Reprogramming *in vivo* produces teratomas and iPS cells with totipotency features

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Background: Previous work

reprogramming 1.0 = iPS cell generation

reprogramming 2.0 = direct transdifferentiation by defined factors (direct reprogramming)

reprogramming 3.0 = direct reprogramming in vivo (in situ induced direct transdifferentiation by defined factors)
Motivation

Reprogramming iPS cells has created many possible therapeutic opportunities.

This paper explores the possibility of reprogramming cells *in vivo* rather than traditionally *in vitro*.

Numerous therapeutic applications to reprogramming *in vivo*, namely regenerative medicine.

- We don’t need to take cells out of natural environment;
- Allows to avoid very resource-consuming and complex step – cell culture;
- Cell therapy without cells – precise manipulation of endogenous cells with therapeutic purpose;
- No induction of pluripotency (bypassing of “cancer phobia”);
- Potential lower cost and less laborious.
Reprogramming Mice

**Transgenic** - organism whose genome has been altered by transfer of gene
1. rtTA: transcriptional activator
2. Rosa26 locus: helps gene expression in mice
3. Lentiviral vectors: gene insertion (Oct4, Sox2, Klf4, c-Myc 15)
4. Addition of Doxycycline

=> Inducible four factors (i4F) capable of expressing iPS cells in vivo.
High dose of doxycycline

1 mg mL$^{-1}$ Doxycycline

1. Weight loss
2. Morbidity of transgenic line
3. Teratomas

i45-A and i45-B

0.2 mg mL$^{-1}$ Doxycycline
2.5 week treatment

1 mg mL$^{-1}$ Doxycycline
1.0 week treatment

Doxycycline Withdrawal

Teratomas

*Teratomas are class of tumors found in pluripotent cells after a process of expansion and disorganized differentiation.

Intestine

Skin
The presence of multiple teratomas in both lines implies that reprogramming into pluripotency is feasible within in vivo condition.
Are in vivo iPS cells from cells of haematopoietic lineage?
Teratomas in multiple organs - immunohistochemistry

CK19 - epithelial maker
NANOG - pluripotency marker

*Reprogramming in vivo is a low efficiency process that likely involves scholastic - random events, similar to in vitro reprogramming.
Reprogrammable mice present iPS cells in blood
Transcriptomic analysis of *in vivo* iPS cells

Hierarchal clustering: divide mRNA transcriptomes to maximize & quantify similarity within groups

Upregulated *in vivo* genes associated with pluripotency
Upregulated in *in vivo* iPS:
Nlrp4f, Etv4, Ppm1j (morula, early development)

Downregulated:
Mmp12, Tnc (extracellular matrix)
*In vivo* iPS cells contribute to the trophectoderm

Teratomas from in vivo iPS cells showed cell resemble trophoblast giant cell (TGC) associated with internal haemorrhages.

PL-1 and CK8 are markers of TGC.

Upregulated markers of trophectoderm lineages in *in vitro* culture (Cdx2).
*In vivo* iPS cells contribute to the trophectoderm

*In vivo* iPS cells contributed to the trophectoderm in blastocyst

GFP+ iPS cells in chimaeric embryos in embryo proper and placenta

“Pluripotency state that cen access the trophectodermal lineage”
*In vivo* iPS cells are injected intraperitoneally into wild-type mice. A fraction of mice injected with *in vivo* iPS cells contained embryo like structures, in contrast to the mice injected with *in vitro* iPS or ES cells.
1) Three germ layers

- **Germ Layer Markers**
  - SOX2
  - T
  - GATA4

2) Extraembryonic Tissues

2.1 Extraembryonic Ectoderm

- CDX2
  - Trophoderm lineage that forms extraembryonic tissues

2.2 Yolk like tissue

- AFP
- CK8

Yolk sac endoderm that is important for early embryonic blood supply
Endothelial cell surface marker
Endothelial cells is a kind of epithelium that lines the interior surface of blood vessels.

Structures looks like yolk sac blood islands.

Nucleated erythrocytes markers because erythrocytes associated with yolk sac
Future Work

Future work will explore the full capabilities of *in vivo* iPS cells.
Extended Data Figures
1: Characterization of four independent i4F transgenic mouse lines.
2: Genomic insertion sites of lentiviral transgenes i4F-A and i4F-B and their effect on the host genes.
3: Histological alterations of the intestine and pancreas upon induction of i4F reprogrammable mice.

Mice were treated with doxycycline (1 mg ml$^{-1}$) for 6 days. Haematoxylin and eosin (H&E) staining and immunohistochemistry of OCT4 in the intestine (a) and pancreas (b). Similar alterations were found in both lines, i4F-A and i4F-B.
4: i4F induction leads to the appearance of tumoral masses and in situ reprogramming events.

<table>
<thead>
<tr>
<th>Mice analyzed with teratomas</th>
<th>Teratomas</th>
<th>Other tumours in the same mice</th>
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<tbody>
<tr>
<td>i4F-A</td>
<td>Multiple per mouse</td>
<td>1 Wilm’s tumour, 1 skin papilloma, 1 mouse with intestinal polyps</td>
</tr>
<tr>
<td>i4F-B</td>
<td>Multiple per mouse</td>
<td>1 urothelial carcinoma, 1 mouse with intestinal polyps</td>
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a, Reprogrammable mouse with multiple tumoral masses in the liver and kidneys (a representative example is shown from 15 mice analysed with teratomas). b, Incidence of other tumours in reprogrammable mice with teratomas. c, Three examples of NANOG-positive tubules in different induced reprogrammable mice.
5: Characterization of in vivo iPS cells.

**a**, Expression of pluripotency markers in the indicated cell types. Data correspond to qRT–PCR from seven independent in vivo iPS cell clones, two in vitro iPS cell clones (no. 1: in vitro reprogrammed i4F MEFs; no. 2: in vitro reprogrammed wild-type MEFs infected with lenti-OSKM), and two ES cell clones (no. 1: C57BL6.10; no. 2: G4). Values correspond to the average ± s.d. of 3 technical replicates. **b**, Silencing of the lentiviral cassette in in vivo iPS cell clones. Upper part, location of the PCR primers used. Lower part, lentiviral RNA levels in in vivo iPS cells (7 independent clones), in an in vitro iPS cell clone (in vitro reprogrammed i4F MEFs), in an ES cell line (C57BL6.10), and in i4F-MEFs induced with doxycycline for 3 days. Values correspond to the average ± s.d. of 3 technical replicates. **c**, Chimaeric E14.5 testis generated with a GFP-labelled in vivo iPS cells. Magnifications show germ cells derived from in vivo iPS cells. **d**, Summary of the isolation of in vivo iPS cells from the bloodstream.
6: Transcriptomic profiles of *in vivo* iPS cells, *in vitro* iPS cells and ES cells.

a, Pearson correlation coefficients among all sequenced samples. The highest and the lowest coefficients are coloured in a blue to red gradient.

b, Principal component analysis of the transcriptomes of *in vivo* iPS cells, *in vitro* iPS cells and ES cells. Data correspond to 6 clones of *in vivo* iPS cells, 5 clones of *in vitro* iPS cells, and 3 lines of ES cells (C57BL6.10, JM8.F6 and Bruce4).

c, Upper part, scatter plots representing the expression of each gene in the indicated pairs of cell types. Middle part, volcano plots representing the *P*-value of the differences in expression of each gene between the corresponding cell types. Significant *P*-values are in blue (that is, indicating differentially expressed genes). Non-significant *P*-values are in red (that is, indicating genes that are not differentially expressed). Lower part, Pearson coefficient correlation among samples. Data correspond to 6 clones of *in vivo* iPS cells, 5 clones of *in vitro* iPS cells, and 3 lines of ES cells (C57BL6.10, JM8.F6 and Bruce4).
7: Validation of RNA-seq data.
Induction of trophectoderm markers (*Fgfr2*, *Eomes*) in the indicated cell types after culture in TS differentiation medium (see Methods) during the indicated period of time. Other markers were used as controls: *Sox1* (ectoderm), *T* (mesoderm) and *Gata6* (endoderm). For each cell type, values are relative to the average levels at day 0. Values correspond to the average and s.d. For ES cells, *n* = 3 (lines C57BL6.10, JM8.F6 and Bruce4); for *in vitro* iPS cells, *n* = 5 clones; and for *in vivo* iPS cells, *n* = 5 clones. Statistical significance was determined using the Student’s *t*-test (unpaired, two-tailed): *P* < 0.05, **P** < 0.01. The lower line of asterisks refers to the comparison with *in vitro* iPS cells, and the upper line of asterisks to the comparison with ES cells.

**b**, Example of a chimaeric blastocyst derived from a Katushka morula injected with GFP-labelled *in vivo* iPS cells. Two different confocal planes are shown containing GFP-labelled cells that have contributed to the trophectoderm and to the inner cell mass, as indicated.

**c**, Chimaerism of GFP-labelled *in vivo* iPS cells in the proper embryo and placenta (E14.5). A wild-type embryo at the same stage of development is shown as a control. Fluorescence pictures were taken with the same settings.
9: Expression levels of 2C marker genes.

Analysis of the expression of genes enriched in the 2C state: the retrotrasposable elements *MuERV-L*, *Zscan4*, and intracisternal A particles (IAP) showed no differences between *in vivo* iPS cells compared to ES cells and *in vitro* iPS cells. For ES cells, \( n = 3 \) (lines C57BL6.10, JM8.F6 and Bruce4); for *in vitro* iPS cells, \( n = 5 \) clones; and for *in vivo* iPS cells, \( n = 6 \) clones. Values correspond to the average and s.d. Statistical significance was determined using the Student's \( t \)-test (unpaired, two-tailed). None of the differences was statistically significant.
10: Immunohistochemical characterization of embryo-like structures.

Haematoxylin and eosin and immunostaining analysis of two examples of embryo-like structures generated upon in vivo iPS cells intraperitoneal injection. The following markers were used: SOX2 (ectoderm), T/BRACHYURY (mesoderm), GATA4 (endoderm), CDX2 (trophectoderm), AFP and CK8 (visceral endoderm of the yolk sac). All lateral panels are at the same magnification.