PHILOSOPHICAL TRANSACTIONS B

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Review

Cite this article: Abu-Dawud R, Graffmann N, Ferber S, Wruck W, Adjaye J. 2018 Pluripotent stem cells: induction and self-renewal. *Phil. Trans. R. Soc. B* **373**: 20170213.
http://dx.doi.org/10.1098/rstb.2017.0213

Accepted: 23 December 2017

One contribution of 18 to a theme issue 'Designer human tissue: coming to a lab near you'.

Subject Areas:
developmental biology, bioinformatics, genomics

Keywords:
iPSC, epigenetics, pluripotency, naive, primed, urine

Author for correspondence:
J. Adjaye
e-mail: james.adjaye@med.uni-duesseldorf.de

Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.4060463.

THE ROYAL SOCIETY PUBLISHING

Pluripotent stem cells: induction and self-renewal

R. Abu-Dawud[1], N. Graffmann[2], S. Ferber[2], W. Wruck[2] and J. Adjaye[2]

[1]Comparative Medicine Department, King Faisal Specialist Hospital and Research Centre, Zahrawi Street, Riyadh 11211, Saudi Arabia
[2]Institute for Stem Cell Research and Regenerative Medicine, Heinrich-Heine-Universität Düsseldorf, Moorenstr. 5, 40225 Düsseldorf, Germany

JA, 0000-0002-6075-6761

Pluripotent stem cells (PSCs) lie at the heart of modern regenerative medicine due to their properties of unlimited self-renewal *in vitro* and their ability to differentiate into cell types representative of the three embryonic germ layers—mesoderm, ectoderm and endoderm. The derivation of induced PSCs bypasses ethical concerns associated with the use of human embryonic stem cells and also enables personalized cell-based therapies. To exploit their regenerative potential, it is essential to have a firm understanding of the molecular processes associated with their induction from somatic cells. This understanding serves two purposes: first, to enable efficient, reliable and cost-effective production of excellent quality induced PSCs and, second, to enable the derivation of safe, good manufacturing practice-grade transplantable donor cells. Here, we review the reprogramming process of somatic cells into induced PSCs and associated mechanisms with emphasis on self-renewal, epigenetic control, mitochondrial bioenergetics, sub-states of pluripotency, naive ground state, naive and primed. A meta-analysis identified genes expressed exclusively in the inner cell mass and in the naive but not in the primed pluripotent state. We propose these as additional biomarkers defining naive PSCs.

This article is part of the theme issue 'Designer human tissue: coming to a lab near you'.

1. Background

The life span of an organism is characterized by continuous development and maturation of early embryonic stages into later stages leading to adulthood. It is a dream to revert or at least halt the ageing process, which is often accompanied by devastating degenerative diseases. Contrary to the normal developmental path, Yamanaka and his colleagues reported in 2006 for the first time the successful reprogramming of adult mouse tail fibroblasts into pluripotent stem cells (PSCs) that closely resembled pluripotent embryonic stem cells (ESCs) derived from the inner cell mass (ICM) of a blastocyst [1]. In other words, they induced the early properties of 3.5-day-old embryos in adult cells, and at face value it seems as if the ageing process or at least maturation had been reversed. The significance of this ground-breaking achievement is that these so-called induced pluripotent stem cells (iPSCs) hold great promise for regenerative therapies that exceeds that of the ESCs, because iPSCs allow personalized medicine and bypass ethical concerns of destroying human embryos.

2. A historical perspective of cellular reprogramming

The foundation underlying the concept of totipotency, pluripotency and multipotency is based on the experiments described by Hans Driesch. In 1891, he showed that separating two-cell stage sea urchin embryos through vigorous shaking in calcium-free water resulted in two new whole embryos [2, pp. 61–63]. Later, in 1962, Sir John Gurdon succeeded in transferring the nuclei of small intestinal epithelial cells into enucleated unfertilized eggs and obtained tadpoles [3]. This

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demonstrated that the adult nuclei do contain the information for the development of tadpoles. Since then, numerous animals have been cloned using somatic nuclear transfer, including Dolly the sheep [4], thus substantiating that somatic nuclei do have the potential and plasticity to adopt other functional nuclear states. Moreover, these experiments suggested that the oocyte contains factors that change the nuclear state back into a totipotent state. Additional clues on totipotency and pluripotency that paved the way for the current protocols for induction of pluripotency were obtained from embryonal carcinoma cells (ECCs) and ESCs. ECCs were isolated in 1964 from teratocarcinomas [5]. When fused with somatic cells, they could reprogram them into a pluripotent state [6]. ESCs were established by isolating cells from the ICM of blastocysts in 1981 [7,8]. Eleven years later, embryonic germ cells (EGCs) were isolated from primordial germ cells [9,10]. All these cell types proved to be capable of reprogramming somatic cells upon fusion [11,12]. These experiments indicated that not only oocytes but also ECCs, EGCs and pluripotent ESCs contained 'reprogramming factors'. Investigation of these cell types enabled the identification and characterization of several key genes (e.g. *OCT3/4*, *NANOG*, *SOX2*, *SALL4*, *ESRRB*, *DPPA4*) associated with the pluripotent state [13–15].

Meanwhile, other groups dedicated their efforts to cell fate conversion studies, which did not employ somatic nuclear transfer or cell fusion, but overexpression of transcription factors. Myogenic transcription factor (MyoD) was the first transcription factor to be overexpressed to achieve a cell fate conversion, also called 'direct reprogramming' or 'trans-differentiation'. Overexpressed in fibroblasts, MyoD trans-differentiated the cells into myoblasts *in vitro* [16].

Many reports followed using various cell types and trans-differentiating them into other cell types (e.g. fibroblasts into hepatocyte-like cells or neural stem cells) [17–19]. It was concluded that in spite of the power of a single transcription factor to alter a specific cell identity, it is still limited within the boundaries of the germ layer of origin. It cannot direct a cell state to cross these boundaries unless overexpressed simultaneously with other key transcription factors.

Based on these insights and those obtained from the field of somatic nuclear transfer, Yamanaka and colleagues speculated that overexpressing key ESC-associated factors could convert somatic cells into PSCs. To identify key factors of the pluripotent state that might revert cell fate, they screened publically available databases of expressed sequence tags in somatic tissues and ESCs [13]. They referred to those expressed uniquely in ESCs as ESC-associated transcripts (ECATs). Among the genes they identified and studied are *NANOG*, *DPPA2*, *DPPA4* [13,20]. Finally, they chose 24 candidates that played important roles in ESCs or were highly expressed in pluripotent ESCs including ECATs. The strategy that they adopted was to use G418-resistant clones among *Fbx15*$^{neo/neo}$ mouse embryonic fibroblasts (MEFs) [1]. *Fbx15 (ECAT3)* is expressed in ESCs, and not in somatic cells [21], which means that ESCs and potential ESC-like cells would be resistant to G418, but not MEFs. They transduced the 24 candidate genes individually and as a cocktail. The individual transduction did not lead to any G418-resistant colonies. The cocktail transduction, however, generated 22 colonies. These colonies were similar to ESCs in terms of morphology, differentiation potential, expression profiles and epigenetic profiles. They named these cells iPSCs. To reduce the necessary factors, Yamanaka and his group eliminated some of them during further rounds of transduction and finally identified *Oct3/4*, *Sox2*, *Klf4*, *c-Myc* (OSKM) as essential and sufficient to generate iPSCs [1]. This combination is routinely referred to as the 'Yamanaka Cocktail'. A year later, Yamanaka and colleagues generated human iPSCs using the same cocktail [22]. Within the same year, James Thomson's group independently also reported the generation of human iPSCs, but using a different cocktail, namely *OCT3/4*, *SOX2*, *NANOG*, *LIN28*—referred to as the 'Thomson Cocktail' [23].

3. Delivery of the reprogramming factors into somatic cells

Ever since the identification of the Yamanaka and the Thomson reprogramming 'Cocktails', researchers have struggled to optimize their delivery into cells (table 1). In the very early protocols, retroviruses or lentiviruses were used as they allow efficient and stable transduction [22,23]. However, in both cases the transgenes integrate into the host genome, potentially inactivating tumour suppressor genes or activating proto-oncogenes. Here, the most critical of the reprogramming factors is c-MYC, an oncogene that can lead to tumour development. Fortunately, it can be substituted by the less-tumorigenic L-MYC [36].

iPSCs derived by integrative methods are not suitable for clinical applications due to the above-mentioned risks, and several non-integrative methods for reprogramming have been developed to circumvent these problems. Some involve quite tedious removal of the transfecting agent, for example sendai virus or transposon-based methods [25,28,30]. Others, such as transfection of minicircle DNA, pSin plasmid, RNA or protein, require repeated transfections of the reprogramming factors, inducing unwanted additional stress to the cells [26,27,32,35]. Episomal-based reprogramming is the most convenient and widely used protocol and iPSCs derived using this system have already entered clinical trials for macular degeneration [37].

Not introducing DNA at all increases the safety of reprogramming, but is usually less efficient. mRNA or proteins are quite unstable and require several rounds of transfection to successfully reprogramme somatic cells [32,35]. Apart from increased cell stress, another drawback of these methods is the fact that the stoichiometry of the reprogramming factors cannot be controlled, which is a crucial factor for successful reprogramming. To bypass this drawback, Yoshioka *et al.* [33] developed a synthetic polycistronic and self-replicating mRNA molecule containing all four factors. Here, one transfection is sufficient and the relative abundance of each factor can be modulated by changing its position within the RNA molecule. The synthetic RNA only remains in the cells as long as their innate immune reaction against it is suppressed. The latest development in reprogramming techniques focuses on a method where the reprogramming factors do not even have to enter the cells. In this regard, Blanchard *et al.* [38] recently described antibodies that are able to stimulate intracellular pathways that ultimately activate the same target genes as the reprogramming factors. However, so far it has not been possible to simultaneously replace all four factors by antibodies.

4. Improving the efficiency of inducing pluripotency

iPSC derivation is a very inefficient process with several bottlenecks. To identify these, we previously analysed

Table 1. Methods for delivering the reprogramming factors.

	reprogramming factor delivery	considerations	references
integrative	retrovirus (ssRNA)	high efficiency integrates into genome: activation of proto-oncogenes/ disruption of tumour suppressor genes possible only infects dividing cells reactivation of viral genes possible	[22]
	lentivirus (ssRNA)	high efficiency infects also non-dividing cells integrates into genome: activation of proto-oncogenes/disruption of tumour suppressor genes possible reactivation of viral genes possible	[23]
not integrative	adenovirus (dsDNA)	low efficiency several rounds of infection necessary	[24]
	sendai virus (ssRNA)	high efficiency replicating virus has to be removed from iPSCs by negative selection	[25]
	pSin plasmid	low efficiency several transfections necessary integrates occasionally	[26]
	minicircle DNA	low efficiency several transfections necessary integrates occasionally does not contain any bacterial genes	[27]
	piggyBac transposon	traceless excision possible integrates into genome at specific integration sites: some effect transcription units excision may affect endogenous piggyBac elements reintegration possible	[28,29]
	sleepingbeauty transposon	traceless excision possible transposase allows efficient removal of transposon integrates into genome at specific integration sites: some effect transcription units reintegration possible	[30]
	oriP/EBNA1 based episomal plasmids	low efficiency self-replicative	[31]
	synthetic modified mRNA	increasing efficiency with more elaborate mRNA synthesis methods repeated transfections necessary because of rapid mRNA degradation	[32]
	VEE RF-RNA	self-replicating RNA that can be easily eliminated	[33]
	miRNA	very low efficiency repeated transfections necessary because of rapid miRNA degradation	[34]
	cell penetrating peptide-coupled protein	very low efficiency repeated transfections necessary because of rapid mRNA degradation	[35]

pluripotency-associated gene regulatory networks and associated biological processes governed by OCT4, SOX2, KLF4 and c-MYC [39]. This analysis unveiled diverse processes such as innate immunity, response to free radical generation-reactive oxygen species (ROS), hypoxia, oxidative DNA damage, p53 activation, senescence, apoptosis, epithelial mesenchymal transition (EMT) and epigenetic modification impinging on the efficiency of iPSC derivation. Distinct cell permeable and non-immunogenic small molecules have been employed to improve the reprogramming process by modulating the above-mentioned biological and metabolic processes, signalling pathways and epigenetic modifications (table 2). These small molecules usually enhance the reprogramming efficiency and can sometimes even replace one of the

Table 2. Small molecule-based signalling pathway modulators and cytokines supporting efficient iPSC derivation.

small molecule	mechanism	function	references
5-azacytidine (AZA)	DNA methyltransferase inhibitor	promotion of reprogramming c-Myc replacement	[40]
8-Br-cAMP	human cAMP-dependent protein kinase activator	promotion of reprogramming	[41]
A83-01	TGF-β receptor ALK5/4/7 inhibitor	promotion of reprogramming	[42]
AM580	retinoic acid receptor a agonist	promotion of reprogramming	[43,44]
compound B6	AKT-mediated inhibitor of GSK3-b	promotion of reprogramming	[45]
compound B4	ALK4 inhibitor	promotion of reprogramming	[45]
compound B8	IP3K inhibitor	promotion of reprogramming	[45]
compound B10	P38 kinase inhibitor	promotion of reprogramming	[45]
DAPT	γ-secretase inhibitor	promotion of reprogramming	[46]
DZNep	histone methyltransferase EZH2 inhibitor	promotion of reprogramming	[47]
DNP	oxidative phosphorylation uncoupler	promotion of reprogramming	[42]
EPZ004777	DOT1 L inhibitor	promotion of reprogramming	[48]
fructose 2,6-bisphosphate	phosphofructokinase 1 activator	promotion of reprogramming	[42]
LiCl	GSK-3b inhibitor, LSD1 inhibitor	promotion of reprogramming	[49]
N-oxaloylglycine	prolyl-4-hydroxylase inhibitor	promotion of reprogramming	[42]
NaB (sodium butyrate)	HDAC inhibitor	promotion of reprogramming	[50]
nicotinamide	inhibition of the H3K79 histone methyltransferase DOT1 L	promotion of reprogramming	[51,52]
PD0325901	potent MEK1 and MEK2 inhibitor	promotion of reprogramming inhibits growth of non-iPS cell colonies	[53]
PS48	PDK1 activator	promotion of reprogramming	[42]
prostaglandin	cAMP agonists	promotion of reprogramming	[47]
quercetin	hypoxia-inducible factor pathway activator	promotion of reprogramming	[42]
rapamycin	mTOR inhibitor activating autophagy	promotion of reprogramming	[54]
RG108	DNA methyltransferase inhibitor	promotion of reprogramming Sox2 (with BIX) or Oct3/4 substitute	[55]
RSC133	DMNT inhibitor	promotion of reprogramming	[56]
rolipram	cAMP agonists	promotion of reprogramming	[47]
SAHA	HDAC inhibitor	promotion of reprogramming	[40]
SB431542	TGF-β receptor ALK5/4/7 inhibitor	promotion of reprogramming	[57]
SGC0496	DOT1 L inhibitor	promotion of reprogramming	[58]
SMER28	autophagy modulator	promotion of reprogramming	[59]
Tranylcypromine (Parnate)	lysine-specific demethylase 1 inhibitor	promotion of reprogramming	[57]
TSA	HDAC inhibitor	promotion of reprogramming	[40]
TTNPB	retinoic acid receptor ligand	promotion of reprogramming	[47]
VPA	HDAC inhibitor	promotion of reprogramming	[60,61]
Y-27632	ROCK inhibitor	promotion of reprogramming	[62]
2-Me-5HT	5-HT3 agonist	Oct3/4 substitute	[47]
BIX-01294	G9a histone lysine methyltransferase inhibitor	Oct3/4 substitute	[53,63]
D4476	mouse CK1 inhibitor	Oct3/4 substitute	[47]
Forskolin (FSK)	PKA activator, mouse cAMP agonist	Oct3/4 substitute	[47]

(*Continued*.)

Table 2. (*Continued.*)

small molecule	mechanism	function	references
04I1 (modifications 1-11)	unknown	Oct3/4 substitute	[64]
04I2 (modifications 1-21)	unknown	Oct3/4 substitute	[65]
OAC1	activates Oct3/4 and Nanog promoters	Oct3/4 substitute	[45,66]
OAC2	activates Oct3/4 and Nanog reporters	Oct3/4 substitute	[45]
BayK	L-channel calcium agonist	Sox2 substitute	[55]
CHIR99021	GSK-3 inhibitor Wnt pathway activator	Sox2 substitute	[60]
Dasatinib	Src family tyrosine kinase inhibitor	Sox2 substitute	[67]
iPY	Src family tyrosine kinase inhibitor	Sox2 substitute	[67]
LY-364947	TGF-b inhibitor	Sox2 substitute	[67]
PP1	Src family tyrosine kinase inhibitor	Sox2 substitute	[67]
Repsox (E-616452)	TGFβ receptor I kinase inhibitor	Sox2 substitute	[68]
Kenpaullone	GSK-3 and CDK1/cyclin B inhibitor	Klf4 substitute	[69]
AMI5	protein arginine methyltransferase (PRMT) inhibitor	Sox2, Klf4 substitute (with A-83-01)	[70]
Hh-Ag 1.5	Smo agonist activating MAPK and SHH pathways	Sox2 and Nestin induction	[59]
Oxysterol	sonic hedgehog signalling activator	Sox2, Klf4, and C-Myc substitute	[71]
Purmorphamine	hedgehog activator	Sox2, Klf4, and C-Myc substitute differentiation inducer to neural stem cells	[71,72]
Shh	sonic hedgehog signalling	Sox2, Klf4 and C-Myc substitute	[71]
(±) BayK 8644	L-type calcium channel agonist	increase in spontaneous beating frequency promotion of reprogramming maintenance of pluripotency	[73]
JNJ-10198409	PDGFR-a and PDGFR-b inhibitor	increase in spontaneous beating frequency differentiation inducer to cardiomyocytes	[74]
SU16F	PDGFR inhibitor	increase in spontaneous beating frequency promotion of reprogramming differentiation inducer to cardiomyocytes	[74]
ATRA	retinoic acid receptor agonist	differentiation inducer to extra-embryonic lineage	[75,76]
AS8351	epigenetic modifications modulation of a specific JmjC-KDM	differentiation inducer to cardiomyocytes	[77]
JAK inhibitor 1 (JI1)	JAK–STAT pathway inhibitor	differentiation inducer to cardiomyocytes	[78]
SC1 (pluripotin)	dual inhibitor of extracellular signal-regulated kinase 1 and RasGAP/inhibitor of the ERK1 and Ras-GAP signalling pathways	differentiation inducer to cardiomyocytes	[73,74]
SU5402	FGFR, VEGFR and PDGFR inhibitor	differentiation inducer to cardiovascular progenitor cells	[79]
Poly(I:C)	toll-like receptor 3 agonist	differentiation inducer to endothelial cells	[80]
Go 6983	broad spectrum protein kinase C inhibitor	differentiation inducer to neurons	[62]
I-BET151	BET bromodomain inhibitor	differentiation inducer to neurons	[81]
ISX9	neurogenic agent	differentiation inducer to neurons	[81]
LDN193189	BMP receptor ALK2 and ALK3 antagonists	differentiation inducer to neurons	[59,82]
Pifithrin-α (PFT-α)	p53 inhibitor	differentiation inducer to neurons	[83]
SAG	hedgehog activator smoothened agonist	differentiation inducer to neurons	[46]
SP600125	JNK inhibitor	differentiation inducer to neurons	[62]
Thiazovivin (Tzv)	selective ROCK inhibitor	differentiation inducer to neurons	[46]

pluripotency factors. However, for most of these molecules we do not know which off-target effects they might have, if they act directly or indirectly to repress or activate known targets and in which additional pathways they might be involved [84]. The advantages, on the other hand, are that small molecules are only needed temporarily and can be removed after successful reprogramming. In addition, they are standardized, cost-effective, and easy to handle and to synthesize [47,62].

5. Phases of pluripotency induction

Key events that were initially discovered in the reprogramming process included the gradual downregulation of *Thy1*, a fibroblast marker, followed by the activation of pluripotency markers like alkaline phosphatase, stage-specific antigen 1 (SSEA-1 mouse; SSEA3&4 human) and, later, the activation of endogenous *Oct3/4* and *Nanog* [85,86]. It has been demonstrated that c-Myc is responsible for the loss of somatic expression patterns, while the function of Oct3/4, Sox2 and Nanog lies in the induction of the pluripotency gene regulatory network, whereby *Sox2* expression occurs only very late in the reprogramming process [87]. Samavarchi-Tehrani *et al*. [88] dissected the reprogramming process in mice into three distinct subsequent phases: the initiation phase, the maturation phase and finally the stabilization phase. The initiation phase is dominated by an immediate mesenchymal-to-epithelial transition (MET) that is driven by bone morphogenetic protein (BMP) signalling and the induction of miRNA-200 family members [88,89]. However, Teshigawara *et al*. [90] showed that during human iPSC induction, the onset of MET occurs later and that it is preceded by *OCT3/4* activation. The authors generated intermediately reprogrammed stem cells (iRSCs), which, in contrast with partially reprogrammed iPSCs, can resume the reprogramming process depending on the cell density. The progression from the intermediate state to the iPSC state is through MET, which appears to be a cell cycle-dependent checkpoint leading to the primed state, rather than the naive state like in mice.

Following the initiation phase, the maturation phase is characterized by the activation of endogenous key pluripotency genes such as *Oct3/4*, *Nanog* and *Sall4*. It is noteworthy that only a subset of pluripotency genes is induced in the maturation phase. Finally, in the stabilization phase pluripotency markers such as *Lin28a*, *Dppa2*, *Dppa4*, *Utf1* and others are induced. It was recognized early on that silencing of the OSKM transgene is crucial for complete and successful reprogramming [91], to release the stabilization phase and express the full pluripotency network as well as its maintenance network. This is in agreement with the observation that key pluripotency-regulating factors such as *Oct3/4* and *Nanog* are dose-dependent and that their overexpression or depletion leads to differentiation [92–94]. In the maturation phase, the cells' (chromatin) state changes to such a degree that transgene expression is essential for colony growth. In the stabilization phase, transgene silencing is essential for consolidation and maintenance of the pluripotent state [95].

It seems that the transition from the maturation phase to the stabilization phase is critical and constitutes the bottleneck of iPSC generation. This limitation will have a direct impact on the percentage of partially reprogrammed cells *in vitro*. The stabilization phase relies on the successful activation of downstream factors of OSKM and on the silencing of the transgenes, which otherwise destabilize the pluripotent state [95]. The activation of downstream targets of OSKM factors is in line with a report from Jaenisch and his colleagues describing that somatic cell nuclear transfer (SCNT) fails when genes such as *Dppa1-5* and *Pramel 4-7* fail to be expressed [96]. The factors identified in both reports are the well-known key pluripotency-regulating markers *Oct3/4*, *Sox2*, *Nanog*, *Klf4*, *Sall4*, etc. as well as genes associated with the cytoskeleton, or involved in chromosomal architecture and segregation, which allow the maintenance of the PSCs [88]. Although few in number, there are increasing reports demonstrating that in addition to the rewiring of the genetic circuitry and epigenetic remodelling, cytoskeletal remodelling is also significant, occurring upon PSC differentiation, upon acquisition of pluripotency of reprogrammed somatic cells or during PSC maintenance [95,97,98].

Another class of factors associated with pluripotency and, hence, requiring reverse regulation upon iPSC induction are miRNAs. Anokye-Danso *et al*. [99] in 2011 were the first to show that the microRNA cluster miR302/367 is capable of reprogramming mouse and human fibroblasts into iPSCs, with miR-367 being involved in activation of OCT4 expression. In addition, miR-302b and miR-372 are involved in the induction of MET, thus promoting one of the first steps during reprogramming [100].

6. Epigenetic basis of cellular reprogramming

Developmental processes are particularly dependent on the coordinated and sequential regulation of genetic programmes, which are reliant on epigenetic mechanisms. PSCs are, in general, euchromatic and become increasingly heterochromatic with progressing cell fate commitment. Hence, the induction of iPSCs requires the reverse process. Several effective reprogramming cocktails have been developed using epigenetic modulators such as SAHA-PiPs [101], NaButyrate [102], Parnate [57], valproic acid (VPA) [41,103,104] or 5-azacytidine [40], all of which induce chromatin de-condensation and/or de-methylation, and thereby activate *Oct3/4*, *Nanog* and *Sox2* expression (table 2). Indeed, the de-methylation of the promoter regions of the endogenous key pluripotency markers such as *Oct3/4* and *Nanog* is essential to enable complete reprogramming [1,105–107].

It has now been widely accepted that the overall euchromatic state of developmental genes in PSCs is bivalent, harbouring active (H3K4me3) and inactive (H3K27me3) histone marks. They resolve into one or the other direction during differentiation according to the needs of the mature cell type [108,109]. Interestingly, it seems that in the naive ground state bivalent domains tend to be occupied by H3K4me3 and only gain the repressive histone mark upon differentiating into naive PSCs [110].

7. Mitochondrial bioenergetics and the induction of pluripotency

The ability of PSCs to self-renew in part occurs via the fine-tuning of pathways associated with cellular senescence, such as the p53 and the mitochondrial/oxidative stress pathways [111–113]. Interestingly, upon the induction of pluripotency in somatic cells, the mitochondria revert to a pre-implantation

embryo and ESC-like state. Mitochondria within PSCs, unlike somatic cells, lack well-defined cristae and generate lower levels of ATP but have an increased lactate production, thus implying a dependence on anaerobic respiration for energy supply and activation of the hypoxia-inducible pathway to overcome the decrease in mitochondria function [114].

8. Pluripotency—naive and primed

Mouse ESCs (mESCs) were derived from the ICM of a blastocyst in 1981. However, it took more than 17 years until James Thomson and his colleagues were able to derive human ESCs (hESCs) in 1998 [115,116]. Such a delay would imply that the two species might have different PSC characteristics based on major mechanistic discrepancies. Indeed, mESC colonies are dome-shaped, while hESCs grow as flat colonies. mESCs depend on Lif signalling to activate the Jak/Stat3 pathway; this pathway seems dispensable for hESCs [117]. Although treatment of hESCs with LIF leads to STAT3 phosphorylation and nuclear translocation, this fails to maintain the pluripotent state. Instead, hPSCs require the addition of FGF2 as well as TGFβ and ACTIVIN A [115,118]. In due course, additional features were discovered to be different, such as X-chromosome activation/inactivation (XaXa/XaXi), methylation status, strength of pluripotency as measured by high versus low chimaera contribution ability, and sensitivity of primed PSCs to single cell dissociation [119–121].

It turned out that such discrepancies between mESCs and hESCs are attributable to different developmental stages, namely naive and primed, rather than species-specific differences. The most striking support for this new concept was provided by Tesar *et al*. and Brons *et al*. who isolated murine PSCs from the epiblasts (EpiSCs) from post-implantation embryos that shared defined features with human ICM-derived ESCs [122,123]. Since then, mESCs have been described as naive, and the mEpiSCs as primed, referring to their distinct developmental stages. This immediately triggered the quest for the human naive pluripotent state, raising the questions: what happens to the human pre-implantation ICM during the process of isolation? In other words, assuming that the human naive state exists, what induces its differentiation from the naive to the primed state upon isolation?

9. Pluripotent sub-states: naive and naive ground state

Austin Smith and his group identified another sub-state of naive pluripotency, which seems to have captured the core regulatory circuit of pluripotency and is, therefore, termed the naive ground state [124]. When cultured with Lif and with the two inhibitors of Mek/Erk signalling and GSK3ß signalling (2i-Lif), murine ESCs are not reliant on external signalling anymore, including JAK/STAT3 signalling. In addition, they do not express, as in the traditional culture medium containing serum and Lif, developmental and differentiation-associated genes, but rather genes related to glycolysis, lipid, vesicle biology and metabolism, which is in agreement with the Warburg-effect of pluripotent cells, and they appear more pluripotent as judged by higher contribution to chimerism [110,124].

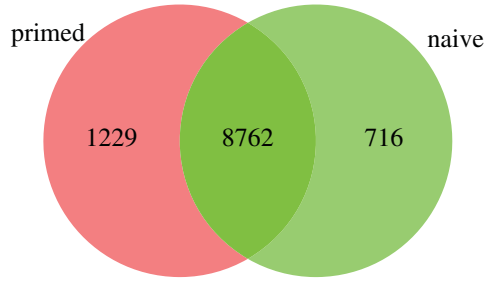
The significance of the Lif-2i culture condition is that it de-differentiates the naive PSCs even further, which are cultured in the Lif-serum conditions into naive ground state PSCs [110]. Naive ground state PSCs do not only exhibit higher chimera contribution, hypomethylation and distinct transcriptome and epigenetic signatures, but most importantly, the Lif-2i culture condition allows the derivation of mESCs from any mouse strain. The serum–Lif condition allowed the derivation only from the 129 strain. This strongly suggests that 2i-Lif allows the pluripotent state to be governed by its core regulatory circuits and is independent of any permissive strain-specific background mutations like in the mouse strain 129. Furthermore, the 2i-Lif culture conditions allowed for the first time the isolation of rat ESCs [125,126].

Indeed, Marks *et al*. [110] investigated the naive sub-states, naive and naive ground state of mESCs in both conditions, serum-Lif and 2i-Lif, respectively. The states are readily interconvertible upon culture medium change. They discovered that in comparison to the cultivation in 2i-Lif, many upregulated genes are involved in the differentiation of the different germ layers. The 2i-Lif, however, revealed the expression of many metabolism-associated and vesicle-associated pathways, in particular lysosome biology, indicating the existence of further unravelled mechanisms associated with the induction and maintenance of pluripotency.

While the murine naive pluripotent state *per se* and its sub-states are subjected to intense investigation, the human naive state has yet to be identified. It is currently being hunted and although several groups have reported to have captured the human naive pluripotent state [127–129], these different groups do not use the same cell culture conditions. This might or might not reflect the plasticity of the supposedly generated naive PSCs.

While at first glance it appeared that species differences account for the different characteristics of murine and human ESCs, which in fact was the difference between the naive and the primed state, it now appears puzzling why murine and supposedly human naive PSCs are very different in their culture condition requirements bearing in mind the astonishing similarity of murine and human primed PSCs. Therefore, it might be justified to expect that human naive stem cells resemble murine naive stem cells very closely, and hence the right culture conditions for robust human naive (ground) state derivation and maintenance have not been found yet. For instance, the murine naive ground state PSCs require 2i-Lif, which results in robust culture across laboratories. Also, new ESCs can be derived by the isolation of the ICM in this condition. So far, however, the culture conditions for human naive PSCs contain factors typical for the primed state such as Rock inhibitor, FGF2, ACTIVIN A or they require the transient and simultaneous overexpression of KLF2 or NANOG [127–132]. In the light of these very diverse culture conditions and the inclusion of factors that are required in the primed state, it seems as if the claimed human naive state is in fact still not fully dedifferentiated. Therefore, key questions remain unanswered: what are the authentic differences between the murine and the human blastocysts in terms of developmental timing? What happens to human ICM in contrast with the murine ICM upon isolation? Why does it appear to differentiate? One potential explanation could be the sensitivity to oxygen. Indeed, it has been reported that there is a reciprocal relationship between body size and oxygen tension in the fallopian

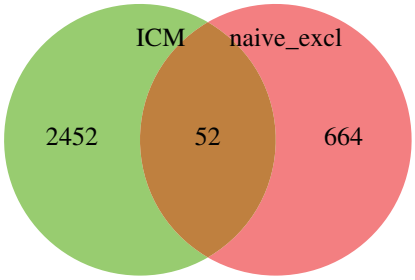
(*a*)



(*b*)

state	kegg_name	p_hyper	q_hyper
primed	focal adhesion	2.1106×10^{-6}	0.00024167
	gap junction	4.4357×10^{-6}	0.00025395
	cell adhesion molecules (CAMs)	0.00041865	0.00798915
	MAPK signalling pathway	0.00057629	0.00942651
	ECM–receptor interaction	0.00145788	0.01963849
	tight junction	0.00551917	0.05744958
	GnRH signalling pathway	0.00678598	0.06215957
	PPAR signalling pathway	0.00819604	0.06824865
	Wnt signalling pathway	0.00834481	0.06824865
	chemokine signalling pathway	0.01738823	0.12443454
naive	pathways in cancer	0.00130423	0.14978918
	glycosaminoglycan degradation	0.00195584	0.14978918
	phagosome	0.0019623	0.14978918
	pancreatic cancer	0.01617302	0.46295271
	B cell receptor signalling pathway	0.02205112	0.53488863
	hedgehog signalling pathway	0.02335758	0.53488863
	Fc epsilon RI signalling pathway	0.02768327	0.57631538
	adipocytokine signalling pathway	0.04830733	0.70485669
overlap	insulin signalling pathway	1.0784×10^{-7}	3.528×10^{-6}
	adherens junction	3.0365×10^{-7}	6.8023×10^{-6}
	metabolic pathways	3.2675×10^{-7}	6.8023×10^{-6}
	mTOR signalling pathway	0.00060744	0.00375953
	ErbB signalling pathway	0.00090692	0.00532527
	notch signalling pathway	0.00187819	0.01024062
	p53 signalling pathway	0.00224803	0.01169996
	TGF-beta signalling pathway	0.00350016	0.01669869
	MAPK signalling pathway	0.00886157	0.03623747
	Wnt signalling pathway	0.01095402	0.039817
	oxidative phosphorylation	0.01291352	0.04549534

(*c*)



(*d*)

naive_exclusive_and_ICM			
CRYBA2	MCF2L	ZFP28	LDHD
PACSIN1	PAPOLB	SLC34A2	MYO1G
PDCL2	GPNMB	C1QTNF3	PTGES
HIST1H2BA	CCL28	DBP	ITGAM
SPINK2	CTSF	SLC12A3	ACPP
MFI2	DDR2	DLX4	HYAL4
FGF18	XYLB	SLC39A4	PKD1L1
MX1	RPL10L	LTBR	FXYD4
RAB3IL1	KCNC4	TFEB	GJA10
SYNJ1	HES2	PCSK1N	BST1
KIF9	ZIC1	CDX1	CDH5
TNFSF9	EPO	HOXC13	CRYAB
CDX2	MSLN	LMO1	TEX11

Figure 1. A gene signature defining the naive state. Genes expressed in the primed and naive state in deep sequencing data from Takashima *et al.* [129] were compared (*a*) and the most relevant over-represented KEGG pathways exclusively in naive, primed and common in naive and primed are listed in (*b*). ECM, extracellular matrix. Genes exclusively expressed in the naive state were compared to genes expressed in the inner cell mass (ICM) from Adjaye *et al.* [134] (*c*) and are proposed as the marker signature of the naive state (*d*).

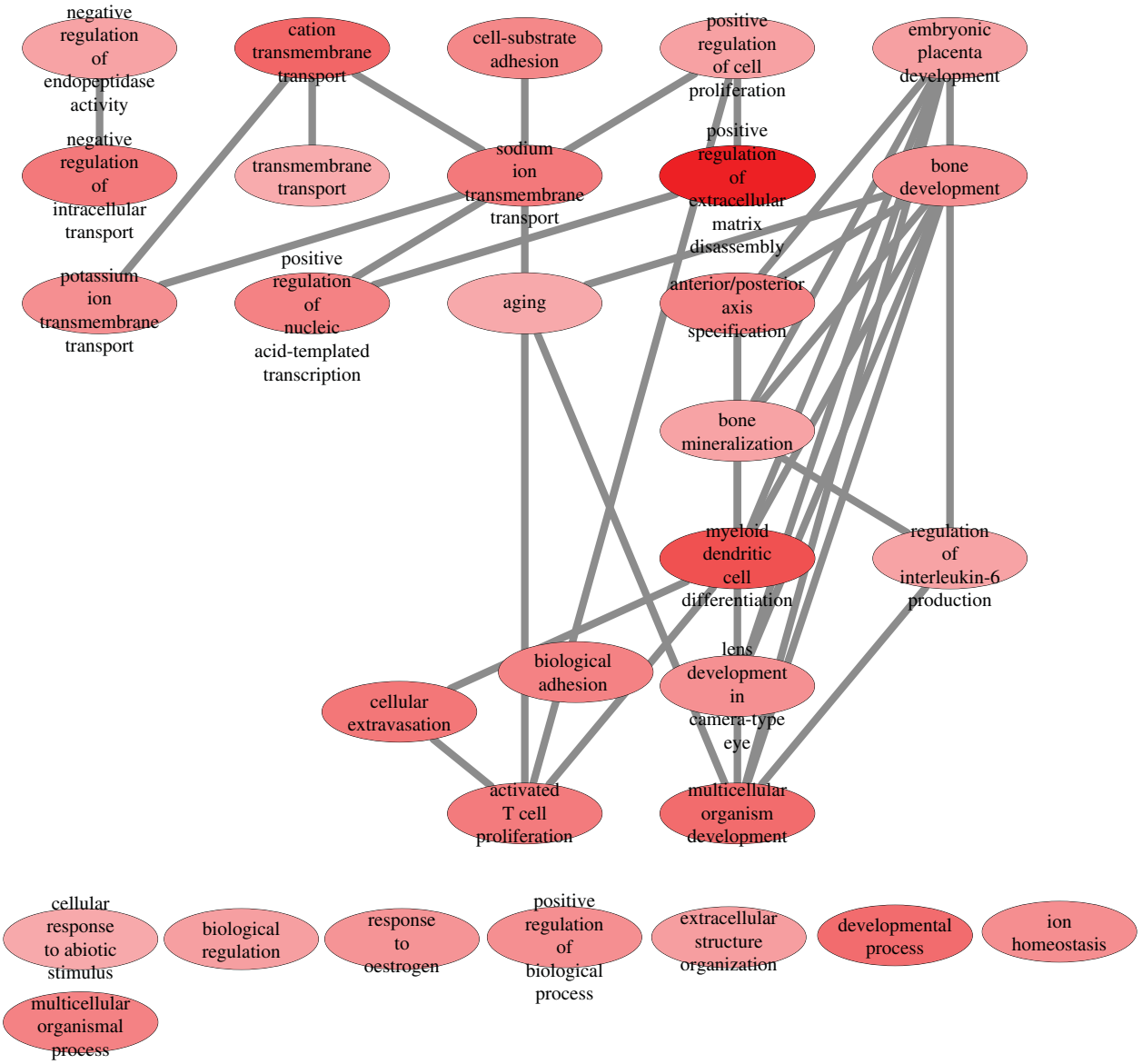


Figure 2. Gene ontology (GO) network of the gene signature defining the naive state. Significantly over-represented GOs for the naive state gene signature were determined via the R package GOstats [137] and summarized to a network via the REVIGO tool [138] and Cytoscape [139]. The colour reflects the significance of over-representation of a GO term. The darker the red of a GO term node, the lower is its *p*-value. GO terms determined as highly similar by the REVIGO tool are connected. In this network, *embryonic placenta development* emerges as a major hub connecting many other developmental GO terms such as *bone development*, lens development in the camera-type eye and *multicellular organism development*. (Online version in colour.)

tubes and uteri of monkeys, rabbits and hamsters [133]. As there is a huge difference in body size in mice and human, one possible explanation could be that the murine embryo is not affected upon ICM isolation *in vitro*, because it was exposed to higher oxygen tension in the murine reproductive tract. By contrast, the human body size makes oxygen diffusion more difficult, resulting in decreased oxygen tension, which could be a differentiation cue upon ICM isolation. Indeed, none of the present studies has applied oxygen tension below 5% [127–132].

10. Comparative meta-analysis of the transcriptomes of naive and primed pluripotent stem cells with isolated inner cell mass cells

To better understand the molecular basis of the naive ground state, we compared it to the ICM in the human blastocyst. For this comparison we used datasets of deep sequencing measurements from human ESCs H9 and 'reset' H9 from Takashima *et al*. [129]. Takashima *et al*. converted human ESCs to a state they call 'reset' via short-term expression of the transcription factors NANOG and KLF2. Reset cells can be kept continuously in a self-renewal state by inhibition of ERK and protein kinase C. We annotated H9 as 'primed' and reset H9 as 'naive'. Figure 1*a* shows a Venn diagram of genes expressed (FPKM: fragments per kilobase of transcript per million mapped reads > 1) in the naive and primed state in the data from Takashima *et al*. [129]. We identified 8762 genes as expressed in common in both states; 1229 genes were expressed exclusively in the primed state and 716 genes exclusively in the naive state. Figure 1*b* shows relevant KEGG pathways [135] over-represented—as calculated via the hypergeometric test—in these subsets of the Venn diagram. Within the overlap were—as expected—many pathways associated with pluripotency such as Wingless-Type MMTV Integration Site Family (WNT) and Notch signalling. In the exclusively primed subset, there is a

tendency for tight junction and adhesion-related pathways, while the naive exclusive subset was enriched with genes associated with cancer. In figure 1*c*, the 716 genes exclusively expressed in the naive state were further compared with genes expressed (background tag: percentile of background spot intensities greater than 0.95) in isolated ICMs [134]. Here, 2452 genes are expressed exclusively in the ICM, 664 exclusively in the naive state and 52 genes are expressed in both (electronic supplementary material, table S1).

Figure 1*d* shows the common signature of genes that we propose as putative and additional markers defining the naive state. Furthermore, associations with pluripotency or differentiation have been ascribed to some of the genes (e.g. *ZIC1*, *TEX11*, *FGF18*, *ZFP28*, *CDX1* and *CDX2*) [136].

The naive state gene signature can be further characterized by significantly over-represented gene ontologies (GOs) as shown in figure 2. These GOs were identified by employing the R package GOstats [137] and summarized to a network using the REVIGO tool [138] and Cytoscape [139]. GO terms in dark red have a greater significance for over-representation; highly similar GO terms are connected. In this network, embryonic placenta development emerges as a major hub connecting many other developmental GO terms such as bone development, lens development in the camera-type eye and multicellular organism development. Though speculative, these GO terms would imply that naive PSCs have a broader developmental potential than their primed counterpart. The full list of GO terms and corresponding genes is presented in electronic supplementary material, table S2.

11. Conclusion and summary

Since the initial publication describing the derivation of iPSCs from fibroblasts, several inroads have been made leading to an increase in our understanding of the biological, molecular and biochemical processes associated with the successful derivation of iPSCs. Distinct states of pluripotency—primed and naive—have been actively debated. To date the naive state of pluripotency, in the human in particular, is still 'work in progress' as there remains a lack of a well-defined robust protocol that works reproducibly across laboratories and somatic cell types, in contrast with the primed state.

The shift from retroviral and lentiviral to non-integrative (episomal-based plasmids) means of delivery of the reprogramming factors has been monumental and even enabled the transfer of iPSC technology to the clinic for treating macular degeneration [37]. We believe the quest now is to identify non-invasive means of obtaining a well-defined and characterized cell population permissible for reprogramming. In this regard, urine has been shown to be an ideal source. For example, retroviruses, episomal-based plasmids and sendai viruses in combination with small molecule-mediated pathway modulations have been employed to successfully derive iPSCs from a not well-defined cell population within urine [140–142]. As a benchmark, SIX2-and SSEA4-positive renal progenitor cells expressing the CYP2D6 *4/*17 variant and of known human leukocyte antigen were isolated from urine and reprogrammed using episomal-based plasmids omitting pathway (TGFβ, MEK and GSK3β) inhibition [143].

Data accessibility. This article has no additional data.

Authors' contributions. J.A. and R.A.-D. conceived the concept of this review. All authors contributed to the writing of the manuscript. N.G. and S.F. provided the tables. W.W. and J.A. performed and interpreted the meta-analysis and provided the figures. J.A. performed final edits and gave the final approval of the manuscript prior to submission.

Competing interests. We declare we have no competing interests.

Funding. J.A. acknowledges funding from the Medical Faculty, Heinrich-Heine-University Düsseldorf. N.G. is funded by the Research Commission of the Medical Faculty, Heinrich-Heine-University Düsseldorf. R.A.-D. is funded by the King Faisal Specialist Hospital and Research Centre Riyadh.

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