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Generation of induced pluripotent stem cells from a small amount of human peripheral blood using a combination of activated T cells and Sendai virus

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Induced pluripotent stem cells (iPSCs) have become important cell sources for genetic disease models, and they have the potential to be cell sources for future clinical therapies. However, invasive tissue sampling reduces the number of candidates who consent to donate cells for iPSC generation. In addition, integrated transgenes can potentially insert at inappropriate points in the genome, and in turn have a direct oncogenic effect. Technical modifications using a combination of activated T cells and a temperature-sensitive mutant of Sendai virus (SeV) can avoid invasive tissue sampling and residual transgene issues in generating iPSCs. Such advances may increase the number of consenting patients for cell donations. Here we present a detailed protocol for the generation of iPSCs from a small amount of human peripheral blood using a combination of activated T cells and mutant SeV encoding human OCT3/4, SOX2, KLF4 and c-MYC; T cell–derived iPSCs can be generated within 1 month of blood sampling.

INTRODUCTION

Generating iPSCs is a prominent recent advance in stem cell biology[1]. iPSCs have become cell sources for genetic disease models and are expected to provide important new cell sources for clinical therapies. Initial studies generated human iPSCs from human fibroblasts obtained from dermal biopsy samples[2,3]. However, although further studies successfully reprogrammed several types of human somatic cells into iPSCs[4–6], the methods and cell sources most suitable for iPSC applications in humans remain undetermined. In particular, the generation of iPSCs for disease research should ideally avoid invasive tissue sampling, which markedly reduces the number of patients who consent to cell donations. In this regard, peripheral blood is an appealing cell source because of the noninvasive collection and easy accessibility of blood cells compared with skin fibroblasts and other types of cells from adult tissues.

We recently demonstrated that transgene-free iPSCs can be efficiently generated from a small amount of human peripheral blood within 1 month of the blood sampling using a combination of activated T cells and temperature-sensitive (TS) mutants of SeV encoding human OCT3/4, SOX2, KLF4 and c-MYC (**Fig. 1**)[7]. We named these T cell–derived iPSCs TiPS cells (TiPSCs). Recombinant SeVs that replicate in the cytoplasm of infected cells in the form of negative-sense single-stranded RNA were originally used to generate iPSCs from human fibroblasts[8]. These recombinant SeVs do not integrate into the host genome[9], and have already been used in human iPSC generation using CD34+ cells from human cord blood[10]. Introducing TS mutations also successfully erased residual genomic RNA of the SeV vectors from the target cells[10], thus generating transgene-free iPSCs with high efficiency. T cells are also an appealing cell source because they are easily proliferated in vitro using a plate-bound anti-CD3 monoclonal antibody and interleukin (IL)-2 (ref. 11). Although it was reported that T cells are not efficiently reprogrammed using only four factors in the mouse[12,13], SeV vectors were efficiently transduced into human activated T cells to express exogenous genes[14]. Thus, the combination of activated T cells and TS SeV mutants made it possible to generate TiPSCs from patients effectively, easily and less invasively.

Advantages of the method

The initial methods for generating human iPSCs used a skin biopsy[2,3], requiring local anesthesia and suturation. In our protocol, iPSCs can be generated from patients without such invasive tissue sampling. Sufficient patient-specific iPSCs can be generated from 1 ml or less of peripheral blood, which contains sufficient terminally differentiated T cells[7]. Our method might therefore decrease the likelihood of patients refusing cell sampling and therefore potentially increase the number of patients who consent to generating iPSCs. In addition, TiPSCs can still be generated from whole blood samples stored at room temperature (20–25 °C) for 24 h and from mononuclear cells stored at − 150 °C. Therefore, transported samples can be easily used for generating iPSCs in any clinical situation. SeV also has the possibility to be used for generating iPSCs from other human blood cells such as monocytes, which, on the basis of existing reports of iPSCs that were generated successfully using SeV from T cells and CD34+ cells[7,10], do not harbor T cell receptor (TCR) or immunoglobulin gene rearrangements.

Comparison with other methods

In the first report of iPSC generation from human peripheral blood cells[6], mobilized CD34+ human peripheral blood cells were successfully reprogrammed into iPSCs. However, this method required extremely large amounts (~300 ml) of blood, an apheresis machine and drug administration before blood sampling to mobilize the CD34+ blood cells, all of which should ideally be avoided because of the possible associated side effects (e.g., bone pain), despite these effects being infrequent. Less invasive methods using peripheral blood have also been reported for

Figure 1 | Overview of the TiPSC generation protocol. PBMCs are activated for 5 d with IL-2 and anti-CD3 antibody, and then transduced with SeV expressing human *OCT3/4, SOX2, KLF4* and *c-MYC*. TiPSC colonies emerge at 20–25 d after blood sampling.
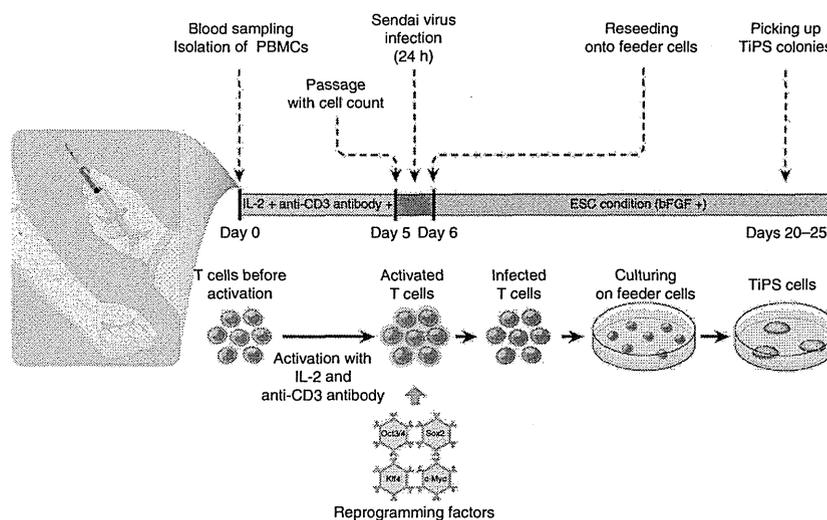
the successful reprogramming of mono-nuclear blood cells[15–17]. In these methods, mononuclear blood cells from donors or frozen samples were infected using retrovirus[15] or lentivirus[16,17] to express four factors, human OCT3/4, SOX2, KLF4 and c-MYC. In these studies, human T cell reprogramming into iPSCs was achieved, but the efficiency of reprogramming was extremely low (approximately 0.0008–0.01%). Although these methods used less peripheral blood and did not require the pharmacological pretreatment of patients, the problems of transgene genomic insertion and low reprogramming efficiency remained, precluding their wide use in the clinical application of iPSCs. Generating iPSCs with TS-mutated SeV easily erases residual genomic viral RNA from the target cells[10], and the method is significantly more efficient (~0.1%) compared with those protocols in which iPSCs were generated from T cells with retrovirus or lentivirus.

Human keratinocytes derived from plucked human hair have also been used as another less invasive method of obtaining iPSCs from patient cells[4,18]. However, in some cases, these reported methods require several hairs to obtain successful cell outgrowth of keratinocytes. Dental tissue has also been explored as a potential source of iPSCs[19]. However, although teeth are routinely removed in many clinics and no further procedures are required with respect to the donor, it is generally difficult to routinely obtain patients' dental tissues—with specific genetic or nongenetic diseases—for the purpose of iPSC studies. In comparison with these outlined methods, our protocol involves harvesting only a small sample of peripheral blood; in addition, T cell proliferation does not need stochastic cell outgrowth. These are clear advantages for clinical application in comparison with the methods reported in the past.

Experimental design
Blood sampling. Our protocol is focused on the simple procedure of peripheral venous blood sampling to obtain the donor cells, using a standard process. Patient somatic cells can then be easily and aseptically obtained from the blood sample. In our protocol, 1 ml of whole blood is sufficient to generate TiPSCs (**Fig. 2a**).

Derivation of activated T cells. Peripheral blood mononuclear cells (PBMCs) can be separated by a Ficoll gradient method from heparinized whole blood samples (**Fig. 2b**). Although PBMCs contain lymphocytes and monocytes, activation with plate-bound anti-CD3 monoclonal antibody and IL-2 selectively proliferates T cells, and clearly increases the proportion of T cells in the cultured PBMCs[11]. CD3 protein exists in the complex of TCR proteins on the surface of T cells, and can therefore be used as a T cell–specific marker. Anti-CD3 antibody modulates the TCR-CD3 complex to induce T cell proliferation and activation[20], whereas IL-2 also activates general T cell signaling pathways and eventually promotes cytokine transcription, cell survival, cell-cycle entry and growth[21]. At day 5 of culture with anti-CD3 monoclonal antibody and IL-2, CD3+ cells increased up to ~95% of cultured PBMCs (**Fig. 2c–e**). With this culture method, users can avoid using a fluorescence-activated cell sorter in which
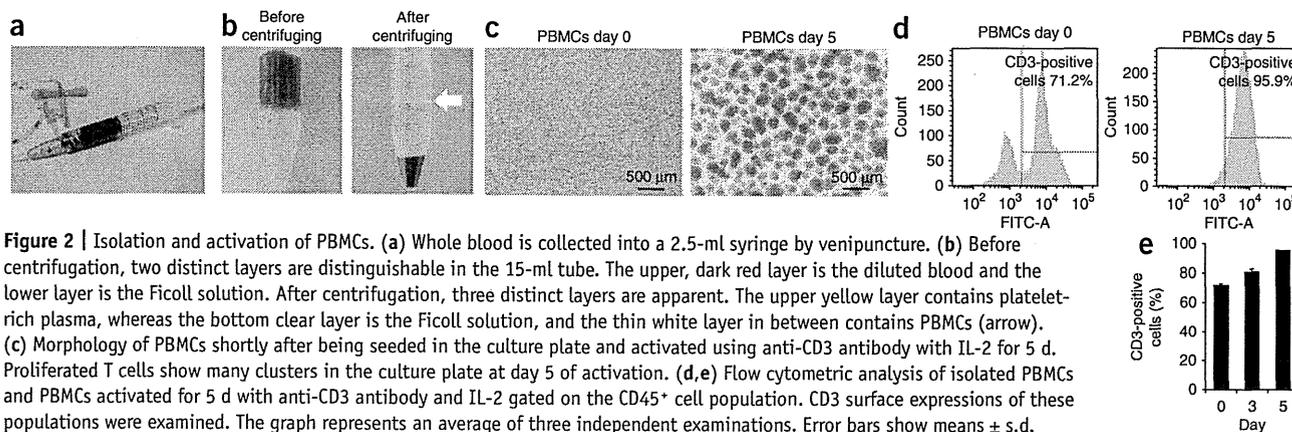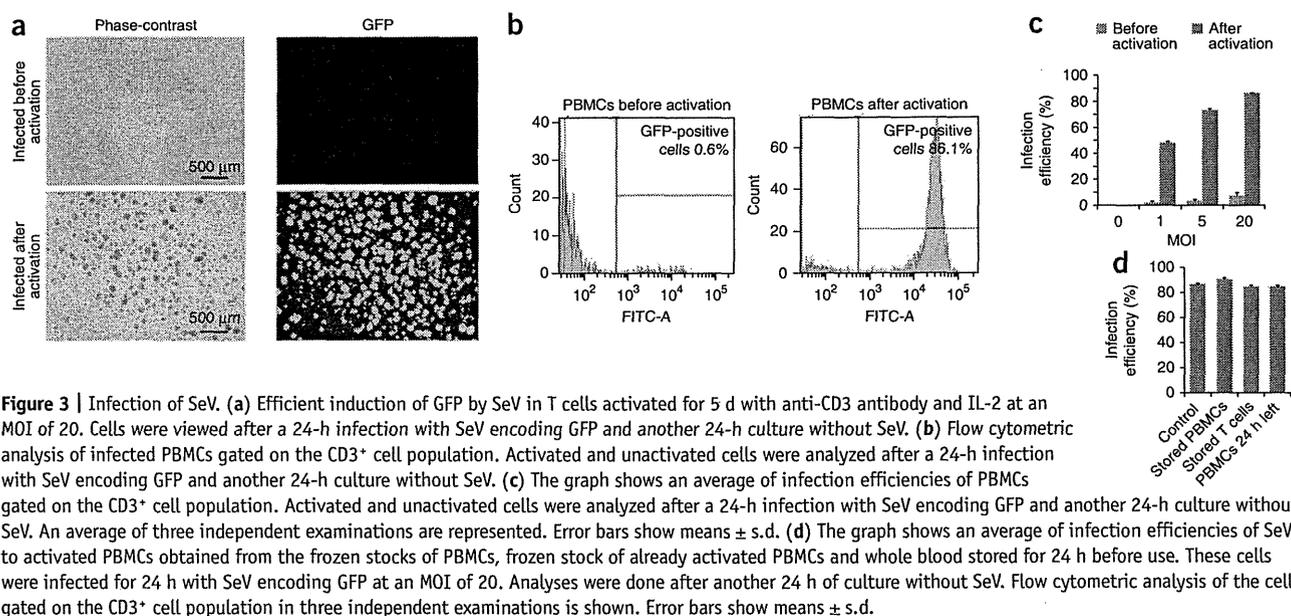

Figure 2 | Isolation and activation of PBMCs. (**a**) Whole blood is collected into a 2.5-ml syringe by venipuncture. (**b**) Before centrifugation, two distinct layers are distinguishable in the 15-ml tube. The upper, dark red layer is the diluted blood and the lower layer is the Ficoll solution. After centrifugation, three distinct layers are apparent. The upper yellow layer contains platelet-rich plasma, whereas the bottom clear layer is the Ficoll solution, and the thin white layer in between contains PBMCs (arrow). (**c**) Morphology of PBMCs shortly after being seeded in the culture plate and activated using anti-CD3 antibody with IL-2 for 5 d. Proliferated T cells show many clusters in the culture plate at day 5 of activation. (**d,e**) Flow cytometric analysis of isolated PBMCs and PBMCs activated for 5 d with anti-CD3 antibody and IL-2 gated on the CD45+ cell population. CD3 surface expressions of these populations were examined. The graph represents an average of three independent examinations. Error bars show means ± s.d.

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Figure 3 | Infection of SeV. (**a**) Efficient induction of GFP by SeV in T cells activated for 5 d with anti-CD3 antibody and IL-2 at an MOI of 20. Cells were viewed after a 24-h infection with SeV encoding GFP and another 24-h culture without SeV. (**b**) Flow cytometric analysis of infected PBMCs gated on the CD3^{+} cell population. Activated and unactivated cells were analyzed after a 24-h infection with SeV encoding GFP and another 24-h culture without SeV. (**c**) The graph shows an average of infection efficiencies of PBMCs gated on the CD3^{+} cell population. Activated and unactivated cells were analyzed after a 24-h infection with SeV encoding GFP and another 24-h culture without SeV. An average of three independent examinations are represented. Error bars show means ± s.d. (**d**) The graph shows an average of infection efficiencies of SeV to activated PBMCs obtained from the frozen stocks of PBMCs, frozen stock of already activated PBMCs and whole blood stored for 24 h before use. These cells were infected for 24 h with SeV encoding GFP at an MOI of 20. Analyses were done after another 24 h of culture without SeV. Flow cytometric analysis of the cells gated on the CD3^{+} cell population in three independent examinations is shown. Error bars show means ± s.d.

the sorted cells are frequently damaged by laser emission and the process of single-cell sorting.

Introduction of SeV vectors. SeV is an enveloped virus with a single-stranded, negative-sense, nonsegmented RNA genome belonging to the paramyxoviridae family. Recombinant SeV vectors replicate only in the cytoplasm of infected cells[9]. SeV vectors containing reprogramming factors were generated by introducing open reading frames for the human *OCT3/4* (official symbol: *POU5F1*) *SOX2* and *KLF4* genes into a fusion protein (F)-deficient, TS SeV vector. SeV vector containing the *c-MYC* gene was also generated with a more TS mutant, the TS15-SeV vector (P2, L1361C, L1558I), so that it could be eliminated rapidly at 37 °C[10].

The seed SeV/ΔF vectors are generated by the transfecting template pSeV/ΔF carrying each transgene and pCAGGS plasmids—varying the genes encoding T7 RNA polymerase, nucleoprotein (NP), phosphoprotein (P), F5R and large protein (L)—into 293T cells. Thereafter, the vector is propagated using LLC-MK2/F7/A cells, which are SeV F–expressing LLC-MK2 cells[10]. SeV solutions can be stored at −80 °C and thawed before use. Activated PBMCs are infected at day 5 of activation culture. For effective reprogramming of T cells, this activation of PBMCs is important because it not only increases the number of T cells, but also significantly promotes the introduction efficiency of SeV. At a multiplicity of infection (MOI) of 20, SeV could infect CD3^{+} T cells at >80% efficiency, which was lower than 10% before activation (**Fig. 3**).

TiPSCs

At around day 15 after transduction of SeV, human embryonic stem cell (ESC)-like colonies emerge on the feeder cells (**Fig. 4**). These T cell–derived TiPSC colonies show monoclonal TCR rearrangement in their genome, which is a hallmark of mature terminally differentiated T cells and indicates that each TiPSC colony is derived from a single mature T cell.
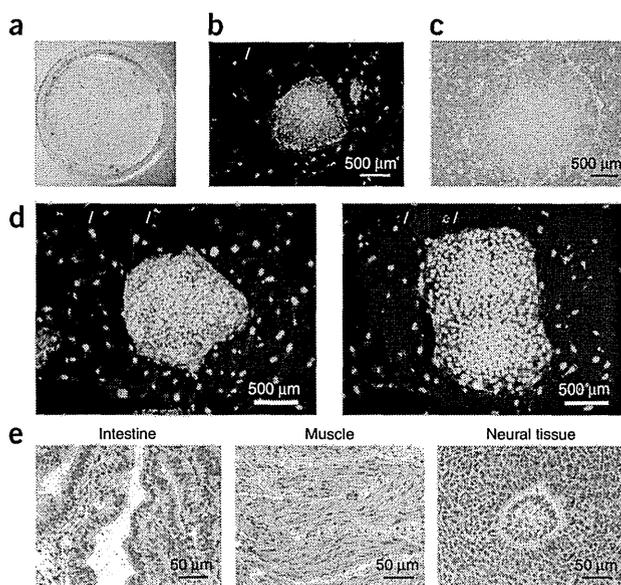

Figure 4 | Characterization of TiPSCs. (**a**) Example of a 10-cm dish stained for ALP on day 20 after SeV infection at an MOI of 20 and seeded at a density of 5 × 10⁴ cells per 10-cm dish. Many ALP-positive T cell colonies that were infected with SeV are visible. (**b**) ALP staining of TiPSC colonies. (**c**) Typical ESC-like TiPS colony on day 20 after SeV infection. (**d**) Immunofluorescense staining for pluripotency and surface markers (Nanog, Tra1-60, Oct 3/4, SSEA3) in TiPSCs. Immunofluorescence staining was performed using the following primary antibodies: anti-NANOG (RCAB0003P, ReproCELL), anti-OCT3/4 (sc-5279, Santa Cruz), anti-SSEA3 (MAB4303, Millipore) and anti-Tra1-60 (MAB4360, Millipore). DAPI (Molecular Probes) was used for nuclear staining. The following secondary antibodies were used: anti-rabbit IgG and anti-mouse IgG and IgM conjugated with Alexa Fluor 488 or Alexa Fluor 568 (Molecular Probes). (**e**) Hematoxylin and eosin–stained representative teratomas derived from the TiPSC line.

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MATERIALS
REAGENTS
- Donors for blood sampling **! CAUTION** Subjects must have given informed consent. **! CAUTION** All experiments involving humans must conform to relevant governmental and institutional ethics regulations.
- Novo-heparin (5,000 units per 5 ml; Mochida Pharmaceutical)
- Ficoll-Paque PREMIUM (GE Healthcare, cat. no. 17-5442-02)
- Purified NA/LE mouse anti-human CD3 (BD Pharmingen, cat. no.555336)
- GT-T502 medium (Kohjin Bio, cat. no. 16025020)
- Fetal bovine serum (FBS; Cell Culture Bioscience, cat. no. 171012)
- Bovine albumin fraction V solution (BSA; Gibco, cat. no. 15260-037)
- DMEM (Sigma, cat. no. D5546)
- DMEM/F12 (Sigma, cat. no. D6421)
- KnockOut serum replacement for ESCs/iPSCs (KSR; Gibco, cat. no. 10828-028)
- GlutaMAX-I (Gibco, cat. no. 35050-061)
- Non-essential amino acid solution (NEAA; Sigma, cat. no. M7145)
- Penicillin-streptomycin (Gibco, cat. no. 15140-122)
- 2-Mercaptoethanol (2-ME; Invitrogen, cat. no. 21985-023)
  ! CAUTION This solution is flammable, harmful if swallowed and toxic when in contact with skin and eyes. Use protective gloves and safety glasses when handling it.
- Recombinant basic fibroblast growth factor, human (bFGF; Wako, cat. no. 064-04541)
- Collagenase type IV (Gibco, cat. no. 17104-019)
- Gelatin powder (Sigma, cat. no. G-1890)
- D-PBS(−) (Wako, cat. no. 045-29795)
- Acetamide (Wako, cat. no. 015-00115)
- Propylene glycol (Wako, cat. no. 16-0499)
- Cell Banker-2 (BIO LABO, cat. no. BLC-2)
- TRIzol reagent (Invitrogen, cat. no. 15596-026)
- Chloroform (Wako, cat. no. 038-02606)
- Ethanol (Wako, cat. no. 057-00456)
- Ethanol (70% (vol/vol); Wako, cat. no. 059-07895)
- Isopropyl alcohol (Wako, cat. no. 166-04836)
- SuperScript double-stranded cDNA synthesis kit (Invitrogen, cat. no. 11917-010)
- Oligo (dT)$_{12-18}$ primer (Invitrogen, cat. no. 18418-012)
- SYBR Premix Ex Taq II (Takara, cat. no. RR081A)
- Sodium acetate (3 M; Wako, cat. no. 316-90081)
- Liquid nitrogen
- CytoTune-iPS reprogramming kit (OCT3/4-SeV/TSΔF, SOX2-SeV/TSΔF, KLF4-SeV/TSΔF, c-MYC (HNL)-SeV/TS15ΔF; Invitrogen, cat. no. A13780-01)
- Mitomycin C–treated mouse embryonic fibroblasts (MEFs; Reprocell, cat. no. RCHEFC003)
- MEF medium (see REAGENT SETUP)
- Human iPSC medium (see REAGENT SETUP)
- Isopropanol (Wako, cat. no. 16604836)
- RNase-free water (Takara, cat. no. 9012)
- DNase I (Invitrogen, cat. no. 18068-015)

EQUIPMENT
- Syringe (2.5 ml; Terumo, cat. no. SS-02LZ)
- Butterfly needle (23 G; Terumo, cat. no. SV-23CLK)
- Needle (23 G; Terumo, cat. no. NN-2325R)
- Alcohol prep pads (Hakujuji)
- Tourniquet (Asone, cat. no. MH-01)
- Millex GV filter unit (0.22 μm; Millipore, cat. no. SLGV033RS)

- C-chip disposable hemocytometer (Digital Bio, cat. no. DHC-N01)
- Trypan blue stain (0.4%; Gibco, cat. no. 15250)
- Microtubes (1.5 ml; Thermo Fisher Scientific, cat. no. 131-615C)
- Tube (50 ml; Corning, cat. no. 430829)
- Tube (15 ml; Corning, cat. no. 430053)
- Tissue culture dish (10 cm; Falcon, cat. no. 353003)
- Tissue culture plate (96 well; Falcon, cat. no. 353078)
- Tissue culture plate (24 well; Falcon, cat. no. 353047)
- Tissue culture plate (12 well; Falcon, cat. no. 353043)
- Tissue culture plate (6 well; Falcon, cat. no. 353046)
- Cryovials (1.5 ml; Sumilom, cat. no. MS-4702X)
- Freezing container (Sanyo, cat. no. MDM-U73V)
- Cell culture incubator set at 37 °C, 5% CO_2 (Sanyo, cat. no. MCO-18AIC)
- Versatile refrigerated centrifuge (Sanyo, cat. no. AX-320)
- NanoDrop 2000 (Thermo Fisher Scientific, cat. no. ND-2000)
- ABI 7500 real-time PCR system (Applied Biosystems, cat. no. 7500-01)
- Inverted microscope
- Phase-contrast microscope

REAGENT SETUP
bFGF Prepare 0.1% (wt/vol) BSA/PBS in a sterile tube and use it to dissolve bFGF for a final concentration of 4 ng ml^{-1}. Prepare 100-μl aliquots in screw-cap microcentrifuge tubes and store them at −20 °C.

Gelatin-coated culture dishes Dissolve 1 g of gelatin powder in 1,000 ml of distilled water, autoclave, filter the solution with a 0.22-μm Millex GV filter unit and store it at 4 °C. Add an appropriate volume of 0.1% (wt/vol) gelatin solution to cover the entire area of the culture dishes to coat. Incubate the dishes for at least 30 min at 37 °C in a sterile environment. Remove the gelatin solution before use.

Collagenase type IV solution Dissolve 1 g of collagenase type IV powder in 1,000 ml of DMEM/F12 medium and filter the solution with a 0.22-μm Millex GV filter unit. Make 50-ml aliquots in 50-ml tubes and store them at −20 °C. Thawed solution can be stored at 4 °C for up to 1 week before use.

Anti-CD3 monoclonal antibody–coated plates Dissolve anti-human CD3 antibody in D-PBS(−) to a concentration of 10 μg ml^{-1}. Add the anti-human CD3 antibody solution to 24-well tissue culture plates to soak the surface of each plate, and then incubate them at 37 °C in a 5% CO_2 incubator for at least 30 min. Remove the anti-human CD3 antibody solution and wash the plates once with D-PBS(−) before seeding the cells.

Human iPSC medium To prepare 500 ml of human iPSC medium, mix 387.5 ml of DMEM/F12 medium with 100 ml of KSR, 5 ml of GlutaMAX-I (1 mM), 5 ml of penicillin-streptomycin, 5 ml of NEAA (10 μM), 500 μl of 2-ME (100 μM) and 50 μl of bFGF (4 ng ml^{-1}). Filter the medium with a 0.22-μm filter unit and store it for up to 1 week at 4 °C.

MEF medium To prepare 500 ml of MEF medium, mix 450 ml of DMEM medium with 50 ml of FBS and 2.5 ml of penicillin-streptomycin. Filter the medium with a 0.22-μm filter unit and store it for up to 2 weeks at 4 °C.

DAP213 solution To prepare 10 ml of DAP213 solution, mix 5.37 ml of human iPSC medium, 1.43 ml DMSO, 1 ml of 10 M acetamide and 2.2 ml of propylene glycol. Store the solution for up to 1 month at −80 °C.

SeV solutions To prepare working stocks of SeV solutions (from the Cyto-Tune kit), thaw the solutions on ice and prepare 50-μl stocks in 1.5-ml tubes. Working stocks can be stored at −80 °C.

PROCEDURE
Blood sampling ● TIMING ~10 min
1| Sterilize the cap from the bottle of heparin with an alcohol prep pad.
! CAUTION Wash your hands before starting venipuncture. Wear gloves when handling blood. Change gloves after venipuncture in each donor or if the gloves become contaminated.

2| Combine a 23-G needle and 2.5-ml syringe and draw up 100–300 µl of heparin.

3| Release the 23-G needle from heparinized 2.5-ml syringe and combine a new 23-G butterfly needle with the heparinized 2.5-ml syringe.

4| Identify the median cubital or cephalic veins of donors' arms; then palpate and trace the vein paths with the index finger.

5| Sterilize the venipuncture site with an alcohol prep pad.
! CAUTION Do not palpate the venipuncture site after sterilization.

6| Apply the tourniquet above the selected puncture site.
! CAUTION Do not place the tourniquet too tightly or leave it on for more than 3 min.

7| Remove the needle shield and perform venipuncture. Insert the needle into the blood vessel and hold still once a backflow of blood is seen in the tube of the butterfly needle setup.
! CAUTION Venipuncture must be done by a person who is well trained and legally certified to carry out the procedure.
! CAUTION Patients' informed consent must be obtained before blood sampling.

8| Draw 1–2 ml of blood into the syringe.

9| Remove the tourniquet.
! CAUTION Do not withdraw the needle before removing the tourniquet.

10| Withdraw the needle fully, apply pressure to the alcohol prep pad over the puncture site and maintain the pressure for 3–5 min until the bleeding stops.

11| Discard the needle of the Vacutainer into a biohazard container without recapping the needle.
! CAUTION Dispose of items that are used for venipuncture immediately and in appropriate containers.
⚠ CRITICAL STEP This step and all subsequent steps should be carried out using sterile reagents and equipment.
▩ PAUSE POINT Heparinized whole blood can be stored for up to 24 h before use (**Fig. 2a**).

Isolating PBMCs using Ficoll gradient ● TIMING ~1 h
12| Add 1–2 ml of heparinized whole blood to a 15-ml tube.
? TROUBLESHOOTING

13| Add 1–2 ml of D-PBS(−) and dilute the blood with D-PBS(−) 1:1.

14| Prepare 3 ml of Ficoll-Paque PREMIUM in a separate 15-ml tube.

15| Pour the diluted blood onto the Ficoll solution carefully so as to form two layers.
! CAUTION Do not mix the blood and the Ficoll solution. The blood must remain on top.

16| Centrifuge for 30 min at 400*g* at room temperature.

17| Collect the layer of PBMCs without touching the Ficoll layer, using sterile pipette tips, into a new 15-ml tube (**Fig. 2b**).
⚠ CRITICAL STEP When collecting PBMCs, carefully avoid obtaining any of the Ficoll solution layer. Contamination of the Ficoll solution reduces the collection rate of the mononuclear cells.

18| Dilute the PBMCs by adding 5 ml of D-PBS(−).

19| Centrifuge for 5 min at 200*g* at room temperature.

20| Discard the supernatant, add 5 ml of D-PBS(−) and dilute the PBMCs with D-PBS(−).

21| Centrifuge for 5 min at 200*g* at room temperature.

22| Discard the supernatant and resuspend the PBMCs with GT-T502 medium to a concentration of 1.25×10^6 cells per ml.
■ **PAUSE POINT** If needed, isolated PBMCs can be frozen and stored at -150 °C by using Cell Banker-2 according to the manufacturer's instructions.

Activating T cells ● TIMING 5 d
23| Add 400 µl of PBMC solution with GT-T502 medium into the wells of a human CD3-specific antibody–coated 24-well plate at a density of 5×10^5 cells per well.
▲ **CRITICAL STEP** Approximately 1 to 2×10^6 PBMCs can be obtained from 1 ml of human peripheral blood with a Ficoll gradient method. This number of PBMCs fills 2–4 wells of a standard 24-well plate. For TiPSC generation, one well of PBMCs is sufficient. Excess cells can be stored using Cell Banker-2, as described at the **PAUSE POINT** in Step 22.

24| Incubate the cells for 5 d at 37 °C in a 5% CO_2 incubator without medium change.
■ **PAUSE POINT** If needed, activated PBMCs can be frozen and stored at -150 °C by using Cell Banker-2 according to the manufacturer's instructions.

SeV infection ● TIMING ~1 d
25| Dilute the SeV solutions containing *OCT3/4*-SeV/TSΔF, *SOX2*-SeV/TSΔF, *KLF4*-SeV/TSΔF and *c-MYC (HNL)*-SeV/TS15ΔF individually on ice as detailed in REAGENT SETUP. This will take ~30 min.
! CAUTION Perform all procedures involving SeV vectors in a safety cabinet while wearing gloves. All waste should be treated first with ethanol, then with bleach, and finally it should be autoclaved.

26| Collect the activated PBMCs by pipetting and transfer them into a 15-ml tube.

27| Centrifuge for 5 min at 200*g* at room temperature.

28| Discard the supernatant and resuspend the cells with GT-T502 medium to a concentration of 7.5×10^5 cells per ml.

29| Add 1 ml of PBMC solution with GT-T502 medium into the wells of a human CD3-specific antibody–coated 12-well plate at a density of 7.5×10^5 cells per well.
▲ **CRITICAL STEP** After 5 d of activation with CD3-specific antibody and IL-2, approximately twofold amounts of activated PBMCs should be obtained. This amount of activated PBMCs fills 2–5 wells of a standard 12-well plate. For TiPSC generation, one well of activated PBMCs is sufficient. Excess cells can be stored using Cell Banker-2.
■ **PAUSE POINT** If needed, activated PBMCs can be frozen and stored at -150 °C by using Cell Banker-2 according to the manufacturer's instructions.

30| Add the virus solutions containing *OCT3/4*-SeV/TSΔF, *SOX2*-SeV/TSΔF, *KLF4*-SeV/TSΔF and *c-MYC (HNL)*-SeV/TS15ΔF individually to the wells, each at an MOI of 20. One well containing all virus solutions is sufficient to generate TiPS colonies.

31| Place the plate in an incubator at 37 °C and 5% CO_2. This plate should be left undisturbed for 24 h for cells to grow; meanwhile, however, immediately proceed with Step 32 (MEF plating).

Plating MEFs ● TIMING ~30 min
32| Place 10 ml of MEF medium in a 50-ml tube. Although MEFs should be ready for use on the following day, plating of MEFs should be done 1 d before plating infected PBMCs for optimal cell viability.

33| Remove a vial of frozen MEFs from freezing container and place the vial into a 37 °C water bath until thawed.

34| Wipe the vial with ethanol, open the cap and transfer the cell suspension to the tube prepared in Step 32.

35| Centrifuge the tube for 5 min at 200*g* at room temperature.
? TROUBLESHOOTING

36| Discard the supernatant and resuspend the cells with 50 ml of MEF medium.

37| Add 10 ml of MEF suspension onto a 10-cm gelatin-coated dish, or add 2 ml into each well of a gelatin-coated six-well plate (6×10^6 cells per 10-cm dish or 1.2×10^5 cells per well of a six-well plate).

PROTOCOL

38| Incubate overnight at 37 °C in a 5% CO_2 incubator.

■ **PAUSE POINT** The MEF dishes can be stored for up to 3–4 d before use.

Replating infected PBMCs ◉ TIMING ~30 min

39| Collect the infected cells (from Step 31) by pipetting and transfer them into a 15-ml tube.

40| Centrifuge for 5 min at 200g at room temperature.

41| Discard the supernatant and resuspend the cells with 1 ml of human iPSC medium.

42| Aspirate the medium from the 10-cm dishes with MEF feeder cells (prepared in Steps 32–38) and add 10 ml of human iPSC medium into the dishes.

43| Use a hemocytometer to count the number of cells in solution prepared in Step 41 and replate 5×10^4 and 5×10^5 cells on a 10-cm dish with the MEF feeder cells prepared in Step 42.

▲ **CRITICAL STEP** After 24 h of infection, approximately 1×10^6 PBMCs can be obtained from one well of a 12-well plate. This number of infected PBMCs is sufficient to replate 5×10^4 and 5×10^5 cells on a 10-cm dish with MEF feeder cells.

▲ **CRITICAL STEP** Excess infected PBMCs can be used as a positive control for assessing the removal of SeV by real-time PCR. Dilute 5×10^4 cells of infected PBMCs with 500 μl of TRIzol and store it at −80 °C if needed.

44| Incubate the dish at 37 °C in a 5% CO_2 incubator.

Culturing infected PBMCs ◉ TIMING ~3 weeks

45| After replating the infected mononuclear cells, change the human iPSC medium every 2 d and maintain cells in culture until the TiPSC colonies emerge.

▲ **CRITICAL STEP** The infected mononuclear cells should be attached to the MEFs within the first 2 d after replating. It is not a problem for TiPSCs generation if unattached cells are lost when changing the human iPSC medium.

46| About 10 d after replating, small colonies should start to appear. At about 15–20 d after replating, colonies showing typical human ESC colony–like morphology should emerge (**Fig. 4c**).

Picking up and expanding the TiPSC colonies ◉ TIMING ~3 weeks

47| Mark the colonies that show ESC-like morphology on the bottom of the dish under an inverted microscope.

48| Prepare the required number of six-well plates with MEF feeder cells.

49| Replace the medium in the six-well plates with MEF feeder cells by human iPSC medium at the amount of 2 ml per well.

50| Add 20 μl of human iPSC medium into the required number of wells of 96-well plates. Colony pick-up needs one well per colony.

? TROUBLESHOOTING

51| Wipe a phase-contrast microscope carefully with ethanol and place it onto a tissue culture clean bench.

52| By using the phase-contrast microscope and a 20-μl pipette, pick up the colonies marked in Step 36 and place each colony into one well of the 96-well plates containing human iPS cell medium (added in Step 50).

53| Use a phase-contrast microscope and a 200-μl pipette to add 200 μl of human iPSC medium into the wells of 96-well plates and break the colonies into small pieces by pipetting.

! CAUTION Do not disperse the colonies into single cells.

54| Transfer the broken colonies into wells of six-well plates containing MEF feeder cells as prepared in Step 48.

55| Incubate at 37 °C in a 5% CO_2 incubator. Change human iPSC medium every 2 d.

56| Every 5–7 d, passage the cells by using collagenase IV solution as follows: when the colonies become confluent, aspirate the medium and add collagenase IV solution (use half the amount you use for maintenance medium; for example, 1 ml for a well of a six-well plate and 5 ml for a 10-cm dish).

57| Incubate for 30–60 min at 37 °C in a 5% CO_2 incubator.

58| Collect the colonies into a 15-ml tube by pipetting when floating colonies appear.

59| Centrifuge for 2 min at 200g at room temperature.

60| Discard the supernatant. Add 1 ml of fresh human iPSC medium and break the colonies into small pieces with a 1,000-μl pipette and by pipetting 1 ml of cell solution four or five times.
! CAUTION Do not disperse the colonies into single cells.

61| Place the broken colonies into a new dish or plate that has been pre-plated with MEFs and filled with fresh human iPS culture medium. The split ratio used for the cells depends on the cell lines and usually ranges from 1:4 to 1:10.

62| If you wish to freeze the iPSCs, follow option A. If you wish to check for the removal of SeV by real-time RT-PCR, follow option B.
(A) Freezing of iPSCs ◉ TIMING ~1 h
   (i) Aspirate the medium and add collagenase IV solution as detailed in Step 56.
   (ii) Incubate for 30–60 min at 37 °C in a 5% CO_2 incubator.
  (iii) Collect the colonies into a 15-ml tube by pipetting when floating colonies appear.
   (iv) Centrifuge for 2 min at 200g at room temperature.
   (v) Discard the supernatant, add 200 μl of DAP213 solution and break the colonies into small pieces with a 200-μl pipette.
     ! CAUTION For good cell viability, perform this step and Steps 62A(vi) and Steps 62A(vii) within 15 s.
   (vi) Transfer 200 μl of the cell suspension to 1.5-ml cryovials.
  (vii) Plunge the vials quickly into liquid nitrogen.
 (viii) Store the cryovials in the freezing container at −150 °C.
(B) Checking for removal of SeV by real-time RT-PCR ◉ TIMING ~8 h
   (i) Prepare primers as shown in **Table 1.**
   (ii) Transfer the excess broken colonies from Step 50 into 1.5-ml tubes. Half of the cells from a 10-cm dish of confluent TiPSCs will supply sufficient mRNA.
  (iii) Centrifuge for 2 min at 200g at room temperature.
   (iv) Discard the supernatant. Dilute the broken colonies by adding 1 ml of D-PBS(−).
   (v) Centrifuge for 2 min at 200g at room temperature.
   (vi) Discard the supernatant. Dilute the broken colonies by adding 500 μl of TRIzol.
     ■ **PAUSE POINT** Cell lysates prepared with TRIzol should be stored at −80 °C.
  (vii) Add 100 μl of chloroform to 500 μl of cell lysate (which should be thawed on ice if stored at −80 °C) and mix vigorously by shaking. To make a positive control, thaw the infected PBMC lysate prepared in Step 43 on ice and use protocol (viii–xxxv) with infected PBMC lysate, as was used for the TiPSC samples.

TABLE 1 | Primer sequences for detecting SeV.

Primers	Sense	Antisense
SeV-hOct3/4	5′-TCTGGGCTCTCCCATGCATTCAAAC-3′	5′-AATGTATCGAAGGTGCTCAA-3′
SeV-hSox2	5′-ACGGCCATTAACGGCACACTG-3′	5′-AATGTATCGAAGGTGCTCAA-3′
SeV-hKlf4	5′-CACCTCGCCTTACACATGAAGAG-3′	5′-AATGTATCGAAGGTGCTCAA-3′
SeV-c-Myc	5′-TAACTGACTAGCAGGCTTGTCG-3′	5′-AAATACGGCTGCACCGAGTCGT-3′
GAPDH	5′-GTGGACCTGACCTGCCGTCT-3′	5′-GGAGGAGTGGGTGTCGCTGT-3′

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(viii) Centrifuge for 15 min at 12,000g at 4 °C.
 (ix) Transfer the aqueous phase (200–250 μl) of lysate to a new 1.5-ml microtube.
  (x) Add 250 μl of isopropanol to the lysate transferred in Step 58 and mix well by pipetting several times.
 (xi) Centrifuge for 5 min at 12,000g at 4 °C.
(xii) Remove the supernatant and add 500 μl of 70% (vol/vol) ethanol.
(xiii) Centrifuge for 2 min at 9,000g at 4 °C.
(xiv) Remove the ethanol completely and air-dry the pellet at room temperature for 5 min.
 (xv) Resuspend the pellet in 50 μl of RNase-free water.
(xvi) Use 2 μl of the sample to determine the RNA concentration of samples by measuring with an optical spectrometer (NanoDrop), and adjust the concentration of each sample to 20 ng μl^{-1} by adding RNase-free water.
(xvii) Transfer 100 μl of lysate (which contains 2 μg of RNA) to a new 1.5-ml microtube.
(xviii) Add 250 μl of ethanol and 10 μl of 3 M sodium acetate, and then mix vigorously by shaking.
(xix) Refrigerate the lysate at −20 °C for 30 min.
 (xx) Centrifuge for 15 min at 12,000g at 4 °C.
(xxi) Remove the supernatant and add 500 μl of 70% (vol/vol) ethanol.
(xxii) Centrifuge for 2 min at 9,000g at 4 °C.
(xxiii) Remove the ethanol completely and air-dry the pellet at room temperature for 5 min.
(xxiv) Resuspend the pellet in 16 μl of RNase-free water.
(xxv) Add 2 μl of 10× DNase I buffer and 2 μl of DNase I to the RNA lysate, mix gently by finger tapping and incubate for 15 min at room temperature.
(xxvi) Add 2 μl of 25 mM EDTA, mix gently by finger tapping and incubate for 10 min at 65 °C.
(xxvii) Transfer the tube onto ice.
(xxviii) Transfer 14 μl of RNA lysate to a new 1.5-ml microtube and then add 2 μl of Oligo dT and 8 μl of 2.5 mM dNTP mix.
(xxix) Incubate for 5 min at 65 °C.
 (xxx) Place the tube on ice.
(xxxi) Add 8 μl of 5× first-strand buffer, 4 μl of 0.1 M DTT, 2 μl of 40 U μl^{-1} of RNase inhibitor and 2 μl of SuperScript II.
(xxxii) Mix gently by finger tapping and incubate for 50 min at 42 °C.
(xxxiii) Incubate for 15 min at 70 °C.
 ▓ **PAUSE POINT** cDNA samples should be stored at −20 °C.
(xxxiv) Prepare 20 μl of real-time PCR mixture by mixing the reagents listed below in a 96-well PCR plate.

Real-time PCR mixture	Per tube (μl)
SYBR Premix ExTaq	10
Primer sense (10 μM)	0.4
Primer antisense (10 μM)	0.4
H$_2$O	4
Rox	0.2
DNA sample	5
Total	20

(xxxv) Perform a SYBR Green reaction in 96-well plates on an ABI 7500 real-time PCR instrument (95 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s).
 ▲ **CRITICAL STEP** By using a cDNA sample obtained from infected PBMCs as positive control and standards, check the expression of transgenes that were normalized to the expression of GAPDH. Check the expression of TiPSC transgenes after 10–15 passages.

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? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
12	The number of PBMCs is insufficient	Ficoll is mixed into the PBMC solution	If more than 1×10^5 cells were obtained, seed them in 400 µl of GT-T502 medium, incubate for 6–7 d, and go to Step 25. If not, repeat Steps 2–12 with new whole blood and collect PBMCs carefully without mixing Ficoll
35	No TiPSC colonies appear	Viral titer is low	Use new SeV solution. Avoid using SeV solution after repeated freeze-thaw cycles
		Most T cells are dead before infection	Ensure the density of T cells in each appropriate step. Try SeV infection on day 4 of activation
50	Signs of iPSC differentiation after expansion	Poor-quality human iPSC medium	Use new human iPSC medium or increase bFGF concentration in the medium

● TIMING

Steps 1–11, blood sampling: ~10 min
Steps 12–22, isolating PBMCs using Ficoll gradient: ~1 h
Steps 23 and 24, activating T cells: 5 d
Steps 25–31, SeV infection: ~1 d
Steps 32–38, plating MEFs: ~30 min
Steps 39–44, replating infected PBMCs: ~30 min
Steps 45 and 46, culturing infected PBMCs: ~3 weeks
Steps 47–62, picking up and expanding the TiPSC colonies: ~3 weeks

ANTICIPATED RESULTS

T cells make up approximately 70% or less of isolated PBMCs, and they are inefficiently infected by SeV vectors before activation. However, after a 5-d activation with IL-2 and anti-CD3 antibody, the T cell proportion increases to become >95% of PBMCs, and it can be effectively infected with SeV vectors. With proper activation, T cells are infected with SeV vectors at >80% efficiency at an MOI of 20 (**Fig. 3a–d**).

From the 5×10^4 cells infected with SeV vectors encoding human OCT3/4, SOX2, KLF4 and c-MYC and seeded onto MEF feeder cells, 50–100 ESC-like colonies grow, on average (**Fig. 4a**). The TiPSC colonies clearly stain positive for alkaline phosphatase (ALP), a stem cell marker (**Fig. 4b**) and express ESC marker transcripts (**Fig. 4d**). The expression of TiPSC transgenes is not always detectable after 10–15 passages. They can also differentiate into three germ layer–derived tissues and generate teratomas *in vivo* (**Fig. 4e**) and embryoid bodies *in vitro*. These TiPSC lines will have TCR rearrangement in their genome, which is a hallmark of mature terminally differentiated T cells, and will show specific peaks for Dβ/Jβ and Vβ/Jβ recombination in capillary electrophoresis of the PCR products.

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Association of genetic variation in *FTO* with risk of obesity and type 2 diabetes with data from 96,551 East and South Asians

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Abstract

Aims/hypothesis FTO harbours the strongest known obesity-susceptibility locus in Europeans. While there is growing evidence for a role for *FTO* in obesity risk in Asians, its association with type 2 diabetes, independently of BMI, remains inconsistent. To test whether there is an association of the *FTO* locus with obesity and type 2 diabetes, we conducted a meta-analysis of 32 populations including 96,551 East and South Asians.

Methods All studies published on the association between *FTO*-rs9939609 (or proxy [r^2>0.98]) and BMI, obesity or type 2 diabetes in East or South Asians were invited. Each study group analysed their data according to a standardised analysis plan. Association with type 2 diabetes was also adjusted for BMI. Random-effects meta-analyses were performed to pool all effect sizes.

Results The *FTO*-rs9939609 minor allele increased risk of obesity by 1.25-fold/allele (p=9.0×10^{-19}), overweight by

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1.13-fold/allele ($p=1.0 \times 10^{-11}$) and type 2 diabetes by 1.15-fold/allele ($p=5.5 \times 10^{-8}$). The association with type 2 diabetes was attenuated after adjustment for BMI (OR 1.10-fold/allele, $p=6.6 \times 10^{-5}$). The *FTO*-rs9939609 minor allele increased BMI by 0.26 kg/m^2 per allele ($p=2.8 \times 10^{-17}$), WHR by 0.003/allele ($p=1.2 \times 10^{-6}$), and body fat percentage by 0.31%/allele ($p=0.0005$). Associations were similar using dominant models. While the minor allele is less common in East Asians (12–20%) than South Asians (30–33%), the effect of *FTO* variation on obesity-related traits and type 2 diabetes was similar in the two populations. *Conclusions/interpretation FTO* is associated with increased risk of obesity and type 2 diabetes, with effect sizes similar in East and South Asians and similar to those observed in Europeans. Furthermore, *FTO* is also associated with type 2 diabetes independently of BMI.

Introduction

Large-scale genome-wide association studies (GWAS) in mainly white Europeans have identified at least 50 genetic loci to be robustly associated with obesity-related traits [1–

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12]. A cluster of common variants in the first intron of the fat mass and obesity-associated gene (*FTO*) was the first obesity-susceptibility locus to be identified by two independent GWAS in 2007 [1, 2] and has since been consistently replicated by many others and for a variety of obesity-related traits [7, 9, 13–15]. Of all currently identified obesity-susceptibility loci, the *FTO* locus has the most pronounced effect on BMI and obesity risk, at least in individuals of European descent. Each minor allele of any commonly investigated variant in *FTO* increases BMI by 0.30–0.40 kg/m^2 (equivalent to 870–1,150 g for a person 1.7 m tall) and risk of obesity by ~20% [7, 15]. The

minor allele of the *FTO* variant is common (minor allele frequency (MAF)=~42%) in white Europeans, such that 66% of Europeans carry at least one risk allele and 18% carry two risk alleles. Because of the high prevalence of the risk allele and its relatively strong effect on BMI, the *FTO* locus explains most (0.34%), yet little, of the variation in BMI in Europeans [7].

FTO has also been examined as an obesity-susceptibility locus in populations of non-white European origin. While the initial replication efforts in East Asian populations were inconsistent [16, 17], a growing number of studies have provided evidence that genetic variation in *FTO* influences

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BMI and obesity risk also in Chinese, Japanese, Korean and Filipino populations [18–27]. A GWAS for BMI in 7,861 Koreans identified variation in *FTO* (rs9939609) as the most significantly associated locus, nearly reaching genome-wide significance ($p=1.5\times10^{-7}$) [28]. Furthermore, literature-based meta-analyses in Asians reported that the minor allele for the rs9939609 *FTO* single-nucleotide polymorphism (SNP) significantly ($p=9\times10^{-9}$) increased the risk of obesity, but no other obesity-related traits were examined [18, 29, 30]. Fewer studies in South Asians have been reported, two of which confirmed the association between the *FTO* locus and obesity susceptibility [31, 32], whereas one did not [33]. The prevalence of the risk allele in East Asians (~20%) and South Asians (~30%) is substantially lower than in Europeans, and the reported effect sizes in both East and South Asians vary widely for BMI (OR 0.13–0.83 kg/m² per minor allele) and obesity risk (OR 1.02–1.48 per minor allele) [16, 18, 20–25, 27, 34–39].

FTO was first identified as a type 2 diabetes-susceptibility gene, but, as further adjustment for BMI abolished the association with type 2 diabetes [1], it was suggested that *FTO* is primarily an obesity-susceptibility locus. However, the BMI-independent role of *FTO* in type 2 diabetes remains a matter of debate, particularly in Asians but also in white Europeans. While several studies have reported that the association between the *FTO* locus and risk of type 2 diabetes remained significant after adjustment for BMI [15, 18, 33, 35, 40, 41], others could not confirm this [21, 30, 32, 37, 42].

To firmly establish the association between the *FTO* locus and obesity susceptibility in East and South Asians and to assess its effect size and potential heterogeneity across Asian populations, we performed a systematic meta-analysis of data from 32 populations, including 96,551 men and women, using standardised study-specific association analyses. Furthermore, we examined whether the *FTO* locus is associated with type 2 diabetes independently of its association with BMI.

Methods

Literature search and study identification We designed a meta-analysis based on de novo analyses of data according to a standardised plan to achieve the greatest consistency possible across studies. We identified all published studies (before September 2010) that had examined the association of genetic variation in *FTO* with risk of obesity and type 2 diabetes and with obesity-related continuous traits in East and South Asian adults (age ≥18 years) by a PubMed literature search using the key words 'FTO', 'fat mass and obesity associated gene' and 'genome-wide association study'. References from the identified papers were subse-

quently screened to identify additional studies and to ensure that the list of eligible studies was complete. The literature search was carried out by two investigators independently, who cross-checked their search results for completeness.

Our literature search identified 38 publications, one of which was excluded because it was a subsample of another identified study. We invited the corresponding authors of the remaining 37 publications to join our meta-analysis, of which 26 agreed to participate and eventually 22 submitted raw data or summary statistics. We also included a Korean population with previously unpublished data (Y. M. Kim, J. Shin, C.B. Lee, M.K. Kim, Y. Tabara, T. Miki and B.Y. Choi), which was presented by a contributing author.

Taken together, our meta-analysis included data for 31 populations from 22 publications and one unpublished study, with 96,551 individuals altogether. The study identification and selection process is illustrated in Fig. 1.
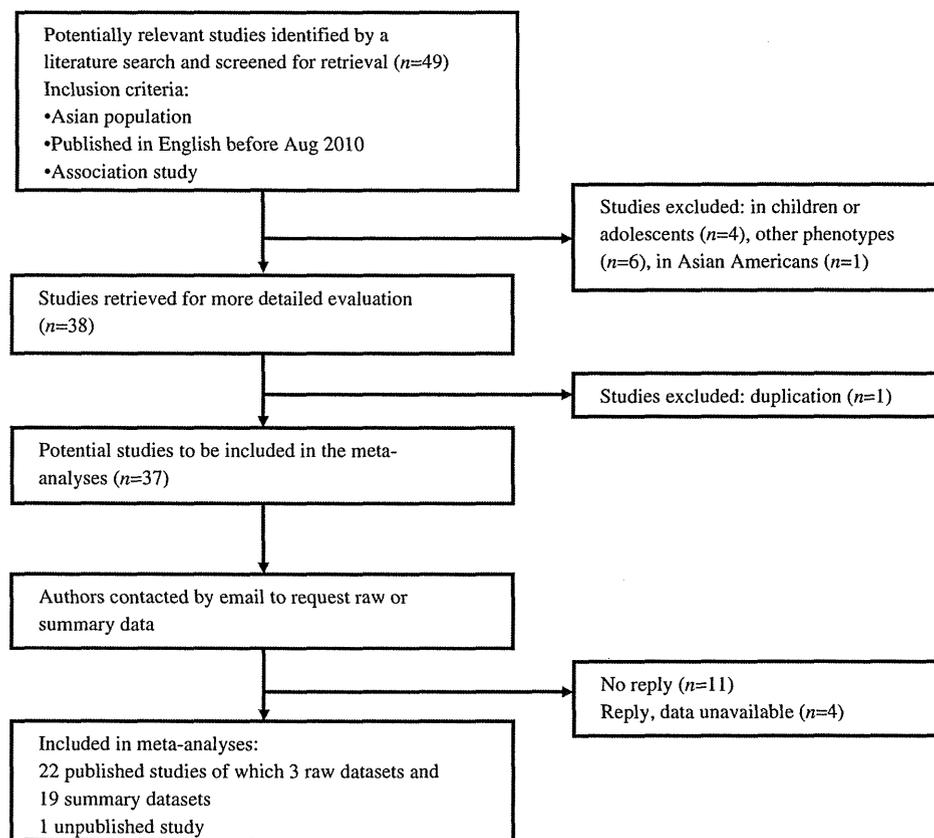
All studies were conducted according to the Declaration of Helsinki. Informed consent was obtained from all participants, and the studies were approved by the ethics committees of the participating institutions.

Genotyping The rs9939609 *FTO* SNP was examined in 18 studies, whereas proxy SNPs were used in 14 studies. More specifically, the rs8050135 SNP was genotyped in 11 studies of East Asians and one of South Asians, and the rs3751812 and rs17817449 SNP were each genotyped once in studies of East Asians (electronic supplementary material [ESM] Table 1). The linkage disequilibrium between rs9939609 and the three proxies (rs8050135, 3751812, rs17817449) is perfect ($r^2=1$) in populations of East Asian origin, based on CHB+JPT data from the HapMap (Rel 24/Phase II). The linkage disequilibrium between rs9939609 and rs8050135 in Indian Asians is very high ($r^2>0.98$), based on a subsample ($n=305$) of the participating Lolipop study.

The genotyping success rate and concordance rate were >95%, and genotype distributions were in Hardy–Weinberg equilibrium ($p>0.01$) in all participating studies (ESM Table 1).

Statistical analysis As case–control definitions and statistical analyses used in the published papers were inconsistent, we asked analysts of each of the participating cohorts to re-analyse their data according to a standardised analysis plan. Summary statistics of each study were subsequently meta-analysed.

Obesity-susceptibility traits and type 2 diabetes Overweight was defined as a BMI ≥24 kg/m², and obesity as a BMI ≥28 kg/m² according to the definition proposed by the Working Group on Obesity in China [43]. Anthropometric data, including weight, height, waist circumference, hip

Fig. 1 Study identification and inclusion in the meta-analyses

Potentially relevant studies identified by a literature search and screened for retrieval (n=49)
Inclusion criteria:
•Asian population
•Published in English before Aug 2010
•Association study

Studies excluded: in children or adolescents (n=4), other phenotypes (n=6), in Asian Americans (n=1)

Studies retrieved for more detailed evaluation (n=38)

Studies excluded: duplication (n=1)

Potential studies to be included in the meta-analyses (n=37)

Authors contacted by email to request raw or summary data

No reply (n=11)
Reply, data unavailable (n=4)

Included in meta-analyses:
22 published studies of which 3 raw datasets and 19 summary datasets
1 unpublished study

circumference and body fat percentage, were collected in each study as described previously (ESM Table 1), BMI was calculated as weight (kg) divided by height squared (m²), and WHR as waist circumference (cm) divided by hip circumference (cm). Raw data were used for analyses.

Type 2 diabetes was defined as meeting one or more of the following criteria: (1) fasting glucose ≥7.0 mmol/l; (2) 2-h glucose ≥11.1 mmol/l; (3) previous diagnosis of type 2 diabetes; (4) HbA₁c ≥6.5% (48 mmol/mol); (5) self-reported type 2 diabetes (ESM Table 1).

Study-specific de novo data analyses Association analyses within each study were performed for the total population and for men and women separately using additive and dominant genetic models. The associations of *FTO*-rs9939609 (or proxy) with risk of obesity and type 2 diabetes were assessed with multiple logistic regression models. Generalised linear models were used to assess the associations of *FTO*-rs9939609 (or proxy) with obesity-related continuous traits. In studies with a case–control design, analyses for continuous traits were conducted in control samples only. All analyses were adjusted for age and sex (sex-stratified analyses were only adjusted for age). The association with type 2 diabetes was also analysed with adjustment for BMI. Adjustments were performed by

including the covariates (age, sex and/or BMI) as a linear term in the association model.

Summary statistics from the study-specific association analyses were reported in a standardised Excel form by the analysts of each study and collected centrally for meta-analyses.

Meta-analyses Data extraction from the forms and meta-analyses was performed independently by two investigators and cross-checked for consistency. All ambiguities were clarified with the respective analysts before the final meta-analyses.

ORs and beta coefficients from the individual studies were pooled using DerSimonian and Laird random-effects meta-analyses [44]. Meta-analyses were performed of all studies combined. Because of differences in genetic background as well as in susceptibility to obesity and type 2 diabetes, meta-analyses were also stratified by East Asian and South Asian origin of the populations. Furthermore, East Asians were further stratified according to their country of origin.

Between-study heterogeneity was tested by Cochrane's Q test and quantified by the I^2 index. I^2 values of <25%, 25–75% and >75% were defined as low, moderate and high heterogeneity, respectively [45]. To examine the sources of

heterogeneity in our meta-analyses, we performed random-effects meta-regressions, where the between-study variance was estimated with the restricted maximum likelihood approach. Meta-regressions included the following study-specific variables as covariates: year of publication, country of origin, sample size, study design, mean age and mean BMI.

A funnel plot, along with Begg's and Egger's tests, was used to test for the presence of publication bias.

Statistical analyses were performed with the Stata 9.0 software (StataCorp LP, College Station, TX, USA). Meta-analyses and meta-regressions were implemented by the *metan* and *metareg* commands of Stata, respectively. $p<0.05$ was considered to be significant, except for Cochrane's Q test for heterogeneity and Begg's and Egger's tests for publication bias, where a level of $p<0.10$ was used.

The variation in obesity-related continuous traits explained by the *FTO* variant was evaluated using the equation $2f(1-f)a^2$, where f is the frequency of the variant and a is its additive standardised effect [5]. Population-attributable risk (PAR) was calculated as PAR $= (X-1)/X$. Assuming a multiplicative model, $X = (1-f)^2 + 2f(1-f)\gamma + f^2\gamma^2$, where γ is the estimated OR, and f is the frequency of risk allele [46].

Results

Characteristics of populations included in the meta-analyses Analyses were conducted in Chinese Hans (China Mainland: $n=10$; Singapore: $n=2$), Japanese ($n=7$), Indians ($n=7$), Koreans ($n=4$), Singapore Malays ($n=1$) and Filipinos ($n=1$; Table 1). Fifteen of the populations were case–control designed for obesity ($n=3$) or type 2 diabetes ($n=8$) or both ($n=4$), whereas 17 populations were population-based. The mean age and BMI of the populations ranged from 27.9 to 66.8 years and from 20.5 to 27.1 kg/m^2, respectively. The prevalence in population-based studies ranged from 3.1% to 37.9% for obesity and from 2.9% to 41.9% for type 2 diabetes.

The MAF of *FTO*-rs9939609 (or proxy) is 12–14% in Chinese Hans and Koreans, 18–20% in Japanese and Filipinos, and 30–33% in Singapore Malays and Indians (Table 1).

Associations with obesity and overweight A total of 24 populations ($n_{obese}=13,032$; $n_{overweight}=22,474$; $n_{normalweight}=35,767$) were available for meta-analyses of the association between the *FTO* variant and risk of obesity and overweight.

Each additional *FTO*-rs9939609 minor (A) allele increased the odds of obesity by 1.25 ($p=9.0\times10^{-19}$) compared with normal weight individuals (Fig. 2), and by 1.17 ($p=7.4\times10^{-11}$) compared with non-obese individuals

(ESM Fig. 1). Each additional minor allele increased the odds of overweight by 1.13 ($p=1.0\times10^{-11}$; ESM Fig. 2). The odds of obesity and overweight were the same in both East Asian and South Asian populations ($p=0.18$ and 0.84, respectively; ESM Table 2). Associations were similar in men and women (ESM Table 3). The heterogeneity across all studies was low (13%$\leq I^2\leq$19%).

When a dominant genetic model was used, the odds were only slightly higher than for the additive genetic model (ESM Table 4).

Association with type 2 diabetes In our meta-analysis of 22 populations ($n_{cases}=33,744$, $n_{controls}=43,549$), each additional *FTO*-rs9939609 minor allele increased the odds of type 2 diabetes by 1.15 ($p=5.5\times10^{-8}$) when adjusted for age and sex (Fig. 3). Further adjustment for BMI attenuated, but did not abolish, the association with type 2 diabetes (OR 1.10, $p=6.6\times10^{-5}$) (Fig. 4). Results were similar in East Asians and South Asians (ESM Table 2), in men and women (ESM Table 3), and when a dominant model was used (ESM Table 4).

The association results across all studies showed moderate heterogeneity (44%$\leq I^2\leq$48%; Figs 3 and 4). Meta-regression analyses revealed that the difference in study design contributed to some of the heterogeneity. Subsequent subgroup analyses showed that the association with type 2 diabetes was more pronounced in studies with a case–control design (OR [95% CI]=1.19 [1.14, 1.23], $p=3.7\times10^{-19}$, $I^2=0.0\%$) than in cohort studies (OR [95% CI]= 1.09 [0.99, 1.20], $p=0.07$, $I^2=54.4\%$), which showed moderate heterogeneity (ESM Table 5).

Associations with obesity-related continuous traits The meta-analyses of the association of *FTO*-rs9939609 with BMI, waist circumference, hip circumference, WHR and body fat percentage included 30 ($n=71,022$), 22 ($n=51,543$), 20 ($n=48,508$), 20 ($n=48,508$) and nine ($n=19,580$) populations, respectively.

Each additional *FTO*-rs9939609 minor allele was associated with a 0.26 kg/m^2 higher BMI ($p=2.8\times10^{-17}$; equivalent to ~750 g/allele for a person 1.7 m tall) (Fig. 5), 0.51 cm larger waist circumference ($p=3.0\times10^{-9}$) (ESM Fig. 3), 0.36 cm larger hip circumference ($p=0.0003$) (ESM Fig. 4), 0.003 greater WHR ($p=1.2\times10^{-6}$; ESM Fig. 5), and 0.31% higher body fat percentage ($p=0.0005$) (ESM Fig. 6). All associations were very similar between East and South Asians (ESM Table 2), between men and women (ESM Table 3), or when a dominant genetic model was used (ESM Table 4).

We observed moderate heterogeneity across studies in the associations with BMI and hip circumference (BMI: $I^2=33\%$; hip circumference: $I^2=51\%$; Fig. 5; ESM Fig. 4). Meta-regression suggested that, for BMI, the heterogeneity was

Table 1 Descriptive information of studies included in the meta-analyses, sorted by ethnicity, study design and publication year

Paper	Study	Publication year	Ethnicity	Country	Study design	Sample size						Mean age (years)	Mean BMI (kg/m^2)	*FTO* SNP	MAF
						Obese	OW	NW	T2DM	NFG	QT analyses				
Li et al. [16]	NHAPC	2008	East Asian	China	Population based	472	1,215	1,503	423	1,893	3,190	58.62	24.43	rs9939609	0.11
Sha et al. [55]	GSBC	2009	East Asian	China	Population based	78	326	1,223	n.a.	n.a.	1,627	34.49	22.21	rs9939609	0.12
Hu et al. [56]	SHDS	2009	East Asian	China	Case–control[b]	n.a.	n.a.	n.a.	1,759	1,791	1,791	57.33	23.57	rs8050136	0.12
Li et al. [35]	WDS	2010	East Asian	China	Case–control[a, b]	243	976	1,368	877	1,405	1,405	44.23	21.45	rs9939609	0.12
Cheung et al. [24]	CRISPS	2010	East Asian	China	Case–control[a]	419	n.a.	691	n.a.	n.a.	691	44.98	21.19	rs8050136	0.12
Liu et al. [18]	n.a.	2010	East Asian	China	Case–control[a, b]	277	794	893	1,767	1,961	1,961	58.09	24.52	rs9939609	0.12
Ng et al. [21]	CUHK	2010	East Asian	China	Case–control[b, c]	1,147	2,293	2,432	5,872	583	583	41.31	22.87	rs3751812	0.12
Shu et al. [42]	SGWAS	2010	East Asian	China	Case–control[b]	n.a.	n.a.	n.a.	1,043	2,170	2,170	49.24	23.30	rs9939609	0.12
Wen et al. [57]	FLSGS	2010	East Asian	China	Case–control[b]	n.a.	n.a.	n.a.	1,160	1,127	1,127	59.09	24.13	rs8050136	0.12
Chang et al. [23]	NTUH	2008	East Asian	Taiwan	Case–control[a, b]	737	677	719	881	1,254	1,254	61.19	21.60	rs9939609	0.14
Cha et al. [25]	Kirin	2008	East Asian	Korea	Population based	252	304	361	n.a.	n.a.	917	27.91	26.39	rs17817449	0.14
Cha et al. [58]	KCMS	2009	East Asian	Korea	Population based	61	261	688	n.a.	n.a.	1,010	43.14	22.77	rs8050136	0.12
Kim et al. (unpublished data)	YangPyeung Cardiovascular Cohort Study		East Asian	Korea	Population based	339	995	1,092	194	2,061	2,426	57.60	24.49	rs9939609	0.12
Ng et al. [34]	Korea SNUH	2008	East Asian	Korea	Case–control[b]	n.a.	n.a.	n.a.	758	629	629	64.70	23.52	rs8050136	0.12
Takeuchi et al. [59]	CAGE-Amagasaki	2009	East Asian	Japan	Population based	388	1,562	3,719	n.a.	n.a.	5,660	48.86	22.99	rs9939609	0.19
Takeuchi et al. [59]	CAGE-Fukuoka	2009	East Asian	Japan	Population based	721	3,763	8,076	n.a.	n.a.	12,560	62.59	23.05	rs9939609	0.19
Takeuchi et al. [59]	CAGE-BMI	2009	East Asian	Japan	Population based	168	607	1,006	n.a.	n.a.	1,781	66.82	23.69	rs9939609	0.20
Karasawa et al. [19]	Takahata	2010	East Asian	Japan	Population based	220	886	1,533	215	2,306	2,639	63.04	23.48	rs9939609	0.20
Hotta et al. [20]	GWASJPN obesity	2008	East Asian	Japan	Case–control[a]	1,559	n.a.	1,541	n.a.	n.a.	1,541	47.52	21.21	rs9939609	0.18
Omori et al. [37]	RIKEN T2D	2008	East Asian	Japan	Case–control[b]	n.a.	n.a.	n.a.	4,584	2,262	2,262	44.84	22.86	rs8050136	0.20
Takeuchi et al. [59]	CAGE-T2DM	2009	East Asian	Japan	Case–control[b]	n.a.	n.a.	n.a.	6,781	7,307	n.a.	64.35	23.47	rs9939609	0.19
Marvelle et al. [27]	CLHNS	2008	East Asian	Philippines	Population based	321	560	836	155	1,463	1,717	48.51	24.31	rs9939609	0.18
Tan et al. [22]	SP2	2008	East Asian	Singapore (Chinese)	Population based	195	624	1,609	145	2,248	2,430	48.11	22.88	rs8050136	0.12
Tan et al. [22]	SiMES	2008	East Asian	Singapore (Malays)	Population based	848	826	846	787	1,248	2,520	59.04	26.38	rs8050136	0.30
Tan et al. [22]	SDCS	2008	East Asian	Singapore (Chinese)	Case–control[c]	426	809	757	n.a.	n.a.	n.a.	64.27	25.34	rs8050136	0.14
Chambers et al. [6]	LOLIPOP (IA317)	2008	South Asian	India	Population based	727	858	536	434	1,651	2,247	48.22	26.83	rs8050136	0.33
Chambers et al. [6]	LOLIPOP (IA610)	2008	South Asian	India	Population based	2,479	2,647	1,423	1,780	4,715	7,060	55.38	27.14	rs8050136	0.32
Tan et al. [22]	SINDI	2008	South Asian	India	Population	760	910	858	974	1,348	2,528	58.01	26.20	rs8050136	0.33

Table 1 (continued)

Paper	Study	Publication year	Ethnicity	Country	Study design	Sample size						Mean age (years)	Mean BMI (kg/m²)	*FTO* SNP	MAF
						Obese	OW	NW	T2DM	NFG	QT analyses				
Yajnik et al. [33]	Parthenon	2009	South Asian	India	Population based	136	320	511	n.a.	n.a.	967	32.44	23.76	rs9939609	0.33
Yajnik et al. [33]	PMNS	2009	South Asian	India	Population based	59	271	1,546	50	1,681	1,876	32.71	20.83	rs9939609	0.31
Sanghera et al. [40]	Sikh Diabetes Study	2008	South Asian	India	Case–control[b]	n.a.	n.a.	n.a.	1,138	765	765	50.85	26.25	rs9939609	0.31
Yajnik et al. [33]	WELLGEN	2009	South Asian	India	Case–control[b]	n.a.	n.a.	n.a.	1,967	1,681	1,681	32.39	20.50	rs9939609	0.31

Individuals from CAGE-T2DM study were selected from other three CAGE population-based studies

[a] Obese case–control study

[b] T2DM case–control study

[c] Obese case–control study conducted in T2DM cases

n.a., data not available or not used in meta-analysis; NFG, normal fasting glucose; NW, normal weight; OW, overweight; QT, quantitative trait; T2DM, type 2 diabetes