Small molecules facilitate rapid and synchronous iPSC generation

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The reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) upon overexpression of OCT4, KLF4, SOX2 and c-MYC (OKSM) provides a powerful system to interrogate basic mechanisms of cell fate change. However, iPSC formation with standard methods is typically protracted and inefficient, resulting in heterogeneous cell populations. We show that exposure of OKSM-expressing cells to both ascorbic acid and a GSK3-β inhibitor (AGI) facilitates more synchronous and rapid iPSC formation from several mouse cell types. AGI treatment restored the ability of refractory cell populations to yield iPSC colonies, and it attenuated the activation of developmental regulators commonly observed during the reprogramming process. Moreover, AGI supplementation gave rise to chimera-competent iPSCs after as little as 48 h of OKSM expression. Our results offer a simple modification to the reprogramming protocol, facilitating iPSC induction at unparalleled efficiencies and enabling dissection of the underlying mechanisms in more homogeneous cell populations.

The generation of induced pluripotent stem cells (iPSCs)1–3 with defined transcription factors such as POU5F1 (hereafter referred to as OCT4), KLF4, SOX2 and c-MYC (OKSM) usually takes weeks to months and gives rise to iPSC colonies at frequencies of less than 5% (ref. 4) with a few notable exceptions5,6. Different approaches have been developed to overcome the low efficiency and slow kinetics of iPSC formation, which constitute major bottlenecks for the mechanistic dissection of the reprogramming process7–9. For example, surface markers have been employed to prospectively identify and isolate those rare cells that are poised to become iPSCs10,11. Although this approach led to the first characterization of defined intermediate stages of cellular reprogramming10, it typically requires time-consuming and costly cell isolation procedures that yield small cell numbers of variable purity. Another approach is based on the manipulation of additional genes to enhance overall reprogramming efficiencies. For instance, loss of the methyl-binding protein MBD3 was recently suggested to oppose cellular reprogramming14. Moreover, both approaches require the introduction of additional transgenes into cells, which is cumbersome.

We therefore set out to test whether efficient and synchronous iPSC formation could be induced from OKSM-expressing somatic cells without further genetic manipulation. We screened for combinations of commonly available cell culture supplements that could improve the speed and efficiency of iPSC formation by using optimized OKSM transgenes, fluorescent reporter systems and clonal reprogramming assays. This effort led to the identification of small molecules that acted synergistically and enabled near-homogeneous iPSC formation from somatic cell types, thus providing a straightforward and affordable approach to study this remarkable cell fate transition in bulk cultures.

RESULTS

A transgenic system to track induced pluripotency

Studying the process of cellular reprogramming with classical tools has been hampered by the inability to monitor exogenous OKSM expression patterns in somatic cells. We therefore generated a transgenic reprogramming system in mice that allowed us to simultaneously induce and track high-level OKSM expression in any target cells (Fig. 1a). To this end, mice homozygous for the doxycycline-inducible, polycistronic tetOP-OKSM construct in the Col1a1 locus15 were crossed to mice homozygous for a cassette containing the coding regions for Oct4, Klf4, Sox2 and an IRES-mCherry reporter in the Col1a1 locus (tetOP-OKSmC) (data not shown) and the M2rtTA allele in the Rosa26 locus (R26-M2rtTA)15. Doxycycline treatment of murine embryonic fibroblasts (MEFs) isolated from this cross induced strong and homogeneous expression of reprogramming factors, as determined by microscopy for mCherry (Fig. 1b), and consistently gave rise to iPSCs from different cell types under conventional culture conditions (Figs. 1c–f and 2a). Cells carrying the
Ascorbate and CHIR-99021 synergize during reprogramming

Extracellular cues enhance the formation of iPSCs by influencing signaling pathways, oxidative state or epigenetic modifications. We therefore tested 16 common cell culture supplements, individually or in combination, for their effect on iPSC generation (see Online Methods for a full list). This effort identified ascorbic acid (AA) and the GSK3-β inhibitor CHIR-99021 as the most effective treatments to boost iPSC generation from MEFs. Notably, combined AA supplementation and GSK3-β inhibitor. Cells were subjected to reprogramming for 9 d, at which point doxycycline and supplements were withdrawn for an additional 3 d. Representative staining of NANOG+ iPSC colonies generated with AGi. Scale bars, 200 µm. The plot shows a quantitative representation of reprogramming efficiency based on transgene-independent NANOG+ clones for the indicated conditions (n = 3 biological replicates; error bars, s.d. for 3 independent experiments). (e) Plating efficiency for clonal reprogramming analyses using MEFs. Values represent the mean for 3 independent time points; error bars, s.d. (f) Clonal analysis of reprogramming efficiency for single MEFs expressing OKSM under the indicated conditions. Reprogramming efficiency was determined by scoring for transgene-independent, NANOG+ colonies.

Stable iPSCs produced after 48 h of OKSM expression

AGi exposure reduced the minimal requirement for exogenous factor expression from 4 d to just 2 d when GMPs were used as starting cells (Fig. 2a); we chose GMPs because they are more susceptible to iPSC formation than more differentiated cell types. iPSC lines, generated after only 2 d of OKSM expression in the presence of AGi, activated an endogenous OCT4-EGFP reporter (Fig. 2b) and could be stably propagated for over 50 passages. AGi treatment had no discernible effect on cell cycle, cell proliferation or apoptosis in the context of OKSM or OKS expression in the first few days of reprogramming, thus excluding the possibility that the enhancement of reprogramming was due to increased cell division or cell loss (Supplementary Fig. 2).

To molecularly characterize colonies generated by OKSM expression with AA, CHIR-99021 or both compounds, we picked ten colonies from each condition and found that every clone exhibited endogenous NANOG expression 7 d after doxycycline withdrawal, suggesting proper activation of the core pluripotency network (Supplementary Fig. 3). Microarray analysis confirmed that iPSC lines, obtained after only 2 d of OKSM expression, exhibited global
Figure 2 | AGi enhances and accelerates iPSC formation across different cell types. (a) Left, effect of AGi on reprogramming potential of different cell types. Doxycycline was withdrawn at the indicated time points (ind., induction), and colonies were assessed for alkaline phosphatase staining 3 d after doxycycline withdrawal. Right, time-course analysis for reprogramming potential of GMPs. Transgene-independent iPSC colonies were obtained from GMPs after 2 d of doxycycline induction in the presence of AGi, followed by 6 d of doxycycline-independent growth. (b) Day 2 iPSCs express OCT4-GFP but no longer express OKSM (mCherry; scale bars, 100 μm) following doxycycline withdrawal. (c) Cluster analysis based on global gene expression analysis of the indicated samples. *Expression data from the ESC2 line were previously published42. (d) Chimeric mouse showing donor-derived agouti coat color contribution from iPSCs generated in b.

gene expression patterns that were highly similar to those of mouse embryonic stem cells (ESCs) (Fig. 2c) and showed activation of key pluripotency genes (Supplementary Fig. 4). Functionally, iPSC lines produced after 2 d of OKSM expression in the presence of AGi contributed to chimeric mice when injected into blastocysts (Fig. 2d). Thus, AGi treatment of reprogramming cultures accelerates iPSC formation, yielding stable and faithfully reprogrammed iPSC colonies after as little as 48 h of OKSM expression (Fig. 2).

AGi exposure triggers rapid and synchronous reprogramming
To accurately quantify reprogramming efficiency and kinetics, we sorted single blood progenitor cells carrying the tetOP-OKSM/tetOP-OKSmC alleles as well as an Oct4-gfp knock-in reporter15 by FACS into individual wells of 96-well plates (Fig. 3a). We then evaluated OCT4-GFP activation at discrete time points (Fig. 3b,c). For GMPs, wells containing 53% or more OCT4-GFP+ cells at any given time point were considered positive hits for reprogrammed iPSCs. We determined this cutoff empirically to eliminate false-positive ‘iPSC calls’ that were prevalent at earlier time points owing to low cell numbers and autofluorescent feeders (Online Methods). By applying these stringent criteria, we noticed a marked difference in reprogramming efficiency with and without AGi at day 6 of induction (Fig. 3b). Examination of reprogramming efficiencies for cells treated with doxycycline alone revealed that roughly 20% of clonal GMP cultures became OCT4-GFP+ by day 8, ~50% by day 20 and close to 100% by day 30 (Fig. 3c). These reprogramming efficiencies were twofold higher than those previously observed by our lab when comparing similar time points5 (25% at day 15), which is likely because of the superior reprogramming system used here15. Exposure of replicate plates to AGi yielded over 20% OCT4-GFP+ clones by day 3, 80% by
day 4 and over 95% by day 5 (Fig. 3c). Similar clonal assays were performed for hematopoietic stem cells (HSCs) and pro-B cells, supporting these results; however, reprogramming efficiency was measured by scoring the relative fraction of OCT4-GFP+ cells per well instead of using an empirical cutoff in the latter experiment. (Supplementary Fig. 5a, b). Thus, AGi treatment facilitates more synchronous iPSC formation from different hematopoietic progenitors when using a clonal reprogramming assay.

Clonal cell populations might activate OCT4-GFP either homogeneously or in a subset of cells within each well, which is not taken into account when measuring iPSC formation efficiency based on a binary system used here and in previous studies12,24. We therefore measured day 4 and over 95% by day 5 (Fig. 3c). Similar clonal assays were performed for hematopoietic stem cells (HSCs) and pro-B cells, supporting these results; however, reprogramming efficiency was measured by scoring the relative fraction of OCT4-GFP+ cells per well instead of using an empirical cutoff in the latter experiment. (Supplementary Fig. 5a, b). Thus, AGi treatment facilitates more synchronous iPSC formation from different hematopoietic progenitors when using a clonal reprogramming assay.

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Figure 4 | AGi rescues the reprogramming defect of refractory cells. (a) FACS analysis of reprogramming intermediates in bulk MEF populations at days 5, 7, 9 and 11 of OKSM or OKSM+AGi expression. Note the more homogeneous shift of intermediates from THY1+ to THY1–/EPCAM+ cells. (b) Graph summarizing the percentages of EPCAM+ cells depicted in a. (c) FACS analysis for OCT4-GFP expression in bulk MEF populations at days 5, 7, 9, 11 and 13 of OKSM or OKSM+AGi expression. PE-Cy7 was used as an autofluorescent control with emission filter of 750 nm. (d) Graph summarizing the percentages of OCT4-GFP+ cells shown in c. (e) Schematic outlining the attempt to restore reprogramming potential in THY1+ refractory cells sorted at day 5 of OKSM expression. (f) Alkaline phosphatase (AP) staining for refractory, THY1+ intermediates after continued OKSM expression (doxycycline exposure) under the indicated conditions. Doxycycline was removed for an additional 4 days before analysis to ensure transgene independence. (g) Quantification for the results shown in f. Replicate analyses and individual treatment with ascorbic acid and GSK3-β inhibitor are shown in Supplementary Figure 6. (h) AP staining for cells expressing OKS under the indicated conditions. Doxycycline was removed for an additional 4 d before analysis to ensure transgene independence. (i) Quantification for the results shown in h (n = 3 technical replicates; error bars, s.d.).
the fraction of GMPs that activated OCT4-GFP within individual wells at different time points of reprogramming. Consistent with the synchronous emergence of clonal iPSC cultures, the majority of cells within individual wells exhibited more homogeneous OCT4-GFP activation (0–10% at day 2, 60–90% at day 4 and 90–100% by day 5) in the presence of AGi, whereas untreated cultures (doxycycline alone) remained highly heterogeneous for OCT4-GFP expression until day 30 (Fig. 3d). We confirmed this observation for other cell types, including HSCs and pro-B cells (Supplementary Fig. 5a,b).

It was previously reported that Aid (Aicda) knockout cells express pluripotency factors earlier in the reprogramming process, although these cells often fail to form stable iPSC colonies25. To ensure that OCT4-GFP activation indeed coincided with the formation of stable iPSC colonies, we withdrew doxycycline (i.e., discontinued transgene expression) and AGi from GMPs at various time points and maintained the cells for at least 4 d in ESC medium before scoring for iPSC colonies (Supplementary Fig. 5c). Consistent with our FACS results, clonal, transgene-independent colonies formed rapidly and efficiently in samples treated with AGi (Supplementary Fig. 5d). We conclude that AGi exposure induces near-homogeneous activation of a key pluripotency gene and concomitant formation of stable, transgene-independent iPSCs in clonal progenitor cells expressing OKSM.

AGi rescues reprogramming potential of refractory cells
To gain mechanistic insights into how AGi treatment may influence the reprogramming process, we analyzed intermediate time points during iPSC formation by FACS using previously described surface markers and a reporter allele10,11. MEFs undergoing successful reprogramming initially downregulate the fibroblast marker THY1, followed by successive activation of the intermediate marker EPCAM, and subsequent expression of OCT4-GFP. In agreement with previous studies, we detected downregulation of THY1 in two-thirds of the population and only a small fraction of intermediates expressing either EPCAM (22%) or OCT4-GFP (0.8%) by day 11 of reprogramming. In contrast, exposure of cells to AGi for the same duration triggered these phenotypic changes in a much larger fraction of cells (92% THY1−, 87% EPCAM+, 48% OCT4-GFP+) (Fig. 4a–d). These results thus suggested that AGi supplementation reduces a high fraction of OKSM-expressing cells that typically fail to form iPSCs. To directly test this hypothesis, we induced OKSM expression in MEFs for 5 d and sorted intermediates on the basis of THY1 positivity and SSEA1 negativity, which identify cells that have become refractory to iPSC induction after day 3 (Fig. 4e)10,11. Whereas THY1+ cells exposed to regular culture conditions failed to yield iPSCs, addition of AGi restored their potential to produce doxycycline-independent iPSCs (Fig. 4f,g and Supplementary Fig. 6). Thus, AGi’s effect on reprogramming is explained at least in part by its ability to prevent cells from arresting at intermediate stages of iPSC induction.

We next examined AGi’s effect on iPSC formation in the absence of exogenous c-MYC expression, which strongly impairs cellular reprogramming26,27. To this end, we derived MEFs carrying the Col1a1-OKSmC and R26-M2rtTA alleles in a heterozygous configuration; exposure of these cells to doxycycline gave rise to extremely few, if any, iPSC colonies. Notably, AGi treatment increased iPSC formation nearly 1,000-fold compared to OKS expression alone (Fig. 4h,i). The combination of ascorbic acid and GSK3-β inhibitor was again synergistic because either compound alone only marginally increased the reprogramming capacity of these cells at the tested time point. We conclude that AGi supplementation provides robustness to the reprogramming process under suboptimal conditions.

AGi modulates somatic, transient and pluripotency genes
Successful reprogramming requires extinction of the somatic program and activation of key pluripotency factors7. Furthermore, cell populations expressing OKSM were reported to transiently upregulate developmental genes10,28; however, the relevance of these changes remains unclear. In an attempt to dissect the molecular consequences of AGi treatment, we performed expression profiling of reprogrammable MEFs exposed to doxycycline for 4, 6, 8, 10 or 12 d in the presence or absence of AGi. Unsupervised clustering of the entire transcripts of these samples showed that bulk MEF cultures exposed to doxycycline and AGi were more similar to established iPSCs, whereas MEFs receiving only doxycycline for the same period were more similar to the starting MEF population (Fig. 5a,b). Notably, reprogrammable MEFs exposed to doxycycline and AGi for only 48 h were already distinguishable from MEFs exposed to doxycycline alone (Supplementary Fig. 7a). Accordingly, functional annotation analysis of downregulated genes in AGi-treated compared to untreated cells showed enrichment for categories related to development and lineage specification (Fig. 5c). For example, 48 h of OKSM expression in the presence of AGi elicited more rapid downregulation of genes associated with FGF signaling, which has been linked to cellular differentiation (Supplementary Fig. 7b). Similarly, MEF-related transcripts (for example, Col5a1, Fbn1 and Mir143) were downregulated more rapidly in AGi-treated cells, whereas key pluripotency transcripts including Nanog, Sox2, Zfp42 and Zfp296 and Mir290–295 were upregulated much earlier in AGi-exposed cells than in control cells treated with doxycycline alone (Fig. 5d).

We further noticed that AGi exposure abolished the transient upregulation of differentiation-associated genes such as Prx and Cxcr4, suggesting that these molecular changes normally resist iPSC formation in the absence of AGi (Fig. 5d). Loss of this transient expression pattern was confirmed at a global level by comparing our results with a previous study that cataloged all transient genes in defined intermediates of reprogramming16 (Fig. 5e). To exclude that the selected gene expression time points may have missed transient upregulation of these genes in the presence of AGi, we performed quantitative reverse-transcription PCR (qRT-PCR) analysis at 12, 24, 36 and 48 h after the induction of reprogramming. Consistent with the microarray results, Prx and Cxcr4 expression remained constant at early time points in AGi-treated samples but spiked in controls (OKS expression alone) (Supplementary Fig. 8). Last, we observed that gene expression patterns of THY1+ refractory cells showed a higher degree of correlation with cells expressing OKSM than with cells expressing OKSM in the presence of AGi (Fig. 5e). This result is in agreement with our earlier finding that AGi treatment rescued the reprogramming potential of THY1+ refractory cells. Collectively, these unbiased genome-wide data provide a partial molecular explanation for our observation that reprogramming occurs more synchronously and rapidly in the presence of AGi.

DISCUSSION
In this study, we provided molecular and functional evidence that exposure of cells to two commonly used tissue culture supplements, ascorbic acid and CHIR-99021, induces more homogeneous reprogramming of different somatic cells into iPSCs at efficiencies and kinetics that have so far been achieved only with genetic manipulation.
of additional genes\textsuperscript{12,13}. Although AGi strongly enhanced and accelerated iPSC induction across diverse cell types, differentiated cells such as MEFs required more time to activate OCT4-GFP and acquire stable pluripotency compared to somatic progenitor cells such as GMPs and HSCs. This observation confirms the previous notion that less differentiated cells are more amenable to iPSC formation than more differentiated cells\textsuperscript{5}, and it further suggests that mature cells have to overcome additional reprogramming barriers that should be identifiable. Mechanistically, ascorbic acid and GSK3-\textbeta inhibition synergize during reprogramming by preventing the generation of refractory cells, blocking the transient activation of developmental regulators and facilitating the early activation of key pluripotency genes necessary to achieve a self-sustaining pluripotent state. It remains to be determined which downstream effectors of ascorbic acid and CHIR-99021 mediate the enhancing effect of AGi on reprogramming. From the observation that ascorbic acid functions as a cofactor for histone demethylases and Tet enzymes implicated in stem cell biology\textsuperscript{20,29–31}, we surmise that this compound contributes to the observed reprogramming phenotype by facilitating epigenetic activation of key pluripotency genes during iPSC generation. In agreement with this idea, we recently showed that ascorbic acid can functionally compensate for the absence of Nanog\textsuperscript{32}, and it prevents aberrant silencing of the Dlk1-Dio3 imprinted cluster during reprogramming\textsuperscript{33}. CHIR-99021, on the other hand, may synergize with ascorbic acid through its destabilizing effect of TCF3, a known repressor of key pluripotency genes\textsuperscript{34}. In addition, CHIR-99021 may counteract inappropriate activation of developmental regulators; this notion is consistent with a recent study by Plath and colleagues, who showed attenuated expression of neural genes in nascent iPSCs depleted for TCF3 (ref. 35).

From a practical point of view, AGi-mediated reprogramming should enable molecular studies of the reprogramming process in bulk cultures, which are confounded by extreme heterogeneity of iPSC intermediates; although experiments in this manuscript were performed with single-copy transgenic tools, AGi enhanced and accelerated iPSC formation also in the context of a lentiviral reprogramming system (data not shown). AGi treatment further offers a simple strategy to generate iPSCs under conditions that impede or resist reprogramming. We have demonstrated this principle by generating iPSCs from THY1\textsuperscript{+} cells, OKS-expressing fibroblasts lacking the c-MYC transgene and IgM\textsuperscript{+} lymphocytes, which are notoriously resistant to reprogramming. We have demonstrated this principle by generating iPSCs from THY1\textsuperscript{+} cells, OKS-expressing fibroblasts lacking the c-MYC transgene and IgM\textsuperscript{+} lymphocytes, which are notoriously difficult to reprogram. We expect this finding to extend to several other cell types that are hard to reprogram and to conditions in which omission of the c-MYC oncogene is warranted. For example, we anticipate that AGi treatment will streamline iPSC generation using integration-free systems that have been hampered by extremely low efficiencies\textsuperscript{36–38}. The strong effect of AGi on mouse iPSC formation also raises the question of whether this treatment enhances human reprogramming. Preliminary data from our lab suggest that AGi does not affect human induced pluripotency (data not shown). However, given the well-established differences in signaling require-

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Figure 5 | Effect of AGi on gene expression patterns in reprogramming intermediates. (a) Unsupervised clustering of global gene expression analysis of indicated samples. “4 d OKSM,” for example, represents reprogrammable MEFs exposed for 4 d to doxycycline in the absence or presence of AGi. (b) Heat map showing expression levels for genes that were more than threefold higher in ESCs than in MEFs. (c) DAVID functional analysis of genes whose expression is downregulated at least 1.5-fold in response to AGi treatment in MEFs expressing OKSM or OKSM+AGi. Note the prevalence of developmental regulators. Benjamini-Hochberg (BH) adjusted \( P \) values are presented (calculated by DAVID as ‘modified Fisher exact \( P \) value’). (d) Examples of somatic, pluripotency, microRNA and transient developmental genes that change expression in response to doxycycline/AGi treatment. (e) Expression patterns of gene sets associated with a refractory phenotype (left) or transient upregulation during reprogramming (right) were compared with expression patterns obtained after OKSM expression in the presence or absence of AGi. Statistical significance is indicated with asterisks (one-tailed Fisher test).
ments between mouse and human pluripotent stem cells, it may be feasible to take a rational approach and identify combinations of small molecules that enhance human iPSC generation in a similar fashion to AGi in mouse cells. Likewise, it will be informative to test whether AGi treatment is beneficial in other settings of cell fate change such as transdifferentiation. Notably, the transdifferentiation of human fibroblasts to induced neurons is strongly enhanced by CHIR-99021 exposure alone. It is therefore conceivable that this and other direct lineage conversion approaches might equally benefit from AGi supplementation.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. NCBI Gene Expression Omnibus (GEO): microarray data have been under accession number GSE57774.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

O.B.-N., J.B. and K.H. conceived of the experiments, interpreted results and wrote the manuscript. O.B.-N. and J.B. conducted all iPS experiments, performed statistical analyses and generated figures; C.V. assisted in experiments; E.A. produced transgenic OKSmC mice; J.B. and R.M.W. generated chimeric animals by statistical analyses and generated figures; C.V. assisted in experiments; E.A. O. B.-N., J.B. and K.H. conceived of the experiments, interpreted results and wrote the manuscript. O.B.-N. and J.B. conducted all iPS experiments, performed statistical analyses and generated figures; C.V. assisted in experiments; E.A. produced transgenic OKSmC mice; J.B. and R.M.W. generated chimeric animals by statistical analyses and generated figures; C.V. assisted in experiments; E.A.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Derivation of reprogrammable cells. Reprogrammable cells were derived from mice carrying a polycistronic cassette containing Oct4, Sox2, Klf4, and c-Myc (OKSM)15, or Oct4, Sox2, Klf4, and mCherry (OKSmC) in the Collal locus. Where indicated, EGFP was knocked into one allele of the Oct4 locus under control of the endogenous Oct4 promoter5. Images in Figures 1b,c and 2a,b are representative of at least 5 experiments, except for IgM+ B cell images, which represent 3 technical replicates. All of the mice carried a Rosa26-M2rtTA allele, completing the inducible system. Murine embryonic fibroblast Rosa26-M2rtTA was experimentally determined for GMPs as follows: GMPs were sorted into one well in which greater than 53% of cells expressed GFP, this threshold was determined for GMPs as follows: GMPs were sorted onto a 96-well plate and treated with AGi for 6 d. Figure 3b is representative of 3 independent experiments. At this time, cells were split and half were analyzed via flow cytometry for OCT4-GFP expression, whereas the other half were replated in the absence of doxycycline. The latter cells were grown for another 10 d in ESC medium alone and analyzed by FACS to determine which wells maintained OCT4-GFP expression. Box plots for single cell reprogramming experiments were plotted using the statistical program R.

Alkaline phosphatase staining. Alkaline phosphatase kits (Vector Labs) were used according to the manufacturer's recommendations to assess pluripotency. Prior to staining, doxycycline and cell culture supplements were removed for a minimum of 3–4 d to eliminate exogenous OKSM expression.

Quantification of alkaline phosphatase staining using ImageJ software. ImageJ was used to process alkaline phosphatase (AP) images for colony counting. A region of interest covering a given well was selected using the oval tool. The process “find edges” was then selected. The threshold tool was applied, with the “default thresholding method,” “red” as the threshold color, “HSB” as the color space, and “dark background” selected. “Analyze particles” quantified the number of AP+ colonies and the area of those colonies using the following parameters: “Size (pixel^2)” = 25–infinity,” “Circularity” = 0–1,” “Exclude on edges” was applied; “Include holes” was applied. The settings were identical for all samples processed.

Cell proliferation, cell cycle and apoptosis assays. Cell proliferation analysis was performed by counting cells on a hemocytometer on consecutive days. Cell-cycle analysis was performed using 5-bromo-2'-deoxyuridine (BrdU). Briefly, BrdU (Sigma) was added directly into culture medium at a final concentration of 10 µM for 30 min. Cells were then dissociated using trypsin-EDTA (Life Technologies) and washed twice in PBS (Life Technologies). The cells were resuspended in 100 µl of normal saline on ice. Ice-cold 70% EtOH was added dropwise to the cells, which were subsequently incubated for 30 min on ice. Then, an equal volume of 4 N HCl was added directly. Cells were then pelleted and resuspended in FACS buffer. Anti-BrdU-FITC was then applied for 30 min at 23 °C before washing the cells and analyzing on a MACSQuant flow cytometer (Miltenyi Biotec). Annexin staining to assess apoptosis and necrosis was performed according to the manufacturer’s recommendations (BD) and analyzed using a MACSQuant flow cytometer.

Cell culture. Mouse ESCs and iPSCs were cultured on irradiated feeders (GlobalStem) in KO-DMEM (Life Technologies) supplemented with 15% FBS (Life Technologies), 1% GlutaMAX (Life Technologies), 1% non-essential amino acids (Life Technologies), 1% penicillin-streptomycin (Life Technologies), 0.5% β-mercaptoethanol (Sigma-Aldrich), and 1,000 U/ml LIF (mESC medium). MEFs were grown in DME (Life Technologies) supplemented with 10% FBS, 1% GlutaMAX, 1% non-essential amino acids, 1% penicillin-streptomycin, 0.1% β-mercaptoethanol. Reprogramming of MEFs and blood progenitors was performed in mESC medium supplemented with 2 µg/ml doxycycline (Sigma). Ascorbic acid (Sigma) was diluted in water and prepared fresh every 10–14 d and added at a final concentration of 50 µg/ml. GSK3 inhibitor CHIR-99021 (from Stemgent).
or Tocris) was administered at 3 µM final concentration for all experiments. Testing for Mycoplasma was routinely performed.

**Doxycycline withdrawal assays.** To test the stability of iPSC colonies formed under different reprogramming conditions, cells were treated for various times with doxycycline in the presence or absence of cell culture supplements. The supplements and doxycycline were removed at the indicated time points, cells were then washed with ESC medium and ESC medium was applied for at least 3 d before analysis. In the case of GMPs, ESC medium contained the following cytokines: 10 ng/ml IL-3, 10 ng/ml IL-6 and 20 ng/ml SCF (Prospec).

**Reprogramming of MEFs and blood progenitors.** In a typical reprogramming experiment, 20,000 MEFs from a reprogrammable mouse were counted and seeded onto six-well plates 1 d before induction. Doxycycline (dox) or Doxycycline + AGi (dox + AGi) was added fresh to the cells every other day. Depending on the experiment, usually 2–9 d for blood progenitors and 6–12 d for MEFs were used for iPSC induction, after which doxycycline and small molecules were removed and replaced with fresh mESC medium. Of note, administration of AGi in mESC medium containing serum replacement instead of FBS showed a marked reduction in the reprogramming efficiency in dox alone or dox + AGi–treated cells. Notably, cell passage highly affects the reprogramming efficiency with AGi. Low-passage, highly proliferative reprogrammable MEFs showed a higher reprogramming efficiency compared to high-passage, less proliferative MEFs in the absence or presence of AGi.

**Candidate chemical screen.** To test which molecules reprogram somatic cells most efficiently into iPSCs, we tested several compounds that we preselected from the literature. The compounds were tested at different concentrations and in different combinations and included: EGF (Life Technologies), bFGF (R&D), Wnt3a (R&D), ascorbic acid (Sigma), GSK3 inhibitor (Stemgent or Tocris), LiCl (Sigma), MEK inhibitor (R&D), Forskolin (Sigma), ALK-4/5/7 inhibitor (Sigma) and RepSOX-616452 (Sigma), VPA (Sigma), 3-deazaneplanocin (Cayman), TTNPB (Sigma), traneykypromine (BPS biosciences), JNK inhibitor (EMD) and BMP4 (Stemgent). Although some other different small-molecule combinations facilitated reprogramming, the dual administration of ascorbic acid and GSK3i showed the strongest effect.

**Blastocyst injections and generation of chimeric mice.** Chimeric mice were generated via blastocyst injections as previously reported. To induce superovulation, five female B6D2F1/J mice (5–6 weeks old; Jackson Laboratory) were treated with pregnant mare serum (Sigma-Aldrich) by intraperitoneal injection. Human chorionic gonadotropin (Sigma-Aldrich) was administered 48 h later by intraperitoneal injection, and the female mice were mated to B6D2F1/J males. Blastocysts were harvested 3 d later and injected with iPSCs that were expanded from a colony generated after 48 h of transgene expression in the presence of AGi. Approximately 12 blastocysts were transferred to each of two pseudo-pregnant Swiss Webster female mice (Jackson Laboratory; approximately 2 months of age) and allowed to develop to term.

**Immunostaining.** To ensure that iPSC colonies generated under different conditions expressed NANOG, we carried out immunofluorescence as follows. Cells were washed once with PBS (Life Technologies). The cells were then fixed in 4% formaldehyde for 20 min at 23 °C and washed again in PBS. Triton X-100 was used at a concentration of 0.5% and applied to cells for 10 min at 23 °C. The cells were washed three times in PBS. Blocking was performed in 10% goat serum with 0.2% Triton X-100 for 30 min at 23 °C. Primary antibody (anti-mouse NANOG; Abcam; Ab80892) was added at a dilution of 1:400 in 1.5% goat serum at 23 °C for 1 h. The cells were washed three times in PBS. Secondary antibody (anti-rabbit, Alexa 555; Life Technologies) was added at a dilution of 1:1,000 in 1.5% goat serum at 23 °C for 1 h. The cells were washed three times in PBS and mounted in mounting medium with 4’,6-diamidino-2-phenylindole (DAPI) (Vectashield; Vector Labs).

**Reprogramming of refractory cells.** Reprogrammable MEFs grown in mESC medium were dox-treated for 5 d and then harvested and stained for various surface markers: Anti-mouse CD326 EPCAM (PE), anti-mouse SSEA1 APC-647 (BioLegend) and anti-mouse CD90 THY1.2 eFluor 450 (eBioscience). THY1+/SSEA1+/EPCAM+/OCT4-GFP– cells (‘refractory cells’) were sorted in equal numbers onto gelatin–coated six-well plates containing mESC medium containing dox, dox + ascorbic acid, dox + GSK3i or doxycycline + AGi. Medium and small molecules were replaced every other day, for a total of 7 d, after which dox and AGi were withdrawn and replaced with fresh mESC medium. Dox-independent iPSCs were scored at least 3 d later by AP staining kit (Vector Labs SK-5100).

**Microarrays and RNA extraction.** DNase–treated (Qiagen) total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Hybridization to the GeneChip Mouse 2.0 ST arrays (Affymetrix) were done according to protocols in the Partners Center for Personalized Genetic Medicine. RNA was performed using Expression Console (Affymetrix). Hierarchical clustering was performed using Expander (EXPression Analyzer and DisplayER). Classification and annotations of selected gene groups was performed using the DAVID online functional annotation tool (http://david.abcc.nicifr.gov/). Heat maps were generated using the statistical software program R. The gene expression data have been deposited to Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) and given the accession number GSE57774. The gene expression data for ESC-2 were downloaded from the GEO study GSE56646 (ref. 42).

**Gene expression analysis: correlation study within refractory and transient gene sets.** We performed a Pearson product-moment analysis to study the correlation between expression patterns from gene sets associated with a refractory/transient upregulation phenotype and OKSM expression with and without AGi. In statistics, Pearson correlation is a measure of the linear correlation (dependence) between two variables, where 1 indicates total positive correlation, 0 is no correlation and −1 is total negative correlation. The statistical significance of the difference between the two correlation coefficients, for a given time point, was calculated using a one-tailed Fisher test; significant differences between two correlation coefficients are indicated by a P value <0.05.

**qRT-PCR analysis.** Total RNA was extracted from one confluent well of a six-well dish using the RNeasy Mini Kit with on-column DNase digestion (Qiagen). cDNA was generated using the Superscript III First Strand Kit via the Oligo(dT) method (Life Technologies).

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qRT-PCR was carried out using the Brilliant III SYBR QPCR Master Mix (Agilent Technologies) on a LightCycler 480 (Roche). Relative expression was calculated using the ΔΔCt method with GAPDH as reference\(^45\). The following validated primers were used for qRT-PCR: Cxcr4\(_{\text{forward}}\), GACTGGCATAGTGGCAATG; Cxcr4\(_{\text{reverse}}\), AAGGCTTACAGTGGCATGACA; Prx\(_{\text{forward}}\), TCAGCGCTTCAACGTA; Prx\(_{\text{reverse}}\), TACTGCGGTAGTCACTG; GAPDH\(_{\text{forward}}\), AGGTGCTTGGAACGAGTTT; GAPDH\(_{\text{reverse}}\), TGTAGACCATGTAGTTGAGTCA.

**Surface marker analysis by flow cytometry.** Cells were harvested using 0.25% trypsin and resuspended in 1× PBS buffer containing 4% FBS (FACS buffer). The appropriate conjugated antibodies were added at similar ratios (1:200): anti-mouse CD326 EpCAM (PE), and anti-mouse CD90 Thy1.2 eFluor 450 (eBioscience). Cells were incubated on ice for 20 min, after which they were washed twice with 1× PBS, resuspended in FACS buffer, filtered and then analyzed on a MACSQuant flow cytometer (Miltenyi Biotec) machine. Analysis was performed using FlowJo software.

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