Stem Cell Research 16 (2016) 1–9

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Elevated levels of ZAC1 disrupt neurogenesis and promote rapid in vivo reprogramming

Vilma Rraklli, Erik Södersten, Ulrika Nyman, Daniel W. Hagey, Johan Holmberg *

Department of Cell and Molecular Biology, Ludwig Institute for Cancer Research, Karolinska Institutet, Nobels väg 3, 171 77, Stockholm, Sweden

ARTICLE INFO

Article history:
Received 26 June 2015
Received in revised form 27 October 2015
Accepted 9 November 2015
Available online 21 November 2015

Keywords:
Neurogenesis
Differentiation
Transcription
Reprogramming
Pluripotency
Imprinting

ABSTRACT

The zinc finger transcription factor *Zac1* is expressed in dividing progenitors of the nervous system with expression levels negatively controlled by genomic imprinting. To explore the consequences of elevated ZAC1 levels during neurogenesis we overexpressed it in the developing CNS. Increased levels of ZAC1 rapidly promoted upregulation of CDK inhibitors P57 and P27 followed by cell cycle exit. Surprisingly this was accompanied by stalled neuronal differentiation. Genome wide expression analysis of cortical cells overexpressing *Zac1* revealed a decrease in neuronal gene expression and an increased expression of imprinted genes, factors regulating mesoderm formation as well as features of differentiated muscle. In addition, we observed a rapid induction of several genes regulating pluripotency. Taken together, our data suggests that expression levels of *Zac1* need to be kept under strict control to avoid premature cell cycle exit, disrupted neurogenesis and aberrant expression of non-neuronal genes including pluripotency associated factors.

1. Introduction

The generation of an organ requires strict control of the number and identity of cells generated during development. *Zac1* (also known as *Plagl1*) belongs to an imprinted gene network (IGN), which has been shown to control embryonic growth (Varrault et al., 2006). Accordingly, *Zac1* knockout mice exhibit reduced body size (Varrault et al., 2006). ZAC1 has also been shown to induce apoptosis and cell cycle arrest (Spengler et al., 1997). The *ZAC1* gene is located at human chromosome 6q24, a locus that harbors a region which exhibits maternal allele methylation in several types of tissue (Abdollahi, 2007). Loss of methylation results in loss of imprinting (LOI) and biallelic expression with higher overall levels of the *ZAC1* transcript. One consequence of *ZAC1* LOI is disturbed pancreas islet development and transient neonatal diabetes mellitus syndrome (Ma et al., 2004; Temple & Shield, 2002).

During central nervous system (CNS) development, neurons, astrocytes and oligodendrocytes are sequentially generated from a pool of progenitors located in the neuroepithelium lining the ventricles. *Zac1* is expressed in this germinal zone during development (Valente et al., 2005) and a recent study shows that ZAC1 is necessary for proper neuronal migration in the developing cortex (Adnani et al., 2015). However, the role for ZAC1 during neurogenesis is not fully understood. Our data reveal that *Zac1* expression in neuronal progenitors is regulated by the SOXB1 (SOX1-3) family of transcription factors and that overexpression of *Zac1* promotes cell cycle exit through induction of the CDK inhibitors (CKIs) P57 in brain and P27 in spinal cord. This is accompanied by a failure to express key neuronal differentiation genes and stalled neuronal differentiation. Genome wide expression data from developing cortices show that ZAC1 regulates a cohort of imprinted genes in forebrain neural progenitors. In addition, there is a rapid induction of genes involved in mesodermal specification and myogenic differentiation. Recent studies have shown that certain cell fate determinants can substitute for core pluripotency factors during the generation of induced pluripotent stem cells (iPSCs) (Takahashi et al., 2014; Shu et al., 2013; Montserrat et al., 2013). Our analysis reveals that ZAC1 induced expression of mesodermal lineage determinants is also accompanied by expression of iPSC associated genes.

Taken together, we demonstrate that it is essential to control *Zac1* expression levels in neural progenitors in order to avoid premature cell cycle exit, failed neuronal differentiation and aberrant activation of determinants of non-neuronal lineages that provoke a rapid process containing features of reprogramming.

2. Experimental procedures

2.1. Cell culture and transfection

293FT cells were seeded out in 9 wells of a 24 well plates at a confluence of 50,000 cells/well. After 24 h, 3 wells were Lipofectamine transfected (LF 2000) with 100 ng of *pCAGG-Zac1* together with 100 ng of P57Luc (Alheim et al., 2003) and 100 ng of a β-galactosidase encoding vector. As control, 3 wells were Lipofectamine transfected with 100 ng of pCAGG empty vector together with 100 ng of P57Luc

* Corresponding author.
E-mail address: johan.holmberg@licr.ki.se (J. Holmberg).

http://dx.doi.org/10.1016/j.scr.2015.11.002

and 100 ng of the β-galactosidase encoding vector. The other 3 wells were not Lipofectamine transfected in order to serve as a control for the luciferase assay. Following transfection, cells were grown for 24 h and then growth media was removed from the cells. 150 μl of cell lysis buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, 150 mM NaCl, 0.65% NP-40, 0.4 mM PMSF, 1 mM DTT) was added and cells were incubated for 20 min at 4 °C.

2.2. β-Galactosidase assay

β-Galactosidase solution (60 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgCl_2$, 40 mM NaH_2PO_4) with β-mercaptoethanol (3.5 μl/ml) and ONPG (0.0025 g/ml) was added to 50 μl of each sample (lysed cells). When samples turned yellow, stop solution was added (1 M Na_2CO_3). Samples were analyzed using spectrophotometry at 405 nm to assess the transfection efficiency.

2.3. Luciferase assay

100 μl of luciferase buffer (H_2O, 1 M $MgCl_2$, 1 M KH_2PO_4 pH 7.8, 0.1 M ATP) were added to each sample (lysed cells). Samples were inserted in a luminometer together with a luciferin working solution (H_2O, 1 M KH_2PO_4 pH 7.8, 0.01 luciferin) that was automatically added to each sample by the machine. Sample luminescence was measured to assess expression of P57luc upon Zac1 overexpression.

2.4. Zac1 electroporation in mouse cortex (in utero) and in chick spinal cord (in ovo)

cDNA encoding mouse *Zac1* was C-terminally fused to a Myc tag and subcloned into a CMV based pCAGGS vector. The *shCdkn1c* vector was a Mission shRNA obtained from Sigma-Aldrich (TRCN0000042590). E14.5 pregnant CD1 mice were anesthetized with isoflurane and uterine horns were surgically exposed. 1–2 μl of plasmids (8 μg/μl) in PBS with fast green (2 mg/ml; Sigma) was injected using pulled glass microcapillaries into the lateral ventricle of the embryos. For each embryo, five electric pulses of 50 V for 50 ms were discharged at intervals of 950 ms across the head using an electroporator (Nepagene CUY21D) and platinum electrodes (Nepagene CUY650P5). Pups were sacrificed at two different time points: after 24 h and after 48 h. 2 h before sacrificing the embryos, BrdU (50 mg/kg) solution in PBS was injected. Heads were collected in cold PBS. After dissection, brains were stored in 4% paraformaldehyde (PFA) overnight at 4 °C and consequently transferred in 30% sucrose at 4 °C overnight. Brains were then cryosectioned (12 μm thickness) onto Superfrost Plus slides (Thermo Scientific) for immunohistochemistry analysis. For the *pCAGG-Zac1/shCdkn1c* in utero *electroporations*, E14.5 cortices were injected with *pCAGG-Zac1* (2 μg/μl) together with *shCdkn1c* (2 μg/μl). Fertilized chicken eggs were incubated at 38 °C for 41–43 h to reach stage HH11. Albumen (5 ml) was withdrawn with a syringe and the top of the shell was opened in order to expose the embryo. The construct of interest (1 μg/μl in PBS; 1 mM $MgCl_2$; fast green) was injected in the neural tube using pulled glass microcapillaries. 5 Electric pulses of 21 V for 50 ms each were discharged at intervals of 1 s. The opening of the eggs was then taped in order to avoid the embryos to dry. Eggs were incubated at 38 °C for 24 h. An hour before sacrificing chick embryos, BrdU (50 μM) solution in PBS was injected underneath the embryos. After dissection, neural tubes were incubated in 4% paraformaldehyde (PFA) for 2 h. After washing with PBS, neural tubes were transferred in 30% sucrose solution overnight and then cryosectioned (10 μm) onto Superfrost Plus slides (Thermo Scientific) where immunohistochemistry was performed.

2.5. Immunohistochemistry

Cryosections were permeabilized with blocking solution (5% FBS, 0.2% Tween, PBS) for an hour at room temperature. Stainings were performed with the following primary antibody incubated overnight at 4 °C: rbZAC1 (aliquot generously donated by L. Journot; 1:500), gtZAC1 (sc17854 Santa Cruz; 1:100), rbMYC (A-14, sc789, Santa Cruz; 1:500), gtGFP (ab6673 Abcam; 1:100), rbSOX2 (AB5603 Millipore; 1:1000), rbKI67 (ab16667 Abcam; 1:500), rbTBR2 (ab23345 Abcam; 1:500), ratBrdU (Abcam; 1:250), mTUJ1 (MMS435P Covance 1:1000), rbP57 (Santa Cruz; 1:100), P27 (1:1000); mPH3 (05-806 Millipore 1:1000), rbDESMIN (ab15200 Abcam 1:250). Secondary antibody Alexa Fluor 488 (1:500) was added for an hour at room temperature to detect ZAC1 overexpression and GFP (control vector) expression. Secondary antibody Alexa Fluor 555 (1:1000) was added for an hour at room temperature to detect the other antibodies. When double staining with another rabbit antibody, the rbMYC antibody was substituted with the gtZAC1 antibody that only detects overexpressed ZAC1 and fails to stain endogenous ZAC1 protein. There was a total and exclusive overlap between gtZAC1 and rbMYC staining in *pCAGG-Zac1-Myc* electroporated brains. To distinguish between endogenous ZAC1 immunodetection and detection of overexpression we have added (gt) in the panels showing *Zac1* overexpression detected by the gtZAC1 antibody.

2.6. Cell cycle labeling index (LI) calculation

Pregnant females undergoing in utero electroporation were exposed to a single pulse label of BrdU at E14.5. 24 h after pulse label the embryos were retrieved according to the procedure described above. Cryosections of electroporated cortices were stained for BrdU and KI67. The proportion of electroporated cells that were $BrdU^+/KI67^+$ was divided by the total number of electroporated $BrdU^+$ cells ($BrdU^+/KI67^+ + BrdU^+/KI67^-$). This LI represents the population of cells in S-phase at E14.5 that still are $KI67^+$ (i.e. in cell cycle) at E15.5.

2.7. Alkaline phosphatase activity assay

Cryosections were washed in a 0.1 M Tris–Cl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$ solution. They were then incubated at room temperature in the same solution with BCIP/NBT (Roche) added according to manufacturer's protocol. After staining the sections were washed in PBS and coverslipped.

2.8. RNA sequencing

24 h after overexpression of Zac1 (5 μg/μl) in mouse cortices via in utero electroporation together with a GFP construct (5 μg/μl) or GFP only as control (5 μg/μl), embryos were sacrificed and brains isolated. A papain neural dissociation tissue kit (Miltenyi Biotech) was used to dissociate brains (*Zac1* $n = 3$, *GFP* only $n = 4$) into single cells. Cells were then sorted for GFP with BD FACSAria II and GFP^+ cells were collected in RLT buffer (+β-mercaptoethanol). RNA extraction was performed using RNeasy Micro Kit (Qiagen). The extracted RNA was then used to make cDNA using SuperScript II Reverse Transcriptase (Life Technologies). RNA Sequencing was performed following the Smart-seq2 protocol (Picelli et al., 2014). Tagmentation was performed using Nextera XT DNA Sample Preparation Kit (Illumina). The samples were sequenced using HiSeq2000 Illumina DNA Sequencing Instrument. Reads were mapped to the mouse genome (mm10 assembly) using STAR (Dobin et al., 2013) and gene expression was calculated using RPKM for genes (Ramskold et al., 2009). Differential gene expression was computed using DESeq2 (Love et al., 2014).