Reprogramming and Induced Pluripotent Stem Cells in Porcine

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Abstract

In 2006, Shinya Yamanaka successfully reprogrammed differentiated mouse somatic cells by ectopic expression of four transcriptionmodulators are needed for overcomin (Oct4, Sox2, Klf4, and c-Myc); this de-differentiation process (or induction of pluripotency) allowed somatic cells to regain the undifferentiated pluripotent state of cells. Those cells are termed induced pluripotent stem (iPS) cells. This work inspired many researchers to discover the underlying mechanisms, and soon iPS cells were reported in multiple species. It took about 3 years to produce iPS cells in porcine. Since 2009, about 70 or more research articles were on porcine iPS cells covering different aspects such as different approaches for derivation of iPS cells and their regenerative biology applications. In this chapter, we attempted to summarize the general concept of reprogramming and elaborated on the current status of porcine iPS cells and discussed the way forward for their future use.

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17.1 Introduction

Shinya Yamanaka and his group published their landmark research article on the induction of pluripotency in mouse fibroblasts in the year 2006 (Takahashi and Yamanaka 2006). In this chapter, the group reported the identification of four specific transcription modulators are needed for overcomin, namely, Oct3/4, Sox2, c-Myc, and Klf4 (also collectively designated as Yamanaka Factors in many recent articles) and demonstrated that after about 2 weeks of overexpression of these factors, mouse fibroblasts gained the features of embryonic stem (ES) cells. Embryonic stem cells are pluripotent stem cells with certain features: (a) they have the ability to self-renew in vitro; (b) they are capable of differentiating in vitro to tissues belonging to three main germ layers (ectoderm, endoderm, and mesoderm); and (c) when injected into developing embryo, ES cells contribute to the embryo proper. Cells produced from mouse fibroblasts in the Yamanaka Laboratory had similar growth, and colony features as that of ES cells had undergone differentiation and produced tissues of three germ layers. In addition, those ES-like cells were shown to produce teratoma when injected subcutaneous to immunocompromised nude mice and were proven to be germline competent. Therefore, Yamanaka termed these ES-like cells as induced pluripotent stem (iPS) cells (Takahashi and Yamanaka 2006). The importance of the work was immediately felt in the community of biomedical researchers. Such impact is evident by continual publications of a number of research articles in iPS cells (till date, PubMed database reports over 19,000 publications since 2006). Induction of pluripotency occurred in the Yamanaka experiment due to reprogramming in the somatic cells. Terminally differentiated somatic cells from adult tissues are usually incapable of multiplying. Still, reprogramming brings them to a developmentally early state, and they regain their ability to self-renew and differentiate. The Yamanaka Laboratory's work impacted the field in several ways. It reaffirmed the work on reprogramming by John Gurdon in the late 1950s (Gurdon et al. 1958), provided tangible tools for reprogramming using those specific transcription factors. The work also devised the method for the generation of patient-specific stem cells to produce the required tissues, and thus, opened up the era for personalized medicine, overcoming the immunological barrier of cell therapy. Also, screening of potential drugs using tissue-specific cells produced from cultured stem cells became a reality in regenerative medicine. It also paved a way to study developmental disorders inaccessible otherwise. In many ways, iPS cells were considered to be another alternative to ES cells. Generation of embryonic stem cells following traditional methods [such as by somatic cell nuclear transfer (SCNT)] is technically more elaborative, involved multisteps, far more complex, and highly challenging. The use of SCNT to generate ES cells also requires more resources than otherwise required for iPS cell generation. Thus, overexpression of a limited number of transcription factors in somatic cells has become an attractive and a pivotal tool for the generation of iPS cells, similar to ES cells.

Authentic ES cell lines could be established from developing blastocysts in a number of species such as mice, humans, and most domestic animals. Unlike those

species, currently available blastocyst-derived porcine cells do not strictly adhere to all the features that define authentic ES cells (Evans et al. 1990; Chen et al. 1999; Alberio et al. 2010). Those porcine ES cells were isolated and maintained following the same methodologies and culture conditions used previously for murine or human ES cells. It is suspected that the procedures possibly have never worked for the porcine species, and this has been considered as the most plausible cause for the failure of the derivation of authentic ES cells in pigs (Telugu et al. 2010). One way to deal with the issue is to find the porcine-specific methodology and derive the ES cells. However, it should take its own course of time to attain the target. In the meantime, the invention of the procedure to reprogram somatic cells to the pluripotent state became very much useful for porcine and other ungulates. Since the first report on mouse iPS cells in 2006 from the Yamanaka Lab, it took about 3 years to apply the same technique and to generate iPS cells in swine. There were simultaneous three publications in 2009: two from China (Esteban et al. 2009; Wu et al. 2009) and the other one from the United States (Ezashi et al. 2009). Those articles from different laboratories clearly demonstrated the universality and effectiveness of the Yamanaka methodology for the generation of iPS cells. Since then, several reports on porcine iPS cells were published from different laboratories. We discuss here briefly the genesis of reprogramming as a tool for iPS cell generation, mechanisms involved in the process of reprogramming. Further, we review the current status of available porcine iPS cells and their therapeutic/other applications.

17.2 Brief History Toward the Generation of Induced Pluripotent Stem Cells

Since the 1950s, seminal advances or breakthroughs of technologies have happened, and these have helped in the understanding of cellular/developmental biology deeper and better. This progress contributed immensely toward the generation of induced pluripotent stem cells. Few remarkable ones may be specially cited here. For example, the invention of the somatic cell nuclear transfer (SCNT) technique allowed the study of the cell's developmental potential (Briggs and King 1952; King and Briggs 1955). Also, the isolation of *pluripotent* embryonal carcinoma cells (ECCs) from a solid tumor of germ cells (teratocarcinoma) was another significant development. It was demonstrated further that the fusion of ECCs with fully differentiated somatic cells such as thymocytes enabled the somatic cells to gain the features of pluripotent cells (Miller and Ruddle 1976).

The next significant development in the field had been the discovery of *assay* determining the developmental potential of putative pluripotent stem cells and their ability to contribute to all three germ layers. This was achieved by injection of cells into blastocysts followed by transfer to foster recipients. Availability of this process also led to successful isolation of other pluripotent stem cells, that is, embryonic stem (ES) cells from mice (Evans and Kaufman 1981; Martin 1981) and human (Thomson et al. 1998) blastocysts. ECCs had abnormal karyotype, mostly aneuploid, but ES cells possessed normal karyotype and also capable of contributing to

all somatic lineages. Further, similar to ECCs, these pluripotent ES cells could be used for the production of fusion hybrids with somatic cells (Tada et al. 2001; Cowan et al. 2005). In these fusion hybrids, somatic cells acquired the biochemical and developmental potential of pluripotent ES cells. Experiments with fusion hybrids suggested the existence of identifiable soluble trans-acting factors in both ECCs and ES cells. The success of experiments with SCNT also demonstrated the presence of similar factors in the cytosol of the unfertilized oocyte, and these factors were capable of conferring pluripotency/totipotency to somatic cells (Wilmut et al. 1997).

Subsequently, improved techniques allowed isolation of a variety of pluripotent stem cells from different sources, such as cloned blastocysts produced by SCNT (Cibelli et al. 1998), and also from other embryonic and adult tissues (for references see Stadtfeld and Hochedlinger 2010). Each of these pluripotent stem cells of different origin expresses a common endogenous transcription factor, Oct4, a key molecule used by the Yamanaka group later for induction of pluripotency in somatic cells.

Long ago, it was established that the ectopic expression of a specific transcription factor might cause a change in cell/lineage fate. For example, the expression of skeletal muscle-specific transcription factor MyoD in fibroblasts resulted in the formation of myofibers (Davis et al. 1987). In this case, both fibroblast and myofibers belong to the same embryonic germ layer, that is, mesoderm. The same concept was extended to produce tissues of different lineage, currently known as trans-differentiation (Vierbuchen et al. 2010; Nizzardo et al. 2013; Cieslar-Pobuda et al. 2017; Flitsch and Brustle 2019), mostly reported after the publication of Yamanaka's 2006 iPS paper (Takahashi and Yamanaka 2006). Most importantly, the concept that transcription factor(s) may regulate cell fate had provided an "intellectual framework" (Stadtfeld and Hochedlinger 2010) and an invaluable contribution toward the generation of induced pluripotent stem cells. Then, the challenge was to identify those trans-acting factors relevant for somatic cell reprogramming.

The Yamanaka group took the challenge to identify those trans-acting factors, presumably involved in reprogramming of both somatic cells and nuclei, respectively, in fusion hybrids and SCNT experiments. To prepare the list of putative transcription factors, the group considered these criteria: (a) genes involved the maintaining pluripotency in early embryos, and ES cells are Oct4, Sox2, Nanog; (b) genes that are highly expressed in tumor cells, and also that contribute in long-term maintenance ES cell phenotype/involved in proliferation such as, Stat3, E-Ras, c-Myc, Klf4, and b-catenin; and (c) ES cell-specific genes such as Nanog, Sox2, and Sox15. Thus, the group tested a total of 24 transcription factors and finally identified four specific factors (Oct4, Sox2, Klf4, and c-Myc or OSKM) that were sufficient to induce pluripotency in somatic cells (Takahashi and Yamanaka 2006). This work, in turn, inspired several groups to investigate the underlying mechanism of reprogramming using these factors. Major breakthroughs that had outstanding contributions to the creation of Yamanaka are summarized in Table 17.1. Now, in the next section, we discuss briefly the basis of reprogramming using transcription factors that allowed the generation of iPS cells.

Table 17.1 Significant milestones in the field of reprogramming leading to the generation of induced pluripotent stem cells

Year	Report	Reference
1952–1955	Establishment of technique: somatic cell nuclear transfer (SCNT)	Briggs and King (1952), King and Briggs (1955)
1958	Sexually mature *Xenopus laevis* from the transplantation of a single somatic cell nucleus	Gurdon et al. (1958)
1962	Demonstration that differentiated amphibian cells retain the genetic information necessary to support the generation of cloned frogs	Gurdon (1962)
1954, 1964	Establishment of immortal pluripotent cell lines called embryonal carcinoma cells from a teratocarcinoma, tumors of germ cell origin	Stevens and Little (1954), Kleinsmith and Pierce (1964)
1967, 1970	Clonally expanded in culture while retaining pluripotency	Finch and Ephrussi (1967), Kahan and Ephrussi (1970)
1987	MyoD overexpression in fibroblast produced myofibers	Davis et al. (1987)
1968	Teratoma assay	R. L. Gardner (1968)
1981	Isolation of embryonic stem cells in mouse	Evans and Kaufman (1981), Martin (1981)
1996	Cloning of Dolly, the sheep, from somatic cells	Wilmut et al. (1997)
1998	Isolation of embryonic stem cells in human	Thomson et al. (1998)
1998	Transgenic bovine chimeric offspring derived from somatic cell-derived stem-like cells	Cibelli et al. (1998)
1998–2005	Identification of transcription factors related to self-renewal and pluripotency in ES cells and in teratocarcinoma cells	Oct4 (Nichols et al. 1998), Sox2 (Avilion et al. 2003), Nanog (Chambers et al. 2003), Stat3 (Niwa et al. 1998), c-Myc (Cartwright et al. 2005), beta-Catenin (Kielman et al. 2002), Klf4 (Li et al. 2005)
2006	Generation of induced pluripotent stem cells	Takahashi and Yamanaka (2006)

17.3 Basis of Reprogramming Using Genetic Factors

17.3.1 Role of Individual Factors in Reprogramming

To understand how Yamanaka factors (OSKM) induce pluripotency in somatic cells, investigators conducted different studies to identify the role of individual factors in reprogramming. Results of these studies reveal interaction and targets of these factors. These factors were found to co-bind to promoters of as many as 565 genes and were involved in regulating (for both repression and stimulation)

16 developmental signaling pathways (Liu et al. 2008; Huang et al. 2009). The four factors had overlapping targets in both iPS and ES cells. Among these factors, c-Myc was found to act prior to activation of pluripotency regulators (Sridharan et al. 2009), and c-Myc was also identified to facilitate engagement of the remaining three factors (Oct4, Sox2, and Klf4) at promoters of genes involved in reprogramming (Soufi et al. 2012).

Thus, out of four factors, three (Oct4, Sox2, and Klf4) were considered as core factors required to bind to activate the pluripotency network. Klf4 interacts directly with Oct4 and Sox2, and these interactions were sufficient to induce pluripotency in somatic cells (Wei et al. 2009). Recently, it is revealed that stoichiometry and cooperativity between Klf4 and Sox2 are necessary for activation of the pluripotency network (An et al. 2019). Furthermore, ectopic expression of all four factors brings about changes not only in the expression of target genes but also remodels chromatin state and genome topology in the cells during reprogramming (Apostolou and Stadtfeld 2018).

It is further found that Oct4 is also required for mesenchymal–epithelial transition (MET), a key event during reprogramming. The open chromatin is facilitated predominantly by Sox2 with an accessory role of Oct4. Oct4/Sox2 heterodimers (Malik et al. 2019) and relevant conformations for the establishment of pluripotency were identified (Tapia et al. 2015). Oct4 alone was sufficient to reprogram human somatic cells to iPS cells when used and some chemicals (Zhu et al. 2010). However, subsequently, iPS cells were generated either with three or two factors but without using exogenous Oct4 (Montserrat et al. 2012; An et al. 2019; Velychko et al. 2019). Cells generated using three factors (Klf4, Sox2, and c-Myc) were free from abnormal imprinting, usually found in iPS cells generated by using Yamanaka factors (Velychko et al. 2019). In summary, each of the factors plays a distinct role during reprogramming. It would be curious to look at how activities identified for individual factors play a role in the global or genome-wide context.

17.3.2 Genome-Wide Sequential Events for Establishment and Maintenance of Pluripotency by OSKM Cocktail

Data from genome-wide chromatin assay experiments, epigenomics, and transcriptomics studies collected during iPS cell generation reveal that the presence of OSKM inside somatic cells first erases differentiated cells' identity. It activates a set of genes needed for the establishment of pluripotency, and finally, reorganization of chromatin structure occurs for maintenance of pluripotency. For a fuller description, it is recommended to refer to Apostolou and Stadtfeld (2018) or to Hochedlinger and Jaenisch (2015). Here, a summary is presented in the entire process.

17.3.2.1 Somatic Program Silencing

It is now known that available OSKM factors' access to chromatin structure around somatic loci and, in turn, turns off somatic gene expression. OSKM can silence the

somatic program directly by (a) displacement of somatic transcription factors (TFs) and/or (b) recruitment of co-repressors, such as HDAC1. OSKM also can reposition/redistribute somatic TFs to newly accessible genome sites. Additionally, just OKS has the ability to activate a co-repressor, such as Sap30. Both these events (somatic TFs redistribution and activation of Sap30) can indirectly cause silencing of the somatic program (Apostolou and Stadtfeld 2018).

17.3.2.2 Stem Cell Program Activation

Initiation of the pluripotency program in the somatic genome is a rare and rate-limiting event. Here, in the absence of critical co-factors, OSKM is either unable or insufficient to bind to the genome, and therefore fails to induce activation of pluripotent genes. Those regions in the genome are "refractory" and thus do not provide access to OSKM. In addition, many stem cell-specific regulators are found in these "refractory" regions, and histone 3 (H3) chromatin protein of these regions is highly methylated in lysine at position 9 (H3K9). Experimental data suggest that downregulation of H3K9 methyltransferases (such as G9A, GLP, SETDB1, SUV39H1, and SUV39H2) improves the efficiency of reprogramming, indicating methylated H3K9 acts as a prohibitory signature against reprogramming. However, too much demethylation also affects reprogramming adversely. Intervention resulting in relaxation of the somatic chromatin enables increased accessibility and binding of SOX2 to pluripotency-related enhancers and super-enhancers. Evidence also strongly suggests that early binding of OSK (Myc to a lesser extent) occurs predominantly on "inaccessible" regulatory elements, including nucleosomal and DNA methylated regions. Further, the co-binding of OSK during early reprogramming activates target gene loci seemingly in a context-dependent manner. OSKM activity also depends on specific critical co-factors linked to chromatin remodeling (e.g., SWI/SNF subunits), epigenetic modulation (e.g., BRD4 and MLL), and factors involved in the release of the paused polymerase (e.g., CDK9, P-TEFb, etc.) (Apostolou and Stadtfeld 2018).

17.3.2.3 Re-organization of Chromatin Architecture

The chromatin topology of cells determines the morphology and functions of cells. During reprogramming, chromatin topology gets reset while somatic cells are induced to become pluripotent stem cells. Evidence suggests that chromatin loops are established around pluripotency-associated loci during reprogramming, and this usually precedes or coincides with transcriptional change. Thus, chromatin reorganization and timing is linked to changes in gene expression, supporting a potential causal role for reprogramming. These topological changes are brought about directly by OSKM binding to reorganized regions. OSKM may also be involved indirectly in histone modification such as H3K4me2, and such changes occur prior to conformational alterations. The role of OSKM in local and global chromatin reorganization would be clear by identifying the OSKM-dependent and OSKM-independent mechanisms during somatic cell reprogramming (Apostolou and Stadtfeld 2018).

It is to emphasize that methylation patterns of both histones and DNA are globally reset during reprogramming. Repressive histone modifications [e.g., histone H3 lysine 27 trimethylation (H3K27me3)] and DNA methylation are responsible for

the silencing of pluripotency genes (such as Oct4 and Nanog) in somatic cells. These repressive marks are replaced by the activating histone marks [such as H3 lysine four trimethylations (H3K4me3)], and also repressive DNA methylation signature in the promoter of Oct4 gene is removed in pluripotent cells. Thus, overall histone modifications and DNA methylation landscapes are restored in authentic iPS cells. Histone lysine demethylase, UTX, removes repressive H3K27 methylation, and WD repeat domain 5 (Wdr5) regulates the activating H3K4 methylation. In addition, the absence of maintenance methyltransferase (Dnmt1) (usually achieved by supplementation of 5′aza-cytidine in culture during derivation of iPS cells) improves the efficiency of reprogramming. However, the lack of activities of de novo methyltransferases (Dnmt3a and Dnmt3b) does not impact reprogramming efficiency. Overall, removal of DNA methylation promotes reprogramming, possibly via the release of repression of pluripotency genes such as Oct4 and Nanog (Hochedlinger and Jaenisch 2015).

Thus, many different kinds of changes take place in a short span of time during reprogramming, yet only a few cells attain the state of pluripotency. One, therefore, wonders how to follow these events during reprogramming and what might be the reasons that restrict the majority of cells to attain the state.

17.3.3 Kinetics of Molecular Signatures During Somatic Cell Reprogramming

Based on studies primarily in mouse fibroblast reprogramming, molecular signatures of some of the key events are currently known. It is now accepted that the expression of four factors is required till fibroblasts reach a stable, self-sustaining pluripotent state. There exist several intermediate stages between somatic fibroblast and fully reprogrammed pluripotent cells. Early intermediates are produced from fibroblasts ($Thy1^+/SSEA1^-$), and these cells at a certain initial point cease expressing Thy1and become $Thy1^-/SSEA1^-$ (Stadtfeld et al. 2008). Downregulation of somatic cell markers is accompanied by changes in cell morphology and occurrence of mesenchymal–epithelial transition (MET). MET is regulated by multiple factors: (a) BMP-dependent miR205 and miR200 family of microRNAs serve as key regulators (Samavarchi-Tehrani et al. 2010) and (b) role of key transcription factors: "Sox2/Oct4 suppress the epithelial–mesenchymal transition (EMT) mediator Snail, c-Myc downregulates TGF-beta1 and TGF-beta receptor 2, and Klf4 induces epithelial genes including E-cadherin" (Li et al. 2010). Subsequently, a subset of $Thy1^-/SSEA1^-$ cells starts expressing SSEA1, and gradually the population is enriched with $Thy1^-/SSEA1^+$ cells (Stadtfeld et al. 2008), expressing other early pluripotency markers such as alkaline phosphatase. Then, late pluripotency markers such as endogenous Sox2/Oct4 and Nanog are reactivated. This event is accompanied by the activity of telomerase enzyme and removal of silencing of X chromosomes (Li et al. 2010; Hochedlinger and Jaenisch 2015). These sequential events with key molecular markers are presented in a schematic diagram (Fig. 17.1a).

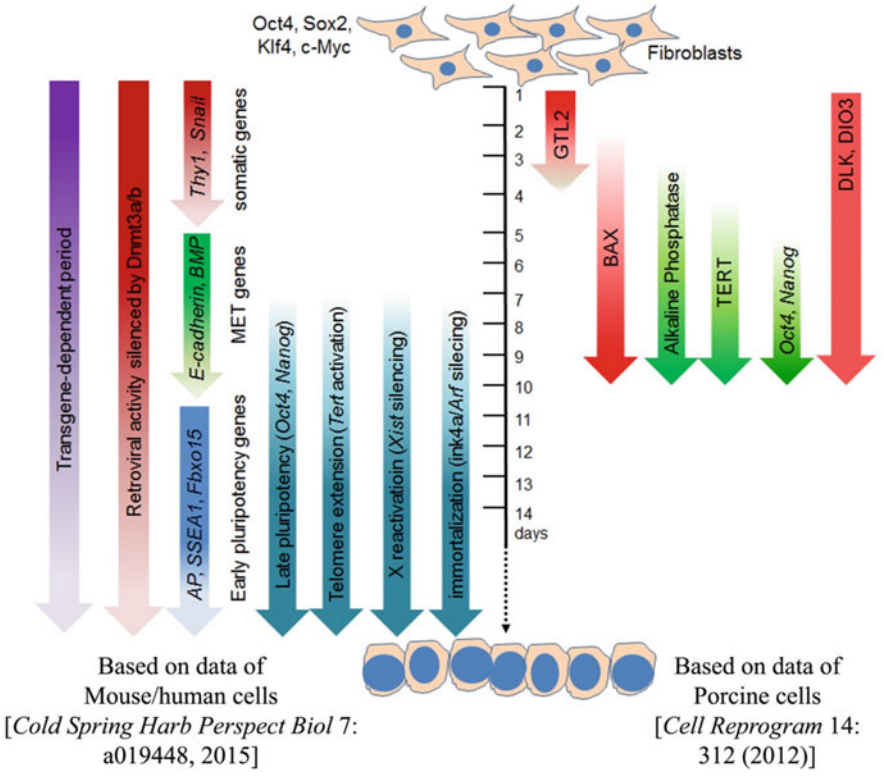

Fig. 17.1 Kinetics of reprogramming events and porcine-induced pluripotent stem cells. (**a**) Schematic diagram showing temporal expression of genes signifying transition of fibroblasts toward generation of induced pluripotent stem cells during reprogramming (left panel shows data from studies on mouse/human cells, right panel represents data from porcine ell reprogramming); (**b**) porcine-induced pluripotent stem cells produced from bone marrow-derived mesenchymal stem cells using doxycycline-inducible system regulating expression of lentivirally transduced four Yamanaka transcription factors

17.3.4 Elite and Stochastic Models for Induced Pluripotent Stem Cell Generation

It is now accepted that reprogramming of somatic cells occurs at a very low efficiency ranging between 0.001% and 0.01%. In order to explain the possible reasons, Yamanaka (2009) proposed two different models: deterministic and stochastic (Yamanaka 2009). The somatic cell requires to undergo a certain number of cell divisions, accumulating the required epigenetic changes to attain the state of pluripotency. In the deterministic model, the number of cell divisions (latency) is considered to be fixed or constant, whereas, for the stochastic model, this number varies from one to the other cells. Under each model, either all or a few selected elite cells would finally reach the state of pluripotency. It is often noted that a few cells in the founder population plated for reprogramming are less differentiated, and these cells are expected to reach the state of pluripotency with a lesser number of cell divisions. Further, within those few cells, the process of reprogramming is likely to be random. Thus, experimental data is consistent with the stochastic model, and the process is influenced by multiple factors like the differentiation stage of the founder cells (e.g., adult stem cells are more amenable than fully differentiated somatic cells), supplementation of other transcription factors (four factors versus six factors), chromatin regulators, growth factors (supporting the cell survival), and the interplay of microRNAs during reprogramming (Theunissen and Jaenisch 2014; Hochedlinger and Jaenisch 2015).

17.4 Porcine-Induced Pluripotent Stem Cells

The innovation of methodology for the generation of induced pluripotent stem (iPS) cells in mice soon resulted in the generation of iPS cells in other species. The different strategies employed could be broadly classified into two categories: (a) transgene-mediated reprogramming and (b) chemical reprogramming. The first approach included the use of transcriptional inducers (such as Yamanaka factors, Nanog, Lin28, Sall4, Glis1, Dppa2, Esrrb, Utf1), epigenetic inducers (Rcor2, TH2A, Tet1), miRNAs as inducers (miR-302, miR-367, miR-200c, miR-302s, miR-369s), and lineage specifiers (such as ectodermal or mesendodermal specifiers). On the other hand, in chemical reprogramming strategy, small-molecule inhibitors of different signaling pathways (such as TGF-b, GSK3, SAHH, and MEK), demethylases (Jhdm1a/b), chemicals that affect methylation (such as vitamin C), and histone deacetylase (HDAC) inhibitors [such as valproic acid (VPA)] were used. For details on all these approaches, including the replacement of classical Yamanaka factors, multiple articles are available (reviewed in Theunissen and Jaenisch 2014).

Having a standard protocol available, three different laboratories attempted to generate iPS cells in porcine. Each group described that porcine ES cells were not available (at the time of publication of their reports), and iPS cells would help meet up the need of porcine ES cells. In a close race, they reported their success between June and July of 2009 (Esteban et al. 2009; Ezashi et al. 2009; Wu et al. 2009). All

three groups used transcription factors of human origin; either six transcription factors (Yamanaka factors plus, Nanog, and Lin28) (Wu et al. 2009) or four Yamanaka factors (Esteban et al. 2009; Ezashi et al. 2009). The transcription factors were delivered to somatic cells [fibroblasts and bone marrow cells (Wu et al. 2009) or just fibroblasts (Esteban et al. 2009; Ezashi et al. 2009)] either by retroviral (Esteban et al. 2009) or by a lentiviral delivery system with (Wu et al. 2009) or without (Ezashi et al. 2009) using doxycycline for triggering the expression of exogenous factors. In fact, Wu et al. abandoned the retroviral system due to failure of transduction of pig ear fibroblasts and switched to a lentiviral delivery system (Wu et al. 2009). Putative iPS cells were cultured on mouse feeder cells in media supplemented with either LIF (Esteban et al. 2009) or FGF2 (Ezashi et al. 2009) or no additional cytokine (Wu et al. 2009). iPS cells produced by three groups expressed alkaline phosphatase, Nanog, and had high telomerase activity. The expression of Oct4 (Ezashi et al. 2009; Wu et al. 2009), Sox2 (Ezashi et al. 2009; Wu et al. 2009), Rex1 (Esteban et al. 2009; Wu et al. 2009), and SSEA-4 (Esteban et al. 2009; Wu et al. 2009) was found in cells from two laboratories. Uniquely, SSEA-3 expression was noted only in cells generated by Wu et al. (2009) and SSEA-1 in cells from Michael Roberts's laboratory (Ezashi et al. 2009). Based on the marker profiles, cells derived by the two Chinese groups were, to some extent, similar to human ES cells, and cells reported by the other group were identical to mouse ES cells. Cells from all laboratories were successfully differentiated to tissues of three germ layers both in vitro and in vivo (teratoma formation). Ezashi et al. reported sustained transgene expression even after differentiation of iPS cells (Ezashi et al. 2009). These authors envisaged the usefulness of porcine ES cells in generating gene-modified pigs, also to study certain human diseases or assess therapeutic applications as large animal models. Further, they can work as model systems for testing the safety and efficacy of stem cell-derived tissue grafts (Esteban et al. 2009; Ezashi et al. 2009; Wu et al. 2009).

Following these three major publications, as of date, a little over 70 research articles are available on porcine iPS cells. Here, we summarize the information available on these newly generated porcine iPS cells in terms of different aspects of reprogramming. Further, we also note here that the citation of literature presented in this article is not exhaustive; instead, it is aimed to reflect the significant progress in the field of research on porcine iPS cells.

Generation of porcine iPS cells reported by different laboratories was achieved using various methods, and each of those iPS lines had features of pluripotent cells to a certain extent but failed to generate stable germline competent chimera. Therefore, many of these iPS cells may not be genuine/authentic iPS cells. In developing the accurate iPS cells, obstacles encountered in the process could be numerous, such as (a) incomplete epigenetic reprogramming, (b) inferior culture conditions, and (c) use of varying serum and supplements, for example, cytokines, LIF, FGF2, epigenetic modifiers, and signal pathway inhibitor. To get an overall idea of these cells, here we discuss some of these issues briefly.

17.4.1 Choice of Reprogramming Factors

Yamanaka transcription factors (Oct4, Sox2, Klf4, and c-Myc) were most frequently used for the generation of porcine iPS cells (Cheng et al. 2012a; Fujishiro et al. 2013; Zhang et al. 2015; Park et al. 2016; Secher et al. 2017). Since Thomson's group reported successful generation of human iPS cells using Oct4, Sox2, Nanog, and Lin28 (Yu et al. 2007), these additional factors (i.e., Nanog and Lin28) were also combined with four Yamanaka factors giving a total of *six* factors for the generation of porcine iPS cells (West et al. 2010; Fukuda et al. 2017; Kwon et al. 2017). One of these iPS lines (produced with six factors and maintained in medium with FGF2 and on mouse feeder cells) was used successfully to create germline-competent chimeric pig (West et al. 2010, 2011), and this remained the only report demonstrating germline-competent pig iPS cells that produced adult animals without any additional treatment (such as differentiation of iPS cells prior to use as nuclear donor or treating nuclear-transferred embryos with HDAC inhibitor) (West et al. 2011). Porcine iPS cells were also produced using *seven* factors by adding the Large T antigen of the SV40 virus to six factors mentioned before (Telugu et al. 2010). Yamanaka factors plus Lin28 (Chakritbudsabong et al. 2017) or TERT (Gao et al. 2014) resulting in *five* factors were also successful in producing porcine iPS cells. Using *three* factors (Sox2, Klf4, and c-Myc), it was possible to generate porcine iPS cells, dispensing Oct4. Apparently, this was the first report of somatic reprogramming in any species without the overexpression, either directly or indirectly, of Oct4. Moreover, cells thus generated could be grown in a feeder cell-free culture system (Montserrat et al. 2012). Knocking down of Klf4 and c-Myc in iPS cells resulted in the loss of pluripotency (Liao et al. 2018a), indicating the crucial roles of those factors.

Small-molecule pathway inhibitors (such as PD0325901 and CHIR99021) were used to improve reprogramming and four Yamanaka factors (Zhang et al. 2015; Secher et al. 2017). PD0325901 is a selective, cell-permeable non-ATP-competitive inhibitor of the MEK/ERK signaling pathway, whereas CHIR99021 is a glycogen synthase kinase (GSK) 3 inhibitor. GSK3 is a serine/threonine kinase, a key inhibitor of the WNT pathway; therefore, CHIR99021 functions as a WNT activator. Both these inhibitors were also used for reducing the number of factors during the reprogramming of fibroblasts. For example, Liu et al. (2012) used only two factors (Oct4 and Klf4) and successfully generated iPS cells (Liu et al. 2012). Fibroblasts electroporated with episomal vectors containing Yamanaka factors resulted in more naïve iPS cells grown in a particular medium containing growth factors (FGF2, LIF) and inhibitors of MAPK14, MAPK8, TGFB1, MAP2K1, GSK3A, and BMP (Yuan et al. 2019).

Blocking MEK signaling enhanced the proportion of NANOG (indicative of the epiblast)-positive cells but did not prevent the segregation of GATA-4 (indicative of the hypoblast)-expressing cells in the inner cell mass (ICM). Interestingly, inhibition of FGF signaling reduced the number of ICM cells without altering the segregation of NANOG and GATA-4 cells, indicating FGF signaling's participation in the formation of the founders of the ICM.

Inhibition of MEK signaling combined with GSK3-beta inhibition and LIF supplementation to culture conditions helped pig iPS cells acquire naive pluripotency features (see detail on types of pluripotency in a later section). Pigs' iPS cells were characterized by the expression of STELLA and REX1, and increased in vitro germline differentiation capacity (Rodriguez et al. 2012). Thus, small-molecule inhibitors can be used to improve the homogeneity of induced pluripotent stem cells and help in the generation of germline-competent stem cells in swine.

Among the growth factors and cytokines, leukemia inhibitory factor (LIF) is of prime interest in stem cell biology. It is accepted that only the naive-type pluripotent stem cells can produce chimeric offspring, and these cells are LIF-dependent. LIF-dependent iPS cells were generated using either four Yamanaka factors (Fujishiro et al. 2013) or six factors (Yamanaka factors plus Nanog and Lin28) (Kwon et al. 2013), and those cells were shown to contribute to fetal development (Fujishiro et al. 2013).

Attempts were also made to find additional factors to substitute or to use in combination with the original Yamanaka factors. Estrogen-related receptor B (ESRRB), an orphan nuclear receptor, is one such factor. It is a direct transcriptional target of Nanog in mouse ES cells and can replace Nanog. Similarly, T box transcription factor, Tbx3, is another factor that improved germline competency of mouse iPS cells. Another nuclear receptor, Nr5a2 (also known as *Lhr-1* or liver receptor homolog-1), could replace exogenous Oct4 during reprogramming of mouse somatic cells. For reprogramming of porcine cells, the addition of ESRRB (Yang et al. 2018) and two other factors (Tbx3 and Nr5a2) (Wang et al. 2013) promoted reprogramming and self-renewal of porcine iPS cells, similar to the case with mouse cell reprogramming. The intracellular domain of epithelial cell adhesion molecule (EpCAM) enhanced reprogramming in porcine fibroblasts via activation of beta-catenin signaling (Yu et al. 2017).

Further addition of epigenetic modifiers such as *Tet1* (Ten-Eleven Translocation) to culture media during reprogramming could significantly enhance iPS cells' derivation with higher levels of expression of pluripotent genes such as *Rex1* (Mao et al. 2017). Long noncoding RNAs (lncRNA) were involved in the transcriptional regulation of somatic reprogramming to pluripotency (Zhong et al. 2018). The reprogramming of cells using miRNA could enhance the generation of porcine iPS. Overexpression of miR-302a, miR-302b, and miR-200c could make the reprogramming more efficient and faster. Therefore, it was suggested to replace c-Myc with these microRNAs (miR-302a, miR-302b, and miR-200c) to reduce porcine iPS tumorigenicity cells (Ma et al. 2014). Similarly, pluripotency could be induced in fibroblasts by epigenetic resetting with extract of porcine germinal vesicle stage oocytes (Bui et al. 2012), though factors present in the extract were not identified.

All in all, porcine-induced pluripotent stem cells were produced with two, three, four (original Yamanaka factors), five, six, or seven factors. One study attempted to dissect the implication of the generation of iPS cells using four (Yamanaka) factors versus six factors (Yamanaka factors plus Nanog and Lin28). Transcriptome analysis revealed that iPS cells, when derived with six factors, belonged to independent

clusters compared to those derived from four factors; those cells produced with six factors were distant from fibroblasts. Further, the expression of various naïve-specific genes was relatively elevated in pig iPS cells derived from six factors (Fukuda et al. 2019), indicating that six factors may preferentially be used for derivation of iPS cells.

Identification of critical transcription factors required for reprogramming also has a certain impact on the derivation of putative ES cells from a porcine blastocyst. In the laboratory of Michael Roberts, Oct4 and Klf4 were overexpressed in the porcine blastocysts, and then LIF-dependent naïve-type mouse ES cell-like cells were isolated from the inner cell mass (Telugu et al. 2011). The attempt carries enormous significance, specifically when authentic ES cells from porcine species are not available.

17.4.2 Choice of the Delivery System

Since the Yamanaka group used retroviruses for delivering Oct4, Sox2, c-Myc, and Klf4 (OSKM) to somatic cells, most groups used the same delivery system to generate porcine iPS cells (Fujishiro et al. 2013; Ji et al. 2013; Zhang et al. 2015; Chakritbudsabong et al. 2017; Mao et al. 2017). Lentivirus delivery system for generation of iPS cells also remained a popular method because of high transduction efficiency (Wu et al. 2009; Fukuda et al. 2017; Kwon et al. 2017; Luo et al. 2017). Pseudo lentiviral particles were suitable to deliver the required factors to a wide variety of cell types. To regulate expression stringently, transgenes were placed under the doxycycline-inducible system (Tet operator), and the same was primarily combined with the lentiviral delivery method (Wu et al. 2009; Luo et al. 2017; Secher et al. 2017), approaching a highly reliable one (Fig. 17.1b, unpublished data from the laboratory of the lead author). As transgenes delivered by retro/lentiviral systems get integrated into the genome, it was speculated to affect the biology of the cells, including disruption of genes involved in pluripotency/differentiation. Further, retroviral elements are considered tumorigenic, and these elements, when integrated into the genome, are known to be targeted for methylation and thereby silencing the transgenes.

Mobile genetic elements transfer DNA to the genome by transposing between vectors and chromosome via a “cut and paste” mechanism using transposase enzyme. Such systems were recognized as alternatives to viral transduction for delivering transgenes into the genome. Both piggyBac (PB) (with four Yamanaka factors) (Kim et al. 2019b) and sleeping beauty (SB) transposon vectors with either six (Petkov et al. 2013) or four Yamanaka (Kues et al. 2013) reprogramming factors were successfully used to deliver and finally to generate porcine iPS cells. Nuclei from porcine iPS cells derived by utilizing the piggyBac system were successfully used to produce transgenic embryos (Kim et al. 2019b). It may be noted that transposase enzymes transpose cargo containing transgenes into the target chromosome at TTAA and TATA site by PB and SB transposases. For SB vectors, CAG or EF1a promoters were adequate, but not with the TetO promoter (Petkov et al. 2013),

and Kues et al., used a single polycistronic construct with all four factors (Kues et al. 2013). Transfection of a CAG-driven polycistronic plasmid expressing Yamanaka factors showed higher efficiency and reprogramming compared with three consecutive retroviral transductions of a similar polycistronic construct (Montserrat et al. 2011). Though these iPS cells were generated without the presence of viral vectors, the transgenes were integrated into the host genome. One could have overexpressed specific transposases to remove the integrated sequences from iPS cells' genome, similar to that done with mouse iPS cells (Yusa et al. 2009). Another alternative to generate transgene integration-free iPS cells is to use episomal vectors. Reports indicate that integration-free porcine iPS cells were produced by using episomal vectors electroporated into pig fibroblasts (Li et al. 2018a; Yuan et al. 2019).

Overall, with the advances in technology, one has several options to choose a suitable delivery system. The specific purpose of the study and utility of the cells should be the prime consideration for determining the delivery system type.

17.4.3 Choice of Somatic Cells to Be Reprogrammed

Fibroblasts are highly active, and one of the most common cells of primitive mesenchyme origin found in connective tissue. These cells are proliferative and easily available from multiple sources, and primary fibroblasts are used in different kinds of biological experiments. For a generation of porcine iPS cells, fibroblasts were most frequently used from multiple sources such as embryonic (Fujishiro et al. 2013; Zhang et al. 2015; Chakritbudsabong et al. 2017; Fukuda et al. 2017; Mao et al. 2017), fetal (Petkov et al. 2013, 2016; Luo et al. 2017; Li et al. 2018a) or postnatal (Kwon et al. 2017) origin. But reprogramming was not highly successful with fibroblasts, and heterogeneity of fibroblasts was suspected to be the reason for the poor efficiency. Li et al. (2017) recently reported that stage-specific embryonic antigen 1 (SSEA1)-positive embryonic fibroblast in Danish Landrace and Gottingen mini pig had a better ability to generate iPS cells compared to SSEA1-negative fibroblasts (Li et al. 2017).

Other than fibroblasts, adult stem cells were also used as somatic donor cells for reprogramming. It was thought that owing to their stem cell characters, adult stem cells would be easier to reprogram. Thus, adult stem cells such as bone marrow-derived mesenchymal stem cells (MSC) (West et al. 2010) and adipose tissue-derived stem cells (ADSC) (Zhang et al. 2014) were successfully used for reprogramming. It was reported that reprogramming of iPS cells from porcine ADSCs was more efficient than from fibroblasts (Zhang et al. 2014). Similarly, for transcription factor-mediated reprogramming, the reprogramming efficiency of ADSCs-derived stem cells was significantly higher than fibroblast collected from embryo or adult ears (Li et al. 2018b). Besides, reports are also available to generate porcine iPS cells from other cell sources such as Sertoli cells (Setthawong et al. 2019) and pericyte (Xu et al. 2019).

Contextually, while planning to generate iPS cells, the availability and purpose of the experiment should get due importance. However, preference should be given to those cells that are known to be easier to undergo reprogramming.

17.4.4 Culture Supplementation with Special Reference to LIF and FGF2

Leukemia inhibitory factor (LIF) is a member of the interleukin-6 family of cytokines, most widely used for the maintenance of undifferentiated state and self-renewal of mouse ES cells (Smith et al. 1988). On the other hand, fibroblast growth factor 2 (FGF2) is a signaling molecule involved in many biological processes, including embryonic development, angiogenesis, and wound healing (Armelin 1973; Gospodarowicz 1974). FGF2 signaling was reported to maintain the growth of human ES cells (Eiselleova et al. 2009). Either LIF (Cheng et al. 2012a; Fujishiro et al. 2013; Zhang et al. 2015; Fukuda et al. 2017; Kwon et al. 2017; Secher et al. 2017) or FGF2 (West et al. 2010; Zhang et al. 2015; Secher et al. 2017) or both were supplemented for culturing porcine iPS cells. Like other species, LIF was identified to activate the transcription factor STAT3 and its target SOCS3 and stimulated cell growth in iPS cells generated in LIF supplemented medium, indicating the existence of a conserved functional signaling pathway across different species in mammals (Thomson et al. 2012). The requirement of LIF and FGF2 for reprogramming of porcine somatic cells was reemphasized but not essential for maintaining self-renewal and pluripotency.

A serum-free 3i medium containing three inhibitors CHIR99021 (GSK-3 inhibitor; acts as Wnt activator), PD0325901 (MEK/ERK inhibitor), and SB431542 (a selective inhibitor of endogenous activin and TGF-β signaling, but has no effect on BMP signaling), plus three cytokines (BMP4, SCF, and IL-6), and human platelet lysates was reported that successfully rescued flattened primed iPS cells to naïve-like cells. This medium maintained the culture for a long-term culture without the use of LIF or FGF2 (Ma et al. 2018), indicating that LIF/FGF2 could be replaced with supplementation of other factors, including different small-molecule inhibitors.

17.4.5 Culture of iPS with or Without Feeder Cells

Porcine iPS cells could be grown in a number of different basal media, such as DMEM/F12 (Secher et al. 2017) with a feeder (Mao et al. 2017) and also without feeder when used proprietary media such as Cellgro (Fujishiro et al. 2013) and mTeSR (unpublished data from the lead author's laboratory).

Is There Any Standard Method to Grow More Naïve-Type Porcine iPS Cells? Only limited data is available dealing with how to isolate and grow naïve porcine iPS cells. As stated in the previous section, the serum-free 3i medium could preferentially support the growth of naïve iPS cells (Ma et al. 2018). Recently, a detail of transcriptome analysis dealt with a similar issue. As per this report, porcine inner cell

mass was identified to have a unique pluripotency transcriptome, distinct from human and mouse ES cells. But it was shown to share more features with human naive-like than primed stem cell states (like an expression of KLF17 but not KLF2). Therefore, to activate specific signaling pathways important for porcine pluripotency, a suitable media was formulated. The media had DMEM/F-12 and Neurobasal medium mixed 1:1 supplemented with Glutamax, 2-mercaptoethanol, N2 supplement, B27 supplement, Pen-strep, human recombinant LIF, L-ascorbic acid, insulin-transferrin-selenium-sodium pyruvate (ITS-A), PD0325901 (MEK/ERK inhibitor), 1 μM CHIR99021 (GSK-3 inhibitor; acts as Wnt activator), Gö6983 (inhibits several isoforms of protein kinase C), and Y-27632 (inhibits both ROCK1 and ROCK2). This media supported human naïve stem culture. The same media improved the efficiency of reprogramming of porcine embryonic fibroblasts, and this culture condition could turn on the expression of important naive stem cell markers such as NANOG, KLF17, and CDH1 in porcine iPS-like cells (Habekost et al. 2019). For scaling up of porcine iPS cells, stirred suspension bioreactors could be effectively used (Burrell et al. 2019) and for improvement of efficiency of cryopreservation of these cells. ROCK inhibitor Y-27632 could be of help (Baek et al. 2019).

Thus, there is a number of media available for culturing porcine iPS cells. One should test and adopt the best one for derivation and propagation of cells.

17.4.6 Expression of Marker Genes in Porcine iPS Cells

Embryonic stem cells are characterized by a number of markers such as Oct4, Sox2, Klf2, Klf4, Rex1, c-Myc, E-cadherin, high level of telomerase, alkaline phosphatase, SSEA1, SSEA3, SSEA4, Tra-1-60, Tra-1-81, Rex1, and CDH1. Porcine iPS cells were reported to express some or most of these ES cell markers, with individual variations. Expression of stage-specific embryonic antigen (SSEA) surface markers remained a reliable tool to differentiate between mouse and human ES cells. Typically mouse ES cells are known to express SSEA1 but not SSEA3 and SSE4, whereas human ES cells express both SSEA3 and SSEA4, not SSEA1. For example, iPS cells derived for the first time in porcine by Wu et al. were shown to express alkaline phosphatase, Tra-1-60, Tra-1-81, Oct3/4, Nanog, Sox2, Rex1, CDH1, and both SSEA3 and SSEA4 (Wu et al. 2009). iPS reported from the Michael Roberts laboratory expressed Oct4, Nanog, Sox2, high telomerase activity, but lacked expression of SSEA3 and SSEA4. However, similar to mouse ES cells, these cells expressed SSEA1 (Ezashi et al. 2009). iPS cells that expressed all three SSEA molecules (detected by PCR) (Fujishiro et al. 2013) or that expressed only SSEA4, not the other two SSEA molecules (Esteban et al. 2009) or not SSEA1 (Thomson et al. 2012), were available too. Porcine iPS cells that expressed SSEA1 but not the other surface markers (such as Tra-1-60, Tra-1-81, SSEA3, and SSEA4) were reported by multiple groups (Kwon et al. 2013; Li et al. 2018a). iPS cells from a resource cell line were shown to express SSEA1 (Chakritbudsabong et al. 2017). Again, cells were also shown to express surface markers in combination such as SSEA4, TRA 1-60, and TRA 1-81 (Yang et al. 2013), or SSEA1, SSEA4, and

TRA-1-60 (Gu et al. 2014). The presence of high telomerase activity was described as a feature in a number of reports, such as of Ezashi et al. (2009) or of Fukuda et al. (2017).

Lastly, since each group chose to test and reported a specific set of markers, and each of the laboratories set up varied, it may not be easier to conclude if conflicting reports of marker expression were due to the cells' inherent characteristics or due to the variation in the setups. However, a panel of markers listed for ES cells was useful and would remain a guide to detect the status of porcine iPS cell marker expression.

17.4.7 In Vitro Lineage Differentiation of Porcine iPS Cells

In each publication, porcine iPS cells were routinely shown to undergo differentiation, producing cells of ectodermal, mesodermal, and endodermal (three germ layers) lineage. A more or less standard procedure is available to differentiate ES cells randomly, and in this procedure, ES cells are allowed to grow as free-floating cell aggregates in a medium without cytokines/growth factors such as LIF/FGF2. Those cell aggregates grow in small clumps/bodies and are termed embryoid bodies (EBs). Tissues of three germ layers are detected in these EBs, usually by PCR or immuno-techniques. By following the similar protocol, iPS cells were allowed to differentiate as EBs randomly, and subsequently, expression of different lineage-specific markers was detected in those EBs, signifying iPS cells, in general, could produce tissues of ectodermal, mesodermal, and endodermal lineage (Zhang et al. 2015). Under special culture conditions, myocardial differentiation of porcine iPS cells was reported, displaying beatings of embryoid bodies in culture (Chakritbudsabong et al. 2017).

However, these were performed as a part of standard procedures to show that porcine iPS cells were pluripotent cells. But in order to extend the usefulness of these cells, specifically for regenerative applications such as cells for transplantation and screening of drugs, lineage-specific differentiation of iPS cells would be required.

Recently, porcine iPS cells were differentiated to skeletal myotubes with coordinated approaches combining two inhibitors and ectopic expression of MyoD1 (Genovese et al. 2017). Using inhibitors of SMAD, TGF-beta, and BMP4, Kim et al. (2019a) developed an efficient method for the production of neural progenitor cells from porcine iPS cells, advancing the application of reprogrammed cells (Kim et al. 2019a). Also, porcine iPS cells underwent neural differentiation when EBs were treated with retinoic acid (Li et al. 2014). Among these limited studies, one study was remarkably reported from the laboratory of Steven L. Stice and Franklin D. West. The group demonstrated that SSEA4-positive porcine iPS cells (compared to SSEA4-negative cells) were more suitable for differentiation into beta III-TUB/MAP2+ neurons, GFAP+ astrocytes, O4+ oligodendrocytes, and motor neurons expressing both HB9 and ISLET1. This work established a link of expression of a specific marker (such as SSEA4) to iPS cells' propensity for a specific lineage differentiation (Yang et al. 2013). It needs to be seen whether SSEA4+ iPS cells

are refractory to differentiation to other lineages. Additionally, more such differentiation-associated iPS markers would help advance the field immensely.

17.4.8 Assay for Testing Developmental Potential

Like differentiation in vitro, pluripotent stem cells are tested in vivo for their ability to contribute to different lineages. This is generally done in more than one way, such as tetraploid complementation, teratoma formation, and chimera formation assays.

17.4.8.1 Tetraploid Complementation Assay

Tetraploid complementation assay is a technique where zonal pellucida-free one tetraploid embryo is electrofused with aggregates of pluripotent stem cells such as ES or iPS cells. Then, lineage development of the resultant tetraploid-pluripotent stem cells chimera is monitored for the contribution of each source. Usually, pluripotent stem cells contribute to fetus proper (ectoderm, endoderm, and mesoderm), and extraembryonic tissues (primitive endoderm and the trophectoderm come from tetraploid embryos). The chimera developmentally progresses if both compartments (embryonic and extraembryonic) of embryos complement each other appropriately (Tam and Rossant 2003). Tetraploid complementation assay is commonly used for testing the pluripotency of any cells, such as ES cells. Blastocysts complemented with mouse iPS cells forming tetraploid embryos were successful in producing viable, fertile, live-born progeny (Kang et al. 2009; Zhao et al. 2009). To our information, porcine iPS cells were used for chimera development by microinjection into blastocyst or morula (Cheng et al. 2012a; Fujishiro et al. 2013), but no such report is available on tetraploid complementation assay using porcine iPS cells.

17.4.8.2 Teratoma Formation with Porcine iPS Cells

Teratoma formation is a tool commonly employed for monitoring pluripotency in stem cell biology. It is applied to assess stem cells' ability to form tissues of three germ layers in vivo (Nelakanti et al. 2016). Teratoma is an encapsulated (or solid) tumor generally formed when iPS (or any pluripotent stem) cells are injected in immunocompromised mice (such as severe combined immunodeficient or SCID mice, lacking B and T lymphocytes). Once grown, tumors are collected, and tissue samples are processed by fixation, followed by staining with H&E (hematoxylin and eosin) dye or for immunodetection of different markers. The stained slide is examined for the presence of ectodermal, mesodermal, and endodermal tissues for ascertaining the differentiation ability of cells injected. Several reports indicated that porcine iPS cells are capable of forming teratoma with tissues of ectoderm, mesoderm, and endoderm lineages (Zhang et al. 2015; Secher et al. 2017; Li et al. 2018a). Cheng et al. (2012b) had success in obtaining teratomas in 8 weeks after injection of porcine iPS cells with SCID mice but had difficulty with nude (NOD-Balb/c) mice (Cheng et al. 2012b), indicating the importance of choosing background of immunodeficient mice for performing teratoma assay. Further, it was

also suggested that porcine iPS cells might take a longer time than mouse iPS cells (Ezashi et al. 2009; Cheng et al. 2012b).

17.4.8.3 Contribution of Porcine iPS Cells to Chimera Formation

The teratoma formation assay described above would indicate if a cell line retains the ability to undergo differentiation under in vivo system. However, the ultimate proof for pluripotency can be tested if those cells contribute to different organs of a growing body. In order to enable this, cells are injected into developing blastocysts and monitored for further development. Advancement in the growing embryos' developmental stages may be monitored with the fluorescent reporter expressed by the cells injected. Further, suppose those chimeric embryos are transferred to surrogates and allowed to complete the term. In that case, injected cells' contribution could also be monitored in animals born out of the procedure.

To our knowledge, to date, only the Stice laboratory succeeded in producing live germline-competent chimeric offspring using porcine iPS cells (West et al. 2010, 2011). Besides this, Fan et al. also produced live offspring using iPS cells as donors for nuclear transfer (NT). However, success was achieved only after silencing the exogenous transcription factors either through spontaneous differentiation of iPS cells before they are used as donor cells; or by treating the constructed embryos with Scriptaid (a novel histone deacetylase inhibitor) to increase histone acetylation (Fan et al. 2013).

Chimeric embryos were formed by using some of these naïve iPS cells, exhibiting fluorescent markers of iPS cell origin (Cheng et al. 2012a; Fujishiro et al. 2013; Secher et al. 2017). Alternatively, a somewhat less stringent test was done by injecting iPS cells into a parthenogenetic embryo and found that iPS cells could continue to contribute to the advancement of embryo growth (Zhang et al. 2015; Fukuda et al. 2017).

These data indicate that most of the laboratories failed to produce live chimera using porcine iPS cells. The reasons for failure were linked to the developmental and pluripotency status of iPS cells. Given the data from murine studies, it was thought that most of the porcine iPS cells were in a primed state of pluripotency, and therefore, they did not yield chimera. Now we look at the concept of the state of pluripotency in the context of porcine iPS cells.

17.4.9 Naïve Versus Primed iPS Cells

Austin Smith from the University of Cambridge introduced and elaborated the concept of two different states of pluripotency ("naïve" and "primed") in the field of developmental biology (Nichols and Smith 2009). This paradigm defined the potential of pluripotent cells isolated from early developing embryos. Naïve pluripotent stem cells are characterized by (a) formation of a compact dome-shaped colony in cell culture, (b) high plating efficiency of dissociated single cell, (c) maintenance requires LIF signaling, (d) BMP4 signaling regulates self-renewal, (e) FGF2/activin/nodal signaling pathway is involved in differentiation, (f) both X chromosomes

remain active in female cells, and (6) naïve cells are capable of forming chimera or even a complete animal and, therefore, these cells constitute competent germline cells (Telugu et al. 2010). Mouse ES cells from inner cell mass (ICM) fulfill all these criteria, and therefore, they are considered as ground state cells or authentic stem cells, or naïve stem cells. On the contrary, stem cells with a "primed" state of pluripotency have the following features: (a) flattened colonies, (b) low plating efficiency, (c) FGF2 signaling (not LIF signaling) maintains pluripotency, (d) BMP4 regulates differentiation, and (e) inactivation of X chromosome and silencing of paternally imprinted Dlk1-Dio3 regions. Mouse ES cells are designated as "primed" stem cells when they fail to generate chimera and lack germline competency. Primed stem cells usually represent cells from the epiblast. Human ES cells require FGF2 to maintain the pluripotency, and those cells are also considered epiblast stem cells or primed stem cells. Both naïve and primed cells undergo differentiation into tissues of three primary germ layers. But ICM-derived authentic or naïve stem cells are fully competent for germline transmission, whereas epiblast-derived primed stem cells have inferior potential and incompetent for germline transmission. A detailed comparison of stem cells with naïve and primed state of pluripotency is presented in Table 17.2.

Most porcine iPS cells reported in the literature are grown with FGF2 rather than with LIF-supplemented cell culture. These iPS cells either lack germline contribution or status not tested, hence unknown. Similar to human ES cells, these iPS cells are also considered to be "primed" or equivalent to cells from the epiblasts stage. One exception was reported where iPS cells were grown in the presence of FGF2 but still contributed to all three germ layers in a chimera (West et al. 2010), and those chimeric animals produced transgenic offspring in the next generation (West et al. 2011). However, it may be noted that those iPS cells were derived and maintained on inactivated mouse embryonic fibroblasts (MEF) feeder cells, and MEF are known to secrete LIF (Lee et al. 2009). Though not supplemented in the media, the availability of LIF from MEF might have maintained a naïve state of pluripotency, resulting in efficient germline-competent iPS cells and producing offspring. This would be interesting to check if other naïve cell criteria/features are found in these iPS cells generated in the Stice laboratory (West et al. 2010).

For testing germline competence in porcine iPS cells, one needs to generate chimera using advanced laboratory and animal facilities. An alternate ready reckoner to detect naïve iPS cells circumventing chimera generation would be useful. To some extent, lessons learned from mouse naïve cells should be of help in setting minimal criteria for determining the state of pluripotency in porcine iPS cells. LIF-dependent growth and expression of SSEA1 (most widely accepted markers of mouse ES cells) could be two prime criteria for detecting naïve iPS cells in swine. To the best of the literature, Telugu et al. attempted for the first time and generated LIF-dependent SSEA1-expressing naïve-type iPS cells that showed characteristics of mouse ES cells (Telugu et al. 2010). Another group also reported porcine iPS cells that met in vitro most criteria of naïve stem cells (such as LIF-dependent growth, negative MHC class I, active X chromosomes, and distinct gene-expression profiles)

Table 17.2 A comparison of naïve and primed iPS cells (Courtesy: Hochedlinger and Jaenisch 2015)

Characters	Naïve iPS cells	Primed iPS cells
Pluripotency state	ICM (inner cell mass) like	Epiblast like
Cell morphology	Compact, small	Large, flat
Colony feature	Dome-shaped	Flattened
Clonogenicity	High	Comparatively low
LIF-dependent to maintain	Yes	No
FGF2 dependent	No	Yes
Active signaling	Stat3	Activin/nodal
High telomerase activity	Yes	?
Short cell cycle interval	Yes	?
Normal karyotype	Yes	Yes
Contribution to chimera	Yes	No
Teratoma formation	Yes	Yes
Amenable to gene targeting	Efficient	Inefficient
X chromosome inactivation status in female cells	Both X chromosomes active (XaXa)	One X chromosome active (XaXi)
Silencing of imprinted genes	No	Yes
Apoptosis when injected in blastocyst	Slow/less	Rapid/high

(Fujishiro et al. 2013). However, it remained unknown if those cells could contribute to generate competent germline chimera.

The marker expression profile of iPS cells varies from report to report. A typical list of some markers for naïve porcine iPS cells includes alkaline phosphatase (AP) staining, expression of Oct4 (Hall and Hyttel 2014; Zhang et al. 2014), Sox2 (Zhang et al. 2014), Nanog (Hall and Hyttel 2014; Zhang et al. 2014), SSEA1 (Telugu et al. 2010; Gu et al. 2014; Hall and Hyttel 2014), SSEA3 (Zhang et al. 2014), SSEA4 (Zhang et al. 2014), and CRIPTO (Hall and Hyttel 2014); upregulation of Stella (Zhang et al. 2014) and Eras (Zhang et al. 2014); low expression levels of TRA-1-60 (Zhang et al. 2014), TRA-1-81 (Zhang et al. 2014), NrOB1 (Hall and Hyttel 2014), REX1 (Hall and Hyttel 2014); MHC I either low (Zhang et al. 2014) or absent (Fujishiro et al. 2013). In addition, naïve porcine iPS cells show LIF-dependency (Fujishiro et al. 2013; Zhang et al. 2014), activation of both X chromosomes (Zhang et al. 2014; Fukuda et al. 2017), normal karyotypes, compact dome-shaped colony, and growth after single-cell dissociation (Zhang et al. 2014). Further, those iPS cells contributed to embryonic and fetal development (Fujishiro et al. 2013). On the other hand, primed stem cells express OCT4 (Hall and Hyttel 2014), NANOG (Hall and Hyttel 2014), SOX2 (Hall and Hyttel 2014), KLF4 (Hall and Hyttel 2014), c-Myc (Hall and Hyttel 2014), REX1 (Hall and Hyttel 2014), CRIPTO (Hall and Hyttel 2014), and KLF2 (Hall and Hyttel 2014). It may be noted that some of these pluripotent markers were overlapping to both kinds of cells, and the list includes OCT4 (Hall and Hyttel 2014), NANOG (Hall and Hyttel 2014), SOX2 (Hall and Hyttel 2014), and CRIPTO (Hall and Hyttel 2014). Thus, it is

evident that both naïve and primed pluripotent stem cells express certain common pluripotent stem cell markers such as Oct4, Sox2, and Nanog.

Since naïve iPS cells have higher potentials compared to their primed counterparts, next comes how to improve pluripotency from primed to naïve state? An only a limited number of reports have dealt with the issue so far. Taking consideration of these publications, the following suggestions could be put forward:

1. One can possibly derive and maintain iPS cells in media supplemented with LIF (Telugu et al. 2010), or LIF and forskolin (Fujishiro et al. 2013).
2. Addition of 2i and LIF in the culture media. It was shown that the addition of two inhibitors (CHIR99021 and PD0325901) in a media containing LIF could support the growth of native-like porcine iPS cells under feeder-independent and serum-free conditions (Zhang et al. 2014), though the use of 2i in culture had also produced conflicting outcome during somatic cell reprogramming (Petkov et al. 2014).
3. Pig iPS cells were grown in LBX medium (LIF + FGF2 + knockout serum replacement + N2B27 supplement) had a small dome-shaped colony, expressed SSEA1, and cells from these colonies were more suitable as donor cells for NT to generate reconstructed embryos (Gu et al. 2014).
4. Additionally, it was demonstrated that treatment of parthenogenetic embryos with lysophosphatidic acid reduced expression of "primed" marker genes such as GATA4 (a marker of primitive hypoblast) in the early development of porcine parthenogenetic embryos (Zhu et al. 2018).

Whether all such approaches would be useful for converting primed iPS cells to their naïve state is yet to be tested. Nonetheless, the field would continue to hunt for finding fully potential naïve iPS cells in porcine.

17.4.10 Other Features of Porcine iPS Cells

In addition to the material presented above, porcine iPS cells also have other features, some of which are already reported for other species. Zhang et al. (2016) reported miRNAs in porcine iPS cells differentially expressed in comparison to embryonic fibroblasts. Multiple miRNAs such as ssc-miR-145-5p and ssc-miR-98 in porcine iPS cells were downregulated, whereas ssc-miR-217, ssc-miR-216, ssc-miR-142-5p, ssc-miR-182, ssc-miR-183, ssc-miR-96-5p, ssc-miR-106a, ssc-miR-363, ssc-miR-146b, ssc-miR-195, ssc-miR-497, ssc-miR-935, and ssc-miR-20b were reported to be upregulated (Zhang et al. 2016). Self-renewal of iPS cells was regulated by common miRNA–mRNA interactions (Zhang et al. 2017) and by the activin-SMAD signaling pathway (Yang et al. 2017). The m(6)A methylation via SOCS3/JAK2/STAT3 pathway was reported to regulate pluripotency of porcine iPS cells (Wu et al. 2019). Paternally imprinted Dlk1-Dio3 gene clusters were found to be aberrantly silent in most murine iPS cells, but in the case of porcine iPS, loss of expression followed by recovery of the clusters occurred.

In most porcine iPS lines, the maternally imprinted GTL2 gene became silent at a very early stage without recovery of expression (Cheng et al. 2012b).

Maintenance of genomic stability is an indicator of good quality cells. Instability in porcine iPS cells was linked to reduced DNA repair and replication capacity (Liu et al. 2017). Maintenance of porcine iPS-like cells for long-term culture, HDAC inhibitors (such as valproic acid, sodium butyrate, and suberoyanilide hydroxamic acid) was useful, but such treatment affected the differentiation ability of those cells (Petkov et al. 2016).

Incomplete reprogramming results in the generation of partially reprogrammed iPS cells, and the growth of these cells requires continuous expression of exogenous factors. In turn, it was demonstrated that the expression of endogenous counterparts was repressed by the sustained expression from exogenous sources (Hall et al. 2012), indicating that additional enhancing factors/modulators are needed for overcoming dependency of exogenous factors during reprogramming.

17.5 Therapeutic and Other Applications of Porcine iPS Cells

For therapeutic and regenerative applications, porcine iPS cells need to undergo rigorous evaluation of immunogenic properties, safety, and identification of the appropriate model. Only limited reports are available for addressing each aspect. Like other pluripotent stem cells, porcine iPS cells either do not express or express a low level of MHC class I molecule, and level of expression gets upregulated upon differentiation (Fujishiro et al. 2013; Park et al. 2013). A detailed study indicates that MHC-matched iPS cells can evade cellular and humoral immune responses but still susceptible to innate immunity in pigs (Mizukami et al. 2014). Therefore, it would be necessary to circumvent major players of innate immunity such as mast cells, macrophages, NK cells, and complement system.

Hence, porcine iPS cells are suggested to be useful for preclinical studies (Park et al. 2013). In one such experiment, functional vascular smooth muscle cells were produced by differentiation of swine iPS cells, and these cells formed readily 3D scaffold-free vascular tissue rings suitable for preclinical applications (Luo et al. 2017). For the first time, transplantation of porcine iPS cell-derived functional endothelial cells could improve cardiac function after myocardial infarction via paracrine activation in a rat model (Gu et al. 2012). Similarly, porcine iPS cells were differentiated to CD31+ functional endothelial cells using GSK3beta inhibitor and BMP4. This approach had potential benefits when evaluating autologous endothelial cell transplantation in pig models (Wei et al. 2020).

Apart from these, porcine iPS cells were used in bone and cartilage-related preclinical studies. Osteoblast-like cells generated from iPS cells could recover bone mass of tibiae in glucocorticoid-induced bone loss in two different animal models, rat (Liao et al. 2018c) and Lanyu pig (Liao et al. 2018b), signifying the usefulness of iPS cell-based therapy. Similarly, iPS-like cells were used successfully for cartilage regeneration in CLAWN miniature pig osteochondral replacement model (Uto et al. 2018).

Further, porcine iPS cells are suitable for generating disease models. Reprogrammed iPS cells were generated that overexpressed two proto-oncogenes, TGF-α, and c-Myc, driven by pig albumin promoter, and this method ensured restriction of transgene expression only in hepatic tissues. Further, these genetically modified iPS cells could be used as NT donors in vitro. These data make a step forward toward the generation of genetically modified pigs as a large animal model suitable for studies of liver cancer and treatment (Park et al. 2016).

Since the reprogramming process overhauls the entire genome landscape to generate iPS cells, the Tönjes laboratory checked if reprogramming had any impact on the expression of porcine endogenous retroviruses (PERV). It was reported that the reprogramming process impacted the expression of PERV in porcine iPS cells (Godehardt et al. 2018). For transplantation purposes, PERV-free somatic cells should be used for generating iPS cells. Pig shares similar anatomy and physiology to humans, and therefore, it is considered as a suitable donor for xenotransplantation in humans. Expression of alpha-1,3-galactosyltransferase (GALT) gene in porcine cells makes them rejected immediately after their transplantation in humans. Knocking out of this gene makes the cells somewhat tolerant, and it indicates the requirement of modifications in additional genes to ultimately make pig tissue immune-compatible with humans. A porcine iPS cell was generated from GALT-knockout fibroblast. It was envisaged that these cells would serve as a resource to dissect the complex phenomena of immuno-rejection, required for xenotransplantation, somatic cell nuclear transfer, or chimera formation (Liu et al. 2013). These are some important advancements toward making the iPS cell safe for preclinical and clinical applications.

Overall, porcine iPS cells attracted the attention of clinical researchers, but a long way to go for utilizing these newly developed cells as resources for clinical applications.

17.6 Concluding Remarks

The discovery of Yamanaka transcription factors for reprogramming has provided an opportunity to generate induced pluripotent stem cells from the pig in various ways in different laboratories. Pigs have contributed immensely to basic research, animal agriculture, and regenerative biology. IPS cells' availability has raised hope for generating transgenic animals for different uses, such as organ transplantation, a genetic model for human diseases, genetically modified animals resistant to certain diseases, and enhancing animal productivity (Cheng and Xiao 2009). Transgenic porcine iPS cells are also available (Liu et al. 2013; Park et al. 2016), and live animals from unaltered iPS cells have already been produced, though with limited success (West et al. 2010, 2011; Fan et al. 2013).

The majority of the porcine iPS cells are not germline competent, primarily due to incomplete reprogramming characterized by sustained expression of exogenous factors. The exogenous factors also prevent the iPS cells from undergoing the desired differentiation. This would remain a great challenge for using porcine iPS

cells in agriculture and biomedical sciences. Finding appropriate differentiation protocol and the generation of precise genome-edited animals need to be prioritized.

Most resources (in terms of tools and manpower) remain engaged in standardizing protocol for generating the porcine iPS cells. In two cases, live animals have been produced (West et al. 2011; Fan et al. 2013), but the achievements are not free from questions. For example, the generation of live animals by nuclear transfer of fully differentiated iPS cells (Fan et al. 2013) can be considered equivalent to the generation of live animals from nuclei of original fibroblast before undergoing reprogramming. Therefore, the generation of live animals could testify to the potential of fibroblasts as a nuclear donor, not of the iPS generated from those fibroblasts. This undermines the need for reprogramming. Further, treatment of reconstructed embryos with HDAC inhibitor to silence the transgene may have an indirect impact and not necessarily on the iPS cells used for cloning. This indicates that the field of iPS biology requires more work to make the technique practically useable.

To use the resources efficiently, the formation of a worldwide network in the form of a consortium may achieve the goal faster and aid in developing standard operating procedure (SOP) for every protocol needed and bank both wild and mutant porcine iPS cells. Consortium should also include finding novel porcine-specific reprogramming factors and reprogramming procedures without using vectors. In conclusion, the technology of reprogramming has a long way to go before harnessing the full potential of porcine-induced pluripotent stem cells in the future.

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