Discovery of Nonsteroidal Anti-Inflammatory Drug and Anticancer Drug Enhancing Reprogramming and Induced Pluripotent Stem Cell Generation

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Discovery of NSAID and anticancer drugs enhancing reprogramming and iPS cell generation

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Abstract

Recent breakthroughs in creating induced pluripotent stem cells (iPSCs) provide alternative means to obtain ES-like cells without destroying embryos by introducing four reprogramming factors (*Oct3/4*, *Sox2*, and *Klf4*/*c-Myc or Nanog*/*Lin28*) into somatic cells. iPSCs are versatile tools for investigating early developmental processes and could become sources of tissues or cells for regenerative therapies. Here, for the first time, we describe a strategy to analyze genomics datasets of mouse embryonic fibroblasts (MEFs) and embryonic stem (ES) cells to identify genes constituting barriers to iPSC reprogramming. We further show that computational chemical biology combined with genomics analysis can be used to identify small molecules regulating reprogramming. Specific down-regulation by small interfering RNAs (siRNAs) of several key MEF-specific genes encoding proteins with catalytic or regulatory functions, including WISP1, PRRX1, HMGA2, NFIX, PRKG2, COX2, and TGFβ3, greatly increased reprogramming efficiency. Based on this rationale, we screened only 17 small molecules in reprogramming assays and discovered that the NSAID Nabumetone and the anti-cancer drug OHTM can generate iPS cells without Sox2. Nabumetone could also produce iPS cells in the absence of c-Myc or Sox2 without compromising self-renewal and pluripotency of derived iPS cells. In summary, we report a new concept of combining genomics and computational chemical biology to identify new drugs useful for iPSC generation. This hypothesis-driven approach provides an alternative to shot-gun screening and accelerates understanding of molecular mechanisms underlying iPS cell induction.

Keywords

NSAIDS; OHTM; iPSC; Sox2; c-Myc

INTRODUCTION

Embryonic stem (ES) cells are not only versatile tools for investigating early developmental events but provide a promising source of tissues potentially useful for regenerative therapies. Recent breakthroughs in generating induced pluripotent stem cells (iPSCs) provide alternative means to obtain ES-like cells without destroying embryos by introducing four

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Conflict of Interest Statement
The authors declare that they have no conflict of interest.

reprogramming factors (*Oct3/4*, *Sox2*, and *Klf4*/*c-Myc or Nanog/ Lin28*) into somatic cells [1–3]. iPS cells share numerous traits with ES cells, such as colony morphology, transcriptome, self-renewal ability and pluripotency [3,4]. Moreover, customized therapeutic applications of iPS cells have been reported [5–7]. Nonetheless, the molecular basis of reprogramming remains unclear.

Reprogramming is a step-wise process moving from differentiated to ES-like stages [8,9], a progression that can be monitored using various cellular markers. The differentiation marker, Thy1, is highly expressed in mouse embryonic fibroblasts (MEFs), and its expression in MEFs decreases within a few days of transduction with transgene Oct3/4, Sox2, Klf4, and c-Myc (denoted here 4F: OSKM). Consequently, expression of the stem cell marker SSEA1 increases, followed by activation of other ES markers, such as endogenous Nanog, Oct3/4, and × reactivation. During this process, iPS cells are enriched or selected [10]. Increasing evidence indicates that the four reprogramming factors cooperatively initiate the transition of cell identity from somatic to iPS cells [11]. Based on these data, we reasoned that signature patterns of gene expression in MEFs constitute a barrier for induced reprogramming and that overcoming this barrier may be a rate-limiting step in the reprogramming process.

Here, for the first time, we describe a systematic strategy to analyze genomics datasets of MEFs and mouse ES (MES) cells to identify barriers to iPSC reprogramming. We show that computational drug screening combined with genomics analysis can identify small molecules that regulate reprogramming. We show that down-regulation by siRNAs of a several key MEF-specific genes encoding proteins with catalytic or regulatory functions, including WISP1, PRRX1, HMGA2, NFIX, PRKG2, COX2, and TGFβ3, greatly increased reprogramming efficiency. Our drug screening results showed that: (a) the non-steroidal anti-inflammatory drug (NSAID) Nabumetone acts as a COX2 inhibitor to enhance reprogramming; (b) the anti-cancer drug OHTM can generate iPS cells without Sox2 during reprogramming by inducing endogenous Sox2 expression; and (c) Nabumetone can produce iPS cells in the absence of c-Myc or Sox2 without compromising self-renewal and pluripotency of derived iPS cells. In summary, our novel strategy combines genomics and computational drug screening to identify new drugs for iPSC reprogramming potentially leading to novel therapies.

MATERIALS & METHODS

MOUSE EMBRYONIC FIBROBLAST (MEF) DERIVATION

Oct4-EGFP MEFs were derived from the mouse strain B6;129S4-*Pou5f1*$^{tm2(EGFP)Jae}$/J (The Jackson Laboratory; stock #008214) following the protocol on the WiCell Research Institute website. In brief, E13.5 embryos were collected from time-mated pregnant female mice and then tested for microbial contamination. Oct4-EGFP MEFs were maintained in MEF complete medium (DMEM with 10% FBS, nonessential amino acids, L-glutamine, and no sodium pyruvate). Cells passaged fewer than 5 times were used for induced reprogramming.

REPROGRAMMING BY RETROVIRUS-MEDIATED TRANSDUCTION OF FACTORS

Reprogramming was conducted as described [2]. In brief, 4×10^4 Oct4-EGFP MEFs were transduced with pMX retroviruses for ectopic expression of *Oct4*, *Sox2*, *Klf4*, and *c-Myc* (Addgene). Three days later, cells were fed ES medium (DMEM with 15% ES-screened FBS, nonessential amino acids, L-glutamine, monothioglycerol, and 1000 U/ml LIF) and the media was changed every other day. Reprogrammed (EGFP+) cells were identified and scored by fluorescence microscopy two to three weeks post transduction, unless otherwise stated. To derive iPSCs, EGFP+ colonies were manually picked under a stereomicroscope

(Leica). In the case of small molecule treatment, indicated small molecules were applied to reprogramming cells on day four post-transduction and fresh medium was added every other day for at least two weeks or until EGFP+ colonies appeared.

siRNA TRANSFECTION

Specific siRNAs were purchased from Dharmacon. 4×10^4 Oct4-EGFP MEFs were transfected with lipofectamine/siRNAs complexes according to the manufacturer's instruction (Invitrogen). Three to five hours later, the transfection reagent was discarded and MEF complete medium was added for culturing. Gene knockdown efficiency was evaluated by semi-quantitative real time RT-PCR. GAPDH served as an internal control to normalize mRNA expression signals.

For reprogramming, retrovirus expressing reprogramming factors (Oct4, Sox2, Klf4, and c-Myc) was added and the medium was then changed to complete medium next day. For overexpression of *COX2* transgene, retroviruses expressing COX2 were added one day after OSKM transduction. siRNAs were introduced at day 5 post-transduction.

IN VITRO DIFFERENTIATION AND TERATOMA FORMATION ASSAY

For in vitro differentiation, iPS cells were dissociated by trypsin/EDTA and resuspended in embryoid body (EB) medium (DMEM with 15% FBS, nonessential amino acid, L-glutamine) to final concentration at 5×10^4 cells/ml. To induce EB formation, 1000 iPS cells in 20 microliter were cultured in hanging drops on inverted Petri dish lids. Three to five days after EB formation, EBs were collected and transferred to 0.1% gelatin-coated 6-well plates at a density of ~10 EBs per well. Two weeks later, beating cardiomyocytes (mesoderm) were identified microscopically. Cells derived from endoderm and ectoderm were identified by α-fetoprotein (R&D; Cat#MAB1368) and neuron-specific βIII tubulin (abcam; Cat# ab7751) antibodies, respectively.

To assay teratoma formation, 1.5×10^6 iPS cells were trypsinized and resuspended in 150 ul of culture medium, and then injected subcutaneously into the dorsal hind legs of athymic nude mice anesthetized with avertin. Three weeks later, mice were sacrificed to collect teratomas. Tumor masses were fixed, dissected and analyzed in the Sanford-Burnham Medical Institute Cell Imaging-Histology Core Facility.

IMMUNOFLUORESCENCE AND ALKALINE PHOSPHATASE (AP) STAINING

iPS cells were seeded and cultured on 0.1% gelatin-coated 6-well plates. Four days later, cells were fixed with 4% paraformadehyde (Electron Microscopy Sciences; Cat# 15710-S). For immunofluorescence, fixed cells were permeabilized with 0.1% Triton X-100 in PBS and blocked with 5% BSA in PBS. SSEA-1 (R&D; Cat# MAB2155), Sox2 (R&D; Cat#MAB2018), and Nanog (R&D; Cat# AF2729) antibodies were used to detect ES markers. Nuclei were visualized by Hoechst 33342 (Invitrogen) staining. For AP staining, fixed cells were treated with alkaline phosphatase substrate following the manufacturer's instruction (Vector Laboratories; Cat# SK-5100).

MICROARRAY ANALYSIS

Total RNAs were isolated from indicated cells using TRIZOL reagent (Invitrogen). Gene expression was detected and normalized in the Sanford-Burnham Medical institute HT screening and genomics core facilities. Heat maps were created using MultiExperiment View (http://www.tm4.org). Scatter plots were created using Excel.

META-ANALYSIS FOR SMALL MOLECULE CANDIDATES

Select individual MEF or MES (Fig. 1A) genes served as queries to perform searches using the NextBio engine. The compounds identified were analyzed for specific activities, such as down-regulation of the PTGS2 gene by Nabumetone. Finally, seventeen molecules (Table S2) were selected as potent inducers of MES genes or inhibitors of MEF genes, as predicted by NextBio meta-analysis.

RESULTS

Silencing MEF-specific genes encoding catalytic or regulatory factors enhance iPS cell generation

To determine quantitatively which genes are specifically expressed in MEF and MES cells, we conducted mRNA a microarray analysis to examine mRNA expression profiles in both cell types. We focused on MEF-specific genes encoding catalytically active or regulatory proteins based on their important roles in cellular function, and selected WISP1, PRRX1, HMGA2, NFIX, PRKG2, COX2, TGFB3, LYZS, and 6720477E09RIK (Figure 1A) for further investigation. These genes are highly expressed in MEF but not MES (Figure 1A & [12]) and play key roles in various biological functions (Table S1). We hypothesized that these factors may negatively regulate reprogramming from an MEF to an ES-like stage by securing identities of fibroblasts and that down-regulation of these genes might enhance the reprogramming process. To test this hypothesis, we examined the effect of knockdown of these genes in Oct4-EGFP MEFs by specific siRNAs. Most genes were knocked down by at least 80% in siRNA-transfected Oct4-EGFP MEFs (Figure 1B), and that down-regulation persisted for at least five days post-transfection (data not shown). Since the duration of down-regulation was sufficient to exert an impact on reprogramming, we introduced the four reprogramming factors (4F or OSKM: Oct4, Sox2, Klf4, and c-Myc) into Oct4-EGFP MEFs followed by siRNA transfection five days later (Figure 1B & 1C). Two weeks later, mature reprogrammed iPS cells were identified based on EGFP-positivity and counted by fluorescence microscopy. Down-regulation of most of the MEF-specific genes encoding catalytic or regulatory factors greatly enhanced reprogramming efficiency by 2 to 6-fold (Figure 1C) compared with non-targeting (NT) control. The genes exhibitng barrier effects on reprogramming play distinct roles in cellular functions, such as signaling molecules (WISP1 and TGFB3), transcriptional regulators (PRRX1, HMGA2, NFIX, and 6720477E09RIK), and catalytic enzymes (COX2 and PRKG2) (Table S1). Most of these identified genes are novel to reprogramming, except TGFβ pathway which has been shown to act as a roadblock during reprogramming [13,14]. Interestingly, LYZS depletion showed reduction of iPS cells (Figure 1C). In addition, we examined quantitative expression of selected set of MEF-specific genes during reprogramming process (Figure S1). All the genes analyzed decreased upon induction of reprogramming, except COX2, which increased at the early stage of reprogramming followed by a dramatic decrease (Figure S1A). Expression levels of all these genes were diminished in late stage of reprogramming (Day 12 or Day 15) as in ES cells. These gene expression patterns indicate that MEF-specific molecular network will be disrupted by 4F to achieve the cell fate transitions during reprogramming. In summary, these results support the idea that MEF-specific catalytic or regulatory proteins can negatively regulate reprogramming and also suggest that it is critical to modulate diverse biological functions during transition of cell identities such as MEFs to iPSCs.

The NSAID Nabumetone enhances iPS cell generation

Next, we developed a genomics database drug discovery strategy to identify small molecules that enhance reprogramming. To shorten the list without extensive shot-gun screening, we focused on candidate molecules that potentially either antagonized MEF-specific genes or upregulated MES-specific/reprogramming genes (Figure 1A). To do so, we conducted

computational screening by utilizing NextBio (www.nextbio.com) data-mining tools to collect information from public data sources [15]. NextBio provides an integrated platform to collect information from public data base, process these data using various pipelines, and then output analyzed results for customized purposes. Using highly enriched genes in either MES or MEF (Figure 1A) as queries, we manually examined the information of meta-analysis and acquired 17 molecules (Table S2) that either negatively regulated MEF genes or positively affected MES genes from various *in vitro* and *in vivo* studies deposited in public data base. We tested all 17 by examining alkaline phosphatase (AP) + colony formation during reprogramming while these molecules were applied. Molecules not showing adverse effect on AP+ colony formation (data not shown) were picked for further study. To the end, we picked 6 molecules—Nabumetone, 4-hydroxytamoxifen (OHTM), Corynanthine, Moclobemide, NiSO4, and lectin—for further analysis (Figure 2A). To evaluate their effect on induction of mature GFP+ iPS cells, we treated OSKM-transduced Oct4-EGFP MEFs four days after transduction with each of these factors separately. Among the six, the NSAID prostaglandin-endoperoxide synthase (PTGS) and the cyclooxygenase (COX) inhibitor Nabumetone greatly increased the number of reprogrammed colonies by at least 2.8-fold (Figure 2B) compared with DMSO controls, while lectin showed minor but consistent improvement on iPS cell formation.

Since MEFs mainly express the COX2 isozyme (verified by RT-qPCR, data not shown & [12]), we proposed that COX2 is the primary Nabumetone target during reprogramming. To test that idea, we knocked down COX2 in Oct4-EGFP MEFs by siRNA with or without Nabumetone during reprogramming with OSKM. In the presence of control siRNA (siNT), Nabumetone alone enhanced reprogramming efficiency by more than 6-fold (Figure 2C) compared with DMSO treatment. Transduction of cells with COX2 siRNA increased the number of GFP+ iPS cell colonies by over 5-fold compared with cells transduced with siNT control (Figure 2C). However, we observed no further enhancement of reprogramming efficiency in the presence of both siCOX2 and Nabumetone (Figure 2C), likely due to the maximal COX2 silencing effects by siRNA. To determine whether the COX2 is the main target instead of COX1, which is constitutively expressed in various tissues, we applied selective inhibitors targeting either COX1 or COX2 during reprogramming with OSKM or OSK [16–18]. Interestingly, only the selective COX2 inhibitors, Celecoxib and NS-398, showed similar effects on iPS cell generation as Nabumetone with OSKM or OSK pluripotency factors (Figure S2). On the other hand, selective COX1 inhibitor, Indomethacin, showed no effect to boost reprogramming with OSKM or OSK (Figure S2), although COX1 greatly decreased upon induction of reprogramming (Figure S1D). To further investigate the role of COX2 in reprogramming, we cloned and overexpressed COX2 along with OSKM during reprogramming. Our results show that overexpression of COX2 compromised reprogramming with OSKM pluripotency factors (Figure S3). Overall, these results support the notion that COX2 is a barrier for reprogramming and that Nabumetone enhances reprogramming by mainly blocking COX2 activity.

Nabumetone can generate iPS cells in the absence of c-Myc

To further analyze Nabumetone reprogramming potential, we asked whether Nabumetone can replace the proto-oncogene c-Myc, which may greatly increase tumorigenesis in vivo. Oct4-EGFP MEFs were reprogrammed using either OSKM or OSK without c-Myc, and induced cells were treated with Nabumetone or DMSO four days later. Nabumetone treatment significantly enhanced reprogramming by OSK by ~2.5-fold as assessed at day 21 (Figure 3A) compared with control OSK+DMSO. This data suggests that Nabumetone not only improves OSKM reprogramming, likely by blocking COX2, but can substitute c-Myc function in the process.

OHTM and Nabumetone can produce iPS cells without Sox2

We next asked whether the small molecules identified in our analysis can replace the need for other reprogramming factors. To do so we tested a pool of the six candidate molecules for their ability to replace any single reprogramming factor. Strikingly, the pool replaced Sox2 during reprogramming of Oct4-EGFP MEF with OKM and significantly increased reprogramming efficiency by more than 10-fold (Figure 3B) compared with controls. To determine which molecule(s) exerted that effect, we individually tested each of the six small molecules in OKM reprogramming protocols. We found that the anti-cancer drug OHTM significantly improved OKM-induced reprogramming, while OKM+DMSO did not produce any mature iPSC colonies (Figure 3C). Similarly, Nabumetone significantly improved OKM-induced reprogramming, which showed comparable effect with OHTM (Figure 3D). Overall, these results indicate that either OHTM or Nabumetone can substitute Sox2 function to generate iPSCs.

OHTM increases endogenous Sox2 expression during OKM reprogramming

To understand the molecular mechanism underlying OHTM's effect on reprogramming, we asked whether OHTM induces endogenous Sox2 expression. To do so, we applied OHTM or control DMSO to Oct4-EGFP MEF four days after transduction with OKM. Cells were harvested at indicated time points for total RNA isolation and real time qRT-PCR analysis (Figure 3E). Strikingly, endogenous Sox2 mRNA was significantly induced by 220% by OHTM in OKM-transduced cells at day 12 and by 400% at day 16 compared with OKM +DMSO controls, indicating that OHTM enhances reprogramming, at least partially, by increasing endogenous Sox2 expression. However, the direct targets of OHTM to affect Sox2 expression are not clear.

OKM+OHTM or OKM+Nabumetone iPS cells attain ES identity and pluripotency

To verify whether iPS cells derived with OKM in the presence of our pooled or individual molecules attain self-renewal and pluripotency, we analyzed iPS cells for these properties. Genomic DNAs were isolated from OKM plus the six-molecule pool (OKM+6), OKM +OHTM, or OKM+Nabumetone iPS cells to verify transgene integration by PCR analysis. OKM iPSC clones showed no Sox2 transgene integration (Figure S4, panel B and C), demonstrating OKM iPS cells could be derived with pool of six molecules, OHTM or Nabumetone alone in the absence of Sox2 transgene. When we cultured OKM iPS cells for at least one month (> 10 passages) and fixed them for immunostaining, OKM+6 and OKM +Nabumetone iPS cells exhibited ES-like dome shape morphology with a clear boundary (Figure 4A, and Figure S4, panel A), and they highly expressed endogenous Oct3/4 (EGFP) and Nanog (Figure 4A, and Figure S4, panel A), indicating establishment of ES-like transcriptional networks. OKM+6 iPS cells expressed SSEA1 (Figure S4A), and OKM +Nabumetone iPS cells also acquired the stem cell marker alkaline phosphatase (AP) (Figure 4A). Importantly, endogenous Sox2 expression was activated in OKM+Nabumetone iPS cells (Figure 4A), suggesting that a full self-renewal circuit was restored. To confirm restoration of an ES-like transcriptome, we examined mRNA expression profiles of OKM +OHTM and OKM+Nabumetone iPS cells by microarray analysis. Representative clones from OKM+OHTM iPS cells showed a high degree of similarity with ES cells, but not MEFs (Figure 4B), as did OKM+Nabumetone iPS clones (Figure 4B).

To determine whether OKM plus small molecule-derived iPS cells show pluripotency comparable to ES cells, we first tested *in vitro* differentiation capacity. OKM+6 iPS cells were induced to form embryoid bodies (EBs) for two weeks, and then fixed for immunostaining. After two weeks of *in vitro* differentiation, cell types typical of all three germ layers were observed (Figure S4D). To further assess differentiation potential, OKM +OHTM and OKM+Nabumetone iPS cells were injected into nude mice and allowed to

differentiate into various tissues. Teratomas, which were observed three weeks post injection, were subjected to histopathological analysis. Tissues originating from all three germ layers were generated (Figure 4C and 4D), confirming that iPS cells obtained pluripotency. To vigorously test pluripotency of OKM iPS cells, OKM+Nabumetone iPS cells were injected into embryonic day (E) 3.5 blastocysts to create chimera. Contributions of OKM+Nabumetone iPSCs to chimera mice were accessed by black coat color at day 17 after birth. We obtained OKM+Nabumetone iPSCs contribution up to 50% (Figure 4E). We next examined the germline transmission capability of OKM+Nabumetone iPS cells. By analyzing E13.5 embryos after injecting OKM+Nabumetone iPSCs into blastocysts, we found strong Oct4-EGFP expression in genital ridge (Figure 4F), showing germline contribution of OKM+Nabumetone iPSCs. In summary, our data demonstrate that small molecule with OKM derived iPS cells do attain ES identity and pluripotency.

DISCUSSION

Based on knowledge of the reprogramming steps, we hypothesized that overcoming MEF-specific networks is the first step in the process. We observed that specific siRNA-mediated knockdown of MEF genes encoding catalytic or regulatory proteins, including WISP1, PRRX1, HMGA2, NFIX, PRKG2, COX2, 6720477E09RIK, and TGFβ3, significantly enhanced reprogramming (Figure 1). To accelerate screening of small molecules, we employed a computational screening method using the NextBio data-mining framework [15] and identified six molecules (Figure 2), including Nabumetone, OHTM, Corynanthine, Moclobemide, NiSO4, and lectin, which function together to reprogram MEFs without Sox2 (Figure 3). One of those factors alone, OHTM, could partially replace the Sox2 transgene during reprogramming by inducing endogenous Sox2 expression (Figure 3). We further showed that Nabumetone enhances reprogramming by inhibiting COX2 activity (Figure 3). Finally, we showed that Nabumetone also promotes reprogramming in the absence of c-Myc or Sox2 function without compromising self-renewal and pluripotency of small molecule-derived iPS cells (Figure 4).

Nabumetone is a non-steroidal anti-inflammatory drug (NSAID) clinically used primarily to treat pain and inflammation associated with osteoarthritis (OA) or rheumatoid arthritis (RA) [19,20]. Nabumetone exerts anti-inflammatory activity by inhibiting COX2 function through its metabolite 6-methoxy-2-naphthylacetic acid (6-MNA). Moreover, it is reported that NSAIDs compromise tumor growth in clinical cases and experimental models of cancer, and also that two isoforms cyclooxygenase-1 and -2 function in a variety of pathophysiological processes, such as modulating apoptosis, angiogenesis, invasion, and carcinogenesis [21–26]. Preliminary *in vitro* and *in vivo* studies show that following COX inhibition, signals regulating cell proliferation and apoptosis networks, including EGFR, KRas, PI3K, JAK1, STAT3, c-jun, PCNA, TGFβ3, BAX, TUNEL, Bcl-2, c-jun, p21, p27, p53, and NM23, are widely altered in tumor cells [27]. However, the roles of COX inhibitors in tumorigenesis remain controversial, since COX2 expression differs widely in different types of cancer cells [23]. In this study we showed that COX2 is highly expressed in MEFs and serves as a barrier to reprogramming. Therefore, further analysis is required to understand the biological function and molecular regulation of COX2 in both cancer and reprogramming biology.

Tamoxifen is a standard chemotherapy used to treat primary and advanced breast cancer by blocking the estrogen receptor (ER) via its metabolites OHTM and endoxifen. OHTM activity has been addressed primarily through its effect on the ER [28]. However, we did not observe detectable levels of ER expression in MEFs (data not shown). OHTM-induced programmed cell death can reportedly be induced through ER-independent pathways in HeLa cells [29], suggesting that other factors respond to OHTM. Moreover, 3, 4-

dihydroxytamoxifen, a more hydroxylated form of OHTM, can interact with both proteins and DNA [28], suggesting the possibility of numerous targets *in vivo*.

Reprogramming of somatic cells to iPS cells by small molecules could facilitate pharmaceutical and medical applications of pluripotent stem cells [30,31]. A number of studies have identified small molecules that enhance reprogramming by targeting various pathways including TGFβ and GSK3 [13,14,32–37]. Although iPS cells can be generated in the absence of Sox2 [13,14,37], only RepSox has been shown to partially induce Nanog expression in partial iPS cells [13]. Here, we are the first to report that Sox2 can be induced by OHTM treatment during reprogramming. Further investigation is required to identify pathways modulated by OHTM in MEFs during reprogramming.

Increasing evidence shows that overcoming the security of somatic cell identity is a critical step initiating the transition from mesenchymal to epithelial status [38–42]. This step requires large-scale regulation of opposing genes within only few days during the first 8 days of reprogramming, including Cdh1, Epcam, Crb3, Ocln, Snail, Slug, Zeb1, Zeb2, BMP, and TGFβ pathways [40,41]. Since TGFβ3 is also on our list and TGFβ3 knock-down greatly enhances reprogramming efficiency, these data support our idea that down-regulating MEF regulatory factors is an effective approach to enhance reprogramming. Furthermore, our study confirms that downregulation of MEF genes encoding catalytic factors constitutes some of the earliest steps of reprogramming and that attenuating key somatic genes is critical to enhance reprogramming efficiency. Further study is needed to reveal how the individual network of these MEF-enriched enzymes functions in the process.

In summary, mouse ES cells express regulatory genes that differ from those expressed in MEFs. Reasoning that the latter encode factors that maintain MEF function and establish fibroblast identity, we manipulated them using either siRNAs or small molecules in an effort to enhance reprogramming efficiency. This hypothesis-driven approach provides an alternative to shot-gun screening, which should accelerate understanding of the molecular mechanism underlying generation of iPS cells and suggest novel therapeutic methodologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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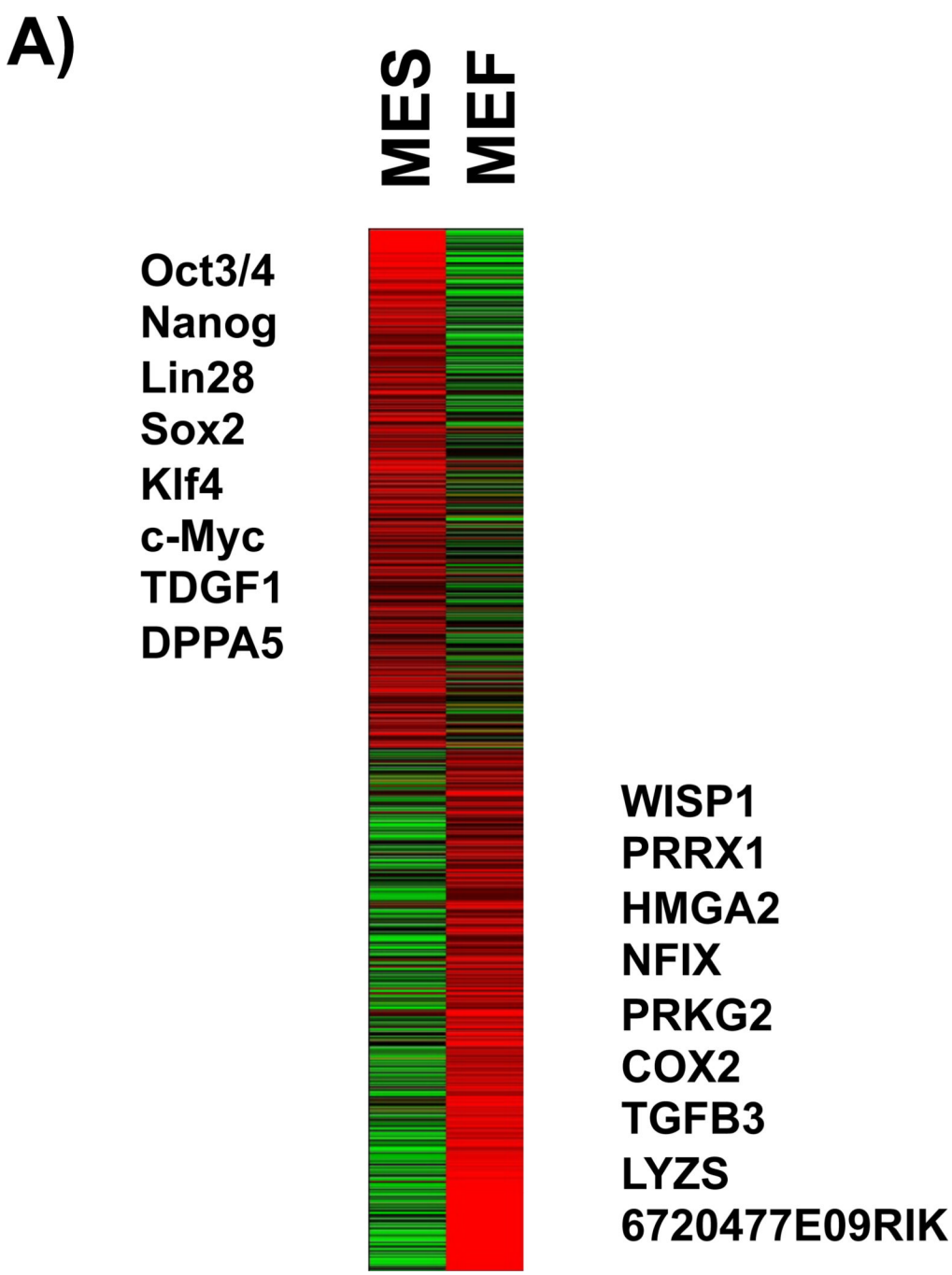


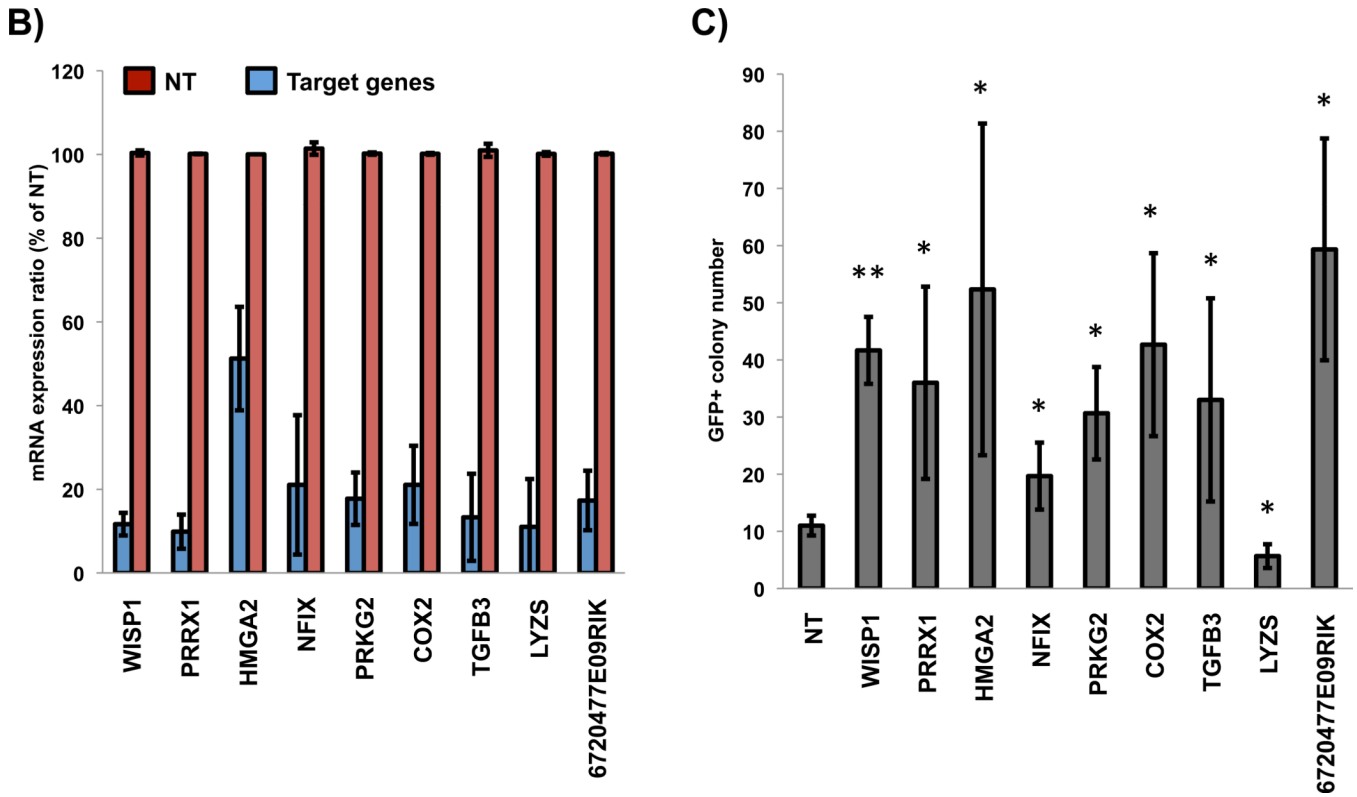


Figure 1. Inhibiting MEF-specific genes enhances iPS cell reprogramming

A) Heat map representing mRNA microarray analysis of mouse ES cells (MES) and MEFs. Total RNA isolated from MEFs and MES cells was used for mRNA microarray analysis. The expression intensity of each gene is shown by colorimeter. Key genes encoding catalytic proteins from MEFs or self-renewal factors from MES cells were selected for further investigation.

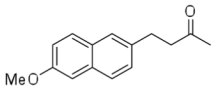
B) Efficient silencing of MEF-specific genes by siRNAs.

MEFs were transfected with siRNAs targeting indicated genes. Cells were harvested ~24 hours post transfection for real time qRT-PCR analysis. Non-targeting (NT) siRNA served as control. Error bars represent standard deviations of six independent experiments.

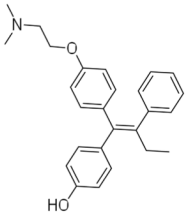
C) Down-regulation of MEF-specific genes significantly improves iPS cell reprogramming. Oct4-EGFP MEFs were transduced with OSKM and five days later transfected with siRNAs targeting indicated genes. Mature reprogrammed iPCS cells were identified as GFP+ colonies and counted by fluorescence microscopy at day 14~16. Error bars represent standard deviations of three independent experiments. * *P* value < 0.05; ** *P* value < 0.005.

A)

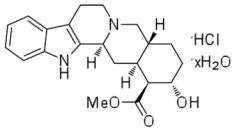
1) Nabumetone



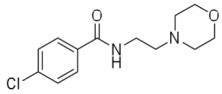
2) 4-Hydroxytamoxifen



3) Corynanthine hydrochloride



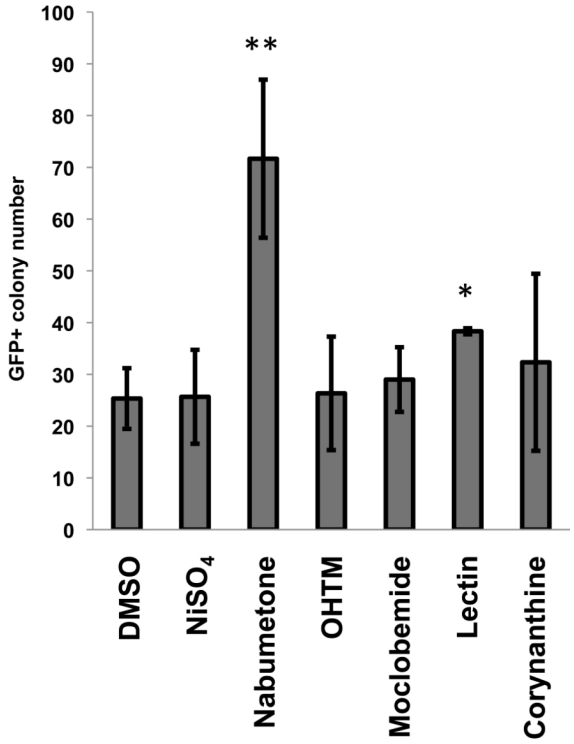
4) Moclobemide



5) NiSO4 · 6H2O

6) Lectin

B)



C)

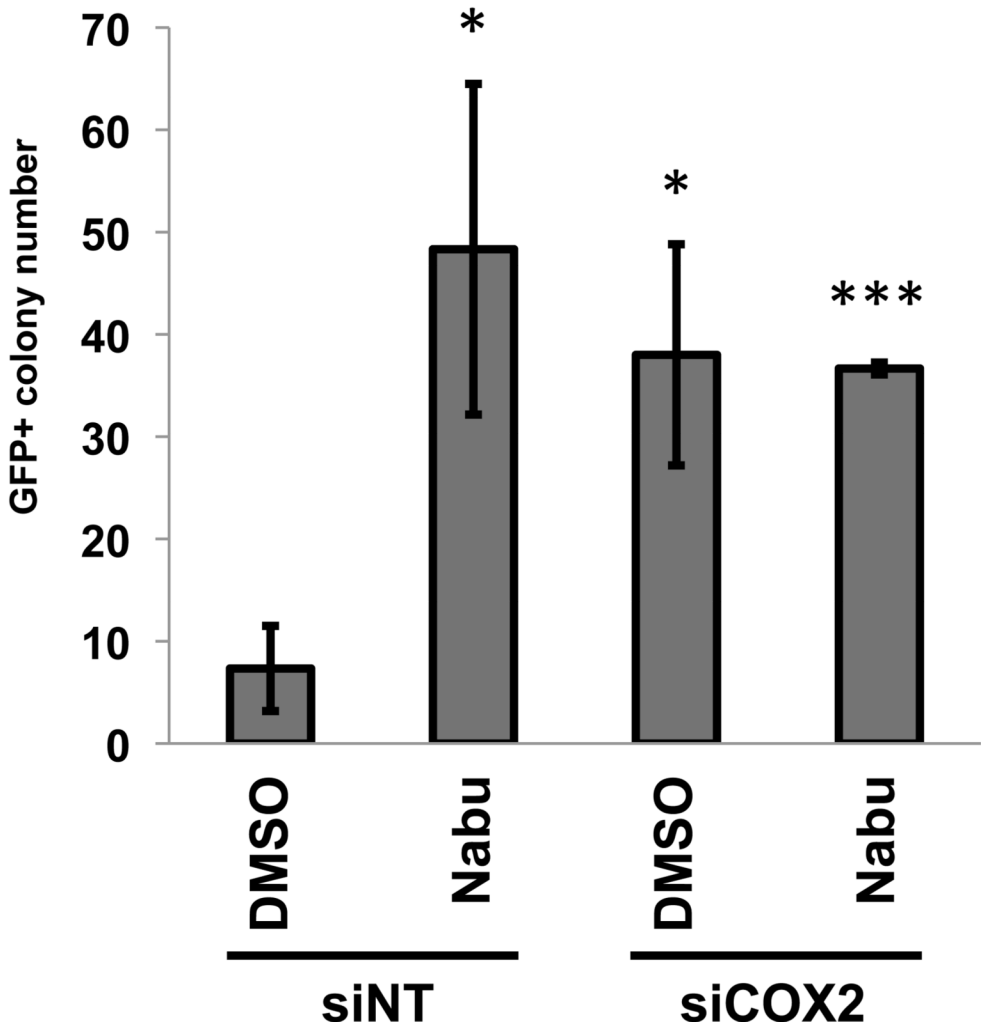


Figure 2. The NSAID drug Nabumetone significantly enhances iPS cell reprogramming by inhibiting COX2

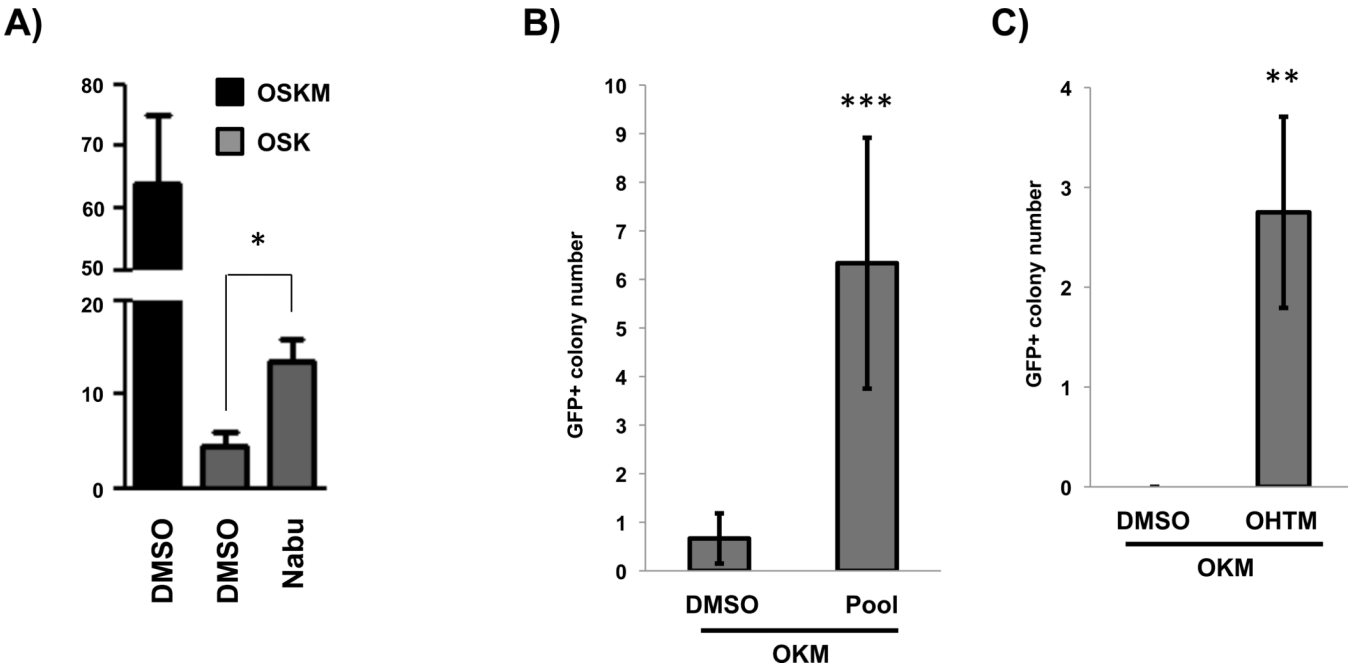
A) Structures of six small molecules used in iPS cell reprogramming.
Small molecules were selected by analyzing MEF and MES genomics data and by cheminformatics as described in text.

B) Nabumetone significantly boosts OSKM-induced reprogramming while lectin showed minor but consistent increase as well.
Oct4-EGFP MEFs were transduced with OSKM and four days later treated with individual small molecules for at least 10 days. GFP+ colonies were identified as described in Fig 1.

Error bars represent standard deviations of three independent experiments. * *P* value < 0.05; ** *P* value < 0.005.

C) Nabumetone improves reprogramming through blocking COX2.

Oct4-EGFP MEFs were transduced with OSKM. Four days later, cells were treated with Nabumetone or DMSO. The next day, cells were transfected with various siRNAs as indicated. GFP+ colonies were identified as described in Fig 1 at day 12 ~ 14. Error bars represent standard deviations of six independent experiments. * *P* value < 0.05; *** *P* value < 0.0005. siNT serves as control. Nabu is abbreviation of Nabumetone.



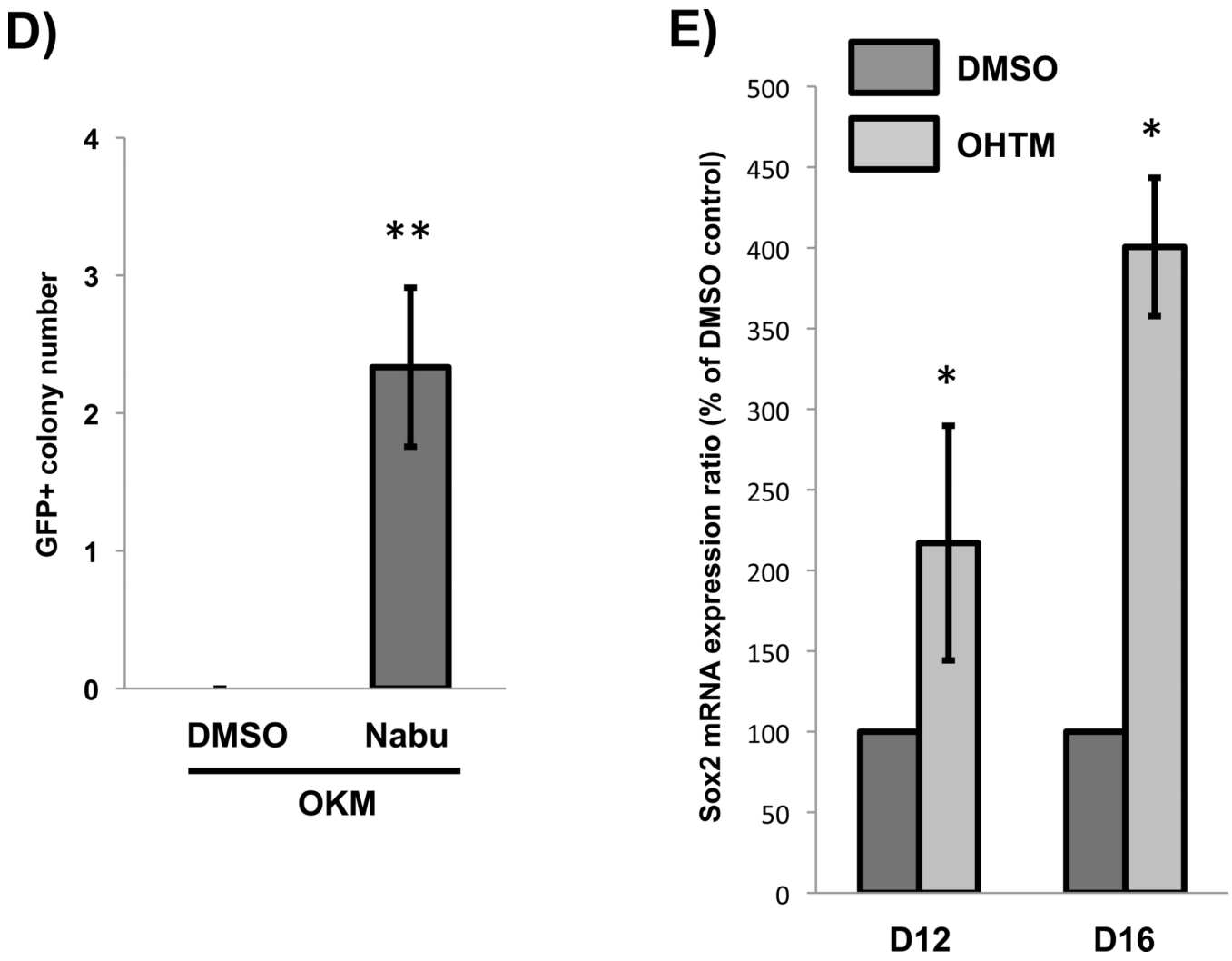


Figure 3. Small molecules can generate iPS cells in the absence of c-Myc and Sox2

A) Nabumetone and OSK reprogram MEF.

Oct4-EGFP MEFs were transduced with OSK without c-Myc and four days later treated with Nabumetone or DMSO for two weeks. Cells transduced with OSKM are shown for comparison. GFP+ colonies were identified as described in Fig. 1 at day 21. Error bars represent standard deviations of two independent experiments. * *P* value < 0.05.

B) A pool of six molecules with OKM reprograms MEFs to iPSCs.

Oct4-EGFP MEFs were transduced with OKM and treated with pool of 6 molecules, including $NiSO_4$, Nabumetone, OHTM, Moclobemide, Lectin, and Corynanthine, at day 4 for at least 10 days. GFP+ colonies were identified and counted as described in Fig. 1 at day 14. Error bars represent standard deviations of six independent experiments. *** *P* value < 0.0005.

C) OHTM and OKM reprogram MEFs to iPSCs.

Oct4-EGFP MEFs were transduced with OKM and four days later treated with 1.25 μM OHTM at least 10 days. GFP+ colonies were counted as described in Fig. 1 at day 15~21. Error bars represent standard deviations of four independent experiments. ** *P* value < 0.005.

D) Nabumetone plus OKM reprograms MEFs to iPSCs.

Oct4-EGFP MEFs were transduced with OKM and four days later treated with 2.18 μM Nabumetone (Nabu) for at least 10 days. GFP+ colonies were counted as described in Fig. 1 at day 17~21. Error bars represent standard deviations of three independent experiments. ** *P* value < 0.005.

E) Sox2 expression is significantly induced by OHTM during OKM-induced reprogramming.

Oct4-EGFP MEFs were transduced with OKM and treated with OHTM four days later. Cells were harvested at indicated days (D) for real time RT-PCR analysis. β actin expression serves as an internal control. Error bars represent standard deviation of 3 independent experiments. * *P* value < 0.05.

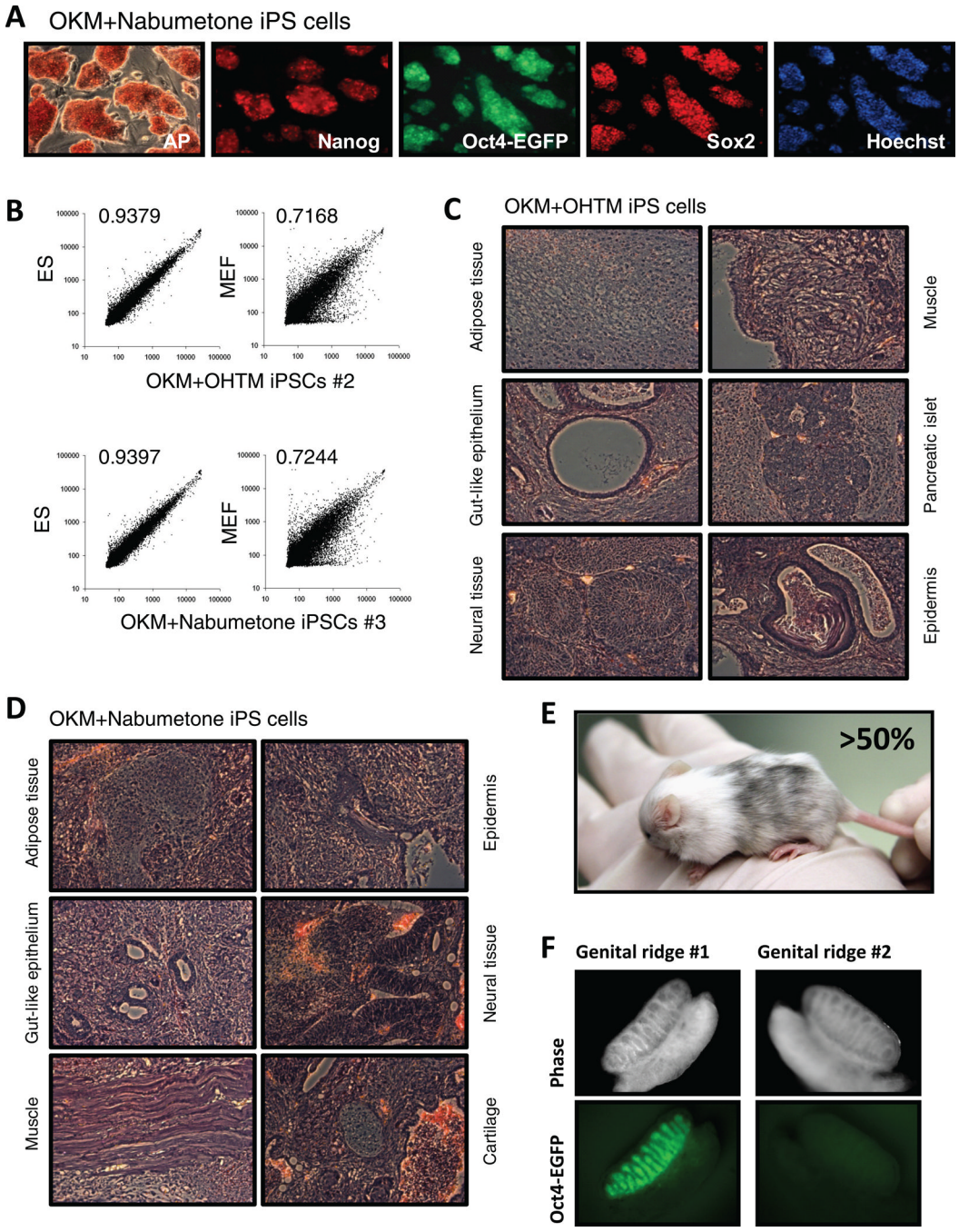


Figure 4. iPS cells derived by OKM + Nabumetone or OKM + OHTM acquire pluripotency

A) Sox2 expression is reactivated in OKM+Nabumetone iPS cells.

OKM+Nabumetone (OKM+Nab) iPS cells were fixed and immunostained for ES cell markers. Representative colonies express ES cell markers Nanog, alkaline phosphatase (AP), and endogenous Oct3/4 (Oct4-EGFP). Endogenous Sox2 was also activated. Hoechst counterstain marks nuclei.

B) OKM+Nabumetone and OKM+OHTM iPS cells share transcriptional profiles similar to MES cells but not MEFs.

Scatter plots show transcriptome comparison of iPS clones with ES or MEF cells. Total RNA was isolated from indicated iPS cells and subjected to mRNA microarray analysis. R^2 values are shown at the top of each panel.

C & D) OKM+Nabumetone and OKM+OHTM iPS cells can differentiate into various cell types.

Teratoma formation analysis of OKM+Nabumetone and OKM+OHTM iPS cells. 1.5×10^6 iPS cells were injected subcutaneously into athymic nude female mice and tumor masses collected three weeks later. Histopathological analysis shows that tissues derived from all three germ layers were identified, including gut-like epithelium and pancreatic islets (endoderm), adipose tissue, cartilage, and muscle (mesoderm), and neural tissue and epidermis (ectoderm).

E) OKM+Nabumetone iPS cells contribute to chimera mice.

OKM+Nabumetone iPS cells were injected into E3.5 blastocysts to create chimera mice. Seventeen days after birth, black coat color was used to determine OKM iPS cells contribution in chimera mice. The representative picture shows >50% contribution of OKM +Nabumetone iPS cells.

F) OKM+Nabumetone iPS cells contribute to germline formation.

OKM+Nabumetone iPS cells were injected into E3.5 blastocysts to create chimera mice. E13.5 embryos were collected from recipient mice for harvesting genital ridge. Germline transmission was determined by Oct4-EGFP expression, indicating contribution of OKM +Nabumetone iPSCs.