

## Chapter 2

# Morphology of Human Embryonic and Induced Pluripotent Stem Cell Colonies Cultured with Feeders

**Abstract** To prolong the stage of undifferentiation, human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) have traditionally been isolated and cultured using feeder layers, such as mouse embryonic fibroblasts (MEFs) or foreskin fibroblasts, with medium supplemented by fetal bovine serum (FBS). For research purposes, these conditions are preferable and are often referred to as the gold standard. This chapter describes the colony morphology of undifferentiated hESCs and iPSCs cultured with MEFs or human foreskin fibroblasts.

## 2.1 Introduction

Traditionally, human ESCs (hESCs) are co-cultured with inactivated mouse embryonic fibroblasts (MEFs) as supporting layers, and the medium is supplemented with a high percentage of fetal bovine or calf serum (FBS) (Thomson et al. 1998). The feeder layer serves a dual role of supporting hESC expansion and preventing spontaneous differentiation. However, such conditions are not appropriate for clinical and industrial purposes due to variations between batches of FBS and MEFs, and to the risk of exposure of the cells to animal pathogens. Modifications that prevent xeno-contamination in the culture system include the use of a defined medium supplemented with serum replacement, without animal products. The MEFs should be replaced by human feeder cells or matrix.

Accumulating data demonstrate that induced pluripotent stem cells (iPSCs) can be cultured in similar conditions as those for hESCs (Takahashi and Yamanaka 2006; Takahashi et al. 2007); therefore, improvements in hESC culture conditions will also apply to those of iPSCs.

Intensive efforts have been invested during the last decade in the search for alternative feeder cells for hESCs. The result is the identification of a number of cell line types that support the culture of undifferentiated hESCs, including human fetal-derived

fibroblasts (Richards et al. 2002), foreskin fibroblasts (Amit et al. 2003; Hovatta et al. 2003), human placenta fibroblasts (Simón et al. 2005; Genbacev et al. 2005), adult human fibroblasts (Tecirlioglu et al. 2010), and adult marrow cells (Cheng et al. 2003). All these cell lines have been demonstrated to support the prolonged culture of hESCs as undifferentiated, while maintaining all hESC features. We found human foreskin fibroblasts (HFF) to equally support the undifferentiated culture of iPSCs (Amit et al. unpublished data).

Human fetal-derived fibroblasts, placenta fibroblasts, adult human fibroblasts, and foreskin fibroblasts have also been found to support the isolation of new hESC lines under animal-free or serum-free conditions (Richards et al. 2002; Hovatta et al. 2003; Simón et al. 2005; Genbacev et al. 2005; Inzunza et al. 2005; Tecirlioglu et al. 2010). Of these, foreskin fibroblast cells are the most common, accounting for 51 of 57 (89%) of the lines reported during recent years (Richards et al. 2002; Ström et al. 2010; Aguilar-Gallardo et al. 2010; Tecirlioglu et al. 2010; Ilic et al. 2009; Valbuena et al. 2006; Ellerström et al. 2006; Genbacev et al. 2005; Simón et al. 2005; Crook et al. 2007). Of these 57 lines, the 7 reported to be clinical-grade lines were all isolated and cultured using foreskin fibroblasts as feeders (Crook et al. 2007; Ellerström et al. 2006). Thus, these cells are not only the most frequently used human feeders, but also those most ensuring a xeno-free culture system.

Though these culture systems promote animal-free conditions for culturing hESCs, they are not well defined, due to variations between batches of feeder layer cells and to the fact that some still use human serum for the feeder cell culture. An additional disadvantage to the use of human feeders is the need to culture the feeder lines, which limits large-scale culturing of hESCs. Therefore, the ideal culture method seems to be a combination of an animal-free matrix and both serum-free and animal-free medium.

## 2.2 Materials

### 2.2.1 *For Mouse Embryonic Fibroblasts (MEFs) and Foreskin Fibroblasts (HFFs)*

#### 2.2.1.1 0.1% Gelatin

0.1% Gelatin (type A, from porcine, Sigma G-1890). All culture dishes should be covered with 0.1% gelatin at least 1 h before MEF plating.

### **2.2.1.2 Culture Medium MEFs**

90% Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS). During the first passage post-derivation, penicillin–streptomycin should be added (Sigma P-3539, final concentration of penicillin 10,000 u/ml and streptomycin 10 mg/ml).

### **2.2.1.3 Culture Medium HFFs**

90% DMEM, 10% FBS, and 2 mM L-glutamine. During the first passage post-derivation, penicillin–streptomycin should be added (Sigma P-3539, final concentration of penicillin 1,000 u/ml and streptomycin 1 mg/ml).

### **2.2.1.4 Feeder Freezing Medium**

60% DMEM, 20% dimethyl sulfoxide (DMSO), and 20% FBS.

### **2.2.1.5 Feeder Splitting**

Trypsin/EDTA (Invitrogen Corporation, type IV C.N 17104019).

### **2.2.1.6 Mitomycin C**

8 µg/ml mitomycin C (Sigma, M-4287) diluted in DMEM.

### **2.2.1.7 Washing**

Phosphate-buffered saline (PBS) with  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ .

## **2.2.2 For hPSC Maintenance**

### **2.2.2.1 hPSC: Serum-Based Medium**

80% DMEM, 20% defined FBS (HyClone), 1% nonessential amino acid, 2 mM L-glutamine, and 0.1 mM β-mercaptoethanol.

#### **2.2.2.2 hPSC: Serum-Free Medium**

85% DMEM/F12, 15% knockout (KO) serum replacement (SR, Invitrogen Corporation, C.N. 10828028), 1% nonessential amino acid, 2 mM L-glutamine, 0.1 mM  $\beta$ -mercaptoethanol, and 4 ng/ml basic fibroblast growth factor (bFGF). For iPSCs, it is recommended to increase the bFGF concentration to 10 ng/ml.

#### **2.2.2.3 Splitting Medium**

The splitting medium consists of 1 mg/ml collagenase type IV (Wordington, type IV C.N 4189, activity of 220–320 u/mg) in DMEM.

#### **2.2.2.4 Freezing Medium**

40% DMEM, 20% DMSO, 20% FBS, and 20% SR.

### **2.3 Methods**

#### **2.3.1 Feeder Culture Methods**

##### **2.3.1.1 Derivation of MEFs from Pregnant Mice**

1. Use of pregnant Imprinting Control Region (ICR) mice (or CD1) on the 13th day of conception is recommended.
2. Sacrifice 1 female mouse by a method approved by the ethics committee of your institution.
3. Wash the abdomen with 70% ethanol and dissect the abdominal cavity to expose the uterine horns.
4. Set the uterine horns in 10-cm<sup>2</sup> petri dishes and wash three times with 10 ml of PBS. A uterine horn is depicted in Fig. 2.1a.
5. Using two pairs of watchmakers' forceps (Dumont 5, Fine Scientific Tolls), open each uterine wall and release each embryo.
6. Wash retrieved embryos three times with 10 ml PBS (see Sect. 2.2.1.7). Embryos released from the embryonic sac are illustrated in Fig. 2.1b.
7. Use the same tools to dissect each embryo from the placenta and membranes, and discard soft tissues as much as possible.
8. Transfer clean embryos into new petri dishes and mince thoroughly using sharp Iris scissors. An example of sufficiently minced embryos is depicted in Fig. 2.1c.
9. Add 6 ml of trypsin/EDTA (see Sect. 2.2.1.5) and incubate for at least 20 min.

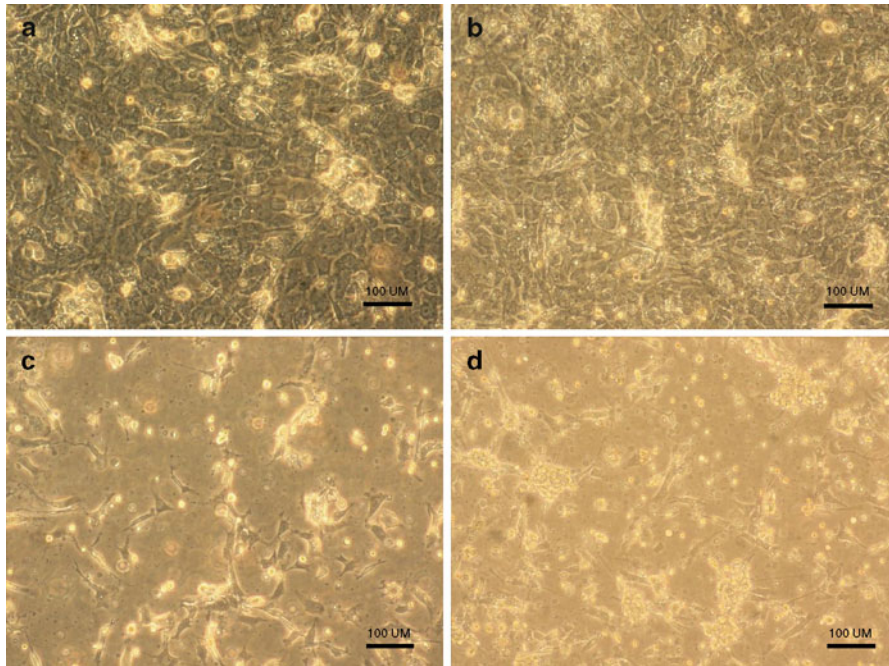


**Fig. 2.1** Mouse embryonic fibroblast (MEF) derivation. (a) Uterine horn of ICR mice on the 13th day of conception. (b) Embryos released from embryonic sac. (c) The same culture after mincing the embryos with sharp Iris scissors to the correct sizes

10. Neutralize trypsin using at least 6 ml of MEF culture medium (see Sect. 2.2.1.2).
11. Transfer the cells into conical tubes.
12. Divide evenly into T75 culture flasks. We recommend a ratio of three embryos per flask.
13. Add 20 ml of MEF culture medium to each flask (see Sect. 2.2.1.2).
14. Grow the MEFs up to 3 days or until the culture is confluent. Change the medium at least once during culturing (do not aspirate any floating clumps). Morphology of derived MEFs during the first days of culture post-derivation is illustrated in Fig. 2.2.
15. Freeze the resulting MEF (see Sect. 2.3.1.4).

### 2.3.1.2 HFF Derivation

1. A newborn human foreskin should be placed in PBS supplemented with penicillin–streptomycin immediately following the circumcision. The foreskin should be maintained at 4–8°C until derivation of fibroblasts, no later than 48 h post circumcision.
2. Unfold the foreskin and wash three times with PBS (see Sect. 2.2.1.7).
3. Cut into small pieces using sharp Iris scissors (about eight pieces per foreskin).
4. Transfer clean pieces into a new petri dish and mince thoroughly using sharp Iris scissors.
5. Add 6 ml of trypsin/EDTA (see Sect. 2.2.1.5) and incubate for at least 30 min.
6. Neutralize the trypsin using at least 6 ml of HFF culture medium (see Sect. 2.2.1.3). Transfer the HFF into conical tubes. Use HFF culture medium to wash the plate.
7. Divide evenly into T25 culture flasks at a recommended ratio of two pieces per flask.
8. Add 6 ml of HFF culture medium (see Sect. 2.2.1.3).
9. Grow the HFF until the culture is confluent. Change medium as needed, every 5 days if not split. HFF morphology is demonstrated in Fig. 2.4.



**Fig. 2.2** MEF primary culture 2 days post-derivation. (a, b) Examples of MEF cultures with expected concentration and fibroblast morphology. (c, d) Examples of MEF cultures with poor recovery; the culture confluence is less than 40%. Bar 100  $\mu$ M

### 2.3.1.3 Feeder Splitting

1. Aspirate the culture medium and wash the flask once with 5 ml PBS (for T75 flask, see Sect. 2.2.1.7).
2. Add 2 ml of trypsin/EDTA (see Sect. 2.2.1.5) and cover the entire culture flask surface.
3. Incubate for 6 min.
4. Tap the side of the flask to loosen the cells. Add 4 ml of culture medium (see Sects. 2.2.1.2 or 2.2.1.3) to neutralize the trypsin.
5. Transfer the cell suspension into a conical tube and centrifuge for 5 min at  $90\times g$ .
6. Remove the suspension from the centrifuge, re-suspend in 2 ml of culture medium (see Sects. 2.2.1.2 or 2.2.1.3), and pipette to fracture the pellet.
7. Distribute the cell suspension to a desired number of culture flasks. For MEFs, we recommend a ratio of 1:5 at passage 1, 1:4 at passage 2, and 1:3 at passages 3–5; and for HFF, a ratio of 1:3.
8. Add culture medium (see Sects. 2.2.1.2 or 2.2.1.3) to reach a final volume of 10 ml.

#### 2.3.1.4 Feeder Freezing

Once inactivated, feeder cells can be frozen at a confluence of at least 80% of the culture dish.

1. Wash flasks once with 5 ml PBS (for T75, see Sect. 2.2.1.7) and remove aggregates as much as possible.
2. Add 2 ml of trypsin/EDTA (see Sect. 2.2.1.5) and cover the entire culture flask surface.
3. Incubate for 6 min.
4. Tap the side of the flask to loosen cells. Add 4 ml of culture medium (see Sects. 2.2.1.2 or 2.2.1.3) to neutralize the trypsin.
5. Transfer the cell suspension to a conical tube. Let the remaining aggregates sink (1–2 min) and transfer the cell suspension to a clean conical tube.
6. Centrifuge for 5 min at  $90\times g$ .
7. Remove the suspension, re-suspend in 2 ml culture medium (see Sects. 2.2.1.2 or 2.2.1.3), and pipette to fracture the pellet.
8. Add, drop by drop, an equivalent volume of freezing medium (see Sect. 2.2.1.4) and mix gently. Adding the freezing medium drop by drop is crucial for cell recovery.
9. Place 1 ml of the medium into 2-ml cryogenic vials. A concentration of 1–2 million cells per vial is recommended.
10. Freeze vials overnight at  $-80^{\circ}\text{C}$  in a freezing box (Nalgene freezing box, C.N.5100-0001) for at least 24 h, but for no more than 1 week.
11. Transfer the vials into a liquid nitrogen container.

#### 2.3.1.5 Feeder Thawing

1. Remove the vial from liquid nitrogen and thaw briefly in a  $37^{\circ}\text{C}$  water bath.
2. When a small pellet of frozen cell remains, clean the vial using 70% ethanol.
3. Pipette the contents of the vial once and transfer the cells into a conical tube.
4. Add, drop by drop, 2 ml of culture medium (see Sects. 2.2.1.2 or 2.2.1.3). Adding the medium drop by drop is crucial for cell recovery.
5. Centrifuge for 5 min at  $90\times g$ .
6. Re-suspend the pellet in culture medium (see Sects. 2.2.1.2 or 2.2.1.3).
7. Transfer the cell suspension to culture flasks and add 10 ml of culture medium (see Sects. 2.2.1.2 or 2.2.1.3). A ratio of 1–2 million frozen cells to one T75 flask is recommended.

#### 2.3.1.6 Preparation of Feeder-Covered Plates

1. Add 8  $\mu\text{g/ml}$  of mitomycin C (see Sect. 2.2.1.6) to culture flasks and incubate for 2 h. Alternatively, feeder cells can be irradiated at 35 grays gamma irradiation.
2. Wash four times with 10 ml PBS (see Sect. 2.2.1.7).

3. Add 2 ml of trypsin/EDTA (see Sect. 2.2.1.5) and cover the entire culture flask surface (T75).
4. Incubate for 6 min.
5. Tap the side of the flask to loosen cells. Add 4 ml of culture medium (see Sects. 2.2.1.2 or 2.2.1.3) to neutralize the trypsin.
6. Transfer the cell suspension to a conical tube.
7. Centrifuge for 5 min at  $90\times g$ .
8. Remove the suspension, re-suspend in 10 ml of culture medium (see Sects. 2.2.1.2 or 2.2.1.3), and pipette to fracture the pellet.
9. Count cells and re-suspend in a medium of the desired volume (see Sects. 2.2.1.2 or 2.2.1.3).
10. Transfer the cell suspension to culture dishes previously covered with gelatin (see Sect. 2.2.1.1). We recommend  $4\times 10^5$  cells per well in 6-well plates ( $10\text{ cm}^2$ ) per 2 ml. Figure 2.3a, b illustrates MEF concentrations; identical concentrations should be used with HFF.
11. Let set for at least 2 h before plating hESCs. It is recommended to prepare the plates 1 day before use. Examples of MEF monolayer morphology are illustrated in Fig. 2.3c–f; and of HFF in Fig. 2.4.

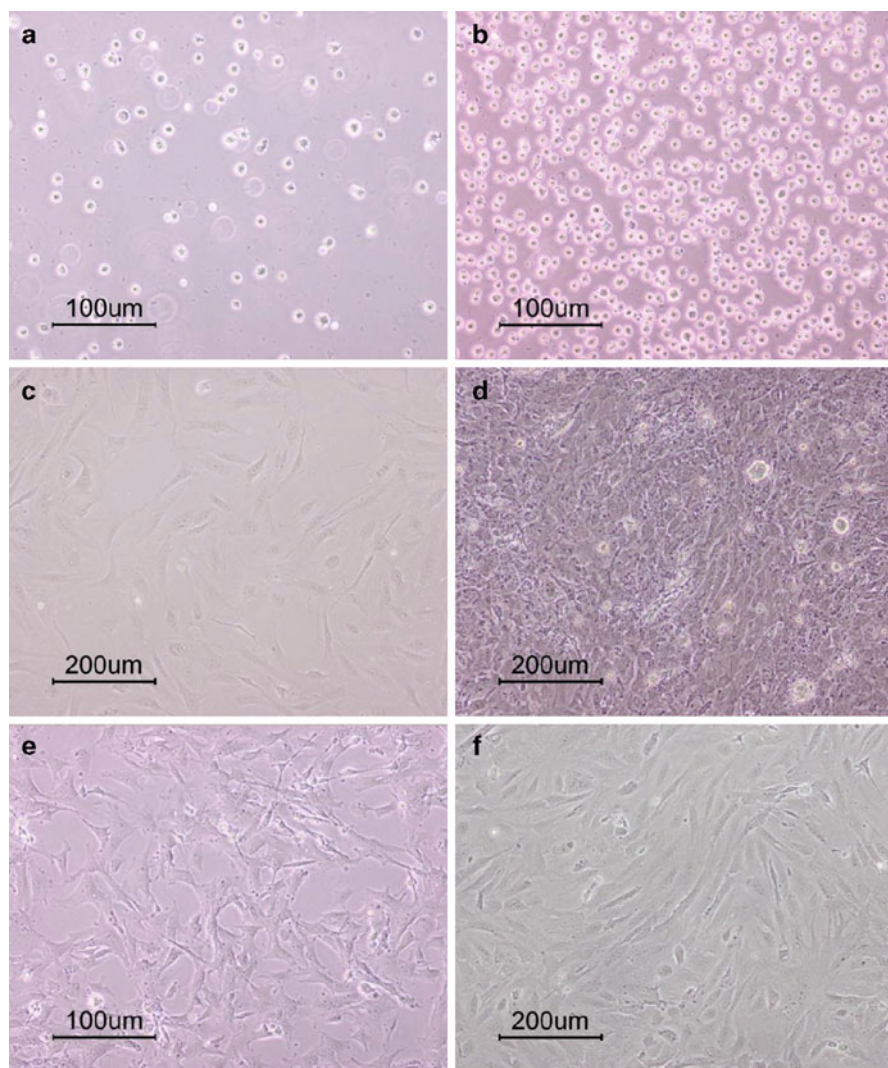
### 2.3.2 *hPSC Culture*

The same culture methodology is used for MEFs and HFFs. Culture methods for hESCs and hiPSCs are also the same, other than the culture medium (see Sect. 2.2.2.2).

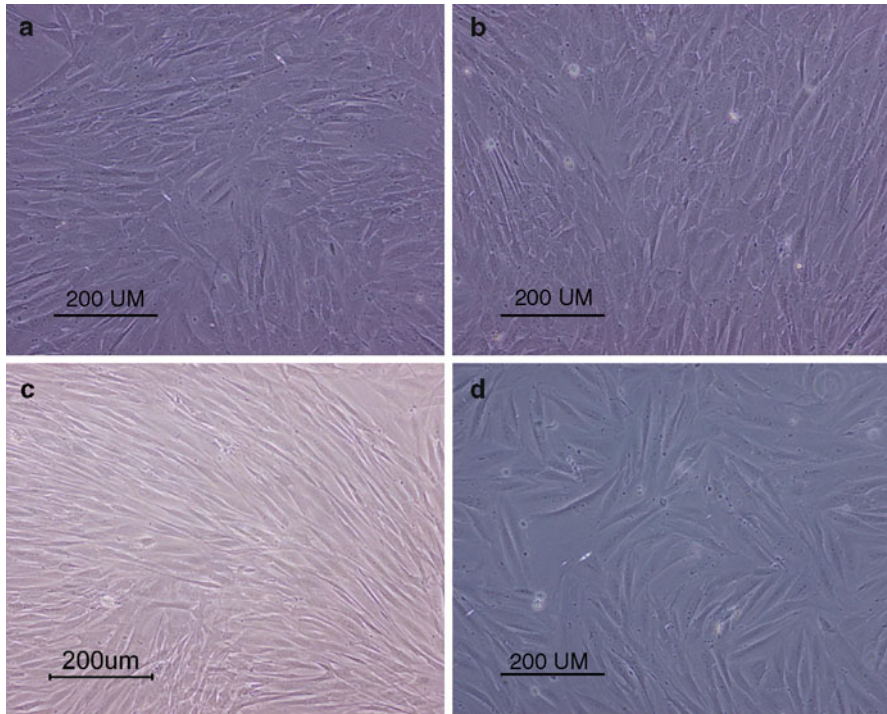
#### 2.3.2.1 *hPSC Splitting*

The culture should be split every 4–6 days when using serum-free medium and every 5–7 days when using serum containing medium. Examples of ready-to-split colonies are depicted in Fig. 2.5.

1. Aspirate the medium from the wells that are to be split. Add splitting medium (see Sect. 2.2.2.3) to cover the wells ( $0.5\text{ ml}$  for  $10\text{ cm}^2$ ) and incubate for 20–40 min. Most colonies will float. The morphology of colonies during incubation is shown in Fig. 2.6.
2. Add 1 ml of culture medium (see Sects. 2.2.2.1 or 2.2.2.2) and gently collect the floating cells. Most feeder cells will remain behind, as exemplified in Fig. 2.7.
3. Collect the cell suspension and place into a conical tube.
4. Centrifuge for 3 min at  $80\times g$  at a recommended temperature of  $4^\circ\text{C}$ .
5. Aspirate the medium from fresh MEF-covered plates, re-suspend cells in medium, and plate. The size of the resulting clumps, including examples of clumps broken to incorrect sizes, is illustrated in Fig. 2.8. Too small clumps may



**Fig. 2.3** Preparation of MEF-covered plates. (a) MEFs after trypsinization, demonstrating a low concentration, which may be insufficient to support pluripotent stem cell (PSC) undifferentiated culture. (b) MEFs after trypsinization, demonstrating a high concentration, which will probably result in a plate suitable to support PSC undifferentiated culture. (c) Inactivated MEF-covered plate, demonstrating low concentration, which may be insufficient to support PSC undifferentiated culture. (d) A plate demonstrating a very high concentration of MEF, which supports the culture of PSCs, but which may detach from the plate after a few days of growth. (e) Inactivated MEF-covered plate, demonstrating a low concentration, which is probably sufficient to support PSC undifferentiated culture. (f) A plate demonstrating the correct concentration of MEF for supporting PSC culture. (a, b, e) Bar 100 μM, (c, d, f) bar 200 μM

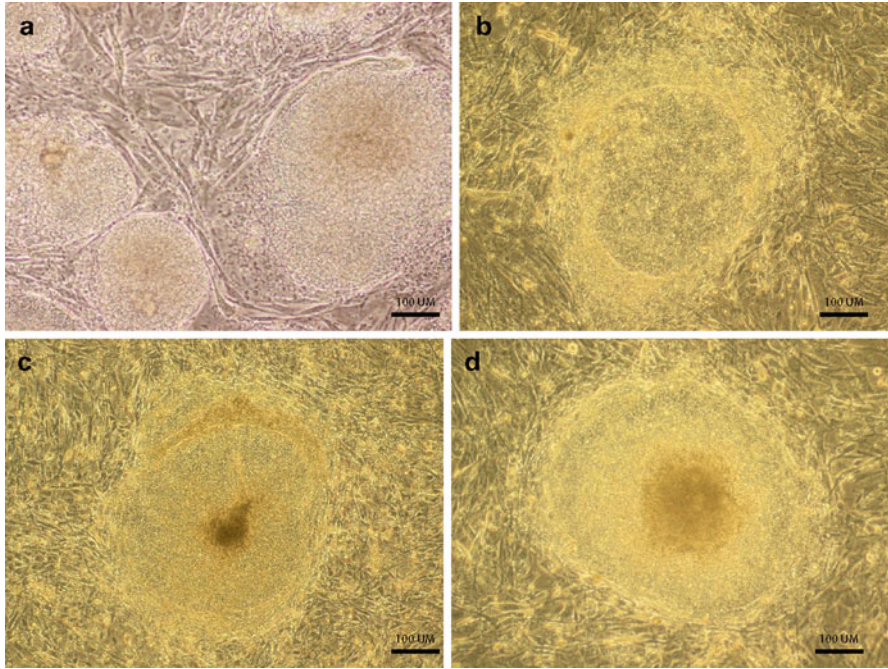


**Fig. 2.4** Preparation of foreskin fibroblast (HFF)-covered plates. (a, b) Cultured HFF; note that the cells have narrow and more homogenous morphology than MEFs. (c, d) Inactivated HFF with sufficient concentration to support PSC culture. Bar 200  $\mu$ m

result in decreased cell survival. If the post-splitting clump size is too large, cell attachment to the feeder cells may be harmed, resulting in increased background differentiation and greater splitting rates. Examples of colony morphology for clumps that are too large are depicted in Figs. 2.9 and 2.10c–f. A general view of colony morphology at 1 day post-splitting is illustrated in Fig. 2.10.

### 2.3.2.2 hPSC Freezing

1. Aspirate medium from wells to be split. Add splitting medium (see Sect. 2.2.2.3) to cover the wells (0.5 ml for 10 cm<sup>2</sup>) and incubate for 20–40 min. Most colonies will float.
2. Add 1 ml of culture medium (see Sects. 2.2.2.1 or 2.2.2.2) and gently collect the floating cells.
3. Collect the cell suspension and place into a conical tube.
4. Centrifuge for 3 min at 80 $\times$ g at a recommended temperature of 4°C.

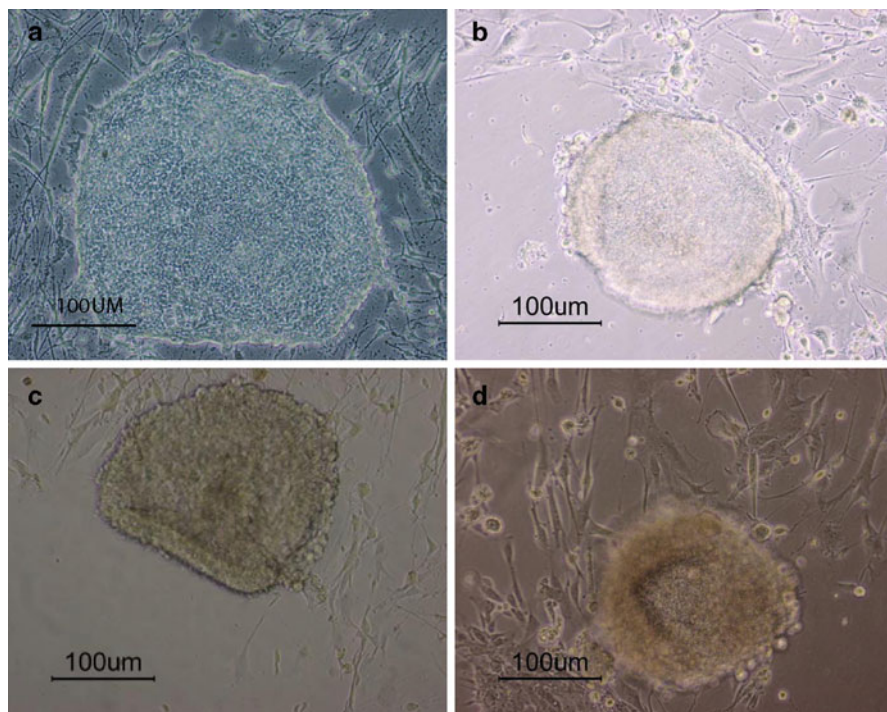


**Fig. 2.5** PSCs ready for splitting. (a) BG01 hESCs demonstrating confluent culture, which should be split to prevent differentiation. The colony size is sufficient to survive splitting. (b–d) iLBWT30m hiPSC colonies (from skin biopsy, O. Brustle, Bonn University) of sufficient size for splitting; the colonies demonstrated in (c) and (d) may differentiate if splitting will be delayed. Bar 200  $\mu$ m

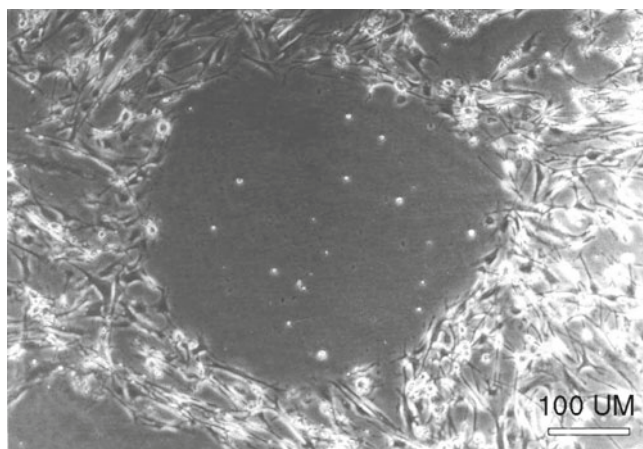
5. Re-suspend cells in a culture medium (see Sects. 2.2.2.1 or 2.2.2.2).
6. Add, drop by drop, an equivalent volume of freezing medium (see Sect. 2.2.2.4) and mix gently. Adding the freezing medium drop by drop is crucial for cell recovery.
7. Pour 0.5 ml into a 1-ml cryogenic vial. A freezing ratio of cells covering 10 cm<sup>2</sup> of culture per vial is recommended.
8. Freeze overnight at  $-80^{\circ}\text{C}$  in freezing boxes (Nalgene freezing box. C.N.5100-0001).
9. Transfer to liquid nitrogen on the following day.

### 2.3.2.3 hPSC Thawing

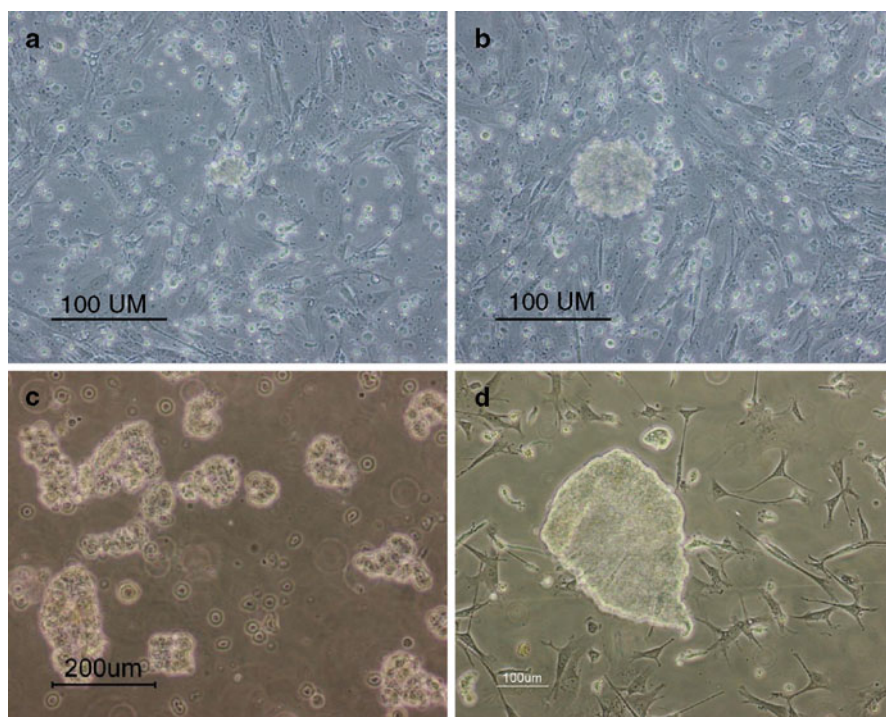
1. Remove the vial from the liquid nitrogen.
2. Gently swirl the vial in a  $37^{\circ}\text{C}$  water bath.
3. When a small pellet of frozen cells remains, wash the vial in 70% ethanol.



**Fig. 2.6** PSCs incubated with collagenase. (a) Colony starting to detach from MEFs. (b, c) Colonies partly separated from MEF feeder layer; at this stage, the incubation with collagenase can be stopped, and the colonies will then easily separate from the feeder layer. (d) Floating colony. Note that most MEFs that remained attached themselves to the culture dish. Bar 100  $\mu$ M



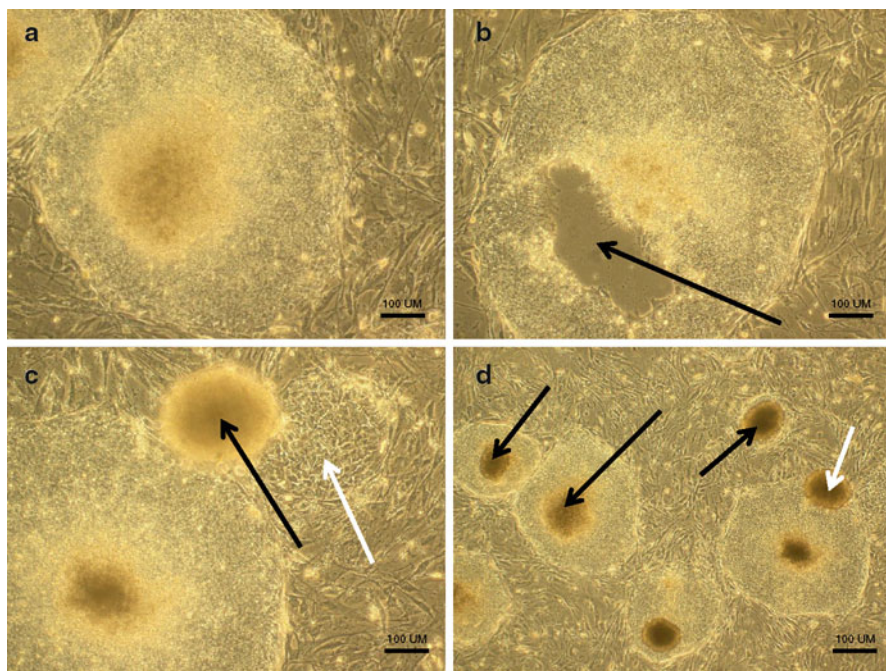
**Fig. 2.7** MEFs post-collagenase splitting. MEFs that were still attached after collagenase splitting. Note the hole in the feeders created by detached PSC colonies. Bar 100  $\mu$ M



**Fig. 2.8** The size of post-splitting clumps. PSC clumps resulting from collagenase treatment. (a) Small clumps may result in poor survival. (b, c) Clump size that ensures good cell recovery. (d) Large clumps may result in cell differentiation. Bar 100  $\mu\text{M}$ , for all but (c), which is 200  $\mu\text{M}$

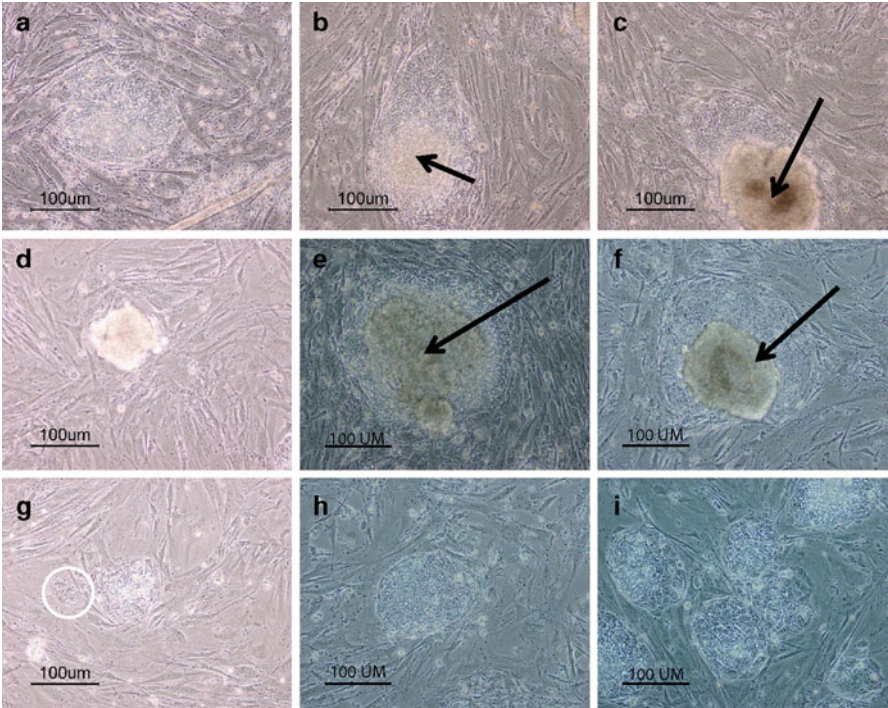
4. To mix, pipette the contents of the vial up and down once.
5. Place the contents of the vial into a conical tube and add, drop by drop, 2 ml of culture medium (see Sects. 2.2.2.1 or 2.2.2.2). Adding the culture medium drop by drop is crucial for cell recovery. Examples of the resultant clumps are illustrated in Fig. 2.11.
6. Centrifuge for 3 min at  $80\times g$  at a recommended temperature of  $4^{\circ}\text{C}$ .
7. Remove the supernatant and re-suspend the cells gently in 2 ml medium.
8. Place the cell suspension in one well of a 6-well plate, or of a 4-well plate, covered with feeder cells (see Sect. 2.3.1.5).

If the thawing procedure succeeds, small colonies should appear 1 day post-thawing (Fig. 2.12). With very good recovery, as demonstrated in Fig. 2.13, colonies will continue to grow, and will not differ from post-splitting cells. However, in some cases, when either freezing or thawing fails, the cells might not survive, as can be seen in the examples illustrated in Fig. 2.14. In some cases, the feeder clumps survive the freeze and thaw cycle, resulting in a feeder cell colony (Fig. 2.14a, c).

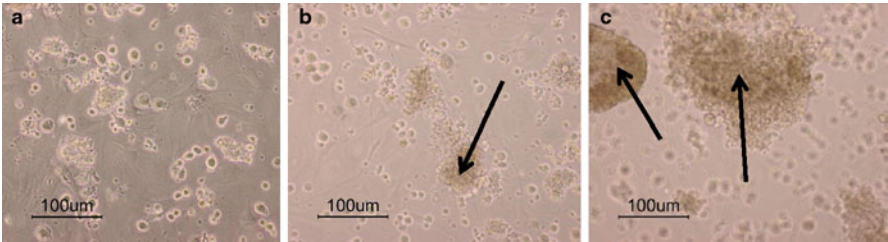


**Fig. 2.9** Colonies of the I3.2 hESC line 2 days post-splitting, after cells were not broken into small enough pieces during the splitting procedure. (a) Since the colonies start from big clumps, they reach sufficient size for splitting 2 days later. (b) The colony diameter at day two is so large that a rapture was formed (marked with *arrow*). (c) The culture also contains colonies with small clumps (marked with *white arrow*), which may not survive the following passage. The passage should be performed when most other colonies of the culture will be suitable for splitting. Some clumps failed to attach to the feeder layer (marked with *black arrow*). (d) Some colonies contain big clumps at the center, which did not attach properly to the feeder layer (marked with *black arrow*). These clumps may differentiate, or the cells may become apoptotic during the passage. Some clumps settle on a different colony (marked with *white arrow*). Bar 100  $\mu$ M

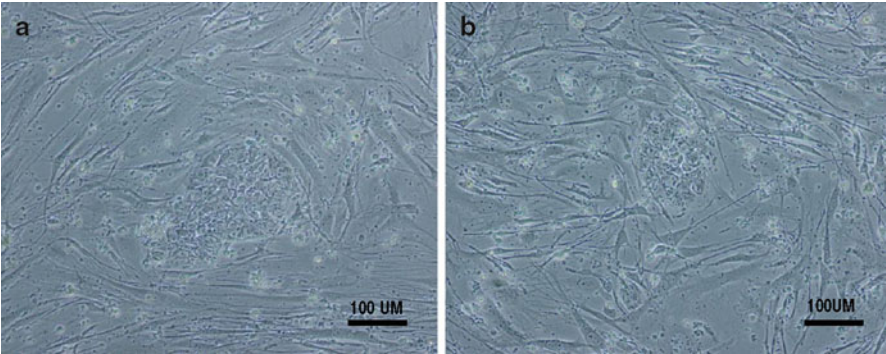
Occasionally, colony morphology is disorganized post-splitting and a normal-looking colony appears only a passage or two later (Fig. 2.15). As with splitting, if during the freeze and thaw cycle the cells are broken into too small clumps, they might not survive, or alternatively colonies will appear only a few days post-thawing (Fig. 2.16). After good recovery, cell concentration will resemble splitting and some clear differentiation may appear (Fig. 2.17). Even after good recovery, some cells within the colony may not survive, and dying cells may appear, usually with no harm to cell recovery (Fig. 2.18).



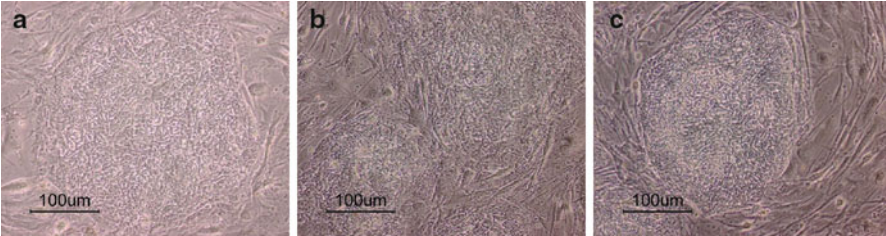
**Fig. 2.10** Resultant PSC colonies 1 day post-splitting. **(a)** BG01 undifferentiated hESC colony. **(b)** BG01 undifferentiated colony with a small clump that did not attach exactly (marked with *black arrow*), and which will probably disappear after a day or two of culture with no harm. **(c)** BG01 hESC colony with a large detached clump at the center (marked with *black arrow*), which may differentiate. **(e, f)** Colony morphology is similar to that of I3 hESC line. **(d)**. Small clump of BG01 hESC attached to the MEFs that have not yet demonstrated outgrowth. **(g)** Small colonies resulting from small clumps of H9.2 hESCs, one of which is hard to recognize (marked with a *circle*). **(h, i)** Additional examples of undifferentiated hESC colonies from H9.2 hESC. Bar 100  $\mu$ M



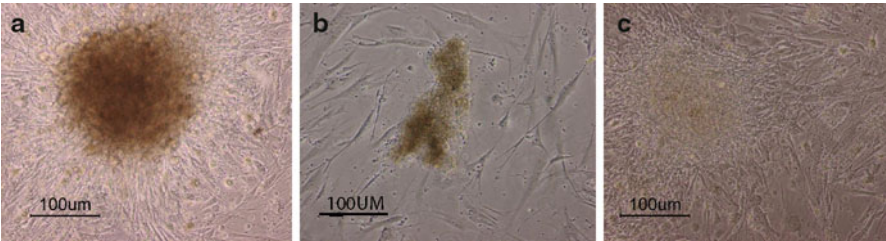
**Fig. 2.11** Post-thaw clumps of I6 hESCs, before attachment to the feeder layer. **(a)** Small clump size and **(b)** good clump size (marked by *arrow*). **(c)** Large clumps with *brown color* (marked by *arrows*), may contain apoptotic cells. Bar 100  $\mu$ M



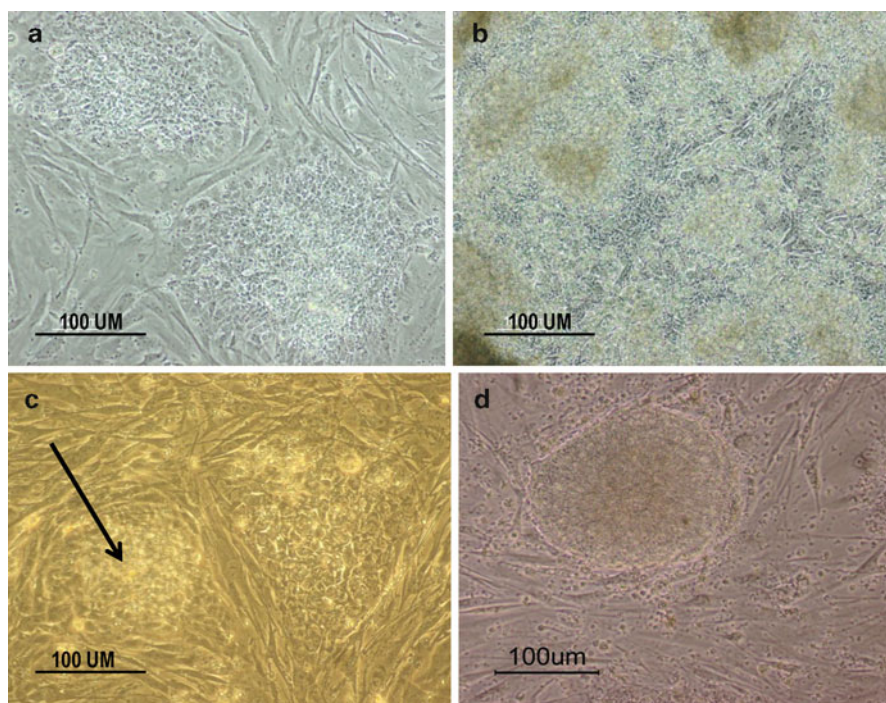
**Fig. 2.12** Undifferentiated hESC colonies 1 day post-thawing. (a, b) H9.2 undifferentiated colonies surviving freeze and thaw cycle. Bar 100  $\mu$ M



**Fig. 2.13** Undifferentiated hESC colonies 2 days post-thawing. (a–c) I6 undifferentiated colonies surviving freeze and thaw cycle, with greater than average recovery rate. Bar 100  $\mu$ M



**Fig. 2.14** MEF post-thawing recovery and dead cells. (a) A large MEF clump that survived thawing; the *brown* area probably contains apoptotic cells. (b) Attached clump containing dead cells; note the *brown* color. (c) MEF clump that survived thawing. Note the edge of the attached clump; only fibroblasts can be recognized at the outgrowth. Bar 100  $\mu$ M

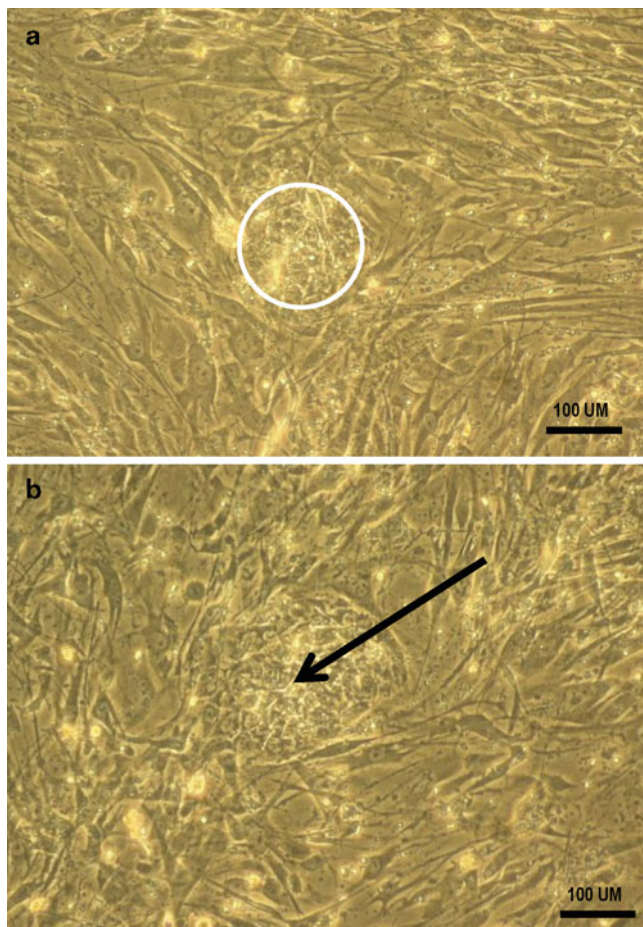


**Fig. 2.15** Post-thawing recovered colonies, with disorganized morphology. **(a)** Two colonies of hESC I4, with unclear status of differentiation. **(b)** Colonies formed from hESC I4, in which all the colonies are located at the center of the dish, without clear borders and cell morphology. This can happen after thawing or splitting if the plates are not tilted before putting them in the incubator. **(c)** A hESC I6 colony with undifferentiated morphology and a second one with unclear morphology (marked with *arrow*). **(d)** A thick colony formed from hESC I6. The multiple layers make it hard to determine the cell morphology. It is not unusual for post-thawing cells to have disorganized morphology; it is, therefore, recommended to culture them for 2–3 passages before assessing and using them. Bar 100  $\mu\text{m}$

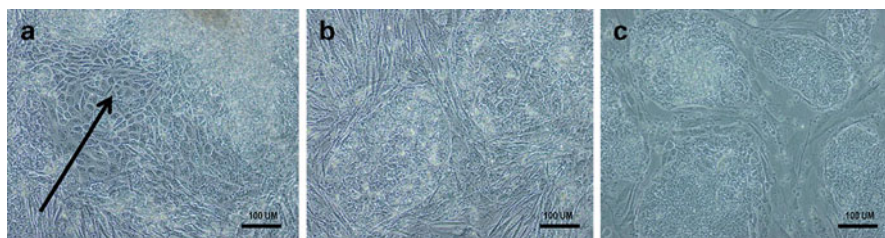
### 2.3.2.4 Routine Culture of hESCs

The medium (see Sects. 2.2.2.1 or 2.2.2.2) should be changed daily. If this is not possible, its quantity should be doubled. hESCs should be passaged (see Sect. 2.3.2.1) directly on fresh feeder-covered plates (see Sect. 2.3.1.6), every 4–6 days if serum-free medium is used (see Sect. 2.2.2.2) or every 5–7 days if serum containing medium is used (see Sect. 2.2.2.1).

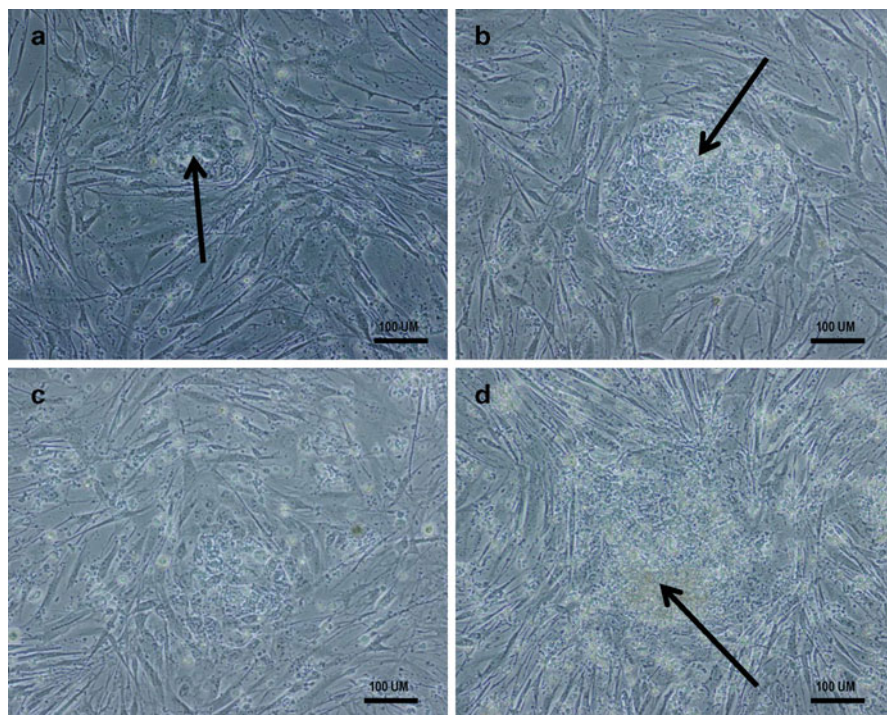
Undifferentiated PSC colonies typically have clear borders from the feeders and contain small round cells, with spaces between them, and large nuclei with notable nucleoli. The undifferentiated morphology of PSCs cultured with MEFs or HFFs is illustrated in Figs. 2.19 and 2.20, respectively. Cell morphology within undifferentiated colonies is demonstrated in Fig. 2.21. In general, any colony with a change in



**Fig. 2.16** Colonies formed from hESC I6.2 4 days post-thawing. (a) A tiny colony that is hardly detectable (marked with *circle*). (b) A clear undifferentiated colony (marked with *arrow*), relatively small for 4 days of culture. If the cells are broken into too small clumps while freezing or thawing, some clumps will not survive; however, the surviving ones will appear a few days post-thawing. It is, therefore, recommended to maintain the culture of thawed cells for 1 week to allow for the appearance of surviving colonies. Bar 100 μM

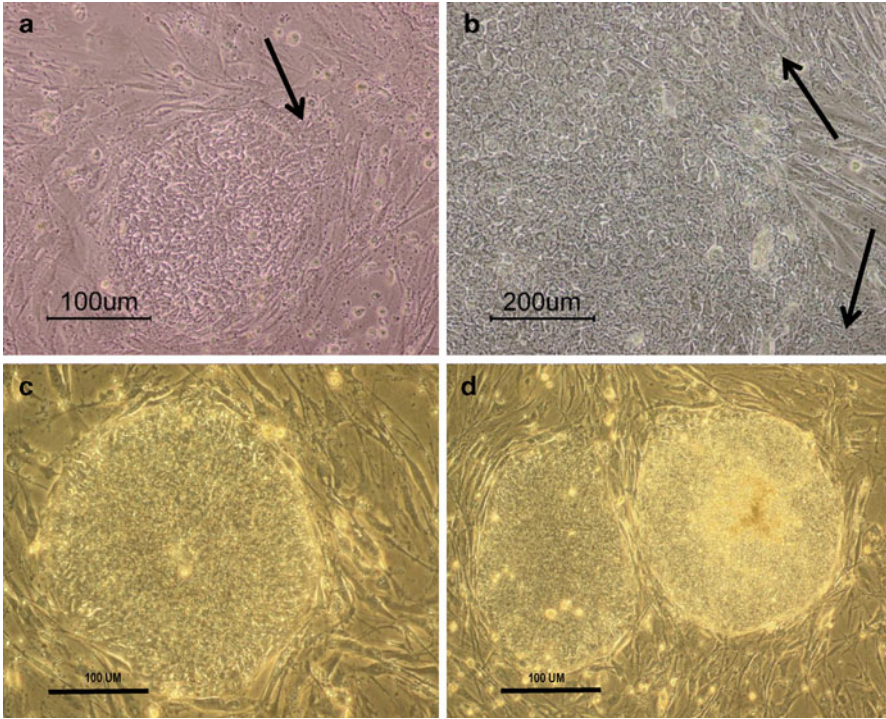


**Fig. 2.17** Colonies from hESC H9 5 days post-thawing. (a) Cells are already differentiated (marked with *arrow*), differentiation probably occurred upon passage to freezing. (b, c) Undifferentiated colonies with good recovery and size. Bar 100 μM

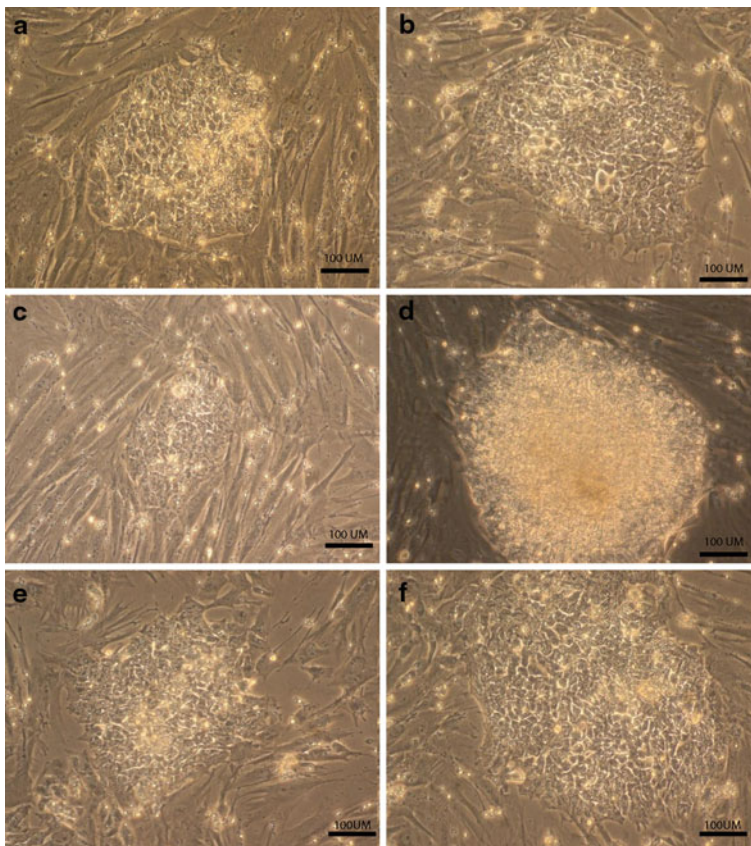


**Fig. 2.18** Recovered colonies 2 days post-thawing from H9.2 hESCs, with disorganized morphology and some apoptotic cells. (a, b, d) Undifferentiated colonies with apoptotic cells (marked by *arrow*) detaching from the colony. In most cases, apoptotic cells will disappear after a few days, and the surviving cells will remain unchanged. (c) Additional examples of colonies with disorganized morphology. Bar 100  $\mu$ m

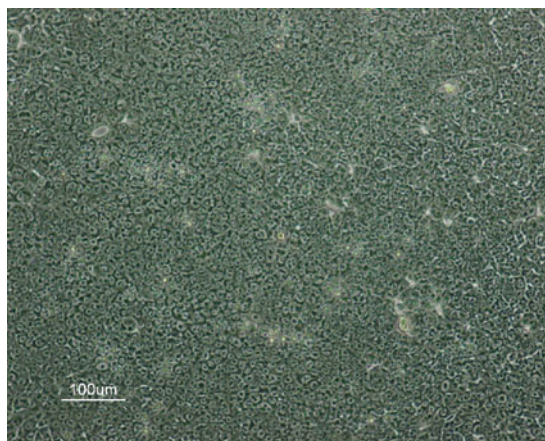
its typical morphology can be considered differentiating; however in some cases, this is hard to determine without pluripotency marker testing (Fig. 2.22). The morphology of differentiated PSCs cultured with MEFs or HFF is illustrated in Figs. 2.23 and 2.24, respectively. Sometimes, a colony remains mostly undifferentiated, with differentiation starting in small areas at its edges (Fig. 2.25). Other colonies have clear differentiation morphology with distinct formation of structures (Fig. 2.26). It is recommended to scrape differentiating colonies after every 5–7 passages.



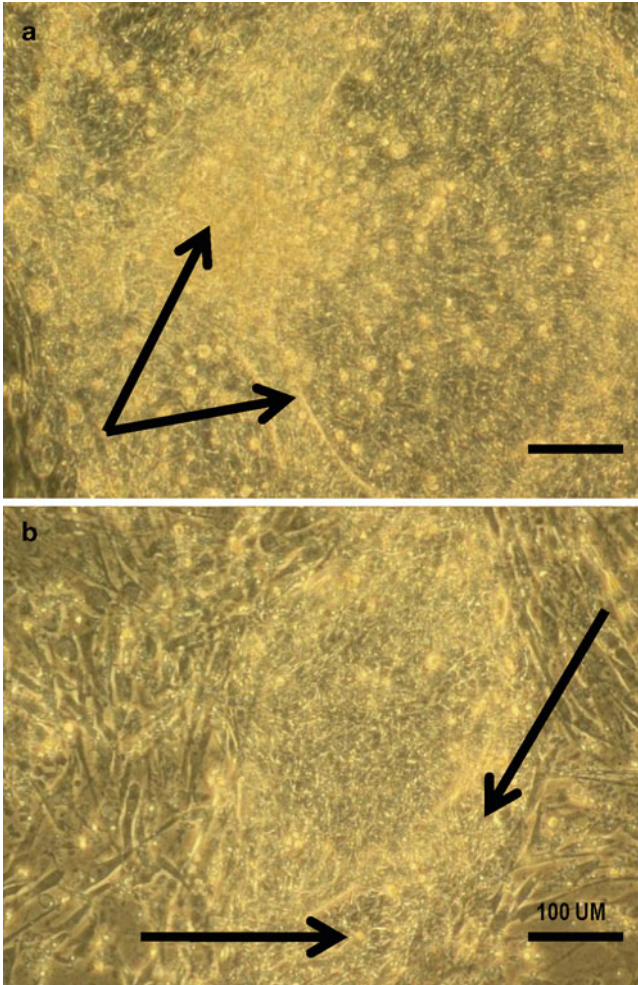
**Fig. 2.19** Undifferentiated colonies. **(a)** A colony 4 days post-splitting formed by hESC BG01, in which spaces between cells can be detected. There is a small area (marked by *arrow*) where some cells slip away from the colony border with the MEFs, but these cells still have clear undifferentiated morphology. **(b)** A colony 3 days post-splitting formed from hESC H9.2, in which spaces between cells can be detected, as well as the typically large nucleus. Similar to the colony presented in **(a)**. There are small areas (marked by *arrow*) where some cells slip away from the colony border, demonstrating still clear undifferentiated morphology. It seems that this large colony was formed by two nearby colonies. **(c, d)** Round undifferentiated colonies formed from hESC I3.2 2 days post-splitting, with clear borders from the MEF feeder layer. **(a, c, d)** Bar 100 μM, **(b)** bar 200 μM



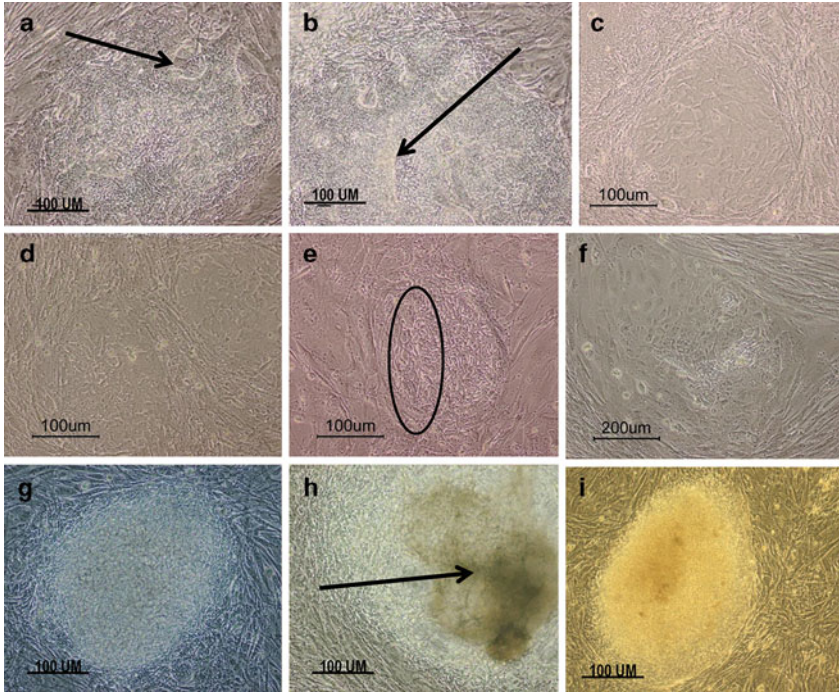
**Fig. 2.20** Undifferentiated colonies formed from hESC I3.2 cultured on HFF and with serum- and animal-free medium (NutriStem™, Biological Industries Ltd). The morphology of most colonies is similar to that of cells cultured with MEFs (**a**, **c**, **d**), but some colonies (**b**, **e**, **f**) do not have clear borders, although the cells within the colony remain undifferentiated. Bar 100 μM



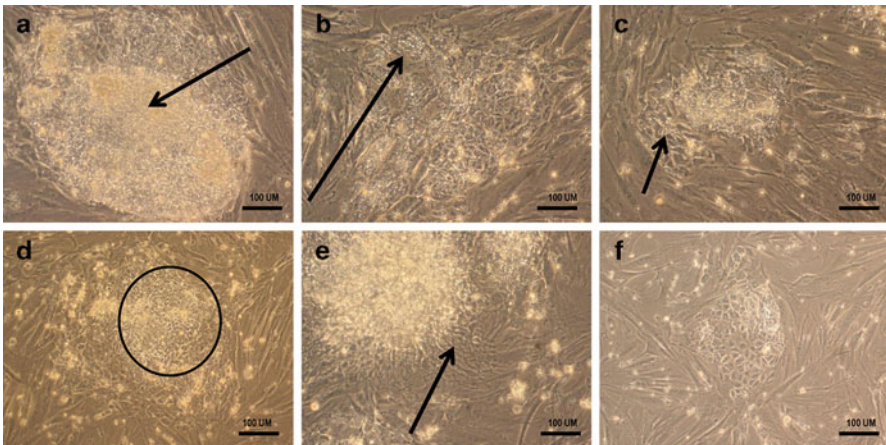
**Fig. 2.21** PSC morphology. Morphology of hESC H9.2 within the colony. Small round cells with large nuclei, notable nucleoli, and spaces between cells. Bar 100 μM



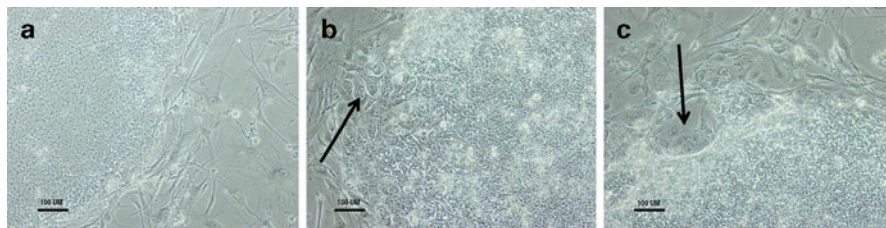
**Fig. 2.22** Colonies formed from hiPSC iLBWT30m with unclear morphology. **(a)** Probably three undifferentiated colonies that combined to one. The colony borders apparently form a differentiated structure (marked by *arrow*). **(b)** Colony with cells that slip away from the colony border with unclear morphology (*arrow*). The cells within the colony have typical undifferentiated morphology. Bar 100  $\mu$ M



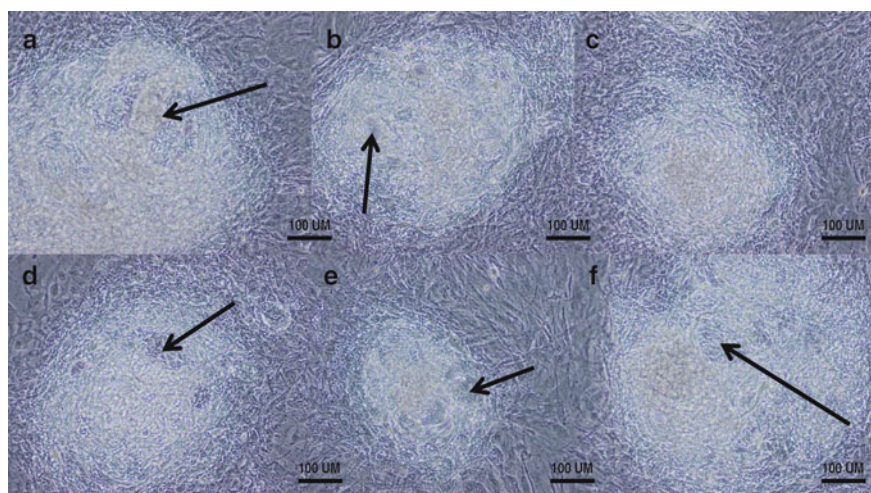
**Fig. 2.23** Differentiated colonies. (a, b, h) Colonies in which differentiating cells formed structures (marked by *arrows*). (c, d) Very flat colonies with unusually large cells. Some cells may still be undifferentiated. (e) Partly differentiated colony; the differentiating cells are marked with a *circle*. (f) A colony containing large cells, with a typical nucleus–cytoplasm ratio. (g, i) Thick colonies with unclear cell morphology; the cells are possibly still undifferentiated. (a–d) hESC BG01, (f) hESC H9.2, and (e, i, g, h) hESCs I3. Bar 100  $\mu$ M for all but (b) which has 200  $\mu$ M



**Fig. 2.24** Differentiated colonies formed from hESC I3.2 cultured on HFF. (a) A colony with a structure (marked with *arrow*) that may contain differentiating cells. However, the structure may be layers of undifferentiated cells. (b) Large lipid-containing differentiated cells (example marked with *arrow*). (c) A colony containing a differentiating area (marked with *arrow*). (d) A differentiating colony with some cells still undifferentiated (in *circle*). (e) Nerve-like differentiating cells at the edge of the colony (marked with *arrow*). (f) Cells with different morphology—sharp shape, larger, and without typical spaces between the cells. Bar 100  $\mu$ M



**Fig. 2.25** Differentiation at the colony borders. hESC H9 colonies 7 days post-splitting (a). With clear border and undifferentiated morphology (b, c). With differentiating cells at the border of the colonies (marked with *arrow*). Bar 100  $\mu$ M



**Fig. 2.26** Differentiating colonies formed from hESC I3 6 days post-splitting. All colonies formed structures within the colony (examples marked with *arrows*). Bar 100  $\mu$ M

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