramming response normally associated with iPSC formation.

Specific Requirements of TGF-β and Wnt Signaling Modulation for Progenitor Cell Reprogramming

Our MEF experiments had shown a strong synergism between AA and TGF-β inhibition and maximal reprogramming efficiencies upon further addition of CHIR (see Figure 1B). We therefore asked whether GMPs and hepatoblasts have similar requirements for signaling pathway modulation and determined the efficiencies of iPSC formation upon reprogramming in the presence of only AA, CHIR, or iAlk5 compared to 3c.

Strikingly, for GMPs, supplementation of reprogramming media with CHIR alone allowed the formation of similar numbers of Dox-independent colonies after 6 days of OKSM expression as with Dox plus 3c (Figures 3A and 3B). In contrast, colony numbers decreased 5- to 10-fold when using only AA or iAlk5 (Figure 3B). By fluorescence microscopy, "CHIR-only" colonies derived from single-seeded GMPs showed homogeneous *Oct4* reactivation (Figure S3A), and quantification by flow cytometry confirmed a GFP-labeling index of over 90% (Figure S3B). Of note, other blood progenitor cell populations including common myeloid progenitors (CMPs), megakaryocyte-erythrocyte progenitors (MEPs), and multipotent lineage-negative Sca1$^+$Kit$^+$ (LSK) cells also formed iPSCs with similar efficiencies in Dox plus CHIR and Dox plus 3c (Figure S3C), suggesting that a strong requirement for Wnt signaling activation is a common feature of blood progenitor cell reprogramming.

Other than for GMPs, CHIR had little beneficial effect on the number of iPSC colonies obtained from hepatoblasts (Figure 3C). In contrast, these cells yielded Dox-independent colonies efficiently in the presence of either iAlk5 or AA alone, reaching 60%–80% of the numbers seen with 3c (Figure 3C). Accordingly, both of these compounds facilitated synchronous reactivation of the *Oct4* locus with their combination being equally efficient as 3c (Figure S3D).

To investigate the reasons for these cell-type-specific differences, we studied the expression of TGF-β and Wnt signature genes in MEFs, GMPs, and hepatoblasts. This revealed pronounced differences, with GMPs exhibiting significantly lower levels of gene products associated with both signaling pathways than hepatoblasts and MEFs (Figure 3D). Among the differentially expressed genes were many core pathway components such as *Tgfbr2* and different ligands (for TGF-β) and *Apc*, *Fzd1*, *Fzd2*, and *Ctnnb1* (for Wnt). Because CTNNB1 is an important mediator of canonical Wnt signaling (Nelson and Nusse, 2004), we further investigated its role. CTNNB1 rapidly accumulated in purified GMPs cultured in CHIR, but not Dox only (Figure S3E), consistent with a possible role in the rapid reprogramming response. Indeed, lentiviral introduction of a stabilized variant of CTNNB1 (Rubinfeld et al., 1997) into blood progenitor cells resulted in efficient reactivation of *Oct4* in the presence of Dox alone, which was indistinguishable from the degree of *Oct4* reactivation seen with empty virus-transduced cells reprogrammed in the presence of Dox plus 3c (Figure 3E). We also obtained similar numbers of Dox-independent colonies with CTNNB1-expressing blood progenitors in Dox alone and control cells in Dox plus 3c, whereas no such colonies were seen with control cells in Dox only (Figures 3F and 3G). This demonstrates that ectopic CTNNB1 accelerates the OKSM-driven acquisition of pluripotency similarly efficient as 3c. Enforced *Ctnnb1*

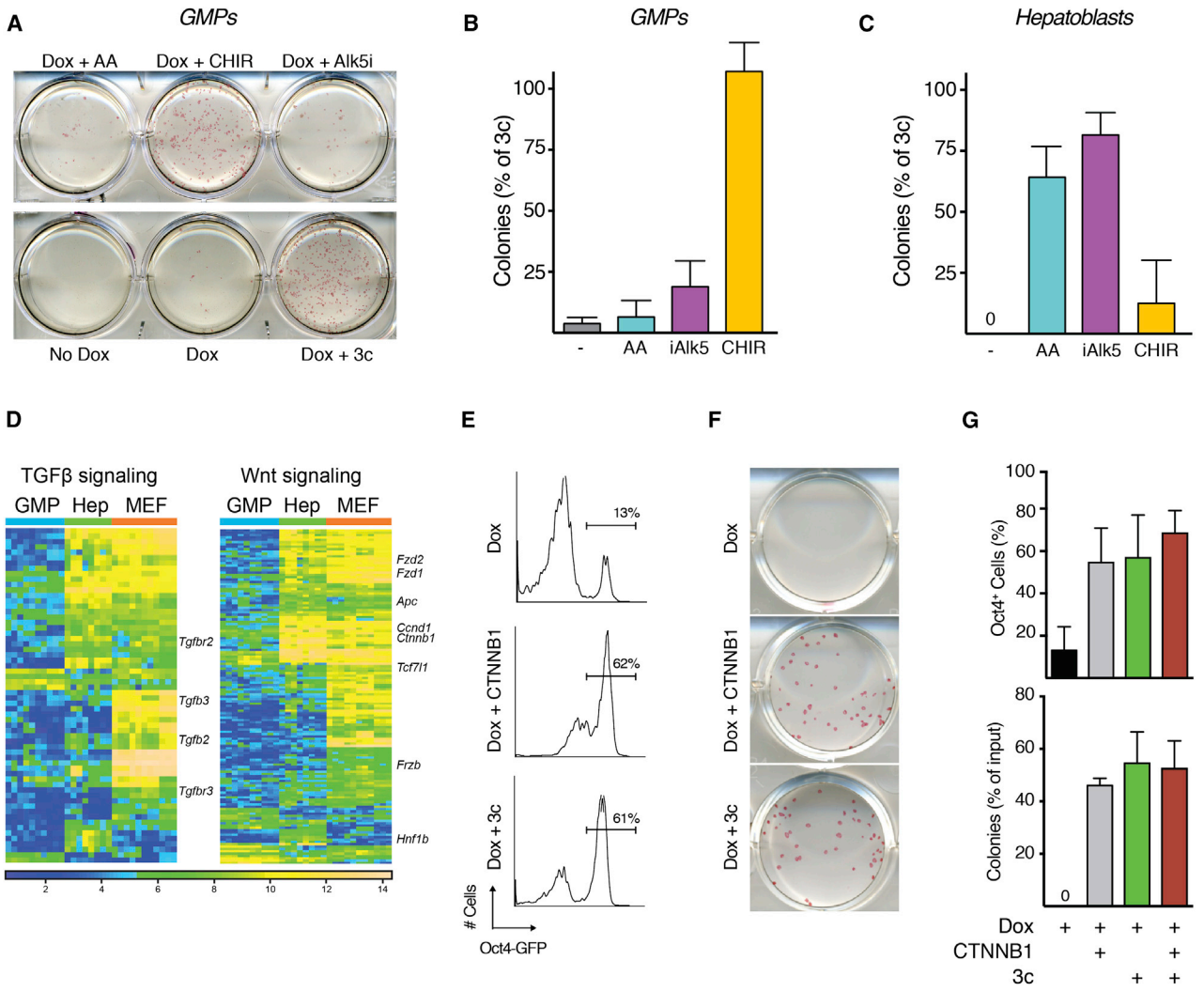

Figure 3. Modulation of Specific Signaling Pathways during Progenitor Cell Reprogramming

(A) AP staining of Dox-independent colonies after 6 days of culture of 100 reprogrammable GMPs in the presence of indicated compounds and 3 days in basal ESC media.

(B and C) Quantification of Dox-independent colonies derived from (B) GMPs and (C) hepatoblasts after 6 days of reprogramming in the presence of Dox and indicated compounds. Colony numbers were normalized to values obtained in Dox plus 3c. Error bars represent the range of 2 independent experiments with at least 50 colonies for GMPs and 14 for hepatoblasts scored in each experiment and condition.

(D) Heatmap showing TGF-β- and Wnt-associated genes differentially expressed (fold change >3; $q < 0.05$) in GMPs, hepatoblasts, and MEFs. Select genes are highlighted.

(E) Representative flow cytometry plots showing *Oct4-GFP* expression after culture of 100 blood progenitor cells transduced with either empty or CTNNB1 virus.

(F) AP staining of Dox-independent colonies formed after 6 days of OKSM expression (in 3 days in basal ESC media) in blood progenitor cells transduced with empty or CTNNB1 virus.

(G) Quantification of *Oct4-GFP* expression and colony formation formed after transducing blood progenitor cells with empty or CTNNB1 virus (n = 3 wells seeded with transduced cells for each condition).

See also Figure S3.

expression in MEFs undergoing reprogramming in Dox alone did not result in early activation of *Oct4* or increased colony numbers (Figures S3F and S3G). Together, these observations show that different cell types require specific signaling pathway modulations for rapid and efficient reprogramming and suggest that CTNNB1-mediated Wnt activation is a major facilitator of blood progenitor cell reprogramming.

Facilitation of Synchronous Reprogramming in Fibroblasts

The results described above provide a rationale for the specific requirements of MEFs, GMPs, and hepatoblasts for TGF-β and Wnt modulation during reprogramming. At the same time, they suggest the existence of additional molecular differences between GMPs and MEFs that facilitate rapid and synchronous iPSC formation from the former. We hypothesized that these differences might encompass other signaling pathways and/or proteins involved in the epigenetic remodeling necessary for reprogramming to pluripotency and subjected the starting cell populations to additional rounds of bioinformatic analyses.

As shown in Figure S4A, we identified mitogen-activated protein (MAP) kinase signaling as significantly more active in MEFs than in GMPs. We therefore reprogrammed MEFs in reprogramming media supplemented with 3c and PD0325901, a specific inhibitor of mitogen-activated kinase kinases 1 and 2 that together with CHIR has been reported to facilitate the conversion of pre-iPSCs into iPSCs (Silva et al., 2008). Reprogramming in such Dox plus 3cM (3c plus MAP kinase inhibition) conditions did not affect the percentage of colonies that contained Oct4-GFP$^+$ cells compared to 3c, but nascent 3cM colonies exhibited a more ESC-like morphology and significantly more homogeneous reactivation of *Oct4* (Figures 4A and S4B). Nevertheless, the majority of colonies in Dox plus 3cM still contained cells that failed to reactivate *Oct4*, suggesting the existence of additional reprogramming barriers in MEFs. Intriguingly, we found that many known epigenetic facilitators of iPSC formation (Apostolou and Hochedlinger, 2013; Papp and Plath, 2013) are expressed at higher levels in GMPs than in MEFs (Figure 4B; Table S3). We therefore used a gain-of-function approach to assess if any of these enzymes might elicit a reprogramming response in MEFs that is similar to GMPs and cloned factors that represent different chromatin-modifying activities into lentiviral expression constructs. We chose KDM2B (a H3K36me2 demethylase) (Wang et al., 2011), KDM6A (a histone H3K27me3 demethylase) (Mansour et al., 2012), WDR5 (a histone methyltransferase complex component) (Ang et al., 2011), EZH2 (a component of polycomb repressive complex 2) (Onder et al., 2012), SMARCC1 (an ATP-dependent chromatin-remodeling complex component) (Singhal et al., 2010), and the catalytic domain of the methylcytosine dehydrogenase TET1, which is functionally equivalent to the catalytic domains of TET2 and TET3 (Hu et al., 2014). When culturing reprogrammable MEFs transduced with these vectors in Dox plus 3c, we observed a strong and highly significant increase in the number of Oct4-GFP$^+$ cells with KDM2B and a more modest response with SMARCC1 (Figure 4C). We did not observe a positive effect on *Oct4* reactivation with any of the other factors (Figure 4C), suggesting that H3K36me2 demethylation but not the other tested enzymatic activities are rate limiting during 3c reprogramming of MEFs. Ectopic expression of *Kdm2b* also shortened the time of Dox exposure required for MEFs to enter a self-sustained pluripotent state (Figures 4C and 4D). Introduction of KDM2B into MEFs cultured in reprogramming media supplemented with 3cM resulted in *Oct4* reactivation in about 80% of day 8 intermediates, suggesting synergism between this enzyme and MAP kinase inhibition (Figure 4D). We also observed homogeneous reactivation of *Oct4*, *Sall4*, and *Nanog* in the majority—about 70%—of nascent colonies in Kdm2b plus 3cM conditions (Figures 4E and S4E). Together, these results are consistent with the notion that reduced MAP kinase activity and elevated expression of specific pluripotency-associated epigenetic regulators contribute to the unique reprogramming response observed in GMPs, which in part can be established in MEFs.

DISCUSSION

In this study, we used a controlled genetic system to investigate to what extent the combinatorial modulation of TGF-β and Wnt signaling together with the enzymatic cofactor AA ("3c conditions") can facilitate iPSC derivation from specific somatic cell types. Although these compounds individually have been reported to moderately facilitate MEF reprogramming, our results demonstrate a strong synergism between them, which was previously not appreciated. By extending our studies to hepatoblasts and blood progenitor cells, we were able to identify cell-type-specific requirements for highly efficient and synchronized reprogramming to pluripotency. Importantly, for each of these cell types, we achieved reprogramming efficiencies of 80% and greater after a week of OKSM expression, which are among the highest reported to date. This suggests that a chromatin state allowing OKSM to engage with appropriate target genes (Soufi et al., 2012) is quickly established during 3c reprogramming. The early upregulation of ESC-specific genes, which are normally subject to silencing mechanisms such as DNA methylation in somatic cells (Papp and Plath, 2013), supports this conclusion. Whether 3c compounds modulate the activity of molecules previously implicated in synchronized iPSC reprogramming responses such as MBD3, C/EBPA or TET2 (Rais et al., 2013; Di Stefano et al., 2014; Doege et al., 2012) warrants further investigation.

Of the somatic cells included in our study, MEFs revealed the most complex requirements for signaling pathway modulation and allowed highly efficient iPSC formation only in the presence of all three compounds. In contrast,

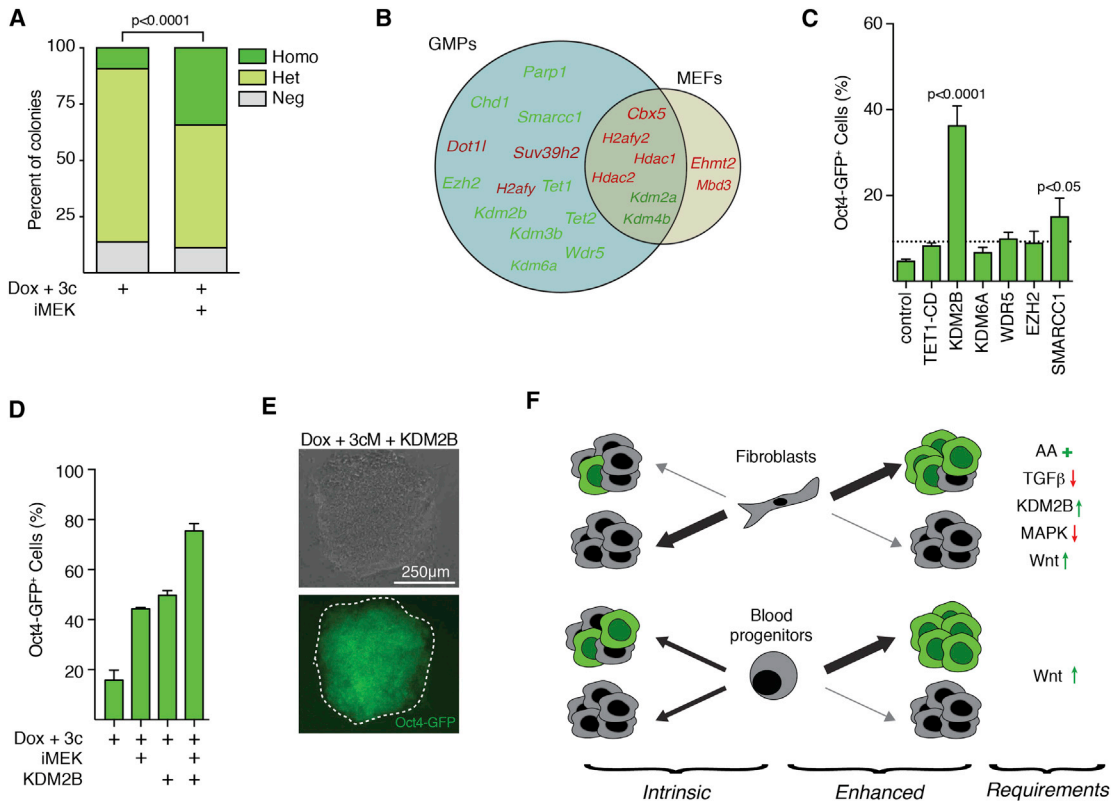

Figure 4. Factors Facilitating Synchronous MEF Reprogramming

(A) Quantification of MEF-derived colonies that express *Oct4-GFP* in a homogeneous or heterogeneous manner or that are negative after 7 days of culture in Dox + 3c or Dox + 3cM. The results are representative of 3 independent experiments with at least 80 colonies scored. p values for homogeneous colonies were determined with Fisher's exact test.

(B) Venn diagram showing whether chromatin regulators implicated in enhancing (green) or repressing (red) reprogramming are expressed at higher levels in GMPs (blue circle) or MEFs (brown circle). Gene names in bold indicate higher expression in pluripotent cells than in MEFs.

(C) Quantification of *Oct4-GFP* expression upon ectopic introduction of indicated chromatin regulators in reprogrammable MEFs cultured in Dox plus 3c for 8 days. p values were calculated with one-way ANOVA (n = 3 or more independent transduction experiments).

(D) Quantification of *Oct4-GFP* expression in colonies derived upon transduction with empty or KDM2B virus and culture for 8 days in Dox + 3c or + 3cM (n = 3).

(E) Representative phase-contrast and fluorescence images showing *Oct4-GFP* expression in a colony obtained after *Kdm2b* expression in Dox + 3cM.

(F) Model depicting characteristic features of the intrinsic and enhanced reprogramming response of fibroblasts and blood progenitor cells upon OKSM expression. Cell-type-specific requirements for achieving enhanced reprogramming, such as repression of TGF-β signaling and increased activity of KDM2B in fibroblasts, are shown. Accumulations of cells represent clonal colonies, with green cells successfully reprogramming and gray cells failing to reprogram. Thickness of arrows indicates the proportion of particular types of colonies that are observed under given conditions.

See also Figure S4.

hepatoblast reprogramming was greatly facilitated by TGF-β inhibition alone, whereas GMPs required only Wnt activation—either by GSK3β inhibition or by enforced expression of *Ctnnb1*—to rapidly reactivate pluripotency loci and to enter a pluripotent state. These cell-type-specific requirements are reflected in the relative strength of TGF-β and Wnt signaling in the starting cell populations, suggesting a way to prospectively identify somatic cell types particularly amenable to factor-mediated reprogramming. The observation that AA, a cofactor of chromatin-modifying enzymes, did not significantly enhance iPSC formation from blood progenitor cells indicates that an epigenetic state favorable for reprogramming might preexist in these cells. However, we cannot rule out that AA modulates GMP reprogramming in a way not measured by our assays.

We observed marked differences between fibroblasts and somatic progenitor cells with respect to the synchronicity of reprogramming, defined as the percentage of cells within an emerging colony that expresses ESC-specific genes. Thus, whereas GMPs and hepatoblasts rapidly gave rise to colonies containing predominantly cells that had reactivated endogenous pluripotency loci, we only infrequently observed nascent MEF-iPSCs with these characteristics (see the model in Figure 4F). This supports the notion that specific molecular features "prime" progenitor cells for efficient reprogramming. Indeed, reducing MAP kinase signaling and elevating the levels of the histone demethylase KDM2B—two intrinsic features of GMPs we identified—facilitated the synchronous reactivation of pluripotency loci in MEFs. This is in agreement with the ability of KDM2B to activate genes during early phases of iPSC formation (Liang et al., 2012) and suggests that rapid removal of epigenetic barriers by this enzyme might be involved in the remarkable reprogramming response of GMPs. It will be interesting to study how OKSM factors, chromatin modulators, and CTNNB1, an interaction partner of pluripotency factors (Kelly et al., 2011), cooperate to achieve rapid iPSC formation.

Our observation that GMPs can readily acquire pluripotency upon OKSM expression is reminiscent of a recent report that describes privileged reprogramming properties of a fast cycling subset within this progenitor cell population (Guo et al., 2014). Although our results do not exclude a role of fast cell-cycle transition in synchronous GMP reprogramming, they imply that the molecular mechanisms underlying this phenomenon are complex and involve additional cellular features. The synchronous reactivation of pluripotency loci in almost all GMP-derived iPSC colonies suggests that 3c conditions override any heterogeneity that might exist within the GMP pool.

The high colony-formation efficiency of close to 100% in less than a week, the rapid and homogeneous reactivation of core pluripotency loci, and the virtual absence of nonreprogrammed cells upon OKSM expression in blood progenitors in the presence of 3c resemble reprogramming kinetics reported after MBD3 ablation in somatic cells (Rais et al., 2013). This suggests that at least some adult cell types can achieve so-called nonstochastic or deterministic reprogramming upon more subtle experimental modulation than the genetic interference with essential endogenous genes (see model in Figure 4F). Collectively, our results define cell-type-specific requirements for highly efficient and synchronous iPSC formation from different somatic cells by combined modulation of signaling pathways and chromatin modifiers. This provides a refined framework for the further exploration of the mechanisms underlying the erasure of the somatic state and the induction of pluripotency.

EXPERIMENTAL PROCEDURES

Mice

Derivation, handling, and genotyping of reprogrammable mice (JAX011001) with the *Oct4-GFP* allele were described previously (Stadtfeld et al., 2010). dsRed transgenic animals (Vintersten et al., 2004) were obtained from Jackson Laboratory (JAX006051). All animal experiments were done in accordance with the guidelines of the NYU School of Medicine Institutional Animal Care and Use Committee.

Cell Culture and Reprogramming

MEFs (Stadtfeld et al., 2008) and hepatoblasts (Gailhouste, 2012) were isolated from embryonic day 14.5 embryos and GMPs, CMPs, MEP, and LSK cells from either fetal liver or adult bone marrow (Akashi et al., 2000). All cell types used for reprogramming experiments were heterozygous for the inducible OKSM cassette, *Rosa26-rtTA*, and *Oct4-GFP*. Freshly isolated MEFs were expanded in reduced-oxygen conditions (4%) and used at passages 1 or 2. For reprogramming, cells were seeded in ESC media (Dulbecco's modified Eagle's medium [DMEM] with 15% fetal bovine serum, L-glutamine, penicillin-streptomycin, nonessential amino acids, β-mercaptoethanol, and 1,000 U/ml leukemia inhibitory factor) at the desired density on a layer of irradiated feeder cells in the presence of 1 μg/ml Dox. If applicable, L-AA (50 ng/μl), CHIR (3 μM), TGF-β RI Kinase Inhibitor II (250 nM), and PD0325901 (0.75 μM) were added. For GMPs and other blood progenitors, cultures were supplemented with Flt3 ligand (10 ng/ml) and stem cell factor (20 ng/ml) for the first 3 days of reprogramming. Media were replenished every 2-3 days. To facilitate detection of *Oct4-GFP* expression in nascent colonies by fluorescent microscopy, reprogramming experiments were conducted in ESC media prepared with FluoroBrite DMEM (Life Technologies) when required. To score factor-independent colonies, Dox (and other compounds) was removed by washing cultures twice with PBS and then maintaining them in ESC media for at least 3 days before colonies were counted by fluorescence microscopy or after alkaline phosphatase staining (Vector Laboratories). For quantification of *Oct4-GFP* expression, cultures were harvested by trypsin digestion, stained with DAPI or 7-aminoactinomycin D to exclude dead cells, acquired on a LSRII cytometer (BD Biosciences), and analyzed with FlowJo software (TreeStar).

Immunofluorescence

Cells were fixed with paraformaldehyde, permeabilized, and stained with primary antibodies against OCT4 (Santa Cruz Biotechnology; sc-8628), NANOG (Abcam; ab808992), UTF1 (Abcam; ab24273), SALL4 (Abcam; ab29112), LIN28 (Abcam; ab46020), and CTNNB1 (Sigma-Aldrich; C7207) followed by staining with appropriate secondary antibodies conjugated to either Alexa Fluor 555 or 647 (Invitrogen). Images were acquired with a Neo 5.5 cSMOS camera (Andor) and processed and analyzed using NIS-Elements and Adobe Photoshop.

Lentiviral Vectors

Vesicular stomatitis virus G protein-pseudotyped lentiviral vectors were produced in 293T cells and concentrated by ultracentrifugation as described in detail before by Sommer et al. (2009).

Transduction of MEFs (3×10^4 cells) and blood progenitor cells (1×10^5 cells) was carried out in the presence of 5 μg/ml polybrene.

Microarray Analysis

For the study of reprogramming intermediates, cultures of reprogrammable MEFs at day 4 of OKSM expression were harvested and stained with biotinylated anti-stage-specific embryonic antigen 1 (SSEA1) antibody (MC-480; eBioscience), followed by allophycocyanin (APC)-conjugated streptavidin and anti-APC microbeads (Miltenyi Biotec). $SSEA1^+$ cells were enriched to about 90% purity using magnetic-activated cell sorting columns (Miltenyi Biotec) according to manufacturer's instructions. Total RNA extracted with the miRNeasy Mini Kit (QIAGEN) with an RNA integrity number value of >8 was subjected to transcriptional analyses with Affymetrix mouse genome 430 2.0 mRNA expression microarrays followed by bioinformatic analyses. For the study of signaling pathways in starting cell populations, expression values of transcripts characteristic for TGF-β (GO:0007179) and Wnt signaling (GO:0016055) were extracted from public data sets.

ACCESSION NUMBERS

The National Center for Biotechnology Information's Gene Expression Omnibus accession number for the microarray analysis of reprogramming intermediates reported in this paper is GSE59865.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.08.003.

AUTHOR CONTRIBUTIONS

S.E.V., B.A., and M.S. conceived, planned, and analyzed all experiments. S.E.V. conducted all reprogramming experiments with MEFs. B.A. conducted reprogramming experiments with GMPs and hepatoblasts. T.C. conducted transduction experiments with blood progenitor cells. A.T. conducted bioinformatic analyses. M.S. supervised the study and wrote the manuscript together with S.E.V. and B.A.

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