Evaluation of porcine stem cells competence for somatic cell nuclear transfer and production of cloned animals

Jan O. Secher^{a,1}, Ying Liu², Stoyan Petkov³, Yonglun Luo⁴, Dong Li⁶, Vanessa J. Hall⁶, Mette Schmidt¹, Henrik Callesen², Jacob F. Bentzon⁵, Charlotte B. Sørensen⁵, Kristine K Freude⁶, Poul Hyttel^{a,6}

a: Corresponding authors: Jan O. Secher (jbsecher@sund.ku.dk) & Poul Hyttel (poh@sund.ku.dk)

1: Veterinary Reproduction and Obstetrics, Department of Large Animal Sciences, University of Copenhagen, DK1870 Frederiksberg C, Denmark

2: Department of Animal Science, Aarhus University, DK8830 Tjele, Denmark

3: Institute for Farm Animal Genetics (FLI), Neustadt, Germany

4: Department of Biomedicine, Aarhus University, DK8000 Aarhus C, Denmark

5: Department of Clinical Medicine, Aarhus University, DK8200 Aarhus N, Denmark

6: Department of Veterinary Clinical and Animal Sciences, University of Copenhagen, DK1870 Frederiksberg C, Denmark

Abstract

Porcine somatic cell nuclear transfer (SCNT) has been used extensively to create genetically modified pigs, but the efficiency of the methodology is still low. It has been hypothesized that pluripotent or multipotent stem cells might result in increased SCNT efficacy as these cells are closer than somatic cells to the epigenetic state found in the blastomeres and therefore need less reprogramming. Our group has worked with porcine SCNT during the last 20 years and here we describe our experience with SCNT of 3 different stem cell lines. The porcine stem cells used were: Induced pluripotent stem cells (iPSCs) created by lentiviral doxycycline-dependent reprogramming and cultured with a GSK3 β - and MEK-inhibitor (2i) and leukemia inhibitor factor (LIF) (2i LIF DOX-iPSCs), iPSCs created by a plasmid-based reprogramming and cultured with 2i and fibroblast growth factor (FGF) (2i FGF PI-iPSCs) and embryonic germ cells (EGCs), which have earlier been characterized as being multipotent. The SCNT efficiencies of these stem cell lines were compared with that of the two fibroblast cell lines from which the iPSC lines were derived. The blastocyst

rates for the 2i LIF DOX-iPSCs were 14.7%, for the 2i FGF PI-iPSC 10.1%, and for the EGCs 34.5% compared with the fibroblast lines yielding 36.7% and 25.2%. The fibroblast- and EGC-derived embryos were used for embryo transfer and produced live offspring at similar low rates of efficiency (3.2 and 4.0%, respectively) and with several instances of malformations. In conclusion, potentially pluripotent porcine stem cells resulted in lower rates of embryonic development upon SCNT than multipotent stem cells and differentiated somatic cells.

Key Words

Pluripotency, induced pluripotent stem cells, embryonic germ cell, transgenic pigs, somatic cell nuclear transfer, handmade cloning.

1 Introduction

Dolly the sheep was the first mammal created by somatic cell nuclear transfer (SCNT) (Wilmut et al., 1997). This achievement paved the way for research within cell plasticity and cell reprogramming, which have been major research areas over the past two decades. SCNT has been implemented with great success to create non-murine genetically modified animals in several species, including the pig (Al-Mashhadi et al., 2013; Callesen et al., 2014; Onishi et al., 2000; Polejaeva et al., 2000). Unfortunately, the efficiency of SCNT is rather low (Liu et al., 2014). In the pig, only 1-5% of the reconstructed embryos transferred to surrogate sows result in live offspring (Lee et al., 2013; Liu et al., 2014; Schmidt et al., 2010). Additionally, a considerable proportion of the born piglets exhibit abnormalities (Schmidt et al., 2015) thereby reducing the efficiency even further (Schmidt et al., 2015, 2010).

Earlier studies have indicated that cells derived from early embryos result in increased developmental rates following SCNT (Obach, 2009). Thus, it was hypothesized that the use of pluripotent stem cells (PSCs) for SCNT would result in higher efficiencies and less abnormalities. Considerable efforts have been invested in deriving PSCs in the large domestic species including the pig, which is generally considered to be a valid model for humans due to physiological and anatomical similarities (Bassols et al., 2014; Lelovas et al., 2014). It has, however, proven to be a great challenge to maintain bona fide porcine embryonic stem cells (ESCs) and also the quest for derivation of porcine iPSCs has been associated with several complications including lack of

transgene silencing and incomplete reprogramming and activation of the endogenous pluripotency network (reviewed by (Telugu et al., 2010)). Some of the problems with piPSC have been sought explained by comparing piPSC to naïve and primed murine embryonic stem cells (mESC). Naïve mESCs can be isolated from the murine ICM just before implantation and depend on leukemia inhibitor factor (LIF) and MEK- and GSK3 β -inhibitors (2i) in vitro (Nichols and Smith, 2009) whereas primed murine ESCs can be isolated from the murine epiblast just after implantation and can be maintained in vitro by addition of bFGF to the media (Nichols and Smith, 2009). Only the naïve mESC can contribute to germline chimeras and they yield a higher efficiency of SCNT than primed mESC (Nichols and Smith, 2009). The first piPSCs generated (Esteban et al., 2009; Ezashi et al., 2009; Wu et al., 2009) resemble the primed type mESCs in regards to colony morphology and dependence on FGF. Subsequently, several groups have established piPSCs and cultured them supplemented with LIF and 2i to mimic the conditions which resulted in naïve murine ESCs (Cheng et al., 2012; Fujishiro et al., 2013; Telugu et al., 2011) and in cattle this has resulted in promising results (Kawaguchi et al., 2015). The use of both naïve-like and primed-like piPSCs for SCNT has been reported (Du et al., 2015; Fan et al., 2013; Yuan et al., 2014). Du et al. used naïve-like porcine iPSCs reprogrammed with a non-integrating episomal vector and cultured with leukemia inhibitory factor (LIF) as well as GSK3 β - and MEK-inhibitors (2i) but was unable to produce live piglets following SCNT (Du et al., 2015). Similar results were obtained by Fan et al. who used primed-like iPSCs reprogrammed with a doxycycline regulated lentiviral construct and cultured with fibroblast growth factor (FGF) (Fan et al., 2013). Interestingly, Fan and co-workers also found that if they submitted the iPSCs to spontaneous differentiation prior to SCNT, they were able to achieve live piglets. This is in contrast to the results obtained in mice where the SCNT efficiency has been demonstrated to increase with differentiation potency of the cells used as nuclear donors (Nichols and Smith, 2009; Okita et al., 2011).

Here, we provide a description of our experience with SCNT using a transgenic male Large White/Landrace perinatal fibroblast line (WL-pNFs) expressing the Venus (green fluorescence) protein, a iPSC line derived from the WL-pNFs using a doxycycline regulated lentiviral construct and cultured with 2i and LIF (2i LIF DOX-iPSCs), a male wild type Göttingen embryonic fibroblast line (G-PEFs), a iPSC line derived from the G-PEFs (2i FGF PI-iPSC) using the non-integrating episomal method (Okita et al., 2011) and cultured with FGF, and finally a Yucatan female transgenic porcine embryonic germ cell line (EGC) overexpressing the human wildtype PCSK9 (for an overview over the experiments and the cell lines please see **Figure 1A-C and 1D**). We compare

97 initial embryonic development and results from embryo transfer to the hypothesis that the capacity
98 of porcine cells to sustain embryonic development upon SCNT is positively correlated with their
99 developmental potency, i.e. that PSCs are more capable of being reprogrammed than somatic cells
100 and that multipotent stem cells might have an intermediate capacity.

102 **2 Material and methods**

104 *2.1 Cells and iPSC reprogramming*

105 Large White/Landrace porcine neonatal fibroblasts (WL-PNFs) carrying a transgene encoding the
106 green fluorescent protein Venus with a constitutive promotor (Garrels et al., 2011) were kindly
107 provided by Wilfried Kues and cultured in high-glucose Dulbecco's modified Eagle's medium
108 (DMEM AQ; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1% Penicillin/Streptomycin
109 (Pen/Strep; Sigma Aldrich) and 10% fetal bovine serum (FBS; Hyclone, GE Health Care Life
110 Sciences, Chicargo, IL, USA). The WL-PNFs were reprogrammed using a lentiviral construct as
111 described previously (Secher et al., 2015). In brief, a doxycycline-regulated CAG promoter
112 containing porcine *OCT4*, *c-MYC*, *SOX2* and *KLF4* was used for lentiviral reprogramming of WL-
113 PNFs into iPSCs (Nethercott et al., 2011). The resulting iPSC (2i LIF DOX-iPSCs) were
114 maintained on mitomycin-C-treated mouse embryonic fibroblasts (MEFs) in DMEM/F12 medium
115 (Sigma Aldrich) supplemented with 20% KnockOut Serum Replacement (Invitrogen, Waltham,
116 MA, USA), 1x Pen/Strep (Sigma Aldrich), 1x nonessential amino acids (Sigma Aldrich), 100 µM
117 b-mercaptoethanol (Life Technologies), 10 ng/mL leukemia inhibitory factor (LIF) (Millipore,
118 Billerica, MA, USA), two kinase inhibitors (1 µM PD0325901 [Sigma Aldrich], 3 µM CHIR99021
119 [Sigma Aldrich]), and 2 µg/mL doxycycline (Sigma Aldrich) (piPSC medium). Cells were
120 dissociated with 1x TrypLE (Gibco, Waltham, MA, USA) and passaged 1:6 every 7 days. Please
121 notice that this cell line differs from the iPSC line reprogrammed with a doxycycline regulated
122 Lentivirus construct made by Fan et al. (Fan et al., 2013) and used for their SCNT experiments by
123 the fact that we used 2i LIF culture conditions and they used FGF. A full characterization of the 2i
124 LIF DOX-iPSC is in press (Secher et al., 2017).

126 Göttingen porcine embryonic fibroblasts (G-PEFs) obtained from embryos at day 25 of gestation
127 were reprogrammed and characterized. In brief, electroporation-based delivery of episomal
128 plasmids (Okita et al., 2011)) purchased from Addgene Inc. (Cambridge, MA), *pCXLE-hOCT3/4-*

129 *shp53* (Addgene plasmid #27077), *pCXLE-hSK* (Addgene plasmid #27078), and *pCXLE-hUL*
130 (Addgene plasmid #27080) were used. The resulting plasmid-based iPSCs (2i FGF Pl-iPSCs) were
131 maintained on mitomycin-C-treated MEFs in DMEM/F12 medium (Sigma Aldrich) supplemented
132 with 20% KnockOut Serum Replacement (Invitrogen), 1x Pen/Strep (Sigma Aldrich), 1x
133 nonessential amino acids (Sigma Aldrich), 100 μ M b-mercaptoethanol (Life Technologies), 20
134 ng/mL human recombinant basic fibroblast growth factor (bFGF) (Prospec, East Brunswick, NJ),
135 and two kinase inhibitors; 1 μ M PD0325901 (Sigma-Aldrich), which inhibits MEK signal pathway,
136 and 3 μ M CHIR99021 (Sigma-Aldrich), a GSK3 β inhibitor. Cells were dissociated with 1x TrypLE
137 (Gibco) and passaged 1:6 every 4-5 days. Please notice that this cell line differs from the iPSC line
138 reprogrammed with an episomal plasmid made by Du et al. (Du et al., 2015) and used for their
139 SCNT experiments by the fact that we used 2i FGF culture conditions and Du et al. used the 2i LIF
140 culture conditions. A full characterization of the 2i FGF Pl-iPSC is pending and will be submitted
141 later this year (Li et al., manuscript in preparation).

143 Yucatan mini-pig EGCs were isolated from individual embryos, maintained, and characterized as
144 described previously (Petkov et al., 2011). Prior to SCNT, the EGCs were genetically modified
145 using a Sleeping Beauty transposon system for insertion of human wildtype PCSK9 comprising a
146 FLAG-tag as described previously (Al-Mashhadi et al., 2013) with the aim of creating a large
147 animal model for hypercholesterolemia and atherosclerosis. The vector pSBT/ApoEHCR-hAATp-
148 WT-PCSK9-FLAG, comprising the apoE locus control region (HCR) and the human α 1-antitrypsin
149 promoter (hAAT), was constructed in essentially the same way as previously described for
150 pSBT/ApoEHCR-hAATp-D374Y-PCSK9 (Al-Mashhadi et al., 2013) except that the human WT
151 PCSK9 cDNA comprising a FLAG-tag was inserted into the vector backbone instead of the mutant
152 D374Y form of human PCSK9. Briefly, EGCs were seeded in six-well plates at a density of 3×10^5
153 cells per well and, on the next day, transfected with 1.0 μ g of pSBT/ApoEHCR-hAATp-WT-
154 PCSK9-FLAG plasmid and 20 ng of pCMV-SB100X (encoding the hyperactive SB100X
155 transposase) using Effectene Transfection Reagent (Qiagen, Hilden, Germany). Three days post-
156 transfection, selection with puromycin (1.0 μ g/ml) was initiated and maintained for 12 days.
157 Puromycin-resistant colonies were subsequently isolated, expanded, and pooled prior to being used
158 for SCNT.

159

160

161 2.2 *Somatic cell nuclear transfer (SCNT)*

162 All cells for SCNT were grown to reach to full confluence and the media was not changed by the
163 EGC for the last 3 days before SCNT to force as many cells as possible into the G1 or G2 phase.
164 The stem cell colonies were controlled for changes in colony morphology as a sign of beginning
165 differentiation, before they were used for SCNT; this revealed that it not was possible to starve the
166 iPSC, and therefore both iPSC and fibroblasts had their media changed every day up to the SCNT
167 to make them comparable. SCNT by handmade cloning was performed as described previously (Li
168 et al., 2013) using donor cells of different developmental potency ranging from fibroblasts (WL-
169 PNFs) over EGCs to iPSCs (2i FGF PI-iPSCs and 2i LIF DOX-iPSCs). The cell lines were from
170 polyclonal origin and at least two replicates were made for each cell line. Reconstructed embryos
171 created from G-PEF, WL-PNF, EGC and 2i FGF PI-iPSC donor cells were cultured in PZM3 (Li et
172 al., 2013) until day 5 to 6 after activation. Reconstructed embryos created from 2i LIF DOX-iPSC
173 were cultured in PZM3 until 4 days after activation followed by division into 3 groups. Group 1
174 was cultured in PZM3 from day 4 to day 6; Group 2 was cultured in PZM3 with 10% fetal calf
175 serum (FCS) (Sigma-Aldrich F7524) from day 4 to day 6; Group 3 was cultured in PZM3 with
176 2µg/ml doxycycline (Sigma Aldrich, Cat. # D9891) from day 4 to day 6. At day 6 all embryos were
177 evaluated for development and quality prior to embryo transfer. SCNT-derived morulae and
178 blastocysts derived from WL-PNFs and EGCs were collected on days 5 and 6 prior to embryo
179 transfer. Blastocyst rates (BR) were compared between donor cell types and culture conditions
180 using the logistic regression function in SAS 9.3. Furthermore, the embryos and piglets derived
181 from the WL-PNFs and 2i LIF DOX-iPSCs were evaluated for Venus fluorescence expression
182 (green fluorescence).

183

184 2.3 *Embryo transfer*

185 General animal welfare guidance was followed and permission for transfer of the cloned embryos
186 was granted by the Danish Animal Experiments Inspectorate (2004-561-925; 2013-15-2934-00943).
187 Embryo transfer was performed surgically to surrogate sows as described previously (Schmidt et
188 al., 2010, 2011) using reconstructed embryos from WL-PNF and EGC donor cells.

189

190

191 2.4 *Molecular genetic analyses and karyotyping*

192 Ear biopsies were taken from all the cloned EGC-derived piglets at birth in order to isolate genomic
193 DNA for molecular genetic analyses. The cloned piglets were genotyped by PCR for the presence
194 of the WT-PCSK9-FLAG transgene using a Pfx Platinum polymerase (Thermo Fischer Scientific),
195 the forward hAAT-specific primer 5'- GGATCTTGCTACCAGTGGAAAC-3', the reverse FLAG-
196 specific primer 5'- ACTTGTCATCGTCGTCCTTG-3', and the PCR protocol: 1 cycle of 94°C for
197 2 mins, 35 cycles of 94°C, 20 secs, 55°C for 30 secs, 68°C for 30 secs, followed by 1 cycle of 68 °C
198 for 7 mins.

199

200 Genomic DNA isolated from the cloned EGC-derived animals was analyzed by Southern blotting.
201 Genomic DNA (15 µg) was digested overnight with *SpeI*, which cleaves at a single site within the
202 transposon. The digested DNA was electrophoresed in a 0.8% agarose gel and transferred to a
203 Hybond membrane (GE Healthcare, Chicargo, IL, USA). The membrane was hybridized overnight
204 using a [α -32P]-dCTP-labeled probe specific for the puromycin resistance gene in the vector. The
205 surviving founder animal was subsequently bred with a wildtype Yucatan boar to generate F1
206 progeny. Genomic DNA isolated from all offspring was subjected to analysis by Southern blotting
207 as described above.

208

209 The surviving EGC-derived founder animal was karyotyped using Q-banding and the software
210 Quips Karytyper version 3.1 (Vysis). Briefly, fibroblasts were isolated from the cloned piglet and
211 cultured in slideflasks. The cells were arrested in metaphase by addition of colcemid and subjected
212 to fixation and staining with DAPI prior to analysis. The DOX-iPSCs and Pl-iPSCs were submitted
213 to karyotyping at Cell Guidance Systems (Babraham Research Campus, Cambridge, UK).

214

215 The EGC-derived founder animal was euthanized at approximately 2.5 years of age and various
216 tissues were isolated and snap-frozen for quantative PCR analysis. RNA was isolated using an
217 RNeasy kit (Qiagen). One µg RNA was used for cDNA synthesis employing a RevertAid First
218 Strand cDNA Synthesis kit (Fermentas, Waltham, MA, USA). Quantative PCR was performed
219 using Maxima SYBR Green qPCR Master Mix (Fermentas) and primers specific for human PCSK9
220 (forward primer: 5'-ACCATGGGCACCGTCAGCTC-3', reverse primer: 5'-
221 AAGGTGGCTGTGGTTCCGTG-3'). The porcine β -actin gene ACTB was used as reference gene
222 (forward primer: 5'-CACGCCATCCTGCGTCTGGA-3', reverse primer: 5'-
223 AGCACCGTGTTGGCGTAGA -3').

3 Results

3.1 Blastocyst rates and quality

The SCNT experiments are summarized in **Figure 1** and the results and statistical analysis are summarized in **Table 1A & 1B**. SCNT based on WL-PNFs as donor cells resulted in a high blastocyst rate (36.7%, n=392) and good blastocyst quality (**Figure 2A & 2F**). SCNT based on G-PEFs yielded a significantly lower blastocyst rate (25.2%, n=190) and impaired quality (**Figure 2B**) compared to the WL-PNFs (p=0.006). The blastocyst rate resulting from SCNT with EGC (34.5%, n=148) was not statistically different from those obtained from the WL-PNFs and the G-PEFs. SCNT based on 2i LIF DOX-iPSC cultured in PZM (Group 1) resulted in significantly lower blastocyst rate (10.7%, n=122) and low quality (small blastocysts with uneven outline and loose blastomeres in the perivitelline space; **Figure 2C, H**). This low developmental capacity corresponds to earlier reported results following iPSC-derived SCNT (Du et al., 2015; Fan et al., 2013). Since we have experienced a positive effect of culturing porcine iPSC chimeric embryos in PZM3 with 10% FCS, we subsequently cultured the iPSC-derived reconstructed embryos in PZM3 with 10% FCS (Group 2), which, however, did not statistically significant improve the blastocyst rate (20.6% n=63, p=0.069) nor quality (**Figure 2D, I**). Also, we added doxycycline to the PZM3 4 days after activation of the reconstructed embryos (Group 3) in order to secure the expression of the pluripotency genes during formation of the ICM (Cao et al., 2014). Again, this did not improve the blastocyst rate (22.2%, n=18, p=0.171) whereas the blastocyst quality was improved (**Figure 2E, J**). Due to the very low blastocyst quality in general, we did not attempt transfer of the 2i LIF DOX-iPSC-derived reconstructed embryos. After the SCNT experiments we discovered that the 2i LIF DOX-iPSC had acquired an additional x-chromosome. Similarly, SCNT based on PI-iPSCs resulted in blastocyst rates (10.1% n=148) and quality (**Figure 2G**) comparable to the 2i LIF DOX-iPSC. The 2i FGF PI-iPSCs were karyotypically normal, but due to the low blastocyst rate and quality no attempts were made to transfer the 2i FGF PI-iPSC-derived embryos to surrogate sows.

3.2 Embryo transfer and birth of piglets

256 Only reconstructed embryos derived from the WL-PNFs and EGCs were used for transfer. A total
257 of 126 WL-PNF-derived embryos were transferred to two surrogate sows (58 and 68 embryos per
258 sow, respectively). One sow became pregnant and birth was induced at day 117 with 1 ml Estrumat
259 vet (MSD Animal Health). One piglet was born vaginally while three were delivered by cesarean
260 section. The four piglets corresponds to a cloning efficiency of 3.2% (live born piglets/embryos
261 transferred; **Figure 3A, C**). All piglets were alive at birth and all expressed the Venus (green)
262 fluorescent protein (**Figure 3B-D**). The piglet that was born naturally, exhibited macroglossia and
263 bilateral cryptorchidism and died within two hours after birth. The other three piglets, delivered via
264 caesarian section, had mild macroglossia and one had bilateral cryptorchidism and was euthanized
265 after 6 hours. Necropsy of the piglets showed that all tissues expressed the Venus protein (for
266 examples see **Figure 3B, D**).

267 A total of 200 EGC-derived embryos were transferred to two surrogate sows (76 and 124 embryos
268 per sow, respectively). Both sows became pregnant and a total of eight liveborn and four stillborn
269 piglets, as well as four mummified fetuses were delivered vaginally. The 12 piglets delivered
270 correspond to a cloning efficiency of 4.0%. Three piglets died within a few days after delivery and
271 4 were euthanized shortly after birth due to failure to thrive and, in two cases, paraplegia and
272 tetraplegia, leaving only one surviving piglet (**Figure 4A**). Necropsy of the dead piglets revealed
273 immature organs, septal defects in the heart, and in the piglet with tetraplegia, cerebellar hypoplasia
274 was observed (**Figure 4B**). Septal defects in the heart are quite common in the Yucatan breed, and
275 we have seen this phenomenon previously when cloning from Yucatan fibroblasts (Schmidt et al.,
276 2015). The transgene itself is unlikely to be the underlying cause of these defects as we have
277 previously cloned Yucatan pigs carrying a mutant form of the PCSK9 gene, or other transgenes,
278 with success and without observing as severe malformations as observed in the pigs derived from
279 EGCs. Apart from one, all EGC-derived piglets were found to comprise the WT-PCSK9-FLAG
280 transgene by PCR analysis (**Figure 4C**). Southern blot analysis performed on genomic DNA from
281 all EGC-derived piglets revealed variable copy number and different integration sites of the
282 transposon in the piglets (see Figure 1, supplementary data). In accordance with the PCR result,
283 Southern blot analysis of genomic DNA isolated from the PCSK9-negative piglet indicated random
284 integration of the entire vector via a breakage point in the PCSK9 part of the vector rather than
285 integration of the transposon by transposition thereby impairing the PCSK9-specific PCR (Figure
286 1, piglet 6, supplementary data). In the surviving piglet, two copies of the transgene were detected
287 by Southern blotting (Figure 1, piglet 2, supplementary data). The piglet was found to be

karyotypically normal (**Figure 4D**) and, as expected, it exhibited abundant liver-specific expression of human WT-PCSK9-FLAG (**Figure 4E**). This founder animal was subsequently bred with a wild type Yucatan boar resulting in normal offspring. Southern blot analysis of genomic DNA isolated from the founder animal and its offspring validated transmission of the WT-PCSK9-FLAG transgene cassette to all offspring (data not shown).

4 Discussion

Our experiments did not sustain the hypothesis that increased cell potency is positively correlated to the ability to sustain embryonic development after SCNT. We found that cells of the expected highest potency (2i LIF DOX-iPSC and 2i FGF PI-iPSC) resulted in the lowest blastocyst rates as well as the poorest embryo quality when compared with cells of medium (EGCs) and low potency (WL-PNFs and G-PEFs). Furthermore, we did not see a higher ability of the 2i LIF DOX-iPSC to sustain embryo development over the 2i FGF PI-iPSC.

Possible explanations for the fact that our piPSC lines have low capacity to sustain embryo development after SCNT can be that the piPSCs are potentially not fully reprogrammed, not cultured under the right conditions (Hall and Hyttel, 2014), exhibit failure of silencing of the transgenes (Takahashi and Yamanaka, 2006), have undergone insertional mutagenesis caused by lentiviral integration of the transgenes (Higuchi et al., 2015) or because it is difficult to force iPSCs into the G1 or G2 phase making them less compatible for SCNT (Yuan et al., 2014); however this last problem should also affect the EGC. Karyotyping results revealed that the 2i FGF PL-iPSC had a normal karyotype, but the 2i LIF DOX-iPSC had acquired an additional X-chromosome. This can potentially explain the low clonogenicity observed with the 2i LIF DOX-iPSC, but since Du and co-workers (Du et al., 2015) reported a similarly low clonogenicity of a piPSC line with normal karyotype and cultured in 2i LIF conditions, this was not pursued any further.

The SCNT experiments with the EGCs showed that it was possible to produce blastocysts at a relatively high rate and quality allowing for transfer and generation of live offspring even though only one out of eight born piglets survived. Thus, we can conclude from our SCNT and embryo transfer experiments using transgenic EGCs and fibroblasts that generation of transgenic pigs is possible from both cell types. Nevertheless, there is no apparent advantage using EGCs over fibroblasts apart from an enhanced in vitro live span of the EGCs. In fact, we observed more severe

developmental defects in the cloned piglets derived from EGCs than when using comparable transgenic Yucatan fibroblasts (see (Schmidt et al., 2015) for a thorough description on developmental defects in piglets made by SCNT). Ahn and coworkers (Ahn et al., 2010) also succeed in creating CD59-transgenic piglets using porcine EGC for SCNT. They transferred 1980 reconstructed embryos to 10 recipient sows resulting in 5 pregnancies and 15 piglets of which 5 died within the first 24 hours (Ahn et al., 2010) and thus also indicating that the EGCs do not have the same clonogenicity as murine embryonic cells (Oback, 2009). Finally, creating and maintaining porcine EGCs are laborious processes that require special technical skills (Petkov et al., 2011)

It should be mentioned that the data for this paper is not collected as part of an experimental set up made to explore the correlation between cell differentiation potency and clonogenicity. It is collected from experiments made to optimize SCNT by using different cell lines. The donor cells used are not isogenic and thus do not comprise an easy comparable background, and factors such as gender, pig breed and genetic modification may affect the clonogenicity of a cell line (Schmidt et al., 2015). Thus, simply comparing cells with regard to differentiation potency should be done with some caution. Despite the lack of a systematic evaluation of only isogenic cell types, however, the data presented clearly shows that reprogramming of the WL-PNFs to 2i LIF DOX-iPSC and the G-PEFs to 2i FGF PI-iPSC in both cases resulted in lower clonogenicity. It also indicate that there is only minor differences in clonogenicity in iPS cells cultured with LIF and FGF and also among cells reprogrammed with an integrating lentiviral construct or a non-integrating plasmid. We therefore believe that this dataset can contribute to the debate regarding the dogma that pluripotent stem cells can increase the SCNT efficiency in comparison to somatic cells as well a to the debate concerning whether it is possible to transfer the effect of the FGF conditions vs the 2i LIF conditions from murine pluripotent stem cells (Nichols and Smith, 2009) to the porcine pluripotent stem cells

In conclusion, in our experiments neither EGC nor iPSC resulted in higher SCNT efficiency compared to the tested fibroblast lines, and similar low efficient clonogenicity was observed when comparing DOX-iPSC cultured with 2i LIF and PI-iPSC cultured with 2i FGF.

Acknowledgements:

351 The authors would like to thank Trine Skov Petersen, Christian Knudsen, Lisa Maria Røge, Tina
352 Christoffersen, Annedorte Valentin Roed, Janne Adamsen, Ruth Kristensen, Anette M Pedersen and
353 Klaus Villemoes for excellent technical assistance.

354 Funding for this research was provided by:

355 The Danish Council for Independent Research, Natural Sciences (FNU), grant number: 11-106627;
356 The Villum Kann Rasmussen Foundation; The US Department of Agriculture Grant number: 2011-
357 67015-30688; The Danish National Advanced Technology Foundation, grant numbers: 047-2011-1
358 and 274-05-0535. Göttingen minipig embryos were kindly provided by Ellegaard Göttingen
359 Minipigs.

360

361 **Author Disclosure Statement**

362 The authors declare that they have no competing financial interests.

363

364

365 **References**

366

- 367 Ahn, K.S., Won, J.Y., Park, J.K., Sorrell, A.M., Heo, S.Y., Kang, J.H., Woo, J.S., Choi, B.H.,
368 Chang, W.K., Shim, H., 2010. Production of human CD59-transgenic pigs by embryonic germ
369 cell nuclear transfer. *Biochem. Biophys. Res. Commun.* 400, 667–672.
- 370 Al-Mashhadi, R.H., Sorensen, C.B., Kragh, P.M., Christoffersen, C., Mortensen, M.B., Tolbod,
371 L.P., Thim, T., Du, Y., Li, J., Liu, Y., Moldt, B., Schmidt, M., Vajta, G., Larsen, T., Purup, S.,
372 Bolund, L., Nielsen, L.B., Callesen, H., Falk, E., Mikkelsen, J.G., Bentzon, J.F., 2013. Familial
373 hypercholesterolemia and atherosclerosis in cloned minipigs created by DNA transposition of
374 a human PCSK9 gain-of-function mutant. *Sci. Transl. Med.* 5, 166ra1.
- 375 Bassols, A., Costa, C., Eckersall, P.D., Osada, J., Sabria, J., Tibau, J., 2014. The pig as an animal
376 model for human pathologies: A proteomics perspective. *Proteomics.Clinical Appl.* 8, 715–
377 731.
- 378 Callesen, H., Liu, Y., Pedersen, H.S., Li, R., Schmidt, M., 2014. Increasing efficiency in production
379 of cloned piglets. *Cell. Reprogram.* 16, 407–410.
- 380 Cao, S., Han, J., Wu, J., Li, Q., Liu, S., Zhang, W., Pei, Y., Ruan, X., Liu, Z., Wang, X., Lim, B.,
381 Li, N., 2014. Specific gene-regulation networks during the pre-implantation development of
382 the pig embryo as revealed by deep sequencing. *BMC Genomics* 15, 4.
- 383 Cheng, D., Guo, Y., Li, Z., Liu, Y., Gao, X., Gao, Y., Cheng, X., Hu, J., Wang, H., 2012. Porcine
384 induced pluripotent stem cells require LIF and maintain their developmental potential in early
385 stage of embryos. *PLoS One* 7, e51778.
- 386 Du, X., Feng, T., Yu, D., Wu, Y., Zou, H., Ma, S., Feng, C., Huang, Y., Ouyang, H., Hu, X., Pan,
387 D., Li, N., Wu, S., 2015. Barriers for Deriving Transgene-Free Pig iPS Cells with Episomal
388 Vectors. *Stem Cells* 33, 3228–3238.
- 389 Esteban, M.A., Xu, J., Yang, J., Peng, M., Qin, D., Li, W., Jiang, Z., Chen, J., Deng, K., Zhong, M.,
390 Cai, J., Lai, L., Pei, D., 2009. Generation of induced pluripotent stem cell lines from Tibetan
391 miniature pig. *J. Biol. Chem.* 284, 17634–17640.
- 392 Ezashi, T., Telugu, B.P., Alexenko, A.P., Sachdev, S., Sinha, S., Roberts, R.M., 2009. Derivation of

induced pluripotent stem cells from pig somatic cells. *Proc. Natl. Acad. Sci. U. S. A.* 106, 10993–10998.

Fan, N., Chen, J., Shang, Z., Dou, H., Ji, G., Zou, Q., Wu, L., He, L., Wang, F., Liu, K., Liu, N., Han, J., Zhou, Q., Pan, D., Yang, D., Zhao, B., Ouyang, Z., Liu, Z., Zhao, Y., Lin, L., Zhong, C., Wang, Q., Wang, S., Xu, Y., Luan, J., Liang, Y., Yang, Z., Li, J., Lu, C., Vajta, G., Li, Z., Ouyang, H., Wang, H., Wang, Y., Yang, Y., Liu, Z., Wei, H., Luan, Z., Esteban, M.A., Deng, H., Yang, H., Pei, D., Li, N., Pei, G., Liu, L., Du, Y., Xiao, L., Lai, L., 2013. Piglets cloned from induced pluripotent stem cells. *Cell Res.* 23, 162–166.

Fujishiro, S.H., Nakano, K., Mizukami, Y., Azami, T., Arai, Y., Matsunari, H., Ishino, R., Nishimura, T., Watanabe, M., Abe, T., Furukawa, Y., Umeyama, K., Yamanaka, S., Ema, M., Nagashima, H., Hanazono, Y., 2013. Generation of naive-like porcine-induced pluripotent stem cells capable of contributing to embryonic and fetal development. *Stem Cells Dev.* 22, 473–482.

Garrels, W., Mates, L., Holler, S., Dalda, A., Taylor, U., Petersen, B., Niemann, H., Izsvak, Z., Ivics, Z., Kues, W.A., 2011. Germline transgenic pigs by Sleeping Beauty transposition in porcine zygotes and targeted integration in the pig genome. *PLoS One* 6, e23573.

Hall, V.J., Hyttel, P., 2014. Breaking down pluripotency in the porcine embryo reveals both a premature and reticent stem cell state in the inner cell mass and unique expression profiles of the naive and primed stem cell states. *Stem Cells Dev.* 23, 2030–2045.

Higuchi, A., Ling, Q.D., Kumar, S.S., Munusamy, M.A., Alarfaj, A.A., Chang, Y., Kao, S.H., Lin, K.C., Wang, H.C., Umezawa, A., 2015. Generation of pluripotent stem cells without the use of genetic material. *Lab. Invest.* 95, 26–42.

Kawaguchi, T., Tsukiyama, T., Kimura, K., Matsuyama, S., Minami, N., Yamada, M., Imai, H., 2015. Generation of Naïve Bovine Induced Pluripotent Stem Cells Using PiggyBac Transposition of Doxycycline-Inducible Transcription Factors. *PLoS One* 10, e0135403. doi:10.1371/journal.pone.0135403

Lee, K., Redel, B.K., Spate, L., Teson, J., Brown, A.N., Park, K.W., Walters, E., Samuel, M., Murphy, C.N., Prather, R.S., 2013. Piglets produced from cloned blastocysts cultured in vitro with GM-CSF. *Mol. Reprod. Dev.* 80, 145–154.

Lelovas, P.P., Kostomitsopoulos, N.G., Xanthos, T.T., 2014. A comparative anatomic and physiologic overview of the porcine heart. *J. Am. Assoc. Lab. Anim. Sci.* 53, 432–438.

Li, J., Li, R., Liu, Y., Villemoes, K., Purup, S., Callesen, H., 2013. Developmental kinetics of pig embryos by parthenogenetic activation or by handmade cloning. *Reprod. Domest. Anim.* 48, 866–873.

Liu, Y., Li, J., Lovendahl, P., Schmidt, M., Larsen, K., Callesen, H., 2014. In vitro manipulation techniques of porcine embryos: a meta-analysis related to transfers, pregnancies and piglets. *Reprod. Fertil. Dev.*

Nethercott, H.E., Brick, D.J., Schwartz, P.H., 2011. Derivation of induced pluripotent stem cells by lentiviral transduction. *Methods Mol. Biol.* 767, 67–85.

Nichols, J., Smith, A., 2009. Naive and primed pluripotent states. *Cell Stem Cell* 4, 487–492.

Oback, B., 2009. Cloning from stem cells: different lineages, different species, same story. *Reprod. Fertil. Dev.* 21, 83–94.

Okita, K., Matsumura, Y., Sato, Y., Okada, A., Morizane, A., Okamoto, S., Hong, H., Nakagawa, M., Tanabe, K., Tezuka, K., Shibata, T., Kunisada, T., Takahashi, M., Takahashi, J., Saji, H., Yamanaka, S., 2011. A more efficient method to generate integration-free human iPS cells. *Nat. Methods* 8, 409–412.

Onishi, A., Iwamoto, M., Akita, T., Mikawa, S., Takeda, K., Awata, T., Hanada, H., Perry, A.C., 2000. Pig cloning by microinjection of fetal fibroblast nuclei. *Science* 289, 1188–1190.

- Petkov, S.G., Marks, H., Klein, T., Garcia, R.S., Gao, Y., Stunnenberg, H., Hyttel, P., 2011. In vitro culture and characterization of putative porcine embryonic germ cells derived from domestic breeds and Yucatan mini pig embryos at Days 20-24 of gestation. *Stem Cell Res.* 6, 226–237.
- Polejaeva, I.A., Chen, S.H., Vaught, T.D., Page, R.L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D.L., Colman, A., Campbell, K.H., 2000. Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* 407, 86–90.
- Schmidt, M., Kragh, P.M., Li, J., Du, Y., Lin, L., Liu, Y., Bogh, I.B., Winther, K.D., Vajta, G., Callesen, H., 2010. Pregnancies and piglets from large white sow recipients after two transfer methods of cloned and transgenic embryos of different pig breeds. *Theriogenology* 74, 1233–1240.
- Schmidt, M., Winter, K.D., Dantzer, V., Li, J., Kragh, P.M., Du, Y., Lin, L., Liu, Y., Vajta, G., Sangild, P.T., Callesen, H., Agerholm, J.S., 2011. Maternal endometrial oedema may increase perinatal mortality of cloned and transgenic piglets. *Reprod. Fertil. Dev.* 23, 645–653.
- Schmidt, M., Winther, K.D., Secher, J.O., Callesen, H., 2015. Postmortem findings in cloned and transgenic piglets dead before weaning. *Theriogenology* 84, 1014–1023.
- Secher, J.O., Freude, K.K., Li, R., Callesen, H., 2015. Optimization of Three-Dimensional Imaging on In Vitro Produced Porcine Blastocysts and Chimeras for Stem Cell Testing: A Technology Report. *Stem Cells Dev.*
- Secher, J.O., Ceylan, A., Mazzoni, G., Mashayekhi, K., Li, T., Muenthaisong, S., Nielsen, T.T., Li, D., Li, S., Petkov, S., Cirera, S., Luo, Y., Thombs, L., Kadarmideen, H.N., Dinnyes, A., Bolund, L., Roelen, B.A.J., Schmidt, M., Callesen, H., Hyttel, P., Freude, K.K., 2017. Systematic in vitro and in vivo characterization of Leukemia-inhibiting factor- and Fibroblast growth factor-derived porcine induced pluripotent stem cells. *Mol. Reprod. Dev.* doi:10.1002/mrd.22771
- Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676.
- Telugu, B.P., Ezashi, T., Roberts, R.M., 2010. The promise of stem cell research in pigs and other ungulate species. *Stem Cell Rev.* 6, 31–41.
- Telugu, B.P., Ezashi, T., Sinha, S., Alexenko, A.P., Spate, L., Prather, R.S., Roberts, R.M., 2011. Leukemia inhibitory factor (LIF)-dependent, pluripotent stem cells established from inner cell mass of porcine embryos. *J. Biol. Chem.* 286, 28948–28953.
- Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., Campbell, K.H., 1997. Viable offspring derived from fetal and adult mammalian cells. *Nature* 385, 810–813.
- Wu, Z., Chen, J., Ren, J., Bao, L., Liao, J., Cui, C., Rao, L., Li, H., Gu, Y., Dai, H., Zhu, H., Teng, X., Cheng, L., Xiao, L., 2009. Generation of pig induced pluripotent stem cells with a drug-inducible system. *J. Mol. Cell Biol.* 1, 46–54.
- Yuan, Y., Lee, K., Park, K.-W., Spate, L.D., Prather, R.S., Wells, K.D., Roberts, R.M., 2014. Cell cycle synchronization of leukemia inhibitory factor (LIF)-dependent porcine-induced pluripotent stem cells and the generation of cloned embryos. *Cell Cycle* 13, 1265–76. doi:10.4161/cc.28176

Figure and table legends

Figure 1 A. Isolation of G-PEFs used to generate 2i FGF pl-iPSC. Both G-PEFs and 2i FGF pl-iPSC were used for Handmade Cloning; B. Isolation of EGC which was genetically modified and used for Handmade Cloning; C. Isolation of (Venus) WL-PNFs used to generate 2i LIF DOX-iPSC. Both WL-PNF and 2i LIF DOX-iPSC were used for Handmade cloning; D. Overview of cells used for SCNT; E. Blastocyst percentage from the different cell lines

presented in a g-plot. * Other refers to reconstructed embryos that did not develop to blastocyst. Embryo transfer is abbreviated ET in the figure.

Table 1. A. Output from Procfreq SAS 5.1. showing number of replicates and calculations for blastocyst percentages; B. Results from Proclogistic from SAS 5.1. showing odds ratio for comparison of cell lines.

Figure 2. A & F 6 days old SCNT embryos from WL-PNF; B, 6 days old SCNT embryos from G-PEFs; G, 6 days old SCNT embryos from 2i FGF PI-iPSCs; C & H, 6 days old SCNT embryos from 2i LIF DOX-iPSC cultured in PZM3 (Group 1); D & I, 6 days old SCNT embryos from 2i LIF DOX-iPSC cultured in PZM3 with 10% FCS (Group 2); E & J, 6 days old SCNT embryos from 2i LIF DOX-iPSC cultured in PZM3 with 2µg/ml doxycycline (Group 3). Arrows indicate blastomers in developmental arrest. Scale bars are 100µm.

Figure 3. Pictures of the newborn SCNT piglets from WL-PNF. A & C, taken in day light; B & D, taken in ultraviolet light; C & D, piglet with macroglosia and green fluorescent eye (arrow) in daylight.

Figure 4: A, Healthy cloned SCNT offspring from EGC; B, Necropsy of euthanized piglet with tetraplegia showing hypoplasia of cerebellum; C, PCSK9-specific PCR performed on genomic DNA isolated from the eight SCNT piglets from EGC (id 1-8); D, Karyotype of the surviving SCNT EGC healthy female founder animal; E, qPCR showing liver-specific expression of PCSK9 in the surviving SCNT EGC founder animal.

Supplementary data

Figure 1. Southern blot detection of transgene integrations in the eight SCNT piglets from EGCs. Both short (panel A) and longer exposure of the blot (panel B) are shown due to high transgene copy number in one of the piglets (piglet 1). The transgene was visualized using a probe recognizing a 1-kb sequence in the puromycin resistance gene cassette. The id numbers (1-8) of the piglets are indicated above the lanes. Plasmid copy number controls were made by spiking genomic DNA from a WT Yucatan mini-pig with different amounts of the pSBT/ApoEHCR-hAATp-WT-PCSK9-FLAG plasmid for detection of 1 (C1) and 10 copies (C10) of the transgene. Genomic DNA from a WT Yucatan mini-pig was used as negative control (WT). All eight piglets were transgenic. The approx. 5 kb band observed for piglet 6, however, is indicative of random integration of the vector rather than transposition which may have occurred via a breakage point within the PCSK9 part of the vector. Arrows indicate the two transgene integrations in the surviving founder animal (piglet 2).

D

Cell line	Breed	Gender	Transgenic	Clonal
WL-PNF	Large White	Male	Venus	Poly
2i LIF DOX-iPSC	Large White	Male	Venus DOX-pOMSK	Poly
G-PEF	Göttingen	Male	-	Poly
2i FGF PI-iPSC	Göttingen	Male	-	Poly
EGC	Yucatan	Female	PCSK9	Poly

A

Cell type/No. of replicates	Blastocyst/reconstructed embryos	Blastocyst rate
WL-PNF/3	144/392	36.7%
2i LIF DOX-iPSC in PZM/5	13/122	10,7%
2i LIF DOX-iPSC in PZM with FCS/3	13/63	20,6
2i LIF DOX-iPSC in PZM with DOX/1	4/18	22,2
2i LIF DOX-iPSC/5	30/203*	14.7%
G-PEF/4	48/190	25.2%
2i FGF PI-iPSC/3	15/148	10.1%
EGC/2	51/148	34.5%

B

Cell type vs cell type**	Pr > ChiSq (proclogistic SAS)	Odds ratio (confidence limits) for blastocyst rate
2i LIF DOX iPSC vs WL-PNF	P<0.0001	0.205 (0.112;0.378)
WL-PNF vs G-PEF	P=0.006	1.718 (1.167;2.528)
2i LIF DOX-iPSC vs 2i FGF PI-iPSC	P=0.889	0.946 (0.432;2.073)
2i LIF DOX-iPSC without FCS vs 2i LIF DOX-iPSC with FCS	P=0.069	0.459 (0.198;1.061)
2i LIF DOX-iPSC without DOX vs 2i LIF DOX-iPSC with DOX	P=0.171	0.418 (0.119;1.061)
EGC vs 2i FGF PI-	P<0.001	4.61 (2.45;8.70)

iPSC		
EGC vs WL-PNF	P=0.587	1.12 (0.771;1.658)

*Pool of 2i LIF DOX-iPSC with and without doxycycline and fetal calf serum.**WL-PNF and 2i FGF PI-iPSC have been used as intercept but the model has not been extended to calculate the odds ratio for all the cell lines, as the model showed no difference between the remaining cell lines.