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## Video Article

# *In vivo* Reprogramming of Adult Somatic Cells to Pluripotency by Overexpression of Yamanaka Factors

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Keywords: Stem Cell Biology, Issue 82, Pluripotent Stem Cells, Induced Pluripotent Stem Cells (iPSCs), Transcription Factors, General, Gene Therapy, Gene Expression, iPS, OKSM, regenerative medicine

Date Published: 12/17/2013

Citation: Yilmazer, A., de Lázaro, I., Bussy, C., Kostarelos, K. *In vivo* Reprogramming of Adult Somatic Cells to Pluripotency by Overexpression of Yamanaka Factors. *J. Vis. Exp.* (82), e50837, doi:10.3791/50837 (2013).

## Abstract

Induced pluripotent stem (iPS) cells that result from the reprogramming of somatic cells to a pluripotent state by forced expression of defined factors are offering new opportunities for regenerative medicine. Such clinical applications of iPS cells have been limited so far, mainly due to the poor efficiency of the existing reprogramming methodologies and the risk of the generated iPS cells to form tumors upon implantation.

We hypothesized that the reprogramming of somatic cells towards pluripotency could be achieved *in vivo* by gene transfer of reprogramming factors. In order to efficiently reprogram cells *in vivo*, high levels of the Yamanaka (OKSM) transcription factors need to be expressed at the target tissue. This can be achieved by using different viral or nonviral gene vectors depending on the target tissue. In this particular study, hydrodynamic tail-vein (HTV) injection of plasmid DNA was used to deliver the OKSM factors to mouse hepatocytes. This provided proof-of-evidence of *in vivo* reprogramming of adult, somatic cells towards a pluripotent state with high efficiency and fast kinetics. Furthermore no tumor or teratoma formation was observed *in situ*.

It can be concluded that reprogramming somatic cells *in vivo* may offer a potential approach to induce enhanced pluripotency rapidly, efficiently, and safely compared to *in vitro* performed protocols and can be applied to different tissue types in the future.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/50837/>

## Introduction

Ethical concerns about the use of embryonic stem cells have been one of the limitations in the development of stem cells research. However, the discovery by Yamanaka and colleagues that the forced expression of four transcription factors (namely: Oct3/4, Klf4, Sox2, c-Myc (OKSM)) could lead to *in vitro* reprogramming of somatic cells into pluripotent cells, named induced pluripotent stem (iPS) cells, has opened new opportunities. Viral<sup>1-5</sup> and nonviral<sup>6-10</sup> gene transfer, protein cytoplasmic translocation<sup>11,12</sup>, and miRNA<sup>13-16</sup> transfection are among the various methods used today to generate iPS cells. Yet, such reprogramming methodologies suffer from various issues that are restricting their translation into the clinic, such as: a) severe limitations in efficiency of cell reprogramming; b) the predominant use of viral vectors; c) long and multi-step protocols of culturing conditions; and d) the risk of tumorigenicity by implantation of the *in vitro* generated iPS cells<sup>17-20</sup>.

Gene transfer of defined transcription factors<sup>3-5</sup> by retroviruses is the most commonly used method to reprogram somatic cells. However, it contains the risks from the possibility of insertional mutagenesis, stable transduction and long-term proto-oncogene expression<sup>21,22</sup>. Nonviral gene transfer vectors such as plasmid DNA<sup>6,7,23</sup> or RNA<sup>10</sup> delivery using liposomes or electroporation have also been explored. While safer compared to viruses, those vectors offer significantly limited transduction and reprogramming efficiency<sup>24,25</sup>.

One of the central dogmas of this emerging field is that *in vivo* implantation of iPS cells will lead to their uncontrolled differentiation and the formation of a tumor-like mass (teratoma), composed of various tissues from the three different germ layers. Therefore, the concept of reprogramming to pluripotency has been primarily focused on *in vitro* manipulations of primary extracted somatic cells (most commonly fibroblasts). However, as described above, this principle suffers from long and complex cell culture protocols, including multiple rounds of treatment (gene transfer, growth factors, antibiotics, antioxidants) that may themselves enhance the risks for teratoma formation or other forms of tumorigenesis upon implantation of the cells<sup>24-28</sup>.

We propose that reprogramming of adult somatic cells *in vivo* by the transient overexpression of the OKSM transcription factors does not lead to subsequent formation of teratomas<sup>29</sup>. The method to achieve that will depend on achieving high levels of transcription factor expression within the target tissue in the safest possible way. In this study, we selected an established virus-free gene transfer technology that has been shown to target efficiently liver in order to reprogram cells to pluripotency *in vivo*. This technology involves large-volume, rapid hydrodynamic tail vein

(HTV) injection of plasmid DNA<sup>30,31</sup>. High expression levels of transfected genes can be achieved in adult hepatocytes following this nonviral gene transfer method. Moreover, this technology minimizes the potential risks from use of viral vectors (e.g. permanent genomic insertion, insertional mutagenesis).

## Protocol

### 1. *In vivo* Reprogramming of Liver Tissue by HTV Administration of pDNA

1. Allow female Balb/C mice, 6 weeks old, to acclimatize to the animal facility for a week before any procedure is performed.
2. Prepare 0.9% saline in distilled water and filter through 0.22  $\mu$ m microfilter.
3. Prepare a solution with 75  $\mu$ g pCX-OKS-2A and 75  $\mu$ g of pCX-cMyc plasmid DNA in 1.5 ml 0.9% saline per mouse. **Note:** Total injection volume must be between 8-10% of the total body weight.

**Note:** prior to the injection, keep the mice in a heating chamber at 37 °C until the tail veins can be easily visualized and appear dilated.

4. Induce anesthesia with isoflurane before (in the heating chamber) and during the injection (with an anesthetic mask).
5. Administer the pDNA solution or saline control with a 27 G syringe by HTV injection. **Note:** for the transfection to be successful, the total volume should be injected in no more than 5-7 sec.
6. Monitor that the animals recover properly from the injection.

### 2. Liver perfusion and Isolation of Primary Hepatocytes for qRT-PCR Studies

1. At different time points including 2, 4, 8, 12, and 24 days after HTV injection, induce deep anesthesia with isoflurane.
2. Spray 70% ethanol on the animal's abdomen, make an incision through the skin on the ventral midline with surgical scissors and dissect away the abdominal muscle layer to expose the viscera.
3. Cut the diaphragm to expose the thoracic cavity with scissors and clamp the cava vein with locking forceps.
4. Displace the viscera to the right and the liver lobes so that both the inferior cava and the portal vein are easily accessible.
5. Cannulate the inferior cava vein with a 22 G catheter and cut the portal vein to avoid excess of pressure.
6. Start the perfusion with 10 ml of HBSS buffer (Ca<sup>2+</sup> and Mg<sup>2+</sup> free, with bicarbonate), prewarmed at 37 °C. Perfuse the buffer slowly with a 10 ml syringe, until the liver whitens (3 min).
7. Continue the perfusion with Liver Digest Medium, prewarmed at 37 °C, at a flow rate of 0.6 ml/min (for 15 min) until the liver becomes swollen and loose. **Note:** Excessive digestion and perfusion can damage hepatocytes or reprogrammed cells.
8. Continue the experiment at 4 °C.
9. Remove the liver by forceps after cutting the falciform and coronary ligaments and wash it with Hepatocyte Wash Medium, while passing it through a 100  $\mu$ m cell strainer to obtain a cell suspension.
10. Collect the cell suspension in 50 ml tubes and adjust the volume to 15 ml with Hepatocyte Wash Medium.
11. Centrifuge the cell suspension at 50 g for 4 min. Keep the temperature at 4 °C. Discard the supernatant (which contains Kupffer and epithelial cells) and resuspend the cell pellet (containing the hepatocytes) in 10 ml of Hepatocyte Wash Medium. **Note:** The cell pellet should be resuspended very carefully, hepatocytes are very fragile cells.
12. Repeat the centrifugation procedure for a total of three times and resuspend the final pellet in 10 ml of Hepatocyte Wash Medium.
13. Estimate the cell number using an optical microscope and a hemocytometer.
14. Prepare aliquots of 2 x 10<sup>6</sup> cells, centrifuge at 300 x g to recover all the cells and discard the supernatant.
15. At this point, the cell pellet can be frozen and kept at -20 °C until further processing or used straight away for the RNA extraction.

### 3. Gene Expression Study of Isolated Hepatocytes for Upregulation of Pluripotent Genes

1. Extract RNA from 2 x 10<sup>6</sup> cells with a spin column kit following the instructions of the supplier.
2. Use 1  $\mu$ g of the extracted RNA to perform two-step qRT-PCR following the instructions of the supplier.
3. Use the hepatocytes of the saline-injected animals as a control to correct the relative gene expression of reprogramming, pluripotency, and hepatocyte markers.

### 4. Flow Cytometry Analysis of Enhanced Pluripotency at the Protein Level

1. On days 1 and 4 after injection, perform liver perfusion and isolate primary hepatocytes as indicated above.
2. Adjust cell density to 1 x 10<sup>7</sup> cells/ml and prepare 100  $\mu$ l aliquots.
3. Fix the cells with BD Cytofix fixation buffer and permeabilize with 1x BD Perm/Wash buffer.
4. Incubate the cells with either anti-mouse OCT4-PerCP-Cy5.5 or anti-mouse Nanog-PE for 30 min.
5. Include negative and isotype controls.
6. Analyze the stained cells by CyAn ADP High Performance Research Flow Cytometer.

### 5. Immunostaining of Liver Sections for Markers of Pluripotency

1. Four days after injection, perfuse the liver as indicated above but only with HBSS buffer in order to remove the blood from the liver tissue.
2. Cut a piece of liver tissue and immerse it in 2-methylbutane (precooled in liquid nitrogen) in order to continue with cryosectioning.
3. Frozen tissues may be kept at -80 °C or directly cut in 14  $\mu$ m thick sections using a cryostat.
4. Air-dry the tissue sections for 1 hr at room temperature and post-fix with methanol, precooled at -20 °C for 10 min. Air-dry for 15 min and then wash the sections twice with PBS (5 min each).

5. Incubate the sections in blocking buffer (5% goat serum-0.1% Triton in PBS pH 7.3) at room temperature for 1 hr, followed by two washing steps with washing buffer (1 % BSA-0.1% Triton in PBS, pH 7.3).
6. Incubate the tissue sections overnight at 4 °C with anti-OCT4, anti-SOX2, and anti-Nanog primary antibodies.
7. The next day, wash the sections 3x (5 min each) with washing buffer and incubate them for 1.5 hr at room temperature with the secondary antibody.
8. Wash liver sections with PBS (3x, 5 min each) and mount them in DAPI and antifade containing medium.
9. Visualize the slides under an epifluorescence microscope.

## 6. Alkaline Phosphatase (ALP) Staining of Liver Tissue Sections

1. Obtain liver tissue sections as described before.
2. Warm the tissue sections at room temperature for 15 min before performing the staining.
3. Fix with 4% paraformaldehyde for 2 min. **Note:** excessive fixation time can decrease ALP activity.
4. Air-dry the sections for 30 min and then incubate with BCIP/NBT liquid substrate at 37 °C for 30 min.
5. Wash the slides with distilled water and mount with aqueous mounting media.
6. Capture random images by light microscopy.

## 7. Quantification of Albumin and Liver Enzyme Levels in Serum to Assess Liver Toxicity

1. On days 4, 8, 12, and 120 after transfection, collect 300 µl of blood directly from the heart ventricle.
2. Keep the blood on ice while the different samples are collected to avoid clotting.
3. Once all samples are ready, incubate for 30 min at 37 °C until blood clots.
4. Centrifuge the samples at 2,000 x g for 5 min.
5. Carefully pipette the serum (in the supernatant) to a new Eppendorf tube and freeze at -80 °C until processing of the samples.
6. Analyze serum levels for albumin, ALT, AST, ALP, and GLDH.

## 8. Hematoxylin and Eosin (H&E) and Periodic Acid-Schiff (PAS) Staining of Liver Sections to Assess Liver Toxicity

1. At different time points, including 2, 4, 8, 12, 24, 50, and 120 days after HTV injection; perform liver perfusion with HBSS buffer only as previously described.
2. Cut a piece of liver tissue and fix in 4% paraformaldehyde.
3. Embed liver tissue in paraffin blocks, section and stain with either H&E or PAS staining.
4. Capture random images by light microscopy.

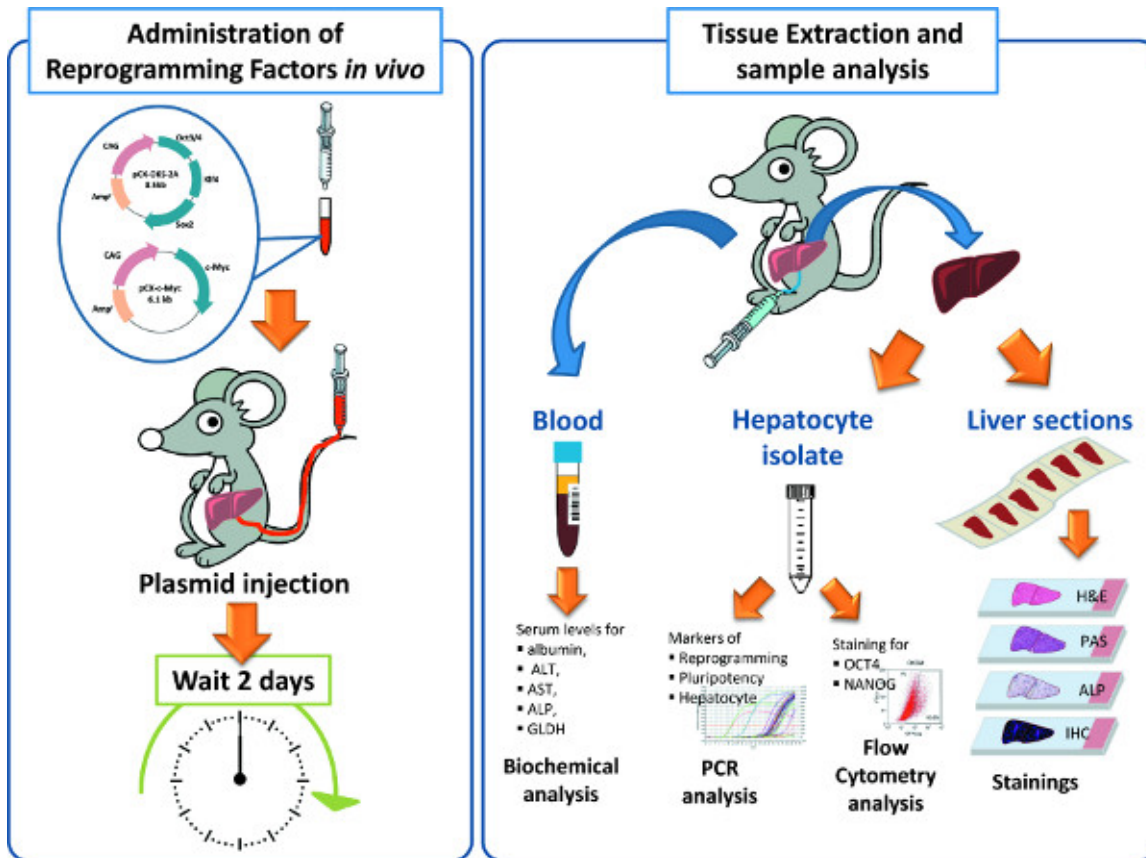
## Representative Results

**Figure 1** shows the overview of the procedure that involves the gene transfer of OKSM encoding plasmids to mice liver and the different techniques performed to observe *in vivo* cell reprogramming. Following HTV injection of the plasmids, a significant increase in gene expression of the transfected reprogramming factors (Oct3/4, Sox2, Klf4, and cMyc) at the mRNA level was observed on day 2 after injection. The expression of these factors decreases over time after injection, as shown in **Figure 2a**. Regarding the expression of endogenous pluripotency markers (Nanog, Ecat1 and Rex1), their levels were significantly upregulated compared to those in hepatocytes from saline-injected animals on day 2 and 4 after injection, and were back to baseline levels from day 8 onward (**Figure 2b**). At the same time dedifferentiation of the hepatocyte population was confirmed by the downregulation of hepatocyte-specific genes (Alb, Aat, and Trf) that was statistically significant on day 4 and reached baseline levels from day 8 onward (**Figure 2d**).

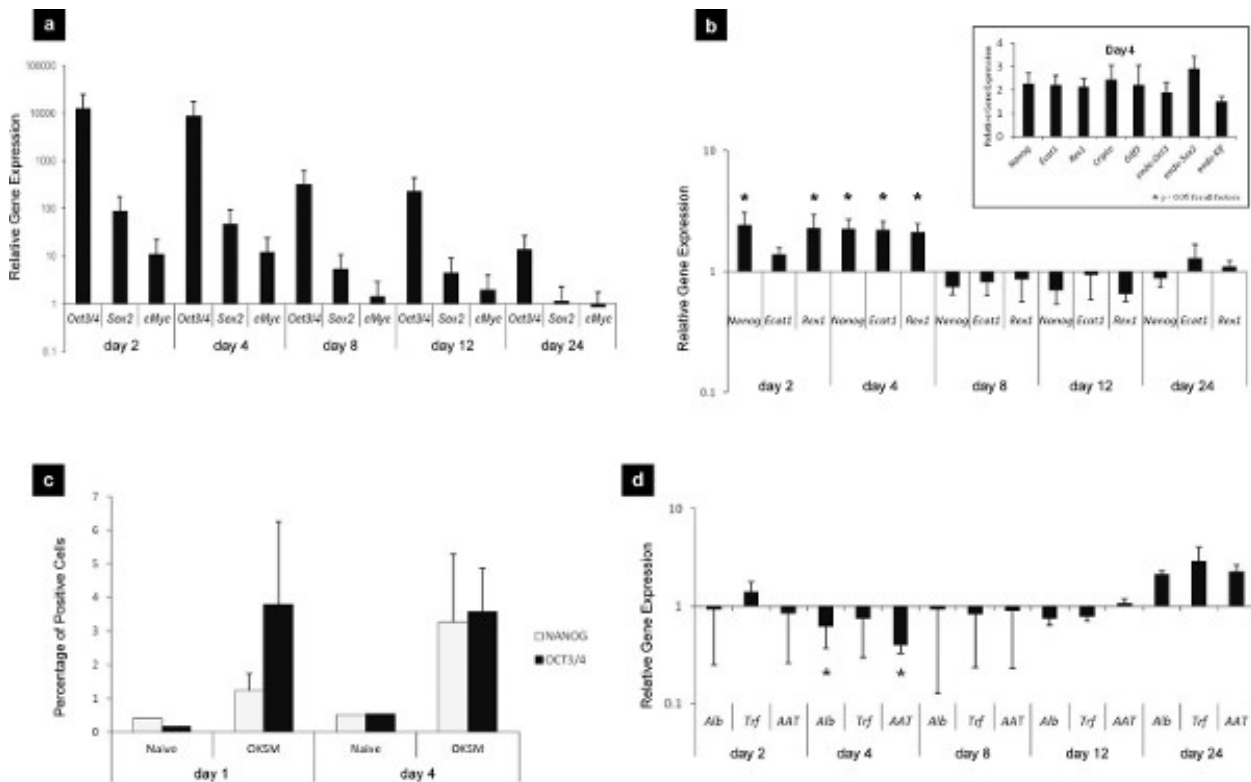
The expression of Oct3/4 and Nanog at the protein level was investigated by flow cytometry. As shown in **Figure 2c**, only Oct3/4 is expressed on day 1 after HTV injection, while for the expression of the endogenous pluripotency marker Nanog it was necessary to wait until day 4.

The occurrence of *in vivo* cell reprogramming was further confirmed by immunohistochemical analysis of liver tissues with anti-OCT4, anti-SOX2 and anti-Nanog antibodies and by specific staining for ALP activity. Positive cells for all markers and enzymatic activity are reproducibly found in the liver tissues from OKSM-injected animals but not in the saline-injected controls (**Figure 3**).

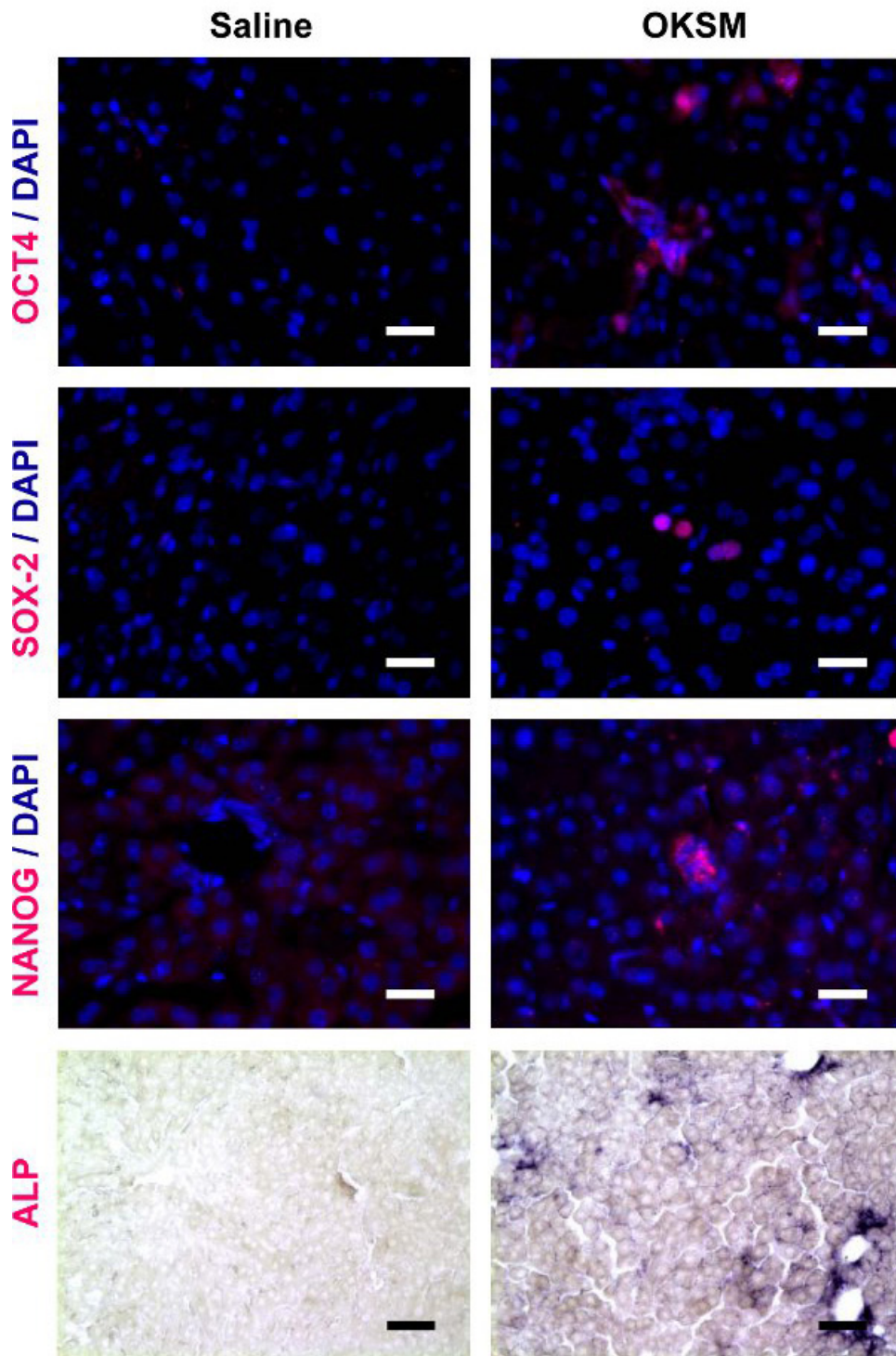
The possible toxicity side-effects and teratoma formation from *in vivo* cell reprogramming by HTV injection of pDNA were investigated by quantification of liver enzyme levels in serum, as well as H&E and PAS staining of liver tissue sections over a period of 120 days. Transient and not severe signs of tissue damage were observed until day 2, but not longer. No formation of teratomas or any sign of dysplasia or morphological alterations were observed for the period of study (**Figure 4**). There were no hepatic structural or functional abnormalities throughout the course of the study for any of the animals, as confirmed by albumin and liver enzyme levels and glycogen staining of tissue sections (**Figures 4b and c**).



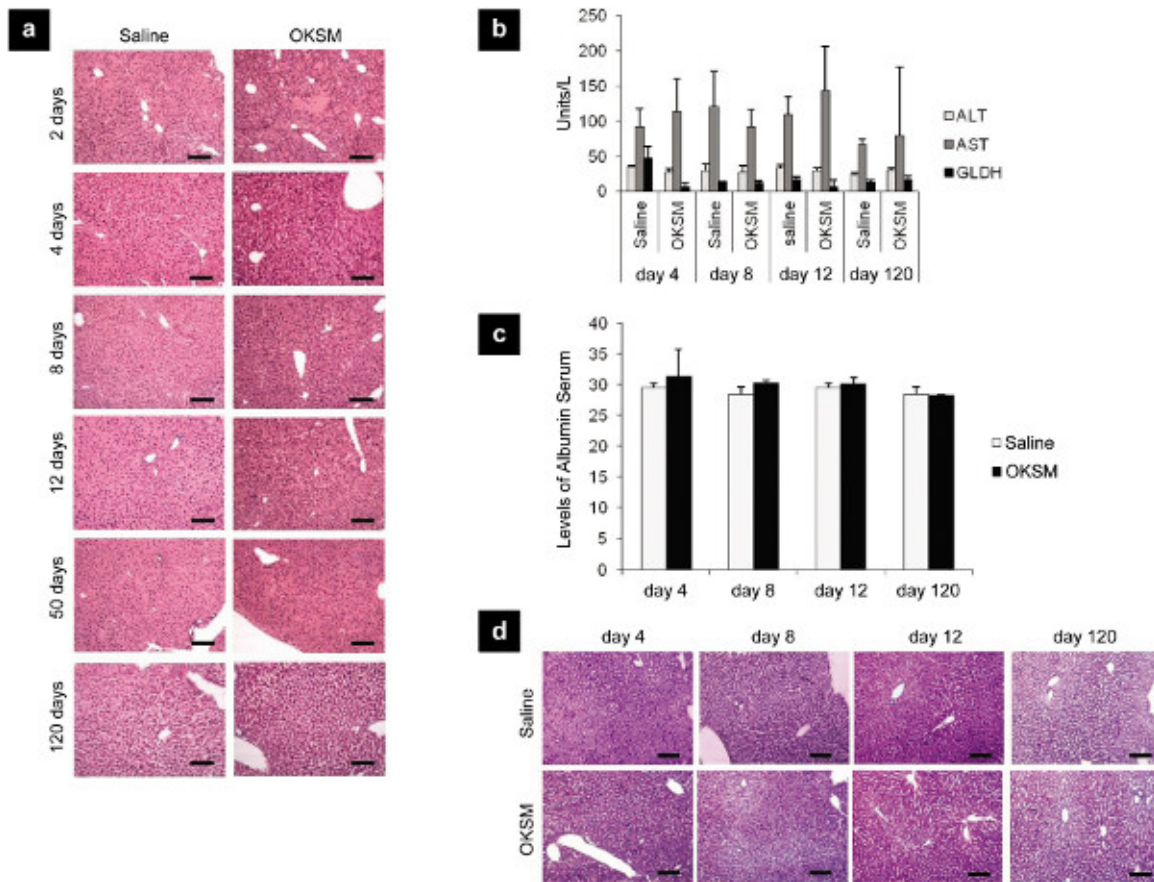
**Figure 1. The schematic overview of *in vivo* reprogramming procedure and its analysis.** The protocol involves two main stages: (i) administration of reprogramming factors *in vivo* and (ii) tissue extraction and sample analysis. [Click here to view larger figure.](#)



**Figure 2. In vivo overexpression of Yamanaka transcription factors in adult mouse liver.** Balb/C mice were HTV injected with 0.9% saline alone, 75  $\mu$ g of pCX-OKS-2A and 75  $\mu$ g pCX-cMyc in 0.9% saline and at days 2, 4, 8, 12, 24, RT-qPCR analysis of hepatocytes was performed to determine the relative gene expression of: (a) transfected transcription factors (OKSM) and (b) endogenous pluripotency markers; (c) flow cytometry analysis of OCT3/4 positive and Nanog positive cells; (d) relative gene expression of hepatocyte markers as determined by RT-qPCR. All gene expression levels were normalized to saline HTV-injected group. (\*  $p < 0.05$  indicates statistically significant difference compared to saline HTV-injected groups, obtained by the analysis of variance and Tukey's pairwise comparison). Figure adapted from Yilmazer *et al.*<sup>29</sup> [Click here to view larger figure.](#)



**Figure 3. *In vivo* cell reprogramming on adult mouse liver tissue by immunohistochemistry.** Balb/C mice HTV injected with 0.9% saline alone, 75  $\mu$ g of pCX-OXS-2A and 75  $\mu$ g pCX-cMyc in 0.9% saline. At day 4, livers were collected and frozen tissue sections were stained with anti-OCT4, anti-SOX2 or anti-Nanog antibodies to assess immunoreactivity, or BCIP/NBT to determine ALP activity in the tissue (40X). Scale bars represent 100  $\mu$ m. Figure adapted from Yilmazer *et al.*<sup>29</sup> [Click here to view larger figure.](#)



**Figure 4. The effect of *in vivo* cell reprogramming on liver functionality and histopathology.** Balb/C mice HTV injected with either 75  $\mu$ g of pCX-OKS-2A and 75  $\mu$ g pCX-cMyc in 0.9% saline or 0.9% saline only. On days 2, 4, 8, 12, 50, and 120 liver tissues and sera were isolated and processed for: (a) H&E staining; (b) levels of liver enzymes; (c) levels of albumin; (d) PAS staining. Representative images were captured with light microscopy (10X). Scale bars represent 100  $\mu$ m. Figure adapted from Yilmazer *et al.*<sup>29</sup> [Click here to view larger figure.](#)

## Discussion

This study provides proof-of-principle evidence of *in vivo* cell reprogramming towards pluripotency following the efficient transfer and overexpression of the OKSM factors to the adult mouse liver. With the help of different techniques such as qRT-PCR, flow cytometry, IHC or serum analysis, the target tissue was investigated to observe *in vivo* cell reprogramming. Very rapidly from the induction of OKSM factor overexpression in the tissue, pluripotency markers were upregulated at the mRNA and protein levels (within 48 hr). Throughout these experiments, between 5-15% of cells extracted from the tissue were shown to be Nanog and Oct3/4 positive. Furthermore, no structural or functional abnormalities were observed in the liver throughout 120 days. Expression of transgenes by HTV injection of plasmid DNA in tissues other than liver (lung, spleen, kidney and heart) has been previously reported, however the levels of gene expression were at least 3 orders of magnitude lower than those in hepatocytes<sup>30</sup>. The data in this study indicates that reprogramming of somatic cells towards a pluripotent state *in vivo* is safe, fast and efficient. In view of the majority of existing *in vitro* cell reprogramming methodologies that generally achieve pluripotent (iPS) cell generation within 3 weeks with less than 1% of efficiency<sup>32</sup>, *in vivo* reprogramming to pluripotency may offer very interesting alternatives. We hypothesize that the concept of *in vivo* reprogramming to pluripotency could be applied to various types of somatic cells with high efficiency (dependent on the vector used), however the level of functional pluripotency achieved in the *in vivo* reprogrammed cells will need to be determined. Moreover, with the development of improved protocols and technologies, *in vivo* reprogrammed cells could potentially be extracted and utilized for regenerative medicine purposes.

There are some critical steps to follow in order to reprogram adult, somatic cells *in vivo* with high efficiency. Achieving high levels of gene expression in the target tissue is a prerequisite. In this study this was illustrated by utilizing the efficient transfer and expression of plasmid DNA to hepatocytes using HTV injection. The most important factors for HTV injection are the volume, which should be between 8-10% of the total body weight of the animal, and the speed of injection, which must be performed within 5-7 sec. Mice younger than 6 weeks old should not be used, as HTV injection can cause more tissue damage in the liver, which can affect the efficiency of cellular reprogramming. The genetic background of the animals had no impact in determining the efficiency of *in vivo* reprogramming, as we have previously reported similar levels of reprogramming in both Balb/C and TNG-A mice, the latter being a transgenic strain that carries the eGFP reporter inserted in the *Nanog* locus and is bred on Sv129 background<sup>29</sup>.

We speculate that *in vivo* cell reprogramming to a pluripotent state (that will need to be determined) can be applied to other tissue types provided that the OKSM factors are efficiently delivered to target cells, allowing high levels of gene expression. A variety of nonviral and viral gene delivery



vectors have been studied in preclinical and clinical trials in order to target different cell and tissue types<sup>33</sup>. For example, for neural or ocular tissues adeno-associated viruses have been the preferred vector choice for efficient *in vivo* gene transfer<sup>34-37</sup>, whereas in muscle tissue naked plasmid DNA injections have been shown to result in relatively high levels of gene expression<sup>38,39</sup>. Therefore, the method of gene transfer vector should be carefully selected depending on the tissue type in order to achieve efficient and rapid *in vivo* cell reprogramming toward a pluripotent state.

## Disclosures

The authors declare that they have no competing financial interests.

## Acknowledgements

This work was partially supported by the Engineering and Physical Sciences Research Council (EPSRC, Swindon, United Kingdom) and the European Commission FP-7 Programs NANONEUROHOP (PIEF-GA-2010-276051) and ANTICARB (HEALTH-2008-20157). AY was supported by a full-time PhD scholarship from the Ministry of Education (Turkey) and IL acknowledges partial support from the British Council and the CAIXA Foundation (Spain). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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