Methods

Note on Protocols for STAP Cell Generation

Three different protocols have been reported for generating STAP cells: 1) mechanical trituration and low pH treatment (BWH protocol employed in the Vacanti laboratory as described here); 2) low pH treatment (RIKEN protocol (found at the following URL: http://dx.doi.org/10.1038/protex.2014.008)); and 3) mechanical trituration and ATP treatment (revised BWH protocol released online September 3, 2014 http://research.bwhanesthesia.org/research-groups/cterm/stap-cell-protocol).

We note that the data in our study comprises the first two protocols. The BWH protocol refers to an unpublished, in-house protocol created and used by the Vacanti laboratory to generate STAP cells. The RIKEN protocol is the published version described in the original STAP reports1,2. The Daley laboratory also attempted the revised BWH protocol without success (data not shown).

The three protocols contain differences and similarities. The major differences concern the distinct combinations of physical and chemical stressors. For example, the BWH protocols emphasized a combination of chemical and physical stressors, such as extensive mechanical trituration, whereas the RIKEN protocol primarily consisted of low pH treatment. However, in all three protocols, somatic cells are treated with a transient acid bath, although the composition of the low pH solution varied across protocols. Whereas the original BWH protocol and RIKEN protocol generate low pH solutions via titration of HBSS with hydrochloric acid, the revised BWH protocol generates a low pH solution via titration with ATP. The experimental procedures followed for the BWH protocol and RIKEN protocol are described below.

Mechanical Trituration and Low pH Treatment of Lung Cells (BWH Protocol)

To trigger STAP cell generation by mechanical trituration and low pH treatment of lung cells, we followed a procedure involving tissue isolation, extensive mechanical trituration, and an acid bath (pH 5.4). The protocol described below was adapted from the website of the Vacanti laboratory, which can be found at the following URL: http://research.bwhanesthesia.org/research-groups/cterm/stap-cell-protocol

Lungs were dissected from seven- to ten-day old neonatal Oct4-GFP mice and wild-type mice. Dissected lungs from multiple animals were pooled and washed twice in 60 mm petri dishes with Hank’s Buffered Saline Solution (HBSS without calcium and magnesium, Life Technologies, 14170-112). To enhance enzymatic dissociation, the whole tissue was minced with scissors for approximately 10 minutes. The minced tissue was treated with collagenase P for 30 minutes and placed in an incubator/shaker for 30 minutes at 37 degrees Celsius at 90 revolutions per minute (rpm). Following
collagenase P treatment, HBSS was added and the total tissue suspension was centrifuged to create a pellet. After aspirating the supernatant, the pellet was re-suspended into a small volume of HBSS (approximately 500 ul to 1000 ul). The resulting suspension was forcefully tritured for five minutes using a Pasteur pipette to further dissociate cell aggregates and debris. The tritured cell suspension was further tritured through a series of flame-pulled Pasteur pipettes. Generally, the trituration was performed for thirty minutes starting with the flame-pulled Pasteur pipette with the largest diameter. After sufficient dissociation was achieved using the large diameter pipette, trituration was performed with pipettes with increasingly smaller diameters.

Following mechanical trituration, erythrocyte depletion was performed. HBSS was added to the tritured suspension to increase the total volume to 5 mL (approximately 3 mL of HBSS). An equal volume of Lympholyte (Cedarlane) (5 mL) was added to the bottom of the tube to create a bilayer. The tube was then centrifuged at 1500 g for 10 minutes. After ten minutes, the tube was rotated 180 degrees and re-centrifuged at 1500 g for an additional 10 minutes. Following the second centrifugation, erythrocytes formed a pellet at the bottom of the tube. The layer between the HBSS and Lympholyte was transferred into a new 50 mL tube and HBSS was added to a total volume of 20 mL. The solution was then centrifuged at 1200 rpm for 5 minutes and the supernatant was aspirated. The cells were re-suspended into acid solution (HBSS titrated to a pH of 5.4 by hydrochloric acid) and placed into an incubator at 37 degrees Celsius for 25 minutes. The pH of the experiment was periodically monitored using a pH meter to confirm that the pH did not rise during the course of the experiment. If the pH increased significantly, the cellular suspension was centrifuged and the resulting pellet was re-suspended into a fresh batch of acid solution. After the 25 minute acid bath, the cell suspension was centrifuged at 1200 rpm for 5 minutes. The supernatant was aspirated and the pellet was re-suspended into sphere medium at a density of approximately 100,000 cells per mL of sphere medium.

Sphere medium consisted of DMEM/F12 medium (Stem Cell Technologies) supplemented with 2% B27 supplement (Life Technologies), penicillin/streptomycin (Life Technologies), bFGF (20 ng/mL, Life Technologies), EGF (20 ng/mL, Peprotech), heparin (0.2%, Stem Cell Technologies), and LIF (1000 U, Sigma). To reduce attachment to the bottom of the non-adhesive plate and facilitate sphere formation, STAP-treated cultures were gently pipetted using a 5 mL pipette, twice per day for 2 minutes. The medium was refreshed every two days, unless otherwise noted.

**Low pH Treatment of Splenocytes (RIKEN protocol)**

We attempted to follow procedures as closely as possible to the original STAP paper published by Obokata and colleagues and the refined protocol published by Obokata, Sasai, and Niwa. The following protocol is adapted from and is essentially identical to
the refined protocol published by Obokata, Sasai and Niwa (found at the following URL: http://dx.doi.org/10.1038/protex.2014.008).

To isolate hematopoietic cells, spleens were dissected and surgically excised from seven- to ten-day old neonatal Oct4-GFP mice and wild-type mice. Spleens from multiple animals were pooled and minced with surgical scissors to facilitate tissue dissociation. We mechanically dissociated the spleen tissues by triturating the minced spleens with Pasteur pipettes. Dissociated spleen cells were re-suspended with PBS (Life Technologies) and strained through a cell strainer (BD Biosciences). Pelleted cells were re-suspended in DMEM or HBSS media (Life Technologies).

For erythrocyte depletion, a similar protocol to the one used for processed lung tissues was applied to minced spleen specimens. An equal volume of Lympholyte (Cedarlane) was added to the bottom of the tube, causing two layers to form. The tube containing two layers was then centrifuged at 1000 g for 20 minutes. The lymphocyte layer was taken out and when indicated, stained with a CD45 antibody (PE-conjugated anti-CD45 antibody (Abcam) or PerCP-Cy5.5-conjugated anti-CD45 antibody (eBiosciences)) and sorted for CD45 on a FACSAria II (BD Biosciences). The resulting cells (unsorted splenocytes or CD45-sorted splenocytes) were treated with 500 ul of low-pH HBSS solution (titrated to pH 5.4 by hydrochloric acid (HCl)) for 25 minutes at 37 degrees Celsius. Throughout the experiment, the pH was periodically monitored to confirm that the pH did not increase significantly during the course of the experiment. If the pH rose significantly, the cellular suspension was centrifuged and the pellet was re-suspended into a fresh batch of acid solution.

After the 25 minute acid bath treatment, treated cells were centrifuged at 1000 rpm for 5 minutes at room temperature. The supernatant was removed by aspiration and pelleted cells were re-suspended in DMEM/F12 medium supplemented with LIF and 2% B27 supplement at a density of approximately 100,000 cells per mL and plated onto non-adhesive culture plates. The medium was refreshed every 2 days until day 7, unless otherwise noted. The spleen data presented in Figure 1B is from unsorted splenocytes because we observed increased sphere-forming efficiencies when the sorting step was omitted.

**Imaging of STAP cultures**

STAP-treated lung cells were imaged every two days until seven days after initiation with the BWH protocol. Genetically matched, homozygous Oct4-GFP mouse ESCs were used as positive controls. In the Vacanti laboratory, for Figure 1A, imaging was performed with a Biorevo Keyence BZ9000 microscope, and BZII Analyzer software was used to acquire and analyze images. For capturing green and red signals, the following filters were used: Green signal: FITC filter; red signal: the TRITC filter.
Flow cytometry

STAP-treated cells were analyzed seven days after STAP treatment by flow cytometry to assess for reprogrammed Oct4-GFP+ cells. To distinguish authentic Oct4-GFP signal from autofluorescence, STAP-treated samples were compared to treated wild-type cells, untreated controls, and positive control mESCs. Positive control mESCs harbored an identical transgenic reporter, originated from the same mouse strain of STAP-treated somatic cells, and were used to calibrate the GFP gates for assessment of authentic reporter reactivation. STAP treatment was performed in the Vacanti laboratory and flow cytometry analyses were performed seven days after STAP treatment. Flow cytometry was performed on an LSR II at Children’s Hospital Boston.

To prepare STAP-treated cells for flow cytometry analyses, cultures were trypsinized to generate a single cell suspension. Single cell suspensions from lungs, splenocytes, STAP-treated cells, and mESCs were pelleted, washed, and stained with PE-conjugated Annexin V antibody (BD Pharmingen, Cat no. 51-65875X; and Biovision, Cat No. K128-100) and DAPI (Life Technologies) to detect apoptotic cells and dead cells, respectively. GFP gates were set based on the GFP excitation/emission profile of positive control homozygous and heterozygous transgenic Oct4-GFP mESCs (cultured on iMEFs) containing the identical reporter present in STAP-treated somatic cells. The data presented in Figure 1B has no singlet/doublet exclusion or live/dead cell exclusion to enable comprehensive analysis for any GFP signal that resembles the GFP signal of Oct4-GFP mESCs. FACS data were analyzed with FlowJo software (TreeStar).

Oct4-GFP Mouse ESC Derivation and Cultivation

To replicate the original STAP papers as closely as possible, we used a transgenic Oct4-GFP reporter (JAX004654). We used this reporter because the identical Oct4-GFP reporter used by Obokata and colleagues was not readily available. The use of homozygous transgenic Oct4-GFP mice facilitated experimental throughput and was suggested by Niwa and colleagues to promote the detection of reprogramming events.

Mouse ESCs were derived from E3.5 blastocysts of mice both homozygous and heterozygous for the transgenic Oct4-GFP reporter to generate positive control cells for STAP replication studies. Because the somatic cells used for STAP replication studies were homozygous, the primary positive control cells used throughout this study were the homozygous Oct4-GFP ESCs.

The transgenic mouse ESCs harboring the GOF-Oct4ΔPE-GFP reporter10 were isolated by explanting the inner cell mass of an E3.5 embryo into KSR-LIF medium on iMEF feeders. Following ICM outgrowth formation, the outgrowth was trypsinized, plated onto irradiated CF1 mouse embryonic fibroblast (iMEF) feeders (GlobalStem) and continuously expanded in classical mESC conditions (serum/LIF medium). Homozygous
and heterozygous Oct4-GFP mouse ESCs were cultured in standard mouse ES cell medium (serum/LIF conditions) on iMEF feeders in gelatinized tissue culture dishes, unless otherwise noted (such as in Extended Data Figure 1 when mESCs were cultured in N2B27-2i/LIF to verify that the batch of B27 supplement used in the Vacanti laboratory can sustain the self-renewal and GFP signal of Oct4-GFP mouse ESCs).

Classical mouse ESC medium (serum/LIF conditions) contains Knockout DMEM (Life Technologies) supplemented with 15% fetal bovine serum, Penicillin / Streptomycin (Life Technologies), nonessential amino acids (Life Technologies), nucleoside mix (Millipore), L-Glutamine (Life Technologies), and 1000 U of mouse leukemia inhibitory factor (LIF) (Gemini Bioproducts).

KSR-LIF medium refers to mouse ESC medium containing Knockout DMEM, 10% Knockout Serum Replacer (Life Technologies), Penicillin / Streptomycin, nonessential amino acids, nucleoside mix, L-Glutamine, and 1000 U of mouse LIF.

N2B27-2i/LIF medium refers to a 1:1 mixture of DMEM/F12 and Neurobasal media (Life Technologies), supplemented with 1X N2 supplement (Life Technologies), 1X B27 supplement (Life Technologies), Penicillin / Streptomycin, L-Glutamine, 1000 U LIF (Gemini Bioproducts), 1 uM PD0325901 (Stemgent), 3 uM CHIR99021 (Stemgent). When indicated, LIF and B27 supplements used in our laboratory were substituted with Vacanti laboratory LIF and B27 supplement to validate their suitability for STAP replication experiments. The mouse LIF used in Vacanti laboratory was Sigma, Catalog No. L5158-5 ug (Lot # 021M1557V). The Vacanti laboratory B27 supplement was Life Technologies, Catalog No. 12587-010 (Lot# 1582961). Notably Vacanti laboratory B27 supplement lacks Vitamin A, whereas the Daley laboratory B27 supplement has Vitamin A.

**Mouse iPSC Generation and Cultivation**

Mouse iPS cells were generated to validate the Oct4-transgenic reporter for STAP replication studies. Mouse iPS cells were generated using standard methods. Retroviruses (pMX-Oct4, pMX-Sox2, pMX-Klf4, Addgene) were packaged in 293T cells using conventional retroviral production methods. MEFs harboring the GOF-Oct4\(\Delta\)PE-GFP reporter were infected with retroviruses encoding reprogramming factors. Infected mouse embryonic fibroblasts were cultivated in KSR-LIF media. Two to three weeks after infection, mouse iPSC colonies were selected based on morphological criteria and GFP reporter activity, expanded and continuously cultivated in serum/LIF conditions on iMEF feeders.

**Teratoma formation analyses**
Positive control mESCs used for teratoma experiments were derived from the same mouse strain used for STAP replication efforts. Teratoma formation assays were performed similarly to previously described methods, unless otherwise noted\textsuperscript{8,9}. Mouse ESCs and STAP-treated cells were re-suspended in Matrigel (BD Biosciences) prior to implantation for teratoma formation. Mouse ESCs grown on irradiated MEFs were collected by trypsin treatment, re-suspended as single cells and injected subcutaneously into the dorsal flanks or into the kidney capsule of NOD-SCID mice. STAP-treated cultures were injected for teratoma generation seven days after being treated with the STAP protocols. STAP-treated cells were not dissociated, and were instead re-suspended as clumps and injected subcutaneously into the dorsal flanks or into the kidney capsule of NOD-SCID mice. For production of teratomas from mESCs, approximately 100,000 mESCs were typically injected. For injection of STAP-treated cultures, approximately 1 – 2 million cells were injected. After 4 – 8 weeks, teratomas were dissected and fixed in 10% formalin. Embedding in paraffin, sectioning of tissue, and hematoxylin and eosin staining were performed by the Rodent Histopathology service of the Dana Farber Cancer Institute.

For subcutaneous transplantation, the following method was used. Briefly, mice were anesthetized with ketamine/xylazine. The dorsal flanks of the mouse were shaved and the skin was swabbed with Betadyne. After the mouse was shaved, mouse ESCs or STAP cells were injected subcutaneously with approximately 100 – 500 ul of the cellular suspension containing either positive control mouse ESCs or experimental STAP-treated cells. Mouse ESC-transplanted mice typically formed teratomas within 3 – 4 weeks, whereas mice transplanted with STAP-treated cells were observed for at least 8 – 12 weeks.

For kidney capsule transplantations, the left flank of the anesthetized mouse was shaved and the skin of the mouse swabbed with Betadyne. After the left kidney was located, a small incision was made in the skin and the peritoneum to expose the kidney. After exposing the kidney, STAP-induced lung cells, STAP-induced splenocytes, or positive control mouse ESCs were injected into the kidney capsule. The peritoneum and skin were then closed using silk sutures. Mouse ESC-transplanted mice typically formed teratomas within 3 – 4 weeks, whereas mice transplanted with STAP-treated cells were observed for at least 8 – 12 weeks.

The original Nature Article describing in vivo differentiation of STAP cells entailed the following: STAP cells were seeded onto a sheet composed of a non-woven mesh of polyglycolic acid fibers (PLGA) and cultured for 24 hours in DMEM containing 10% FBS. This mixture was implanted subcutaneously into the dorsal flanks of immune-compromised NOD-SCID mice. To replicate the in vivo differentiation experiments described by Obokata and colleagues, we obtained PLGA from a member of the Vacanti laboratory. We seeded STAP-treated GFP+ splenocytes onto a sheet of PLGA
mesh, cultured the mixtures for 24 hours in DMEM + 10% FBS, and subcutaneously implanted the mixture into the dorsal flanks of NOD-SCID mice. For subcutaneous implantations, the left flank of the anesthesized mouse was shaved and the skin of the mouse swabbed with Betadyne. An incision was made. The mixture containing the STAP-treated cells and PLGA fibers was implanted subcutaneously, and the incision was closed using silk sutures. The implant was typically placed near the incision site to facilitate implant recovery. After approximately 3 weeks or three months, the implant was recovered from the implantation site. Similar data was obtained between three week and three month STAP-PLGA implant experiments. We dissected the opposite side of the mouse to confirm that the same implant structure was not present. The implants were fixed with 10% formalin. Embedding in paraffin, sectioning, and stainings (H & E staining and Masson’s staining) were performed by the Rodent Histopathology service of the Dana Farber Cancer Institute. Additional GFP immunohistochemistry was performed in-house or by the Histology Core at the Harvard Stem Cell Institute. Detailed procedures for the GFP staining of paraffin-embedded STAP masses are described below in the immunofluorescence section of the methods.

For validating the pluripotency of GOF18-iPSCs by teratoma formation capacity, similar methods to ESC-based teratoma generation were used to generate GOF18-iPSC-derived teratomas.

**Morula aggregations and blastocyst injections**

The developmental potential of STAP-treated cells was further evaluated in vivo by two methods: morula aggregation and blastocyst injection. Morula aggregations were performed similarly to previously described methods used for mouse ES cells. Briefly, STAP cells were aggregated as small clumps with E2.5 morula stage embryos in depression wells. The aggregates were cultured in KSOM embryo medium overnight and incorporation/contribution was assessed 24 hours later (~E3.5) by fluorescence microscopy.

Methods for blastocyst injection were similar to those described in the Nature STAP article with minor modifications. Briefly, the STAP-treated spheres were mechanically “crushed” or disaggregated to generate aggregates of reduced size that can fit into a blastocyst injection needle. Following immobilization of an E3.5 blastocyst using a holding pipette, the end of the injection pipette containing the STAP-treated sphere was juxtaposed against the blastocyst surface and swiftly introduced into the blastocyst cavity. The STAP-treated aggregates were released from the injection needle slowly into the blastocyst cavity. Following withdrawal of the needle from the blastocyst, the injected blastocyst was allowed to recover in an incubator. Recovered embryos were transferred into the uterus of 2.5 dpc pseudopregnant females. Seven to eight days
later, females were sacrificed by CO₂ asphyxiation and embryos were harvested at ~E10.5 for analysis.

**Tetraploid complementation (iPS cells)**

For validating the pluripotency of GOF18-iPS cells, tetraploid (4N) blastocysts were placed into KSOM embryo medium. iPS cells were injected into the blastocyst cavity using a microinjection pipette. After blastocyst injection, blastocysts were returned to KSOM medium and cultured at 37 degrees Celsius until transferred to recipient females.

**qPCR analysis**

Total RNA was isolated as described in the RNeasy Kit (Qiagen) and reverse transcribed using the Superscript III First Strand Synthesis Kit (Invitrogen). Quantitative RT-PCR analysis was performed in triplicate with FAST SYBR Green Mix (Applied Biosystems). Gene expression was normalized to GAPDH. The primer sets used to generate the data in Extended Data Figure 2 are listed below. We note that alternative primer sets also showed lack of ESC-like Oct4, Sox2, and Nanog induction (data not shown).

**GAPDH-F:** GGTTGTCTCCTGCGACTTCAACAGC  
**GAPDH-R:** CGAGTTGGGATAGGGCCTCTTGC  
**Oct4-F:** TTGGGCTAGAGAAGGATGTGGTT  
**Oct4-R:** GGAAAAGGGACTGAGTAGTGTGG  
**Nanog-F:** GGTTGAAGACTAGCAATGGTCTGA  
**Nanog-R:** TGCAATGGATGCTGGGATACTC  
**Sox2-F:** GCACATGAACGGCTGGAGCAACG  
**Sox2-R:** TGCTGCGAGTAGACATGCTGTAGG

**Immunofluorescence**

Standard immunofluorescence procedures were followed. To stain mouse ES cells and STAP-treated cells, cells were fixed with 4% paraformaldehyde, washed and permeabilized with PBS containing 0.1% Triton-X-100. Next, samples were blocked with PBS containing 5% FBS solution. After overnight incubation in primary antibody solution (PBS containing 1% FBS solution) at 4 degrees Celsius, cells were washed three times, and then incubated with antibody solution containing secondary antibodies. Cell nuclei were visualized with DAPI. The following primary antibody dilutions were used: mouse monoclonal against Oct4 (1:100) from Santa Cruz Biotechnology (C:10, Cat No.: sc5279), rat monoclonal against Nanog (1:500) from eBiosciences (eBioMLC-51, Cat No.:14-5761-80).
GFP immunostaining of paraffin-embedded STAP masses was performed using the following protocol. Slides with paraffin-embedded sections were de-waxed with xylene and rehydrated through a series of washes with decreasing percentages of ethanol. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) by placement in a de-cloaking chamber at 95 degrees Celsius for 30 minutes. Immunohistochemistry was performed with Elite ABC kit and DAB substrate (Vector Laboratories) according to the manufacturer’s protocol.

Genomic data analysis

Datasets

We used the RNA-seq and ChIP-seq data (input control) from Obokata and colleagues\(^1\), available in the Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra) with accession number SRP038104.

Copy number variation analysis

Input (control) samples from the ChIP-seq datasets were used to infer copy number profiles. Array CGH experiments were performed in the original publication but the data were not deposited; our attempt to obtain data through the editor was not successful. Reads were aligned to the mm9 genome using BWA\(^18\) (v.0.5.9-r16) and filtered to select uniquely aligned reads ("XT:A:U" flag). Copy number analysis was performed on the read counts using BIC-seq\(^19\) after normalization by GC-content and sequence context, with parameters lambda=3 and bin-size=10000. We used 10kb bin size since we expect the read coverage in ChIP input to be uneven due variation in global chromatin status and accessibility.

Single nucleotide variant analysis

As exome or whole-genome sequencing data were not available, single nucleotide sequence variants (SNVs) were inferred from RNA-seq data. RNA-seq data were aligned to the mouse genome mm10 using Tophat 2.0.10\(^{20,21}\) allowing two mismatches and two indels. The ENSEMBL GRCm38 gene annotation was used. On average 14.6M reads were aligned (12.9M-16.4M), which corresponds to 84.3% of total reads. Uniquely aligned reads were used, and duplicates were removed. Variants were called using Genome Analysis Toolkit\(^{22}\) 3.0 UnifiedGenotyper, with local indel realignment and base quality score recalibration\(^23\). Only those variants assigned PASS by variant quality score recalibration (VQSR)\(^23\) and with quality score ≥100 were retained. Known mouse SNPs for various strains were obtained from the Wellcome Trust Sanger Institute Mouse Genomes Project\(^{24}\) and used during the recalibration steps. To remove the effect of variable read coverage (due to varying levels of gene expression), only the 9,739
variants with at least 30X coverage across all samples were considered for downstream analyses.
Supplementary References


