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OSKM Induce Extraembryonic Endoderm Stem Cells in Parallel to Induced Pluripotent Stem Cells

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SUMMARY

The reprogramming factors OCT4, SOX2, KLF4, and MYC (OSKM) can reactivate the pluripotency network in terminally differentiated cells, but also regulate expression of non-pluripotency genes in other contexts, such as the mouse primitive endoderm. The primitive endoderm is an extraembryonic lineage established in parallel to the pluripotent epiblast in the blastocyst, and is the progenitor pool for extraembryonic endoderm stem (XEN) cells. We show that OSKM induce expression of endodermal genes, leading to formation of induced XEN (iXEN) cells, which possess key properties of blastocyst-derived XEN cells, including morphology, transcription profile, self-renewal, and multipotency. Our data show that iXEN cells arise in parallel to induced pluripotent stem cells, indicating that OSKM drive cells to two distinct cell fates during reprogramming.

INTRODUCTION

The pluripotency-promoting role of the reprogramming factors OCT4, SOX2, KLF4, and MYC (OSKM) is widely appreciated. However, these reprogramming factors also promote expression of non-pluripotency genes. For example, OCT4 (*Pou5f1*) directly promotes expression of genes important for mouse primitive endoderm (Aksoy et al., 2013; Frum et al., 2013; Le Bin et al., 2014), an extraembryonic lineage present in the blastocyst, SOX2 indirectly promotes expression of primitive endoderm genes in the mouse blastocyst (Wicklow et al., 2014), KLF4 may regulate expression of primitive endoderm genes in the mouse blastocyst (Morgani and Brickman, 2015), and MYC regulates endodermal genes in fibroblasts and embryonic stem cells (ESCs) (Neri et al., 2012; Smith et al., 2010). These observations raise the possibility that OSKM induce expression of endodermal genes in somatic cells. In support of this idea, several groups have reported that endodermal genes, such as *Gata6*, *Gata4*, and *Sox17*, are upregulated in protocols used to reprogram fibroblasts to induced pluripotent stem cells (iPSCs) (Hou et al., 2013; Serrano et al., 2013; Zhao et al., 2015).

However, there is no consensus as to whether endodermal gene expression promotes or antagonizes the acquisition of pluripotency. GATA4 and GATA6 can reportedly substitute for OCT4 to produce iPSCs (Shu et al., 2013, 2015), arguing that endodermal genes promote acquisition of pluripotency. Consistent with this, endodermal genes are reportedly expressed by cells as they become pluripotent during chemical reprogramming (Hou et al., 2013; Zhao et al., 2015). By contrast, other evidence suggests that endodermal genes oppose pluripotency during reprogramming. For example, *Gata4* interferes with the acquisition of pluripotency during *OSKM* reprogramming (Serrano et al., 2013), *Gata6* is expressed in some partially reprogrammed cells (Mikkelsen et al., 2008), which are thought to be trapped in a state between differentiated and pluripotent (Meissner et al., 2007), and *Gata6* knockdown led to increased expression of *Nanog* in these cells (Mikkelsen et al., 2008). Thus, endodermal genes have been described as indicators of incomplete reprogramming. Here, we show that OSKM drive cells along two distinct and parallel pathways, one pluripotent and one endodermal.

RESULTS AND DISCUSSION

iXEN Cells Display XEN Cell Morphology and Gene Expression

We infected mouse embryonic fibroblasts (MEFs) or adult tail tip fibroblasts (TTFs) with retroviruses carrying *OSKM* (Takahashi and Yamanaka, 2006). Eighteen days after infection, we observed domed colonies with smooth boundaries (Figure 1A), which could be propagated as stable iPSC lines (16 out of 28 colonies) and could contribute to normal development in chimeras (Figure S1A). In addition, we observed colonies that were large and flat, with ragged boundaries (Figure 1A), and roughly three times more abundant and three times larger than presumptive iPSC colonies (Figure 1B). These colonies were visible as early as 6 days after *OSKM* infection (Figure S1B). Here, we demonstrate extensive similarity between blastocyst-derived extraembryonic endoderm stem cell (XEN) cell lines and the MEF-derived cell lines that we hereafter refer to as induced XEN (iXEN) cells.

We manually isolated putative iXEN cell colonies and cultured these in ESC medium without leukemia inhibitory

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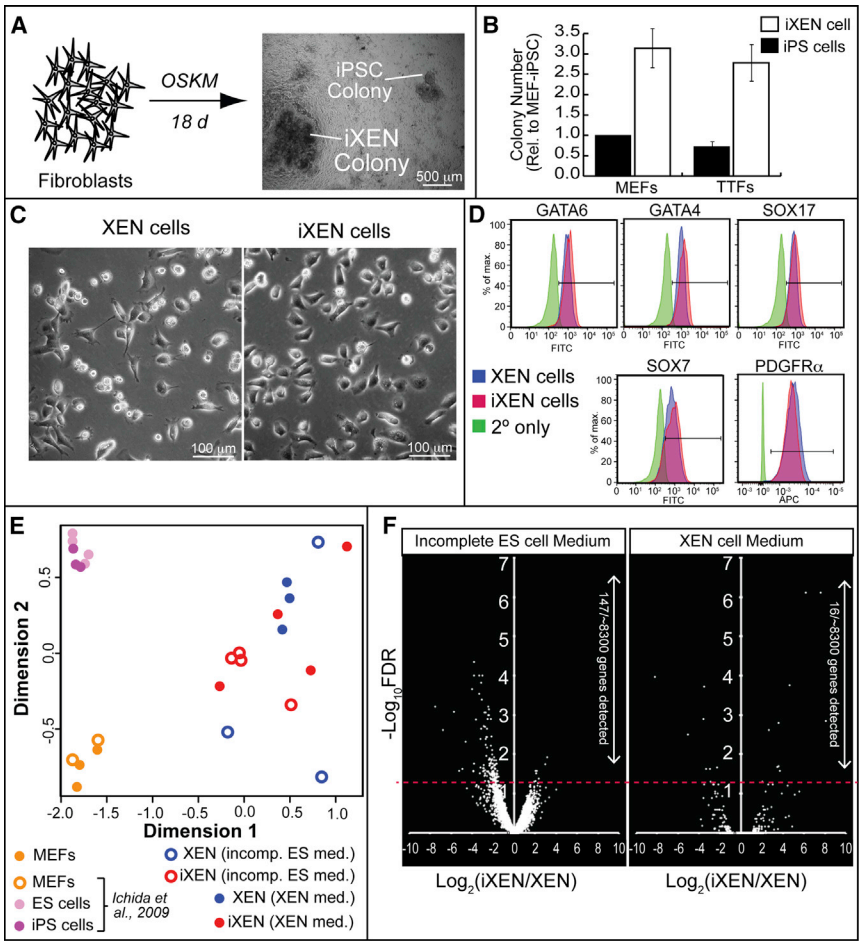

Figure 1. OSKM-Induced XEN Cells Arise during Reprogramming

(A) Fibroblasts were reprogrammed (Takahashi and Yamanaka, 2006), and examined 18 days after *OSKM* infection.

(B) Frequencies at which iPSC and iXEN cell colonies were observed. Error bars denote SE among three reprogrammings each.

(C) Morphology of iXEN cells is similar to that of blastocyst-derived XEN cells.

(D) Flow cytometric analysis shows that endodermal proteins are detected in essentially all XEN and iXEN cells (representative of three independently derived XEN and iXEN cell lines; brackets, see Figure S1C).

(E) Multidimensional scaling analysis of the 100 most variably expressed genes shows that iXEN and XEN cell lines are highly similar, regardless of culture medium, and dissimilar to MEFs and pluripotent stem cell lines (Ichida et al., 2009).

(F) Volcano plots show genes whose average expression level differs significantly (FDR > 0.05, red dotted line) between XEN and iXEN cell lines in each cell culture medium.

See also Table S1.

factor (LIF) (incomplete ESC medium) or in XEN cell medium, which includes FGF4 and HEPARIN, because both media support the expansion of blastocyst-derived XEN cells (Kunath et al., 2005). Most iXEN cell colonies maintained XEN cell morphology, growing as individual, dispersed, and apparently motile cells, in either medium (40 of 51 colonies) (Figure 1C). A minority of non-iPSC colonies (11 of 51 colonies) displayed a mixed mesenchymal morphology (not shown), reminiscent of partially reprogrammed or transformed cells (Meissner et al., 2007; Mikkelsen et al., 2008; Sridharan et al., 2009).

Next, we evaluated the expression of endodermal markers, including GATA6, GATA4, SOX17, SOX7, and PDGFRA, which were expressed to a similar degree in both XEN and iXEN cell lines (Figures 1D, S1C, and S1D). Notably, NANOG was not detected in iXEN cells (Figure S1D), indicating that iXEN cells are distinct from F-class ("fuzzy") cells, which exist in a state of alternative pluripotency (Tonge et al., 2014). These observations show that iXEN cells express XEN cell markers.

Finally, we compared iXEN and XEN cell transcriptomes by RNA sequencing independently derived cell lines, as well as MEF, iPSC, and ESC lines. Multidimensional scaling (MDS) analysis of the 100 most variably expressed genes showed that iXEN and XEN cell transcriptomes are more similar to each other than to MEF, ESC, or iPSC transcriptomes, regardless of the medium in which XEN/iXEN cell lines had been cultured (Figure 1E). Comparing XEN with iXEN cell lines, we observed significant (false discovery rate [FDR] < 0.05) differences in the expression levels of few (146) genes between XEN and iXEN cells cultured in incomplete ESC medium, and even fewer (16) differences in XEN cell medium (Figure 1F and Table S1). Expression of *OSKM* was not detected in iXEN cells, consistent with transgene silencing. Pathway and gene ontology (GO) term analysis of the differentially expressed genes identified deficiencies in expression of oxidative phosphorylation and glutathione metabolism genes in iXEN cells cultured in incomplete ESC medium relative to those grown in XEN cell medium (Table S1), which could indicate deficient iXEN cell proliferation in the absence of growth factor. No pathways were significantly enriched among the differentially expressed genes when XEN and iXEN cells had been cultured in XEN cell medium. Thus, while

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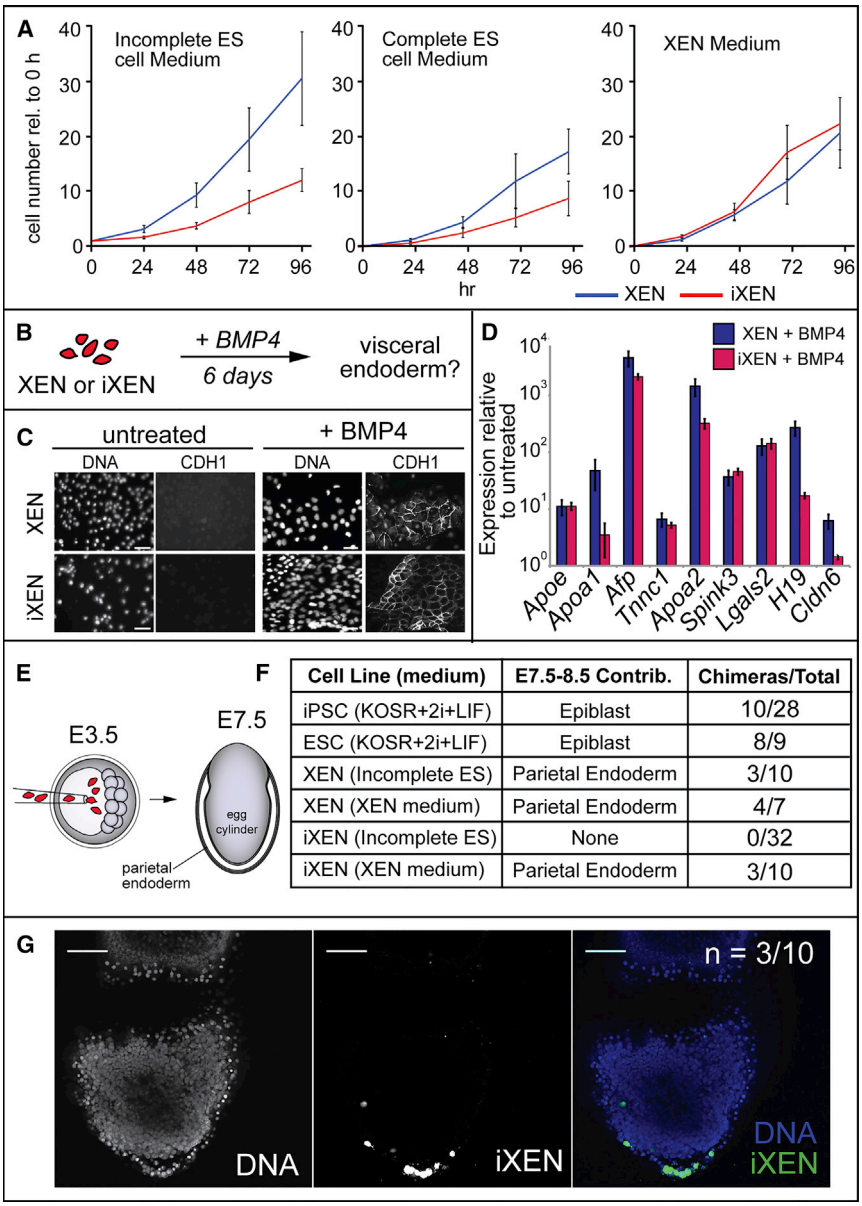

Cell Line (medium)	E7.5-8.5 Contrib.	Chimeras/Total
iPSC (KOSR+2i+LIF)	Epiblast	10/28
ESC (KOSR+2i+LIF)	Epiblast	8/9
XEN (Incomplete ES)	Parietal Endoderm	3/10
XEN (XEN medium)	Parietal Endoderm	4/7
iXEN (Incomplete ES)	None	0/32
iXEN (XEN medium)	Parietal Endoderm	3/10

Figure 2. iXEN Cells Are Self-Renewing and Multipotent

(A) Proliferation rates for cell lines grown in each cell culture medium. Error bars denote SE among three XEN and iXEN cell lines.

(B) VE differentiation assay.

(C) Immunofluorescence shows CDH1 at cell junctions in differentiated iXEN and XEN cells, but not in untreated cells (representative of five independent XEN/iXEN cell lines, DNA = DAPI). Scale bar, 100 μm.

(D) qPCR analysis of VE gene expression in differentiated XEN/iXEN cells, relative to untreated cell lines. Error bars denote SE for two differentiations and four qPCRs each.

(E) In vivo differentiation assay.

(F) Summary of chimera results.

(G) iXEN cells contribute to ParE (see Figure S2 for control chimeras). Scale bar, 100 μm.

more transcriptional differences between iXEN, XEN, MEF, and pluripotent cell lines could become apparent with deeper biological sampling, we conclude that iXEN and XEN cell transcriptomes are extremely similar, and that XEN cell medium better supports conversion of MEFs to XEN-like cells, consistent the role of FGF4 signaling in promoting primitive endoderm development in vivo (Chazaud et al., 2006; Kang et al., 2013; Nichols et al., 2009; Yamanaka et al., 2010).

MEF-Derived XEN Cells Exhibit Stem Cell Properties

Next, we evaluated the self-renewal and multipotency of iXEN cell lines. In terms of self-renewal, iXEN cell lines could be passaged >35 times in either medium. However, iXEN cells grew more slowly than XEN cells in incomplete ESC medium than in XEN cell medium (Figure 2A), consistent with transcriptional profiling predictions. Because LIF supports the expansion of totipotent ESCs that possess XEN-like properties (Morgani et al., 2013), we also examined the proliferation rate of iXEN cells in ESC medium with LIF, but iXEN cells did not proliferate as rapidly as XEN cells in this condition (Figure 2A).

Since blastocyst-derived XEN cells can differentiate into visceral endoderm (VE) or parietal endoderm (ParE) (Artus et al., 2012; Kunath et al., 2005; Paca et al., 2012), we evaluated the multipotency of iXEN cells. During the VE

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differentiation assay (Figure 2B), iXEN cell lines were able to differentiate to VE, evidenced by epithelialization, localization of E-cadherin (CDH1) at cell boundaries (Figure 2C), and upregulation of VE markers (Figure 2D). To evaluate differentiation to ParE, we made chimeras with XEN and iXEN cell lines. ESC and iPSC lines were used in parallel positive controls. In chimeras examined between embryonic days 7.5 and 8.5, ESCs and iPSCs contributed to the epiblast lineage, while XEN cells contributed to ParE with expected degree and frequency (Figures 2F and S2) (Kunath et al., 2005; Wamaitha et al., 2015). iXEN cells cultured in incomplete ESC medium did not contribute to chimeras, even though XEN cells cultured in incomplete ESC medium did. However, iXEN cell lines cultured in XEN cell medium contributed to ParE (Figures 2F and 2G) to a similar extent as XEN cells, indicating that iXEN cells cultured in FGF4/HEP have XEN cell-like developmental potential in vivo. These observations underscore the importance of FGF4/HEP for acquisition of iXEN cell function. These results also indicate that iXEN cells are distinct from totipotent cells isolated from pluripotent cell cultures (Canham et al., 2010; Macfarlan et al., 2012; Morgani et al., 2013), because iXEN cells did not contribute to epiblast or trophoblast lineages.

iXEN Cells Are Not Derived from Pre-existing iPSC Colonies

In monolayers, ESCs can differentiate to XEN-like cells at low frequency in the presence of LIF (Niakan et al., 2010), or at high frequency in the absence of LIF and presence of retinoic acid and activin (RA/activin) (Cho et al., 2012; Niakan et al., 2013). These observations raised the possibility that iXEN cells were derived from iPSCs. However, this possibility seemed unlikely for several reasons. First, we derived iXEN cells in the presence of LIF and absence of RA/activin, and rare XEN-like cells that arise under these conditions arise adjacent to, or encircling, the ESC colony from which they are derived (Niakan et al., 2010). By contrast, iXEN cell colonies were often located far ($\geq$50 μm) from the nearest iPSC colony (29 of 48 colonies). In addition, we routinely observed nascent iXEN cell colonies on the sixth day of OSKM infection (Figures 3A and S1B), which is before we observed iPSCs. These observations argue that iXEN cells are derived from MEFs in parallel to iPSCs.

To query the cellular origins of iXEN cells experimentally, we infected ~100 wells each containing around ten tdTOMATO-labeled MEFs per 20,000 unlabeled MEFs with *OSKM* retroviruses (Figure 3B). Because MEFs were labeled sparsely, we predicted that labeled iPSC or iXEN cell colonies would be relatively rare, enabling us to discern iXEN cell origins. For example, if iXEN cells were derived from iPSC colonies, labeled iXEN cell colonies would always be coincident with labeled iPSC colonies. Alternatively, if iXEN cells were derived from MEFs, labeled iXEN cell colonies would be observed in wells lacking labeled iPSC colonies. As expected, most of the wells (85 of 93 wells) contained unlabeled colonies after 18 days of OSKM infection (Figure 3B). Of the wells containing labeled colonies, most (7 of 8) contained one labeled iXEN cell colony and no labeled iPSC colonies. Only in one well did we observe a labeled iXEN cell colony and a labeled iPSC colony (1 of 93 wells). Therefore, the majority of iXEN cells were not derived from iPSC colonies. We do not exclude the possibility that iXEN cells could be derived from a cell that transiently expressed pluripotency genes (Bar-Nur et al., 2015; Maza et al., 2015). Nevertheless, the presence of iXEN cells in conventional reprogramming experiments could influence the interpretation of reprogramming outcomes, and underscores the importance of evaluating cell fates at the clonal level.

All Four Reprogramming Factors Induce XEN Cell Fate

Next, we investigated whether iXEN cells and iPSCs are induced by similar or different combinations of OSKM. We evaluated the copy numbers of each transgene by qPCR analysis of genomic DNA from multiple iXEN cell and iPSC lines. We observed that the number of *OSKM* copies tended to be lower in iXEN cell than in iPSC lines, although average copy numbers did not differ significantly (Figure 3C). To determine whether the trend was meaningful, we overexpressed equal levels of *OSKM* by deriving MEFs by carrying a doxycycline (dox)-inducible *OSKM* cassette (Carey et al., 2010). Interestingly, we observed an increase in the efficiency of forming both iPSC and iXEN cell colonies (Figure 3D), indicating that all four reprogramming factors can induce formation of iXEN cells.

GATA6 and GATA4 Facilitate iXEN Cell, but Not iPSC, Formation

Endodermal genes are reportedly upregulated prior to pluripotency genes in cultures of MEFs undergoing small-molecule reprogramming, but not during *OSKM* reprogramming (Hou et al., 2013; Zhao et al., 2015). However, we observed GATA6-positive cells 6 days after *OSKM* infection (Figure 3A). Moreover, qPCR analysis showed that endodermal genes, like pluripotency genes, were increasingly upregulated during the 20-day time course of *OSKM* reprogramming (Figure 4A), but this did not resolve whether endodermal genes were expressed within iPSC progenitors or within a distinct population. We therefore used flow cytometry to determine whether NANOG-positive, pre-iPSCs (Bar-Nur et al., 2015) expressed endodermal (GATA6 or SOX17) proteins during reprogramming. We detected NANOG and endodermal proteins in two largely distinct populations that increased in size during reprogramming (Figures 4B and S3A–S3C). Neither

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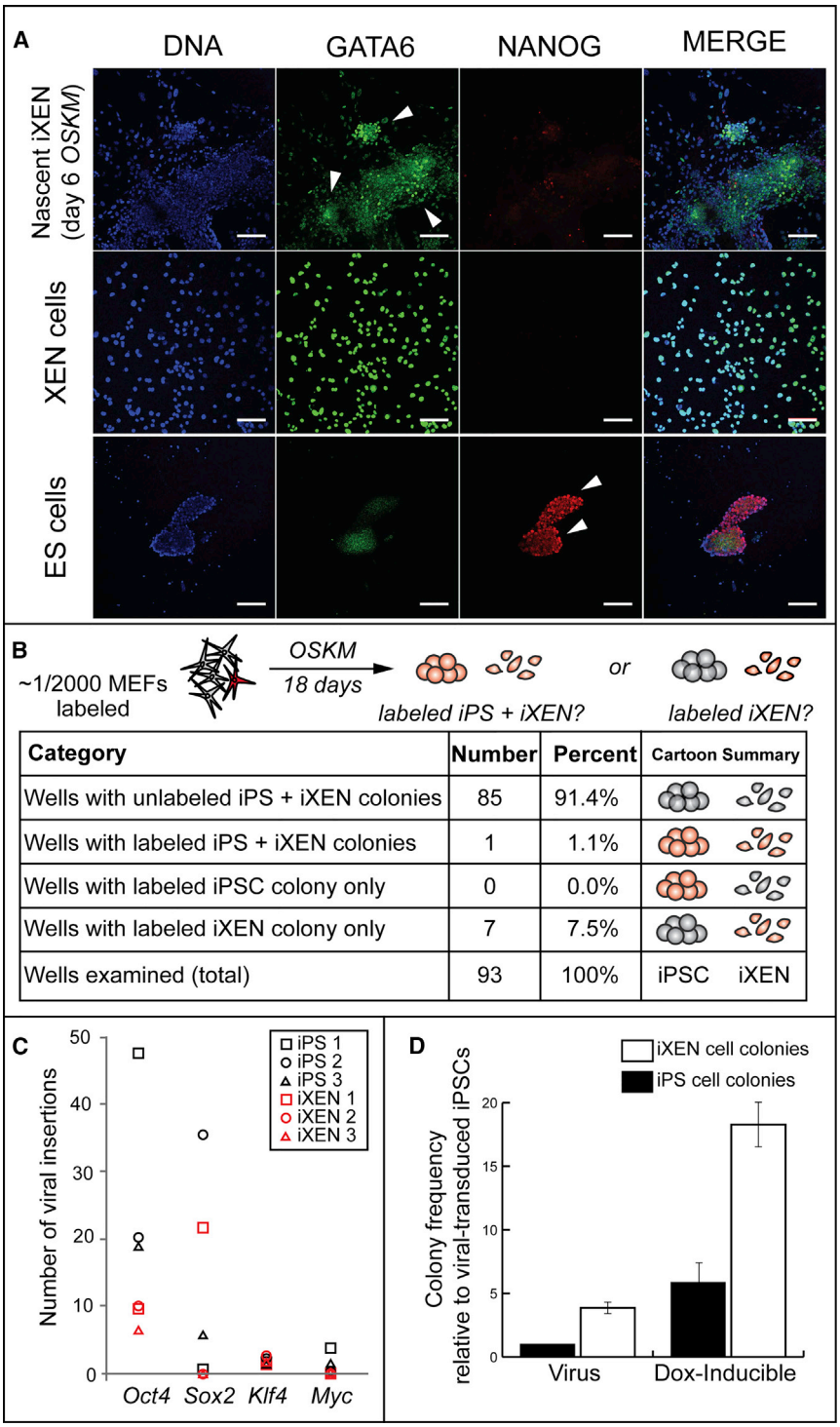

Category	Number	Percent	Cartoon Summary
Wells with unlabeled iPS + iXEN colonies	85	91.4%	
Wells with labeled iPS + iXEN colonies	1	1.1%	
Wells with labeled iPSC colony only	0	0.0%	
Wells with labeled iXEN colony only	7	7.5%	
Wells examined (total)	93	100%	iPSC iXEN

Figure 3. OSKM Induce iXEN Cell Fate in MEFs

(A) Nuclear GATA6, but not NANOG, in nascent iXEN colony on day 6 of *OSKM* reprogramming (compared with XEN cell and ESC controls). Arrowheads point to nuclear proteins. Scale bar, 100 μm.

(B) Lineage tracing shows that iXEN cells are not derived from iPSC colonies during *OSKM* reprogramming of MEFs (representative of two experiments).

(C) Absolute qPCR measurement of *OSKM* copy numbers in XEN/iXEN cell genomic DNA.

(D) Comparison of the frequency of iXEN cell/iPSC colonies after retroviral or transgenic overexpression of *OSKM* on day 18 of reprogramming. Error bars denote SE; two cell lines, four experiments each.

population was prevalent in MEFs undergoing mock reprogramming (Figure S4A), but both populations were present and distinct in TTFs during *OSKM* reprogramming (Figure S4B). These observations suggest that endodermal genes are not expressed in pluripotent cells during *OSKM* reprogramming, in contrast to evidence that MEFs undergoing chemical reprogramming transition through a XEN-like state (Zhao et al., 2015).

To investigate further whether iPSCs transition through a XEN-like state during *OSKM* reprogramming, we used

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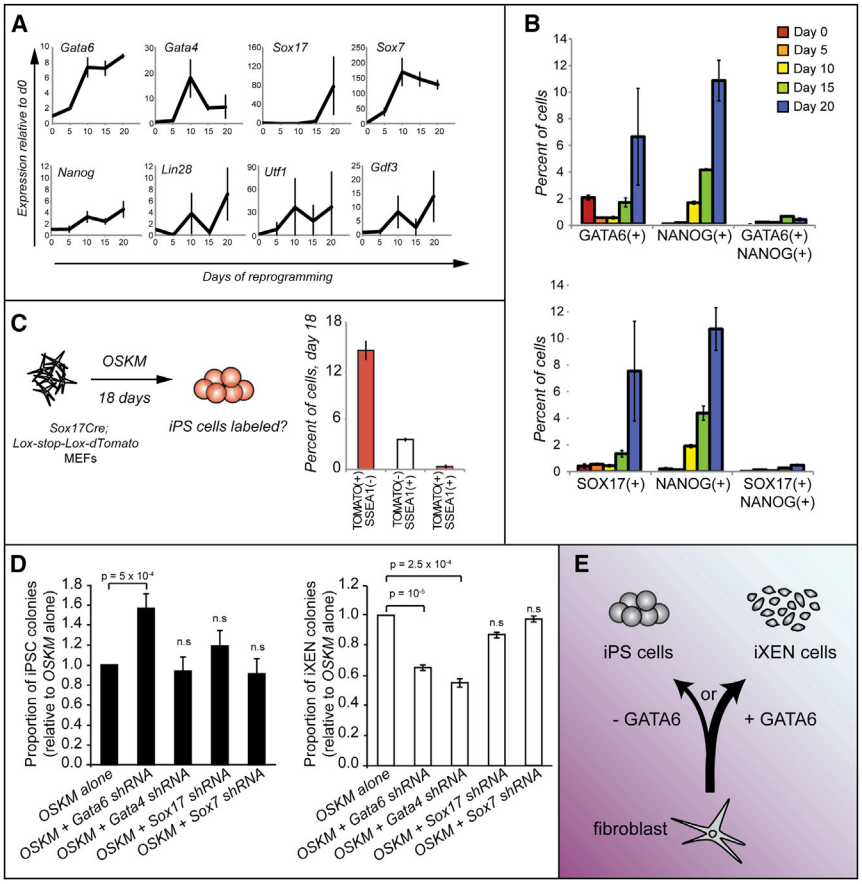

Figure 4. MEF-Expressed Endodermal Genes Promote iXEN Cell Fate

(A) Endodermal (top row) and pluripotency (lower row) genes are upregulated during retroviral *OSKM* reprogramming of MEFs, measured by qPCR. Error bars denote SE among three reprogrammings and four qPCRs.

(B) Flow cytometry analysis of MEFs during *OSKM* reprogramming shows that cells expressing endodermal and pluripotency proteins are largely distinct. Error bars denote SE between two experiments (see also Figures S3A–S3C and S4).

(C) *Sox17Cre* lineage tracing and flow cytometry analysis of cells 20 days after *OSKM* infection of MEFs, showing that most pluripotent (SSEA1-positive) cells never expressed *Sox17*. Error bars denote SE among three reprogrammings (see also Figure S3D).

(D) Proportions of iPSC and iXEN cell colonies after coinfection of MEFs with *OSKM* and shRNA constructs. Error bars denote SE among three reprogrammings (see also Figure S3E).

(E) Model proposing that endogenous GATA6 expression can push cells toward either iPSC or iXEN fate during reprogramming.

lineage tracing. We retrovirally reprogrammed MEFs carrying *Cre* under the control of the *Sox17* promoter (Liao et al., 2009) and a CRE-sensitive *lox-stop-lox-tdTomato* reporter (Madisen et al., 2010). We predicted that if iPSCs had expressed endodermal genes during reprogramming, then most iPSCs would be tdTOMATO-positive 20 days after *OSKM* infection because SOX17 is highly and homogeneously expressed in iXEN/XEN cells (Figure 1D). However, we observed that almost all SSEA1-positive cells were tdTOMATO negative (Figures 4C and S3D), indicating that most pluripotent cells had not expressed *Sox17* during reprogramming. Taken together, our observations indicate that during *OSKM* reprogramming, endodermal genes are upregulated in cells that are largely distinct from those becoming pluripotent. In addition, our observations indicate that pluripotency and XEN pathways are parallel during *OSKM* reprogramming, in contrast to the serial, XEN-to-iPSC pathway that predominates chemical reprogramming (Zhao et al., 2015). Moreover, XEN-like cells derived during chemical reprogramming cannot be maintained in XEN cell medium (Zhao et al., 2015), highlighting fundamental differences in cells produced by chemical and *OSKM* reprogramming.

Finally, we tested the requirement for endodermal genes in the formation of iXEN cells, with the expectation that decreasing endodermal gene expression would decrease the proportion of iXEN cells. We first confirmed substantial knockdown of *Gata6*, *Gata4*, *Sox17*, or *Sox7* in established XEN cells by transfection of small hairpin RNA (shRNA)-encoding plasmids (Figure S3E). We then infected MEFs with shRNAs during reprogramming. Knockdown of *Gata6* or *Gata4* led to a 2-fold decrease in the number of iXEN cell colonies obtained (Figure 4D), indicating that these genes are required for iXEN cell fate. Notably, knockdown of *Gata6* also led to a significant increase in the number of iPSC colonies. Thus, endodermal gene expression interferes with pluripotency during *OSKM* reprogramming. We propose that heterogeneous expression of GATA6 within the MEFs (Figure 4B) could contribute to different outcomes during reprogramming (Figure 4E). Alternatively, stochastic differences in the timing of translation or nuclear localization of the reprogramming factors could influence cell fates. Finally, our observations suggest that the parallel pluripotency and XEN pathways compete with, rather than support, each other during reprogramming. By contrast, paracrine signaling

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from pluripotent epiblast cells supports the formation of XEN cell progenitors in the blastocyst (Frum and Ralston, 2015), but our evidence does not support this model during reprogramming. We anticipate that identification of additional mechanisms regulating the balance between iXEN cell and iPSC fates will inform future efforts to characterize the molecular steps of cell fate specification, and lead to establishment of new genetic models of reproductive disorders.

EXPERIMENTAL PROCEDURES

Mouse Work

All animal work conformed to the guidelines and regulatory standards of the Michigan State University Institutional Animal Care and Use Committee. See Supplemental Experimental Procedures for strains.

Cell Culture

See Supplemental Experimental Procedures for media recipes. *OSKM* retroviruses were produced by transfecting 293T cells with pCL-ECO and pMXs plasmids encoding *OSKM* (Addgene). 48 hr later, supernatant was harvested and qPCR was used to quantify virus (for primer sequence see Supplemental Experimental Procedures). Approximately 6×10^7 copies OSKM per 40,000 MEFs were added for 24 hr, and medium was then replaced with MEF medium, then ESC medium on days 2 and 4, and finally reprogramming medium on day 6 and every other day thereafter. For dox-induced reprogramming, dox-inducible MEFs were plated at a density of 50 cells/mm^2 on gelatin in MEF medium. After 24 hr and every 2 days for 16 days thereafter, wells received ESC medium with 2 μg/ml dox (Sigma). For sparse labeling, ten tdTOMATO-labeled MEFs and 20,000 unlabeled MEFs were seeded in each well of 24-well dishes, then infected with *OSKM* and examined 18 days later. For lineage tracing, MEFs carrying *Sox17*$^{tm1(icre)Heli}$ and *Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$ were infected with *OSKM* retrovirus, as described above. shRNAs were cloned into pMXs and titrated by qPCR (for sequence see Supplemental Experimental Procedures).

Single-Cell Analyses

Immunostained cells were analyzed on a Becton Dickinson LSR II or Olympus Fluoview FV1000. Details are available in Supplemental Experimental Procedures.

RNA Sequencing and qPCR

cDNA libraries were sequenced to a depth of 25–50 million 50-bp single-end reads using an Illumina HiSeq 2500. See Supplemental Experimental Procedures for details on library preparation and data processing. The accession number is GEO: GSE77550.

XEN/iXEN Cell Differentiation

In vitro differentiation followed previously described techniques (Artus et al., 2012; Paca et al., 2012). To create chimeras, we injected ~15 fluorescently labeled cells into each blastocoel of unlabeled CD-1 blastocysts, and embryos were then transferred into the uterus of E2.5 pseudopregnant recipient females. See Supplemental Experimental Procedures for detailed protocols.

Statistical Analyses

Unless otherwise stated, t tests were performed for pairwise comparisons and ANOVA for multiple pairwise comparisons.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.02.003.

AUTHOR CONTRIBUTIONS

A.P., M.A.H., K.W., K.L., and A.R. designed the experiments. A.P. and K.W. performed experiments, and M.A.H. performed the computational analysis. A.P., M.A.H., and A.R. interpreted the experiments. A.R. wrote the manuscript, with input from A.P., M.A.H., K.W., and K.L.

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