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Supplemental Information

**Non-viral, Tumor-free Induction of Transient
Cell Reprogramming in Mouse Skeletal Muscle
to Enhance Tissue Regeneration**

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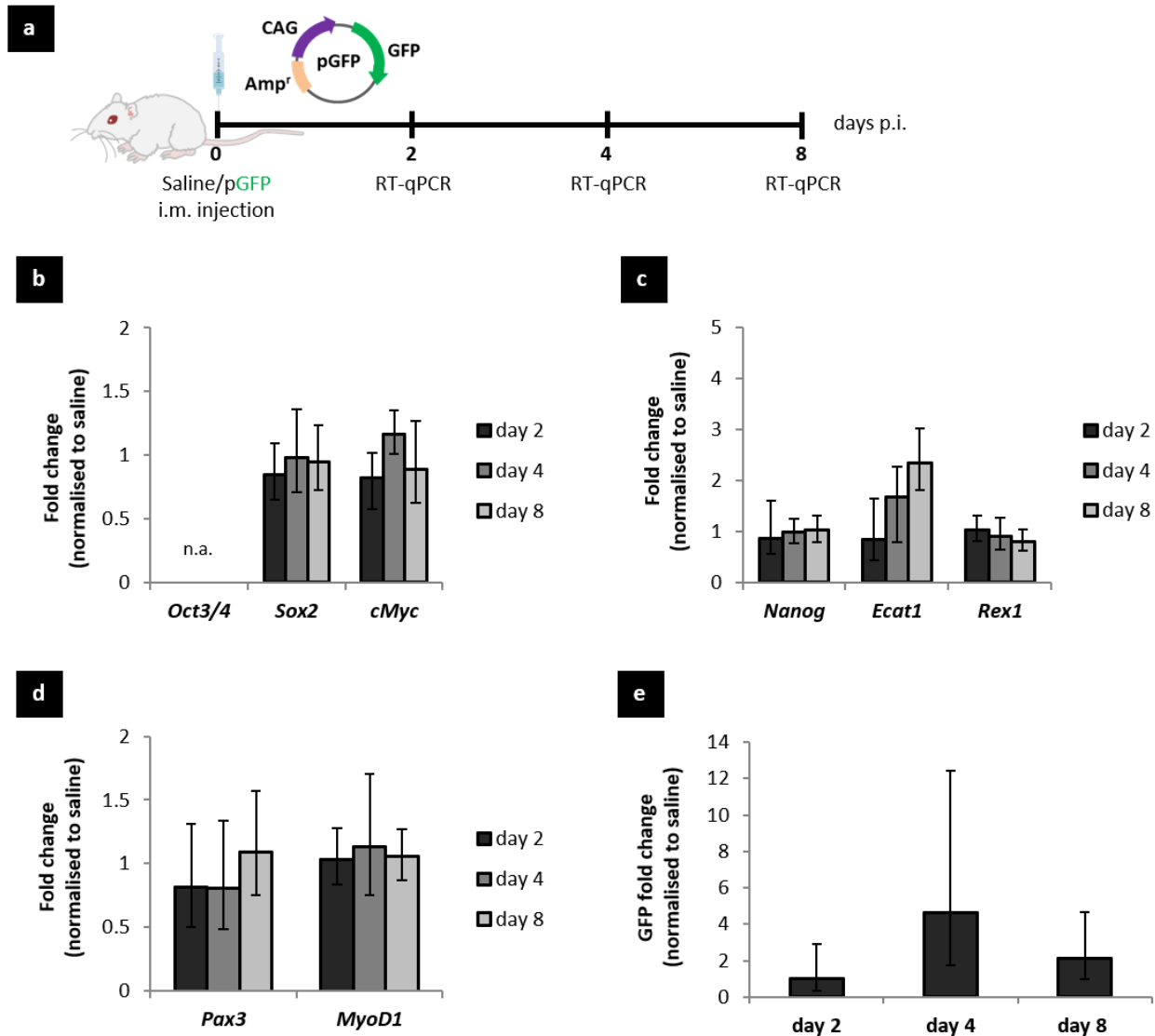


Figure S1. Gene expression after i.m. administration of pGFP in mouse GA. (a) BALB/c mice were administered 50 μ g pGFP in 50 μ l 0.9% saline or 50 μ l saline alone in the GA. 2, 4 and 8 days after injection GA were dissected and real-time RT-qPCR was performed to determine changes in gene expression of (b) OKSM reprogramming factors, (c) endogenous pluripotency markers, (d) myogenesis-related genes and (e) GFP transgene. GFP mRNA was normalised to the values from day 2. Expression levels of other mRNAs were normalised to saline-injected controls. No statistically significant differences in gene expression were detected between groups, assessed by one-way ANOVA, n=3, n.a. indicates no amplification of the target.

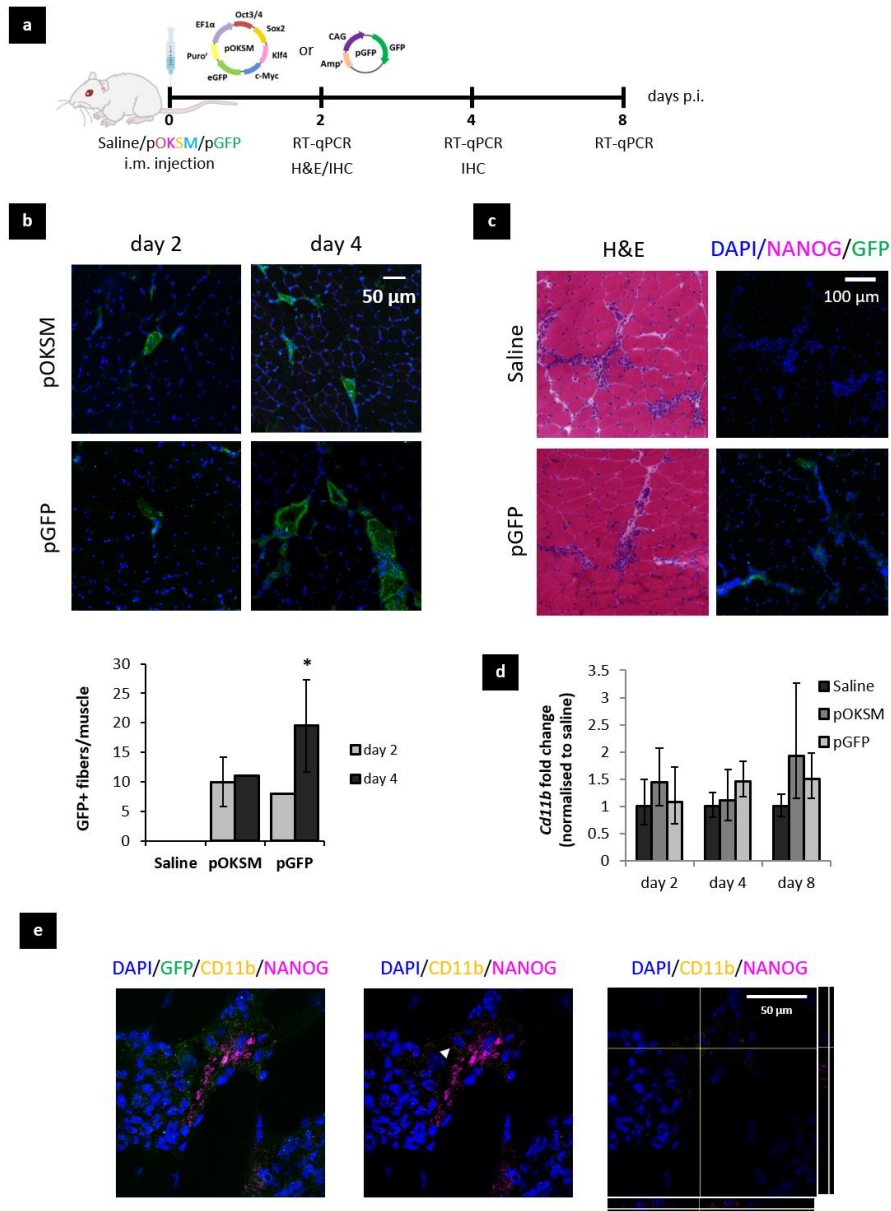


Figure S2. Characterisation of pOKSM and pGFP injected GA tissues. (a) BALB/c mice were administered 50 μ g pOKSM or pGFP in 50 μ l 0.9% saline, or 50 μ l saline alone, in the GA. 2, 4 and 8 days p.i., muscles were dissected for gene expression and histological analysis. (b) Representative images of GFP+ muscle fibers located in pOKSM and pGFP injected muscles, scale bar represents 50 μ m. * $p < 0.05$ indicates statistically significant differences in the number of GFP+ fibers compared to saline-injected controls, assessed by one-way ANOVA and Tukey test, ($n = 2$ GA, 3 (whole) sections/muscle). (c) Representative images of the needle tract left by i.m. injection in pGFP and saline-injected controls. Left panel shows H/E staining and right images show green fluorescence, scale bar represents 100 μ m. (d) Cd11b expression was assessed by real-time RT-qPCR in pOKSM, pGFP and saline-injected muscles. No statistically significant differences were found among groups, assessed by one-way ANOVA, $n = 4$. (e) IHC of a cluster containing reprogrammed cells, 2 days after pOKSM administration. Left and middle images show maximum intensity projections of Z stacks (DAPI/GFP/Cd11b/NANOG and DAPI/Cd11b/NANOG staining, respectively). White arrowhead points to a Cd11b+ NANOG- cell. The right panel offers an orthogonal view of a specific section, DAPI/Cd11b/NANOG IHC. Images were taken with a confocal microscopy at 100X magnification, scale bar represents 50 μ m.

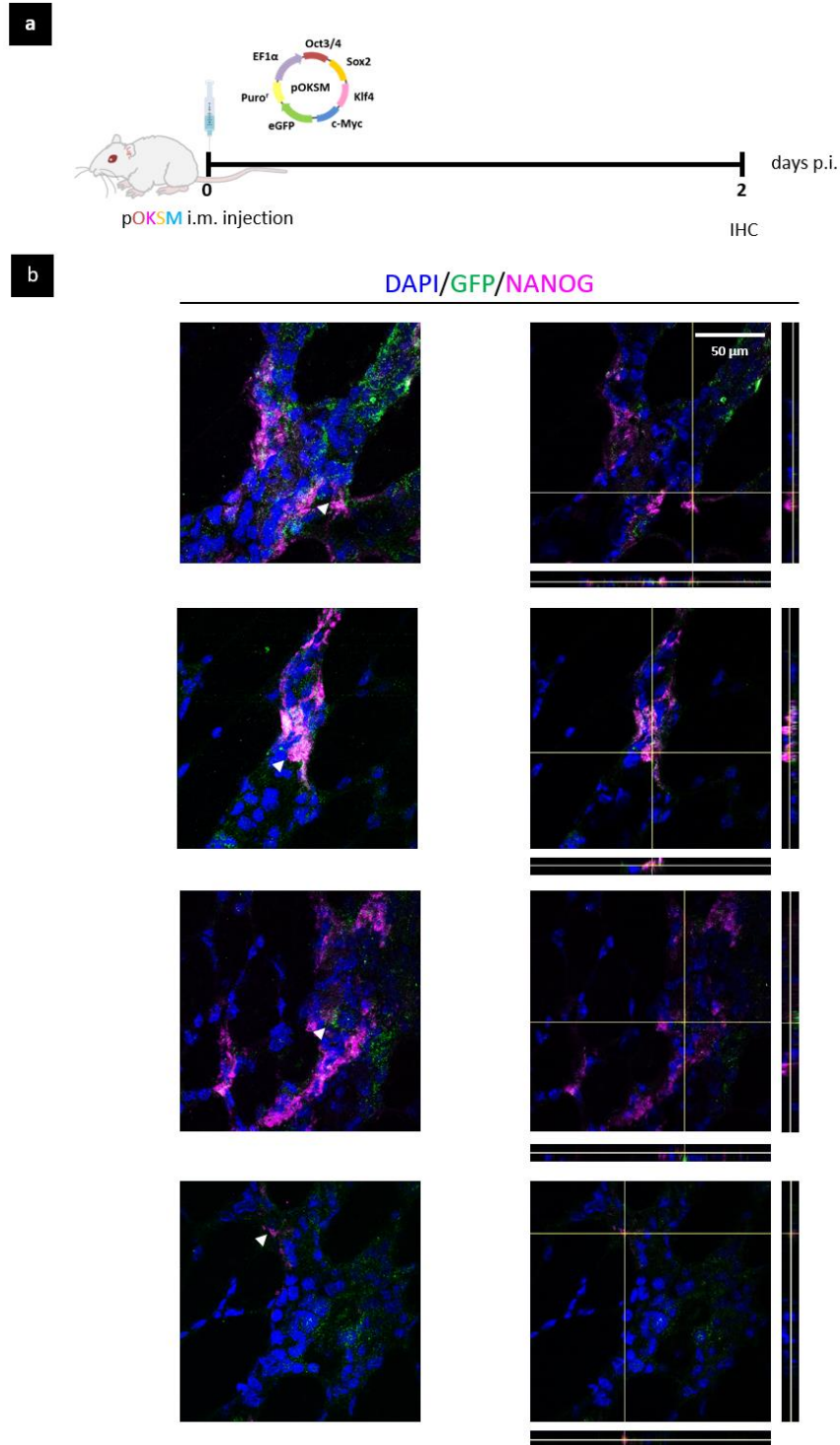


Figure S3. Characterisation of NANOG⁺/GFP⁺ cells clusters in pOKSM injected muscles. (a) BALB/c mice were administered 50 μg pOKSM in 50 μl 0.9% saline in the GA, dissected 2 days p.i. for histological analysis. **(b)** Representative images of NANOG⁺/GFP⁺ cell clusters obtained by confocal microscopy. Left panels show maximum projection intensity of the Z-stack while right panels offer the orthogonal view of a specific section. Axes in right panels cross at NANOG⁺GFP⁺ double positive cells, which are tagged by white arrowheads in the left images. Scale bar represents 50 μm.

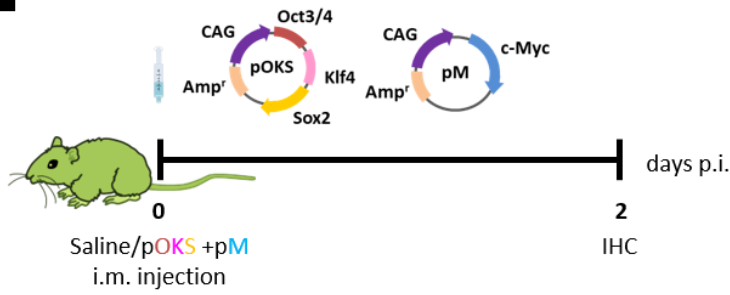
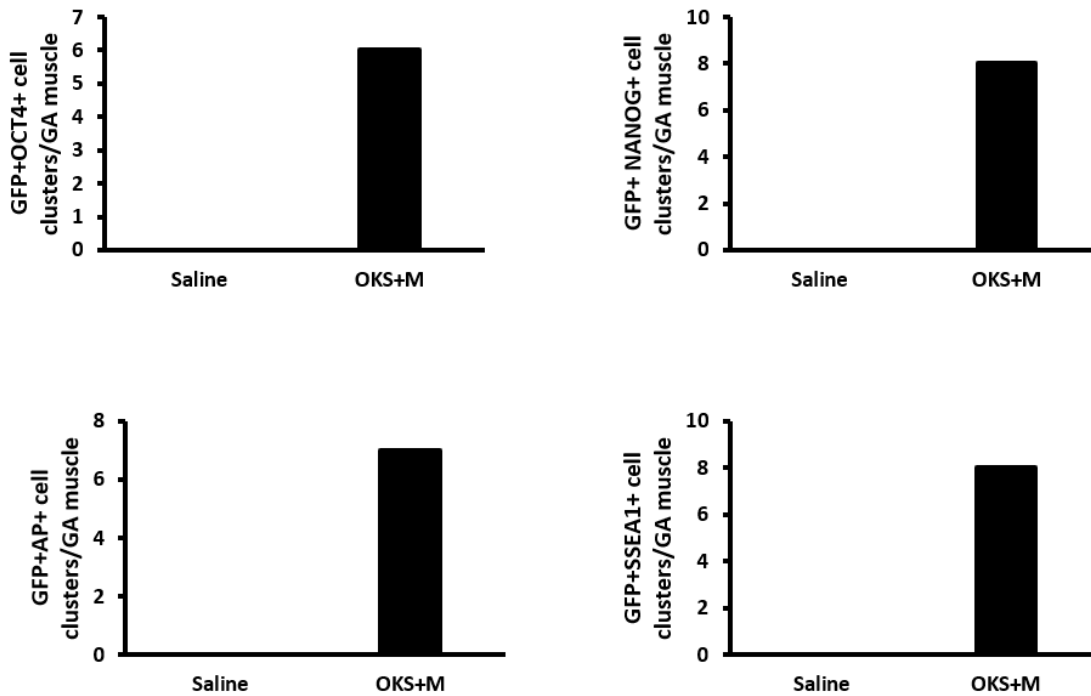
a**b**

Figure S4. Characterisation of *in vivo* reprogrammed cell clusters in Nanog-GFP mice GA. (a) Nanog-GFP mice were administered 50 µg pOKS and 50 µg pM in 50 µl 0.9% saline, or 50 µl saline alone, in the GA. Muscles were dissected 2 days p.i. for histological analysis. (b) Counting of GFP+/NANOG+, GFP+/OCT4+, GFP+/AP+ and GFP+/SSEA1+ cell clusters in reprogrammed and saline-injected tissues, n=1.

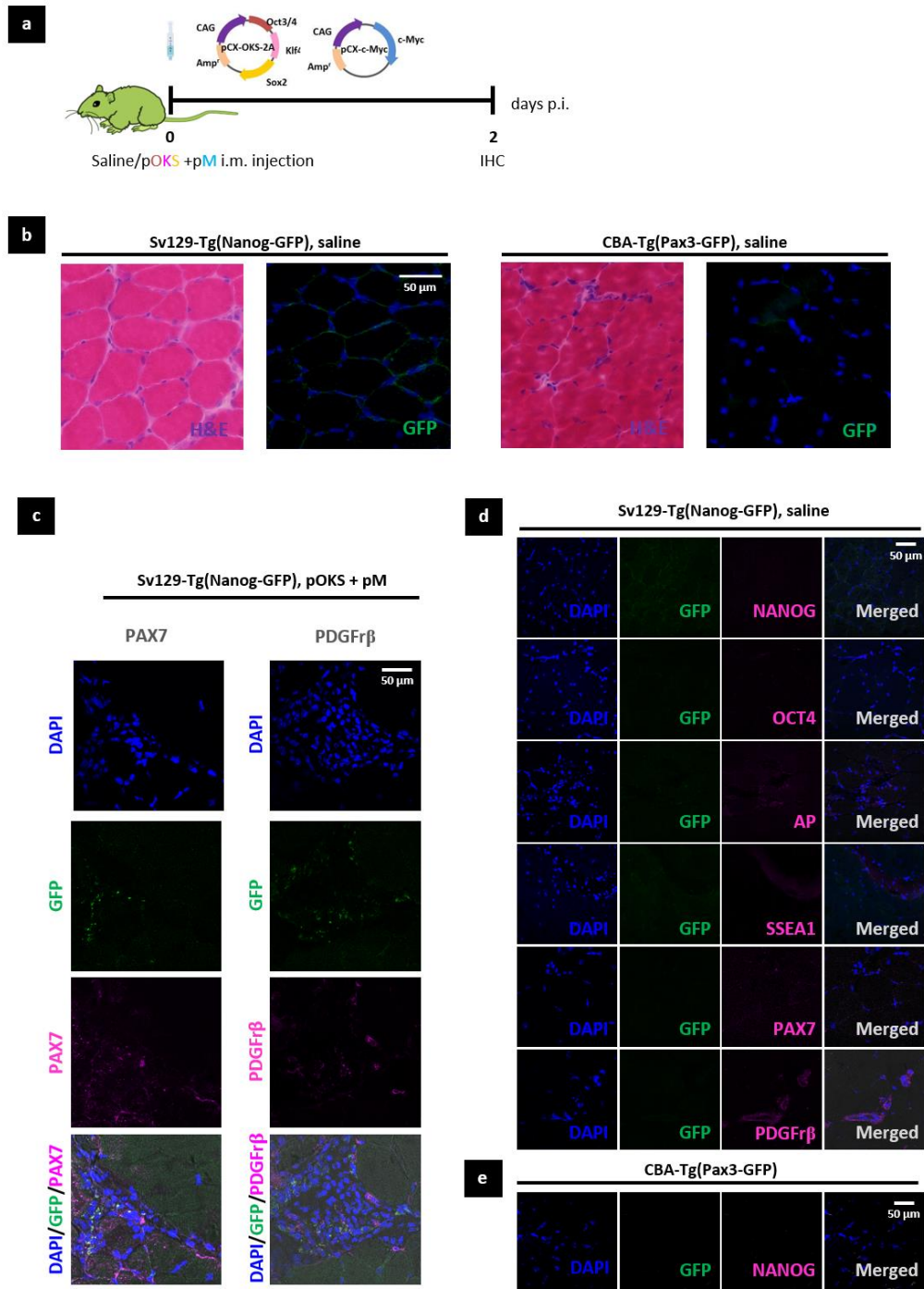


Figure S5. IHC in Nanog-GFP and Pax3-GFP mice GA tissue sections. (a) Nanog-GFP and Pax3-GFP mice were i.m. administered 50 μ g pOKS and 50 μ g pM in 50 μ l 0.9% saline, or 50 μ l saline alone, in the GA. Muscles were dissected 2 days p.i. for histological analysis. (b) Absence of GFP⁺ cell clusters in tissue sections from saline-injected Nanog-GFP and Pax3-GFP mice (100X, scale bars represent 50 μ m). Brightfield and fluorescence images show the same region within the tissue. (c) IHC for the expression of PAX7, a marker of satellite cells, and PDGFr β , a marker of pericytes, in Nanog-GFP mice administered with reprogramming pDNA (100X, scale bar represents 50 μ m). (d) IHC for the expression of pluripotency, satellite cell and pericyte markers in Nanog-GFP mice administered with saline solution (100X, scale bar represents 50 μ m). (e) IHC for the expression of the pluripotency marker NANOG in saline-injected Pax3-GFP mice (100X, scale bar represents 50 μ m).

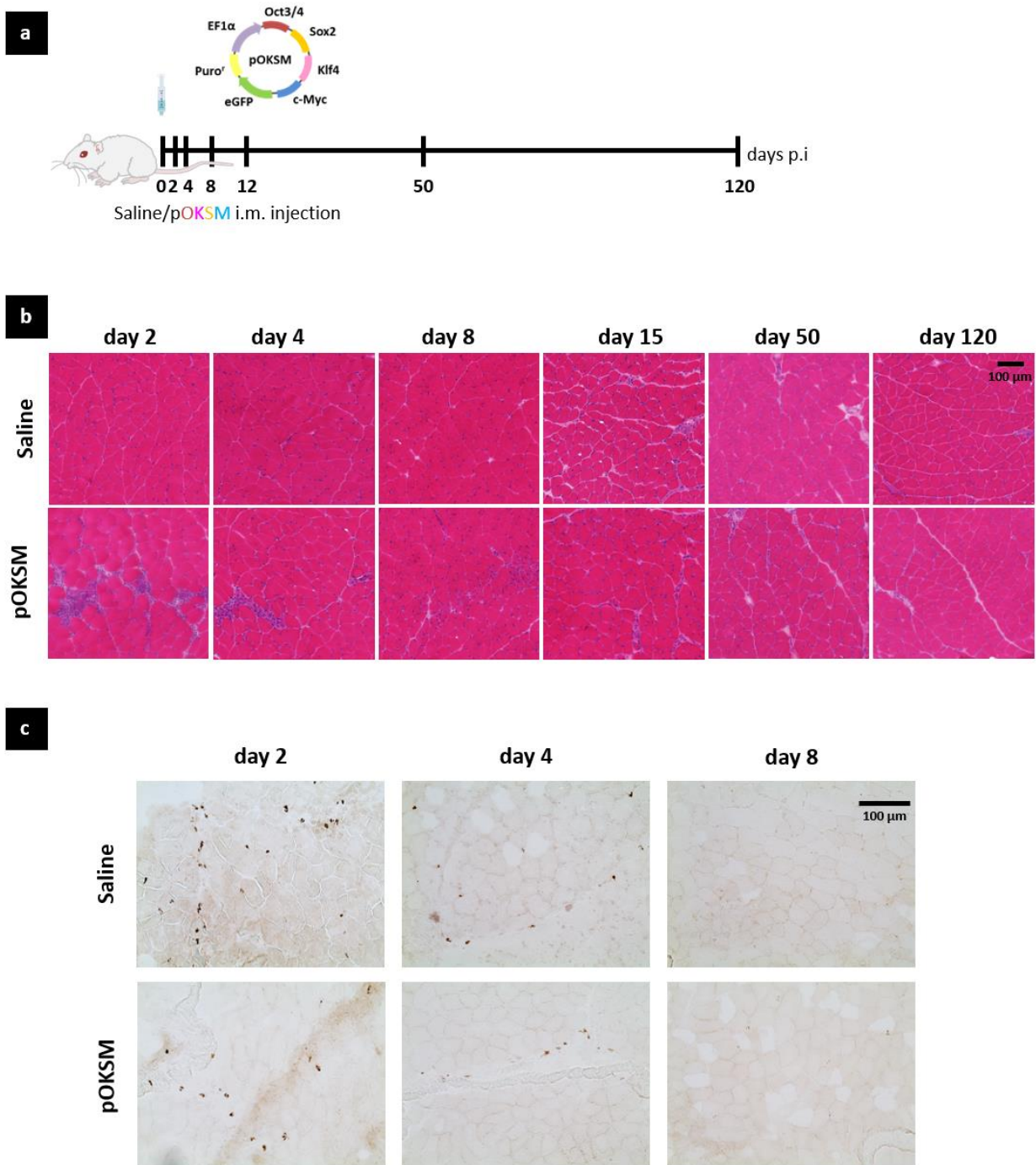


Figure S6. Effects of *in vivo* reprogramming towards pluripotency in healthy skeletal muscle. (a) BALB/c mice were administered 50 μ g pOKSM in 50 μ l 0.9% saline, or 50 μ l saline alone, in the GA. Muscles were dissected 2, 4, 8, 12, 50 and 120 days after injection for histological analysis. (b) H&E staining focusing on the evolution of clusters of mononucleated cells (40X magnification, scale bar represents 100 μ m). (c) TUNNEL staining to detect apoptotic nuclei, stained in brown (40X, scale bar represents 100 μ m).

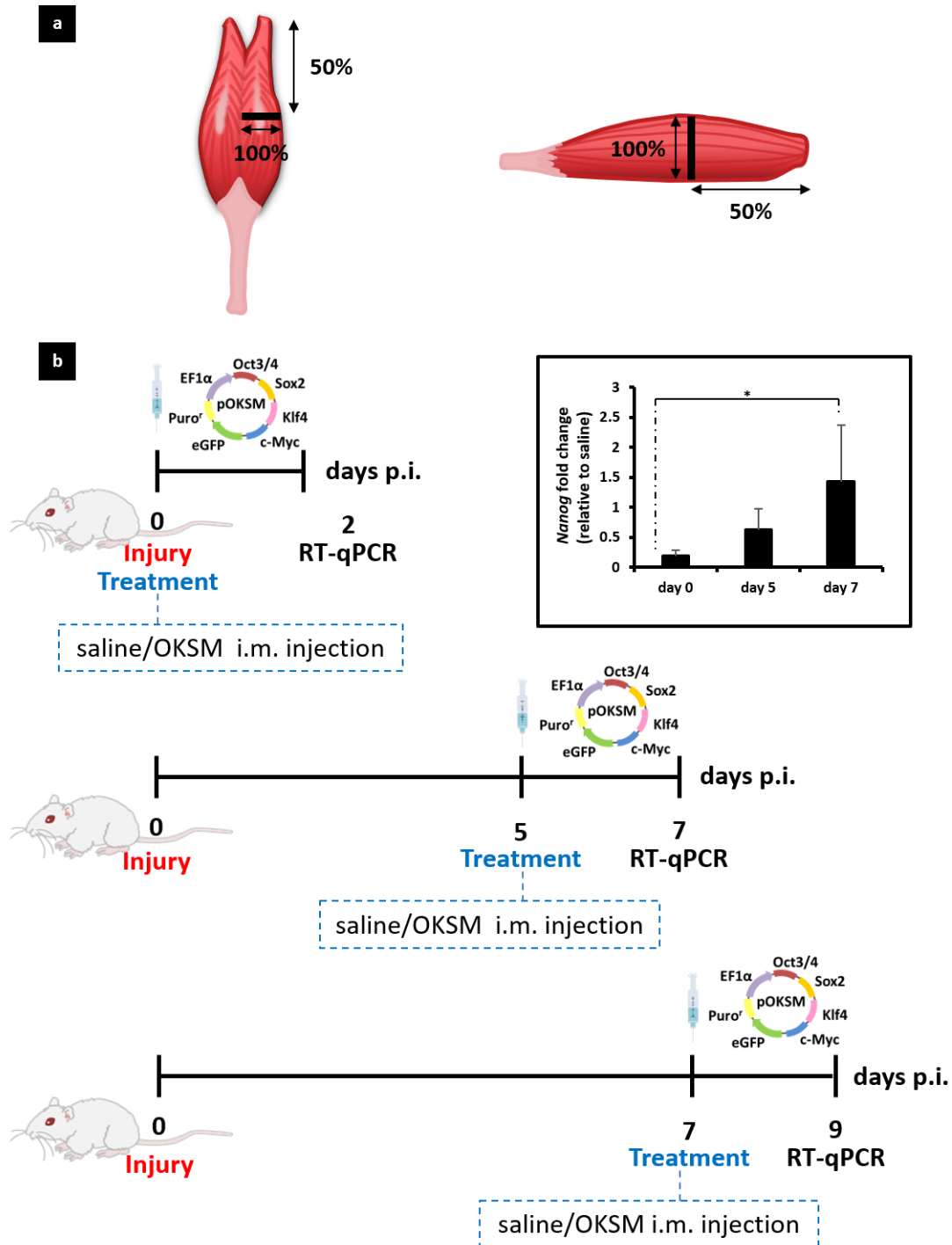


Figure S7. Effects of *in vivo* reprogramming towards pluripotency after transection of the medial head of the GA. (a) Diagram of the anatomical localisation where the injury was created in the medial head of the GA: coronal (left) and sagittal plane (right). The incision was performed mid-length in the medial head of the GA and covered the 100% of its width and depth. (b) *Nanog* gene expression upon i.m. administration of 100 μ g pOKSM at the time of injury or 5 or 7 days after surgery. Relative gene expression was normalised to saline-injected controls. * $p < 0.05$ indicates statistically significant differences in the expression of *Nanog* when the reprogramming pDNA was administered at the time of injury or one week later, assessed by one-way ANOVA and Tukey's test. Data are presented as $2^{-\Delta\Delta Ct} \pm$ propagation of error, $n=3$.

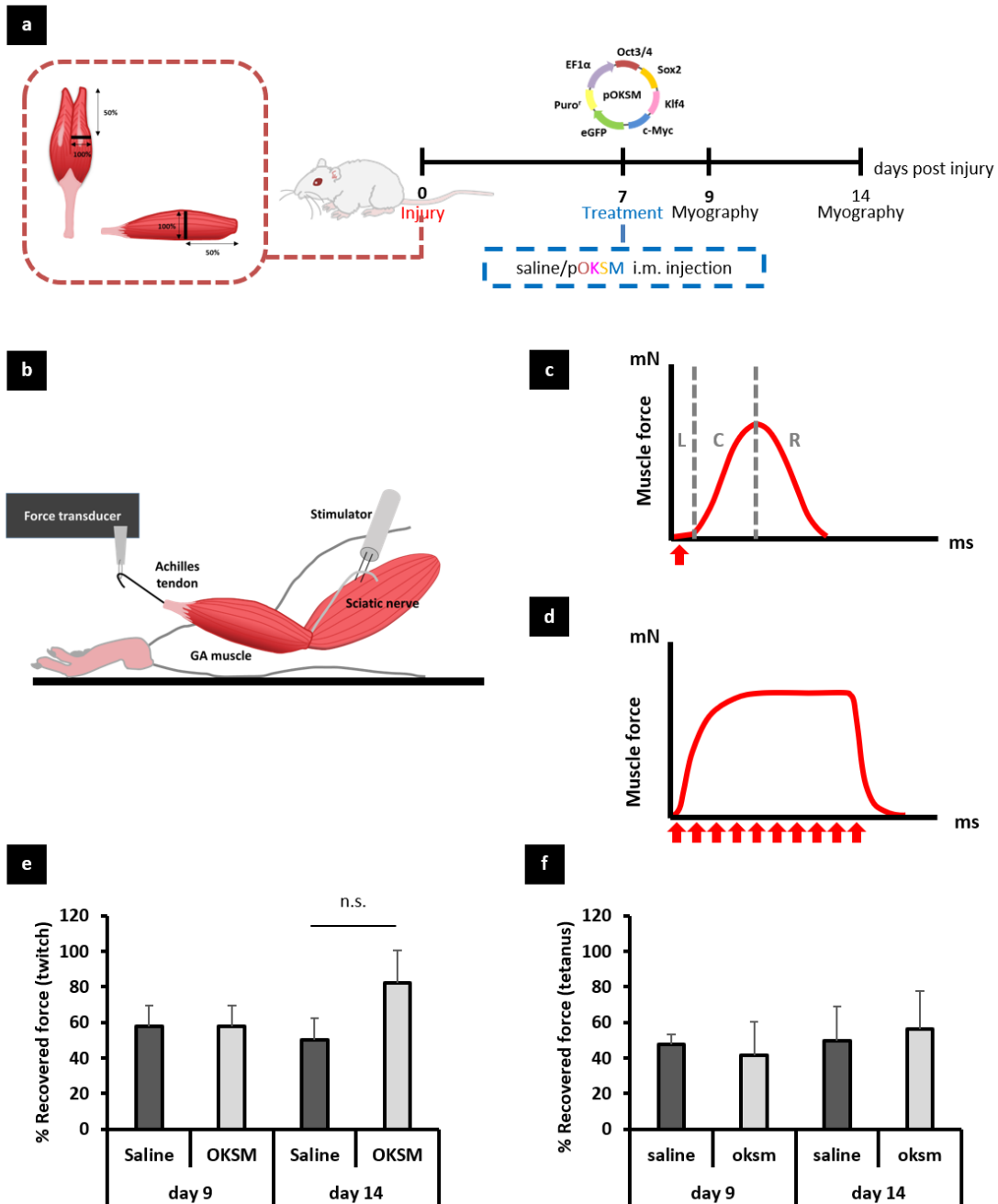


Figure S8. Functional assessment of muscle regeneration. (a) The medial head of the GA of BALB/c mice was surgically lacerated and 100 μ g pOKSM in 40 μ l 0.9% saline or 40 μ l saline alone were i.m. administered 7 days after injury. Functional recovery was assessed 9 and 14 days after surgery. (b) *In vivo* myography setup. The GA was exposed, an electrode was placed on the sciatic nerve for direct stimulation while the Achilles tendon was tied to a force transducer. (c) Twitch contraction was produced by a single stimulation (red arrow) at the optimal current (L= latent period, C= contraction, R=relaxation). (d) Tetanus contraction was produced by repeated stimulations (red arrows) with a 150 Hz frequency that did not allow the relaxation of the muscle between single contractions. Muscle forces under (e) twitch and (f) tetanus contractions were recorded on days 9 and 14 after injury and expressed as a % of the force produced by the contralateral (intact) hind limb. Data are presented as mean \pm SE, n=4. No statistically significant differences were found between groups.

Supplementary tables

Table S1. Primer sequences used in the study.

	Forward Primer	Reverse Primer
<i>β-Actin</i>	GACCTCTATGCCAACACAGT	AGTACTTGCGCTCAGGAGGA
<i>Oct3/4</i>	TGAGAACCTTCAGGAGATATGCAA	CTCAATGCTAGTTCGCTTTCTCTTC
<i>Sox2</i>	GGTTACCTCTTCTCCCACTCCAG	TCACATGTGCGACAGGGGCAG
<i>C-Myc</i>	CAGAGGAGGAACGAGCTGAAGCGC	TTATGCACCAGAGTTTGAAGCTGTTCG
<i>Nanog</i>	CAGAAAAACCAAGTGGTTGAAGACTAG	GCAATGGATGCTGGGATACTC
<i>Ecat1</i>	TGTGGGGCCCTGAAAGGCGAGCTGAGAT	ATGGGCCCCATACGACGACGCTCAACT
<i>Rex1</i>	ACGAGTGGCAGTTTCTTCTGGGA	TATGACTCACTCCAGGGGGCACT
<i>eGFP</i>	GACGGCGACGTAAACGGCCA	CAGCTTGCCGGTGGTGCAGA
<i>Pax3</i>	GGGAAGTGGAGGCATGTTTA	GTTTTCCGTCCAGCAATTA
<i>MyoD1</i>	AGCACTACAGTGGCGACTCA	GCTCCACTATGCTGGACAGG
<i>Pax7</i>	GACGACGAGGAAGGAGACAA	CGGGTTCTGATTCCACATCT
<i>Caveolin1</i>	AGCAAAAGTTGTAGCGCCAG	TGGGCTTGTAGATGTTGCC
<i>Integrin α7</i>	CAATCTGGATGTGATGGGTG	CTCAGGGGACAAGCAAAGAG
<i>Jagged1</i>	AGCTCACTTATTGCTGCGGT	CCGCTTCCTTACACACCAGT
<i>TN-AP</i>	GTGGATACACCCCGGGGC	GGTCAAGGTTGGCCCAATGCA
<i>Pdgfβ</i>	AAGTTTAAGCACCCCATGACAAG	ATTAATAACCCTGCCCACTCT
<i>Rgs5</i>	GCTTTGACTTGCCAGAAA	CCTGACCAGATGACTACTTGATTAGC

Table S2. Percentage of centronucleated myofibers after transection and administration of reprogramming factors or saline control.

<i>Group</i>	<i>day 9</i>	<i>day 14</i>
<i>Injury d0 saline d7</i>	33.9 ± 12.9	45.6 ± 6
<i>Injury d0 OKSM d7</i>	56.0 ± 23.4	18.2 ± 7.7

Data are expressed as Mean ± SD.

Table S3. Percentage of fibrotic area after transection and administration of reprogramming factors or saline control.

<i>Group</i>	<i>day 9</i>	<i>day 14</i>
<i>Injury d0 saline d7</i>	15.4 ± 5.2	22.6 ± 6.7
<i>Injury d0 OKSM d7</i>	12.3 ± 5.1	15.5 ± 6.5

Fibrotic area is calculated as the percentage of the muscle cross-section that stained positively for collagen. Data are expressed as Mean ± SD.

Supplementary Methods

TUNEL staining. 10 µm thick frozen tissue sections were processed with DeadEnd Colorimetric TUNEL Assay kit (Promega, G7130, UK) according to manufacturer's specifications. In brief, tissue sections were fixed in 4% paraformaldehyde (15 min, RT) and permeabilised with Proteinase K (20µg/ml, 15 min, 37°C). Sections were then incubated with TUNEL reaction mixture containing recombinant terminal deoxynucleotidyl transferase (rTdT) and biotinylated nucleotide for 1 h at 37°C. After several washes in 2X SSC and PBS, slides were blocked with 0.3% hydrogen peroxide in PBS (5 min) and incubated with horseradish peroxidase-labelled streptavidin (Streptavidin-HRP) antibody diluted 1:500 in PBS. Reaction with diaminobenzidine (DAB) was observed by light microscopy (Leica, UK) and representative images were taken at 40X magnification.

Optimisation of the timing of *in vivo* reprogramming towards pluripotency after physically-induced skeletal muscle injury. Transection of the medial head of the GA was performed as described before and 100 µg pOKSM or 40 µl saline solution were administered at the time of injury, 5 or 7 days after transection. GA muscles were dissected 2 days after injection and gene expression was investigated by real-time RT-qPCR as previously described.

Electromechanical evaluation of muscle force. Functional recovery of the GA after injury was measured by recording the force produced under fast twitch and tetanus contraction, via direct stimulation of the sciatic nerve. Such measurements were taken with an Aurora 1300A myograph (Aurora Scientific Inc, Canada) that allowed *in vivo* recordings. In brief, mice were anaesthetised with isoflurane and the GA was exposed and released from the fascia as previously described. The GA and sciatic nerve were bluntly dissected. The femur's head was prepared free from surrounding tissue and fixed to the platform clamp as shown in Figure 7b preventing movement of the leg upon the stimulation. The Achilles tendon was connected to the force transducer and an electrode was placed on the sciatic nerve. The nerve and muscle were kept moist with paraffin oil at 37°C throughout the measurements. Since we aimed to record isometric contractions, the optimal muscle length was first determined by repeating the twitch measurements with a fixed current of 5V and while adjusting the length of the muscle. The maximum twitch force was then assessed by increasing the voltage of stimulation. Finally, tetanus contractions were produced by repeated stimulation at the optimal length and current identified from the twitch measurements, with a 150 Hz frequency. 1 and 5 minutes were allowed between twitch and tetanus measurements, respectively, to avoid muscle fatigue that could influence the results. We calculated the percentage of recovered force as the ratio between the force produced by the left (injured) and right (intact) GA, n=4.