Induced pluripotent stem cells (iPSCs) are an attractive cell source for regenerative medicine and the development of therapies, as they can proliferate indefinitely under defined conditions and differentiate into any cell type in the body. Large-scale expansion of cells is limited in adherent culture, making it difficult to obtain adequate cell numbers for research. It has been previously shown that stirred suspension bioreactors (SSBs) can be used to culture mouse and human stem cells. Pigs are important preclinical models for stem cell research. Therefore, this study investigated the use of SSBs as an alternative culture method for the expansion of iPSCs. Using an established porcine iPSC (piPSC) line as well as a new cell line derived and characterized in the current study, we report that piPSCs can grow in SSB while maintaining characteristics of pluripotency and karyotypic stability similar to cells grown in traditional two-dimensional static culture. This culture method provides a suitable platform for scale-up of cell culture to provide adequate cell numbers for future research applications involving piPSCs.

**Keywords:** bioreactor, porcine, iPSC cells

**Introduction**

Pluripotent stem cells (PSCs) are defined by two main features: the ability to proliferate indefinitely in an undifferentiated state under defined conditions, and the potential to become any cell type arising from the three germ layers, endoderm, ectoderm, and mesoderm, in the body. Human and mouse embryonic stem cell (ESC) lines have significantly contributed to the progress in biomedical research and cell therapy development [1,2]; however, the ethical and safety concerns surrounding the use of human embryonic stem cells (hESCs) have limited their potential applications [3]. The emergence of induced pluripotent stem cells (iPSCs) as an alternative source of cells that recapitulate the phenotype and function of ESCs, helped overcome these concerns and energized the stem cell research field [4,5].

Outbred, large animal models that are closer in size, longevity, and physiology to humans are essential to expand our knowledge gained from rodents and facilitate translation of stem cell-based approaches to human applications. iPSCs have been derived from large ungulate species, including pigs that share many physiological characteristics with humans, making the pig a powerful model for biomedical research and drug testing [6].

Large numbers of cells are required for many research and therapeutic applications, which can be challenging to obtain through conventional two-dimensional static culture systems in tissue culture plates and flasks. Two-dimensional culture systems have limited surface area, often require a large number of culture vessels, and can be labor intensive. This additional handling can lead to cell loss and increased risk for contamination. Moreover, the use of numerous plates and flasks may introduce culture heterogeneity between plates and less consistency between cells. Stirred suspension bioreactors (SSBs) have been used to alleviate the problems associated with scale-up of two-dimensional static culture systems [7].

The purpose of this study was to establish an alternative, scalable culture method for the expansion of piPSCs using SSBs, based upon previous work involving human and murine PSCs [8–13]. In this study, we demonstrate that piPSCs from an established line and a newly generated line grown in static and SSB culture exhibit similar characteristics in terms of apparent doubling time, gene expression, and differentiation potential, illustrating the utility of SSBs as an alternative culture method for the expansion of piPSCs.
Materials and Methods

piPSC lines and culture media

The piPSCs (BT3p17) that constitutively express GFP [14] were generously donated by Drs. Telugu, Ezashi, and Roberts (University of Missouri). Briefly, these piPSCs were derived from inner cell mass cells of porcine embryos transduced with a tetracycline-inducible hKLF4 lentiviral vector. Single-factor piPSCs were further transduced using a tetracycline-inducible bicistronic lentiviral vector containing hKLF4 and hPOU5F1 (also known as hOCT4) [14]. The BT3p17 line was maintained in N2B27-3i medium as previously described [15] with slight modification [1:1 ratio of DMEM/F12 (D6421; Sigma) and Neurobasal medium (21103049; Gibco)], 0.5 × N2 supplement (17502048; Thermo Fisher), 0.5 × B27 supplement (17504044; Thermo Fisher), 1.000 U/mL human leukemia inhibitory factor (hLIF, L5283; Sigma), 3i cocktail [30 mM CHIR99021 (GSK3 Inhibitor, 130-103-926; Miltenyi), 40 mM PD0325901 (MEK Inhibitor, 130-104-170; Miltenyi), 10 mM PD173074 (FGFR3 Inhibitor, P2499; Sigma)], 0.5 × GlutaMAX (35050-061; Gibco) supplemented with 0.1 mM β-mercaptoethanol (M3148; Sigma), 1 × MEM nonessential amino acids (MEM NEAA, 11140050; Gibco), 0.01% bovine serum albumin (BSA) (A7906; Sigma), and 2 μg/mL doxycycline (DOX, D9891; Sigma). We will refer to these cells as LIF-independent piPSC line.

To test the utility of SSB culture on another cell line, a second piPSC line was generated for this study. Briefly, the cells were derived from dermal fibroblasts of a 2-year-old Hampshire pig (Kewanee Farm, Dudley, GA). The cells were transduced using viPS lentiviral vectors (Thermo Scientific, West Palm Beach, FL) that express human OCT4, NANOG, SOX2, LIN28, KLF4, and C-MYC open-reading frames under the control of the human elongation factor-1α promoter in the presence of 1.2% GeneJammer (a polyamine-based transfection reagent; Stratagene, La Jolla, CA). Cells were maintained in mTeSR1 medium ([14]) containing various other factors, recombinant human basic fibroblast growth factor (FGF) and recombinant human TGFβ3. We will refer to these cells as LIF-dependent piPSC line.

piPSC static culture

For static culture, 10^4 cells/mL from the initial pre-piPSC culture of LIF-dependent and LIF-independent cells were plated onto six-well culture plates coated with PDL/LN or Matrigel, respectively, with a full medium change at day 2 and passage at day 3 using Accutase. Each subsequent passage used piPSCs generated from previous static culture up to passage 8.

piPSC SSB culture

For SSB culture, 50 mL magnetically driven suspension bioreactors (Spinner Flask Assembly Complete, 264500-50; NDS Technologies, Inc.) were siliconized as per manufacturer’s instructions (SL-2; Sigma) and used with a working volume of 50 mL (Table 1 and Fig. 1). Fifty milliliters of SSBs were inoculated with 1.406 × 10^6 piPSCs from the pre-piPSC culture resuspended in 50 mL N2B27-3i medium or mTeSR1. The piPSCs were agitated at 104 rpm, corresponding to a maximum shear stress of 3.0 dyne/cm^2. The cells were passaged on day 3 using Accutase. Subsequent
TABLE 1. FIFTY MILLITERS STIRRED SUSPENSION BIOREACTOR AND MEDIUM PARAMETERS

| Parameter                  | Value | Units | Symbol |
|----------------------------|-------|-------|--------|
| Working volume             | 50    | mL    | VL     |
| Impeller width             | 0.95  | cm    | Wi     |
| Impeller diameter          | 3.5   | cm    | Di     |
| Vessel diameter            | 3.88  | cm    | Dt     |
| Medium density<sup>a</sup> | 1.006 | g/mL  | ρ      |
| Medium viscosity<sup>a</sup>| 0.85  | cP    | M      |
| Medium kinematic viscosity<sup>a</sup> | 0.008449 | cm<sup>2</sup>/s | v |

<sup>a</sup>Denote values used from previous study [18].

SSB cultures were inoculated using piPSCs generated from the previous SSB culture up to passage 8.

Apparent doubling time calculations

The piPSCs collected from the static culture and the SSB culture were counted at 72 h after the start of each passage. Apparent doubling times were calculated using Trypan Blue exclusion and the following formula [15]:

$$T_d = \frac{(t_2 - t_1) \log(2)}{\log(\frac{q_2}{q_1})}$$

(1)

where \(T_d\) represents apparent doubling time, \(q_1\) is the initial viable cell number at \(t_1\), and \(q_2\) is the final viable cell number at \(t_2\) (72 h). \(t_1\) (0 h) represents the initial inoculation time. Live cell counts were used for this calculation.

Cell viability calculation

Live and dead cells were counted based on the Trypan Blue exclusion, and cell viability was determined using the following formula:

$$CV = \left(\frac{C_L}{C_L + C_D}\right) \times 100$$

where \(CV\) represents cell viability as a percentage, \(C_L\) is the total live cells counted after 72 h that did not stain with Trypan Blue, \(C_D\) is the total dead cells counted 72 h after the start of every passage that did stain with Trypan Blue.

Shear stress calculation

Parameters, variables, and equations to calculate the maximum shear stress that the piPSCs may experience during SSB culture are listed in Table 1 and the following equations were used:

The maximum shear stress (\(\tau_{\text{max}}\)) that a single cell is exposed to, on the surface of an aggregate can be estimated using the following equations [16]:

$$\tau_{\text{max}} = 5.33 \rho (\varepsilon W)^{\frac{1}{2}}$$

where power dissipated per unit mass (\(\varepsilon\)) is calculated using the following equation:

$$\varepsilon = \frac{P}{V_L \rho}$$

\(P\) is representative of the consumed power (W) and can be estimated using the equation below [17]:

$$P = N_p N^3 D^5 \rho$$

Whereas the power number (\(N_p\)) can be estimated through the following equations [17]:

$$N_p = \frac{K_1}{R} + \frac{K_2}{(10^3 + 1.2K_3^{0.66})^{K_4}}$$

$$K_1 = 14 + \frac{W}{D_T} \left[670 \left(\frac{D_T}{D_T} \right)^{-0.6} + 185 \right]$$

$$K_2 = 10^{K_1}$$

$$K_3 = 1.3 - 4 \left(\frac{W}{D_T}\right)^2 - 1.14 \left(\frac{D_D}{D_T}\right)$$

$$K_4 = 1.1 + 4 \left(\frac{W}{D_T}\right)^2 - 2.5 \left(\frac{D_D}{D_T}\right)^2 - 7 \left(\frac{W}{D_T}\right)^4$$

The Reynolds number (Re) can be calculated using the following equation [18]:

$$R = \frac{(D_T^2) (N)}{\nu}$$

Immunocytochemistry analysis

piPSCs were collected from preculture, passage 8 (P8) static, and P8 SSB cultures, washed and fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature (RT). Cells were immobilized onto cytospin slides by centrifugation at 1,000 g for 5 min, and permeabilized with 0.1% Triton X (9410; Millipore Sigma) in PBS for 10 min. Permeabilized cells were washed with PBS, blocked with CAS block (008120; Life Technologies) at 4°C overnight. Cells were washed and incubated with secondary antibody (Supplementary Materials and Methods section) at 4°C for 15 min with CAS block, followed by primary antibody or isotype incubation for 25 min at RT. Cells were washed and

Flow cytometry

piPSCs from preculture, P8 static, and P8 SSB cultures fixed in 2% paraformaldehyde in PBS for 20 min at RT were prepared by permeabilization with 0.1% Triton X in PBS for 10 min. Permeabilization was not required to detect the surface antigen SSEA-4. Cells were washed and blocked for 15 min with CAS block, followed by primary antibody or isotype incubation for 25 min at RT. Cells were washed and
incubated with secondary antibodies for 20 min at RT in darkness. Cells were washed, resuspended in 400 μL of 1% BSA in PBS, and transferred to 5-mL round-bottom FACS tubes for analysis (FACSCalibur; Becton Dickinson).

**Spontaneous differentiation in embryoid bodies**

LIF-dependent piPSCs from preculture, P8 static, and P8 SSB cultures were collected as single cells, transferred into differentiation medium [piPSC medium supplemented with 20% fetal bovine serum (FBS) and without LIF; 3i cocktail; with and without DOX] and plated onto 0.2% gelatin-coated (G1393; Sigma) 12-well plates (665180; Greiner Bio-One) at an inoculation density of 1 × 10^5 cells per well. Spontaneously formed embryoid bodies (EBs) were collected after 3 days of culture.

LIF-independent piPSCs from preculture, P8 static, and P8 SSB cultures were collected as single cells and transferred into low-attachment plates and kept in mTeSR1 medium for 24 h to allow aggregates to form. Dulbecco’s modified Eagle’s medium (DMEM)/F12 (D6421; Sigma) supplemented with 20% Knockout serum medium (10828010; Thermo Fisher) was added to the formed aggregates to support differentiation. The medium was changed every 48 h, and EBs were collected after 10 days of culture.

The samples from both types of cells were subjected to immunocytochemistry and RNA extraction for gene expression analysis.

**RNA extraction and cDNA synthesis**

piPSCs from pre-, static, SSB cultures, and spontaneously formed EBs were collected in PBS and transferred into 1.5-mL centrifuge tubes. The samples were centrifuged at 1,000 g for 5 min to pellet cells that were snap frozen in liquid nitrogen and stored at −80°C until RNA extraction. RNA was recovered from piPSCs (74106; the Qiagen RNeasy Mini Kit) and differentiated cells (74004; the Qiagen RNeasy Micro Kit) as per the manufacturer’s protocol. The RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). A total of 1 μg of RNA was used for cDNA synthesis, using the High-Capacity cDNA Reverse Transcription Kit (4368814; Thermo Fisher) according to the manufacturer’s instructions. cDNA was used at 0.1 ng/μL for primer testing and standard curve generation and 0.01 ng/μL for quantitative polymerase chain reaction (qPCR).

**Quantitative polymerase chain reaction**

qPCR was performed on cDNA generated from piPSCs, spontaneous EBs, and pig fetal fibroblasts to assess relative gene expression of pluripotency-associated genes SOX2, OCT4, cMYC, KLF4, NANOG, TERT, as well as genes specific for the three germ layers [NESTIN (ectoderm), ACTA2 (mesoderm), and GATA4 (endoderm), as well as genes specific for the three germ layers [NESTIN (ectoderm), ACTA2 (mesoderm), and GATA4 (endoderm), as well as genes specific for the three germ layers [NESTIN (ectoderm), ACTA2 (mesoderm), and GATA4 (endoderm), as well as genes specific for the three germ layers [NESTIN (ectoderm), ACTA2 (mesoderm), and GATA4 (endoderm), as well as genes specific for the three germ layers [N...
and addition of microcarriers indicated that piPSCs formed aggregates rather than attached to microcarriers. A shear stress of 3.0 dyn/cm² was chosen as it supported high cell viability in piPSC aggregates. This shear stress maintained pluripotency in mESCs comparable to 6.0 dyn/cm² in the presence of LIF, without cell loss due to necrotic centers in cell aggregates [9]. Therefore, we used the LIF-dependent piPSC line as a first target to evaluate these culture conditions.

Static- and SSB-cultured iPSCs maintain similar cell growth rates and stable karyotype

Since piPSCs have never been exposed to the dynamic conditions observed with SSB culture, a 28-day comparative culture was designed to compare the growth of LIF-dependent piPSCs in SSBs to the static culture. Our first objective was to determine if the dynamic SSB environment changed the apparent doubling time, cell viability, and karyotype stability of piPSCs when compared with the static culture system (Fig. 2). Aside from passage 1 (Fig. 2A; P < 0.05), there was no difference between culture conditions at any passage when considering apparent doubling times (Fig. 2A) nor overall cell viability (Fig. 2B).

Cytogenetic analysis showed a stable karyotype (>95%) throughout the duration of the culture for preculture, static culture, and SSB culture (Fig. 2C; visual representation using piPSCs cultured in SSB culture). Each sample showed no evidence of any structural chromosome abnormality, however, all cell cultures had a 2n=39, XYY karyotype constitution (disomy of the Y sex chromosome) (Fig. 2C). Freshly thawed cells showed a similar karyotype, including disomy of the Y sex chromosome.

Samples were collected from SSB culture at each passage to visualize aggregate formation in suspension culture. Aggregates formed freely after inoculation of piPSCs as single cells (Fig. 3B, C, respectively) while maintaining GFP expression (Fig. 3BII, CII, respectively). piPSCs cultured statically also maintained GFP expression (Fig. 3A–AII).

SSB-cultured iPSC line maintains the expression of pluripotency markers

To determine if dynamic culture conditions affect the maintenance of pluripotency in piPSCs, we assessed the expression of pluripotency-associated genes by qPCR, flow cytometry, and immunocytochemistry. Preculture piPSCs were used as a control to identify any differences observed in gene or protein expression attributed to long-term culture. No statistically significant difference was detected by qPCR in expression of SOX2, OCT4, KLF4, and cMYC between cells from pre-, static, and SSB culture conditions (Fig. 4A). The similarity of piPSCs from static and SSB cultures was also apparent using flow cytometry analysis, which determined that there were no significant differences in the percentage of cells expressing the pluripotency-associated markers, SSEA-4, SOX2, and OCT4, as well as the proliferation marker Ki67 (Fig. 4B). These similarities were qualitatively confirmed by immunocytochemistry for SSB-cultured piPSCs (Fig. 4C) and piPSCs cultured statically (Fig. 4D).

As controls, isotype controls and unstained piPSCs were used to validate staining of piPSCs from pre-, static, and SSB culture conditions (Supplementary Fig. S1).

SSB-cultured iPSC line differentiates into three germ layers in vitro

The ability to differentiate into derivatives of all three germ layers is a characteristic of pluripotency. To assess the differentiation potential of the piPSCs in vitro, differentiation assays were performed. The LIF-dependent piPSCs used in this study were reprogrammed to a pluripotent state using a tetracycline-inducible lentiviral vectors [14], and the

![FIG. 2. Apparent doubling time, viability, and cytogenetic analysis. Apparent doubling (A) and cell viability (B) comparing SSB to static cultures over eight passages. Cells were passaged every 3 days, and cells were collected at day 3 for analysis n = 5. Error bars = SD. Cytogenetic analysis of piPSCs (C; image shows piPSCs from SSB culture). Cytogenetic analysis was performed on preculture piPSCs, P8 SSB piPSCs, and P8 static piPSCs; n = 5. piPSCs, porcine induced pluripotent stem cells. Color images are available online.](image-url)
continued expression of these lentiviral vectors depends on the presence of tetracycline, or DOX, in the culture medium. Beside its role in controlling the expression of pluripotency markers in DOX-inducible iPSCs, DOX has been proven to trigger phosphoinositide 3-kinase-Akt signaling pathways involved in maintaining survival and promoting the self-renewal of ESC and iPSC [20]. Upon removal of DOX, cells are expected to differentiate. The assessment of the differentiation potential of iPSC in the presence of DOX will address the question whether DOX alone is able to prevent the differentiation of iPSCs by maintaining the expression of pluripotency markers or additional factors present in 3i culture medium. This explains more robust differentiation of the iPSCs upon the removal of DOX in addition to removing the 3i cocktail, LIF, and the addition of 20% FBS.

At the same time, analysis of the expression of pluripotency-associated genes demonstrated downregulation of cMYC (Fig. 5D; $^{ab}P < 0.05; n = 5$), KLF4 (Fig. 5E; $^{ab}P < 0.05; n = 5$), and SOX2 (Fig. 5F; $^{ab}P < 0.05; n = 5$) upon differentiation in the absence of DOX (Fig. 5C). Expression of OCT4 was not analyzed under these conditions. Downregulation of pluripotency-associated genes is consistent with the data demonstrating upregulation of differentiation-associated genes as pluripotency-associated genes are expected to become downregulated upon differentiation to lineage-specific cells. Quantification of the differentiation- and pluripotency-associated gene expression is relative to preculture piPSCs, which were used as a control.

Supporting the gene expression data, lineage-specific protein expression was observed by staining for αSMA and NESTIN in piPSCs from pre-, static, and SSB cultures allowed to spontaneously differentiate for 3 days (Fig. 6).

**LIF-independent piPSCs cultured in SSB retain their pluripotency characteristics**

Since the first porcine induced pluripotent stem cell (piPSC) line examined in the current study relies on LIF to self-renew and proliferate, we sought to determine if a dermal fibroblast-derived piPSC line that is LIF independent would maintain iPSC key characteristics under the same dynamic environmental conditions. Applying SSB culture to this cell line addresses the question whether a possible interaction exists between the signaling pathways triggered by the shear stress and added growth factors that would affect piPSCs self-renewal and pluripotency. Similar to results obtained with the LIF-dependent piPSCs, SSB culture of the newly derived LIF-independent piPSCs (Supplementary Fig. S2B) resulted in viability (Fig. 7A) and doubling times (Fig. 7B) comparable to those of the static culture (Supplementary Fig. S2A) after four passages and cells maintained a stable karyotype (Fig. 7C).

Assessing the expression of pluripotency-associated genes, showed a downregulation in porcine SOX2 (pSOX2) and cMYC gene expression in SSB culture as compared with static culture, whereas no significant difference in the expression of OCT4, cMYC, KLF4, or NANOG was observed between static and SSB cultures (Supplementary Fig. S2C). In contrast, immunocytochemistry did not detect a difference between SSB and static culture in the expression of exogenous hSOX2, OCT4, and SSEA-4, or Ki67 used in this study to assess cell proliferation (Supplementary Fig. S2D). These observations further support the dependency of piPSCs on the exogenous reprogramming genes to maintain their pluripotency.

To fully explore the potential of this newly generated LIF-independent piPSC line to differentiate into the three germ layers, we performed in vitro and in vivo differentiation assays. When compared with undifferentiated piPSCs, both static and SSB culture-derived EBs (Supplementary Fig. S3A) showed an upregulation of ACTA2, SOX17, and NESTIN expression, whereas they maintained GATA4...
expression (Supplementary Fig. S3B). The gene expression of NESTIN differed in EBs between the two culture conditions. There was no noticeable difference in the expression of αSMA, GATA4, or NESTIN observed between the static and SSB-derived EBs (Fig. S3C). Surprisingly, EBs derived from static and SSB cultures maintained the expression of pluripotency markers, OCT4, cMYC, and KLF4, during the course of this experiment (Supplementary Fig. S3D), and further showed a significant increase in pSOX2 expression, as compared with undifferentiated cells (Supplementary Fig. S3D).

We then performed teratoma assays by injecting piPSCs cultured under static and SSB conditions subcutaneously to NOD-SCID mice. Histology analysis showed the presence of mesodermal, endodermal, and ectodermal structures within the tissue formed from both static culture piPSCs (Supplementary Fig. S4A) and SSB culture piPSCs (Supplementary Fig. S4B). The expression of NESTIN and β III TUBULIN (as ectoderm markers), αSMA (as mesoderm marker), and GATA4 and α fetoprotein (AFP) (as endoderm markers) was assessed and confirmed the differentiation of piPSCs into ectoderm (Supplementary Fig. S4C) and mesoderm (Supplementary Fig. S4C), but not endoderm based on the staining for GATA4 or AFP. To what extent the type of reprogramming strategy (lentiviral reprogramming vs. DOX-inducible transgene activation) affects the differentiation potential of piPSC deserves further investigation.

Discussion

In the present work, we aimed to determine whether SSBs offered an efficient alternative culture platform to the two-dimensional static culture system for the expansion of piPSCs. In this study, we provide evidence that piPSC, similar to mESCs, can be expanded in SSBs using a shear force of 3.0 dyn/cm² while maintaining their pluripotency potential. Our study demonstrated that LIF-dependent piPSCs cultured in SSBs showed no difference in cell viability or apparent doubling time after the first passage, when compared
with static culture. With the exception of P7 from static culture, which may have been due to an issue during piPSC dissociation and/or collection, the percent viability ranged between 90% and 95% for both static and SSB cultures. Our findings are consistent with previous studies using either similar or different SSB culture systems. hiPSCs cultured in a pendulum-stirred system showed >95% viability [21], whereas a study employing a triangular impeller and glass-etched baffles achieved a viability between 85% and 95% [10,22]. Two murine iPSC lines cultured in SSBs with an impeller similar to a magnetic stirrer bar that resembles the one used in the current study, also showed viability higher than 85% and 93% [13].

The proliferation of piPSCs in static and SSB cultures as assessed by Ki67 expression further validated the apparent doubling time data. For the LIF-dependent piPSCs, with the exception of the first passage, no difference was observed between the two types of cultures, which was consistent with previous work on human cells [23]. Similarly, viability of the LIF-independent piPSCs increased with subsequent passages and was comparable to that observed in static culture. These data suggest that piPSCs can adapt rapidly to

**FIG. 5.** Gene expression analysis. Upregulation of genes indicative of differentiation (A–C) and the subsequent down-regulation of pluripotency-associated gene expression (D–F) on day 3 of spontaneous embryoid body differentiation. Differentiation medium with or without DOX was used to determine the differentiation capacity of the piPSCs. Significant differences in gene expression were observed for (A) ACTA2 \((a,bP<0.05)\); (B) GATA6 \((a,bP<0.05)\); (C) NESTIN \((a,bP<0.05; a,bP<0.05; b,cP<0.05)\); (D) cMYC \((a,bP<0.05)\); (E) KLF4 \((a,bP<0.05)\); and (F) SOX2 \((a,bP<0.05)\). Gene expression was normalized to GAPDH and represented in log2 scale; \(n=5\). Error bars = SD. DOX, doxycycline. Color images are available online.
SSB culture, possibly through selection for a subpopulation able to survive under these conditions. Our results contrast, however, previous work on hiPSCs [12] and mESCs [8], where cell yield in SSB increased or decreased when compared with static controls, respectively. It is worth noting that optimization was required in both studies to circumvent the reduction of SSB-cultured cell yield compared with static culture, such as increasing agitation rate or adding supplements to culture medium. We speculate that the differences between our study and the aforementioned studies may be due to species-specific differences, inoculation density, agitation rate, or cell-specific responses to shear and the dynamic environment.

It is important to note that maintenance of the two piPSCs lines under dynamic conditions for an extended period of time had no impact on chromosomes stability. The disomy of the Y chromosome observed in the karyotype of LIF-dependent piPSC was already apparent in the starting cell population, which appeared to have arisen before the onset of the current experiments.

Expanding the LIF-dependent piPSCs line under dynamic conditions did not affect their pluripotency potential, as clearly confirmed by the sustained expression of OCT4, SOX2, cMYC, and SSEA-4, which is consistent with previous reports on SSB culture of miPSCs [13] and hiPSCs [12]. These piPSCs resemble hESCs in the expression of

**FIG. 6.** Differentiation-associated protein expression in day 3 spontaneously formed embryoid bodies from piPSCs. Intact embryoid bodies were collected and stained for ectoderm (NESTIN; purple) and mesoderm (α-SMA; purple) proteins to illustrate differentiation into the respective germ lineages. Cells are constitutively expressing GFP (green). Scale bar 50 µm. Color images are available online.
SSEA-4 and SSEA-1 [23], presumably due to the presence of LIF. piPSCs grown in the presence of LIF express SSEA-4, while piPSCs grown in the presence of FGF express SSEA-1 [24–26]. This was further supported by the low expression of SSEA-4 observed in the LIF-independent piPSC line used in the current study. However, downregulation of SOX2 and cMYC was observed in the LIF-independent piPSCs cultured in SSBs as compared with static conditions. SOX2 controlled the proliferation of adult stem cells by regulating the expression of cMYC [27]. Although speculative, it is possible that the piPSCs used in the current study regulate the expression of SOX2 to maintain their stemness. As Golden et al. reported, ESCs regulate the levels of SOX2 by activating a negative feedback loop through Akt signaling to retain their stem cell properties [28]. Whether Akt signaling can be triggered as a result of the expression level of SOX2 alone or combined with other shear force-induced effects deserves further investigation, as a drastic change in morphology and function of human endothelial cells due to fluid shear stress-induced Akt phosphorylation has been reported [29].

One of the hallmarks of pluripotent cells is their ability to differentiate into three germ layers. Spontaneously formed EBs from pre-, static, and SSB culture of LIF-dependent piPSCs expressed proteins representative of ectoderm, mesoderm, and endoderm. NESTIN represents a reliable marker for neural progenitor cell differentiation from porcine cells [30,31] ACTA2, the gene encoding for the protein αSMA, was selected as a reliable marker for mesodermal differentiation for piPSCs [26], as well as hESC differentiation toward smooth muscle cells [32]. GATA6 has been shown in previous studies to be expressed upon endodermal lineage differentiation of piPSCs [30,33] and is considered to play an integral role in the formation of human definitive endoderm from PSCs [34]. Our findings showed gene expression corresponding with ectoderm (NESTIN), mesoderm (ACTA2), and endoderm (GATA6) differentiation within 3 days of spontaneous EB differentiation in cells cultured in both conventional adherent culture conditions and SSB. As expected, with the removal of DOX, pluripotency-associated gene expression was downregulated, offering further evidence of the differentiation of the piPSCs toward the germ lineages.

Overall, SSB culture conditions maintained the differentiation potential of both piPSC lines tested in the current study. Some differences, however, were noted between the two piPSC lines regarding the expression of pluripotency markers during the differentiation process. Perhaps an intrinsic feature of the LIF-independent piPSC, there was an upregulation of SOX2 along with the differentiation markers in both static and SSB-derived EBs. A two-fold upregulation in SOX2 expression induced differentiation of ESC into cells that express ectoderm, mesoderm, and trophectoderm but not endoderm [35]; however, the LIF-independent piPSCs reported here differentiated into derivatives of all three germ layers in vitro.

Formation of teratomas in NOD-SCID mice is considered a gold standard of pluripotency. The LIF-dependent iPSC failed to form teratomas. In the previous work, LIF-dependent piPSCs were only able to form teratomas upon administration of DOX through drinking water [36]. Dependence on DOX is consistent with the fact that the pluripotent state of piPSCs still requires the persistence of exogenous genes to maintain pluripotency, possibly due to the inadequate activation of endogenous genes to maintain pluripotency [37]. However, the newly derived LIF-independent iPSCs differentiated into ectoderm and mesoderm in vivo, and this potential was maintained in the SSB culture.

In this study, we demonstrate that piPSCs grown in static and SSB cultures are similar in their functional characteristics. The downstream application of using SSBs is the potential for scale-up. Considering the experimental
parameters set by this study, such as seeding density and culture duration, the theoretical cell yield can be calculated to illustrate the number of piPSCs generated if all piPSCs were seeded in SSBs after each passage (Supplementary Data). Using $10^5$ cells as a “therapeutic” dose, 1,406 x $10^5$ piPSCs can be inoculated into a 50 mL SSB and scaled up over the following 3 passages yielding a total cell number of $5.91 \times 10^7$ piPSCs if scaled up to 2–10 L SSBs (Supplementary Table S2). If this theoretical experiment was carried out using the static six-well tissue culture plates, a “therapeutic” cell yield would be achieved by the third passage, however, 143 six-well tissue culture plates would be required. The scalability of these dynamic cultures makes them an important tool to meet increasing needs of translational cell-based therapy models [38,39], drug-testing platforms [40], vaccine manufacturing [41], and animal-derived products [42], such as meat. This approach would benefit both medical research and the livestock industry by overcoming some ethical, environmental, and economical challenges. As this study illustrates that the piPSCs cultured statically or in SSBs are functionally equivalent, scale-up would be a more feasible option using SSBs rather than static tissue culture plates for future studies.

**Conclusions**

We present, in this study, proof-of-principle for SSB culture of piPSCs, providing a scalable method of propagating piPSCs in vitro.

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**Author Disclosure Statement**

Dr. Ina Dobrinski was a member of the Scientific Advisory Board of Recombinetics, Inc.

**Supplementary Material**

Supplementary Data  
Supplementary Figure S1  
Supplementary Figure S2  
Supplementary Figure S3  
Supplementary Figure S4  
Supplementary Table S1  
Supplementary Table S2

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