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Methods and Reagents

# An improved reprogrammable mouse model harbouring the reverse tetracycline-controlled transcriptional transactivator 3

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ABSTRACT

Reprogrammable mouse models engineered to conditionally express *Oct-4*, *Klf-4*, *Sox-2* and *c-Myc* (*OKSM*) have been instrumental in dissecting molecular events underpinning the generation of induced pluripotent stem cells. However, until now these models have been reported in the context of the m2 reverse tetracycline-controlled transactivator, which results in low reprogramming efficiency and consequently limits the number of reprogramming intermediates that can be isolated for downstream profiling. Here, we describe an improved *OKSM* mouse model in the context of the reverse tetracycline-controlled transactivator 3 with enhanced reprogramming efficiency (>9-fold) and increased numbers of reprogramming intermediate cells albeit with similar kinetics, which we believe will facilitate mechanistic studies of the reprogramming process.



## 1. Introduction

Ten years ago it was shown that mature cell types can be reverted back towards a pluripotent state by the forced expression of *Oct-4*, *Klf-4*, *Sox-2* and *c-Myc* (*OKSM*) with tremendous implications for the regenerative medicine field (Takahashi & Yamanaka, 2006; Stadtfeld & Hochedlinger, 2010). However, only in recent years we have started understanding the molecular mechanisms that underpin the reprogramming process into these so called induced pluripotent stem cells (iPSC) (Woltjen et al., 2009; David & Polo, 2014; Alaei-Shehni et al., 2014; Firas et al., 2015; Polo et al., 2012). The establishment of reprogrammable mouse models, genetically engineered to conditionally express the four factors from a defined locus, have been instrumental in these efforts (Carey et al., 2009; Stadtfeld et al., 2010). Arguably the most widely used reprogrammable mouse model is the *m2rtTA-OKSM* mouse (Stadtfeld et al., 2010) which harbours (I) a multicistronic *OKSM* cassette with an inducible promoter at the *Collagen1a1* locus, (II) the m2 reverse tetracycline-controlled transactivator (*m2rtTA*) constitutively expressed from the *Rosa26* locus and (III) a GFP reporter under control of the endogenous *Oct-4* promoter. Reprogramming is induced in cells from these mice by exposure to doxycycline (dox) reversibly activating OKSM expression (Stadtfeld et al., 2010). Compared to traditional methods of iPSC generation using lenti- and retroviruses, the *m2rtTA-OKSM* mouse model has proven advantageous as it is more efficient (increased reprogramming frequency) and allows temporal control over transgene expression from a defined locus (Stadtfeld et al., 2010). Nevertheless, reprogramming is still a rare event that the majority of cells are refractory to (Stadtfeld et al., 2010). Accordingly, in order for the molecular mechanism of the reprogramming process to be studied, only cell populations poised to becoming iPSC have to be isolated and used for profiling. However, due to the low reprogramming efficiency, the collection of intermediates is currently a very cost and labour intensive process (Polo et al., 2012; Hansson et al., 2012).

Thus far, the molecular events of the reprogramming process have been studied most extensively for mouse embryonic fibroblasts (MEFs). Successfully reprogramming MEFs from the *m2rtTA-OKSM* mouse model initially loose identity cell surface marker THY1 followed by reactivation of pluripotency associated cell surface marker SSEA1 (Polo et al., 2012; Stadtfeld et al., 2008). A subset of SSEA1 positive cells is able to activate late stage reprogramming marker EPCAM, followed by reactivation of the endogenous pluripotency network (Oct4-GFP expression) (Polo et al., 2012; Stadtfeld et al., 2010). Cells refractory to reprogramming on the other hand, distinguish themselves from reprogramming intermediates by their inability to downregulate THY1 expression and the fact that they contain less OKSM protein (Polo et al., 2012). We have recently shown that these refractory cells can be rescued by superfection with additional *OKSM* virus (Polo

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et al., 2012), demonstrating that expression levels of *OKSM* can be rate-limiting. Consequently, we hypothesized that cells from a mouse model with higher expression levels of *OKSM* at the cell population level should reprogram more efficiently. To achieve this, we exchanged the *m2rtTA* for the reverse tetracycline-controlled transactivator 3 (rtTA3), which has been shown to be more efficient in activating dox inducible promoters (Das et al., 2004). The resulting *rtTA3-OKSM* mouse model is characterized by higher protein levels of the reprogramming factors and an over nine-fold increased reprogramming efficiency compared to the *m2rtTA-OKSM* mouse model, while reprogramming kinetics remained conserved between both models.

## 2. Materials and methods

### 2.1. Breeding

Embryos hetero/hemizygous for the *OKSM*, *rtTA3* and *Oct4-GFP* locus were generated by crossing animals homozygous for the *OKSM* cassette with animals both hemizygous for the *CAG-rtTA3* locus and homozygous for the *Oct4-GFP* locus (Fig. 1a). Embryos heterozygous for the *OKSM*, *m2rtTA* and *Oct4-GFP* locus were generated by crossing animals homozygous for the *OKSM* cassette with animals homozygous for both the *m2rtTA* and the *Oct4-GFP* locus. Founder animals with the *OKSM* cassette (https://www.jax.org/strain/011001), *rtTA3* cassette (https://www.jax.org/strain/016532),* *m2rtTA* cassette (https://www.jax.org/strain/006965) and *Oct4-GFP* reporter (https://www.jax.org/strain/008214) can be purchased from the Jackson Institute (Bar Harbor, USA). *Please note that the *CAG-rtTA3* cassette has to be maintained hemizygously to not impact negatively on fertility.

### 2.2. Generation of mouse embryonic fibroblasts

Mouse embryonic fibroblast (MEFs) were generated from embryonic day (E) 13.5 embryos as described previously (Nefzger et al., 2014).

### 2.3. Western blot

Nuclear extracts were prepared from approximately one million cells as described previously (Andrews & Faller, 1991). Protein samples were separated on 4–12% Bis-Tris gels (NuPAGE Cat. No. NP0335BOX, Life Technologies) under reducing conditions with 14 μg nuclear extracts loaded per lane. They were then transferred onto nitrocellulose membranes (NuPAGE; Invitrogen) followed by immunoblotting with the primary antibodies (rabbit anti-OCT-4 Cat. No. ab19857, Abcam and mouse anti-GAPDH Cat. No. MAB374, Millipore) and fluorescently labelled secondary antibodies (IRDye 800RD Donkey anti-rabbit Cat.

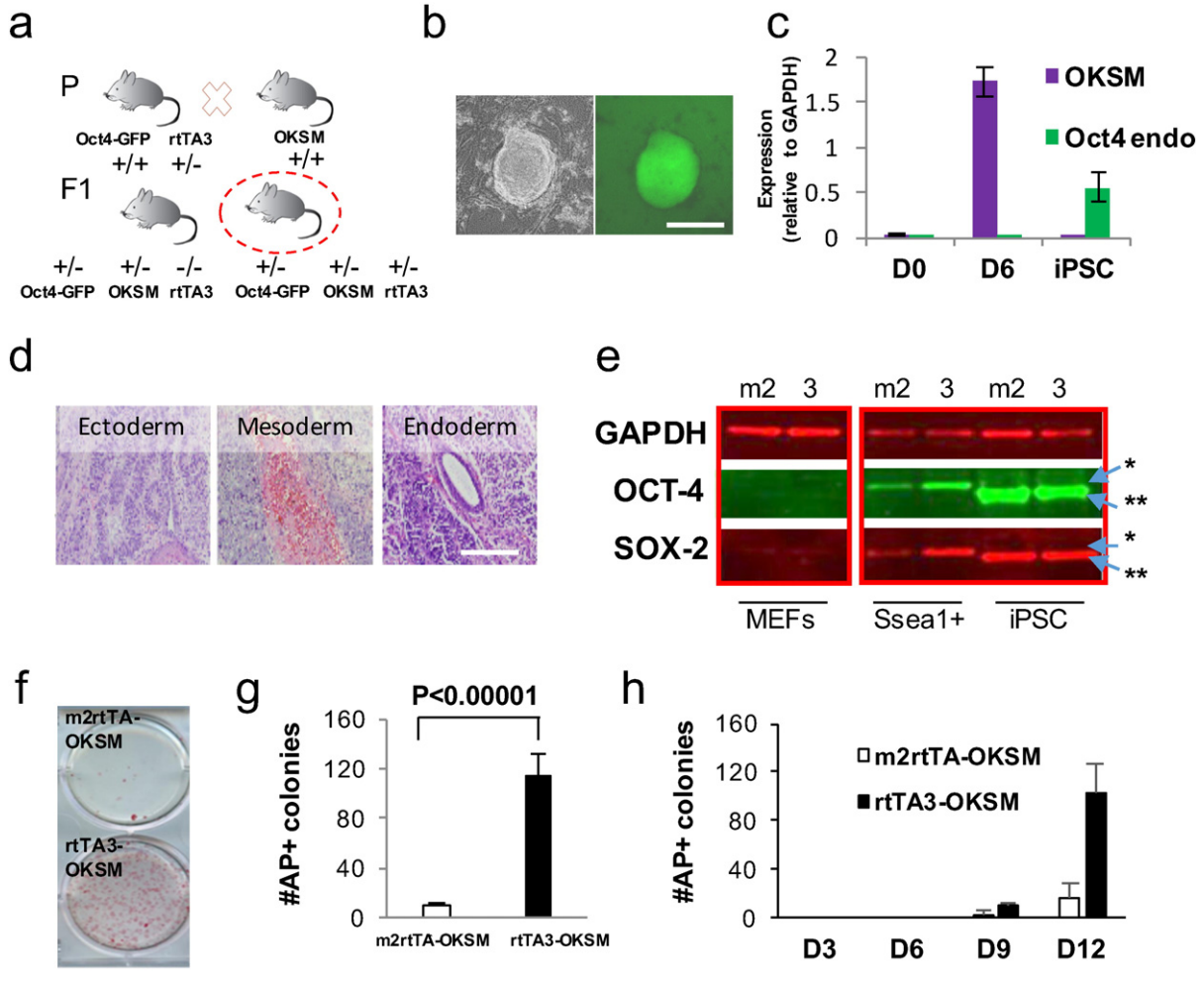


**Fig. 1.** Reprogramming potential: (a) Schematic of breeding strategy for the *rtTA3-OKSM* mouse model. (b) White light and fluorescent image of *rtTA3-OKSM* derived iPSC colony; Scale bar: 100 μm (c) qPCR to test expression levels of *OKSM* cassette and endogenous Oct4 transcripts relative to housekeeping gene Gapdh for *rtTA3-OKSM* model in MEFs, on day 6 of reprogramming (+dox) and in established iPSC (−dox) (n = 3, biological replicates). (d) Representative histology sections from an *rtTA3-OSKM* derived teratoma confirming the presence of derivatives of all three germ layers; Scale bar: 50 μm. (e) Western blot analysis for GAPDH, OCT-4 and SOX-2 in MEFs, day 6 intermediates and iPSC of the *m2rtTA* and *rtTA3-OKSM* mouse model (*exogenous protein, **endogenous protein). (f) Alkaline phosphatase (AP) labelling on day 16 and (g) quantification of AP positive colonies (n = 3, biological replicates). (h) Point of no return experiment: Quantification of number of AP positive colonies on day 16 with varying periods of dox exposure as indicated on x-axis (n = 2, biological replicates).