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LETTER TO THE EDITOR

Efficient and rapid generation of induced pluripotent stem cells using an alternative culture medium

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Dear Editor,

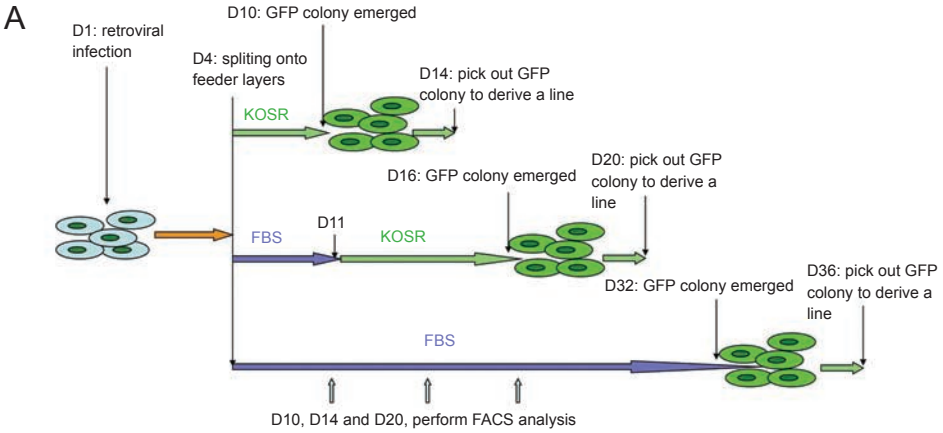
Mouse and human somatic cells can be induced to become pluripotent stem (iPS) cells by retroviral transduction with defined transcription factors [1-3]. Reprogrammed pluripotent stem cells have great potential for regenerative medicine and also provide a good experimental system for studying epigenetic reprogramming and differentiation. However, at present, the reprogramming process is still somewhat slow and ineffective. Research to improve reprogramming efficiency has included co-expression with more factors or treatment with chemical compounds [4-6]. Here we report that the pace and efficiency of reprogramming can be greatly improved by using a high concentration of knockout serum replacement (KOSR) instead of fetal bovine serum (FBS) in the culture medium. While reprogramming efficiency is increased more than 100-fold, the iPS cell lines generated with this modified culture condition maintain normal karyotype, hold full developmental potential including germ-line contribution in diploid chimeric mice, and support full-term development of tetraploid complementation embryos.

We generated iPS cells as described by the Yamanaka group, by infecting MEF cells carrying an Oct4-enhanced green fluorescent protein (GFP) reporter gene (Oct4-GFP) with four factors, pMXs-Oct4, Sox2, Klf4 and c-Myc [7, 8]. These cells were cultured in 10% FBS DMEM medium until day 4 post infection, when the MEFs were split and replated onto new feeder-coated dishes. The efficiency of iPS cell induction was compared between cultures in different induction media. Specifically, we cultured cells using a modified medium with 20% KOSR instead of the commonly used 15% FBS. The use of KOSR was reported to facilitate the generation of ES cells from specific inbred mouse strains [9], and so we hypothesized that KOSR may also improve the efficiency of iPS cell generation. We tested cultures in FBS, FBS followed by KOSR, or KOSR alone (Figure 1A), and observed notable differences between media for alkaline phosphatase (AP) expression as an indicator of early reprogramming events. At day 10 of culture, the number of AP^+ colonies was almost two-fold higher in the group treated with KOSR compared to the FBS group, and 1.6-fold higher at day 14 (Figure 1B). The presence of Oct4-GFP allowed us to directly screen for positive clones under a fluorescence microscope, detecting GFP as early as day 10 for the KOSR group, but only around day 32 for the FBS group. FACS analysis more accurately quantified GFP^+ cells at days 10, 14, and 20 (post-infection). Interestingly, for cells grown in KOSR medium beginning at day 4, GFP^+ cells increased to 0.99% ± 0.19% (mean ± SE), 3.26% ± 0.34%, and 24.04% ± 7.6% at days 10, 14, and 20, respectively, compared to the FBS cultures, which showed only 0.02% ± 0.01% GFP^+ cells at day 20 (Figure 1C). This improvement of greater than 1 000-fold more cells was dependent upon early culturing in KOSR medium; at 20 days post-infection, 3.57% ± 1.34% GFP^+ cells were identified in the sequential FBS-KOSR treatment, a 35-fold increase over the FBS-only medium (0.10% ± 0.08%) (Figure 1D). Clearly, these modifications greatly increased the efficiency of the reprogramming procedure.

iPS cell lines were derived at days 20 and 36 to cover the same time course used previously [10], and at day 14, when we can first reliably pick colonies with an ES cell morphology (Figure 1B). From multiple experimental runs, 34 GFP^+ colonies were obtained from KOSR-treated cells and all yielded stable cell lines, demonstrating the efficiency of cell line derivation in this system.

We tested the pluripotency of the KOSR iPS cell lines by examining specific stem cell markers and performing *in vivo* and *in vitro* differentiation tests as previously reported [1]. Similar to the previous study, the standard pluripotency markers were activated for lines derived from each time point and the iPS cell lines have normal karyotypes, with about 70% showing 40 chromosomes. Nanog and Oct promoters were shown to be demethylated in these iPS cells compared to the original MEF from which they were derived.

Endogenous Oct4, Sox2, Klf4, and c-Myc were reactivated and the exogenous transgenes were silenced, in-



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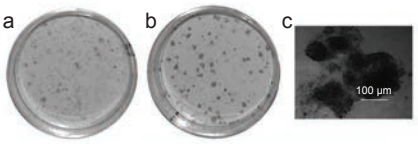
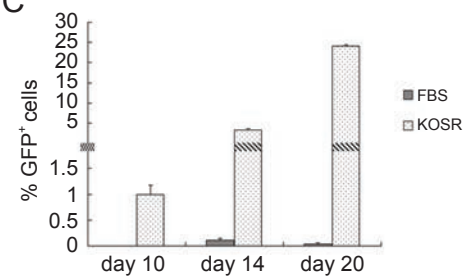
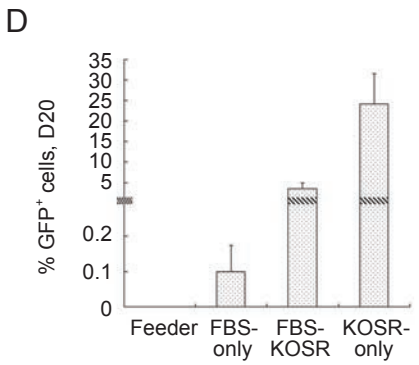
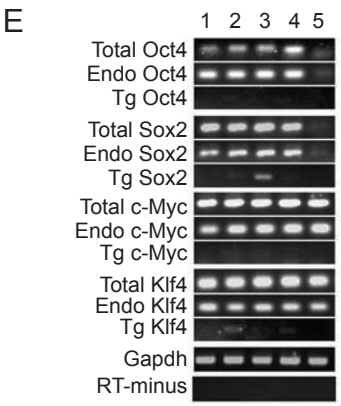
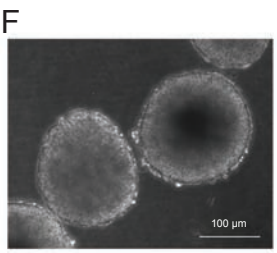


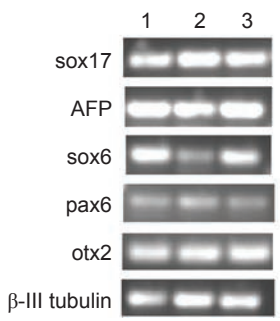
	FBS	KOSR
D10	82 ± 2	168 ± 4
D14	117.2 ± 7.6	188 ± 37.2

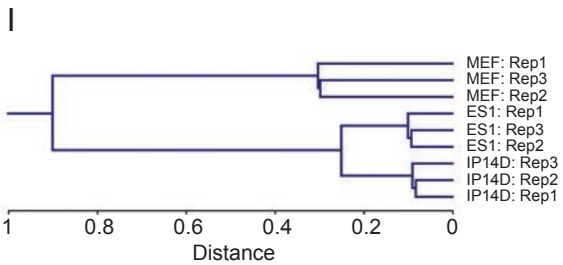












dicating that the pluripotent state was not maintained by continuous expression of exogenous factors (Figure 1E). After suspension in medium without leukemia inhibitory factor and feeder cells, the cells formed embryoid bodies and expressed markers from the three germ layers (Figure 1F). Teratoma assays were used to test the pluripotency of these iPS lines. iPS cell lines were injected into SCID mice, and produced teratomas with all three germ layers and with appropriately differentiated cells as determined by histological analyses (data not shown).

A more stringent test for pluripotency is germline transmission, so we injected KOSR iPS cells into CD-1 blastocysts and transferred the reconstructed embryos to CD-1 pseudopregnant recipient females. Germline transmission was noted for two of the four lines tested (Figure 1G). Finally, tetraploid complementation is considered the most stringent test for pluripotency, because any resulting embryo must develop exclusively from donor diploid cells [8, 11]. When iPS cells derived from one of the 14D KOSR iPS lines (which originated from black-coated mice) were injected into tetraploid blastocysts from a CD-1 genetic background (white coat), we observed full development resulting in the birth of live-born pups with completely black coats (Figure 1H). Global transcript profiling further confirmed that these iPS cells expressed genes similar to those from pluripotent ES cells of the same genetic background (Figure 1I). Together, these results clearly demonstrate that the iPS cells generated in KOSR-based medium have the same developmental potential as ES cells.

We have shown that just by modifying an existing culture system, reprogramming efficiency and the pace of stem cell generation can be greatly improved. Other approaches use either co-transfection of more genes or addition of small molecule compounds [6, 12], or alternative induction systems to produce pluripotent cells without genetic modification [13], but the efficiency and quality of these iPS cells are still unclear. For example, Blelloch *et al*. [13] reported that a mixture of FBS and KOSR in the induction medium improves iPS cell generation, but this work utilized different induction and culture conditions, and no statistical significance was reported. Our results indicate that culture conditions significantly affect reprogramming. A medium containing a high concentration of KOSR, which is a widely used commercial reagent, may specifically support the growth of iPS cells. Compared to FBS, which is believed to contain many factors that promote the growth and differentiation of multiple cell types, KOSR contains fewer such factors and has less ability to support growth other than for embryonic stem cells. Previous reports have shown that KOSR can improve embryonic stem cell line derivation by negative selection against trophoblast cells and differentiated cells from the inner cell mass. The presence of differentiated cells in mixed cultures may induce early-stage reprogrammed cells to develop into other cell types rather than achieve full pluripotency. We found that the total number of live cells in culture is lower in KOSR than in FBS after 10 days, although the number of iPS colonies formed was significantly greater from the KOSR culture system, suggesting that transfected MEFs that do not eventually become iPS cells proliferate much more slowly in KOSR medium than in FBS medium. KOSR may selectively support the growth of reprogrammed cells and thus directly enrich them in culture, while also selecting against non-reprogrammed cells and thereby indirectly promoting stem cell proliferation by reducing differentiation signals from other co-cultured cells.

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Figure 1 Generation of high-quality pluripotent iPS cell lines using modified culture conditions. **(A)** Schematic outline of the experimental design comparing media. Specifically, 2.5 × 10^4 MEFs transduced with four factors were plated on 35-mm dishes coated with feeder cells. On day 4 (D4) post-infection, dishes were divided into three groups with different culture media, and cells were monitored or sampled for the indicated assays. **(B)** Number of AP-positive clones obtained from transduced MEF cells. The media conditions are *a. FBS*, *b* and *c. KOSR*. Bar is 100 μm. **(C**, **D)** Percent of GFP-positive cells by FACS analysis. **(E)** The gene expression profiles of the four Yamanaka factors. 1: Embryonic stem cells with Oct4-GFP marker gene, 2: a KOSR iPS cell line from D36, 3: a KOSR iPS cell line from D20, 4: a KOSR iPS cell line from D14, 5: MEFs with Oct4-GFP marker gene. **(F)** Top: embryoid bodies (EB) at day 8 from KOSR iPS cells; bottom: gene expression profile for six germ-layer-marker genes assayed at day 15 after differentiation. 1 and 2: two iPS-14D cell lines; 3: an ESC cell line. **(G)** Germ-line transmission of D14 KOSR iPS cells when injected into CD-1 blastocysts. **(H)** Four 5-months-old mice generate by D14 KOSR iPS cells injected into a CD-1 tetraploid embryo. **(I)** Hierarchical clustering of expression profile data for differentially expressed genes [8] from MEF, an iPS-14D cell line, and an ES cell line. Rep, replicate.

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