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Atlas of Human Pluripotent Stem Cells in Culture

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In loving memory of Professor Donald S. Munro, who taught me tissue culture and always believed in me.

Ludmila Ruban

This book is dedicated to my Mum and Jude, who have supported me always, and it is in memory of my Dad, who was the reason that I went into science.

Lyn Healy

Preface by Lyn Healy

The derivation of human embryonic stem cells was described in "Embryonic stem cell lines derived from human blastocysts" by James A. Thomson et al. in 1998. Shortly after this discovery, work on this cell type in the U.K. commenced in the laboratories of Professor Peter Andrews and Professor Harry Moore in the University of Sheffield. Indeed, by 2003, the groups in Sheffield had derived their own lines and were running training courses in the growth and characterization of human embryonic stem cell lines.

It was during the preparation for one of these courses that Ludmila and I first met. We both had vast experience in cell culture, and Ludmila had the good fortune to have been involved in both the derivation and extended culture of these lines and their complex characterization. I had come into the area from a haemopoietic stem cell culture background with some experience in mouse embryonic stem cell culture, and I was eager to broaden my skill set to include human embryonic stem cells. I had eight weeks to be trained so that I could help with the practical training course. Ludmila was keen to train me, and among the first things that she did was to introduce me to her extensive collection of printed images of human embryonic stem cells displaying various levels of the differentiated state. With the aid of these images, she carefully explained to me what I should look for in the colonies in order to make a morphological judgment on their status.

I took this learning back with me to the U.K. Stem Cell Bank when we were starting our banking processes, and many of our staff have passed through the portals of the University of Sheffield Laboratories and have received training from Ludmila. When Ludmila moved to University College London in 2006, we were able to work more closely, and she suggested that we put together an atlas. We both believe that a picture really is "worth a thousand words." It is still the case that human pluripotent stem cells, including the human ES-like induced pluripotent stem cells, are generally assessed during their growth and expansion purely on morphology. This atlas is intended as a laboratory reference manual for scientists new to the field as well as for those with more experience, and we hope that it will be of value to those at the bench who rely on morphology as the basis for judging, subjectively and in isolation, the quality of pluripotent stem cells in culture.

We hope that this atlas will prove to be a valuable resource in the laboratory for researchers at all levels of experience.

Potters Bar, Hertfordshire, UK — Lyn Healy

Preface by Ludmila Ruban

Saying that I am not well known would be an overstatement. I am not known in the exquisite world of stem cell research. Nevertheless, in 2001, I was fortunate to join Peter Andrews' and Harry Moore's laboratory in Sheffield University, which later became the Centre for Stem Cell Biology. We were one of the first in the U.K. and Europe to start working with the Wisconsin human embryonic stem cell lines. The Centre was awarded one of the first two licenses granted by the U.K. Human Fertilisation and Embryology Authority (HFEA) for derivation of human embryonic stem cells. I was also actively involved in the training of national and international researchers within the Centre and coordinated, organized, and taught at the Centre for Academics' annual practical training course, "Working with Human Embryonic Stem Cells."

I met Lyn eleven years ago when she came to help us run the first practical course. I remember it like it was yesterday: We were passaging mouse embryonic fibroblasts (MEFs) together the first day we met, and after centrifuging the cells, we looked at the pellet and at the same time said "five million." I knew straight away we were talking the same language. We have been collaborating since then, working with stem cells, very often looking at the same problem from a different angle. We complement each other's knowledge. After looking in the microscope and watching stem cells "being" for more than thirteen years, we now feel we have a story to tell. It is what this book is about: the story of stem cells in culture. Step by step, day after day, month after month, thirteen years on a story that is really fascinating, intriguing, and sometimes frustrating. We were always passionate and very often emotional about it. I am absolutely sure anyone going into stem cell research should feel the same: passionate and determined.

In 2006, I joined the Department of Biochemical Engineering at University College London. Every day in the lab, I mentor undergraduate, master, and PhD/EngD students, helping them to understand the practical complexity of stem cell research, and I act as an adviser on numerous collaborative research and development programs. With the rest of my time, I manage the Cell Therapy Research Facilities. I run stem cell practicals and tissue culture courses for undergraduate and postgraduate students who very often have only an engineering, mathematical, or physics background. The lack of a stem cell atlas was really clear from the beginning. From day one, I have had all my images printed in a folder, and before going to the tissue culture practical, I would introduce everyone to "stem cells in pictures" first, discussing all the pros and cons. Only after that would we start the practicals, with a basic knowledge. Every participant would have copies of the pictures. We would compare cells in real culture to cells in the images. It was always very productive this way.

Sometimes, I found it easy to teach students without any experience in stem cells or even without any tissue culture experience. Most importantly, I found that I was learning along with them. Most of the participants are students with a biochemical engineering background, and their questions are unexpected for a biologist, coming from a totally different angle. Stem cells are very unpredictable. They are challenging but exciting to work with. My students are always surprised by my excitement and enthusiasm when I am talking about stem cells. I am very often surprised myself, and sometimes I wonder why. I guess it happened after I realized that human pluripotent stem cells are like humans: they resemble each other, and at the same time they differ, each in their own way, with their own special character. Lively or moody, easygo-

ing or not, they want to be treated with great care, love, and respect like any human being. One of their very important characteristics is that they want to be together all the time like most of us. They are not happy to be single and lonely, especially for a long time. They usually grow as colonies very close to each other. I can definitely say this: They are very friendly and like company not just of themselves but of other species too. Their best friend in this case would be mouse fibroblasts/feeders, but good matrix is very often a suitable replacement. Again, this is not very far from human existence.

Some of them are very easygoing and adapt to new conditions; others are very slow and can make your life very difficult. They have a taste for expensive "food"—specially designed media with lots of different growth factors. These cells are very conservative, sometimes selfish and needy, and they demand attention 365 days a year! Yes, you understand correctly: you will not have any more weekends or relaxing holidays. At the same time, they are very loyal: They want only you and no one else to look after them. That is why when you come back from holidays, they never look the same as before you left. Like some of us, they definitely do not cope well with stress and absolutely hate change. They take time to get used to any new conditions. If the conditions are very tough and they still manage to adapt, then you may be unlucky—these cells can become totally different characters: still pluripotent, still stem cells, but karyotypically abnormal. It's what happens when you select a survivor, and an adaptation of this kind can unfortunately be the first step in neoplastic transformation.

One very important suggestion to everyone who wants to become a stem cell biologist: You must fall in love with stem cells and everything around them. That is the only way to be successful.

London, UK Ludmila Ruban

About the Authors

Dr. Lyn Healy started her training at the Paterson Institute for Cancer Research in Manchester, England, in the experimental hematology laboratory of Professor Dr. T. M. Dexter, where she obtained her master's degree. From there, she moved to London's Institute of Cancer Research, where she worked in the laboratory of Professor M. F. Greaves in the Leukemia Research Fund Centre and obtained her Ph.D. from the University of London on stem cells in normal and leukemogenic hemopoiesis. She joined the United Kingdom Stem Cell Bank (UKSCB) at the National Institute for Biological Standards and Control as the Senior Stem Cell Biologist. Dr. Healy leads the research and development program at the UKSCB and plays an active role in training scientists in pluripotent stem cell culture. She has collaborated on training courses hosted by the Centre for Stem Cell Biology in Sheffield and has run courses at the UKSCB. In addition, she set up the UKSCB Technical Forum, where scientists discuss and resolve practical challenges in the field of stem cell culture. Dr. Healy has published more than forty peer-reviewed papers on stem cell biology.

Ludmila Ruban obtained her MSc in physiology at the University of Kiev, Ukraine. She began work on human embryonic stem cells (hESC) in Peter Andrews' and Harry Moore's laboratory in Sheffield University, which later became the Centre for Stem Cell Biology. This group was one of the first in the United Kingdom and Europe to start working with the Wisconsin human embryonic stem cell lines. The Centre has been awarded one of the first two licenses granted by the U.K. Human Fertilisation and Embryology Authority for derivation of hESC, and she has helped derive several new stem cell lines. She was also actively involved in the training of national and international researchers within the Centre and coordinated, organized, and taught at the Centre's annual practical training course for academics, "Working with Human Embryonic Stem Cells." She joined the Department of Biochemical Engineering at University College London, and her research interests lie primarily in the area of human embryonic stem cells, specifically optimization of tissue culture conditions for stem cell lines. She also has an interest in stem cell bioprocessing.

Acknowledgements

The authors would like to thank Professor Peter Andrews and Professor Harry Moore, University of Sheffield, Centre for Stem Cell Biology, for the dissemination of their practical experience and knowledge in the field of stem cell biology. The authors would also like to thank the UK Stem Cell Bank, National Institute for Biological Standards and Control (NIBSC); colleagues at the Department of Biochemical Engineering, University College London (UCL); and other colleagues in the stem cell community, especially the post-graduate students in the Department of Regenerative Medicine, UCL, for their help and support.

Contents

Before We Begin: An Introduction to the Culture of Pluripotent Cells

1

Cell culture is a very diverse discipline that covers many biological systems. Although it is anticipated that most readers of this atlas will be experienced cell culture technologists, it is still worthwhile to review and revise the assumed knowledge base so that all readers are in agreement with the definitions and the terminology used by the authors (see glossary). For the most part, the material covered in this atlas is mainly qualitative and therefore subjective.

The aim of this book is not to deliver a set of protocols for the culture of pluripotent stem cells (PSCs), but rather to provide a pictorial resource as a guide and an *aide-mémoire*. Currently, morphology is the fundamental characteristic used to assess the quality of growing cells from day to day. It is non-invasive and relatively accurate on the whole, but as previously stated, morphology is a subjective measure of quality and is not a standardised procedure. There are a number of ways that other parameters can be added to provide more information on the state of the growing cells. These include such processes as 'live' staining and morphometric analysis of cell growth, but these require more sophisticated equipment than just a standalone bright field microscope.

Readers who are new to cell culture are advised to familiarise themselves with the basic techniques of cell culture and to adhere to the principles of good cell culture practice (GCCP). The maintenance and expansion of PSCs requires a skill set that is best acquired through training. If you are not deriving the cell lines yourself, it is of fundamental importance to obtain the lines or other biological materials from a reputable provider, to enable traceability and instil confidence that the materials have been ethically sourced. Doing so also will allow for the replacement of the cells, if required, with equivalent material, for consistency in cell culture work and any associated assays. In general, cell lines will be provided with a certificate of analysis containing data such as safety testing and characterisation specific to the cell line obtained. The safety testing information will enable a risk assessment to be performed before the cells are brought into the laboratory and used, allowing appropriate safeguards to be put in place if required. Cell lines should be quarantined upon arrival and kept in isolation until it has been established that they are free from contamination.

Lines should be authenticated upon receipt, usually by short tandem repeat analysis, and this analysis should be repeated prior to the publication of datasets, to ensure that the cell lines have not been cross-contaminated or misidentified. It is advisable to produce a bank of each cell line received, to facilitate the replenishment of cells at defined intervals in the cell culture process and thereby reducing the risk of genomic changes in the cell culture, which are generally a result of extended passaging of the cells. With this in mind, cells should be assessed for their genetic stability by karyotyping. It is suggested that this be performed every ten passages. A panel of genotypic and phenotypic characterisation tests should be performed on the cell line at defined intervals as part of quality control. Routine phenotypic analysis of the cell line should include morphological assessment, which should be part of the daily best practice cell culture regimen.

This atlas aims to provide a simple resource for general, day-to-day laboratory activity with respect to the visual assessment of the status of cell cultures. The authors have endeavoured to concentrate solely on the provision of a range of images that will be seen in the routine PSC workflow. Also included is a chapter on infections, which we believe to be unique and of great value to researchers, especially in an environment where antibiotics are seldom used in routine culture and it may be difficult to distinguish between, for example, the shedding of cells and the identification of a low-grade yeast infection.

With regard to protocols and other cell culture-related issues, the lists of suggested readings should provide the reader with a good source of reference information.

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Suggested Reading

General Cell Culture and Best Practice

Freshney RI. Basic principles of cell culture. In: Vunjak-Novakovic G, Freshney RI, editors. Culture of cells for tissue engineering. Hoboken: Wiley; 2006. p. 3–22.

Bal-Price A, Coecke S. Guidance on good cell culture practice (GCCP). Neuromethods. 2011;56:1–25.

Coecke S, Balls M, Bowe G, Davis J, Gstraunthaler G, Hartung T, et al. Guidance on good cell culture practice. A report of the second ECVAM task force on good cell culture practice. Altern Lab Anim. 2005;33:261–87.

International Stem Cell Banking Initiative. Consensus guidance for banking and supply of human embryonic stem cell lines for research purposes. Stem Cell Rev. 2009a;5:301–14.

Pauwels K, Herman P, Van Vaerenbergh B, Chuong DD, Berghmans L, Waeterloos G, et al. Animal cell cultures: risk assessment and biosafety recommendations. Appl Biosafety. 2007;12:26–38.

Stem Cell Protocols

Amit M, Itskovitz-Eldor J, editors. Atlas of human pluripotent stem cells: derivation and culturing series, Stem cell biology and regenerative medicine series. New York: Springer; 2012.

Diekmann U, Spindler R, Wolkers WF, Glasmacher B, Müller T. Cryopreservation and quality control of mouse embryonic feeder cells. Cryobiology. 2011;63:104–10.

Harvard Stem Cell Institute, University of Massachusetts Medical School, National Institutes of Health, MassGeneral Institute for Neurodegenerative Disease. StemBook. Available at: http://www.stembook.org.

Hernandez D, Ruban L, Mason C. Feeder-free culture of human embryonic stem cells for scalable expansion in a reproducible manner. Stem Cells Dev. 2011;20:1089–98.

International Stem Cell Banking Initiative. Consensus guidance for banking and supply of human embryonic stem cell lines for research purposes. Stem Cell Rev. 2009b;5:301–14.

Johkura K, Cui L, Asanuma K, Okouchi Y, Ogiwara N, Sasaki K. Cytochemical and ultrastructural characterization of growing colonies of human embryonic stem cells. J Anat. 2004;205:247–55.

Lakshmipathy U, Vemuri MC, editors. Pluripotent stem cells: methods and protocols. Methods in molecular biology, vol. 997. New York: Humana Press; 2013.

Luong MX, Auerbach J, Crook JM, Daheron L, Hei D, Lomax G, et al. A call for standardized naming and reporting of human ESC and iPSC lines. Cell Stem Cell. 2011;8:357–9.

Nieto A, Cabrera CM, Catalina P, Cobo F, Barnie A, Cortés JL, et al. Effect of mitomycin-C on human foreskin fibroblasts used as feeders in human embryonic stem cells: immunocytochemistry MIB1 score and DNA ploidy and apoptosis evaluated by flow cytometry. Cell Biol Int. 2007;31:269–78.

Oh SK, Kim HS, Park YB, Seol HW, Kim YY, Cho MS, et al. Methods for expansion of human embryonic stem cells. Stem Cells. 2005;23:605–9.

Peterson S, Loring JF, editors. Human stem cell manual: a laboratory guide. 2nd ed. Boston: Academic; 2011.

Sathananthan H, Pera M, Trounson A. The fine structure of human embryonic stem cells. Reprod Biomed Online. 2002;4:56–61.

Schwartz PH, Wesselschmidt RL, editors. Human pluripotent stem cells series: methods in molecular biology, vol. 767. New York: Humana Press; 2011.

Mouse and Human Fibroblasts

2

Fibroblasts are cells found in abundance in the connective tissue of an organ. They are active cells producing extracellular matrix and collagen. These cells are isolated from tissues by dissociating the cells using enzymatic treatment and culturing them in the appropriate cell culture medium. These are primary cultures and as such have a limited lifespan. This is the case with both mouse and human fibroblasts.

Antibiotics are not used in many pluripotent stem cell (PSC) laboratories, so it is imperative that you observe the cell cultures from these primary cells to ensure that there is no observable contamination (Chap. 11), as these cells will be used to produce batches of cells. Ultimately, the size of a batch of primary cells relates to the amount of cells isolated from the original source tissues, so it is important that these cells are free from contamination; an appropriate testing regimen should be put in place.

The production of primary fibroblasts in general is an infrequent activity in the laboratory setting because fibroblasts will be expanded to make batches of cells, which may be frozen, inactivated and used, or inactivated and frozen. A number of laboratories routinely freeze cells at passage 0. This cell passage number can be defined as the cells harvested from the primary vessel in which the original cells dissociated from the source tissue were first grown in culture. When thawed from this passage number, a large batch of cells can be generated.

Fibroblasts in their inactivated state are used as feeder cells (Chap. 3) both to derive and to maintain PSCs. The use of fibroblasts for these purposes is well documented and historically originates from mouse embryonic stem cell culture. Many sources of fibroblasts have been demonstrated to support the growth of undifferentiated cells, but the sources most frequently used in the laboratory are mouse embryonic fibroblasts (MEFs) and human foreskin fibroblasts. To date, maintenance of PSCs on feeder cells (especially mouse) is still the standard practice in most laboratories around the world.

Fibroblasts display biological variability even when isolated from the same source, and this attribute is reflected in the batch-to-batch differences observed when it comes to the support of PSCs. Quality control of batches is important, as poor-quality fibroblasts produce poor-quality feeder cells. This variation is recorded in this chapter for three independent batches of mouse fibroblasts. Batches 1 and 2 show a good morphology from passage 1, whereas batch 3 does not display an optimal morphology until passage 3 but goes on to produce a batch of robust feeders. As stated previously, primary fibroblasts have a limited lifespan, and their ability to produce feeders that support the growth of undifferentiated PSCs is also limited. In the case of mouse feeders produced from primary fibroblasts subject to a split ratio of 1–3 or 1–5, this limit is usually around passage 3 or 4, although with a robust batch, it can go out to passage 7 (Figs. 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 2.10, 2.11, 2.12, 2.13, 2.14 and 2.15).

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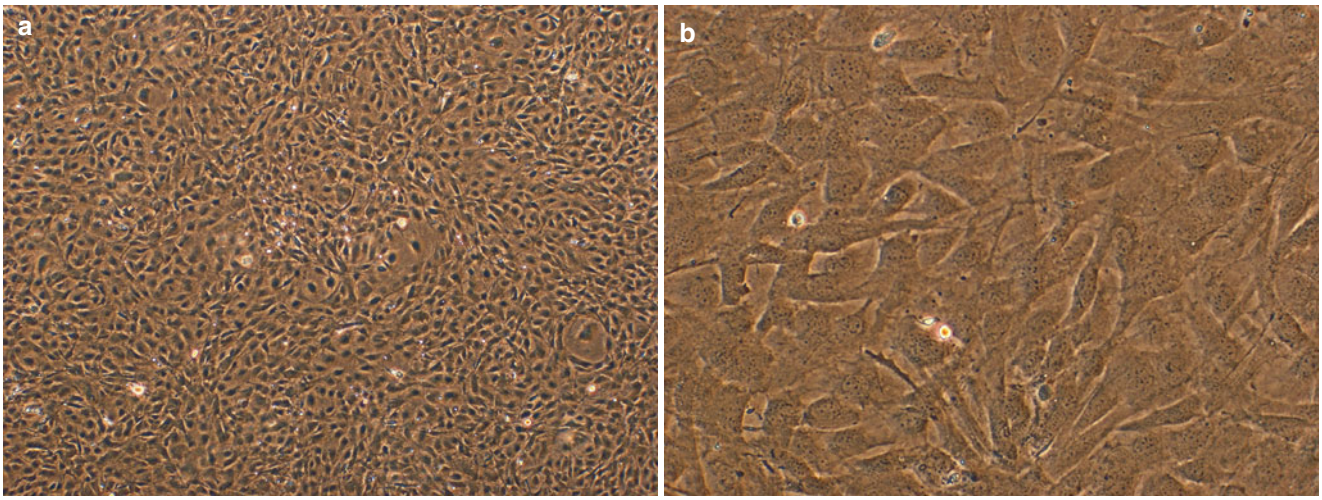

Fig. 2.1 Confluent culture of mouse embryonic fibroblasts (MEFs) exhibiting the typical elongated morphology of healthy fibroblasts. **a**, ×4 magnification. **b**, ×20 magnification

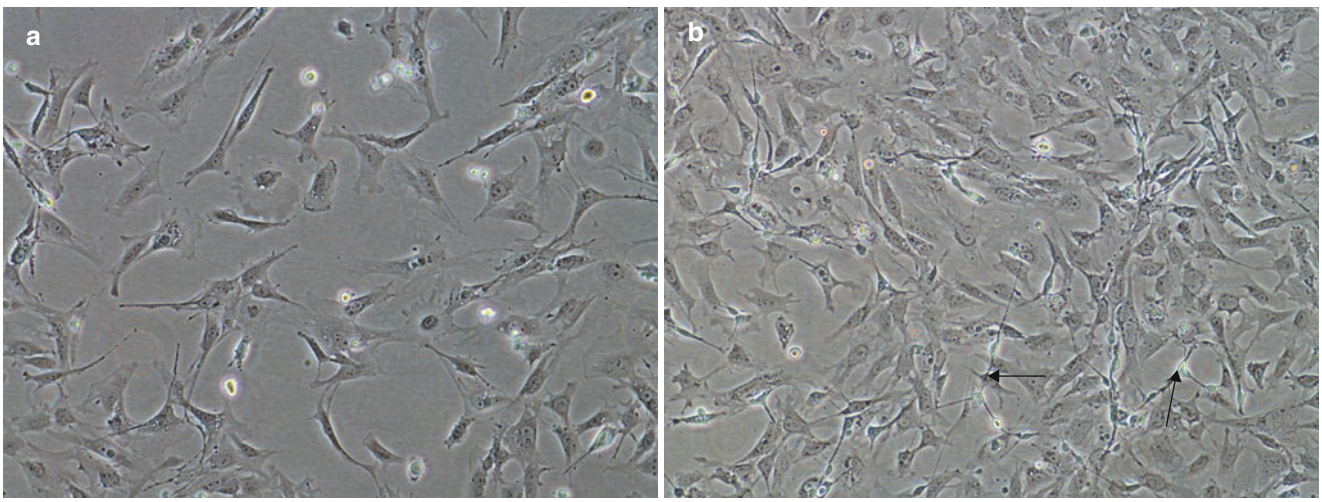

Fig. 2.2 Good quality Batch-1 of MEF-1 at passage 1. (**a**) At 24 h after passaging, the cells display a confluency of 40–50 % (×10 magnification). (**b**) At 48 h after passaging, the cells display a confluency of 90–95 % (×10 magnification). Different cell types, such as neural cells present in the culture, are visible (*black arrow*). After two passages, the culture appears more uniform, with only an occasional neural cell present

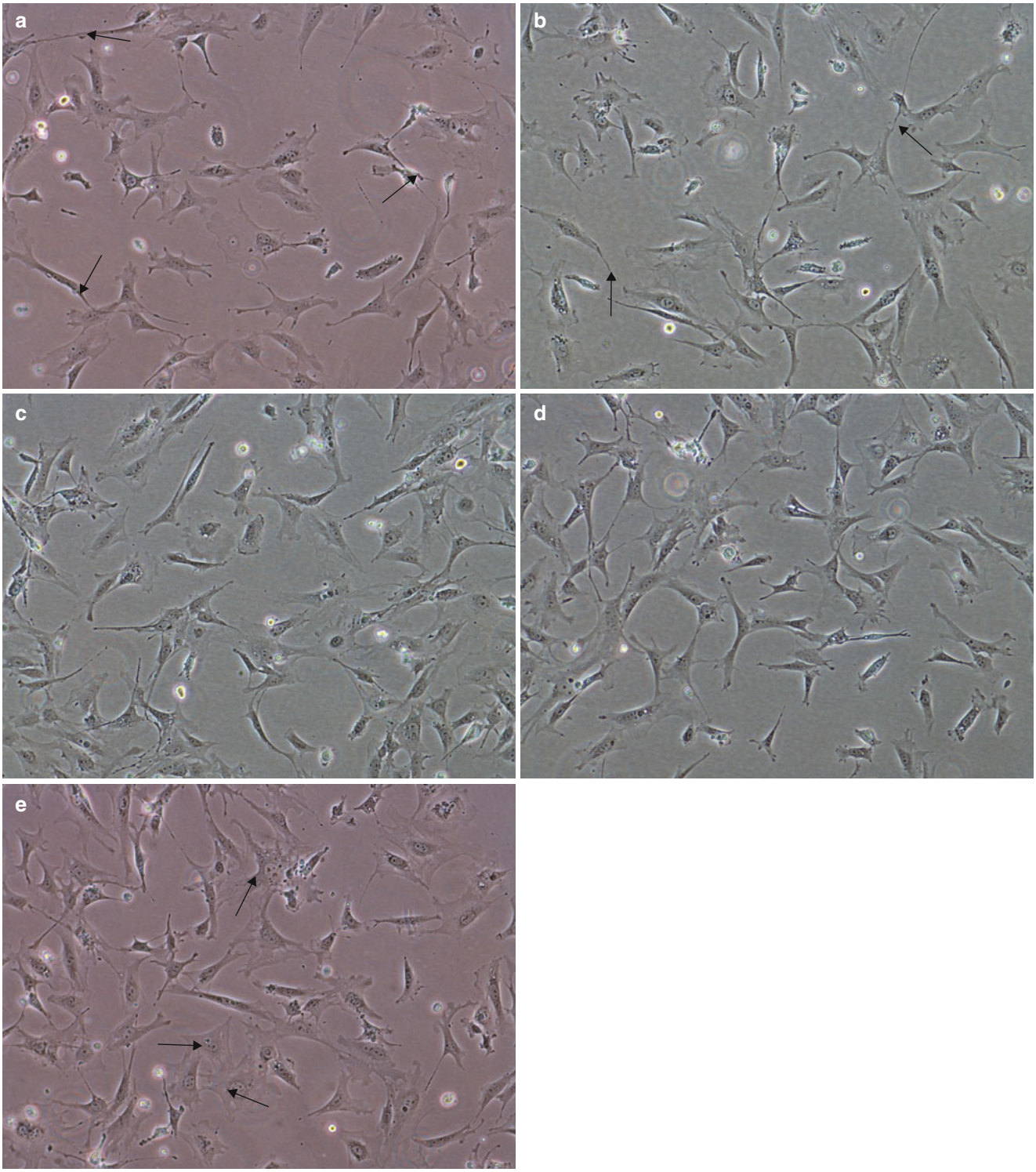

Fig. 2.3 The Batch-1 MEFs in these images show a slightly different confluency, 24 h after passaging. The fibroblasts look very similar at first, but the trained eye notes the difference (×10 magnification). (**a**, **b**) Cells are struggling to touch each other. They are stressed as indicated by their thin spindle-like appearance with long processes (*black arrows*) (×10 magnification). (**c**, **d**) These cells are at their best. They exhibit a good morphology and confluency (×10 magnification). (**e**) At first glance, this looks like a good culture, but if this culture is carefully examined, there are too many cells with spread bodies (*black arrow*) (×10 magnification). These cells are often senescent, and they become extra large (see also Fig. 2.12) and take up a large amount of space in the tissue culture dish, thereby reducing the number of cells that can proliferate in that dish

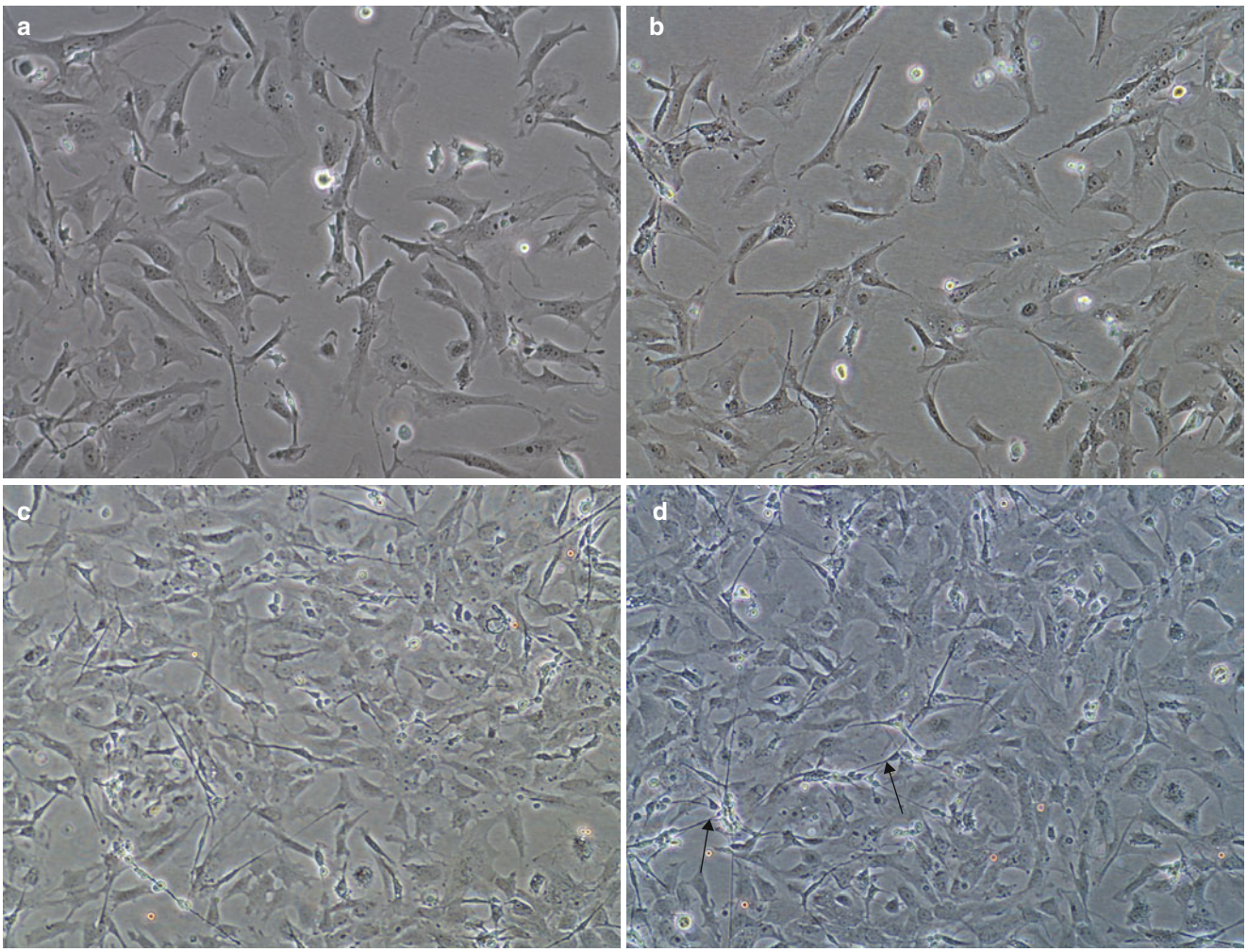

Fig. 2.4 Another example of Batch-1 MEFs at passage 1. (**a**, **b**) At 24 h after passaging (×10 magnification). (**c**, **d**) Three days after passaging, these cells display 100 % confluency. Note the presence of a number of neural cells in the culture (*black arrows*) (×10 magnification)

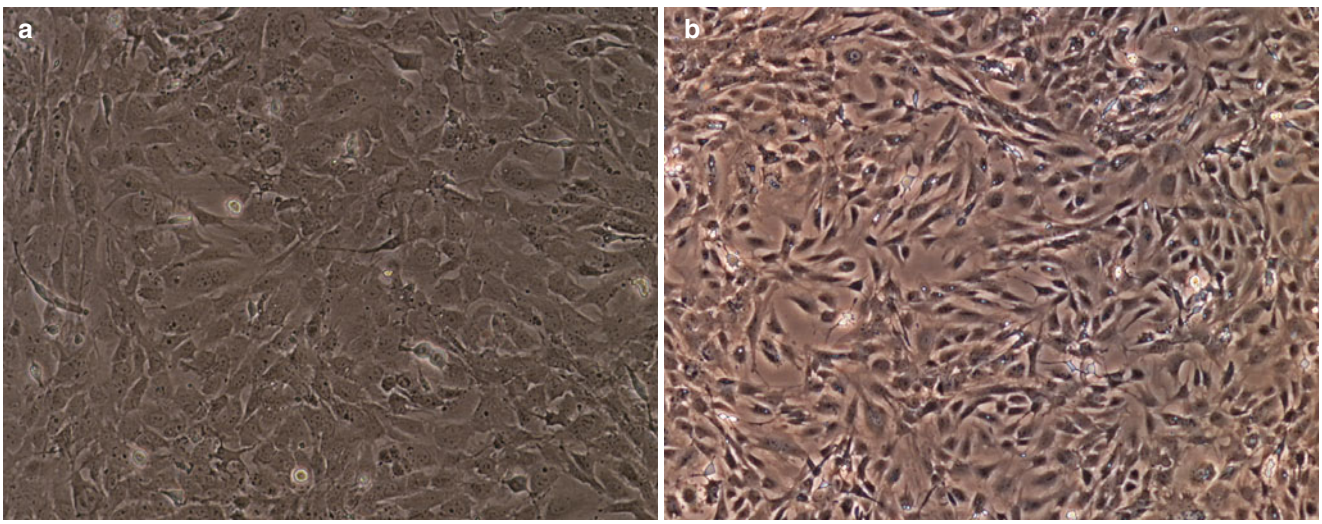

Fig. 2.5 Comparison between two different batches of confluent MEFs, Batch-1 and Batch-2. These cultures exhibit a slightly different morphology, but both are of an equally good quality (×10 magnification). (**a**) Batch-1 MEFs. Cells are spread and round, at 100 % confluency. (**b**) Batch-2 MEFs. Cells are spindle shaped at 100 % confluency

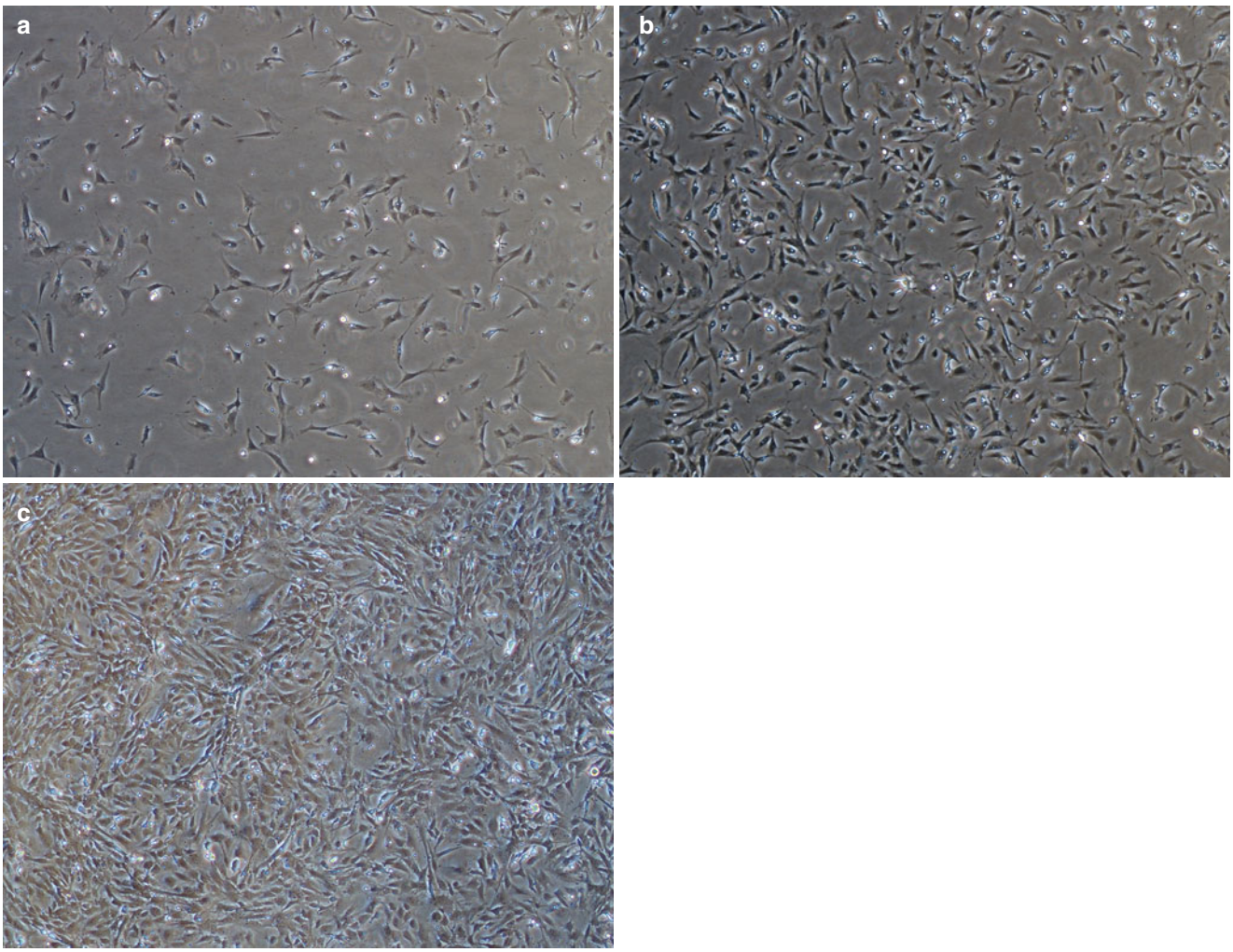

Fig. 2.6 Batch-2 MEFs at different confluences. (**a**) At 24 h after plating, the cells are 40–50 % confluent (×4 magnification). (**b**) At 48 h after plating, the cells are 60–70 % confluent (×4 magnification). (**c**) At 72 h after plating, the MEFs are 100 % confluent (×4 magnification)

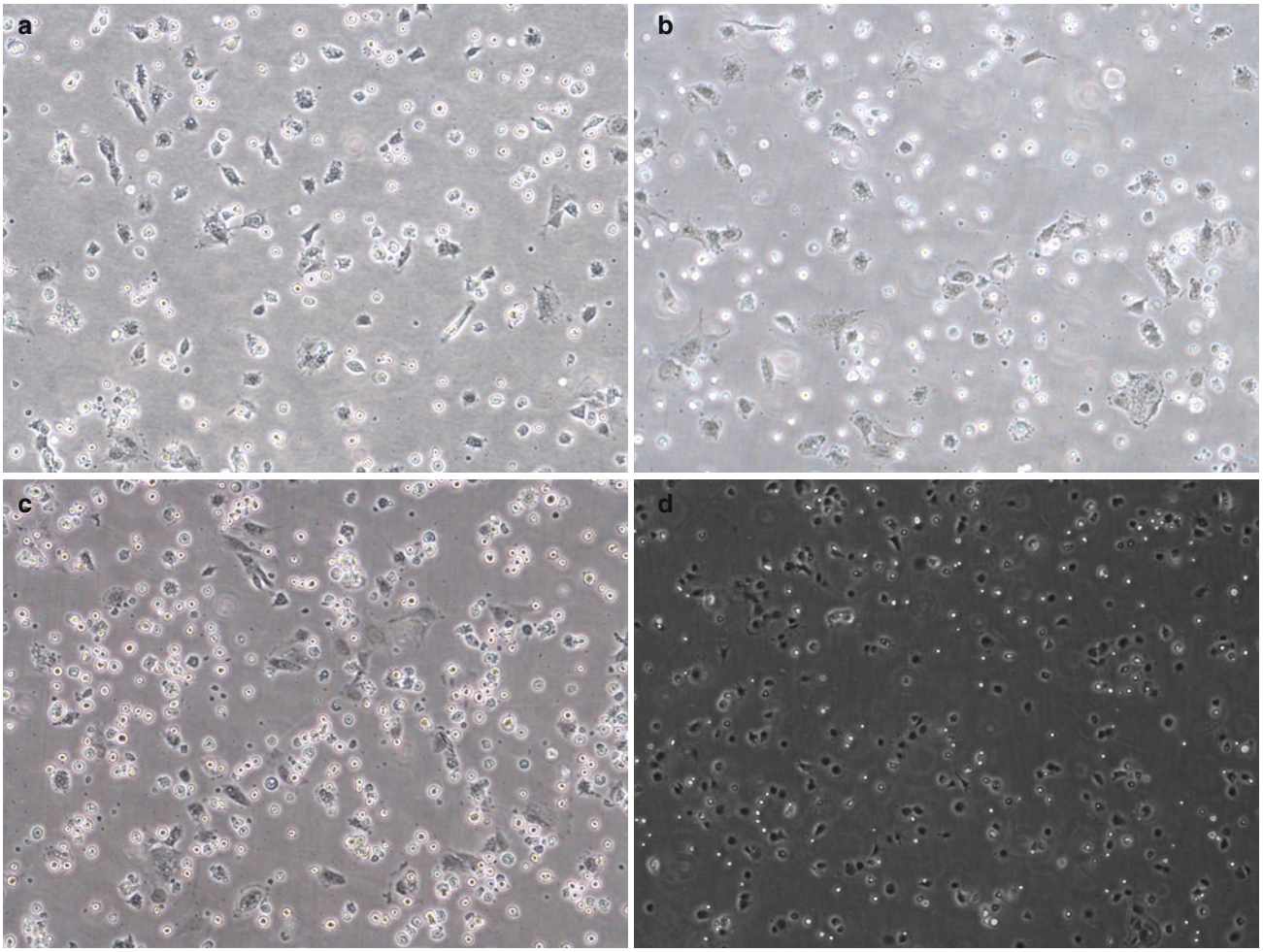

Fig. 2.7 (**a**–**c**) Batch-3 MEFs 2 h after thawing. The cells in this batch are not in the best condition; the morphology differs because the freezing process for these cells was not optimal. In all pictures, the cells are displaying the signs of stress: they are not attaching and spreading. There are also too many floating, apoptotic cells (×10 magnification). (**d**) By comparison, these Batch-2 MEFs attached to the culture dish in less than an hour, and there are not many cells floating in the culture medium (×4 magnification)

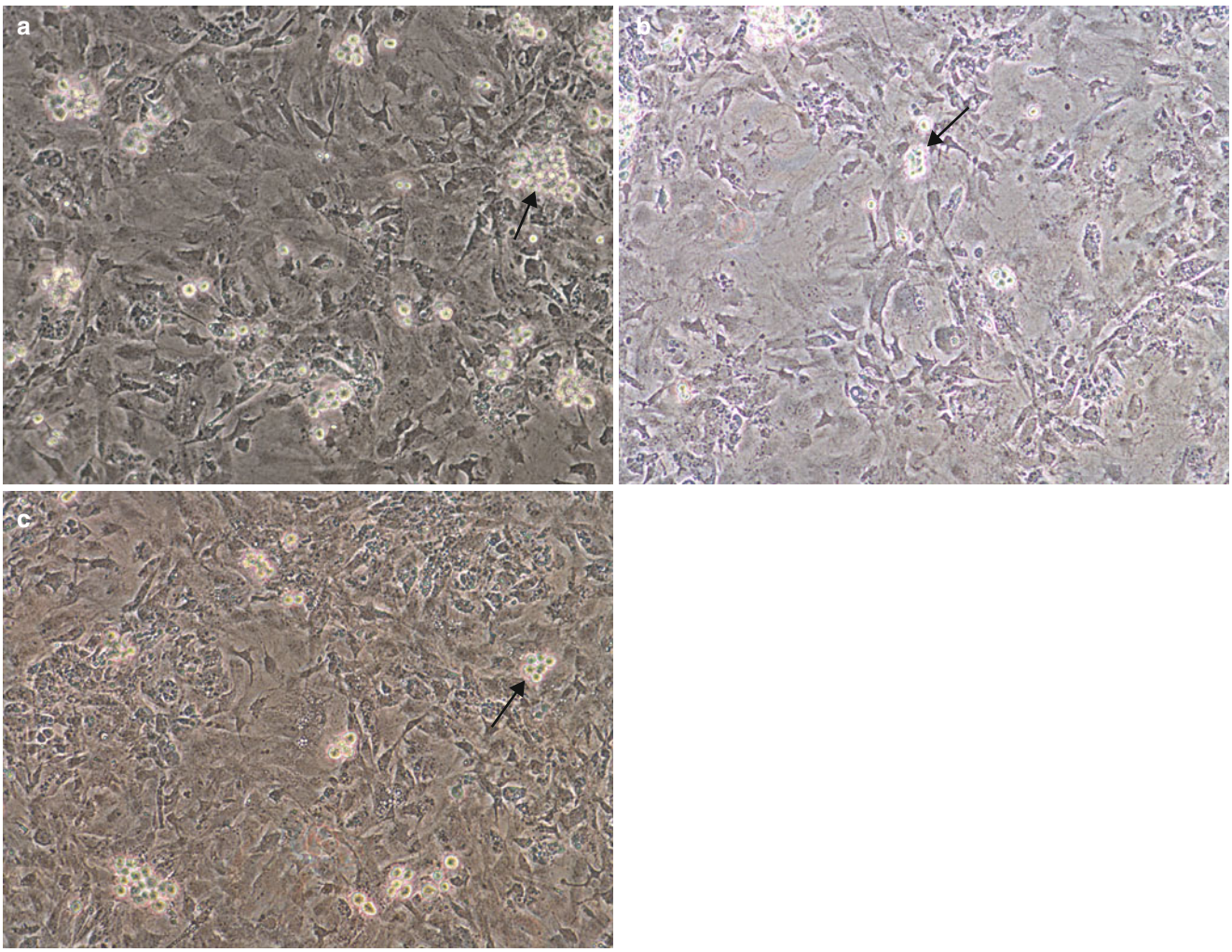

Fig. 2.8 (**a**–**c**) Batch-3 MEFs, passage 1, 3 days after plating. The cells are overconfluent and are not displaying a homogeneous morphology, which indicates the presence of different cell types. A number of floating cells are seen above the adherent fibroblasts (*black arrows*) (×10 magnification)

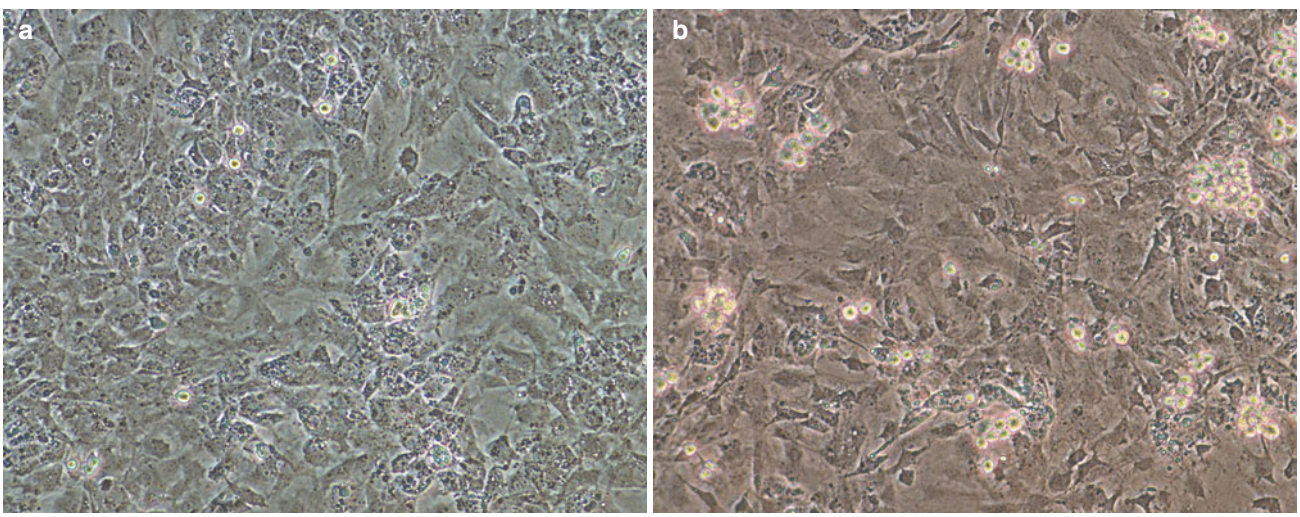

Fig. 2.9 Batch-3 MEFs, passage 2 (**a**) show an apparent improvement in comparison to passage 1 cells (**b**). The culture at passage 2 is more homogeneous (both ×10 magnification)

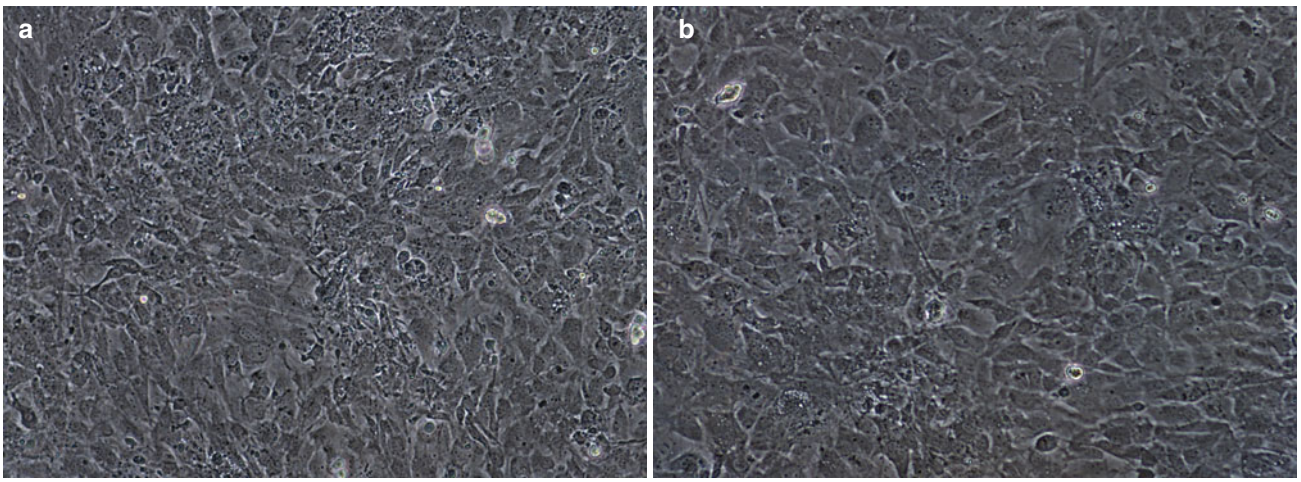

Fig. 2.10 (**a**, **b**) Batch-3 MEFs, passage 3 (×10 magnification). The cell morphology is improved greatly from that seen in Fig. 2.9a. The culture is 100 % confluent. With the improvement in morphology observed between passage 1 and passage 3 of the Batch-3 fibroblasts, these fibroblasts could be used to produce feeders from the passage-3 cells. In addition, it should be noted that with Batch-3, the production of supportive feeders lasted longer than the feeders prepared from Batch-1 and Batch-2. At passage 7, the Batch-3 cells were still proliferating at a steady rate and the feeders produced at this passage supported undifferentiated stem cell growth. Batch-1 and Batch-2 MEFs were supportive only until passage 5

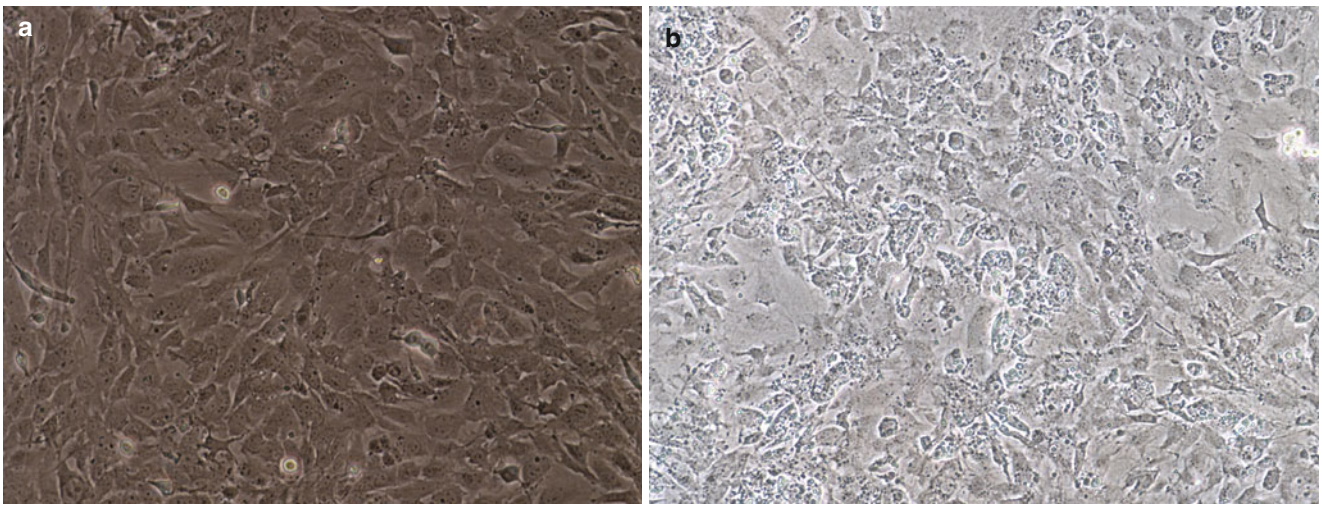

Fig. 2.11 Comparison between Batch-1 MEFs designated as having a good morphology (**a**) and Batch-3 MEFs with a suboptimal morphology (**b**) at passage 1, showing a clear difference between the two cell cultures (×10 magnification). (**a**) The morphology and nucleus of every single cell in the field of view can be easily distinguished. (**b**) In contrast, the morphology of these cells is not well defined and vacuoles are present inside cells, indicating that the cell is under stress

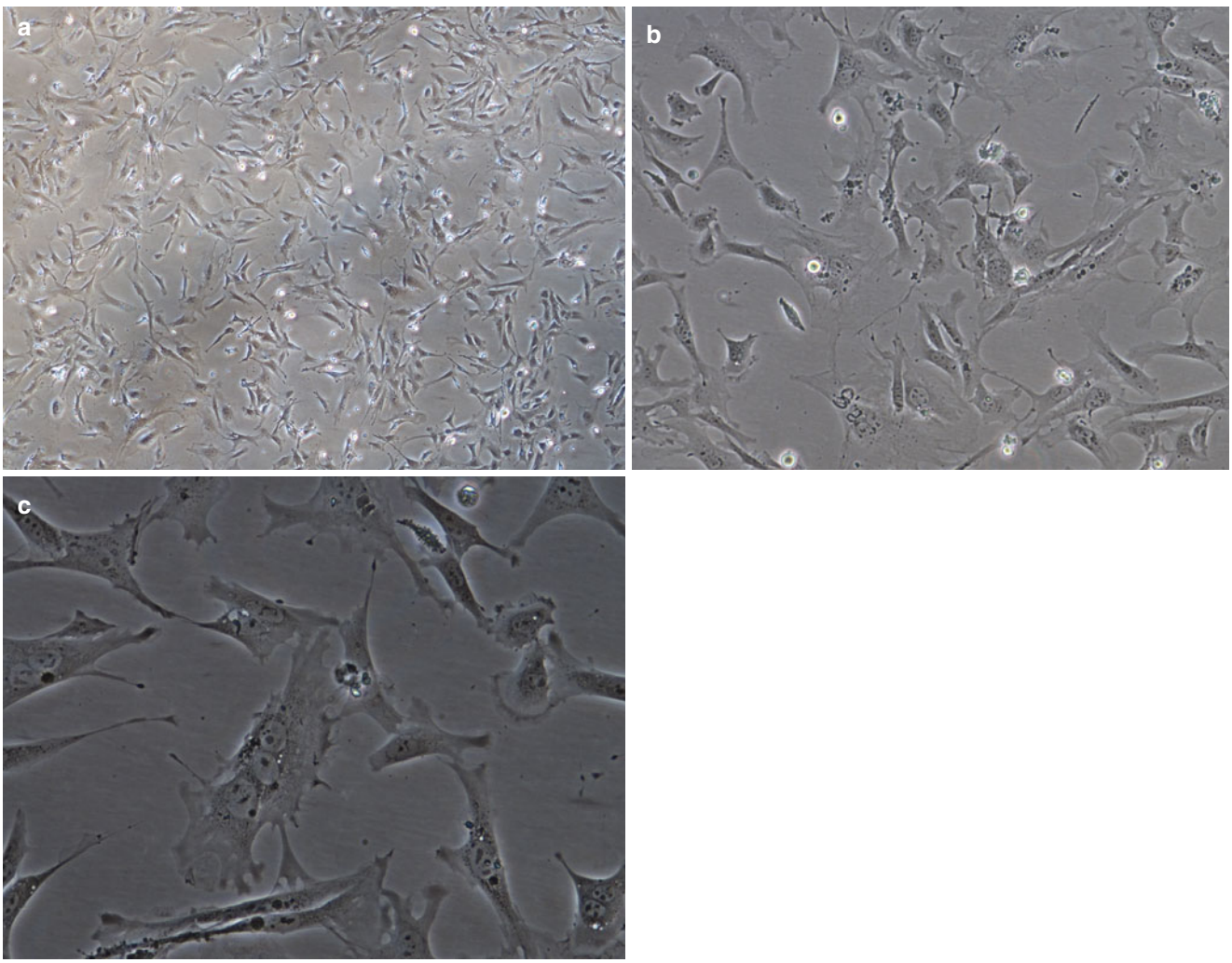

Fig. 2.12 (**a**–**c**) MEFs, Batch-2, passage 4, 24 h after passaging. The cells are attached, spread, actively proliferating and display a good morphology. There are not too many floating cells in the medium. **a** ×4 magnification; **b**, ×10 magnification; **c**, ×20 magnification

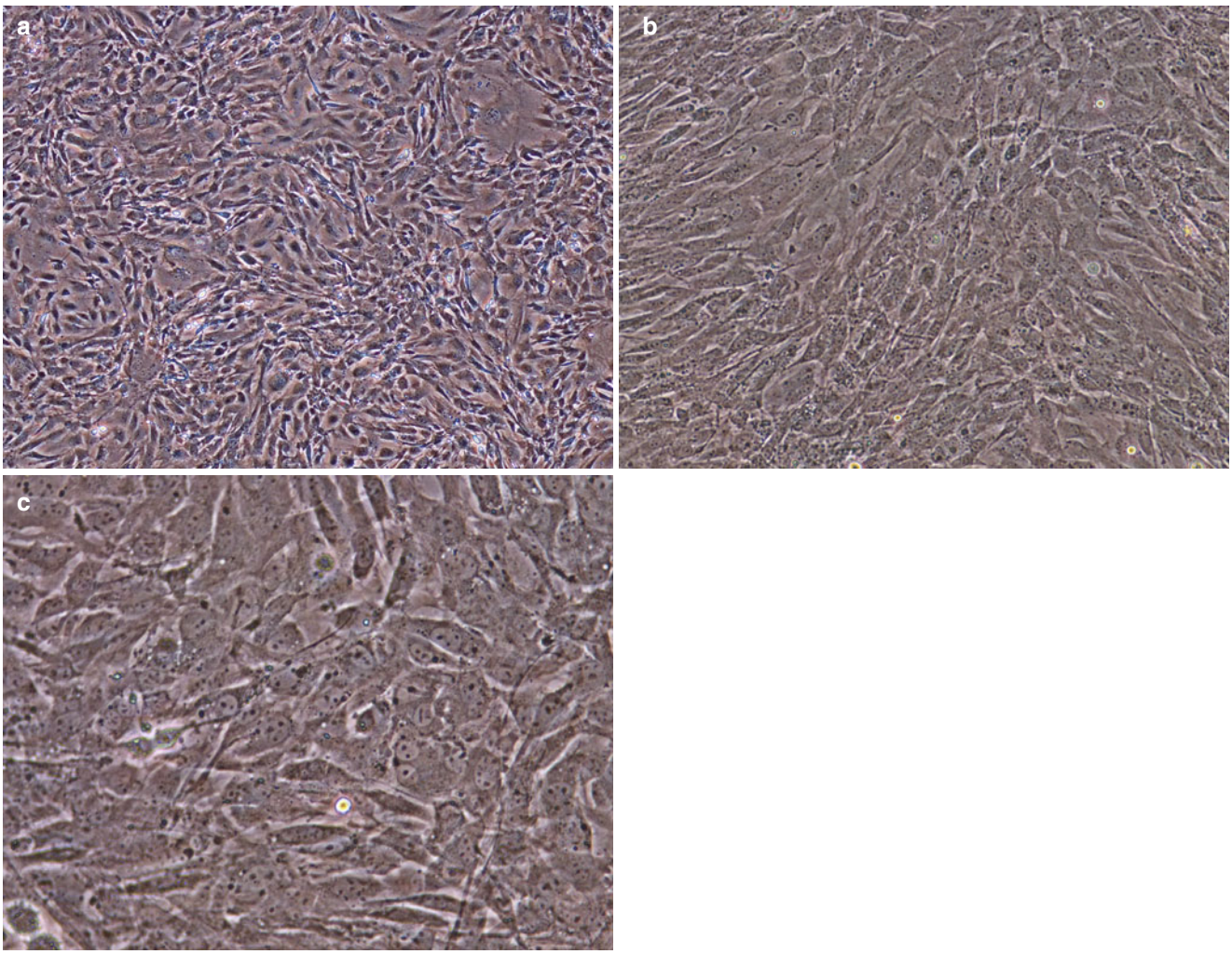

Fig. 2.13 (**a**–**c**) MEFs, Batch-2, passage 4, 72 h after passaging. These cells display 100 % confluency. There are not too many floating cells in the medium. From a T-75 flask in this condition, expect a yield of about 6–7 × 10^6 cells. **a**, ×4 magnification; **b**, ×10 magnification; **c**, ×20 magnification

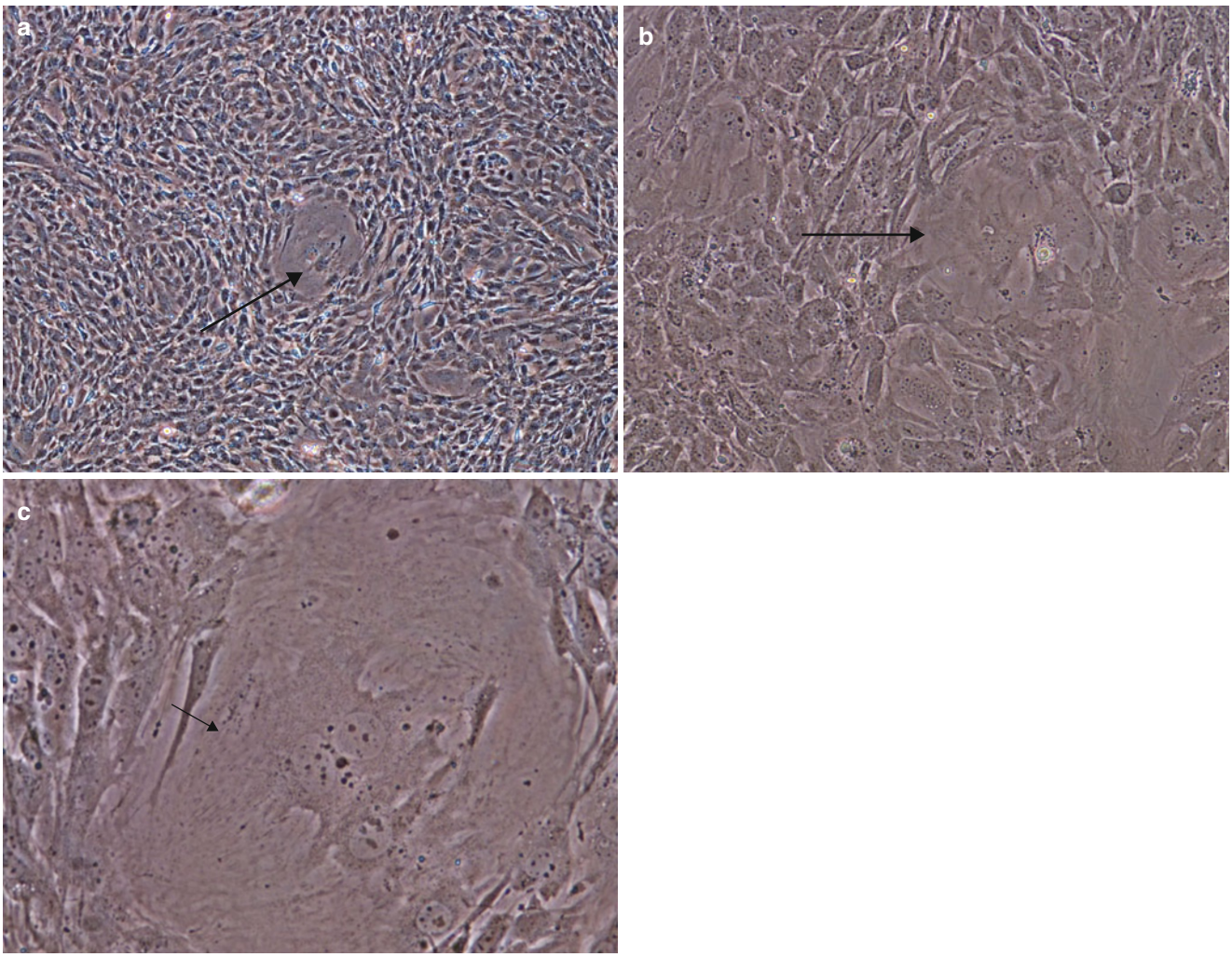

Fig. 2.14 In primary cultures of MEFs, sometimes extra-large cells (*black arrows*) can be observed growing within the homogeneous fibroblast cultures. These extra-large cells display a different morphology to the fibroblasts; they appear to have a flattened cytoplasm and are sometimes mistaken for spaces in the cell cultures. If these cells are observed, do not worry about their presence. If there are too many of them in the culture; however, the number of MEFs generated will be reduced greatly because these large cells take up more space in the culture vessel than a normal fibroblast. **a**, ×4 magnification; **b**, ×10 magnification; **c**, ×20 magnification

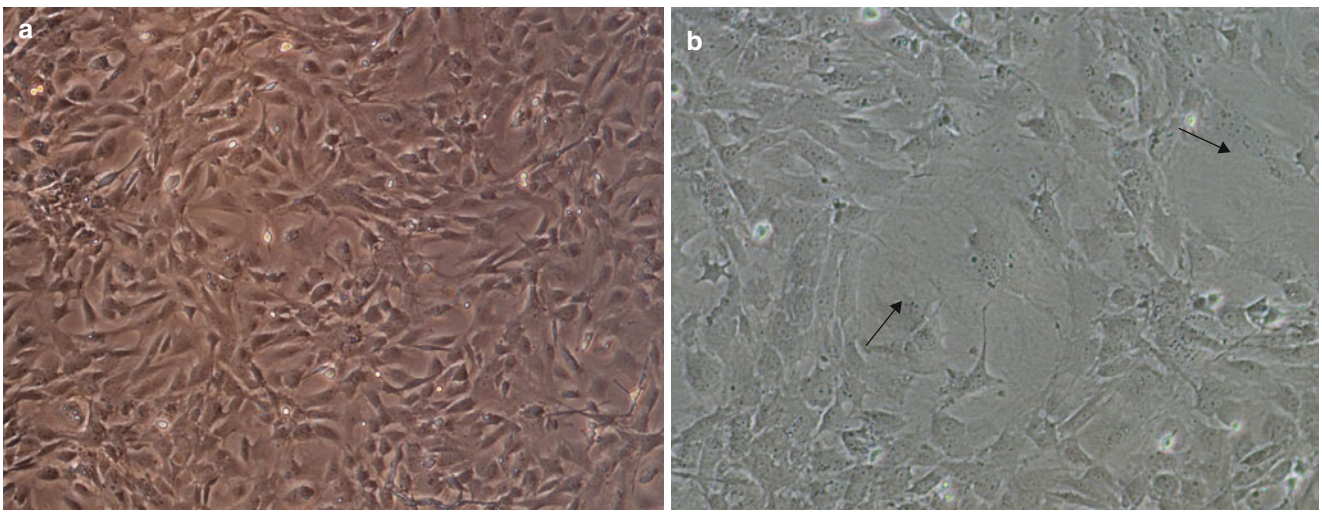

Fig. 2.15 Comparison of MEF cultures showing the difference in confluent cultures between cultures in the presence of large cells and the absence of these cells. This demonstrates why an anomaly in the cell count can arise (×10 magnification). (**a**) From a normal culture of confluent MEFs, expect a yield of about $5–7 \times 10^6$ cells from a T-75 flask. (**b**) From the same T-75 flask of MEFs with two or three extra-large cells in the field of view (*black arrows*), expect a yield of about $2–3 \times 10^6$ cells

In the case of human fibroblasts, which generally have a longer lifespan in culture, the passage number and range of supportiveness of the undifferentiated cells varies. For both mouse and human fibroblast sources, the feeders generated require a robust testing procedure to establish over what range of passages and cell density the feeders will support the maintenance of undifferentiated PSCs. A number of fibroblast cell lines have also been shown to maintain undifferentiated PSCs, but although referred to as cell lines, these are usually not immortal and as such have a limited lifespan.

The quality of the cultures of fibroblasts is assessed morphologically. When passaged, the cells should adhere and should start to spread out. In a good culture, the morphology should be more or less homogeneous. In the first two passages, it is evident that other cell types (such as neural cells) are present, but these cells are outcompeted by the growth of the fibroblasts and are soon lost. Under the microscope, the cells of a good-quality culture should be attached to the culture vessel, and the individual fibroblasts in culture have an elongated spindle shape with a clear cytoplasm and a flat, oval nucleus. The fibroblasts in culture also display processes extending from the cell body. The cultures should contain few unattached, floating cells. If many floating cells are observed or the cells start to detach, then the culture is not optimum. As cells grow, the culture becomes more confluent, but the morphology remains the same. It is best to inactivate the cells whilst they are still actively growing—that is, before the cells become completely confluent. Good-quality feeders are produced from these subconfluent cultures. It is usually recommended that the cells should cover 80 % of the area of the culture vessel. This guideline is of course subjective, unless you are in a laboratory with sophisticated imaging equipment. It is best not to let the cells grow past this point, but feeders can be made from more-confluent cells if a large number of cells are required for a batch. In this compromise, however, quality is effectively reduced for increased quantity (Figs. 2.16, 2.17, 2.18, 2.19, 2.20, 2.21 and 2.22).

Some human fibroblasts, when confluent, form swirl patterns. Overconfluent fibroblasts eventually become fragile and detach when left in cell culture. The cell morphology can change, and the cells can appear very long and thin. The cytoplasm can acquire a grainy appearance, and in some cases the cell membrane degrades.

Fibroblasts are critical biological substrates for the maintenance of good-quality, undifferentiated PSCs. They form the basis on which robust, reliable, reproducible research will be founded, and it is well worth the time and effort to ensure that they are well sourced, free from contamination and appropriately frozen and banked, to guarantee their fitness for purpose.

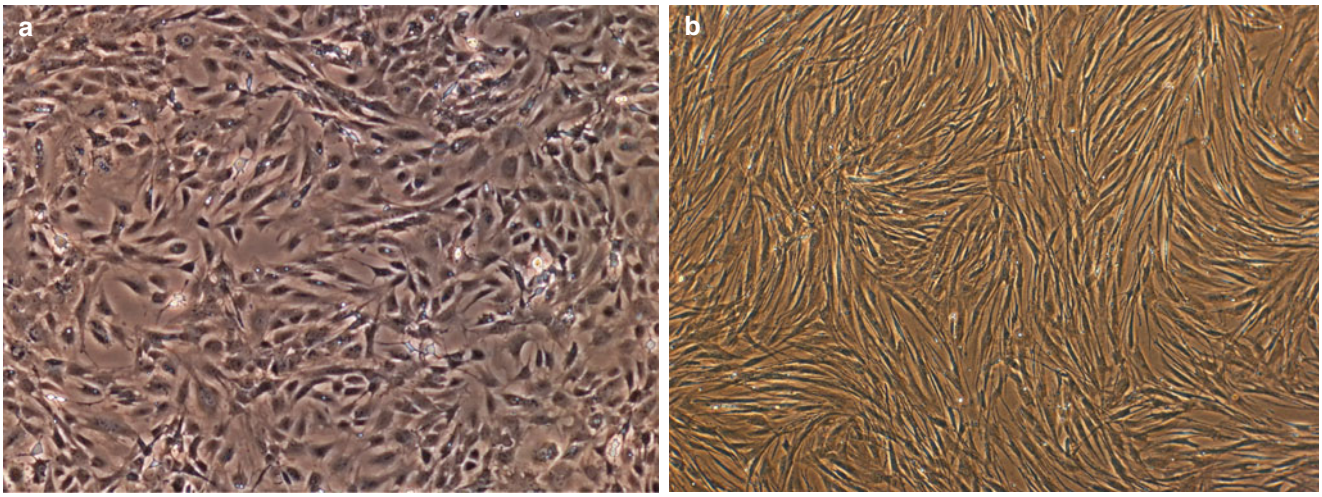

Fig. 2.16 Comparison between 100 % confluent culture of mouse embryonic fibroblasts (**a**) and human skin fibroblasts (**b**) (×10 magnification). The difference between the shape and size of the cells is clear: Human fibroblasts are much longer, more spindle shaped and are more robust in culture

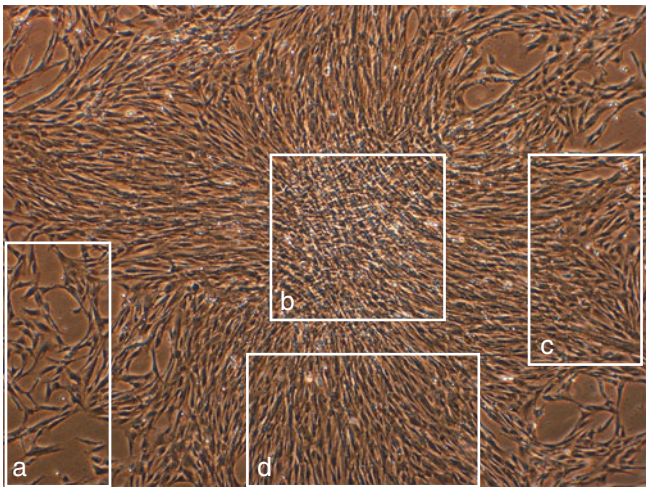

Fig. 2.17 Human fibroblasts growing in culture (×10 magnification). This image is an example of a cell culture containing areas of different cell densities within the identified rectangles. (**a**) Approximately 50 % confluent. (**b**) Overconfluent, with cells growing on top of each other. (**c**, **d**) 100 % confluent culture, with no space between cells

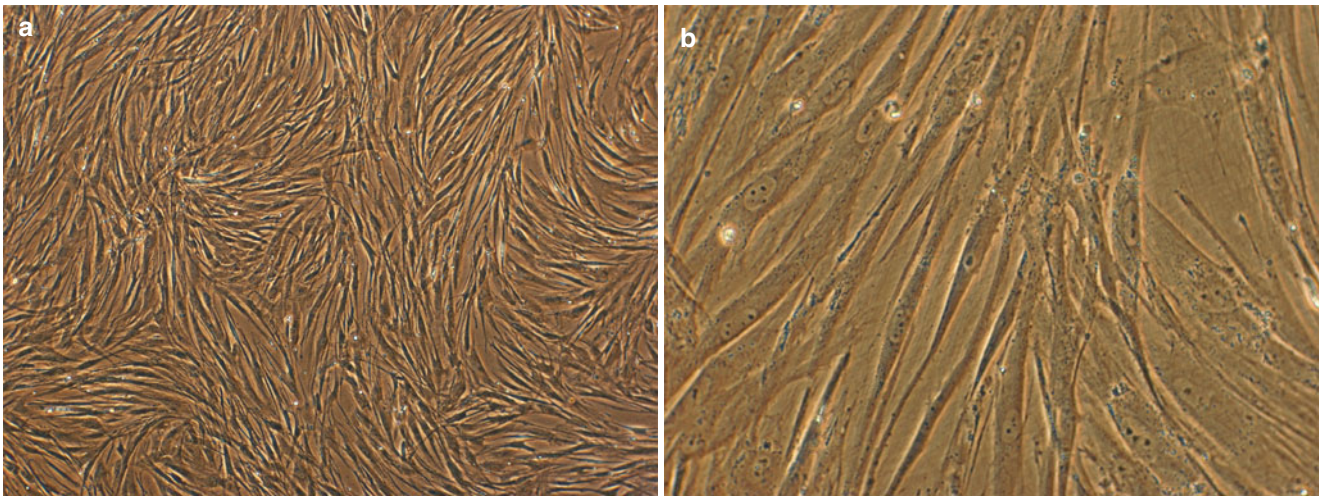

Fig. 2.18 A 100 % confluent culture of human fibroblasts. In this example of a very good-quality culture, every cell body is visible, as are the nucleoli in the cell nuclei. There are no floating cells in the culture (**a**, ×10 magnification; **b**, ×40 magnification)

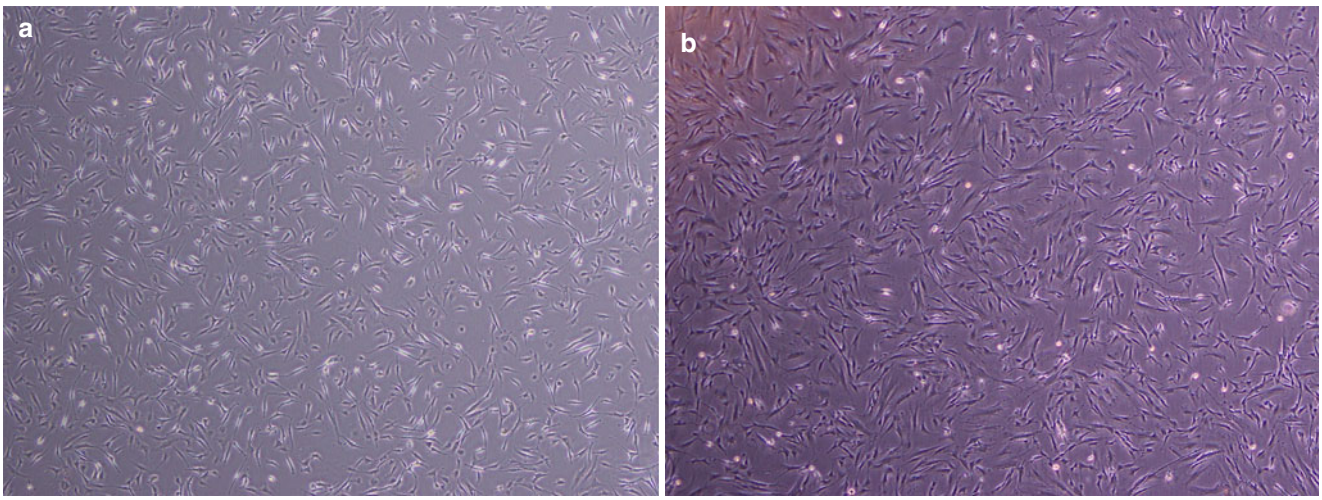

Fig. 2.19 These images of human fibroblasts at 24 h after passaging display different confluences: 50–60 % (**a**) versus 70–80 % (**b**). The cells display the typical elongated morphology of healthy fibroblasts (×4 magnification)

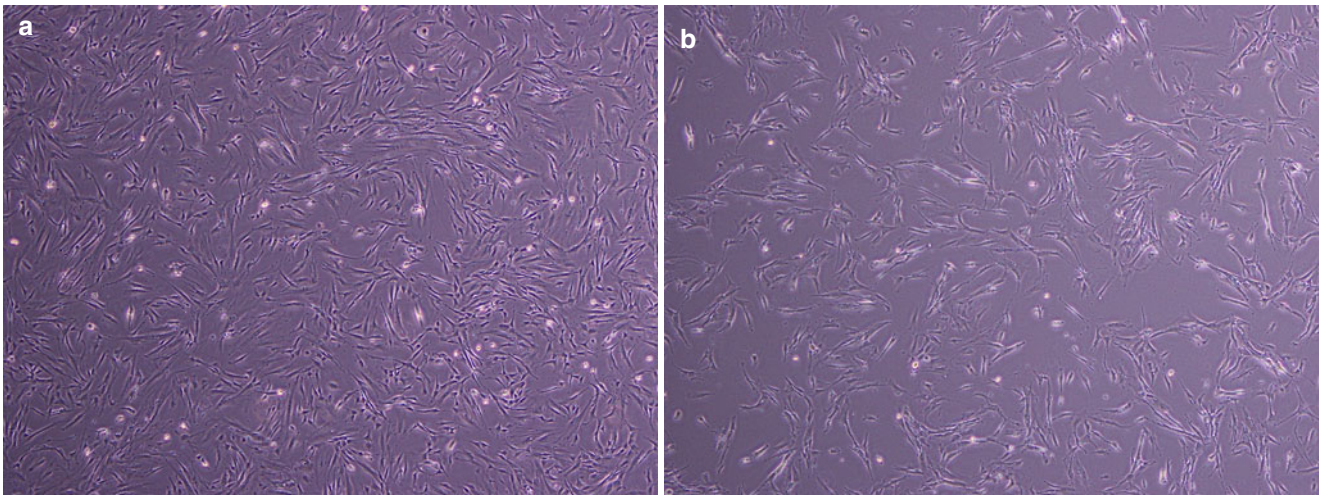

Fig. 2.20 In these images of growing human fibroblasts at differing magnification, the cells display the typical elongated morphology of healthy fibroblasts (**a**, ×4 magnification; **b**, ×4 magnification)

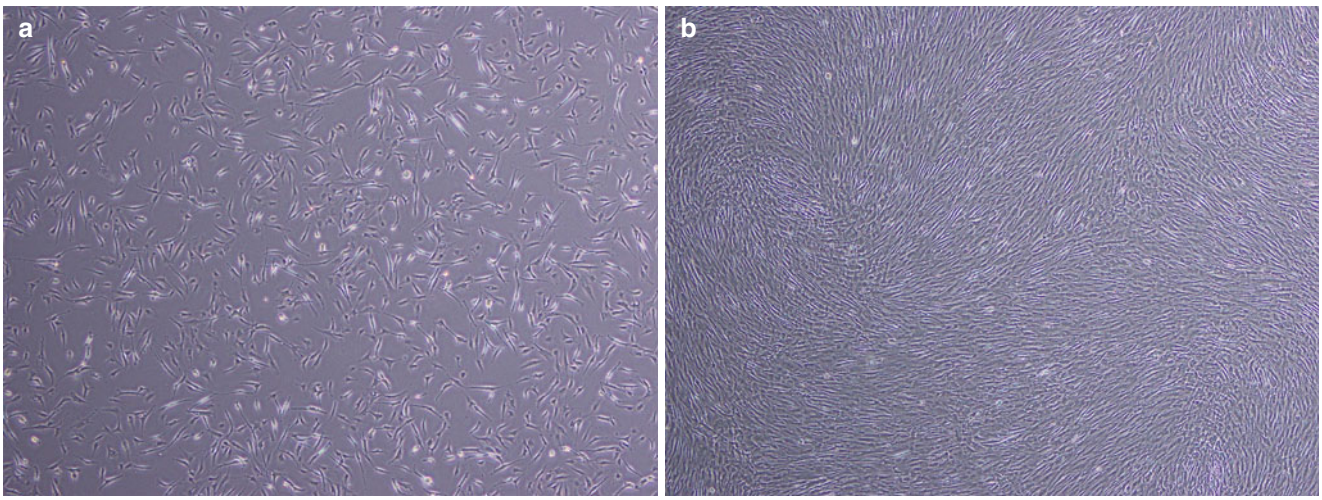

Fig. 2.21 (**a**) A culture of human fibroblasts 24 h after passaging. (**b**) The same culture of cells is confluent by 96 h post-passaging. Note that a few apoptotic cells (small round cells) are starting to appear in the culture (Both ×4 magnification)

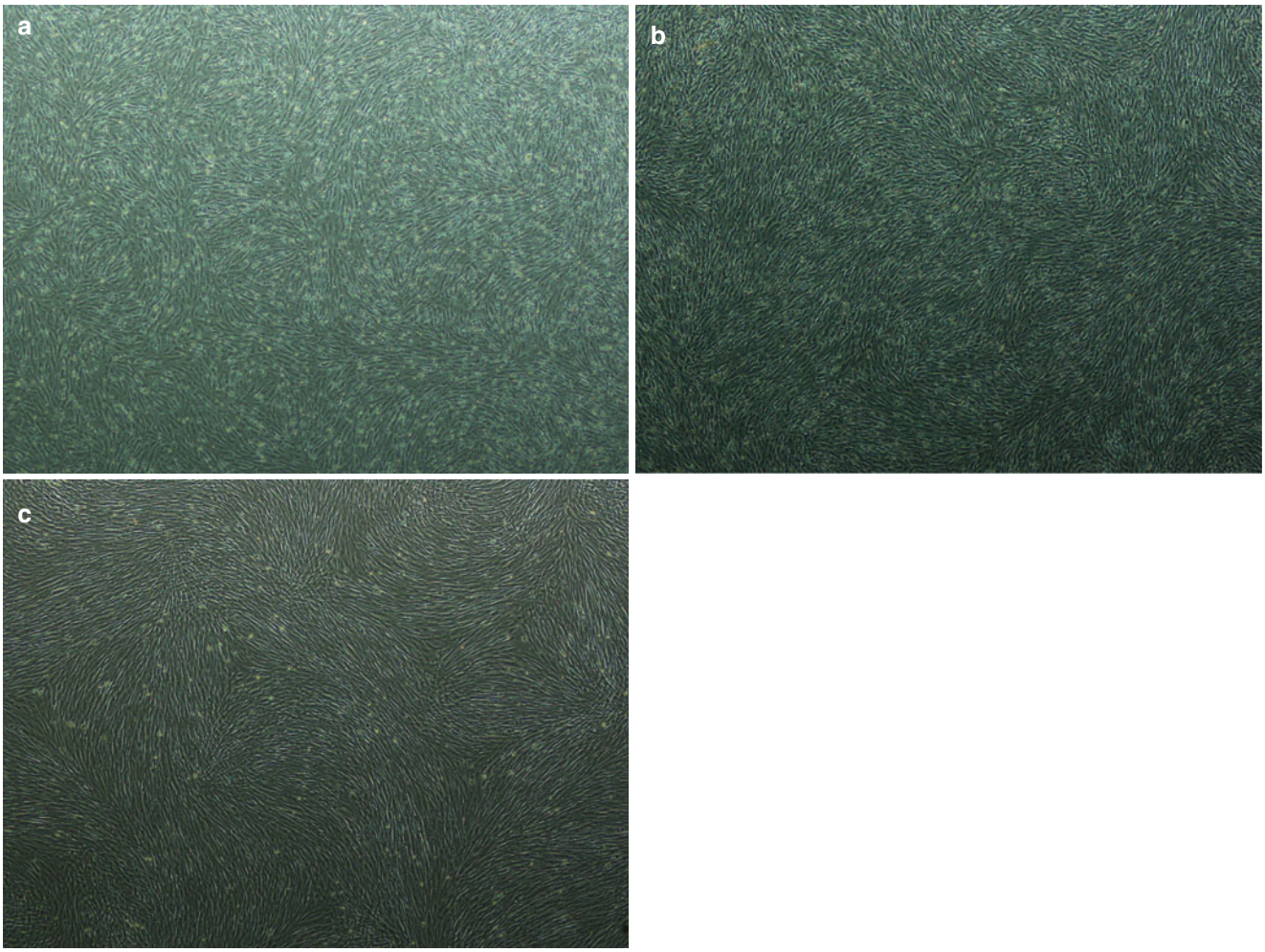

Fig. 2.22 (**a**–**c**) Three different human fibroblast cultures, all confluent (×4 magnification). These cells will no longer be proliferating because there is no space for new cells to occupy after mitotic cell division. (**a**, **b**) Overconfluent cells; a large number of apoptotic cells can be identified. (**c**) 100 % confluent cells with very few apoptotic cells

Suggested Reading

Amit M, Itskovitz-Eldor J. Morphology of human embryonic and induced pluripotent stem cell colonies cultured with feeders. In: Amit M, Itskovitz-Eldor J, editors. Atlas of human pluripotent stem cells: derivation and culturing, Stem cell biology and regenerative medicine. New York: Humana Press; 2012. p. 15–39.

Eiselleova L, Peterkova I, Neradil J, Slaninova I, Hampl A, Dvorak P. Comparative study of mouse and human feeder cells for human embryonic stem cells. Int J Dev Biol. 2008;52:353–63.

Fernandes AM, Meletti T, Guimarães R, Stelling MP, Marinho PA, Valladão AS, Rehen SK. Worldwide survey of published procedures to culture human embryonic stem cells. Cell Transplant. 2010; 19:509–23.

Jaccard N, Griffin LD, Keser A, Macown RJ, Super A, Veraitch FS, Szita N. Automated method for the rapid and precise estimation of adherent cell culture characteristics from phase contrast microscopy images. Biotechnol Bioeng. 2014;111:504–17.

Leonardo TR, Schell JP, Tran HT, Peterson SE. Culturing human pluripotent stem cells on a feeder layer. In: Loring JF, Peterson SE, editors. Human stem cell manual. 2nd ed. London: Elsevier; 2012. p. 3–14.

Inactivated Mouse and Human Fibroblasts

3

To date, the use of inactivated fibroblasts in the derivation and culture of pluripotent stem cells (PSCs) remains the most widely used culture system despite the availability of other systems that are feeder-free. In general, the feeder-based systems tend to be less expensive than their feeder-free equivalents, and feeders have a proven ability to maintain PSCs stably in an undifferentiated state, so in most cases the cost-effective feeder system is the system of choice. This chapter looks at the morphology of mouse and human feeder cells in order make a subjective assessment of the quality of the feeders and their suitability to support the growth and expansion of PSCs (Figs. 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8 and 3.9).

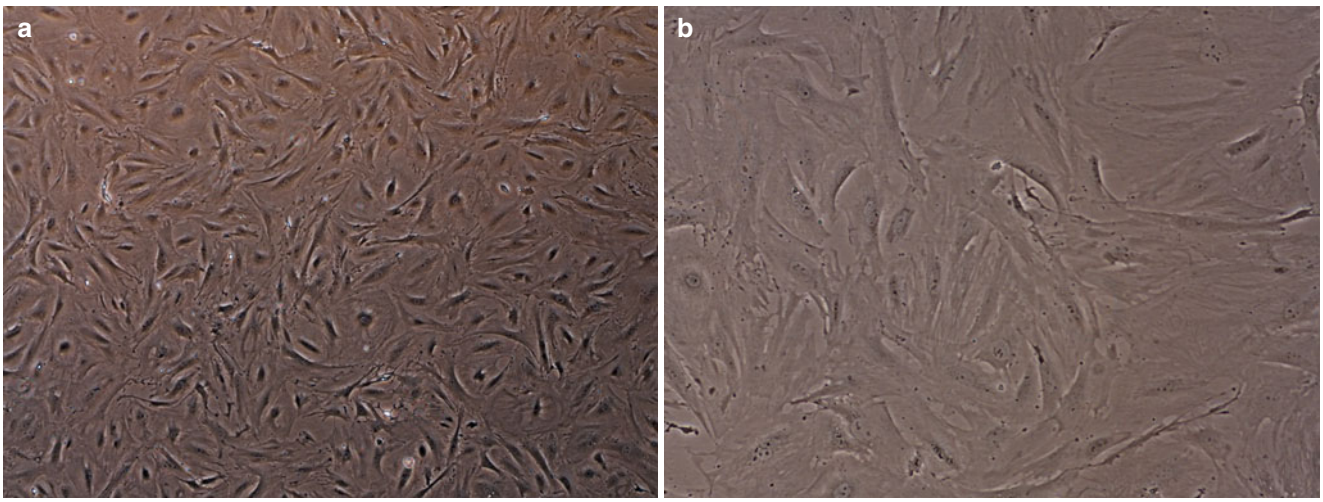

Fig. 3.1 (**a**, **b**) Mouse embryonic fibroblast (MEF) feeder layers of very good quality. The cells are uniform and evenly spread, with few apoptotic or floating cell debris. This is an ideal feeder cell culture for co-culture with pluripotent stem cells (PSCs) (**a**, ×4 magnification; **b**, ×10)

L. Healy, L. Ruban, *Atlas of Human Pluripotent Stem Cells in Culture*,
DOI 10.1007/978-1-4899-7507-2_3, © Springer Science+Business Media New York 2015

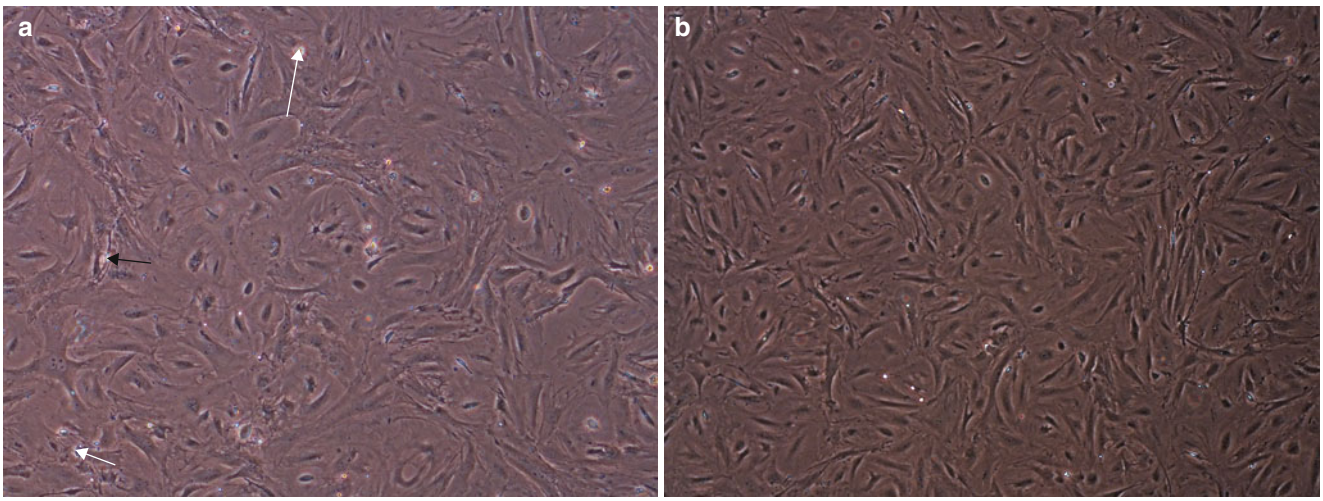

Fig. 3.2 Comparison of two MEF feeder cultures. (**a**) This culture contains occasional neural cells (*black arrow*) and a few apoptotic cells (*white arrow*), but the cells are still of a good quality. (**b**) The cells in this culture are more uniform than those in a (Both ×4 magnification)

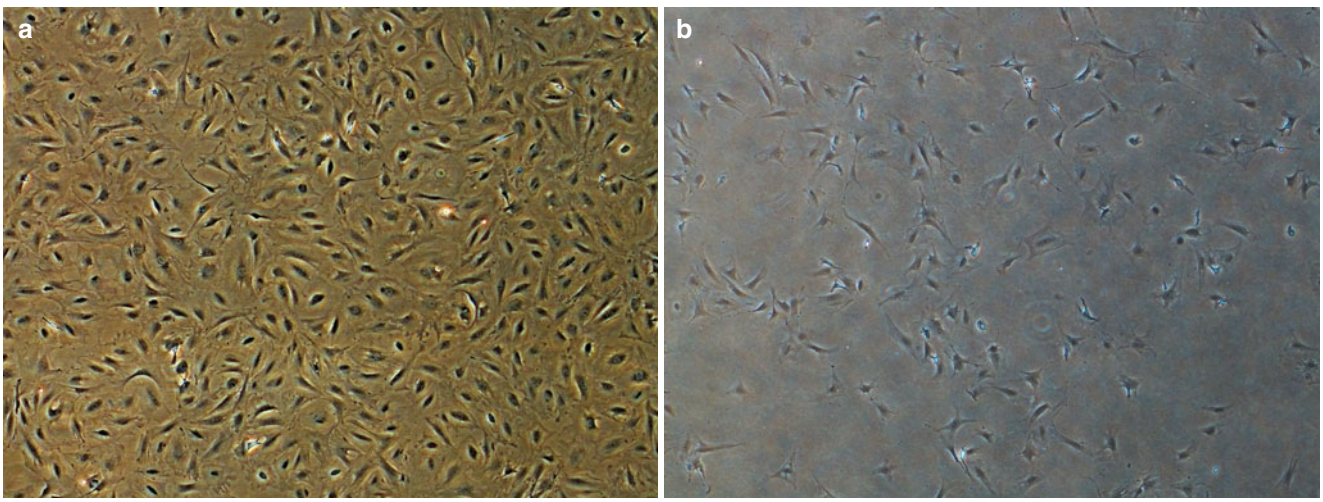

Fig. 3.3 (**a**, **b**) Comparison of two different densities of mouse feeder cultures. The cells in b are sparse and too distant from each other to provide an optimal environment for co-culture; these cells would promote differentiation and would not support the growth of undifferentiated hPSCs (Both ×4 magnification)

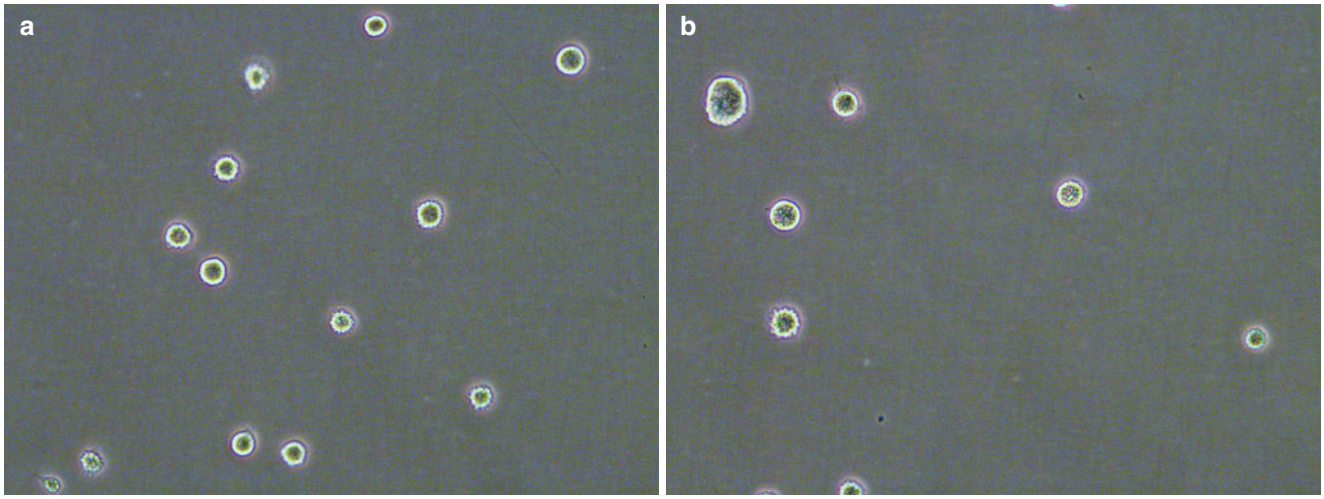

Fig. 3.4 (**a**, **b**) Dissociated MEFs in culture following inactivation for the preparation of feeders. The dissociated cells should be round, single and refractile (bright ring around the outside of the cell) (Both ×10 magnification)

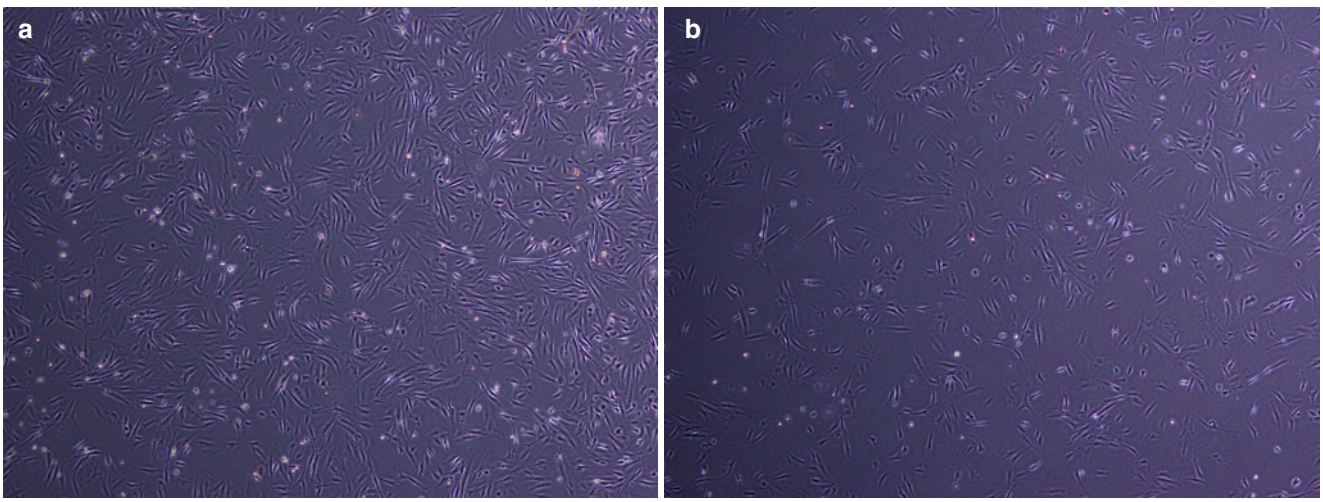

Fig. 3.5 (**a**, **b**) Comparison between two different densities of human fibroblast feeders (derived from human dermal fibroblasts), 24 h after passaging. Both cultures are of good quality. As with their murine counterparts, the cells are uniform and evenly spread, with few apoptotic cells and little floating debris (Both ×4 magnification)

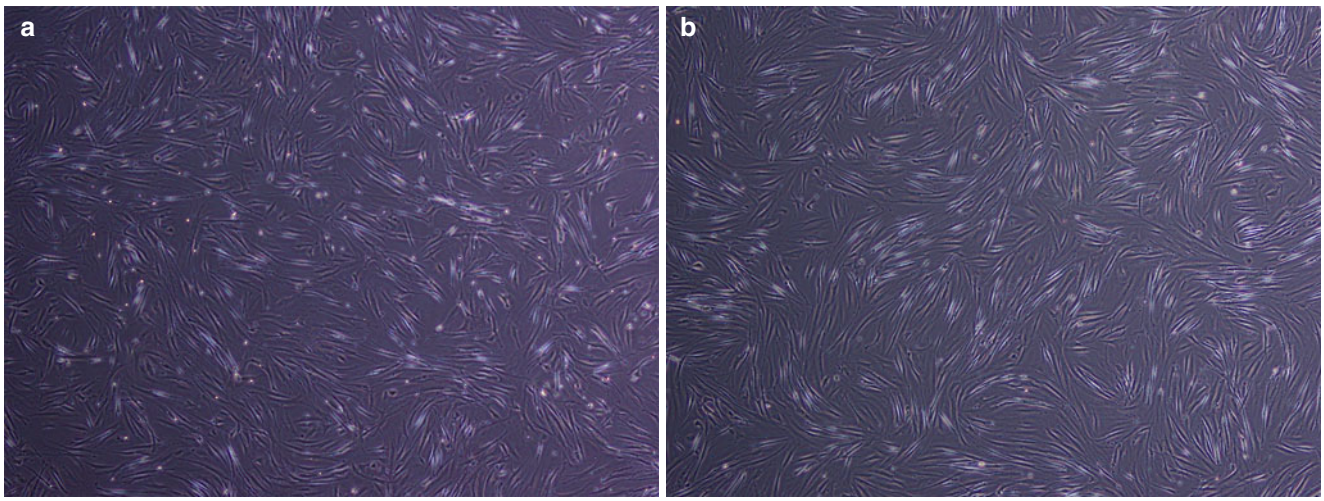

Fig. 3.6 Human feeders at Day 2 after passaging (**a**) and Day 4 (**b**). Again, both cultures are of good quality. The cells are evenly spread, with few apoptotic cells and floating debris. In practice, we would use the cells on Day 1 or Day 2 after passaging to ensure that they provide the maximum support for the undifferentiated cells (Both ×4 magnification)

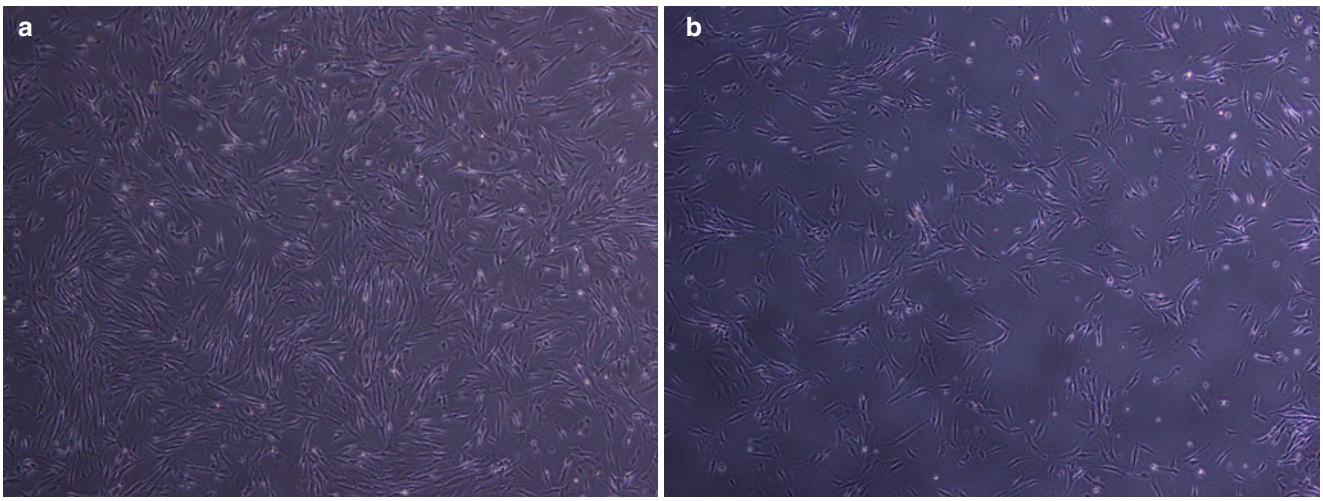

Fig. 3.7 Comparison between two different densities of human fibroblast feeders (MRC-5) 24 h after passaging. Both cultures are of good quality. As with their murine counterparts, the cells are uniform and evenly spread, with few apoptotic cells. (**a**) This culture is at an optimal density (approximately 70 %) to support PSCs. (**b**) This culture is suboptimal, at approximately 50 % density. However, the density of feeder cells required to maintain PSCs in an undifferentiated state can differ from cell line to cell line, so it is best practice to try a few different feeder densities in order to determine which density is optimal for the maintenance of the PSC line (Both ×4 magnification)

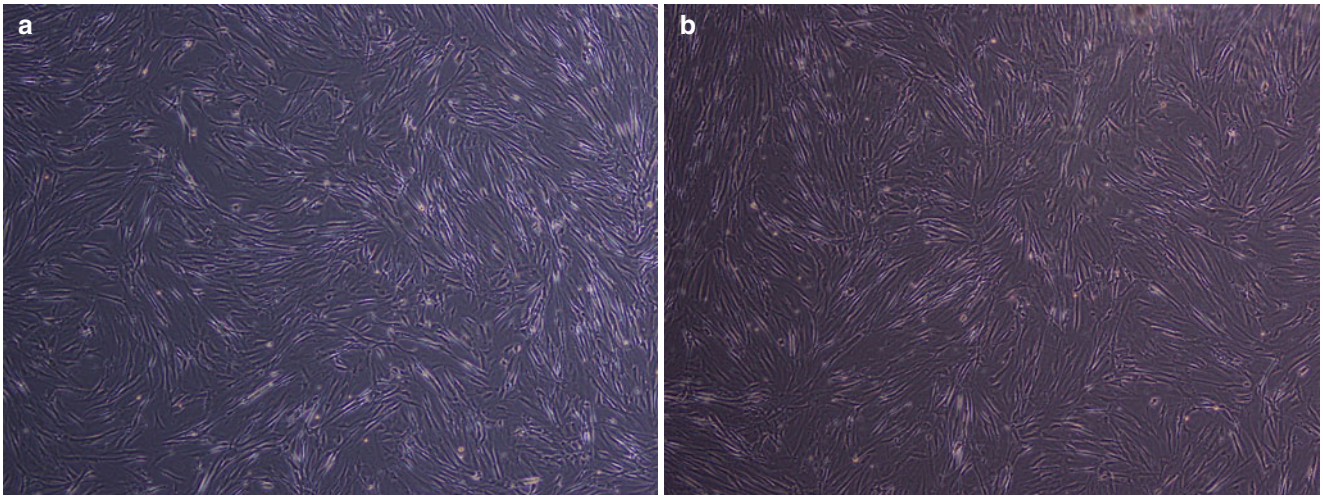

Fig. 3.8 Human feeders (MRC-5) at Day 2 after passaging (**a**) and Day 4 (**b**). The cells are uniform and evenly spread, with a few apoptotic cells and floating debris (Both ×4 magnification)

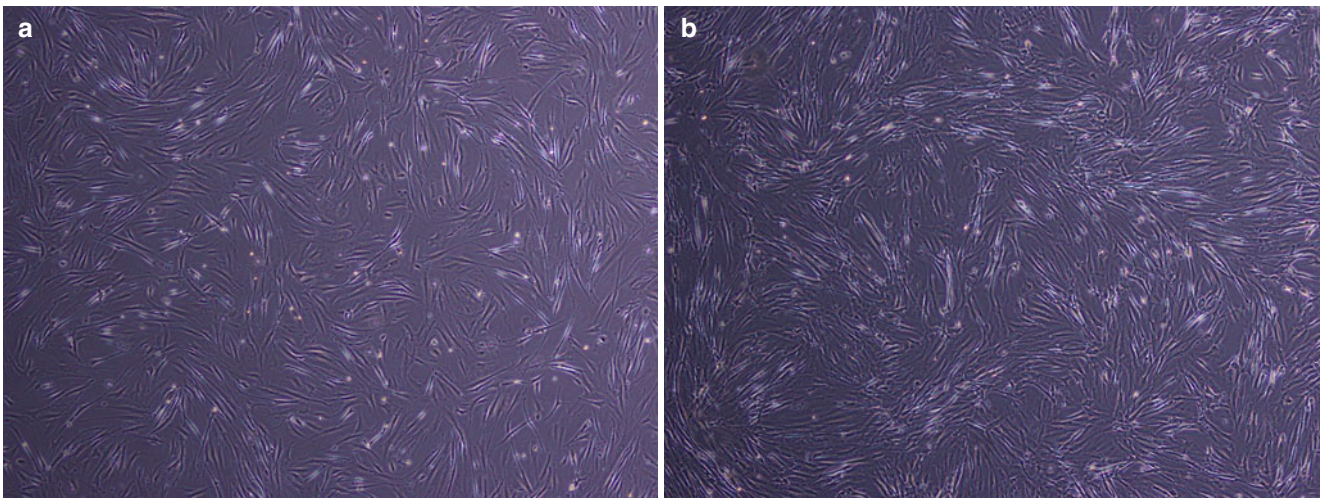

Fig. 3.9 Comparison of human feeders derived from human dermal fibroblasts (**a**) and human feeders from an MRC-5 cell line (**b**). The morphology of the two lines is indistinguishable (Both ×4 magnification). In practice, we would use the cells on Day 1 or Day 2 after passaging to ensure that they provide the maximum support for the undifferentiated cells

As stated in Chap. 2, good-quality fibroblasts produce good-quality feeders, which in turn produce good-quality, undifferentiated PSCs. Feeders that support the growth of PSCs can be derived from a range of different fibroblasts. The feeders secrete growth factors and provide the PSCs with extracellular matrix to support their undifferentiated growth. Feeders are generated by the mitotic inactivation of the fibroblasts, traditionally using either mitomycin C or irradiation. This inactivation prevents the fibroblasts from overgrowing the PSCs in culture. The amount of radiation or mitomycin C required to inactivate the fibroblasts should be established as part of quality control before batches of cells are prepared. It should be noted that in general, human fibroblasts are more difficult to inactivate than mouse fibroblasts.

Once inactivated, the cells should be counted and either used immediately to prepare feeder layers or frozen and thawed when needed. There is a loss in cell number when the cells are frozen after inactivation. On average, about 70 % of the cells originally frozen are recovered, and this should be borne in mind when establishing feeders at a certain number of cells per square centimetre in a culture vessel.

The density of fibroblasts used for each cell line is cell-line specific and needs to be determined on a case-by-case basis. If the density is too high or too low for a specific cell line, spontaneous differentiation may occur. A range of densities should be tested for each line to determine the optimum density. In an appropriate tissue culture vessel, a typical density for mouse feeders is 150,000 cells per square centimetre, and for human feeders, 50,000 cells per square centimetre (Figs. 3.10, 3.11, 3.12, 3.13, 3.14, 3.15, 3.16, 3.17, 3.18 and 3.19).

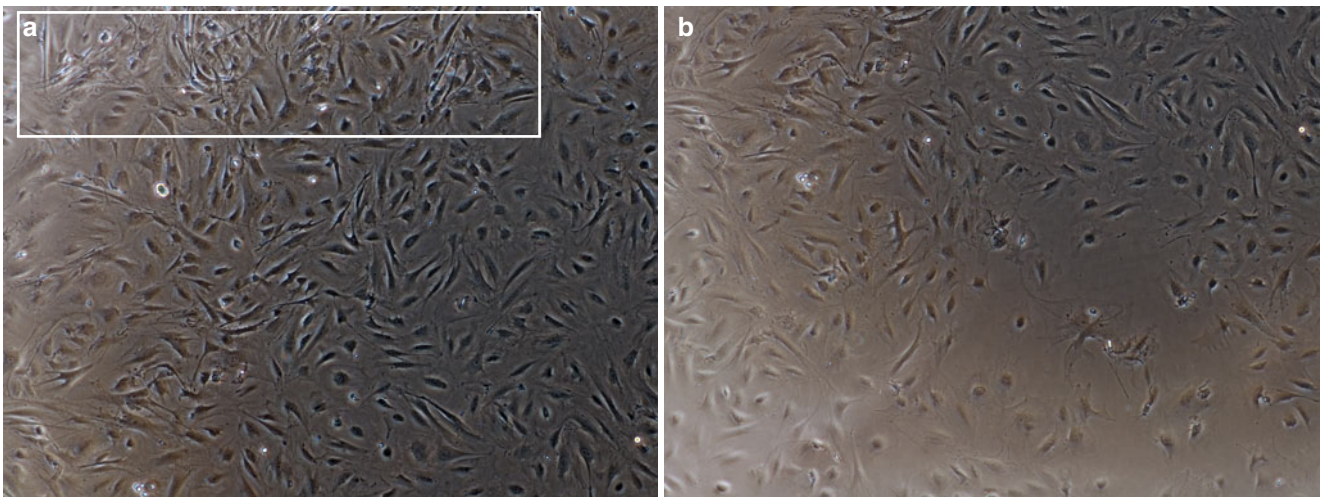

Fig. 3.10 Examples of mouse feeders that have not spread evenly in the culture dish. (**a**) This image shows a typical spread of feeders when using an in vitro fertilisation (IVF) dish for culture. The cells tend to pool in the middle of the dish even when gently shaken to disperse the single cells following their addition to the IVF dish (*in rectangular area*). (**b**) Cells often are sparse around the edges of the IVF dish, compared with the centre of the dish. Because of this distribution of feeders, the PSCs grown in these dishes can display a range of differentiation within the colony, depending on where the PSC adheres in the dish (Both ×10 magnification)

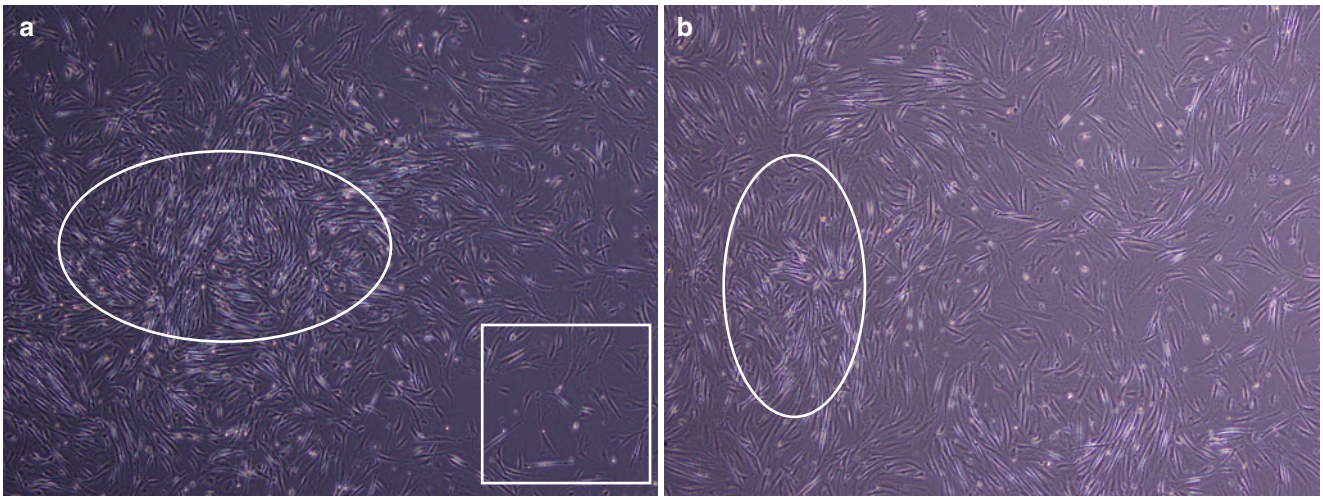

Fig. 3.11 (**a**, **b**) Examples of human feeders that have not spread evenly in the culture dish (in a 6-well plate). In both images, there are areas where the cells have not spread evenly. In some areas, the cells are too dense (*oval*) or too sparse (*box*). As with the MEFs, this type of spreading could have an effect on the outcome of the culture of PSCs (Both ×4 magnification)

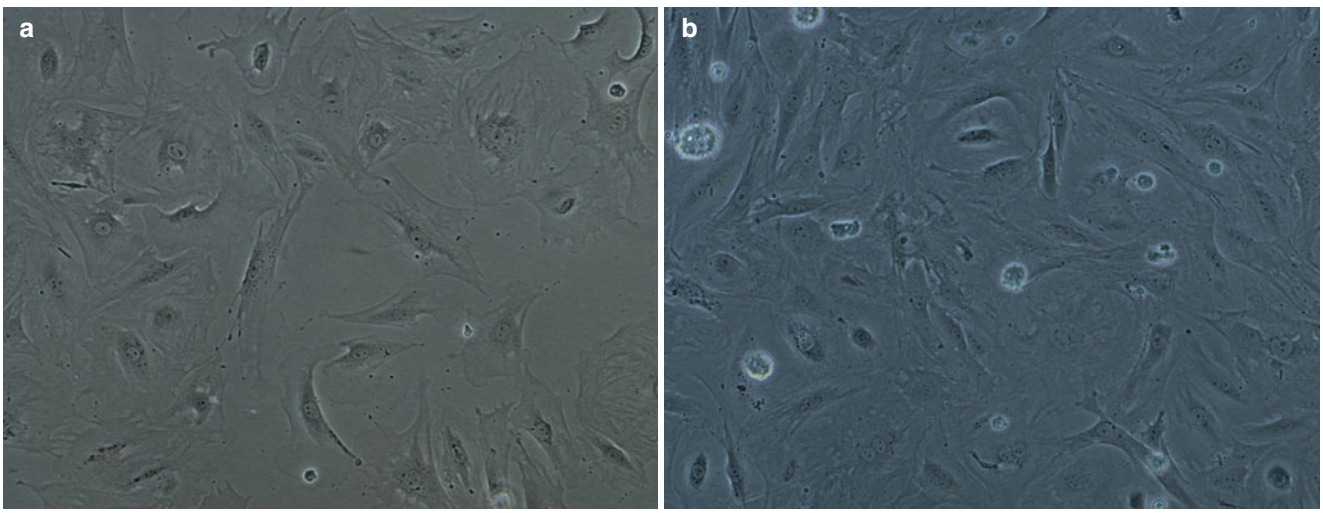

Fig. 3.12 Another example of a good culture of mouse feeders. In (**a**) the cells are less confluent and more spread than in (**b**), simply because they have more space in the culture vessel. Feeder cells are normally fit for the purpose when the cells are dense enough to touch each other, but some PSC lines have been adapted to a very low feeder density. This is often the case with karyotypically abnormal human embryonic stem cell (hESC) lines (Both ×10 magnification)

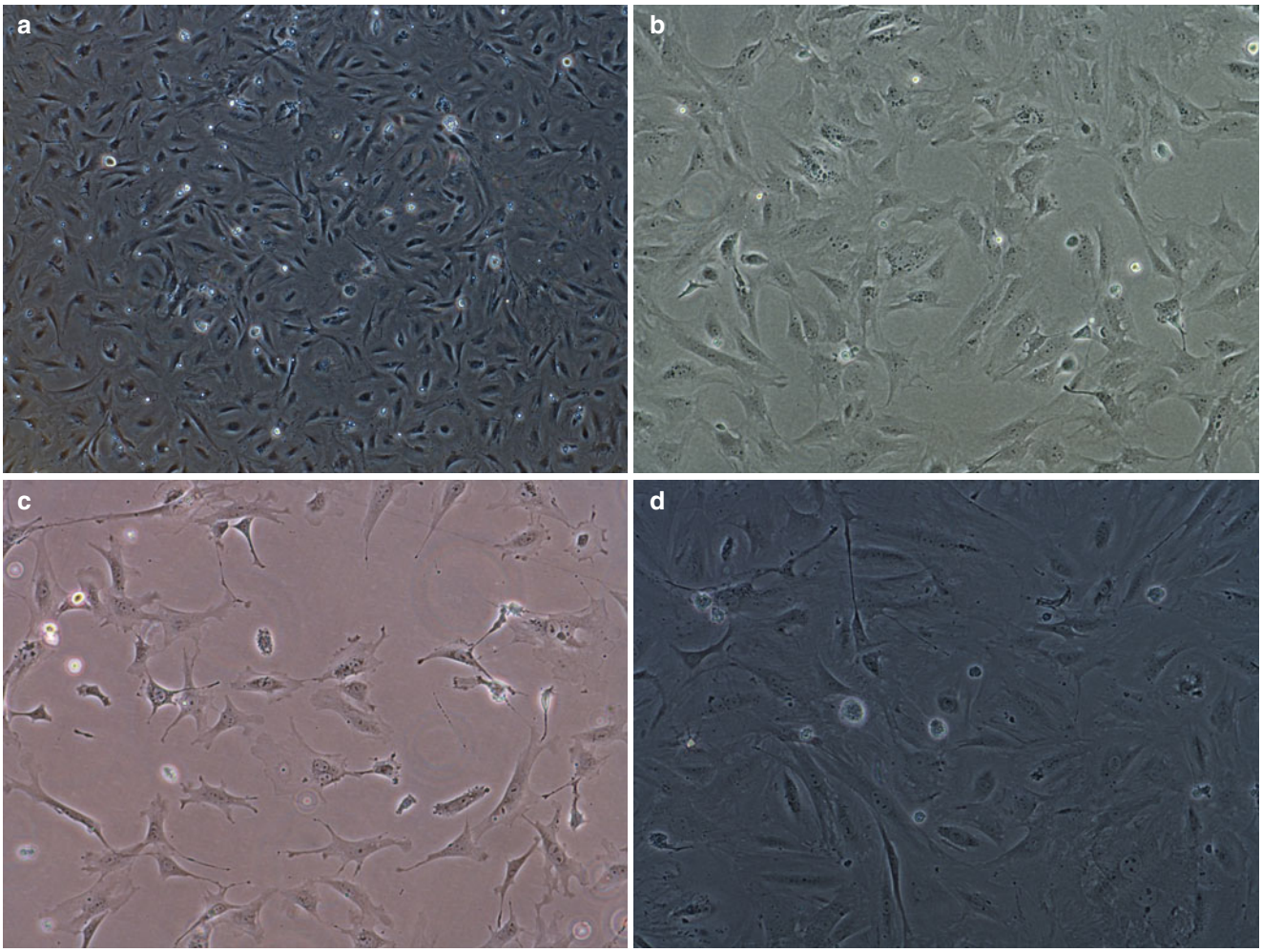

Fig. 3.13 Images of MEF feeders. (**a**, **d**) Optimal density, good-quality feeders with only a few apoptotic cells and little debris. (**c**) These feeder cells are too far away from each other; the cell bodies are too spread. This culture will not support PSCs in an undifferentiated state. (**b**) These feeders are not evenly spread, and the culture is not uniform (**a**, ×4 magnification; **b**, **c**, ×10. **d**, ×20)

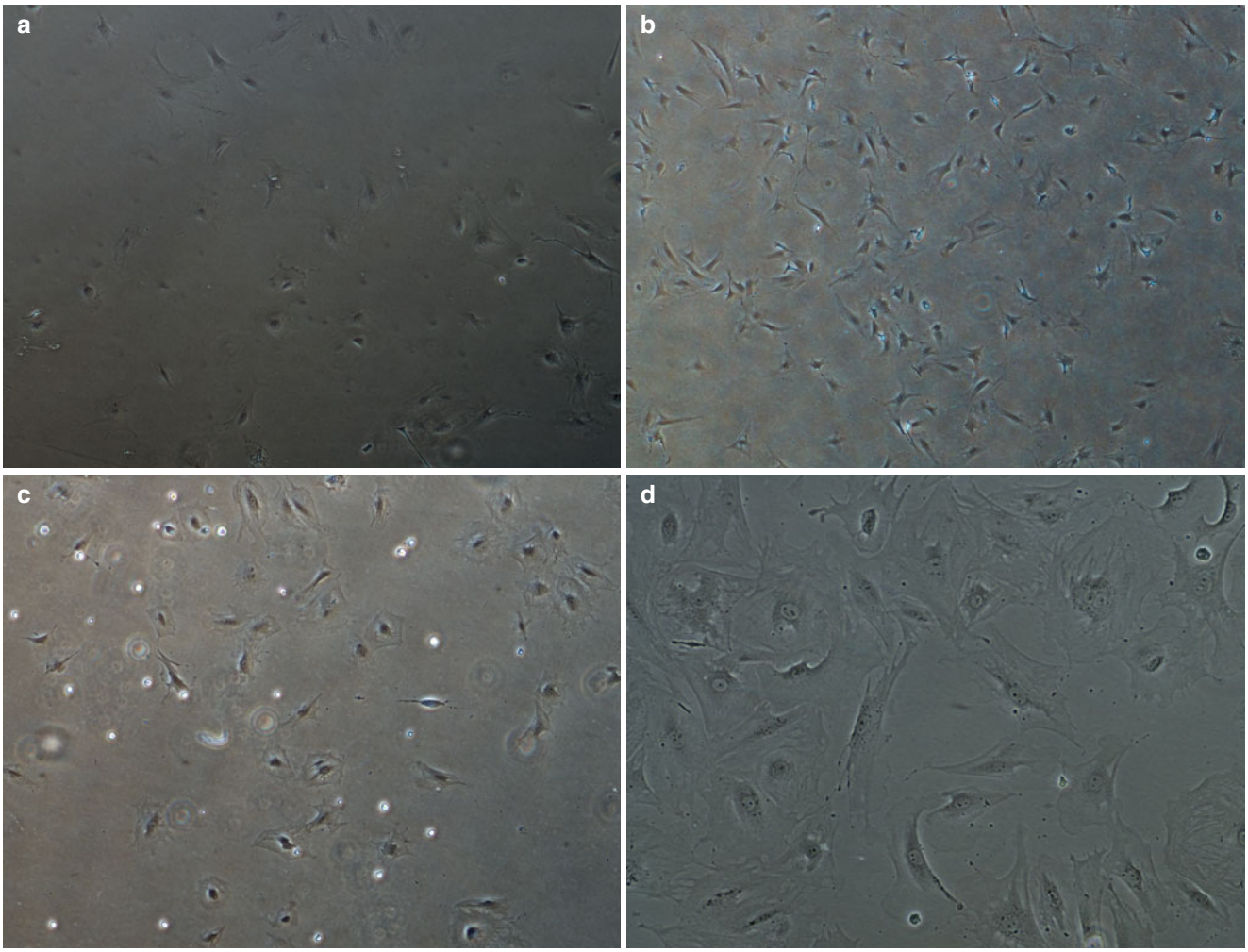

Fig. 3.14 (**a**–**d**) Mouse feeder cultures at a low density. These images show cells at a density that is not optimal to support the growth and expansion of undifferentiated PSCs. The morphology of the cells is too spindly, indicating that the cells are stressed. Amongst the possible reasons for this stress are that the feeders could have been produced from a high passage of MEFs, the freezing conditions may not have been optimal, or the wrong thawing procedure could have been used. (**a**–**c**, ×4 magnification; **d**, ×10)

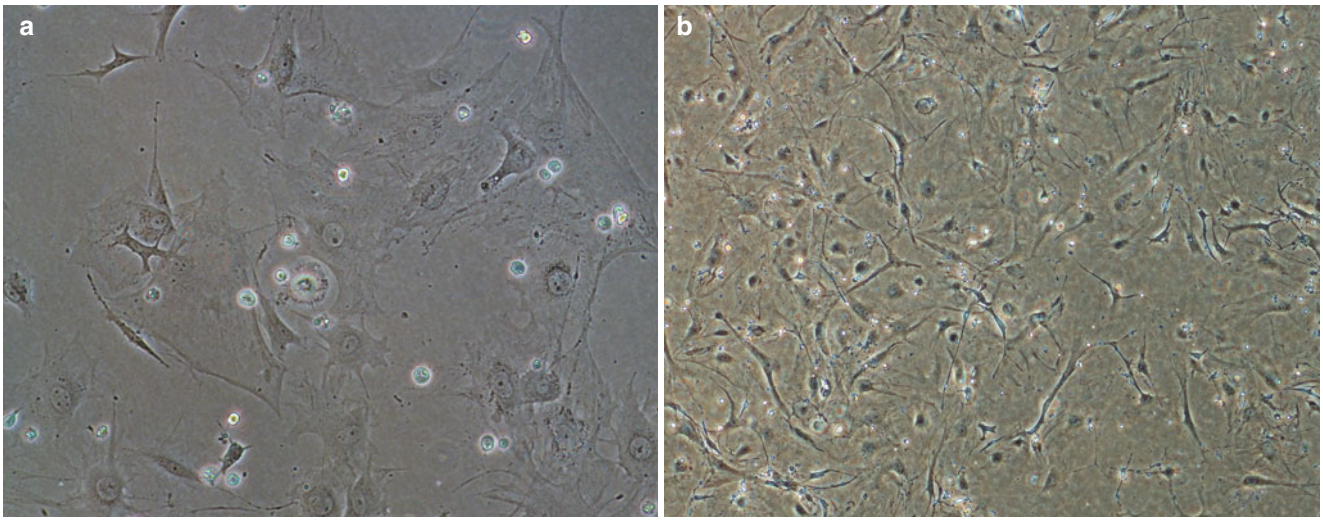

Fig. 3.15 Images of Day 10 feeders. (**a**) Note the large cells in the low-density culture. These cells display the morphology of senescent cells. (**b**) This culture shows cells displaying a heterogeneous morphology, with many large cells present. These cells would not support the growth and expansion of undifferentiated PSCs (Both ×10 magnification)

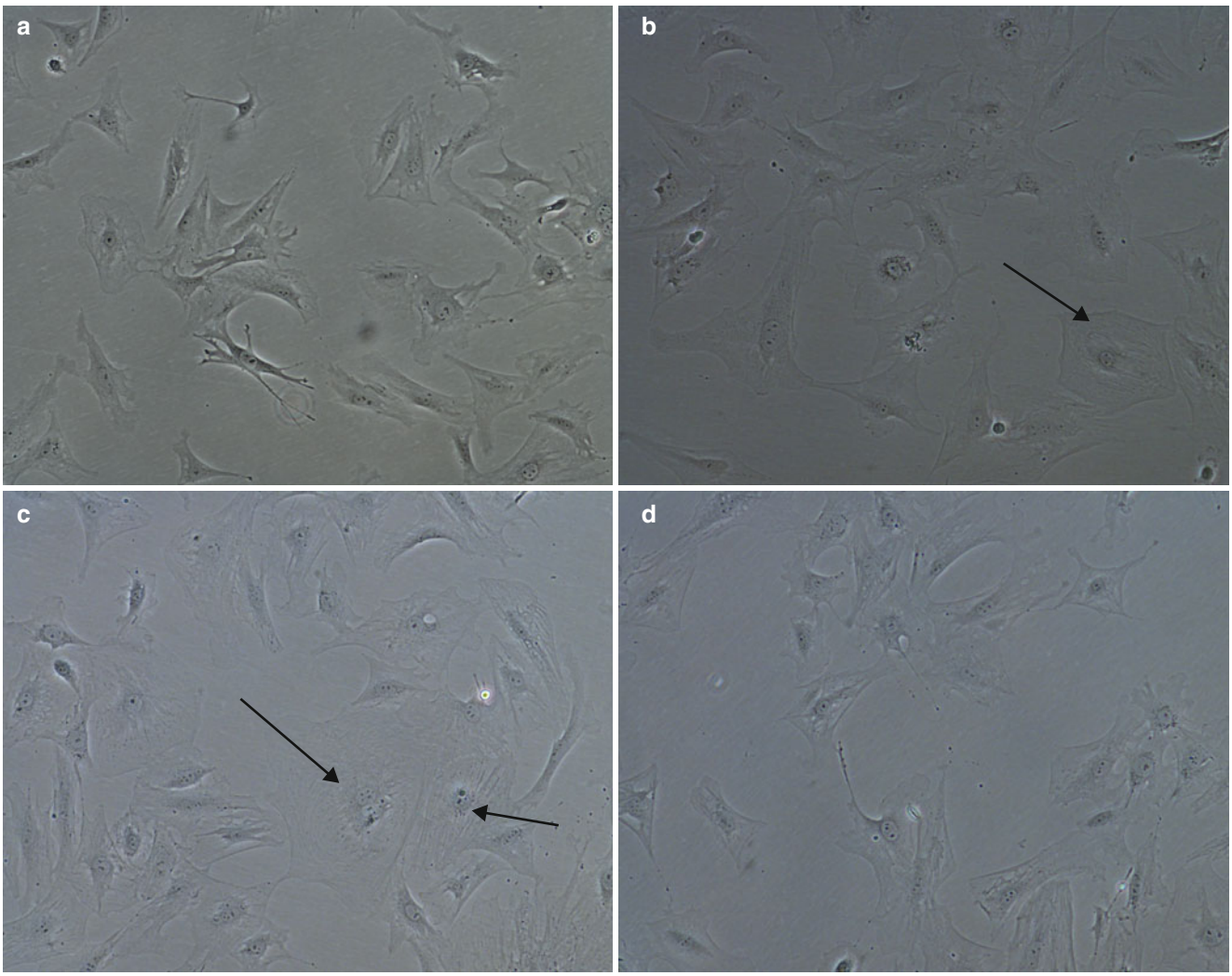

Fig. 3.16 (**a**–**d**) Images of very low-density feeders, 24 h after plating. Note the large spaces between the cells and the emergence of large cells (*black arrows*), indicating that these cultures are not optimal. In general, these cells would not support the growth and expansion of undifferentiated PSCs (All ×10 magnification)

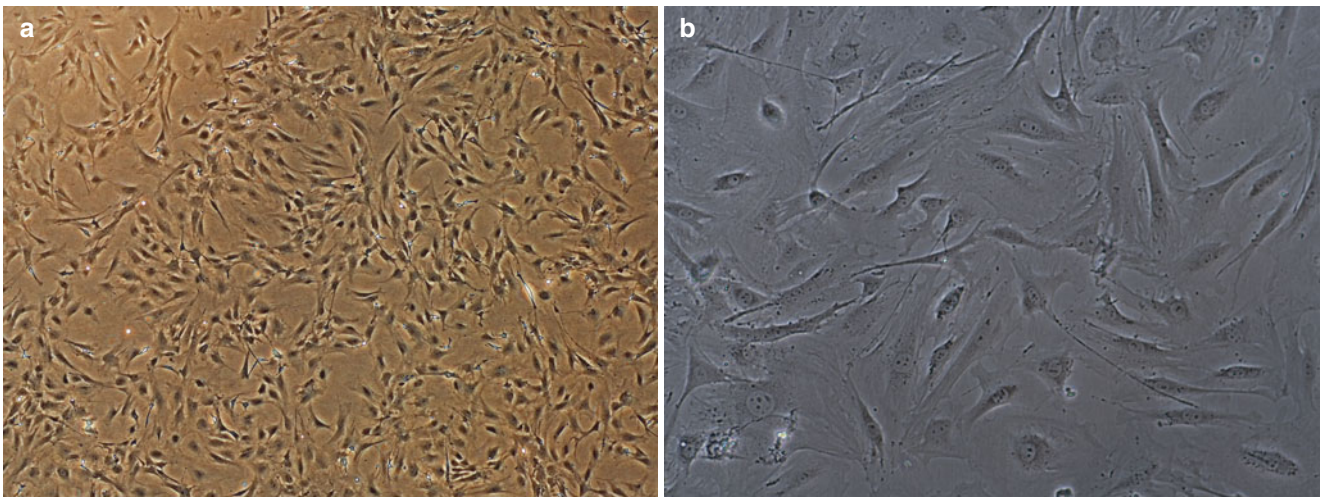

Fig. 3.17 (**a**, **b**) Optimal density of good-quality mouse feeders. These cells would support the growth and expansion of undifferentiated PSCs (**a**, ×4 magnification; **b**, ×10)

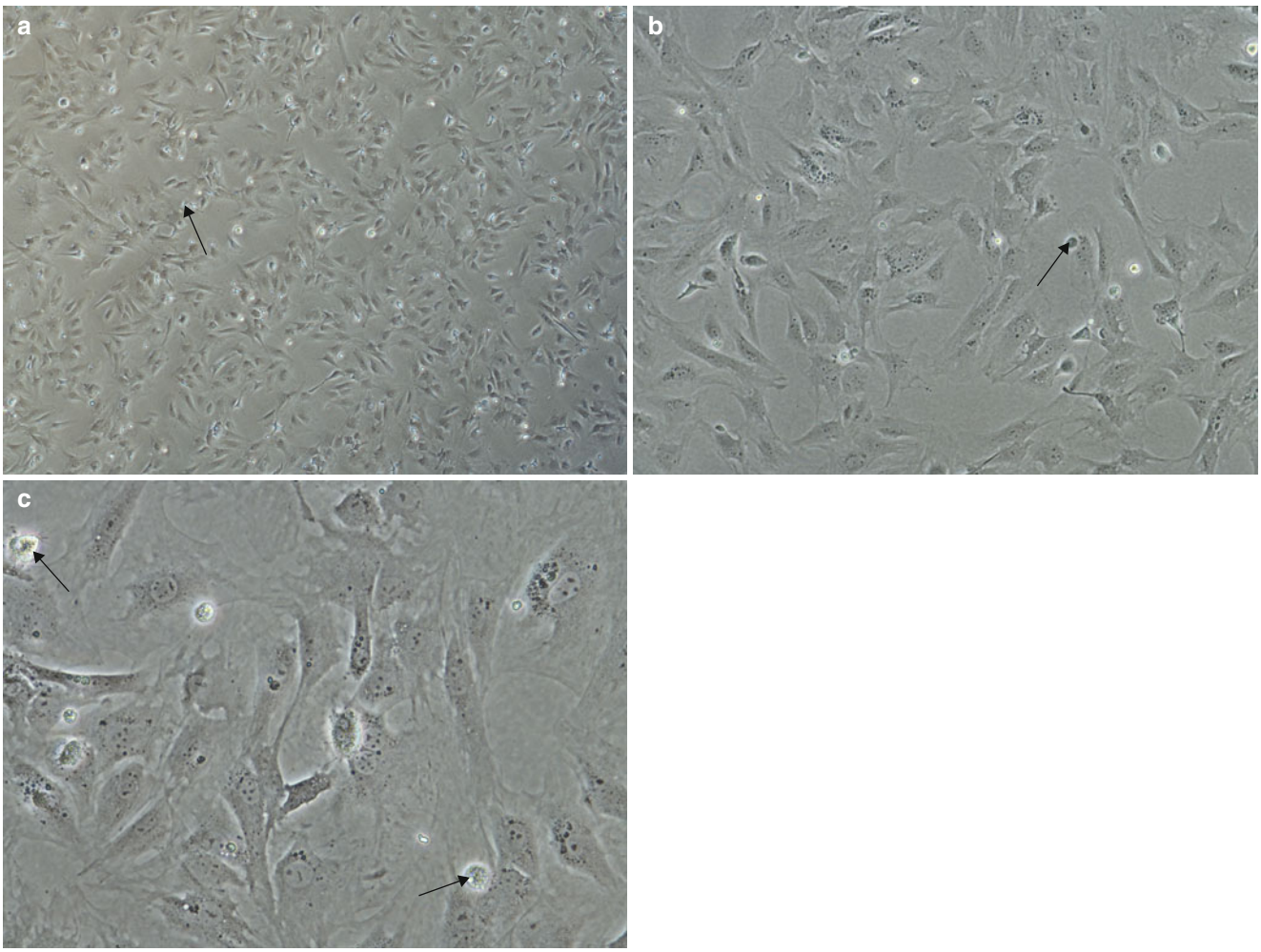

Fig. 3.18 (**a**–**c**) Images of an optimal density mouse feeder culture with a few apoptotic cells present (*black arrow*) (**a**, ×4 magnification; **b**, ×10; **c**, ×20)

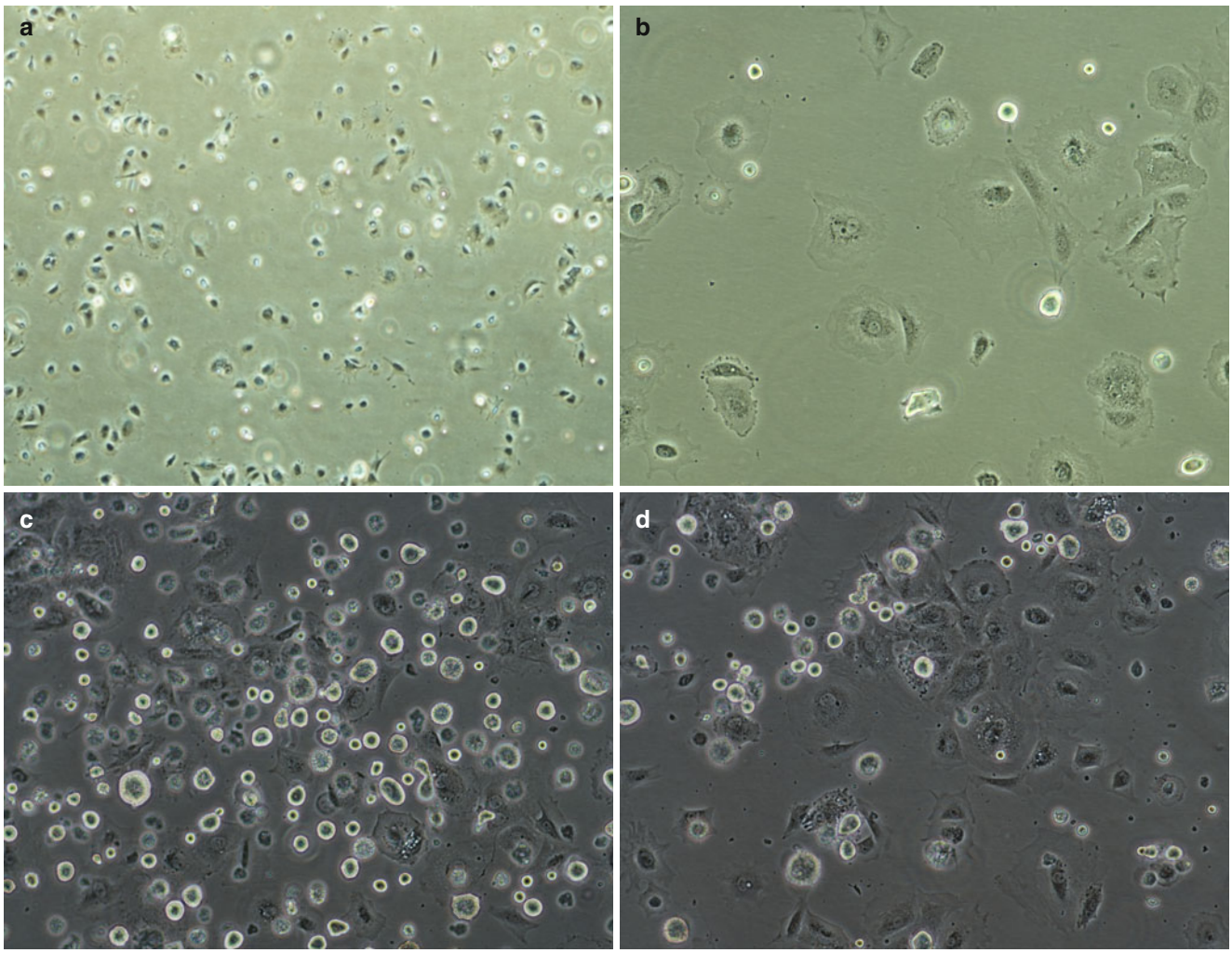

Fig. 3.19 A comparison between two different cell densities of mouse feeder cells, 3 h after plating. (**a**, **b**) Low density. (**c**, **d**) High density. Note that the cells have adhered to the culture vessel and are beginning to spread out (**a**, ×4 magnification; **b**–**d**, ×10)

Quality control of feeders should also include an assessment of the ability of a batch of feeders to support the growth and proliferation of undifferentiated PSCs over at least three, preferably five successive passages. Feeders should also be proven to be free of contamination with mycoplasma (Chap. 11). Evaluation of the cells should also be performed to demonstrate that the mitotic inactivation has indeed been successful (Figs. 3.20, 3.21 and 3.22)

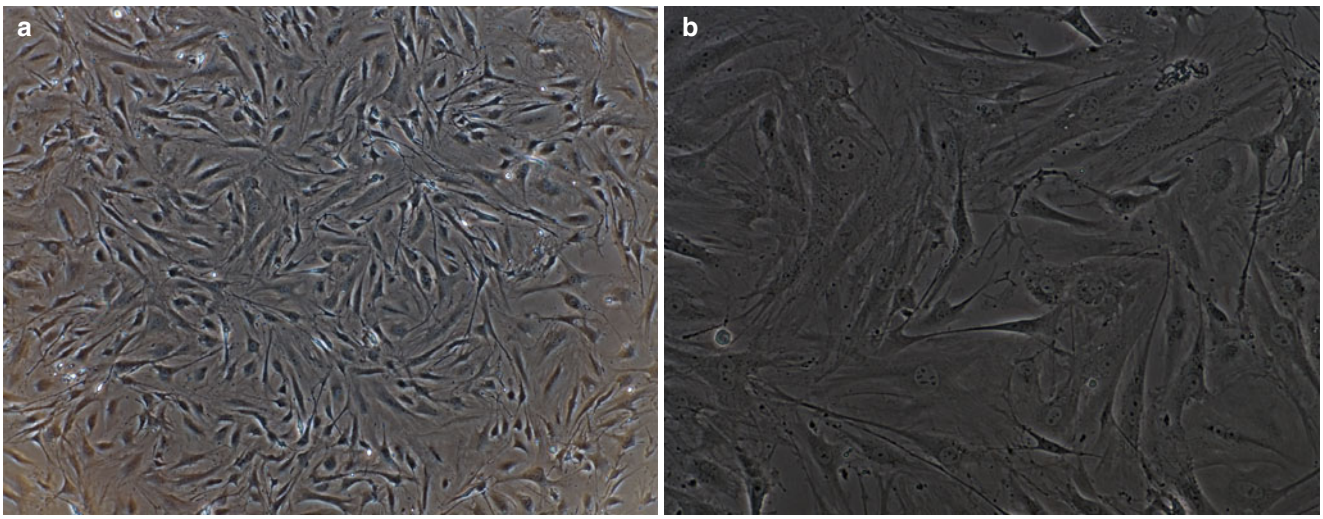

Fig. 3.20 (**a**, **b**) Mouse feeder cells 48 h after plating. Initially, these cells were cultured for 24 h in feeder media; then the medium was removed and replaced with hESC medium and incubated for a further 24 h in culture. The feeder cells appear almost identical to those grown in feeder medium. The cells show a slight morphological change, becoming more elongated and spindle-shaped. Feeder cells do not maintain their morphology in hESC media (**a**, ×10 magnification; **b**, ×20)

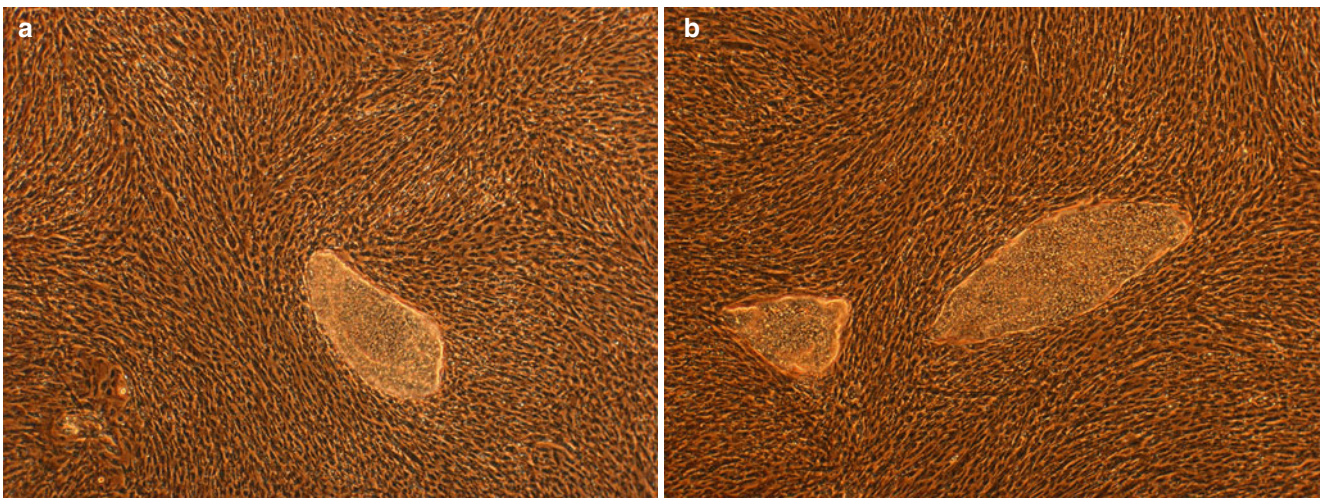

Fig. 3.21 (**a**, **b**) These two images depict inactivated human feeders plated at a density optimal for co-culture with a particular PSC. It is clear from the growth profile of these cells; however, that the inactivation was not successful and the feeders have overgrown. This has an adverse effect on the PSCs in co-culture, as these fibroblasts grow quickly, outgrowing the stem cells and preventing their expansion and proliferation. If possible, the culture should be started again with a new batch of inactivated feeders and new PSCs. If starting again is not possible, manually dissect the colony and co-culture the PSCs on a new batch of inactivated feeders. Be aware that any inactivated feeders that are carried over from the initial co-culture will again grow and outcompete the PSCs (Both ×4 magnification)

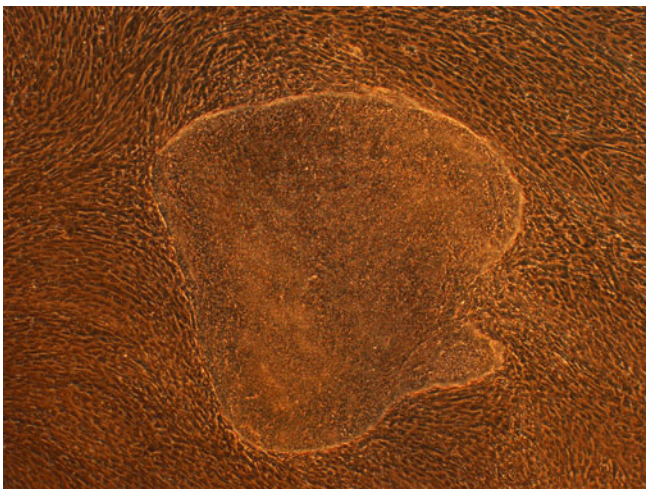

Fig. 3.22 Another image of incomplete inactivation of human fibroblasts in co-culture with PSCs (×4 magnification). This problem can also be observed with murine feeders, but it is less common, as mouse fibroblasts appear more sensitive to inactivation

It is best to use feeders between 1 and 2 days after plating in the culture vessel. If they are left for too many days after plating, they lose their ability to maintain cells undifferentiated. However, in co-culture with appropriate media and media changes, the PSCs can remain undifferentiated for up to 2 weeks. As feeder layers age, the feeder cells start to deteriorate and lose their ability to prevent the differentiation of PSCs. Feeder cells maintained in media formulations optimised for use in co-culture with PSCs show a change in morphology over time, acquiring an elongated, spindle-shaped phenotype indicative of stress. Feeder cells are therefore maintained in the appropriate media until used in co-culture with PSCs (Fig. 3.23).

Feeders also can be obtained from a number of commercial sources, but these should be subject to the same quality control as feeders prepared in the PSC laboratory setting.

Feeder cells are fundamental to the growth of good-quality PSCs in feeder-dependent cell culture systems, and they should be prepared in a manner that recognises the quality attributes of feeder cells and the impact of these attributes on the production and maintenance of undifferentiated PSCs.

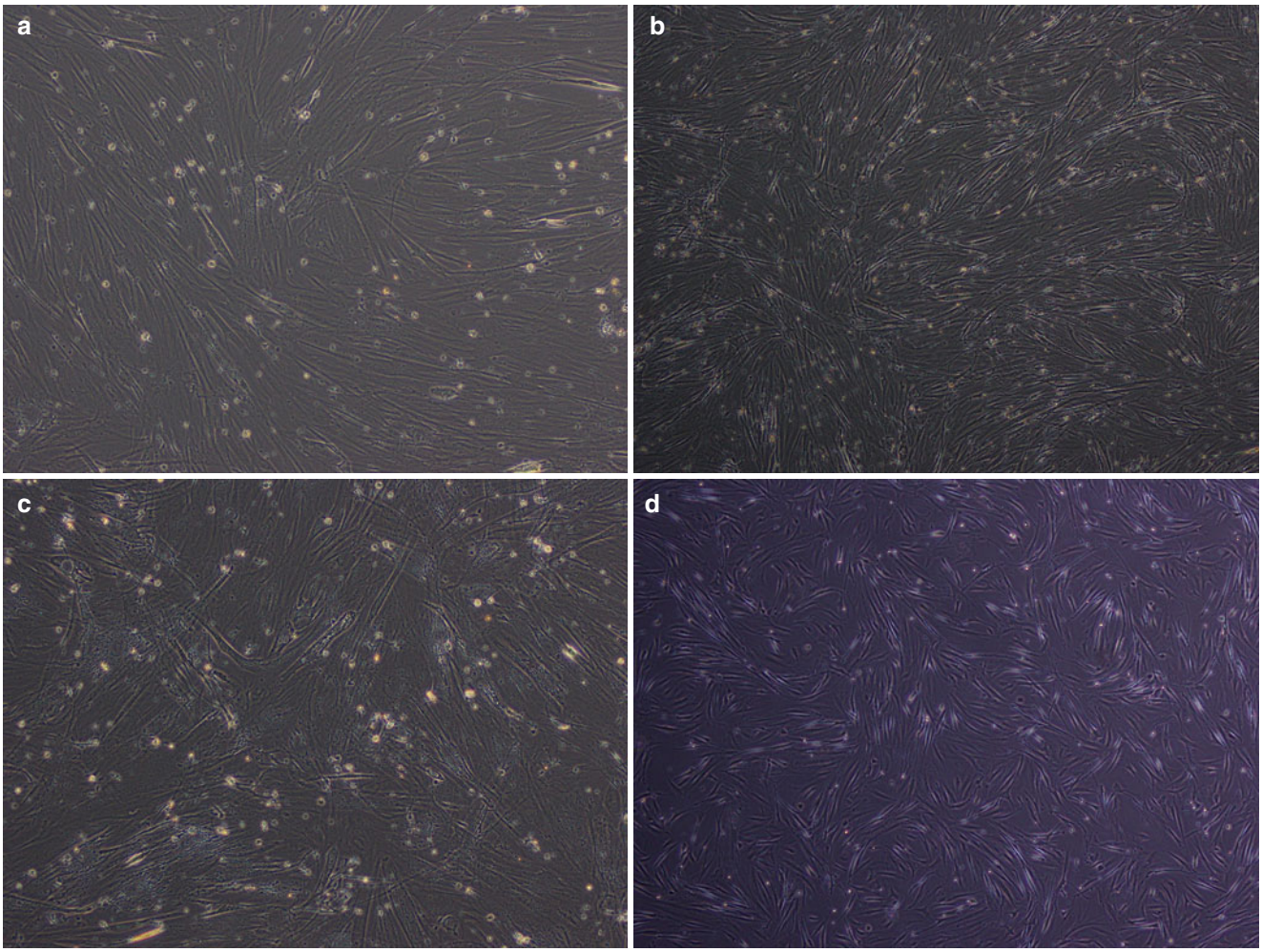

Fig. 3.23 Images of old human feeders (**a**–**c**) compared with an optimal human feeder (**d**). **a** (Day 7 post-plating), **b** (Day 10) and **c** (Day 14) show a change over time in the morphology of the cells to an elongated, stretched, fragile cell morphology with evidence of cellular degeneration, unlike the optimal, robust fibroblast morphology seen in (**d**). Apoptotic cells are also evident in the old feeders (All images ×10 magnification)

Suggested Reading

Amit M, Itskovitz-Eldor J. Morphology of human embryonic and induced pluripotent stem cell colonies cultured with feeders. In: Amit M, Itskovitz-Eldor J, editors. Atlas of human pluripotent stem cells: derivation and culturing, Stem cell biology and regenerative medicine. New York: Humana Press; 2012. p. 15–39.

Diekmann U, Spindler R, Wolkers WF, Glasmacher B, Müller T. Cryopreservation and quality control of mouse embryonic feeder cells. Cryobiology. 2011;63:104–10.

Eiselleova L, Peterkova I, Neradil J, Slaninova I, Hampl A, Dvorak P. Comparative study of mouse and human feeder cells for human embryonic stem cells. Int J Dev Biol. 2008;52:353–63.

Fernandes AM, Meletti T, Guimarães R, Stelling MP, Marinho PA, Valladão AS, Rehen SK. Worldwide survey of published procedures to culture human embryonic stem cells. Cell Transplant. 2010;19:509–23.

Lee JB, Song JM, Lee JE, Park JH, Kim SJ, Kang SM, et al. Available human feeder cells for the maintenance of human embryonic stem cells. Reproduction. 2004;128:727–35.

Leonardo TR, Schell JP, Tran HT, Peterson SE. Preparation of mouse embryonic fibroblast feeder cells. In: Loring JF, Peterson SE, editors. Human stem cell manual. 2nd ed. London: Elsevier; 2012. p. 15–27.

Michalska AE. Isolation and propagation of mouse embryonic fibroblasts and preparation of mouse embryonic feeder layer cells. Curr Protoc Stem Cell Biol. 2007;3:1C.3.1–17.

Nieto A, Cabrera CM, Catalina P, Cobo F, Barnie A, Cortés JL, et al. Effect of mitomycin-C on human foreskin fibroblasts used as feeders in human embryonic stem cells: immunocytochemistry MIB1 score and DNA ploidy and apoptosis evaluated by flow cytometry. Cell Biol Int. 2007;31:269–78.

Prathalingam N, Ferguson L, Young L, Lietz G, Oldershaw R, Healy L, et al. Production and validation of a good manufacturing practice grade human fibroblast line for supporting human embryonic stem cell derivation and culture. Stem Cell Res Ther. 2012;3:12.

Roy A, Krzykwa E, Lemieux R, Néron S. Increased efficiency of gamma-irradiated versus mitomycin C-treated feeder cells for the expansion of normal human cells in long-term cultures. J Hematother Stem Cell Res. 2001;10:873–80.

Unger C, Felldin U, Nordenskjöld A, Dilber MS, Hovatta O. Derivation of human skin fibroblast lines for feeder cells of human embryonic stem cells. Curr Protoc Stem Cell Biol. 2008;5:1C.7.1–10.

Human Embryonic Stem Cells

4

In 1998, the derivation of the first three human embryonic stem cell (hESC) lines from the inner cell mass of a blastocyst was reported. The cells grew as colonies and were derived and maintained on mouse feeder cells. The cells displayed a normal karyotype over the reported 8 months in continuous cell culture and displayed cell surface markers characteristic of undifferentiated non-human primate embryonic stem cell lines (Chap. 8) and human embryonal carcinoma cells. These cells were shown to be pluripotent in nature through their ability to form teratomas in immunocompromised mice, composed of tissues derived from all three germ layers. The potential utilisation of these cells for disease modelling, toxicology studies and regenerative medicine heralded a new era in the field of biology.

The routine culture of established human cell lines in the laboratory under the basic principles of good cell culture practice (GCCP) is regarded as straightforward; in general, their culture conditions are robust, as are their morphological characteristics. However, the culture of undifferentiated hESC lines is not easy, as these cells are very sensitive to alterations in their culture conditions and respond by changing their growth characteristics and morphology. Each cell line responds differently to these alterations, and morphological change is the easiest way to monitor the status of the cell culture. Cell cultures should be monitored under the microscope every day; this monitoring should be part of the best-practice activity employed in the maintenance and expansion of these cell lines. This chapter includes an extensive portfolio of images depicting the culture of different hESC lines on mouse and human feeders and describes the changes seen in these cultures when cells are grown in optimal and suboptimal conditions (Figs. 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 4.10, 4.11, 4.12, 4.13, 4.14, 4.15, 4.16, 4.17, 4.18, 4.19, 4.20, 4.21, 4.22, 4.23, 4.24, 4.25, 4.26, 4.27, 4.28, 4.29, 4.30, 4.31, 4.32, 4.33, 4.34, 4.35, 4.36, 4.37, 4.38, 4.39, 4.40, 4.41, 4.42, 4.43, 4.44, 4.45, 4.46, 4.47, 4.48, 4.49, 4.50, 4.51, 4.52, 4.53, 4.54, 4.55, 4.56, 4.57, 4.58, 4.59, 4.60, 4.61, 4.62, 4.63, 4.64 and 4.65).

L. Healy, L. Ruban, *Atlas of Human Pluripotent Stem Cells in Culture*,
DOI 10.1007/978-1-4899-7507-2_4, © Springer Science+Business Media New York 2015

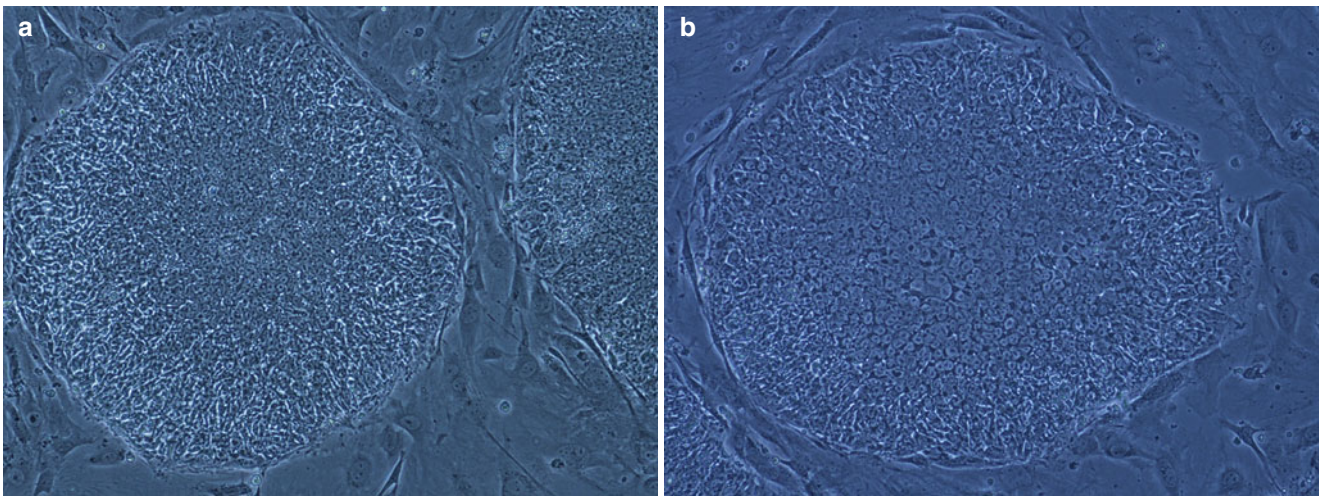

Fig. 4.1 (**a**, **b**) Human embryonic stem cell (hESC) colony cultured on mouse embryonic fibroblast (MEF) feeder cells. These colonies display the typical morphology of a good-quality colony (both ×10 magnification)

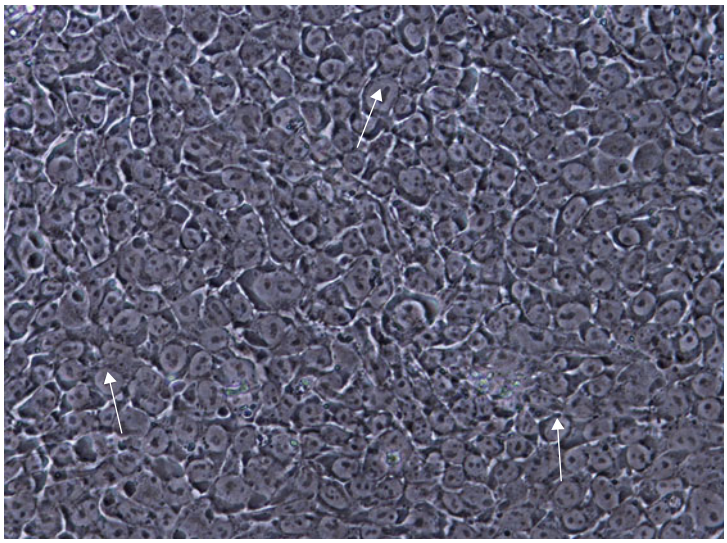

Fig. 4.2 Typical hESC morphology seen under high magnification: small, round cells with prominent nucleoli. High ratio of nucleus to cytoplasm (*white arrows*), with narrow spaces observed between the cells (×40 magnification)

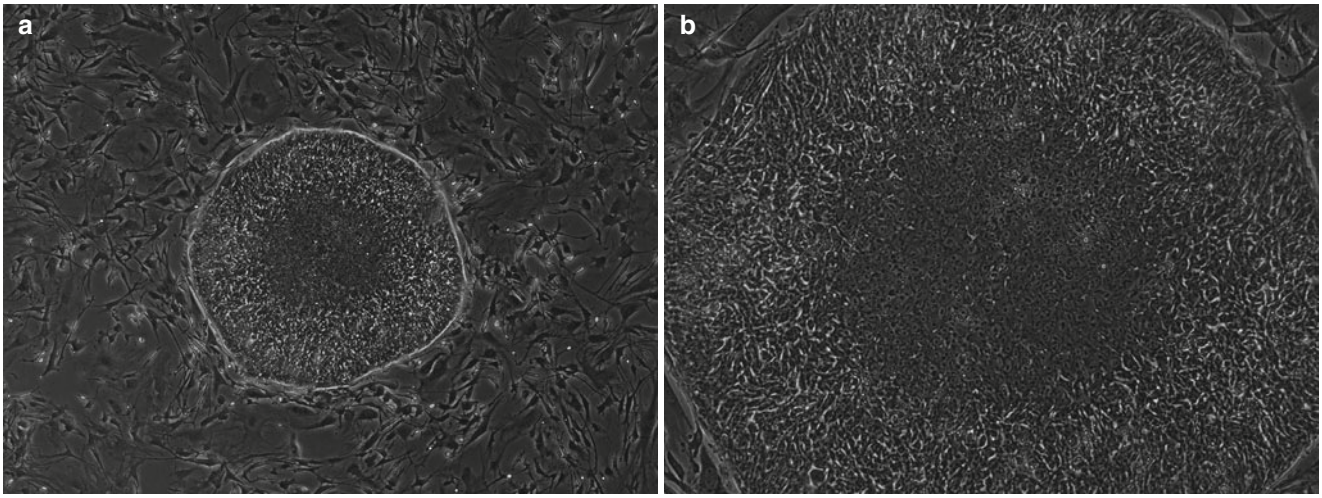

Fig. 4.3 Typical hESC colony. (**a**) A small "young" colony, 24 h after passage (×4 magnification). Note the compacted centre, actively proliferating cells around the edges, and defined borders. Spaces are typically observed between the cells. This morphology would be considered to represent a good undifferentiated colony and would be optimal when expanding undifferentiated cells in a culture system. (**b**) The same colony at ×10 magnification

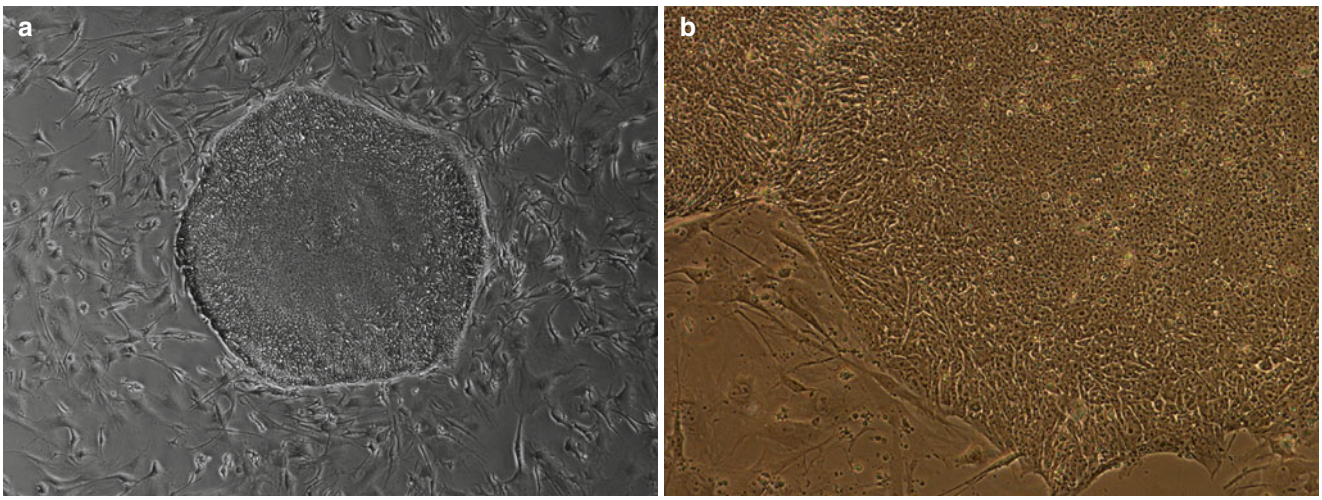

Fig. 4.4 Two images of good-quality colonies. (**a**) Small, undifferentiated, compacted colony with clear borders. Under phase contrast, these colonies appear to be growing in three dimensions. (**b**) Two colonies merge and spread to form a perfect monolayer, ready for passaging. Both colonies are compacted and ready to passage (**a**, ×4 magnification; **b**, ×10)

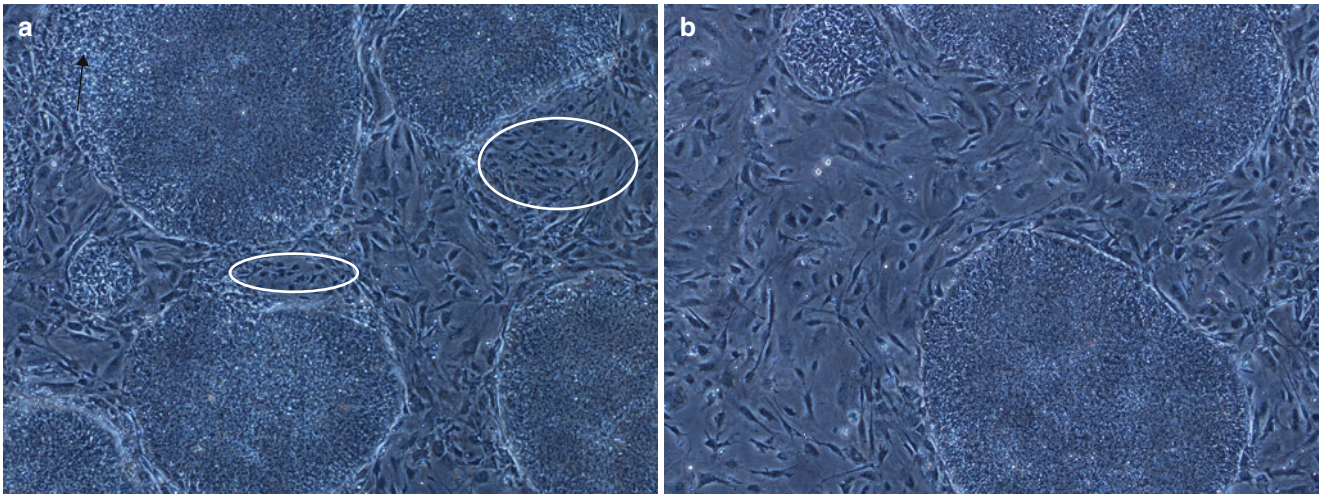

Fig. 4.5 (**a**, **b**) Relatively good-quality, high-density culture. The colonies are close together and cells within the colonies are compacted. The feeders are still of a good quality, so this culture could be kept for another day, but it would be advisable to passage at this stage. This culture is described as "relatively good" because spontaneous differentiation (*white ovals*) can be seen (**a**). Morphologically, the cells look like fibroblasts, but they are much smaller and rounder. The large colony in the top left of image **a** shows slight differentiation (*black arrow*), but in general it is a good culture. It is advisable to passage this culture at this stage (both ×4 magnification)

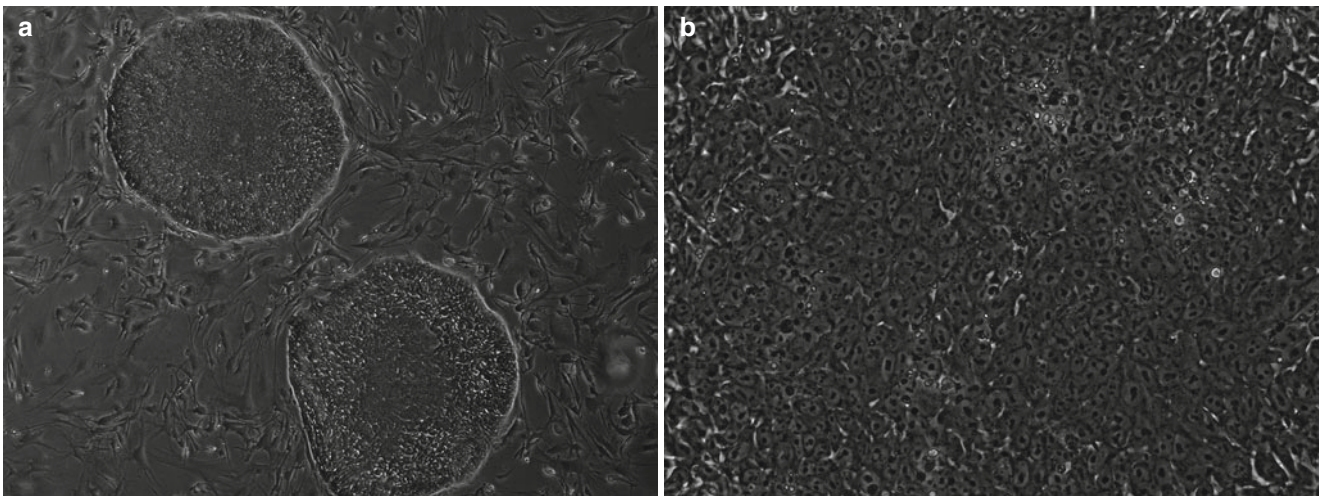

Fig. 4.6 (**a**) Two small colonies compacted in the middle, 24 h after passage. (**b**) The compacted centre of the same colony (**a**, ×4 magnification; **b**, ×20)

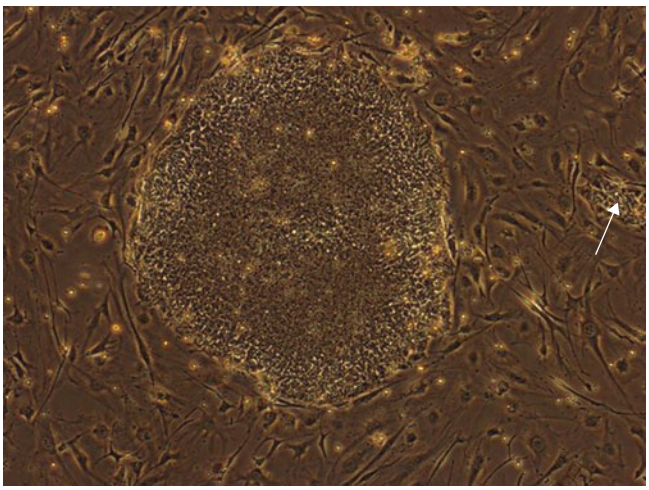

Fig. 4.7 Example of a good colony with fresh feeders surrounding it. This colony should be passaged immediately, because it could begin to spontaneously differentiate in the centre if it is left for another 24 h. The small colony (*white arrow*) is too small to maintain in an undifferentiated state in this culture system and will probably spontaneously differentiate within a few days (×10 magnification)

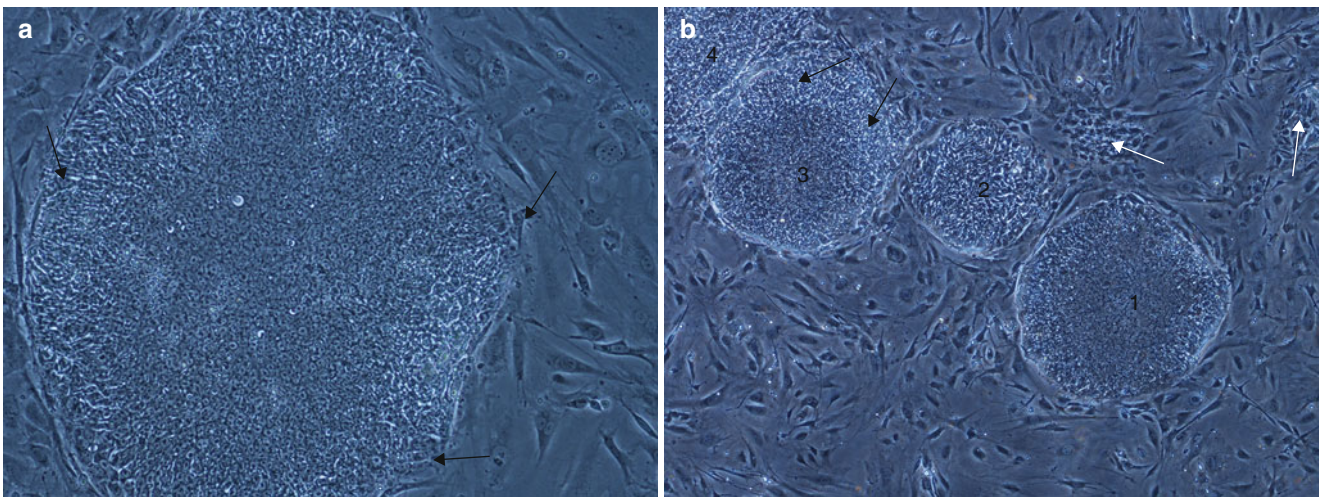

Fig. 4.8 (**a**) An example of a good-quality colony. If left for another 24 h, the colony could spontaneously differentiate around the areas shown with *black arrows*. (**b**) Four small colonies displaying different morphologies: Colony number *1* is perfectly round and compacted in the centre; it is a good example of an undifferentiated colony. Colony number *2* is small, not compacted, and displays a low degree of spontaneous differentiation around the edges. Colony number *3* is mainly undifferentiated in the centre, except along the top edge (*black arrows*). Colony number *4* is totally differentiated. It should be noted that small clumps of hESC very rarely form a good colony. In this image, all of these small clumps have differentiated (*white arrow*) into a cell type of unknown morphology, possibly epithelial (**a**, ×10 magnification; **b**, ×4)

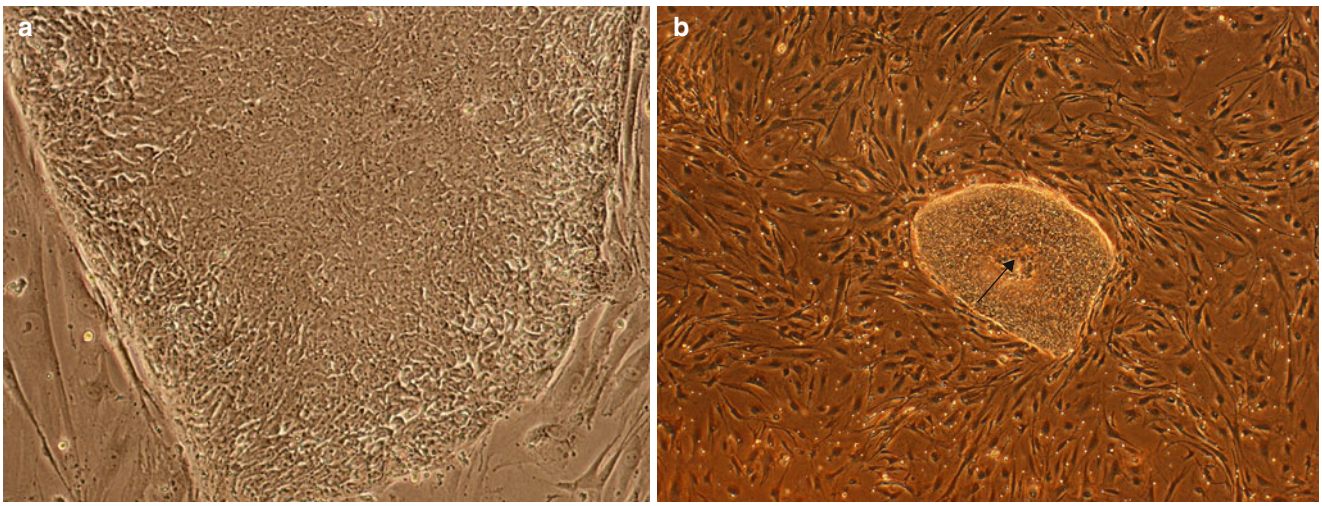

Fig. 4.9 (**a**) A good-quality, slightly three-dimensional colony, under phase contrast. (**b**) A small hESC colony, on relatively high-density feeders, with spontaneous differentiation in the centre (*black arrow*). For this cell line, one of the reasons for this differentiation could be that the feeders are of higher-than-usual density and it was difficult for the colony to push them away. Another reason could be that the original clump of stem cells was too thick and could not spread out into a good monolayer, and this condition promoted differentiation (**a**, ×10 magnification; **b**, ×4)

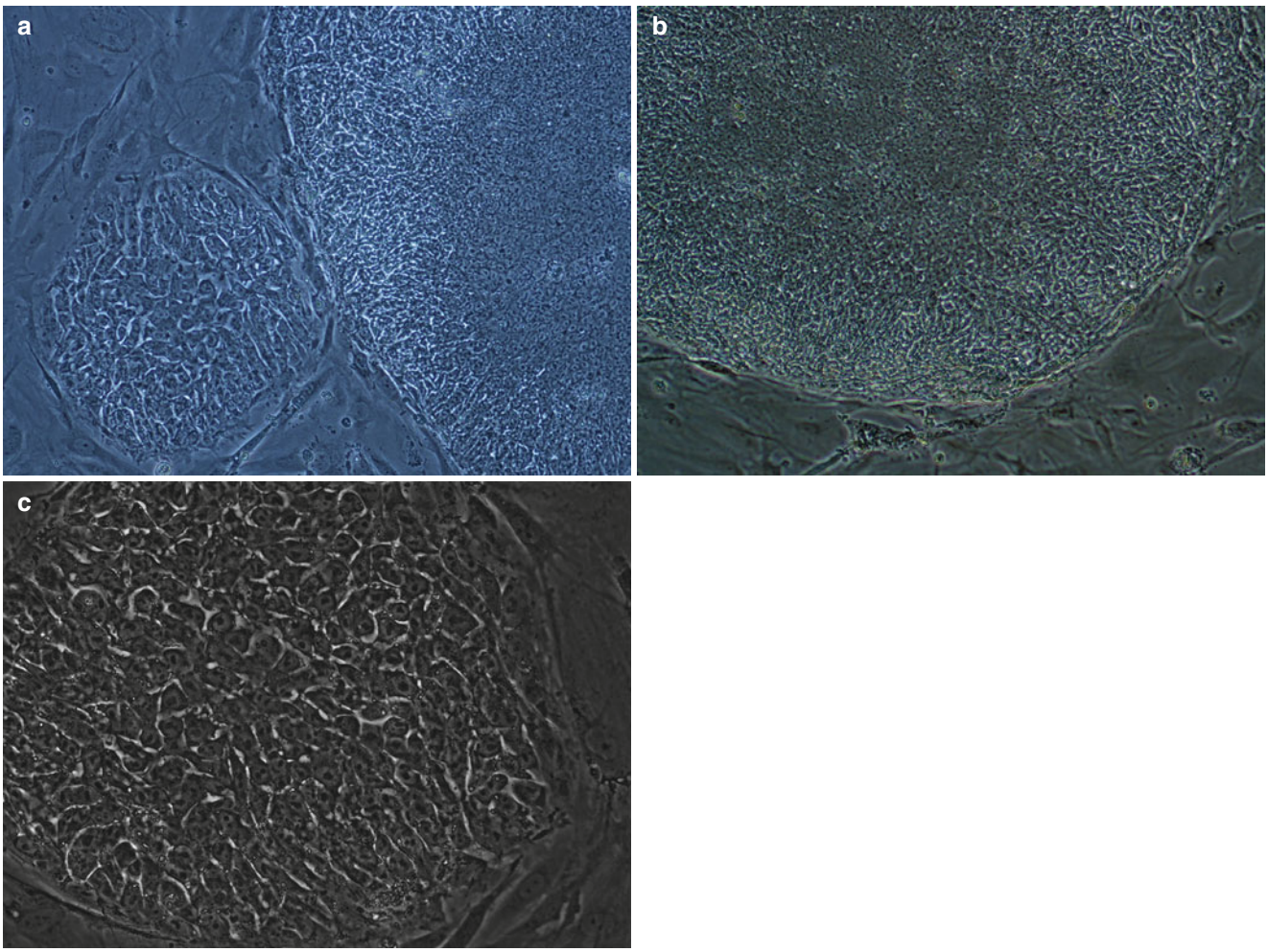

Fig. 4.10 (**a**) Two good colonies merging. The one on the *right side* is large and compacted in the middle. The small colony on the *left side* is not yet compacted; every cell within the colony can be seen. It is also actively proliferating. (**b**) A large, undifferentiated hESC colony compacted in the middle and actively proliferating around the edges. (**c**) A small colony, not yet compacted (**a**, **b**, ×10 magnification; **c**, ×20)

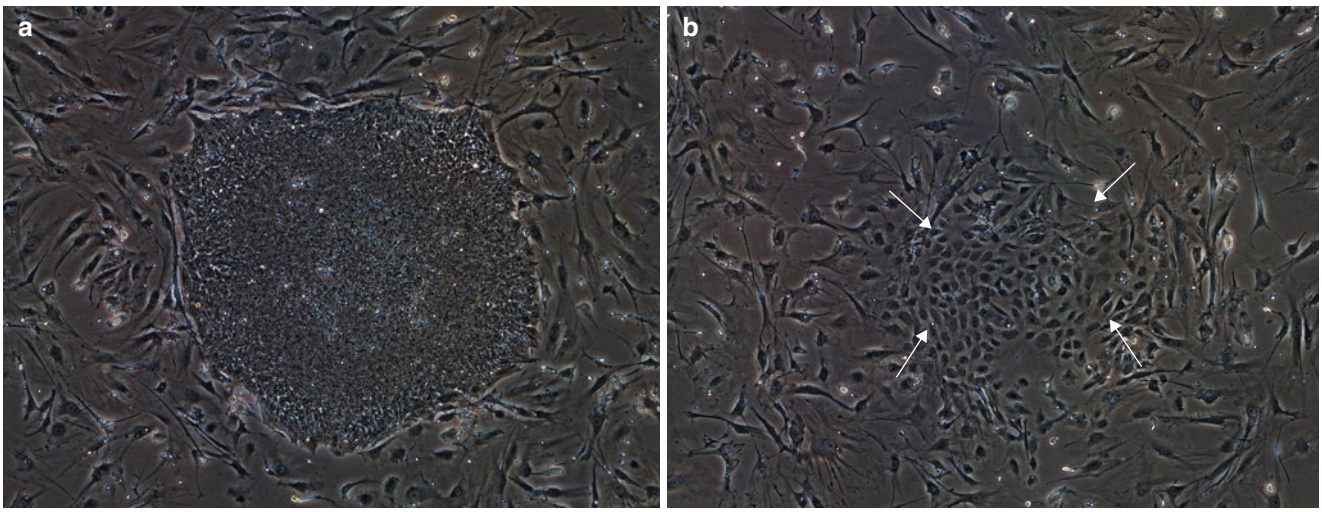

Fig. 4.11 Comparison of a colony with a good, undifferentiated hESC colony morphology (**a**) and a totally differentiated colony (**b**). It is important to be able to distinguish the differentiated cells in the centre of the image (*white arrow*) from the more elongated feeders surrounding the differentiated colony (both ×10 magnification)

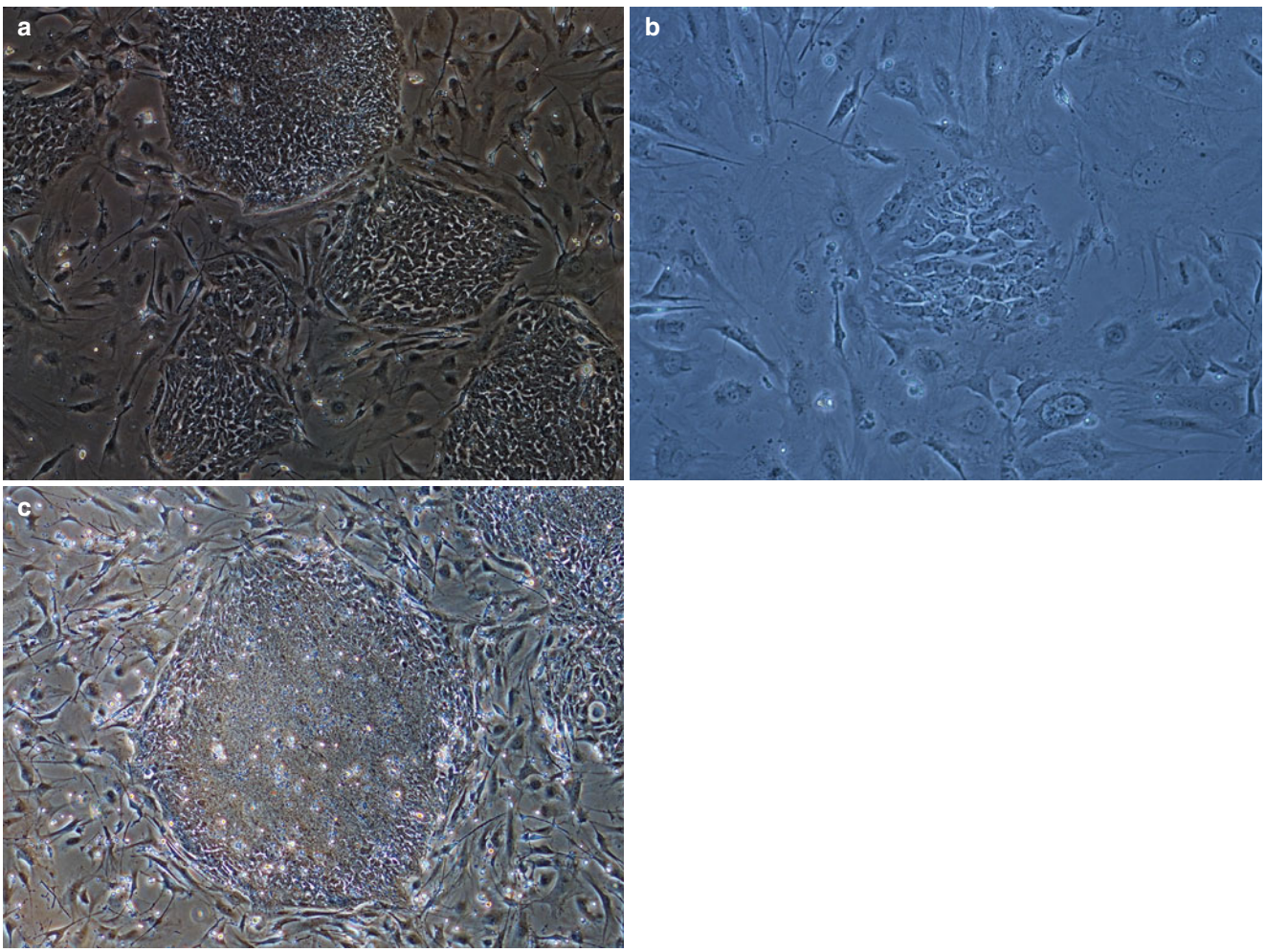

Fig. 4.12 (**a**) High-density small colonies 24 h after passaging, with good-quality, healthy feeders evident by their morphology (×4 magnification). (**b**) A small colony, not compacted and without clear borders, having a low density of feeders surrounding the colony (too few feeders to promote maintenance of the undifferentiated state) (×10 magnification). A colony like this is not likely to make a good colony. (**c**) A small, compacted colony surrounded by feeders that are less than optimum (too many apoptotic cells) (×10 magnification)

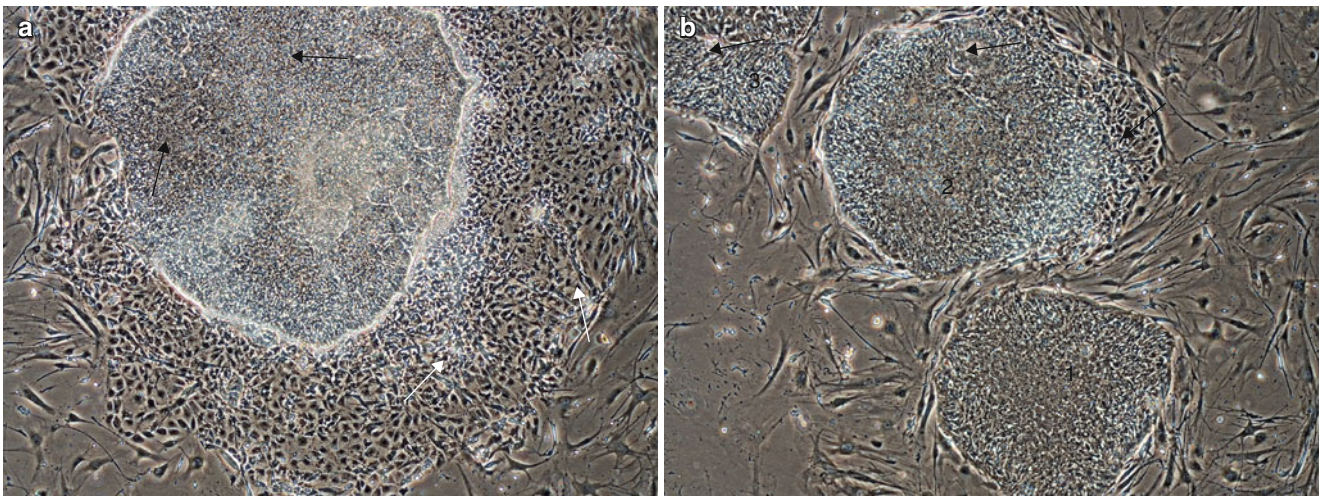

Fig. 4.13 (**a**) Typical pattern of differentiation observed when using old feeders (>3 days old). Some areas in the centre of the colonies are still undifferentiated (*black arrows*). Outside of the colony is a wide border of totally differentiated cells (*white arrows*). (**b**) Three colonies: Number *1* has a perfect morphology. Number *2* is partially differentiated (*black arrows*). Number *3* is differentiated and is beginning to form a neuronal rosette (both ×4 magnification)

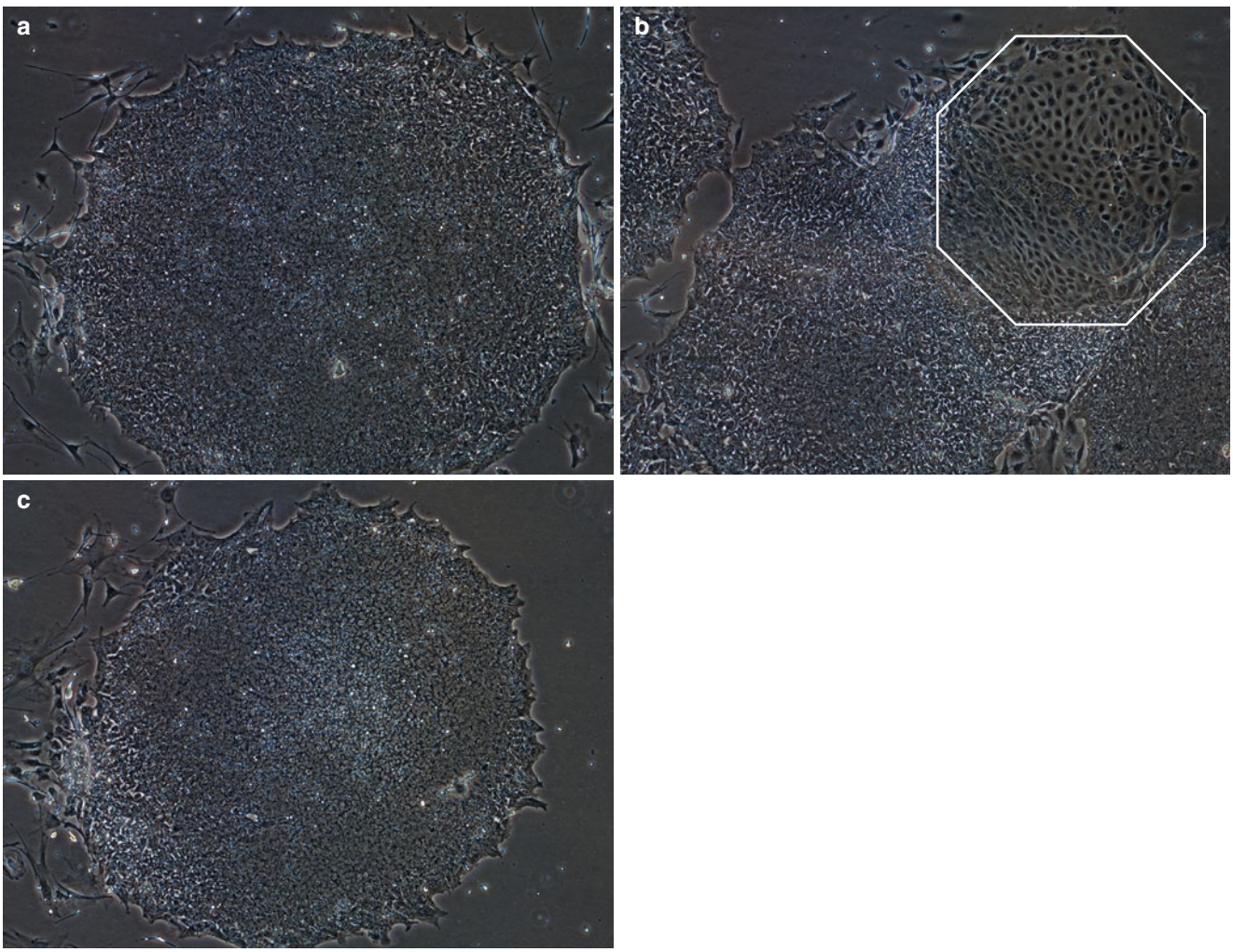

Fig. 4.14 In these images, hESC colonies have grown into the empty spaces created by removing or scraping away colonies. This has proved to be a very useful method during the derivation of cell lines for regrowth of colonies; it can be observed in old culture dishes kept and maintained after mechanical removal of some colonies. Leftover small clumps of cells grow into spaces covered with matrix produced from feeders and stem cells. (**a**) Large, undifferentiated colony surrounded by a low number of feeders. (**b**) A large part of the colony has spontaneously differentiated (*white octagon*). (**c**) Another good colony similar to the one in **a** (all ×10 magnification)

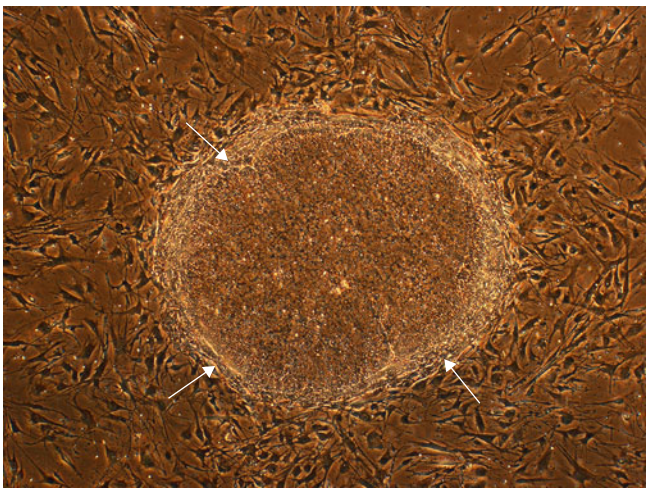

Fig. 4.15 There is spontaneous differentiation (*white arrows*) on the edges of this colony, but the rest of the colony is undifferentiated (×4 magnification)

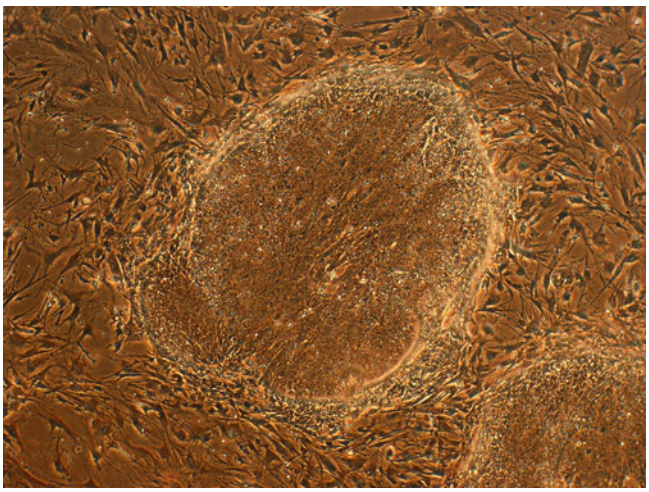

Fig. 4.16 At first glance, this colony appears to have a typical individual stem cell morphology (small cells with nucleoli). However, this colony displays differentiation at the edges and inside the colony, and all the cells are lined up. It is beginning to differentiate (×4 magnification)

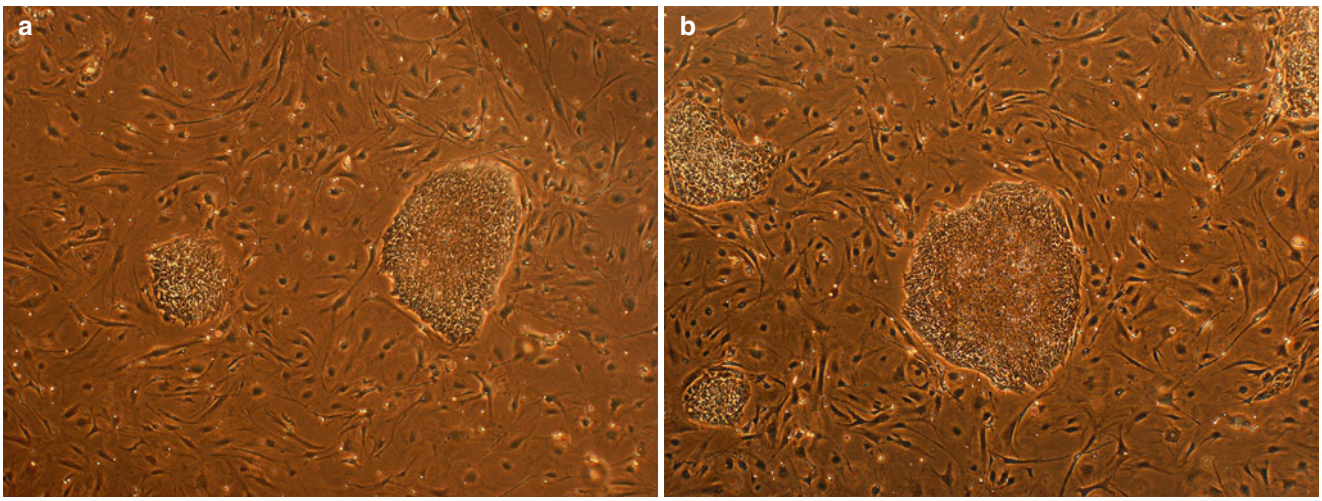

Fig. 4.17 (**a**) The large colony on the *right* side is of a relatively good morphology. The smaller colony on the *left* is not compacted, does not have smooth edges, and the cells are disorganised. Because of these features, the colony is probably going to differentiate within a day or two. (**b**) The large colony in the middle of the image is undifferentiated, but all of the smaller colonies in the image are too small to form any decent compacted colonies (both ×4 magnification)

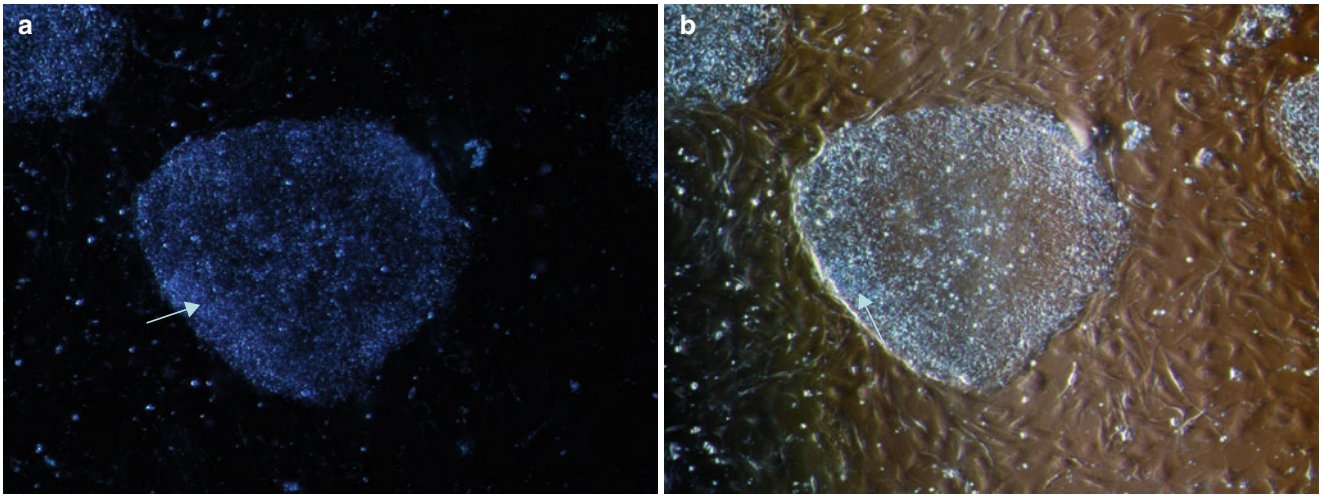

Fig. 4.18 (**a**) Darkfield microscopy of a good colony under a dissecting microscope. (**b**) The same colony with a little bit of illumination behind it. This is the preferable way of illuminating culture plates for mechanical passaging. Under these conditions, it is much easier to recognise differentiation or areas of potential differentiation. In this image, multilayered cells appear whiter than the rest of the colony (*white arrow*). In a few days, these multilayer areas are the areas where the cells will differentiate (both ×4 magnification)

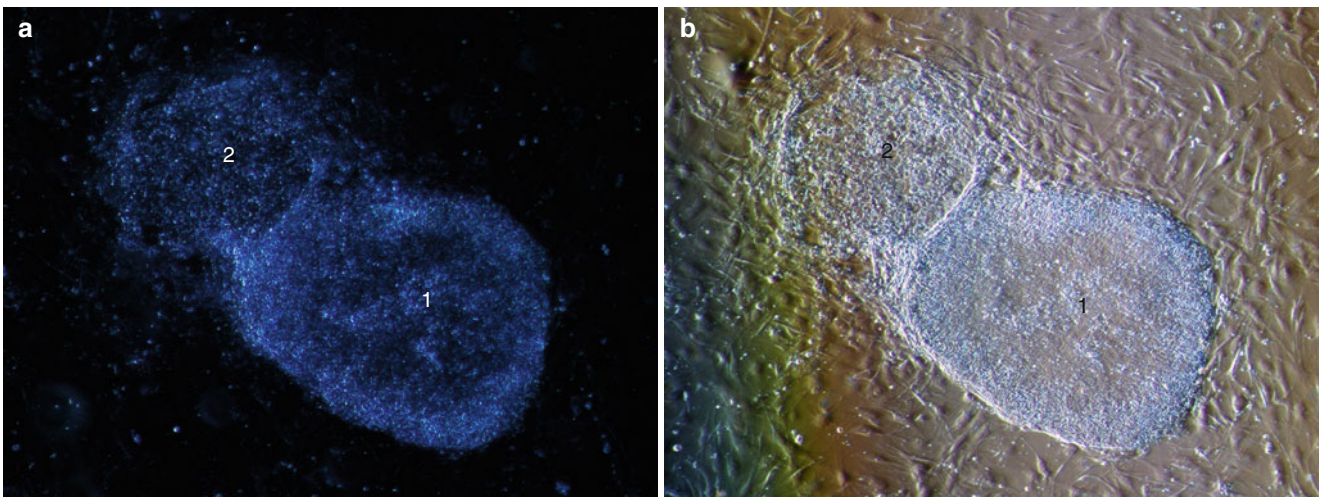

Fig. 4.19 (**a**, **b**) Darkfield microscopy of two colonies that have merged. Colony number *1* is undifferentiated, and the smooth, perfect edge of the compacted colony can be seen. The smaller colony, number *2*, is fully differentiated, without smooth edges (both ×4 magnification)

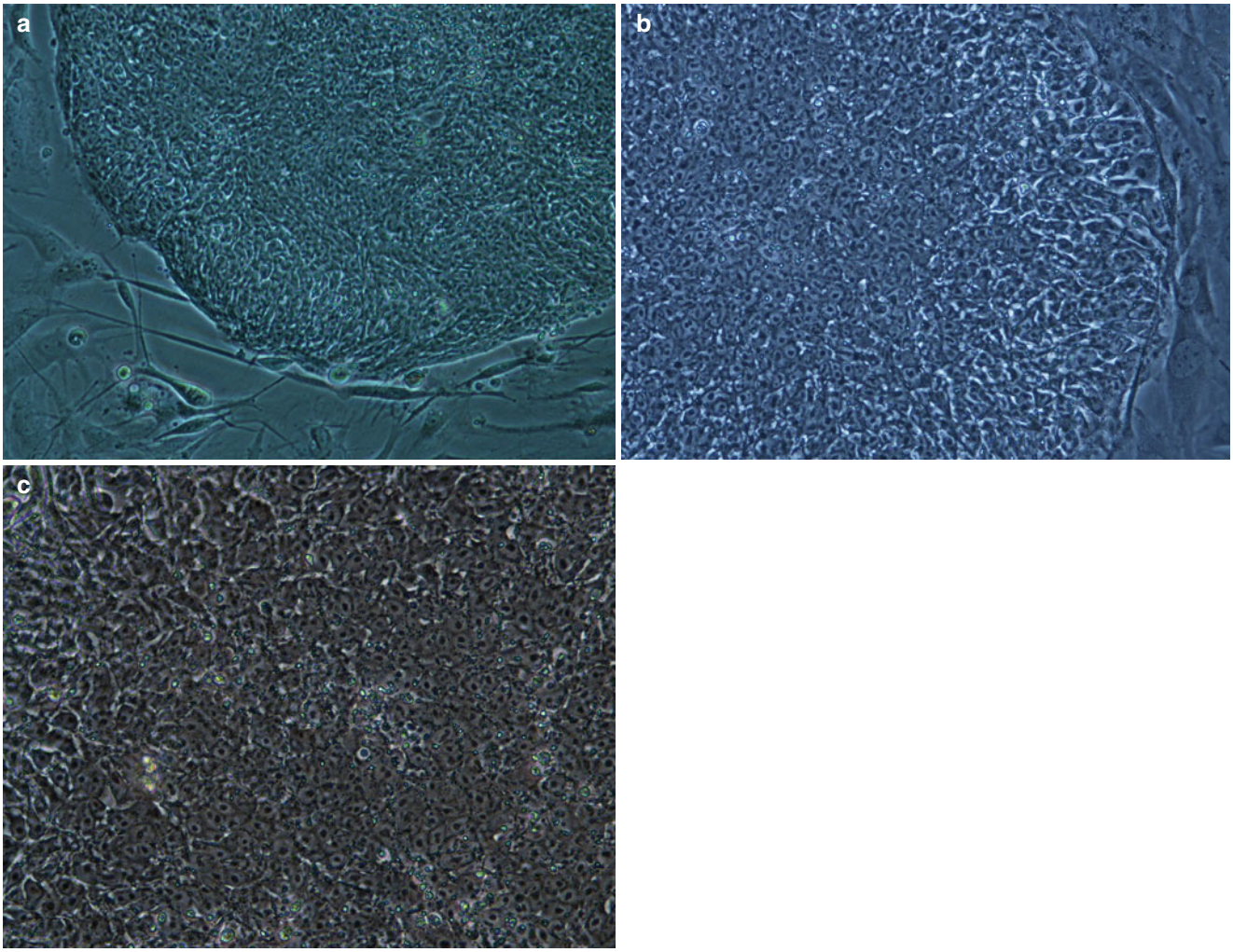

Fig. 4.20 (**a**) An undifferentiated colony, with defined edges. The colony contains round, compacted cells with large nucleoli. (**b**) Higher magnification of the edges of the colony. The cells are elongated, with spaces between them, as is typical for actively proliferating stem cells. It is very important to know difference between actively proliferating and differentiating cells. (**c**) Higher magnification of the middle of the colony, showing the cells becoming more compact (**a**, ×10 magnification; **b**, **c**, ×20)

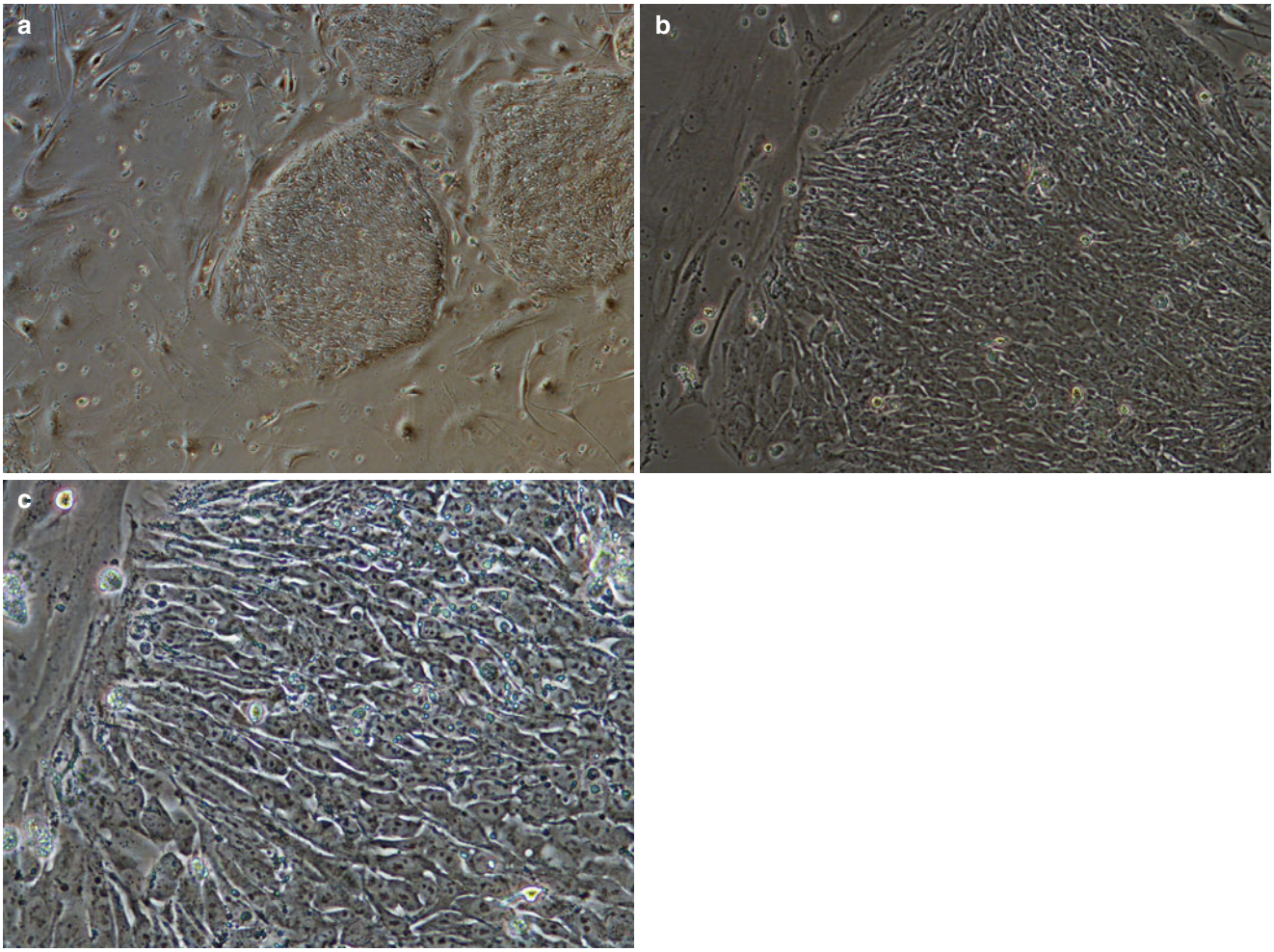

Fig. 4.21 Small stem cell colonies. At low magnification (**a**), the colonies look relatively normal and undifferentiated, but under higher magnification (**b**, **c**) it is clear that the colonies are differentiating. Almost all of the cells are elongated and lined up, and the colony is poised to differentiate. It is evident from the image that differentiation would be a result of the low density of feeders, which is insufficient to maintain the colony in the undifferentiated state (**a**, ×4 magnification; **b**, ×10; **c**, ×20)

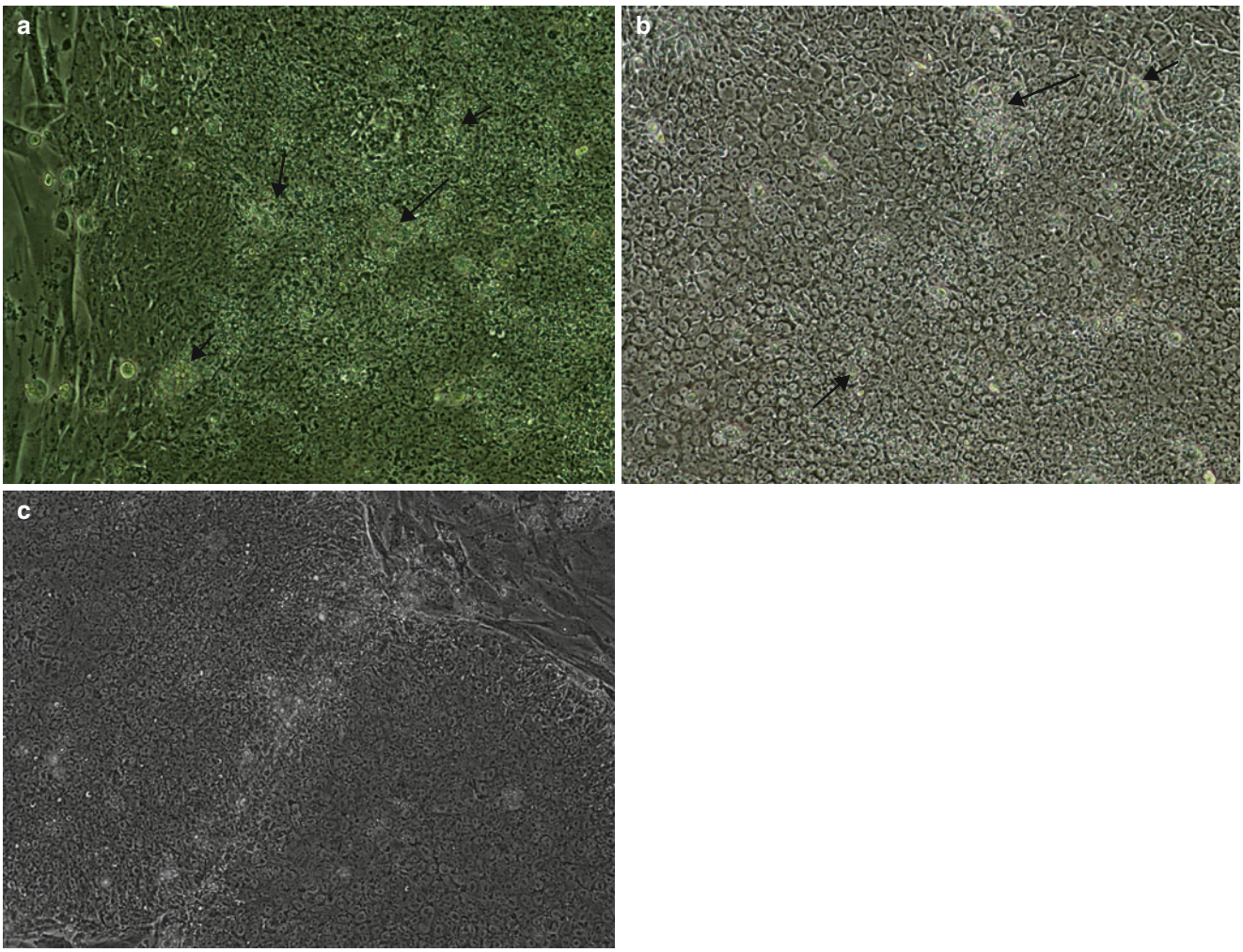

Fig. 4.22 (**a**, **b**) To the untrained eye, this colony may appear to be a good one, but it is not. The colony shows areas differentiation (*black arrows*). The areas appearing as white patches of cells are typical of the beginning of the formation of neural rosettes. If left for a further 48 h, the culture would look like the images depicted in Fig. 4.23. (**c**) Two colonies with a good, undifferentiated morphology that have merged (all ×10 magnification)

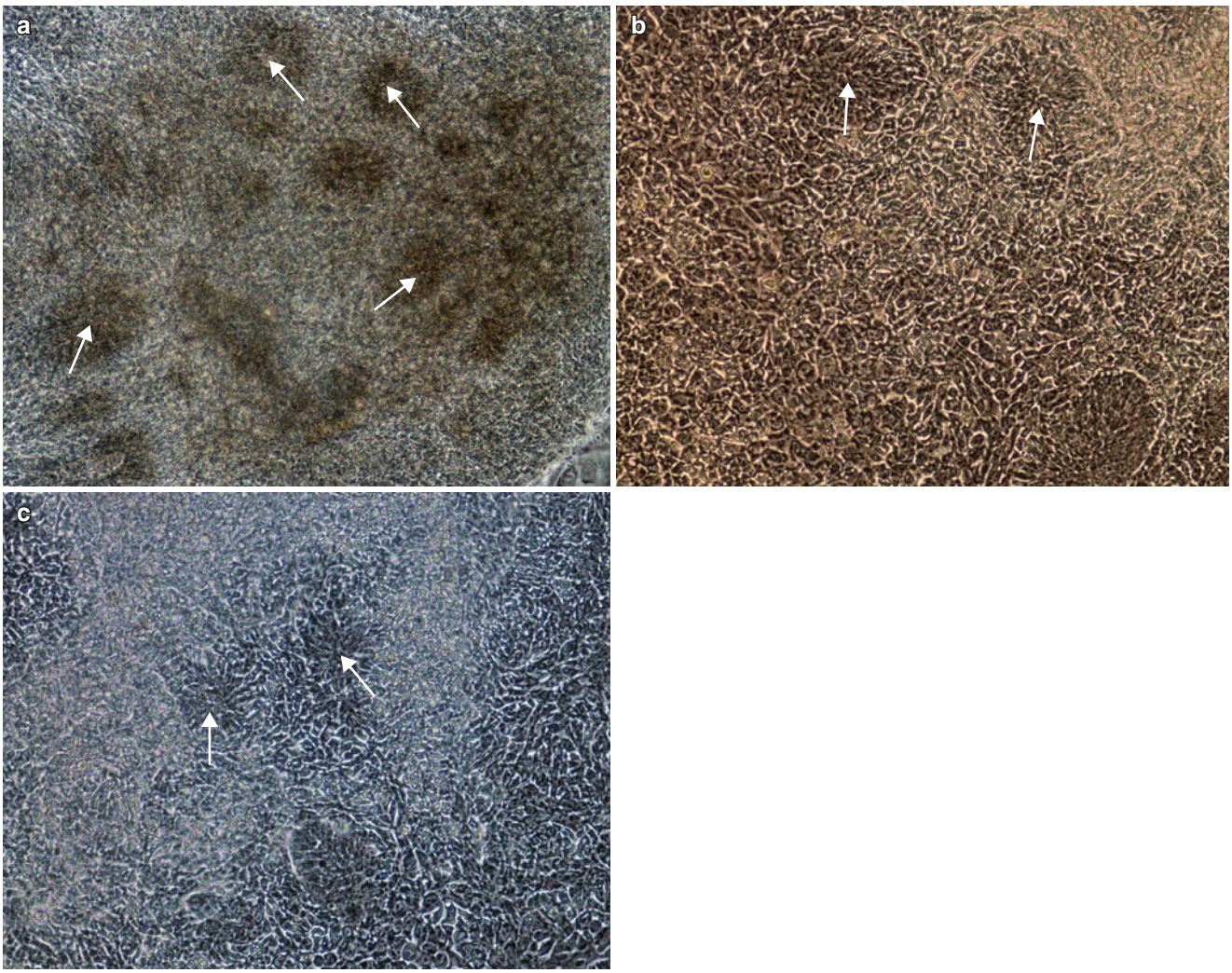

Fig. 4.23 (**a**–**c**) Neural rosettes (*white arrows*) forming from differentiated areas in the hESC colonies. (**a**, ×4 magnification; **b**, **c**, ×10)

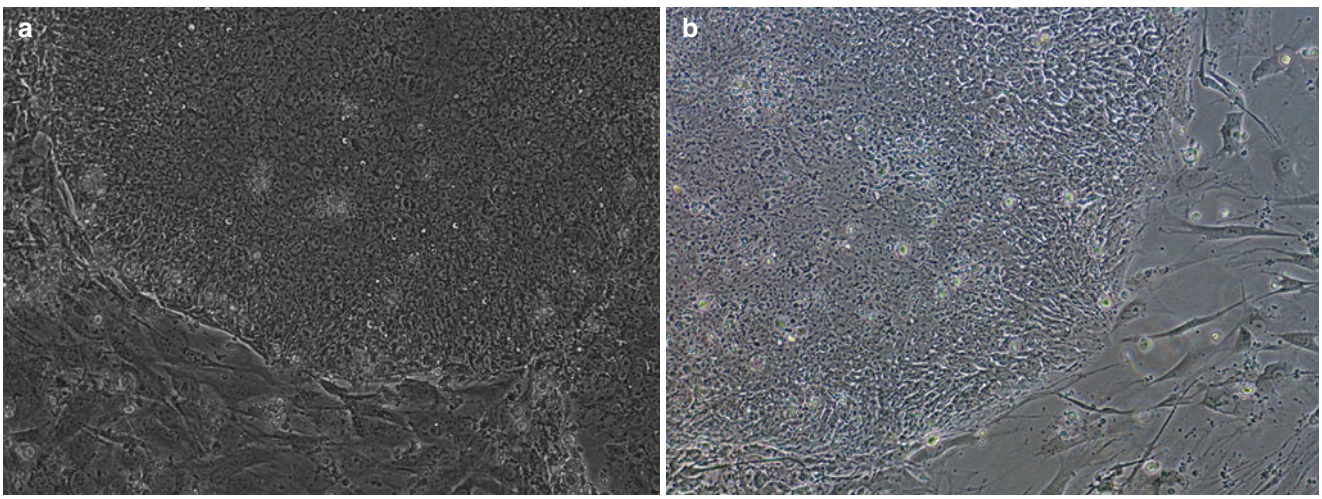

Fig. 4.24 (**a**) A good, compact colony displaying small, round cells with prominent nucleoli. (**b**) A compacted relatively good colony, ready to be passaged (both ×10 magnification)

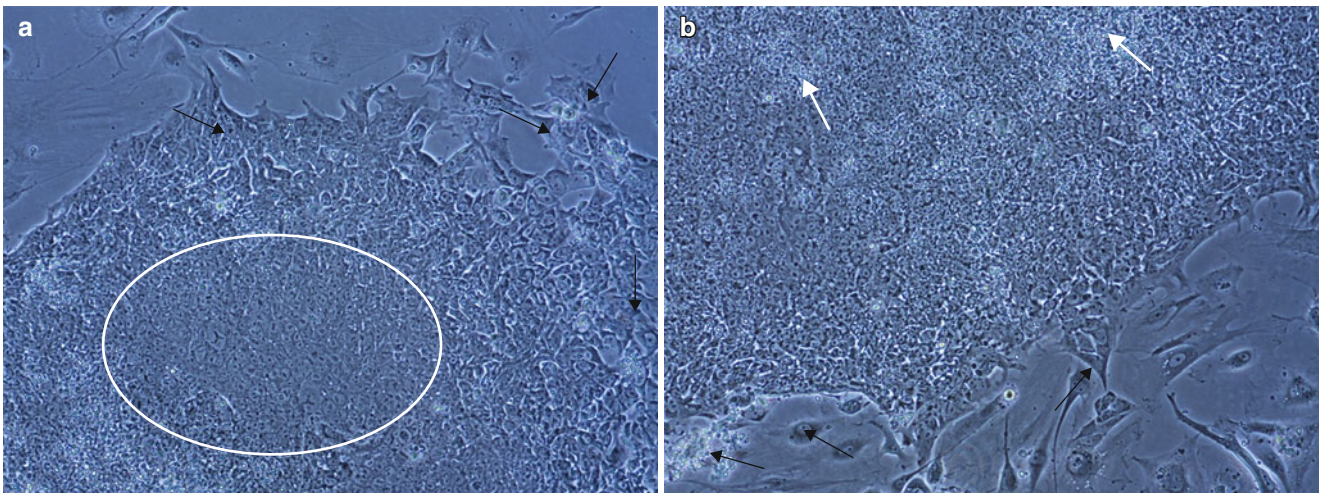

Fig. 4.25 (**a, b**) Two colonies without defined, smooth edges. (**a**) Some cells within the colony (*inside oval shape*) are undifferentiated, but cells around the edges and outside of the both (**a, b**) colonies are spontaneously differentiated (*black arrows*). (**b**) A small group of cells differentiating into neural rosettes (*white arrows*). This culture should be passaged. If left for another 24 h, it would contain much differentiation. One reason for this differentiation could be that there are not enough feeders in the culture to support these colonies in the undifferentiated state. Another reason could be that the culture is just too old, and the feeders have lost their supportiveness (both ×10 magnification)

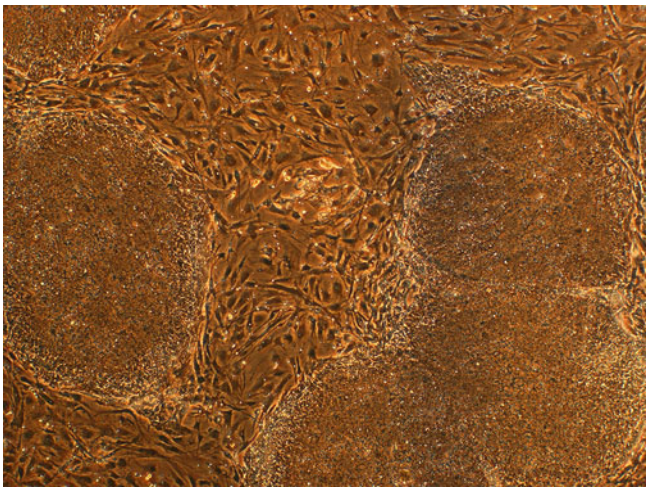

Fig. 4.26 Challenge: what can you say about this figure? See page 199 for answer

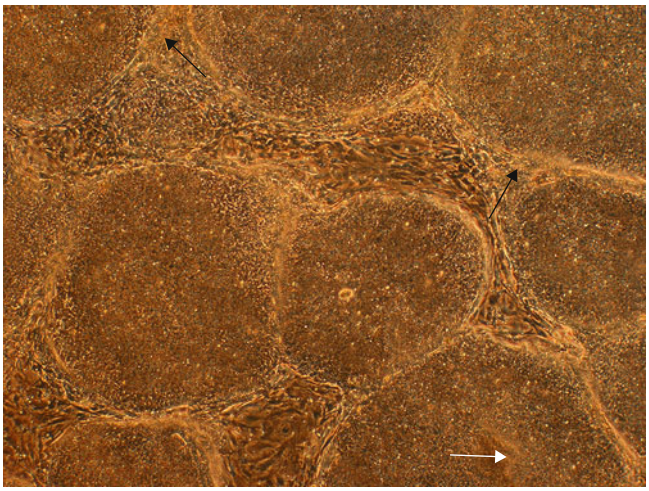

Fig. 4.27 Challenge: what can you say about this figure? See page 199 for answer

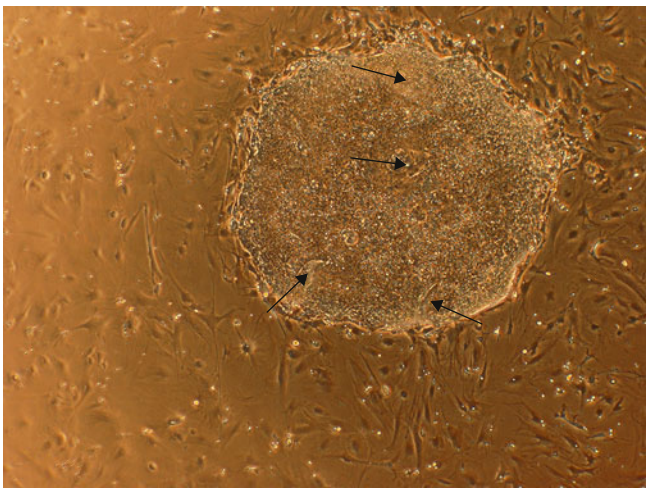

Fig. 4.28 To the untrained eye, this colony would look undifferentiated, but it is "bumpy" (not flat any more) and there are a number of areas of differentiation. In 2 or 3 days, these areas will have differentiated further, and the colony would be full of neural rosettes (*black arrows*) (×10 magnification)

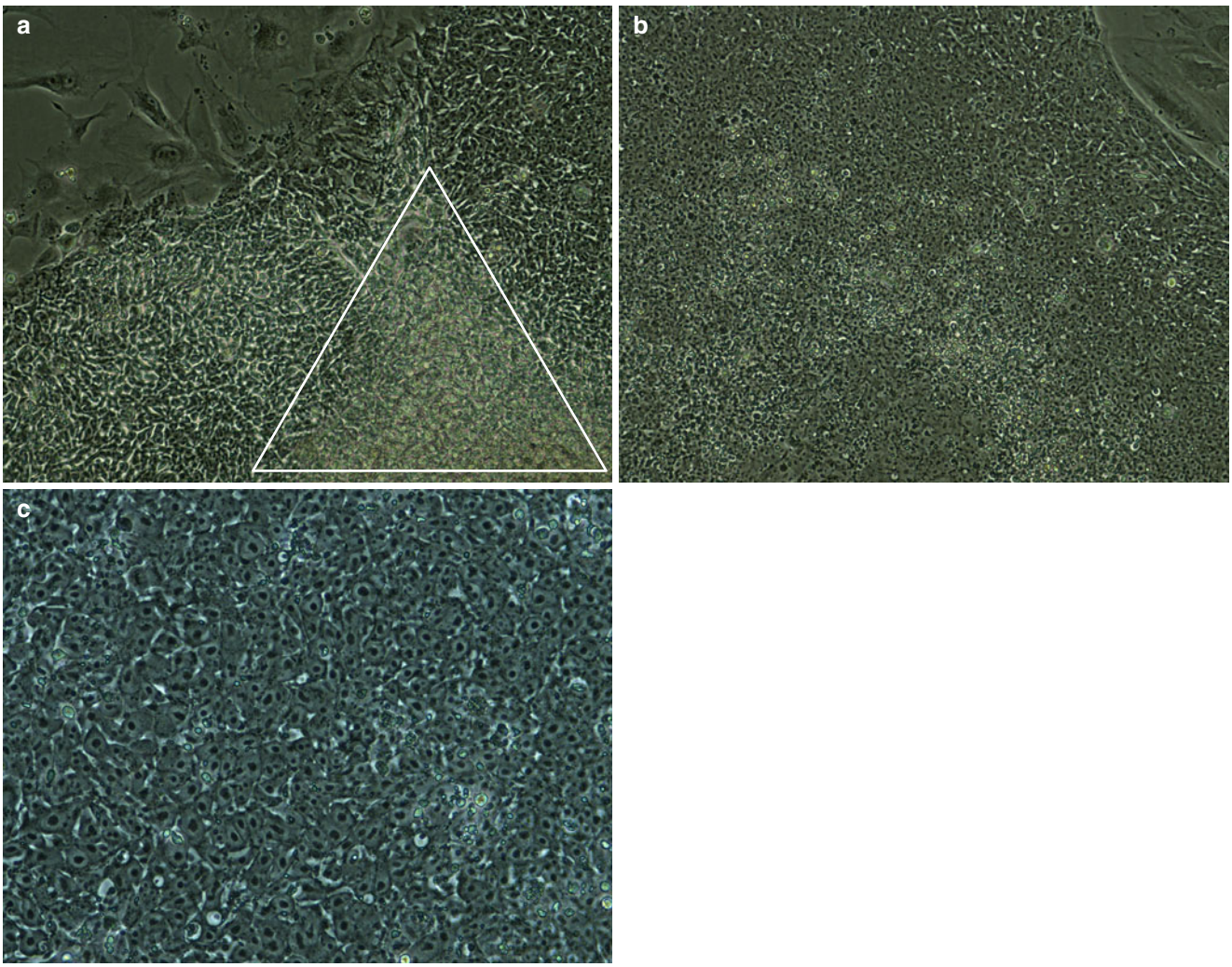

Fig. 4.29 (**a**) Example of two colonies that have merged. The cells within the larger colony (*on the right*) are compacted, and grow flat as a monolayer. The cells within the smaller colony *(on the left*) are not compacted, and the typical spaces between cells in an undifferentiated colony that has not yet compacted can be seen. Within the *triangle*, a multilayer of cells that have "piled up" can be observed, but they could still be undifferentiated. (**b**, **c**) Both of these images display a monolayer of undifferentiated stem cells within a large colony exhibiting perfect, smooth borders. These colonies are ready to be passaged (all ×10 magnification)

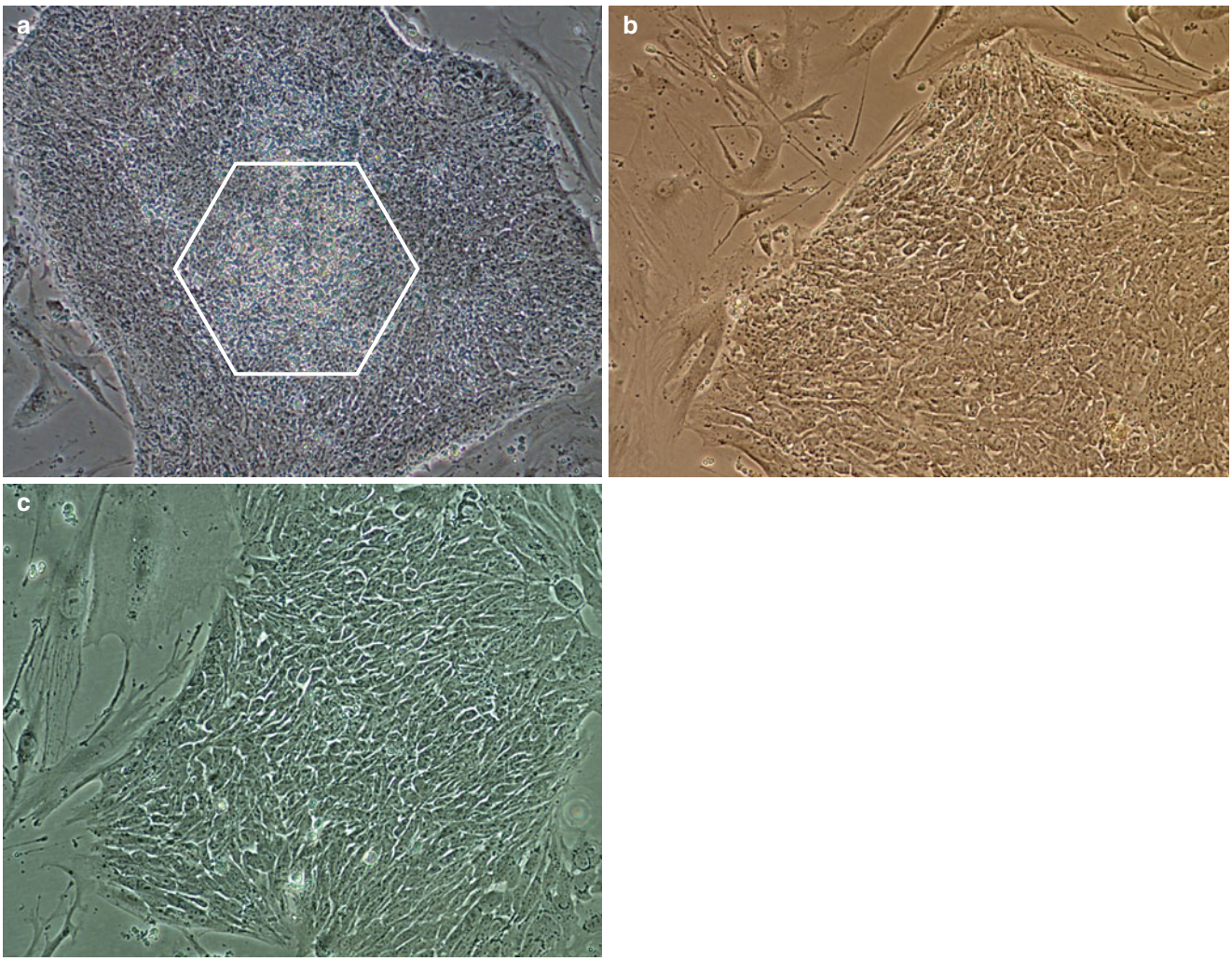

Fig. 4.30 (**a**) A compacted colony with some differentiating cells (*within the octagon*). The rest of this colony is still a monolayer, with the typical uniform morphology of hPSCs. (**b**) The cells within this colony are not yet compacted. They are actively proliferating and therefore display a slightly elongated shape. The quality of this colony during next 24 h will depend on the quality and quantity of feeders in its locality. With feeders of good quality and optimal density, the cells will remain undifferentiated. In the case of this particular colony, however, there are only a few feeder cells around it, and it is highly likely that the cells within the colony will line up and begin to differentiate during the next 24 h (similar to the cells in **c**). (**c**) A colony without defined edges but with some cells lining up. All the cells within the colony are of a different shape; some are stretched and poised to differentiate (all ×10 magnification)

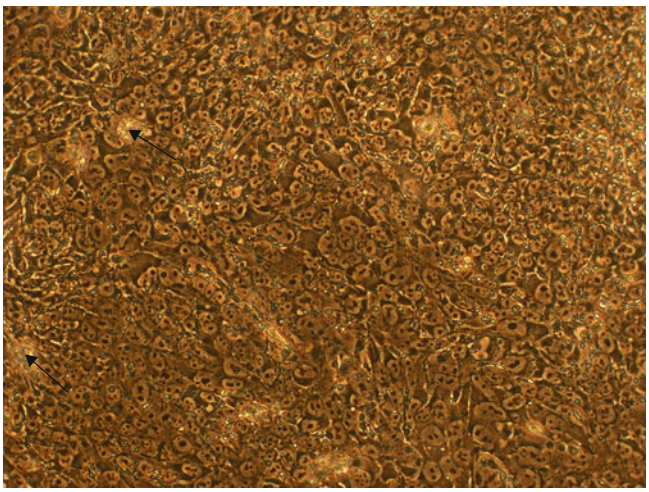

Fig. 4.31 Challenge: what can you say about this figure? See page 199 for answer

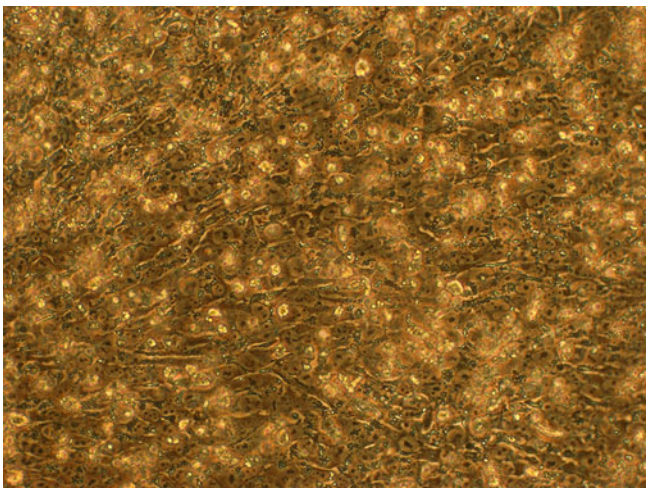

Fig. 4.32 Challenge: what can you say about this figure? See page 199 for answer

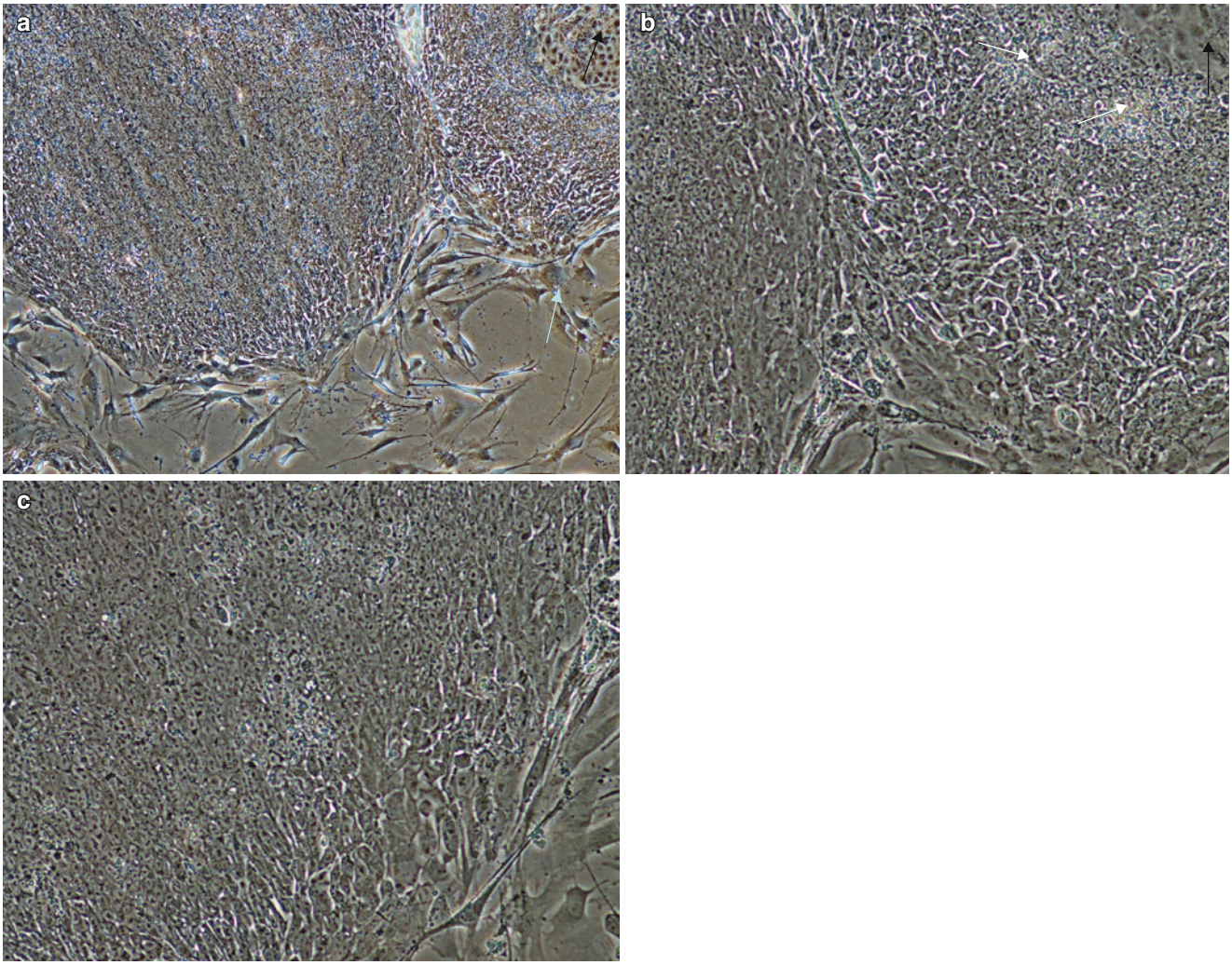

Fig. 4.33 This series of images is from two colonies merging. (**a**) The cells are still in a monolayer, but cells are lined up within the larger colony on the *left side*; the colony is no longer smooth and it is easy to see that the morphology of the cells is not perfect. The colony on the *right side* displays an area of overt differentiation (*black arrows*). (**b**, **c**) higher-magnification images of the same colonies, show that the cells are lined up. In (**b**) the smaller colony on the *right side* is differentiated in the centre of the colony (*black arrow*), and the white patches of cells are morphologically typical of the beginning of the formation of neural rosettes (*white arrows*). In (**c**) the cells appear to be compacted and normal, but looking at the overall colony morphology of this same colony in image (**a**), it is apparent that the monolayer is not smooth, and the colony is probably going to differentiate. It is important to examine cells under different magnifications in order to distinguish subtle differences between colonies (**a**, ×4 magnification; **b**, **c**, ×10)

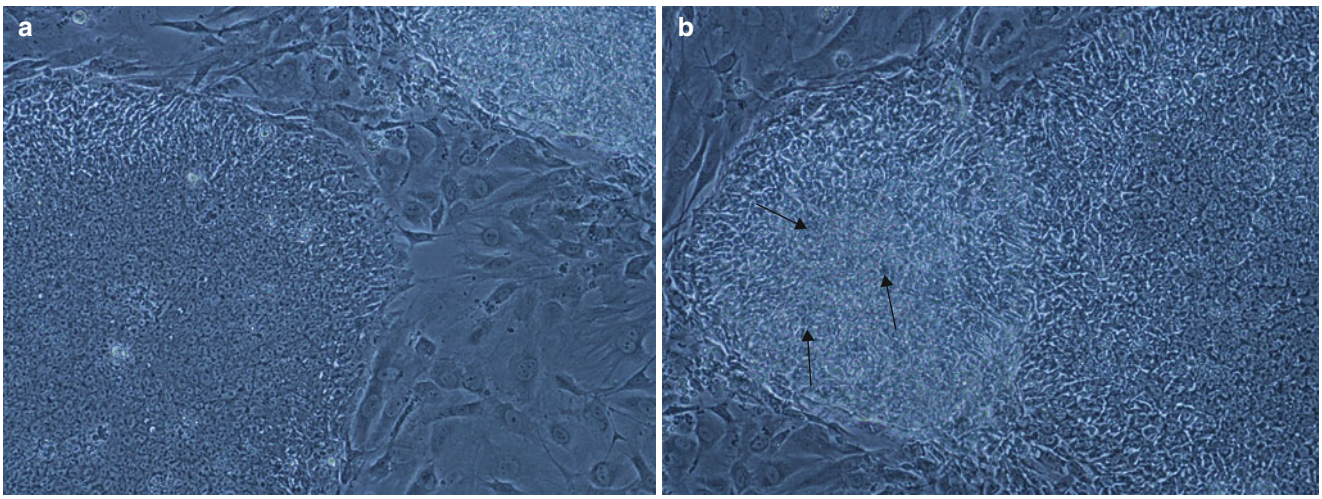

Fig. 4.34 (**a**) This image shows two colonies. In the *bottom left-hand side*, the large colony is perfectly compacted. All the cells within the small colony on the *right side* are multilayered and the cells are piled on top of each other. It is hard to determine whether they are differentiated or not. Sometimes, it is not easy to distinguish between differentiated and undifferentiated cells in this context. (**b**) These two colonies are merged. The large one on the *right* displays a good, undifferentiated morphology, but the small one on the *left* is multilayered and is forming structures that typically will develop into neural rosettes (*black arrows*) (both ×10 magnification)

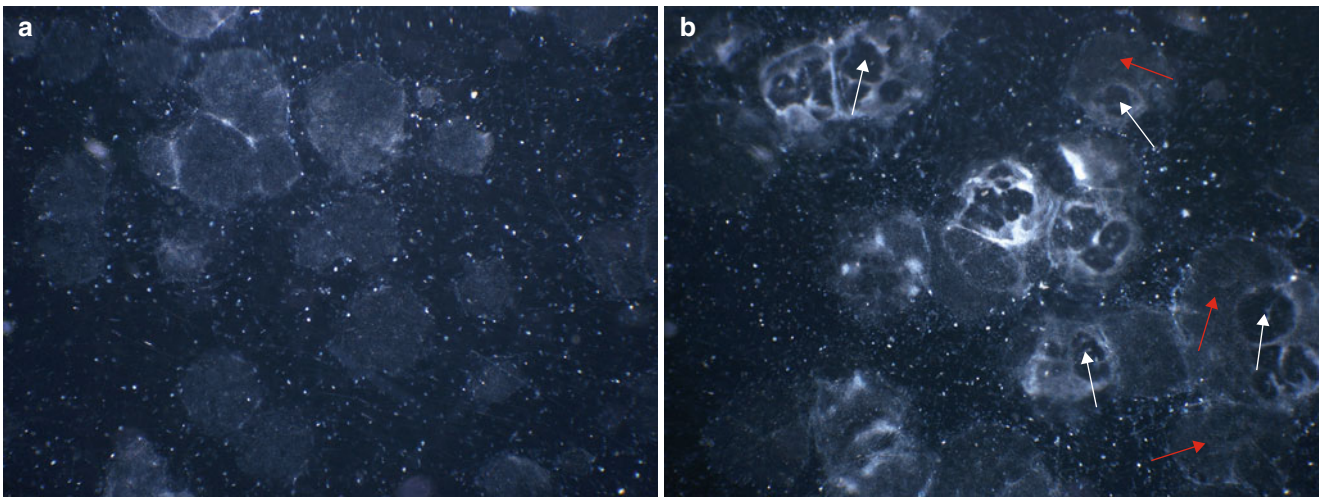

Fig. 4.35 (**a**) Dark field of hESC colonies under the dissecting microscope (×2 magnification). This image shows a good, high-density culture of undifferentiated stem cell colonies. (**b**) A high-density culture of differentiated colonies that have been in culture for more than 10 days. *White arrows* indicate the stem cell colonies. Some parts of the colonies are still undifferentiated (*red arrows*) (×2 magnification). If only this dish was available for subculture, it would still be possible to rescue the undifferentiated cells by dissecting out the parts of the colony that were still morphologically undifferentiated

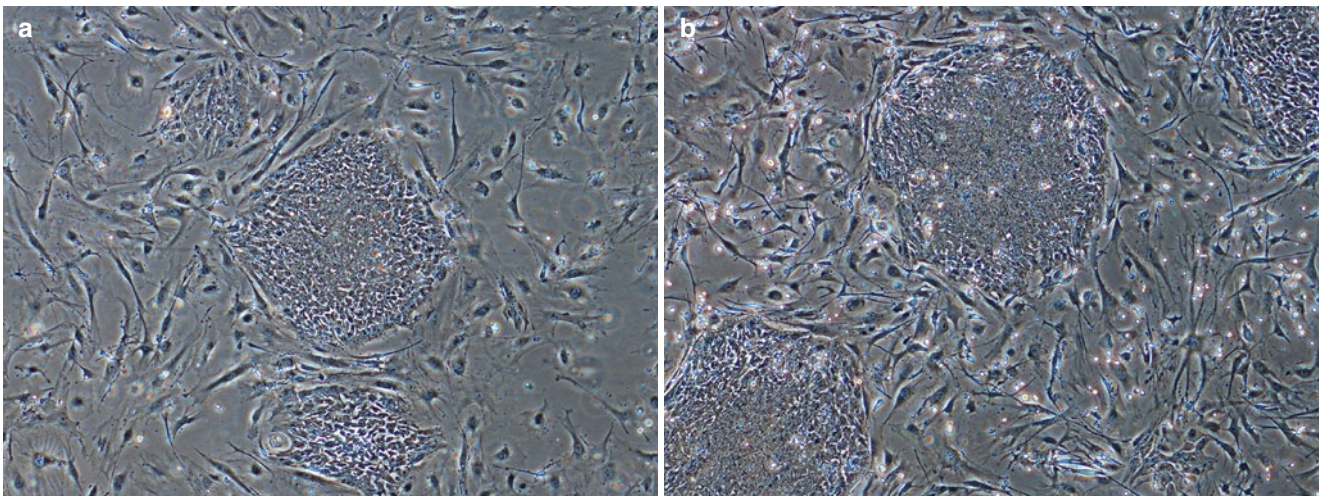

Fig. 4.36 (**a**, **b**) A relatively good culture of hESCs, 24 h after passage (both ×4 magnification)

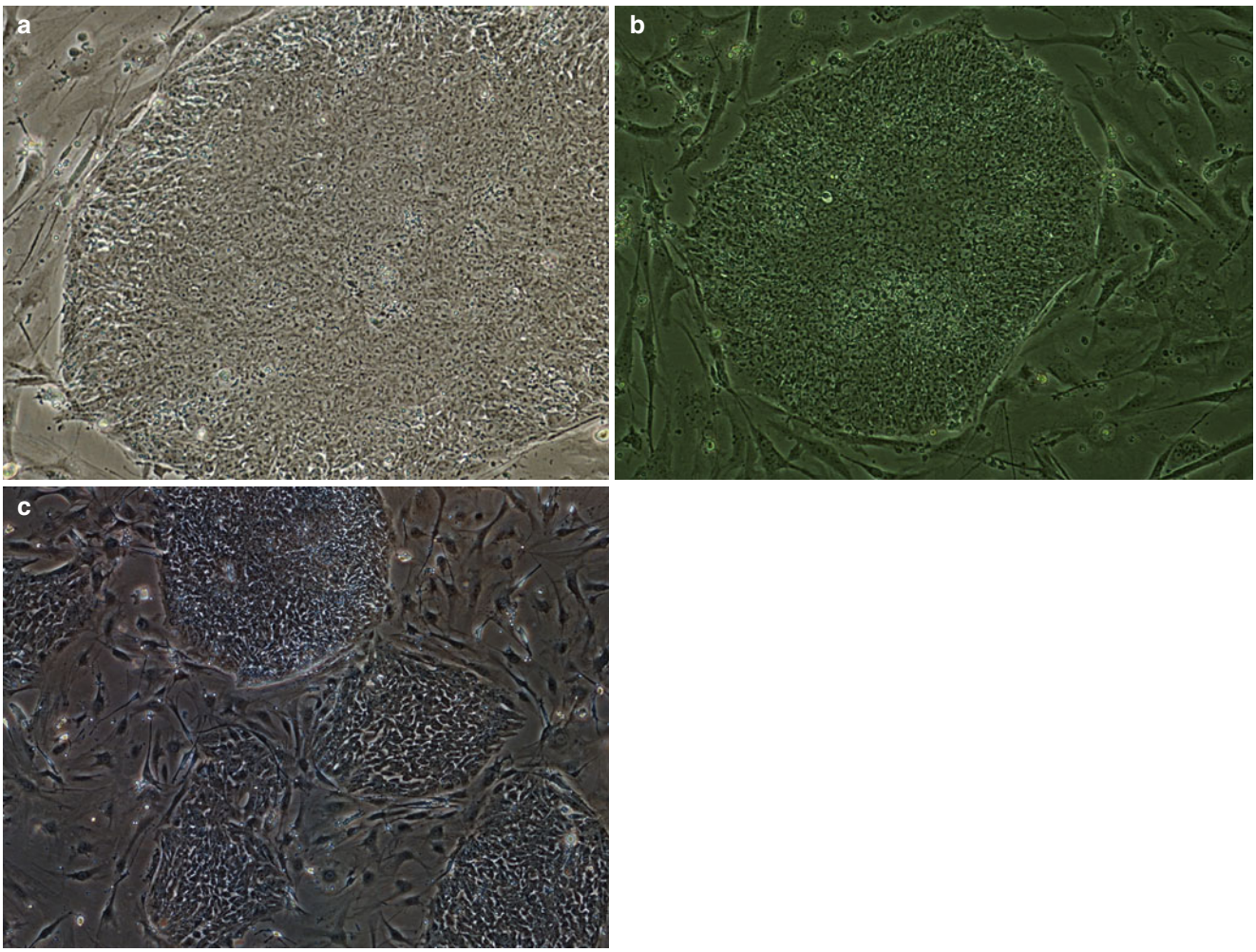

Fig. 4.37 (**a**–**c**) Good-quality colonies, 48 h after passage. Note the defined borders (all ×4 magnification)

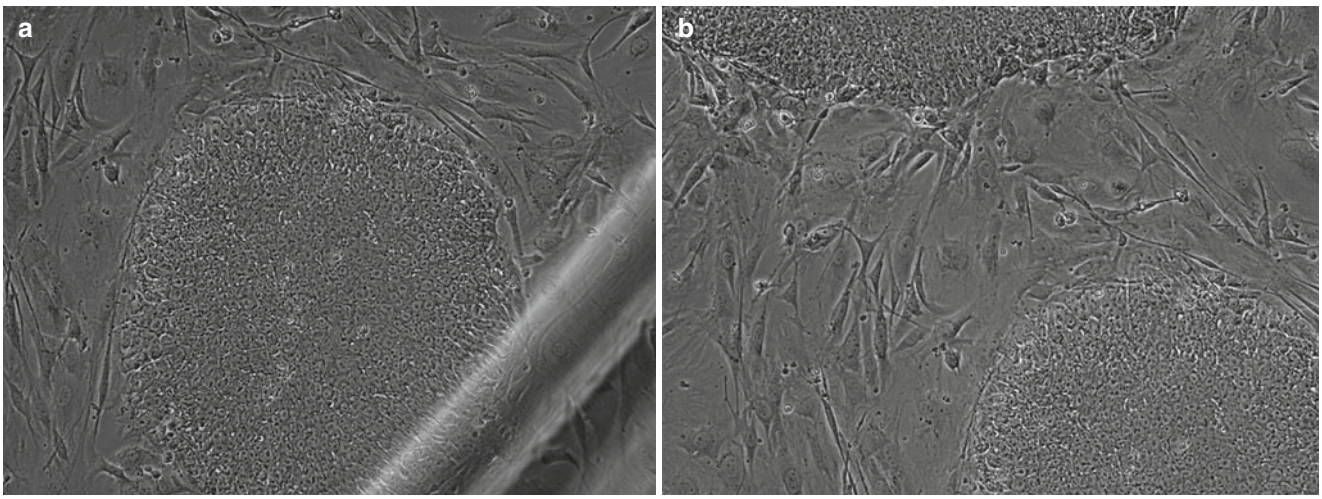

Fig. 4.38 (**a**, **b**) Good colonies growing in a single-well IVF dish. The morphology of the colony is perfect, with well-defined edges and compact cells. There is no evidence of differentiation (both ×10 magnification)

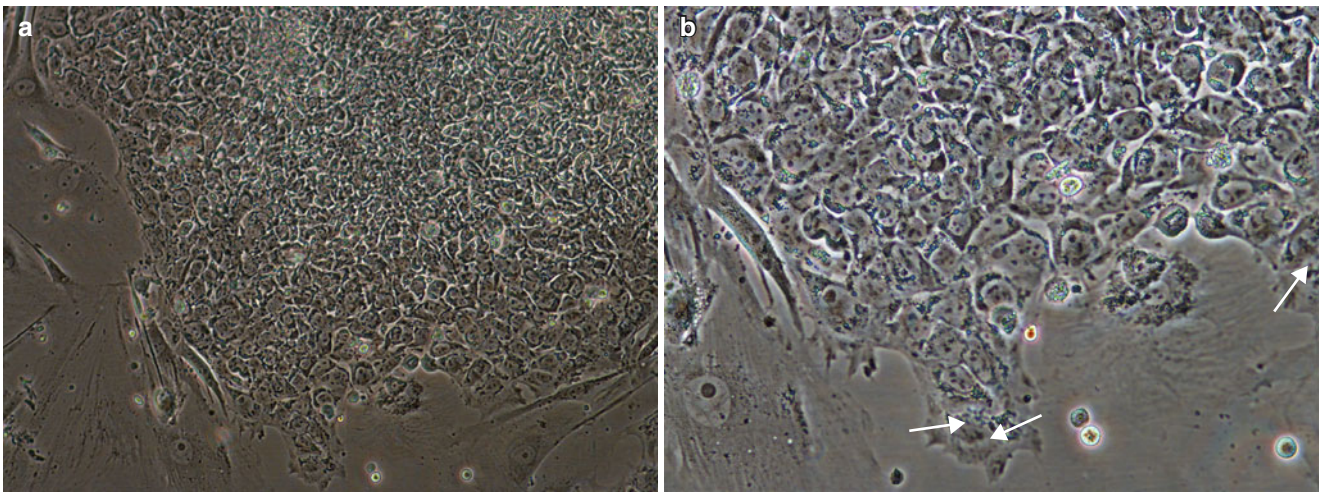

Fig. 4.39 (**a**) This colony is not perfect. It does not display smooth borders, simply because there are not enough feeders around the colony. (**b**) An image of the same colony at higher magnification. Undifferentiated stem cells with prominent large nucleoli can be seen. Cells (*white arrows*) are going to differentiate (**a**, ×10 magnification; **b**, ×20)

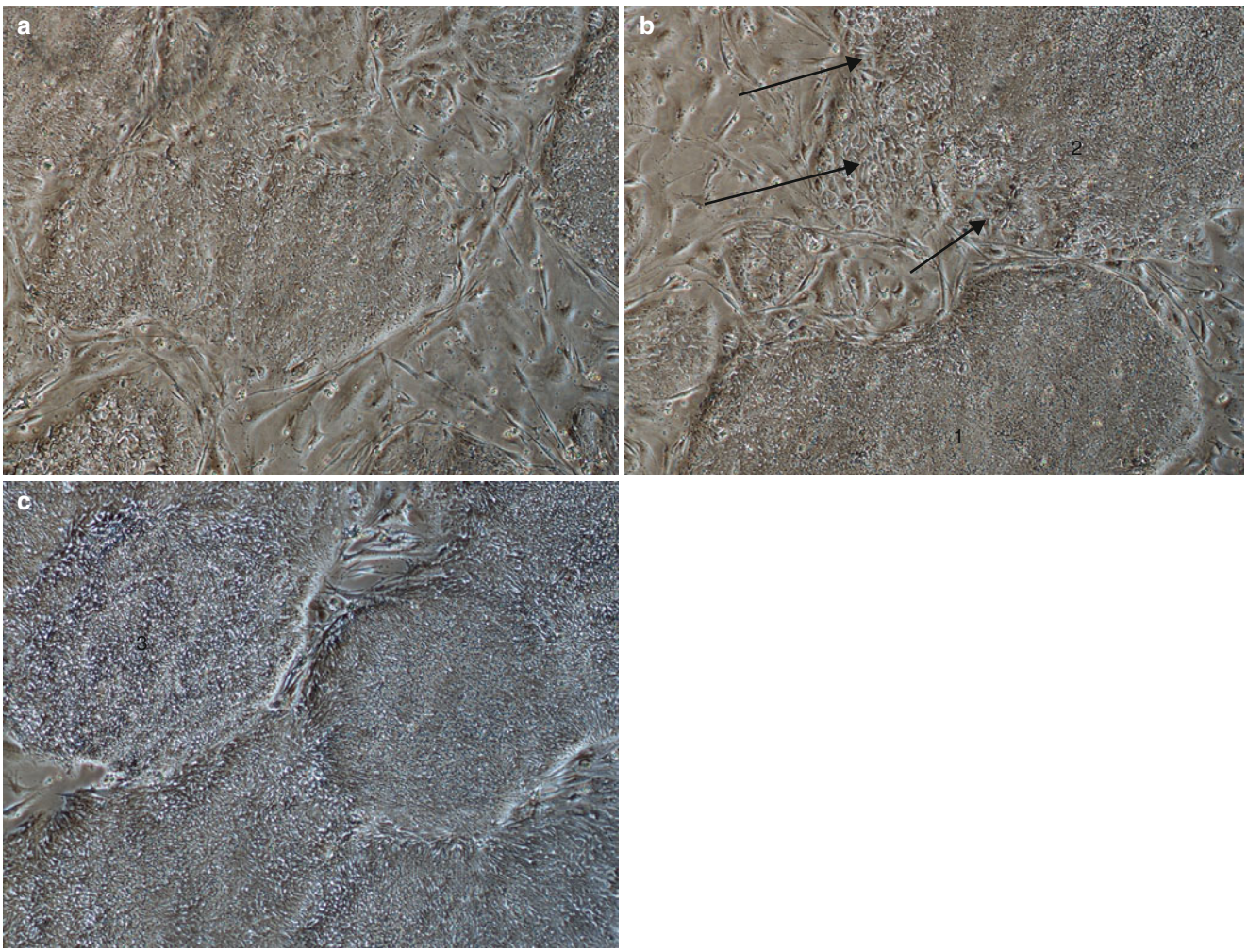

Fig. 4.40 (**a**) A high-density hESC culture that is ready to be split with a ratio of 1:3 into a new culture vessel containing feeders. (**b**) A high-density hESC culture displaying a small amount of differentiation. Colony number *1* is undifferentiated and compacted. Colony number *2* is "bumpy" (not a smooth monolayer) and exhibits differentiation around the edges (*black arrows*). (**c**) Colony *3* is not smooth and flat. It appears to be undergoing differentiation (all ×4 magnification)

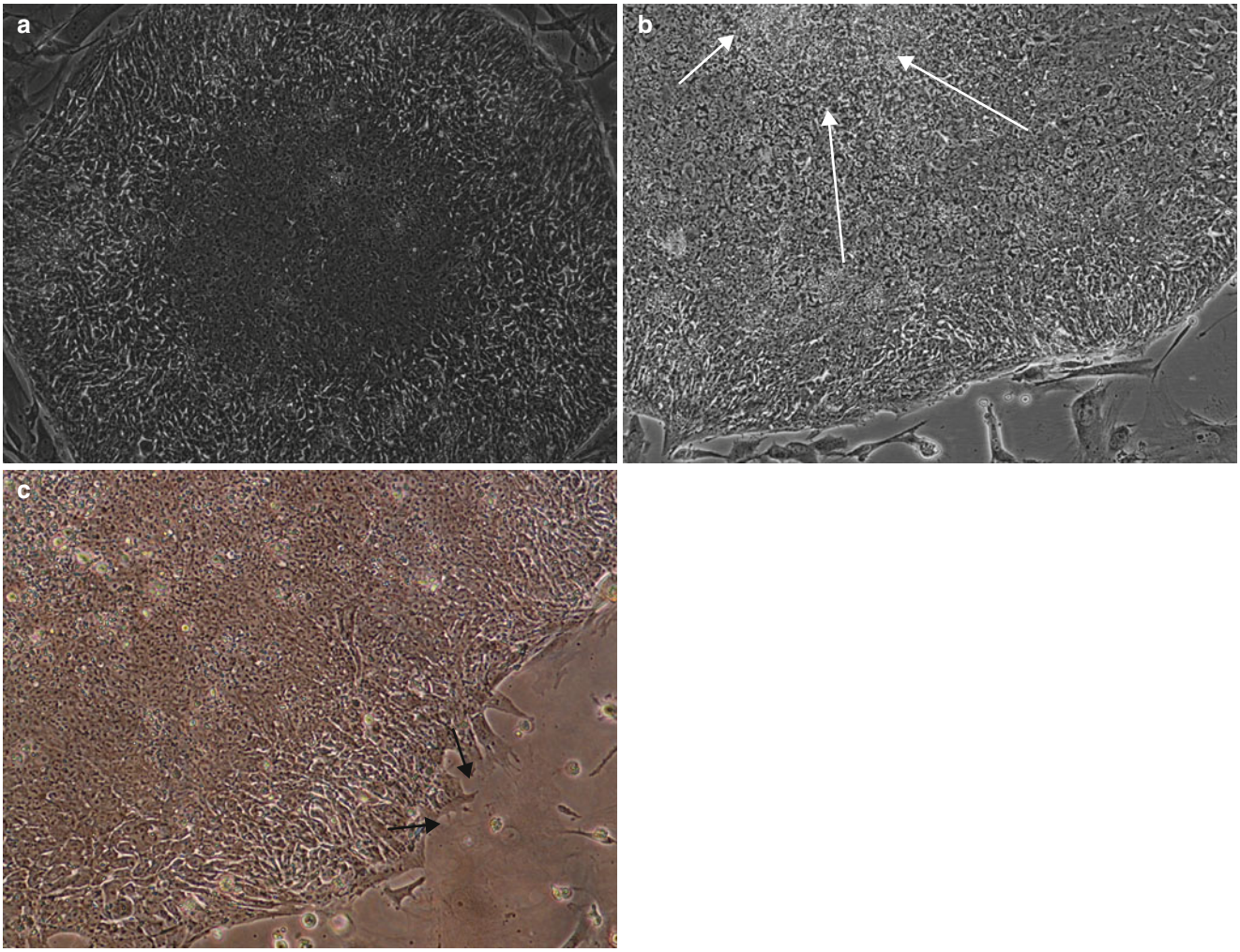

Fig. 4.41 (**a**) Good-quality colony, compacted and with defined borders. (**b**) Good-quality colony, over compacted in the centre (*white arrows*), with defined borders. (**c**) This colony is beginning to differentiate around the edges (*black arrows*), and no defined borders are observed. Typical elongated cells are seen around the perimeter of the colony. It is easy to see the differences in the morphology of the borders in images **b**, **c** (**a**, ×4 magnification; **b**, **c**, ×10)

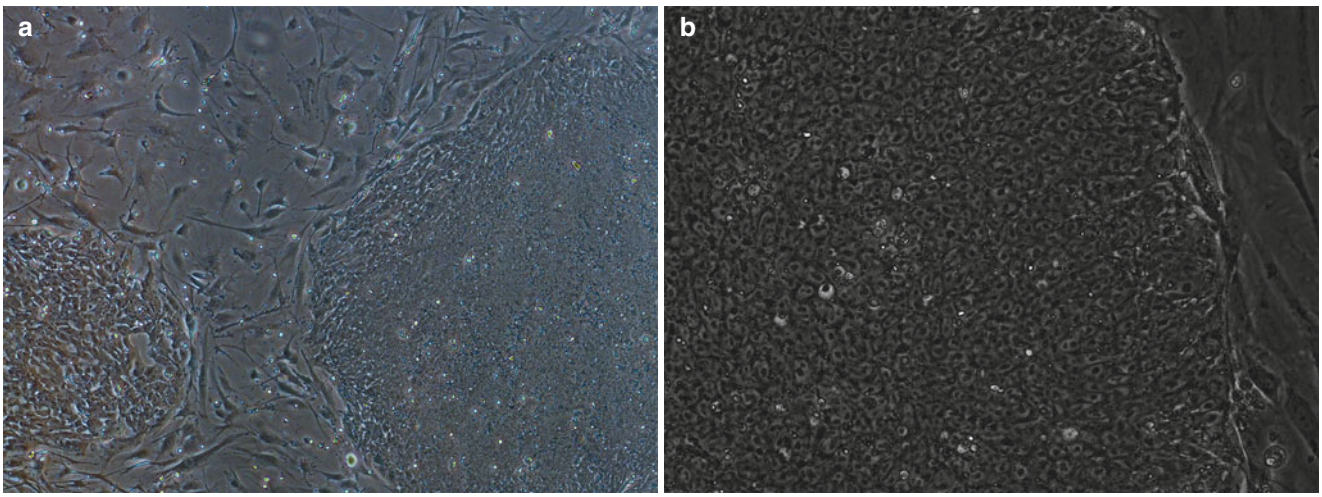

Fig. 4.42 (**a**) The colony on the *right side* is compacted, with the distinct borders around the edges of the colony. The cells within the smaller colony *(on the left*) are not compacted, they are lined up and probably going to differentiate during the next 24 h. (**b**), A large, compacted colony displaying prominent nucleoli: a good morphology for an undifferentiated colony (**a**, ×10 magnification; **b**, ×20)

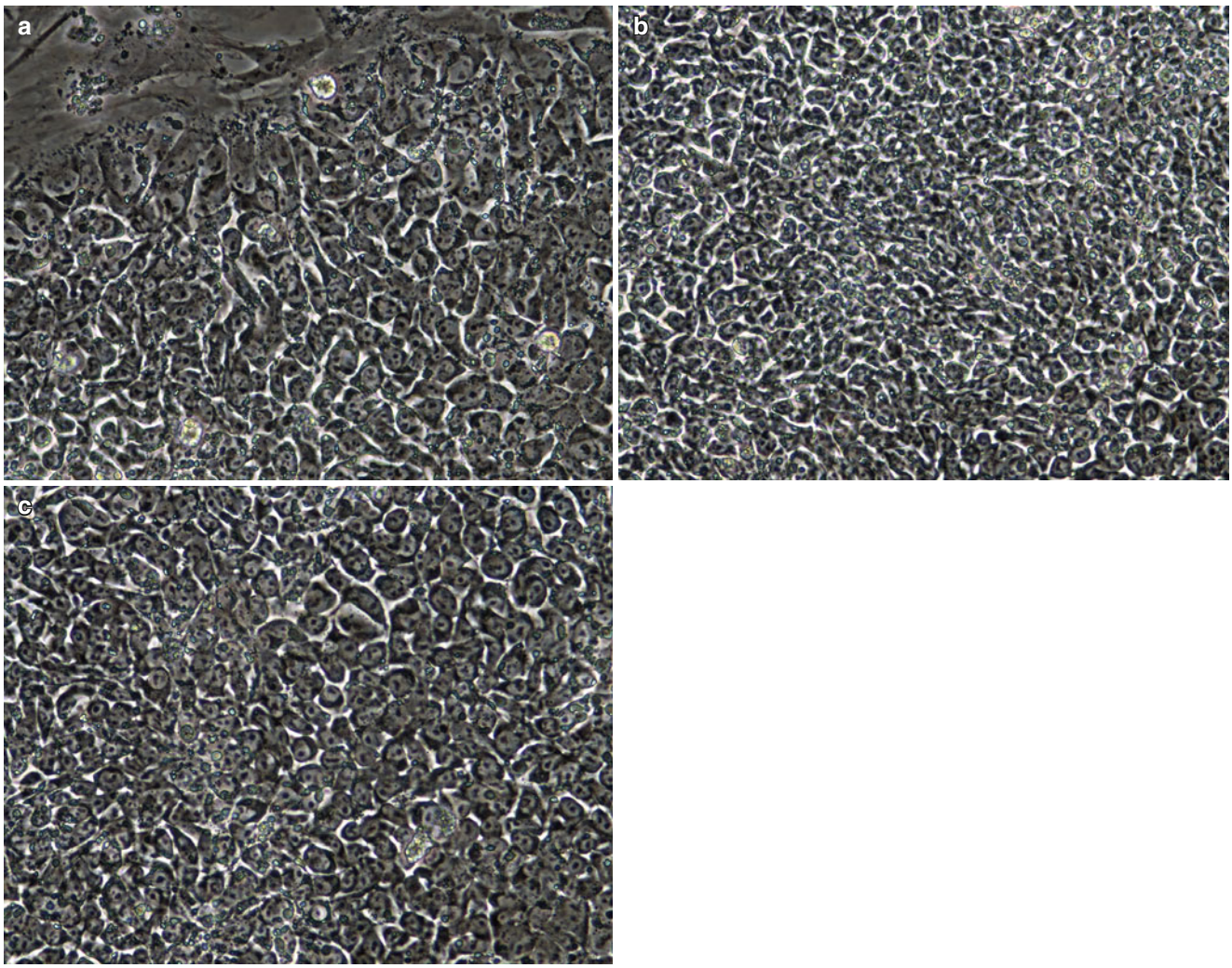

Fig. 4.43 (**a**–**c**) All three images display good, undifferentiated colony morphology with prominent nucleoli and characteristic spaces between the cells before compaction. (**a**) The edge of the colony. (**b**, **c**) The centre of the same colony (all ×10 magnification)

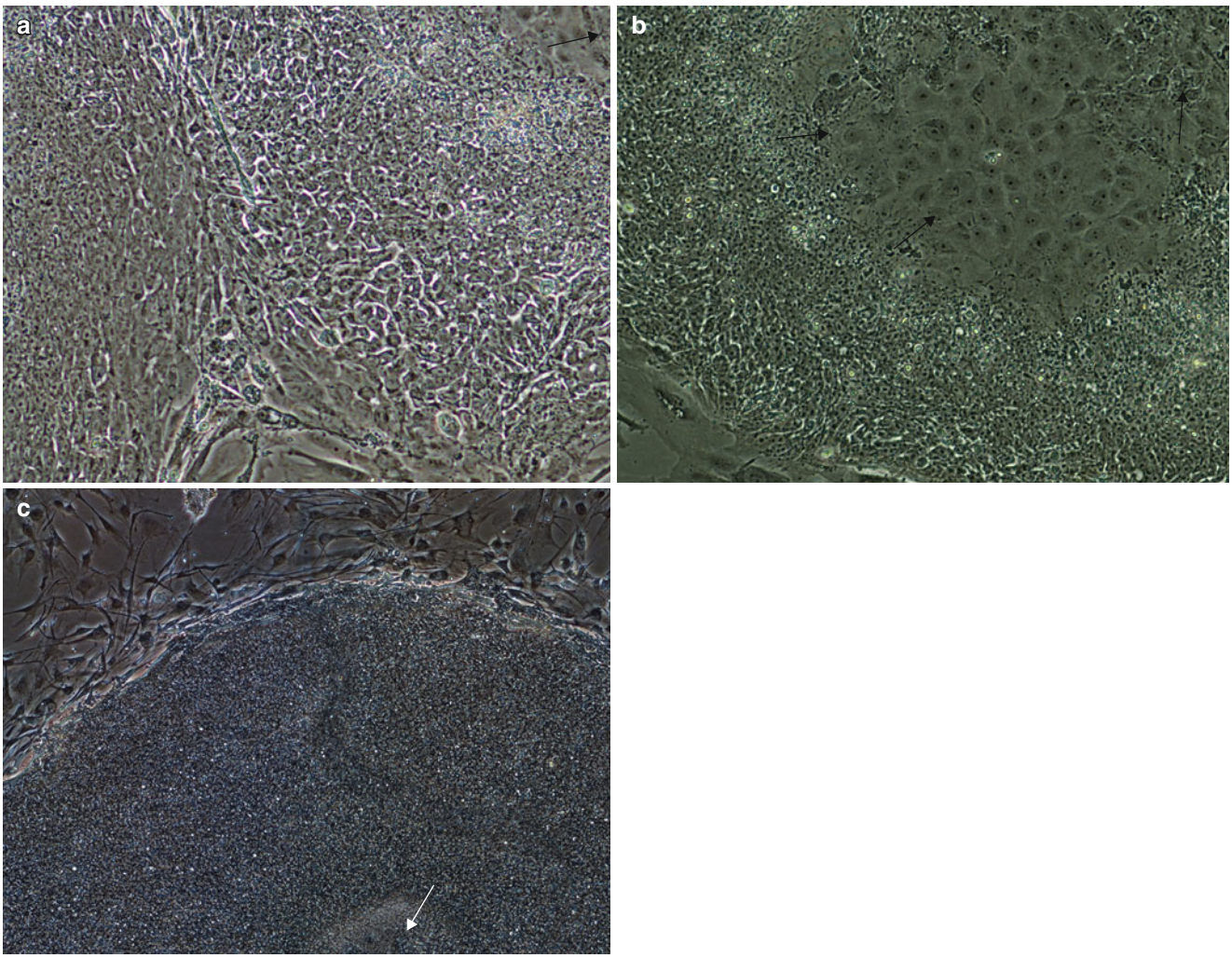

Fig. 4.44 (**a**) Example of two colonies that have merged. The colony on the right demonstrates spontaneous differentiation in its centre (*black arrow*). (**b**) This colony as well demonstrates spontaneous differentiation in its centre (*black arrows*). (**c**) This colony is 5–6 days old. It is overcompacted and multilayered, with differentiation in the middle (*white arrow*). When the density of feeders is higher than usual, sometimes the feeders do not allow the colony to spread and form a good, flat monolayer (all ×10 magnification)

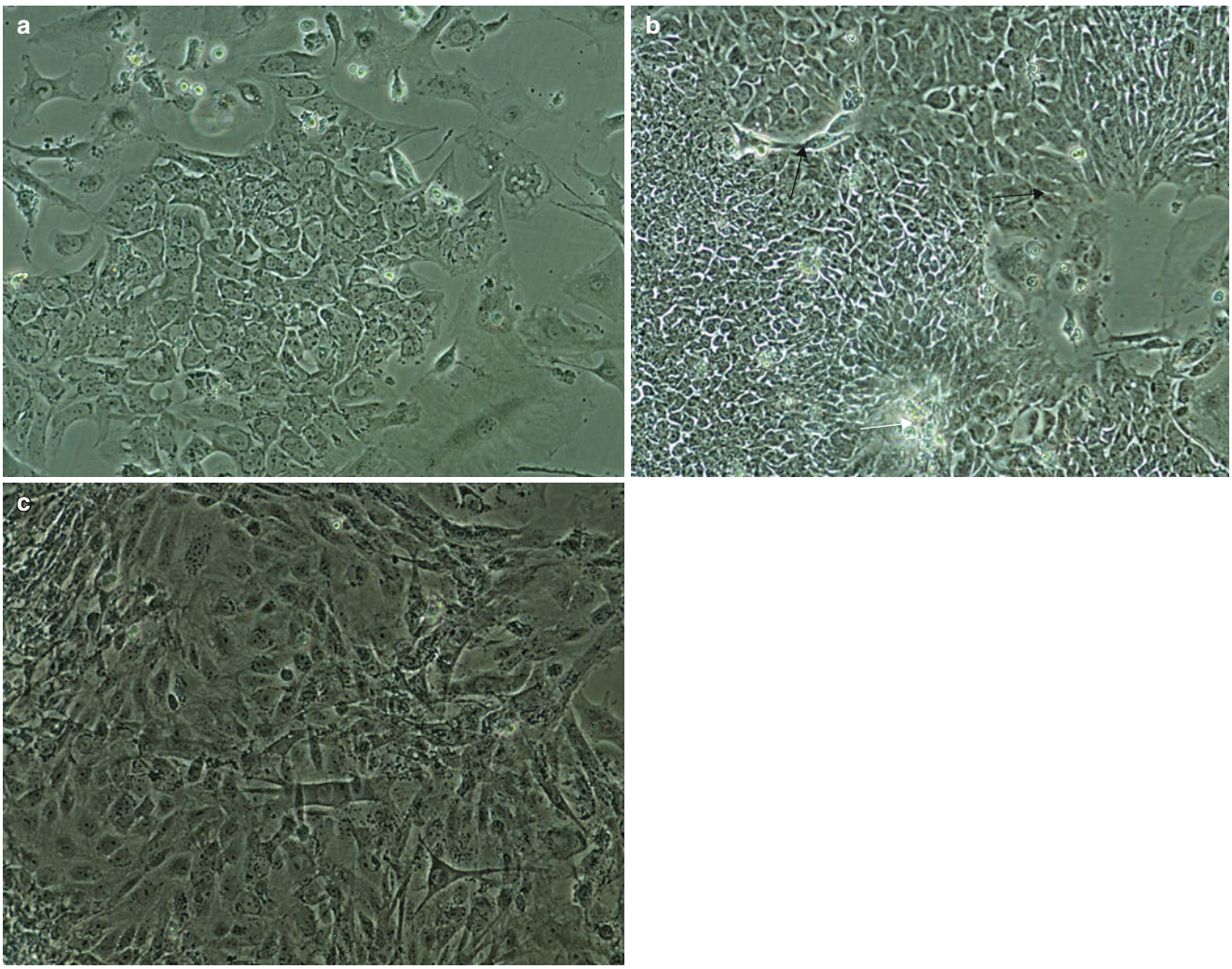

Fig. 4.45 (**a**) This colony has not yet compacted, and it lacks smooth, defined borders. The cells within the colony are of different shapes and sizes. There are not enough feeders around to support the colony in an undifferentiated state. (**b**) This overgrown, high-density hESC culture shows spontaneous differentiation (*black arrows*) and the formation of a rosette-like structure (*white arrow*). (**c**) A differentiated colony. Note the difference in morphology of the individual cells compared with those in **a** (**a**, ×10 magnification; **b**, **c**, ×20)

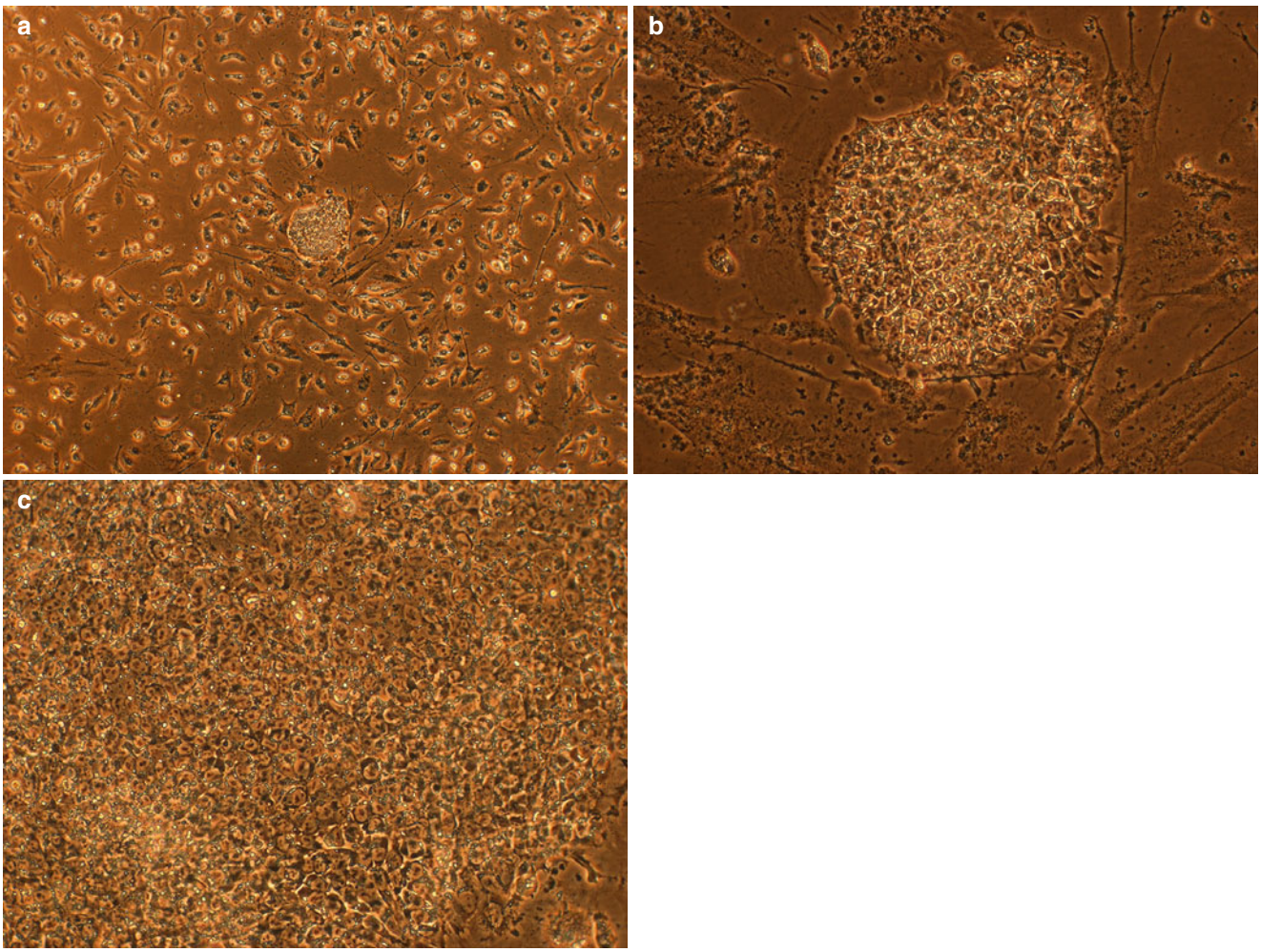

Fig. 4.46 (**a**–**c**) Images of a single colony at different magnifications. This colony is small, compacted, and stressed, and the cells within the colony are looking disorganised. It is hard to see the clear typical morphology of cells and large nucleoli. The reason for the condition of this colony is that the feeders around it are of extremely poor quality. They are not spread out, and there are abundant apoptotic cells. Remember that only good-quality feeders produce good-quality stem cell cultures (**a**, ×4 magnification; **b**, ×10; **c**, ×20)

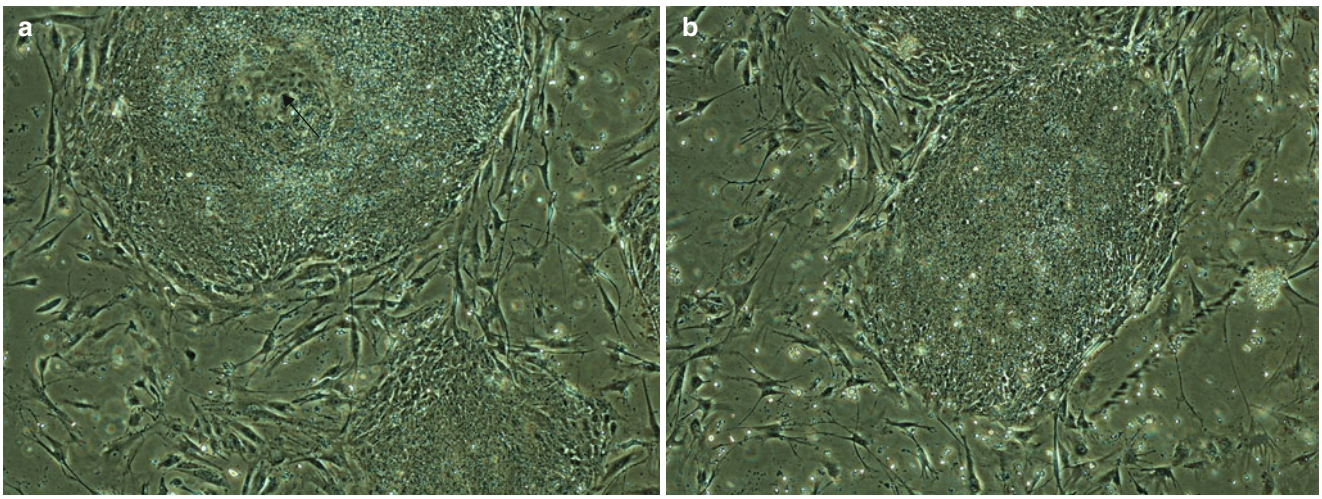

Fig. 4.47 (**a**) The large colony on the top left-hand side is differentiated in the middle (*black arrow*). (**b**) These two merged colonies are of relatively good quality. They are compacted, with a few elongated cells within the border. These cultures should be passaged immediately, as the feeders are too old and their density is too low to support undifferentiated cells. The culture is not optimum (both ×4 magnification.) (*See* Chap. 2 for comparisons of feeders that are old and new, good and bad.)

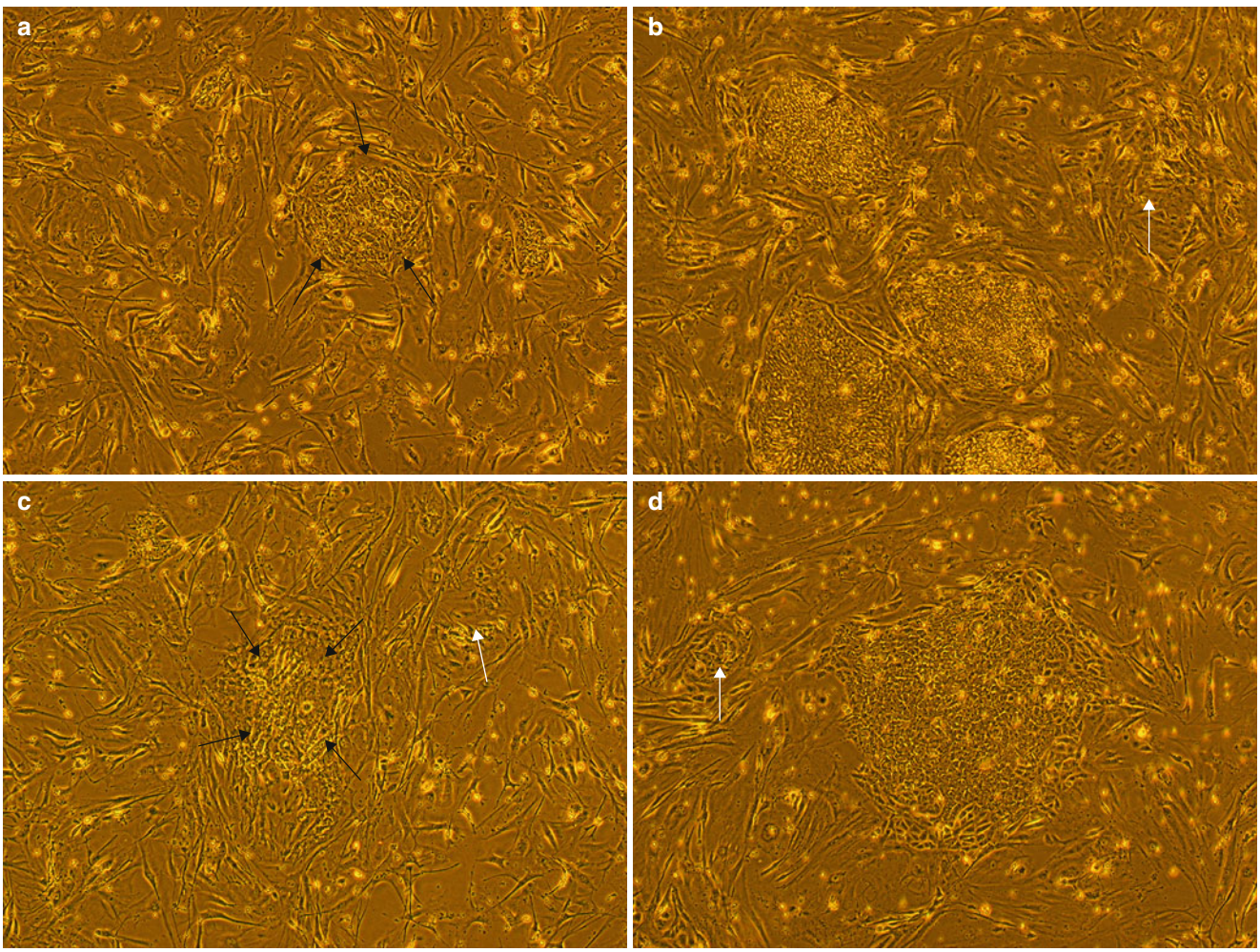

Fig. 4.48 (**a**–**d**) Images representing a 24-h culture of hESC. In all four pictures, small differentiated colonies (*white arrows*) are visible. The feeders are not at their best because there are too many apoptotic cells. (**a**) The colony in the centre (*black arrows*) is quite fragile. It is small and its cells are heterogeneous in size. The colony is not compacted. (**b**) These colonies are stressed and small, but they could still make a good culture. Quite a few apoptotic cells are present. (**c**) This hESC colony spontaneously differentiated (*black arrows*). (**d**) This larger colony shows a relatively good monolayer of flat cells but lacks defined borders (all ×4 magnification)

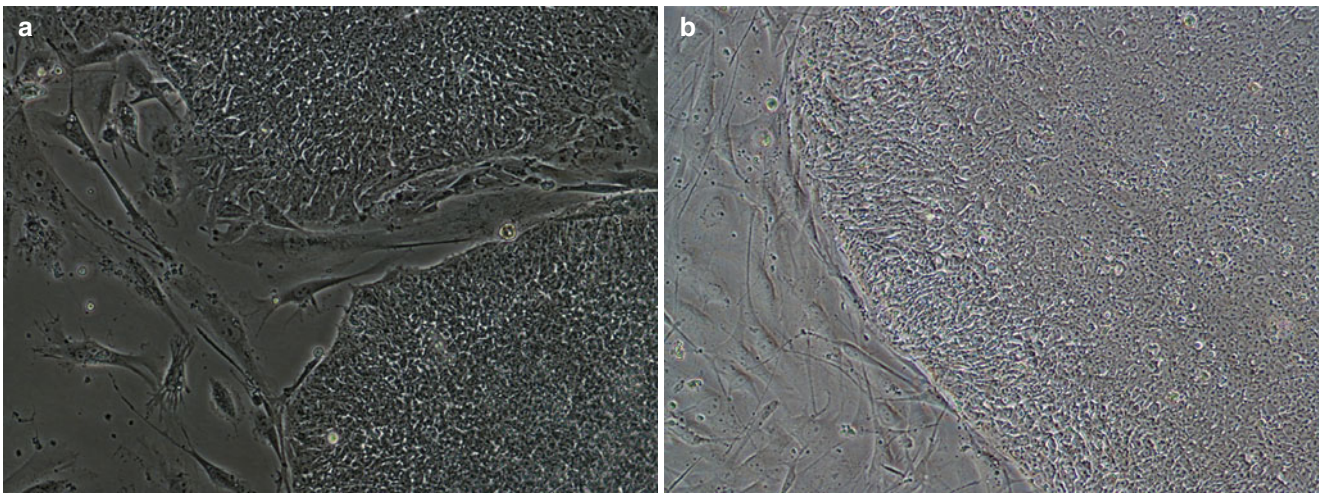

Fig. 4.49 Challenge: what can you say about this figure? See page 199 for answer

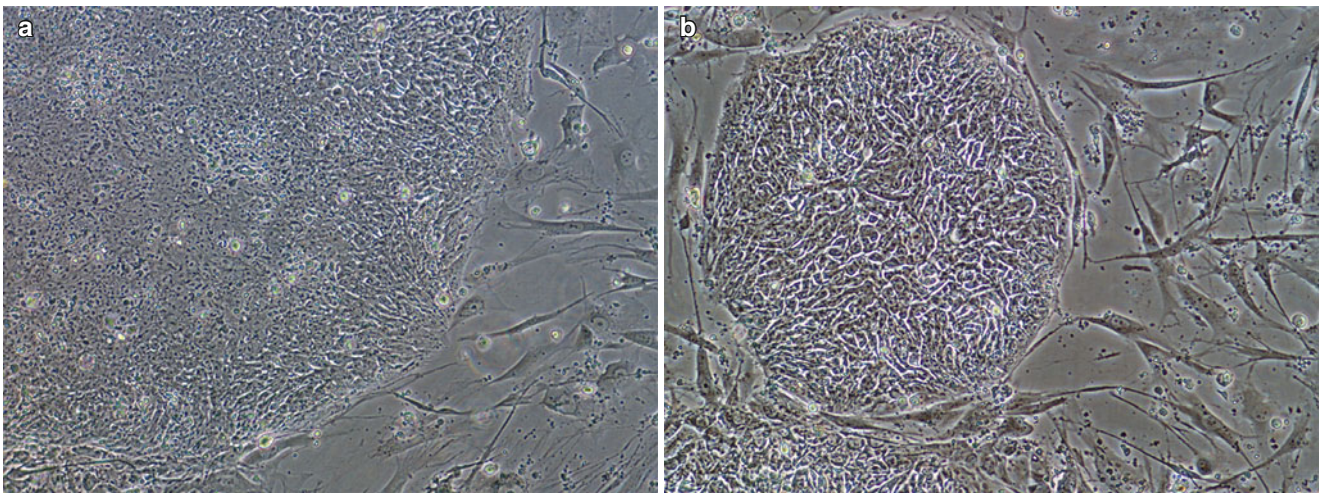

Fig. 4.50 Challenge: what can you say about this figure? See page 199 for answer

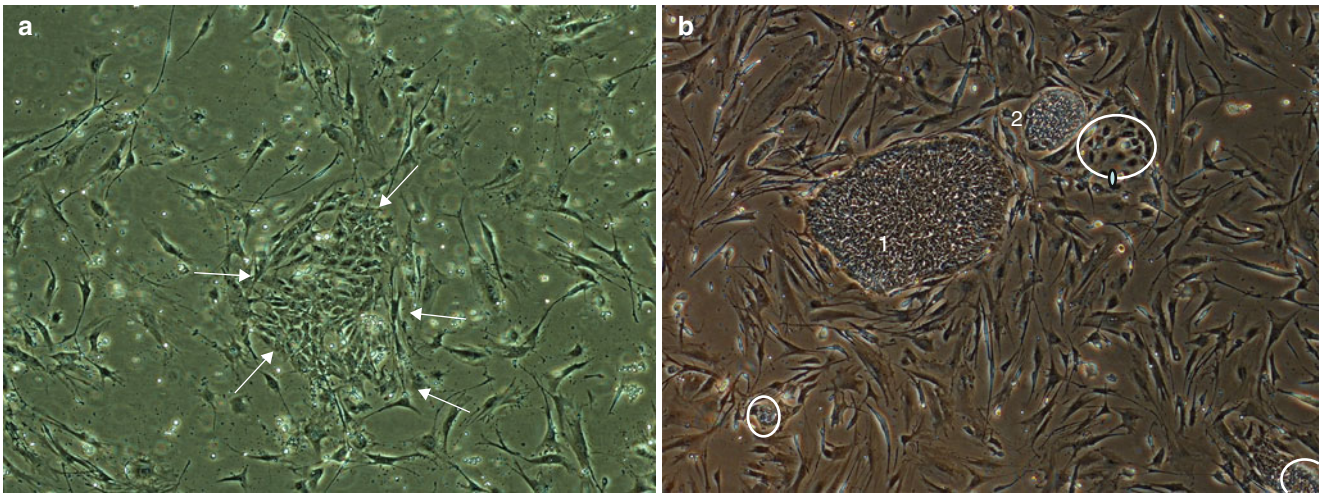

Fig. 4.51 (**a**) The hESC colony (*white arrows*) has spontaneously and completely differentiated. All of the cells are of different shapes and the colony has no defined borders. (**b**) Colony *1* is large and compacted. Colony *2* is small and compacted; it has not yet started to spread and flatten out. The colonies shown within *white circles* are too small to be maintained undifferentiated (both ×20 magnification)

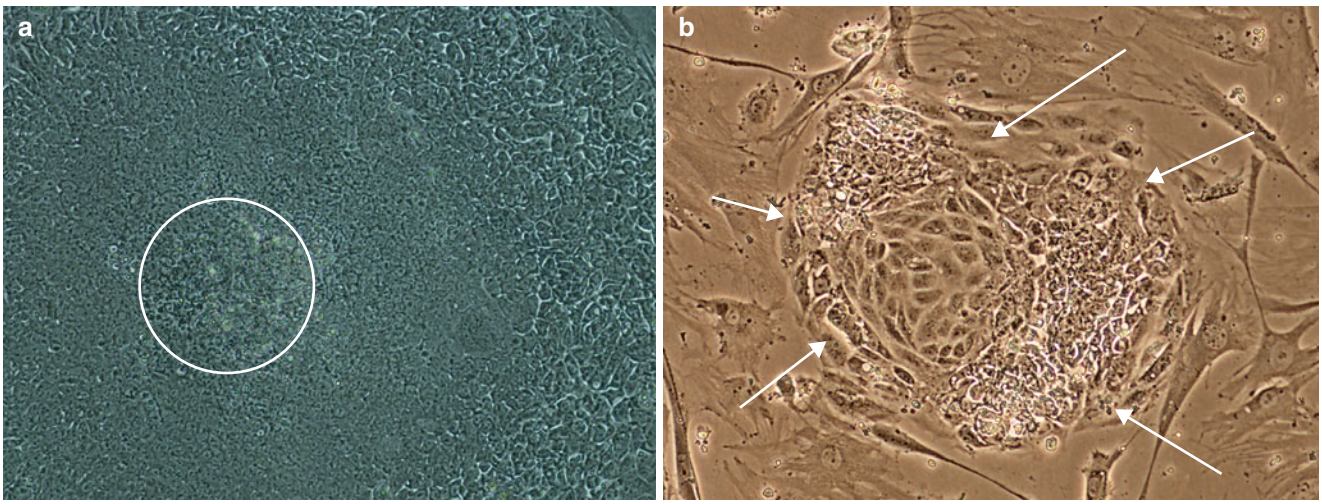

Fig. 4.52 (**a**) A large, compacted colony with good morphology, which is ready to be passaged. A small area of differentiation is visible within the *white circle*. (**b**) This colony is totally differentiated (*white arrows*) (both ×10 magnification)

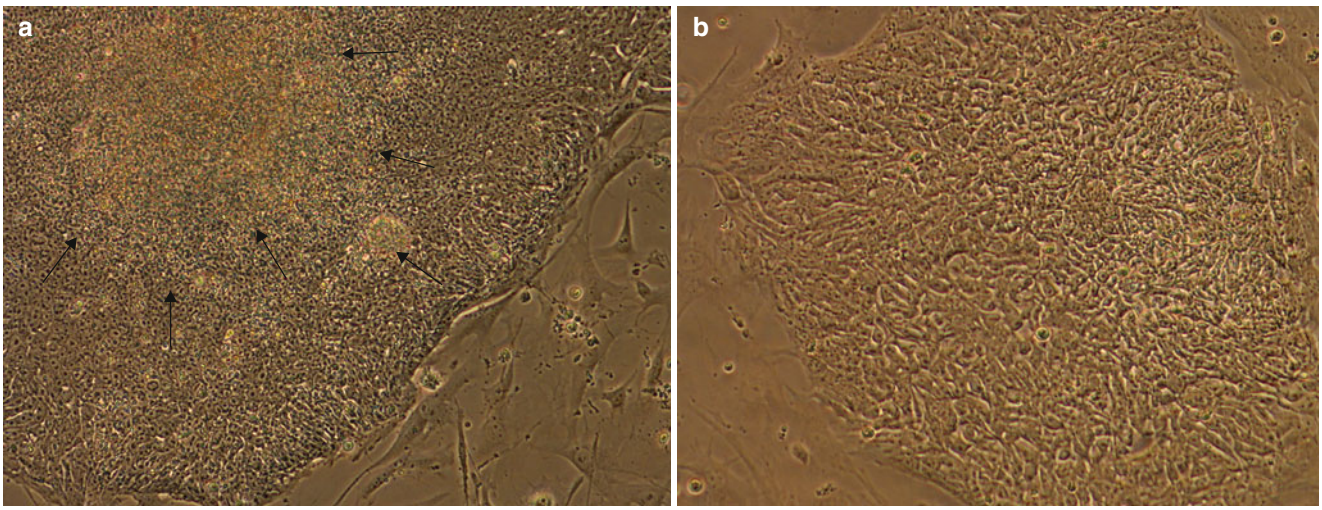

Fig. 4.53 (**a**) This colony is overcompacted, exhibiting smooth, defined borders; it is starting to differentiate in the centre (*black arrows*). (**b**) This colony with a disorganised morphology will differentiate next 24 hours (both ×10 magnification)

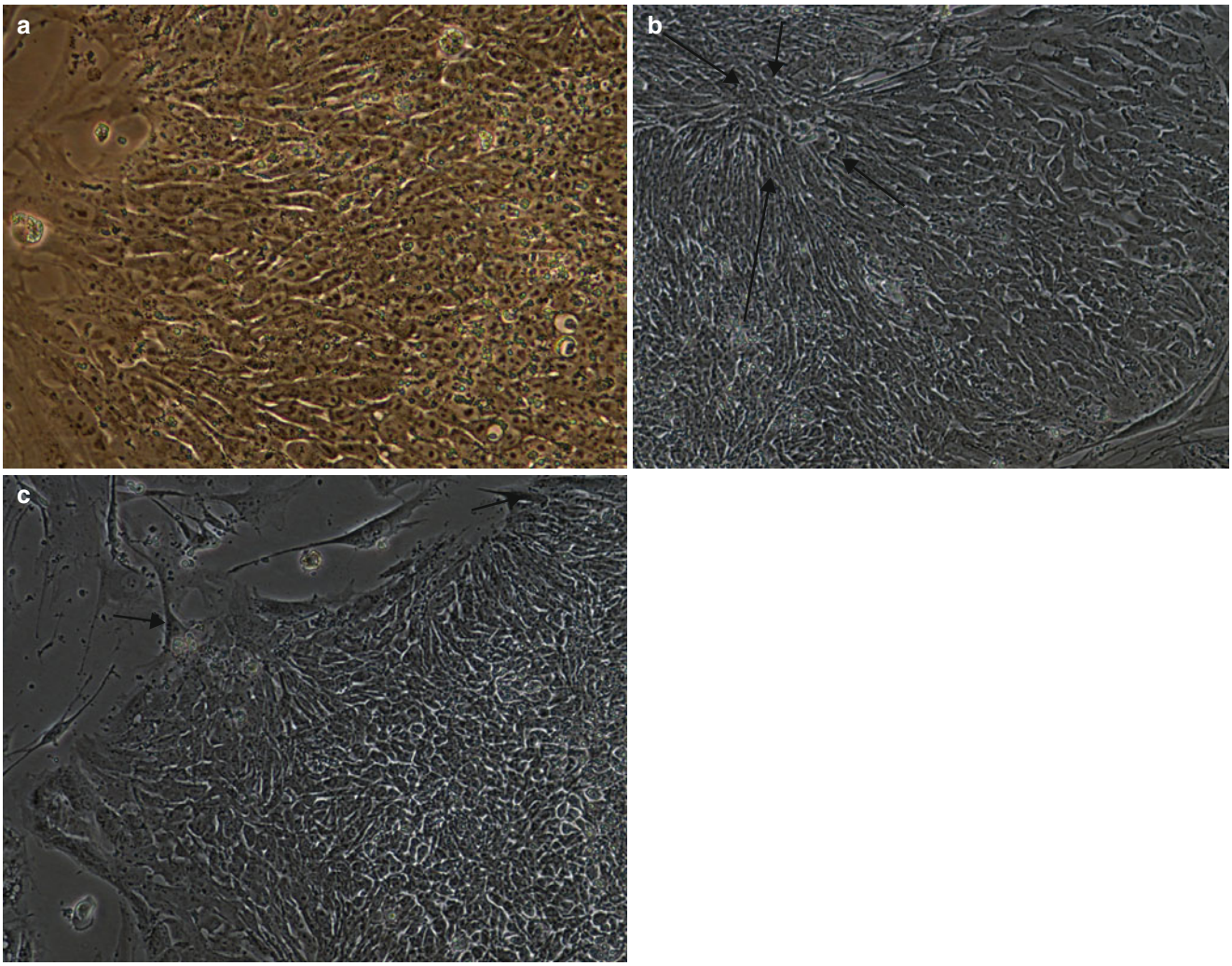

Fig. 4.54 (**a**, **b**) Examples of colonies containing elongated cells. (**b**) is forming a rosette (*black arrows*). (**c**) A colony with a disorganised morphology, which is beginning to differentiate (all ×10 magnification)

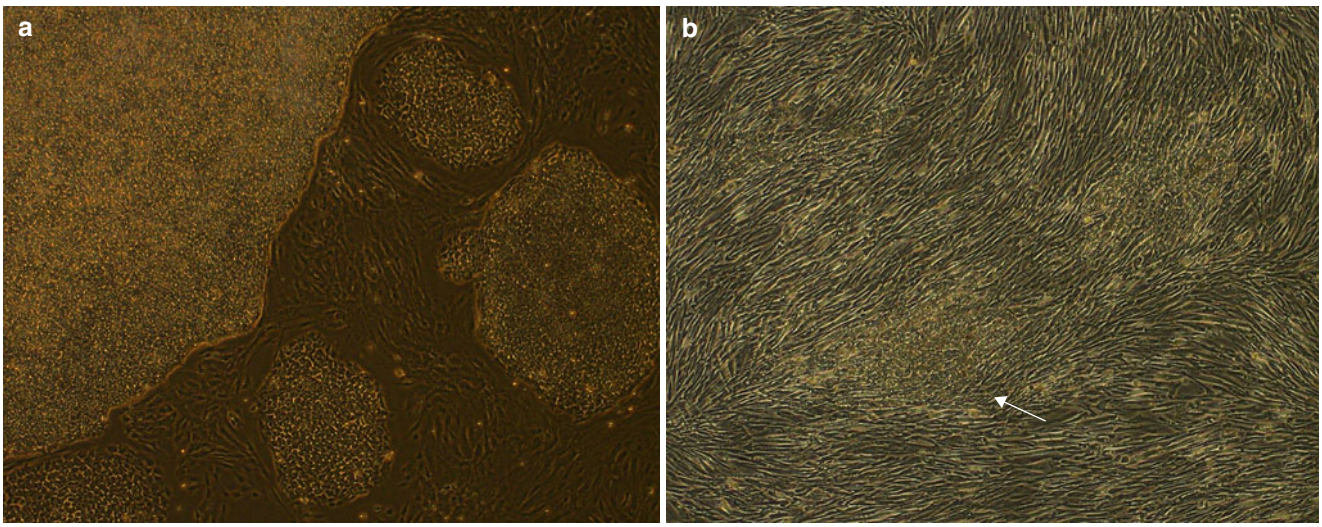

Fig. 4.55 (**a**) Colonies of hESCs growing on low-density human feeders. The colonies display an ideal undifferentiated morphology—that is, they are compact colonies with well-defined edges. (**b**) A 2-day-old human hESC colony (*white arrow*) developing on high-density human feeders. The high-density feeders restrict the expansion of the cells, which quickly display a compact colony morphology (both ×4 magnification)

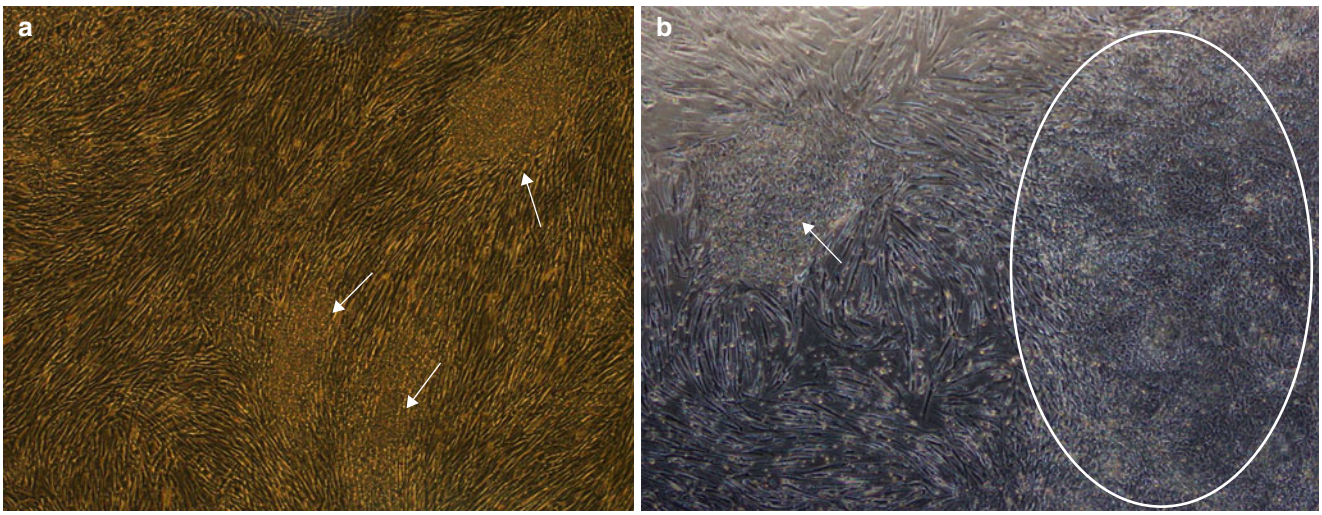

Fig. 4.56 (**a**) Colonies of Day 3 hESCs (passaged enzymatically) growing on high-density human feeders. Again, note the compact colonies emerging. In general, it takes longer for colonies to emerge and be detected in cultures where the cells have been enzymatically dissociated and passaged than when the cells are manually dissected and passaged, because in the latter case, fragments containing many cells are passaged, whereas with enzymatic dissociation the cells are treated to form small clumps (or even single cells) prior to passaging onto fresh feeders, so it takes longer for the cells to form identifiable colonies. (**b**) These colonies have been growing on human feeders for 5 days. There is a colony displaying good morphology, with compact undifferentiated cells (*white arrow*), and a colony with a number of areas of differentiation and undefined borders (*in the oval*). After removal of differentiated areas from the culture, these cells could be passaged onto fresh human feeders (both ×4 magnification)

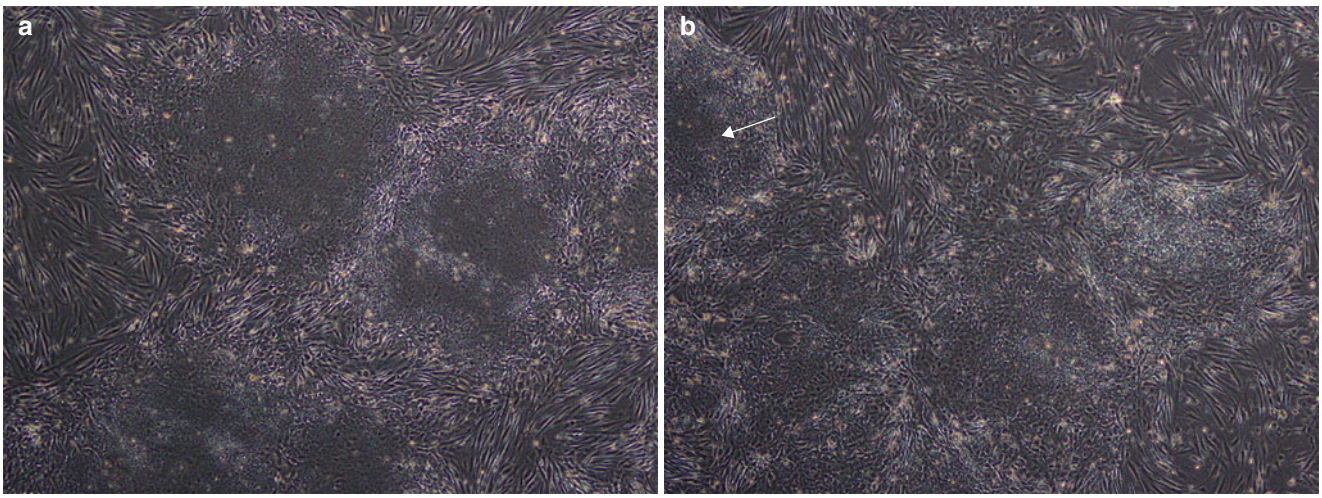

Fig. 4.57 (**a**, **b**) The colonies in these images have been enzymatically treated, passaged at a low split ratio, and have been growing on human feeders for 5 days. There are colonies in both images that have fused together. (**a**) Colonies that are compact can be seen, with some differentiation around the edges of the colonies. (**b**) The majority of one colony (*white arrow*) is compact and undifferentiated, but the rest of the colonies show a disorganised morphology with heterogeneous cell types typical of differentiation (both ×4 magnification)

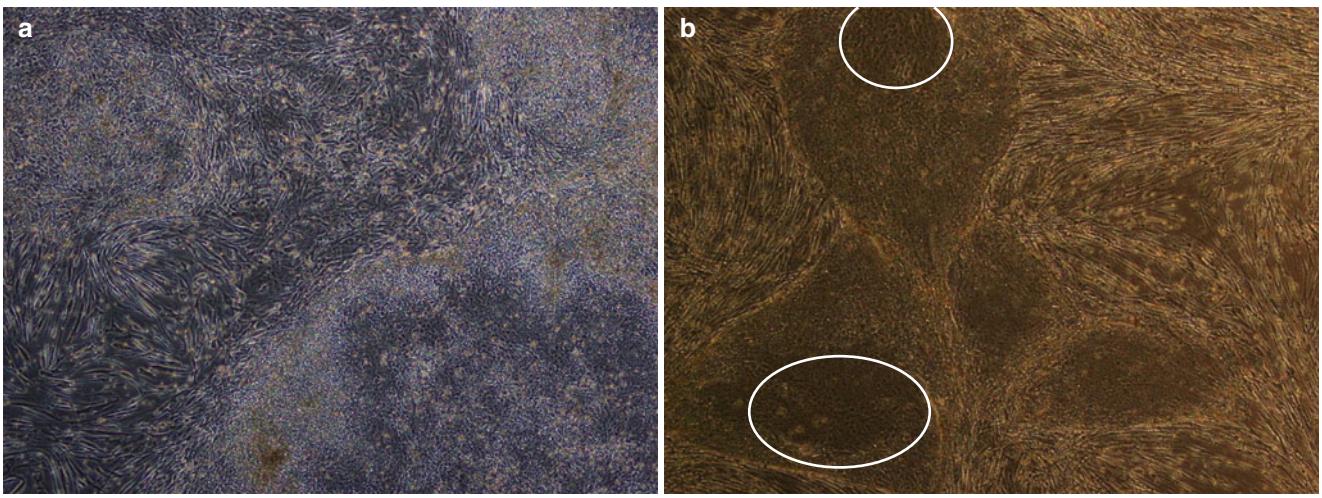

Fig. 4.58 (**a**) These colonies, growing on human feeders, are 7 days old and are derived from enzymatically treated cells. The cells have begun to pile on top of each other and are mostly disorganised and differentiated. (**b**) This image shows manually passaged hESC growing on human feeder cells. This culture is 10 days old. A number of differentiated areas are seen in the colonies (*ovals*); in other areas, the cells are still compacted and undifferentiated. The human feeders look old and granular (both ×4 magnification)

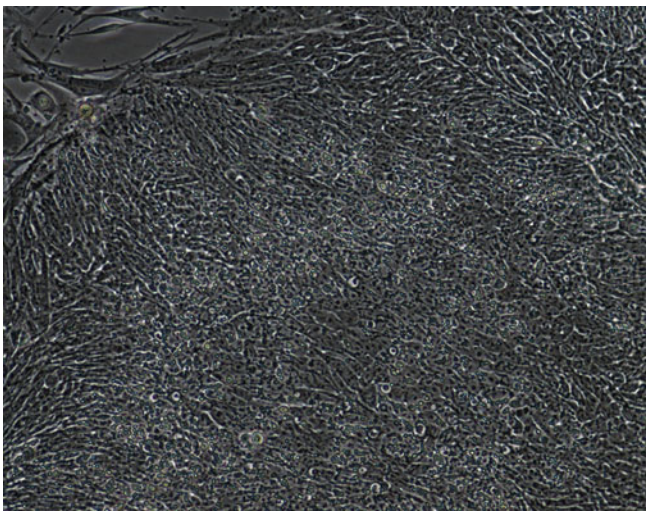

Fig. 4.59 Challenge: what can you say about this figure? See page 199 for answer

Fig. 4.60 Challenge: what can you say about this figure? See page 199 for answer

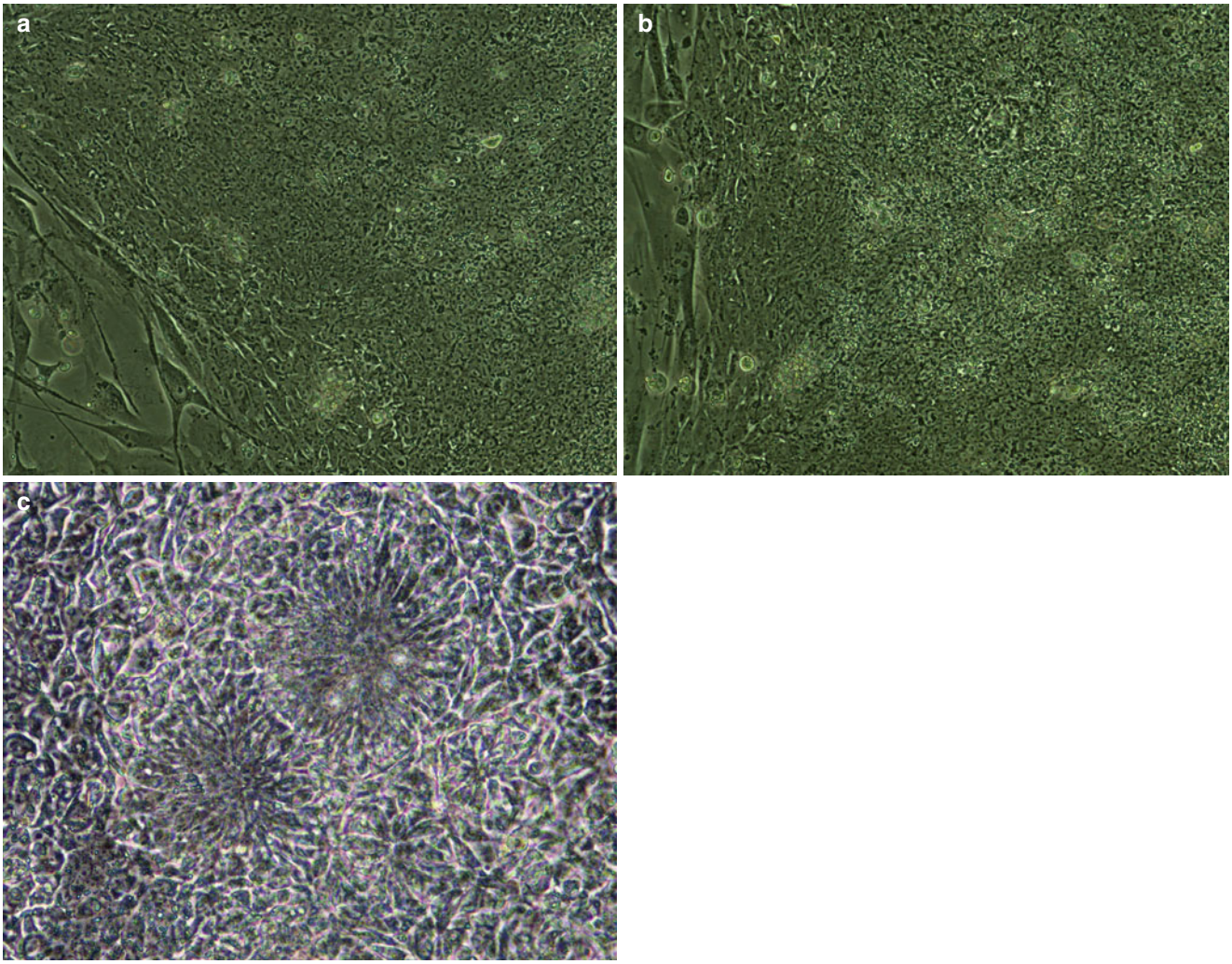

Fig. 4.61 Challenge: what can you say about this figure? See page 199 for answer

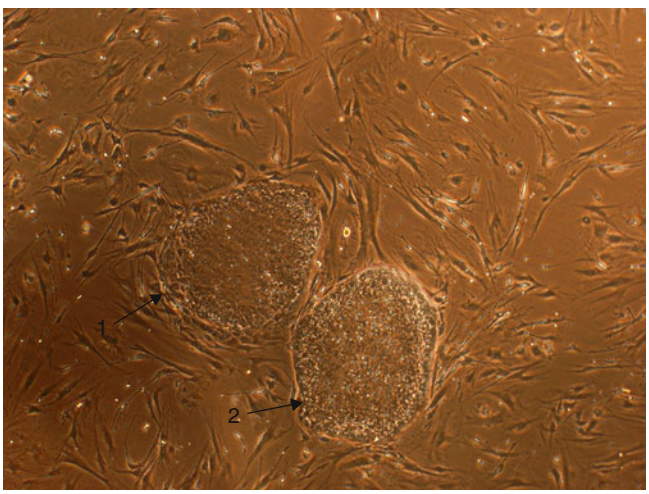

Fig. 4.62 Challenge: what can you say about this figure? See page 199 for answer

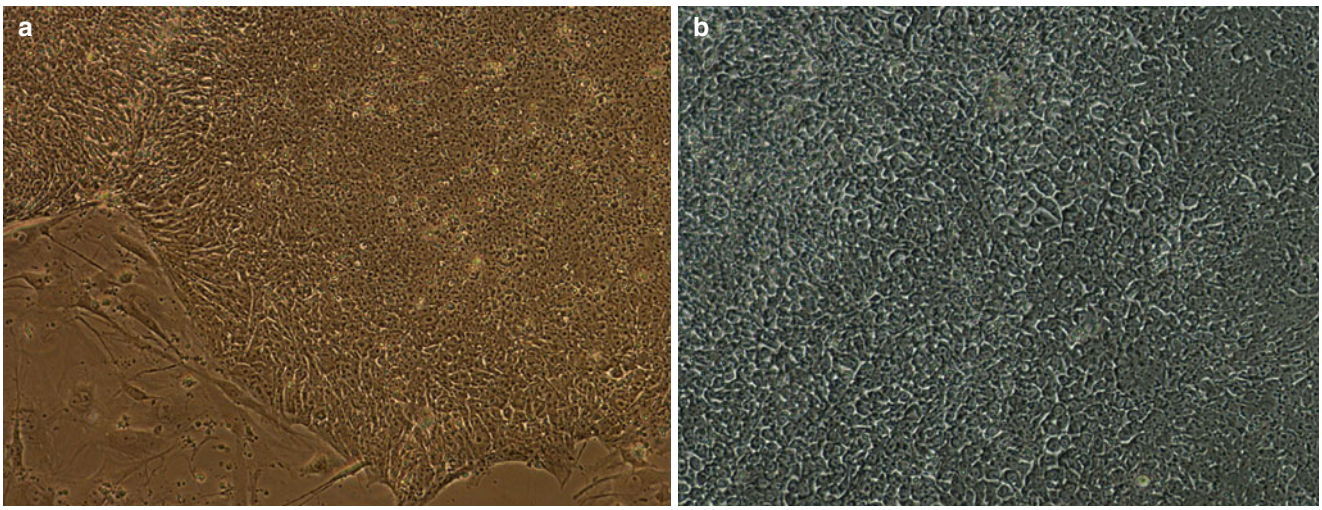

Fig. 4.63 (**a**) The colonies in this image display well-defined borders. Individual stem cells with large nucleoli are evident. Both colonies are compacted and ready to passage. (**b**) This colony is slightly disorganised. It is poised to differentiate (both ×10 magnification)

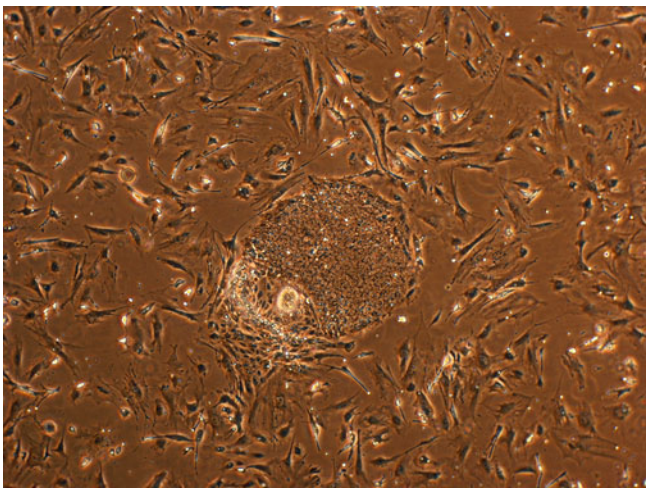

Fig. 4.64 The majority of this colony is undifferentiated, with defined borders. One-third of the colony (*bottom left*) is spontaneously differentiated (×4 magnification)

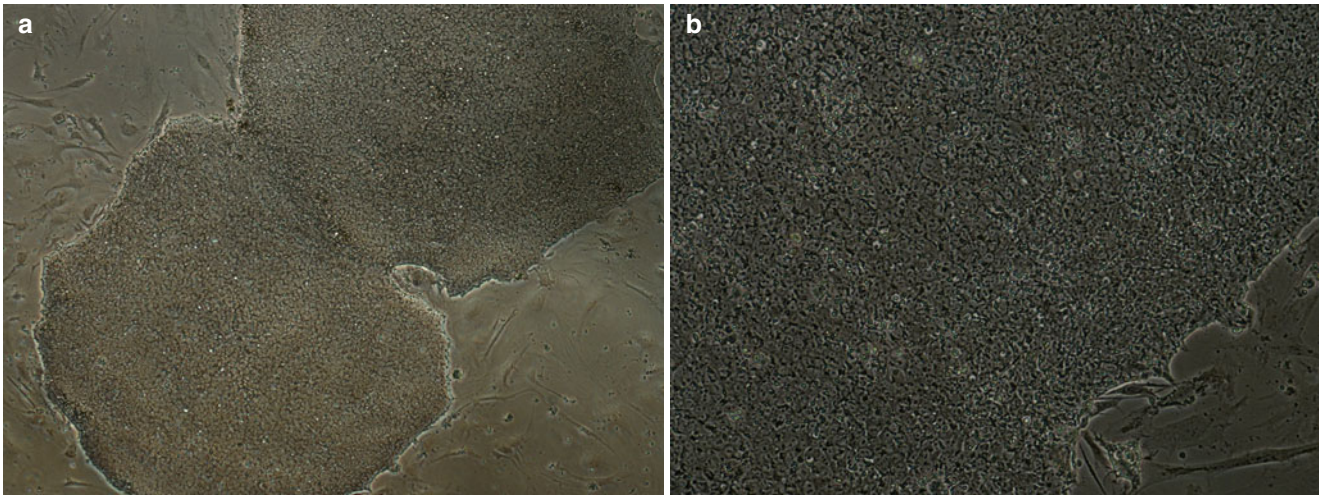

Fig. 4.65 This is an example of what happened to a culture after accidentally adding media with 2.5 times more basic fibroblast growth factor (bFGF) than usual for this cell line. The colonies became overcompacted, and any trace of differentiation was totally absent (**a** ×4 magnification; **b**, ×10)

In general, cells grown on mouse and human feeders grow in colonies, but what morphological characteristics indicate that the cells are growing optimally? A good-quality cell culture just prior to passaging contains compact colonies with defined borders. The colonies are formed of cells with a high ratio of nucleus to cytoplasm and prominent nucleoli. These good-quality cultures look identical on both mouse and human feeders.

The colony morphology evolves over the days after passage, and the change is readily observed under the microscope. At first, the colonies can appear as loose structures where borders are not defined and the cells are large, with spaces that appear white under brightfield microscopy. With each day, as the cells within the colony proliferate, the morphology changes and the cells in the centre of the colony become smaller and more compact. The border of the colony also becomes more defined. When a culture is ready to passage, the colonies look compact and are filled with small, uniform cells. The white spaces have been reduced and there is little evidence of apoptosis in the colony. If the colonies are allowed to grow beyond this stage, they begin to overcompact, and areas of thickening appear in the centre of the colonies. This condition generally promotes differentiation, whereby the undifferentiated hESCs become committed to germ layer differentiation. The borders of the colonies also begin to lose their definition in this situation.

If cells are passaged when they are not in an optimal condition, the result can be spontaneous differentiation within a short period of time, typically less than 48 h. All of these morphological changes can be observed in culture and are indicative of the physiological quality of the cells. With hESC culture, each cell line can respond differently to the same culture conditions, so the culture routine must be tailored to the individual cell line. It is important to note that if you do not optimise the culture conditions for each line, you will be unable to maintain undifferentiated cultures of cells.

Suggested Reading

Amit M, Itskovitz-Eldor J. Morphology of human embryonic and induced pluripotent stem cell colonies cultured with feeders. In: Amit M, Itskovitz-Eldor J, editors. Atlas of human pluripotent stem cells: derivation and culturing, Stem cell biology and regenerative medicine. New York: Humana Press; 2012. p. 15–39.

Johkura K, Cui L, Asanuma K, Okouchi Y, Ogiwara N, Sasaki K. Cytochemical and ultrastructural characterization of growing colonies of human embryonic stem cells. J Anat. 2004;205:247–55.

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol. 2000;18:399–404.

Sathananthan AH, Trounson A. Human embryonic stem cells and their spontaneous differentiation. Ital J Anat Embryol. 2005;110:151–7.

Sathananthan H, Pera M, Trounson A. The fine structure of human embryonic stem cells. Reprod Biomed Online. 2002;4:56–61.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145–7.

Human Induced Pluripotent Stem Cells on Feeders

5

In 2006, it was reported that pluripotent stem cells (PSCs) could be generated by reprogramming mouse fibroblasts to a pluripotent state using four transcription factors delivered via a retroviral infection system. These cells were shown to be embryonic stem cell like with respect to their functional, genotypic and phenotypic properties and were named induced pluripotent stem cells (iPSCs). In 2007, human iPSCs (hiPSCs) were generated. Since then, many different delivery systems have been used to generate cell lines from a broad array of somatic cell types with widely varying efficiency.

The morphology of hiPSCs is identical to that seen for hESCs. When grown in feeder-based systems that have been optimised for the line, undifferentiated cells form colonies that differ in size depending on the density of the feeders. The iPSC lines appear, in general, to remain undifferentiated on lower-density feeder cells than their hESC counterparts, but this difference is dependent on the cell line and feeder source. In some hiPSC lines, the colonies are not as compact as those seen with hESCs. The maintenance and passaging of these lines is also carried out using the same procedures as for hESCs and with the same considerations as described in Chap. 7, although with some cell lines, smaller hiPSC clumps can be passaged to form undifferentiated colonies, compared with hESC clumps.

This chapter presents images of hiPSCs grown on mouse feeders (Figs. 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 5.10, 5.11, 5.12, 5.13, 5.14, 5.15, 5.16, 5.17, 5.18, 5.19, 5.20 and 5.21) and human feeders (Figs. 5.21, 5.22, 5.23, 5.24, 5.25, 5.26, 5.27, 5.28, 5.29, 5.30, 5.31, 5.32, 5.33, 5.34, 5.35, 5.36, 5.37, 5.38, 5.39 and 5.40) and describes their morphological features with time, at various stages in the growth of the colonies. The images also include observations on the growth of colonies following the use of different types of passaging techniques. As with hESCs, the feeder-based system must be optimised for each iPSC line, a statement that seems obvious considering the diversity of somatic cell backgrounds from which the cell lines are derived.

L. Healy, L. Ruban, *Atlas of Human Pluripotent Stem Cells in Culture*,
DOI 10.1007/978-1-4899-7507-2_5, © Springer Science+Business Media New York 2015

5.1 Human Induced Pluripotent Stem Cells on Mouse Feeders

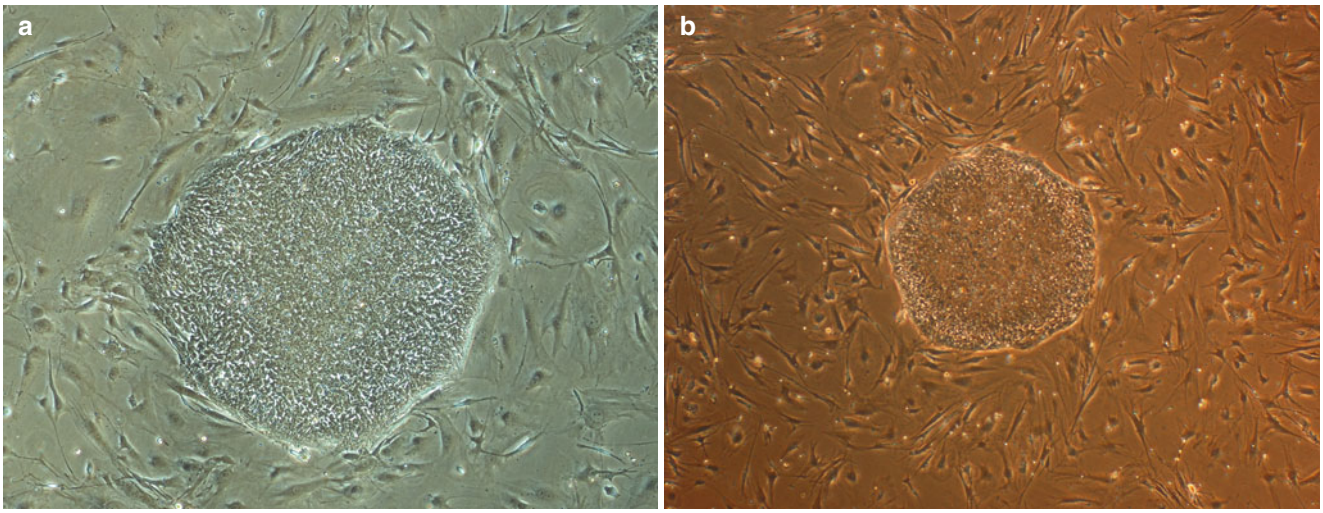

Fig. 5.1 Comparison of the morphology of undifferentiated induced pluripotent stem cells (iPSCs) (**a**) and human embryonic stem cells (hESCs) (**b**) cultured on mouse feeders. Note that the morphology of the colonies in the two pictures looks identical. The individual cells making up each colony are small and compacted (both ×4 magnification)

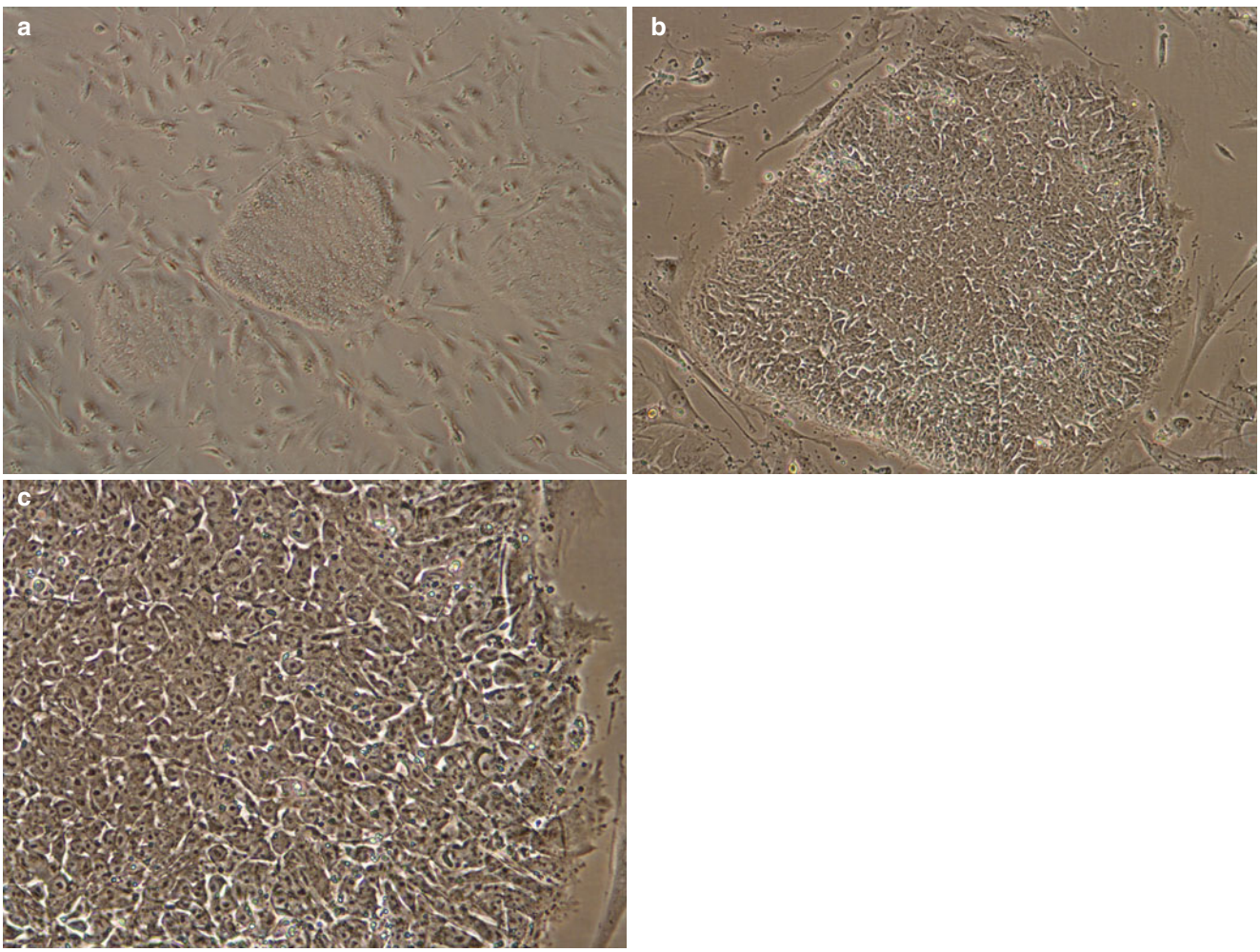

Fig. 5.2 (**a**–**c**) Small colony, 24 h after passaging onto feeders. Note the prominent spaces between cells prior to compaction of the colony (**a**, ×4 magnification; **b**, ×10; **c**, ×20)

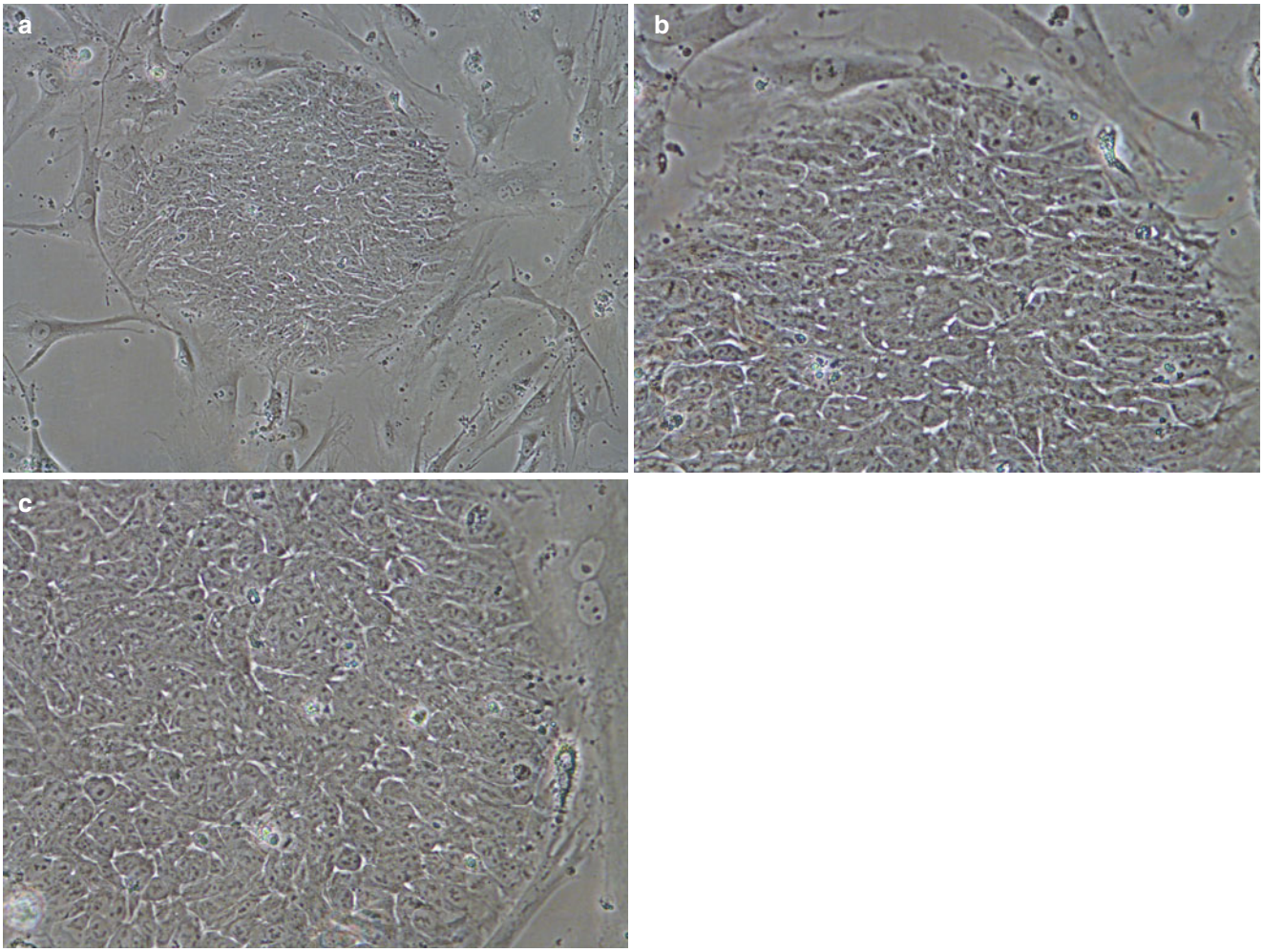

Fig. 5.3 (**a**–**c**) Another example of a well-formed small colony, actively proliferating with defined borders, 24 h after passaging onto feeders (**a**, ×4 magnification; **b**, ×10; **c**, ×20)

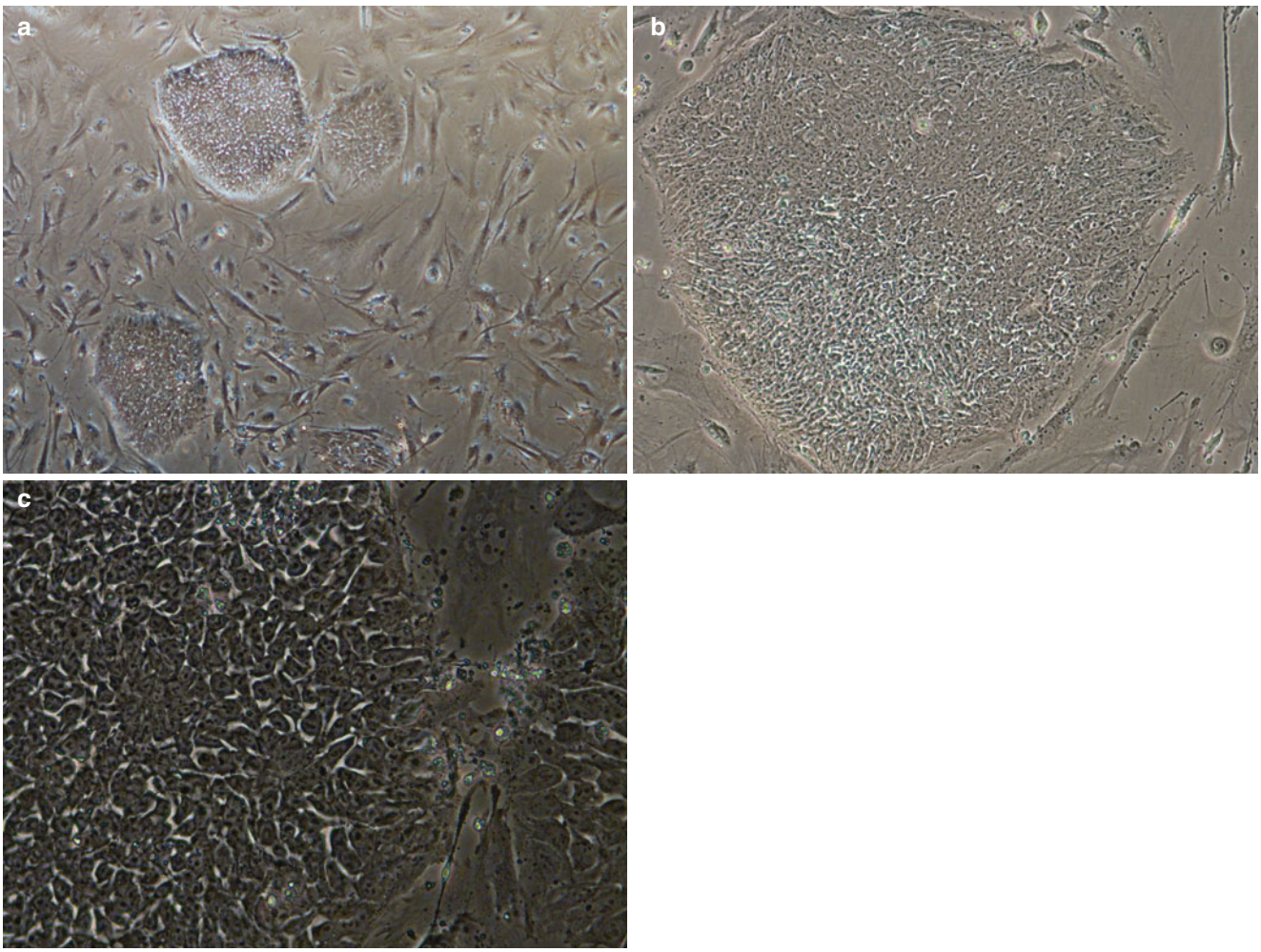

Fig. 5.4 (**a**–**c**) Small colonies 24 h after passaging. Note the prominent spaces between cells prior to compaction of the colony (**a**, ×4 magnification; **b**, ×10; **c**, ×20)

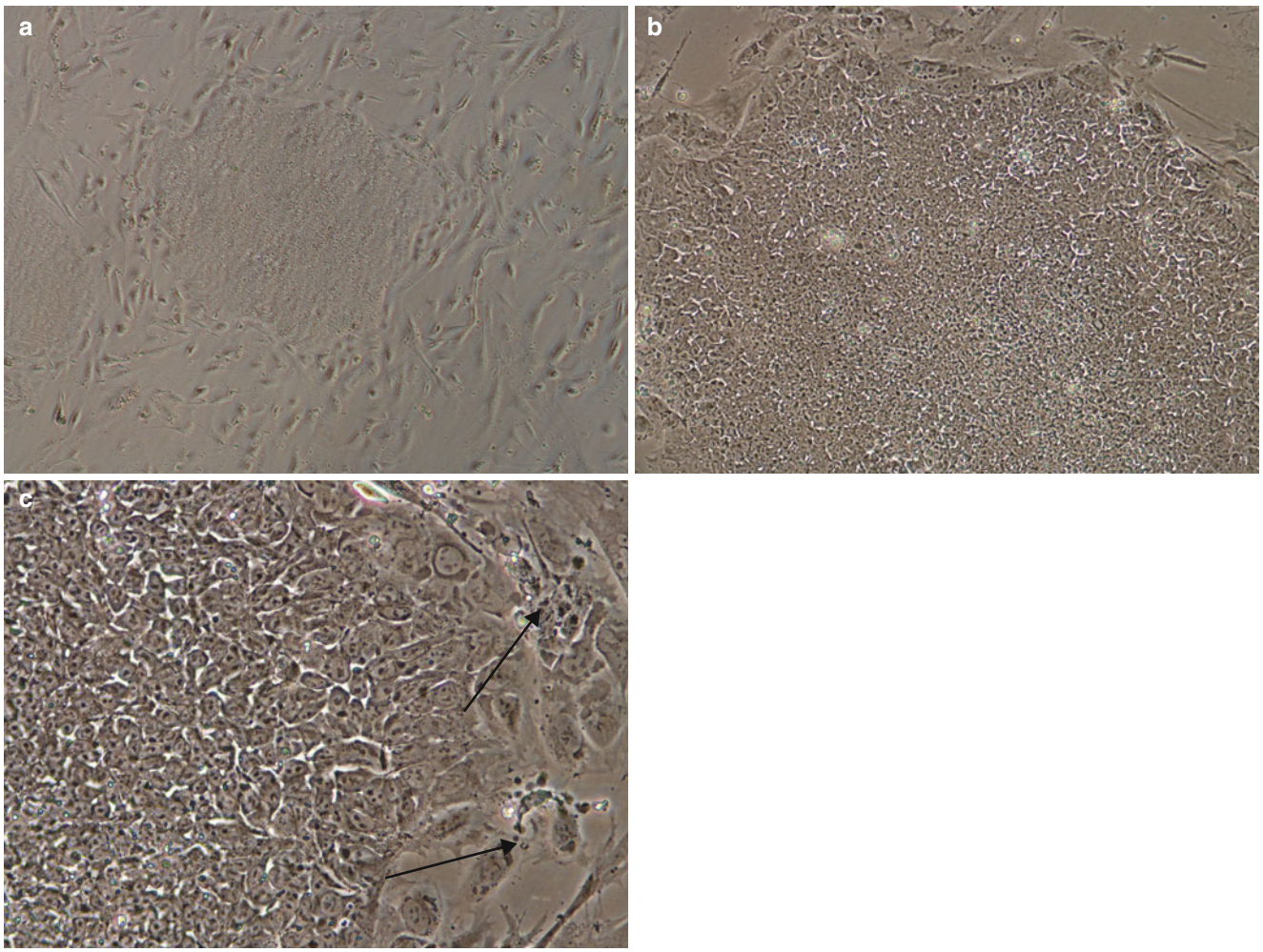

Fig. 5.5 (**a**–**c**) A relatively small, compacted colony 48 h after passaging onto feeders. The borders are not well defined, owing to the presence of differentiated cells. The feeder density in this culture is not optimal for the cell line, so differentiated cells (*black arrows*) are present. This colony is ready to be subcultured (**a**, ×4 magnification; **b**, ×10; **c**, ×20.)

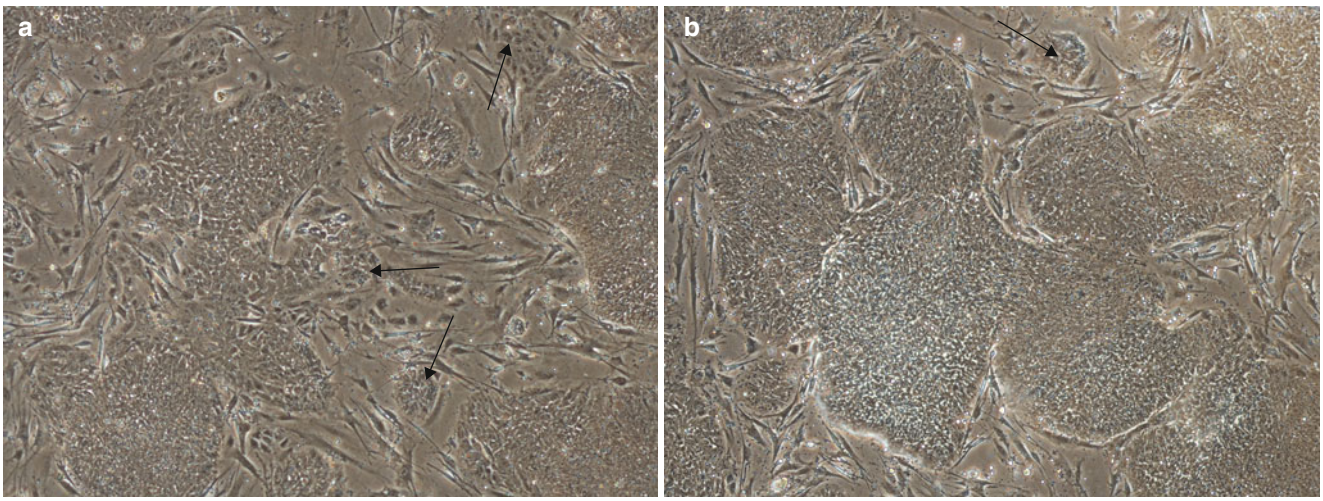

Fig. 5.6 (**a**, **b**) High-density culture of iPSC colonies grown on fibroblasts, showing the typical morphology of undifferentiated cells, with well-defined borders and compact colonies. Note the small group of cells forming a very small colony. A colony of this size will often spontaneously differentiate (*black arrows*) (both ×4 magnification)

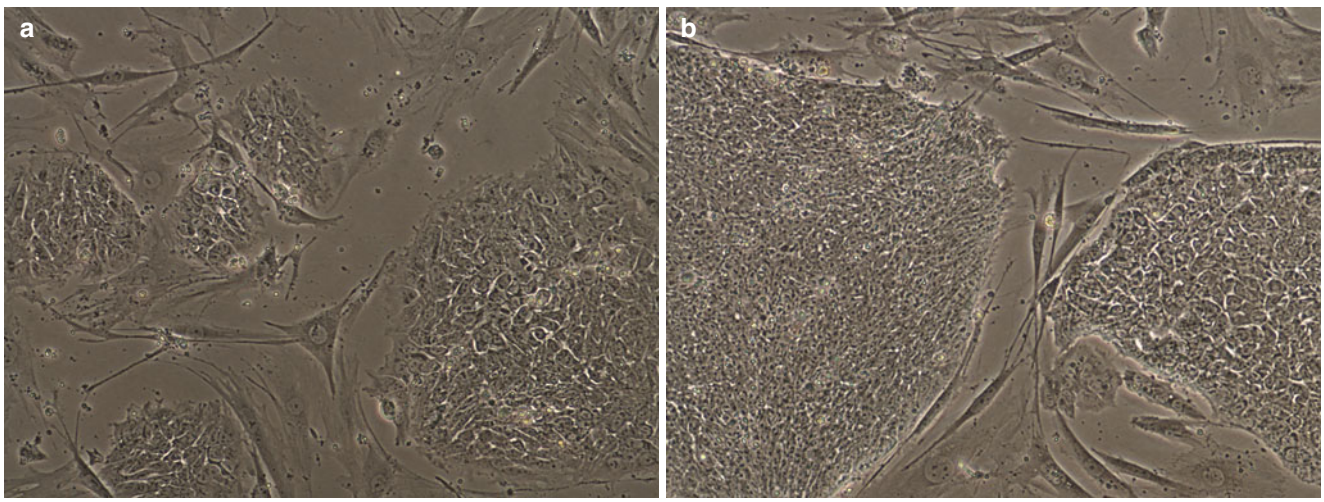

Fig. 5.7 Example of a well-formed small colony showing good morphology 24 h after passaging onto feeders (**a**), and 48 h after passaging (**b**) (both ×10 magnification)

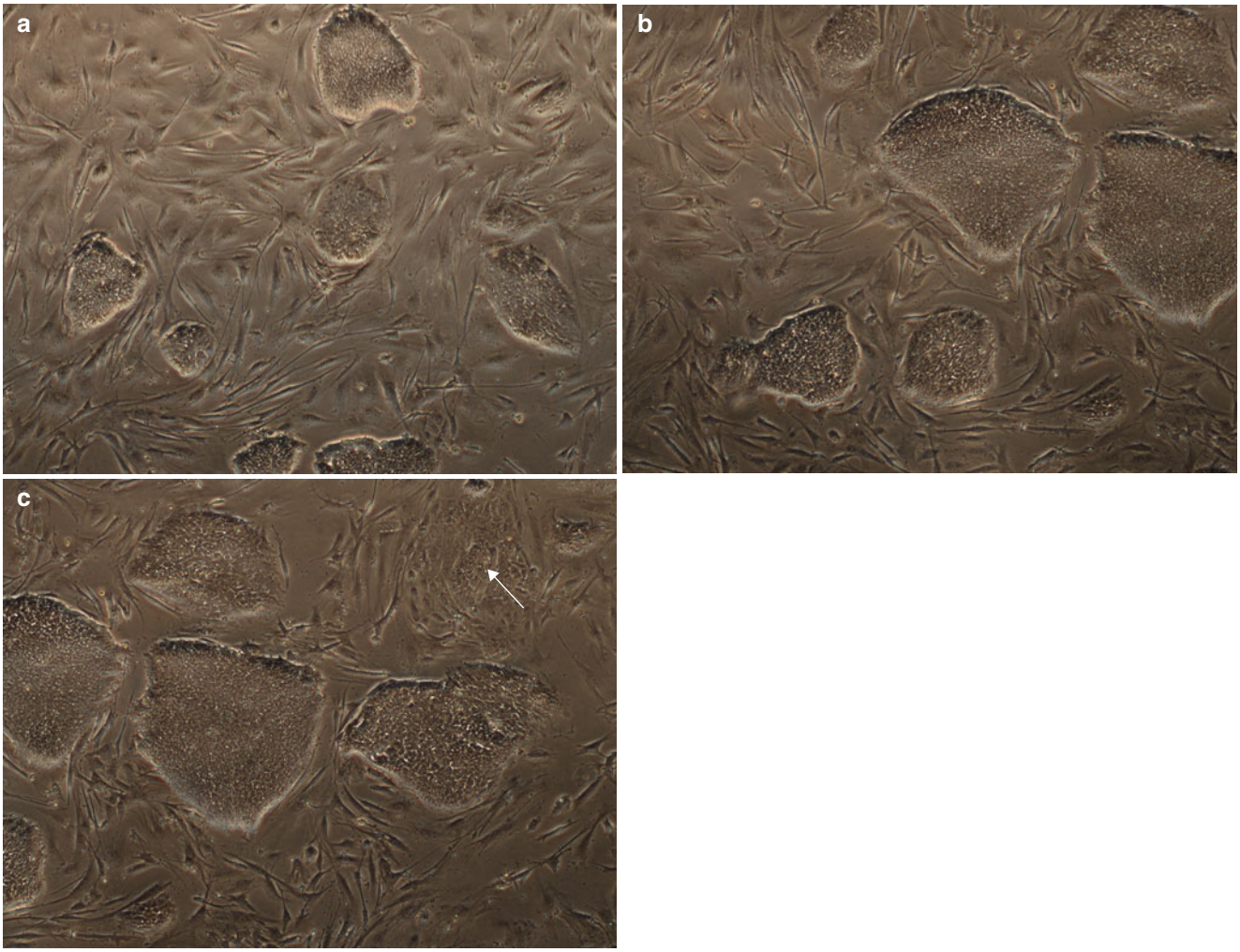

Fig. 5.8 (**a**–**c**) Colonies of iPSCs grown on fibroblasts in three individual cell cultures showing typical morphology of undifferentiated cells, with well-defined borders and compact colonies. Note that the small colonies are also compact. In hESC cultures, these small colonies will often spontaneously differentiate, but this phenomenon is less frequently observed in iPSC cultures, in which small clumps of cells can produce good-quality, undifferentiated colonies. The *arrow* in **c** indicates a colony that has differentiated (all ×4 magnification)

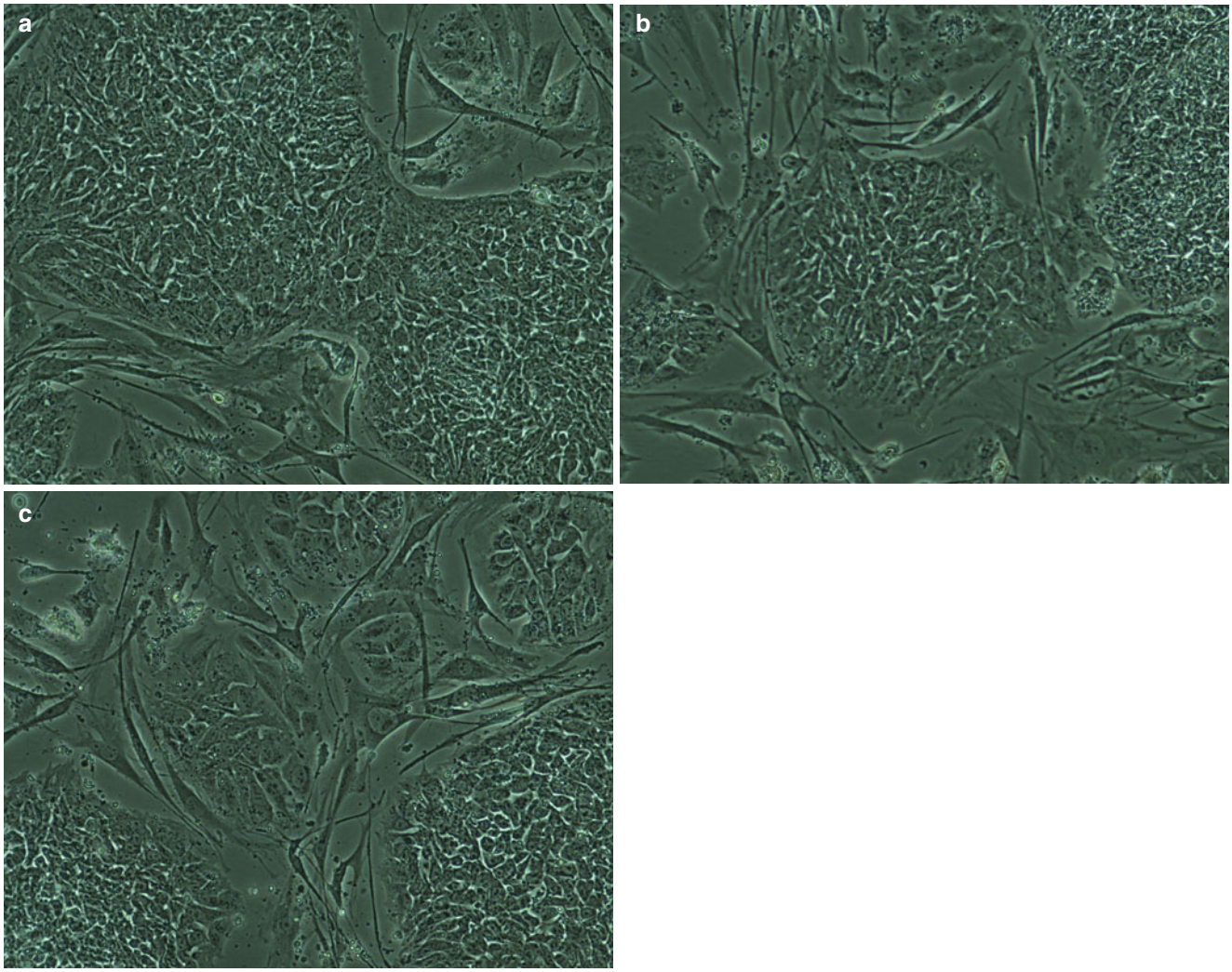

Fig. 5.9 (**a**–**c**) Early colonies 24 h after passaging onto feeders. Note the prominent spaces between cells prior to compaction of the colony. Colonies can be seen merging, but this is not a problem, as it does not necessarily promote differentiation of the colony (all ×10 magnification)

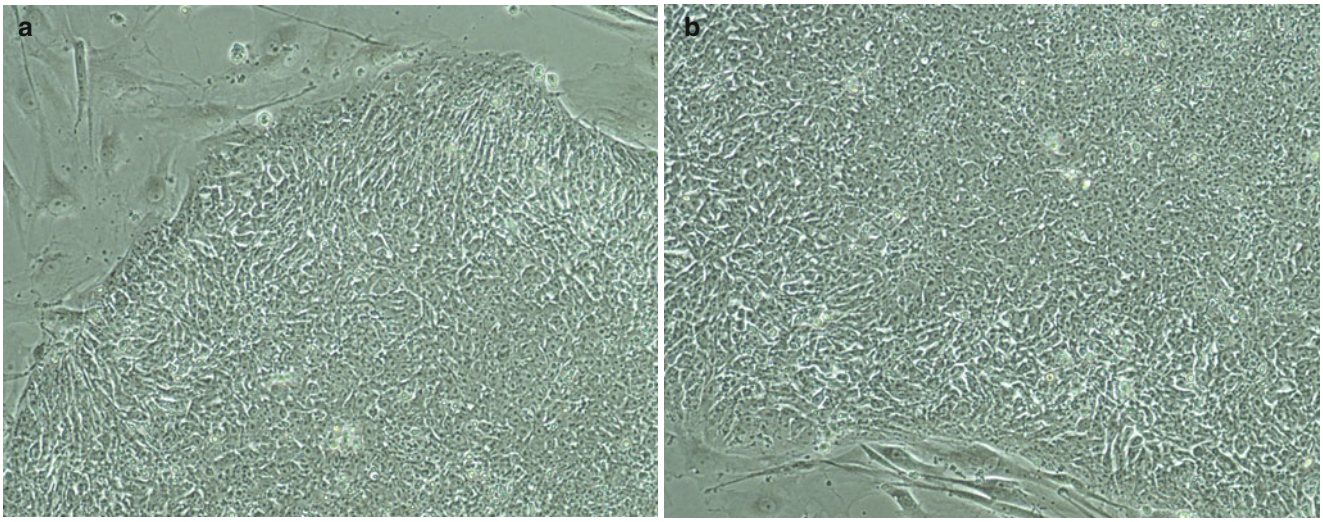

Fig. 5.10 (**a**, **b**) These colonies are compacted and are ready to be subcultured (both ×10 magnification)

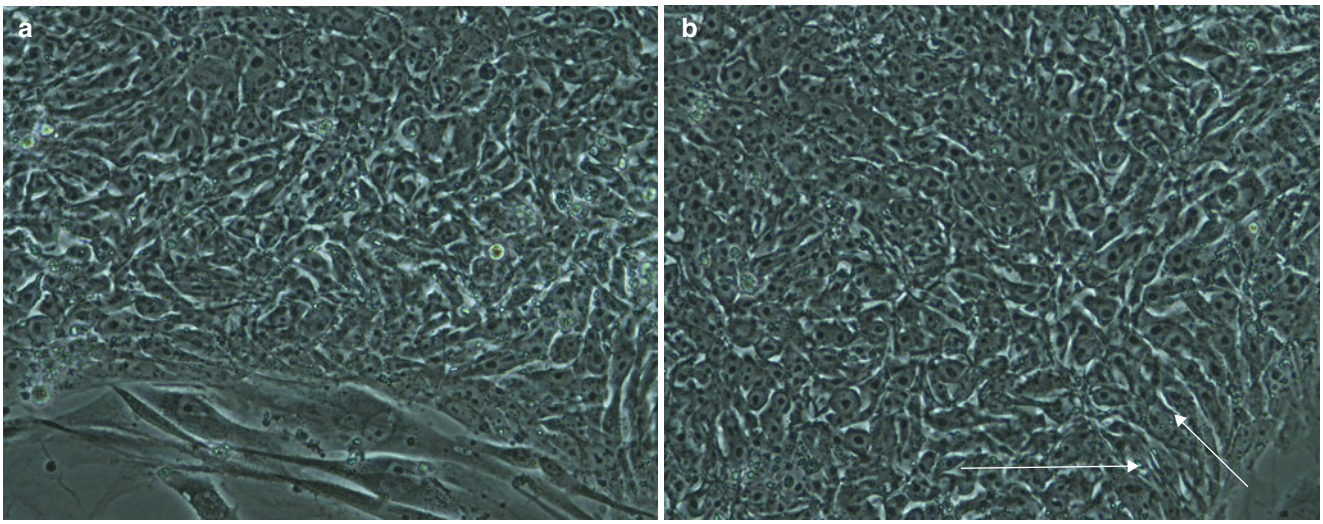

Fig. 5.11 (**a**, **b**) Magnification ×20 of the iPSC colony in Fig. 5.10 (**b**) reveals spaces between cells that display large nucleoli. (**b**) Large, proliferating cells (*white arrows*) can be seen at the edges of the colony

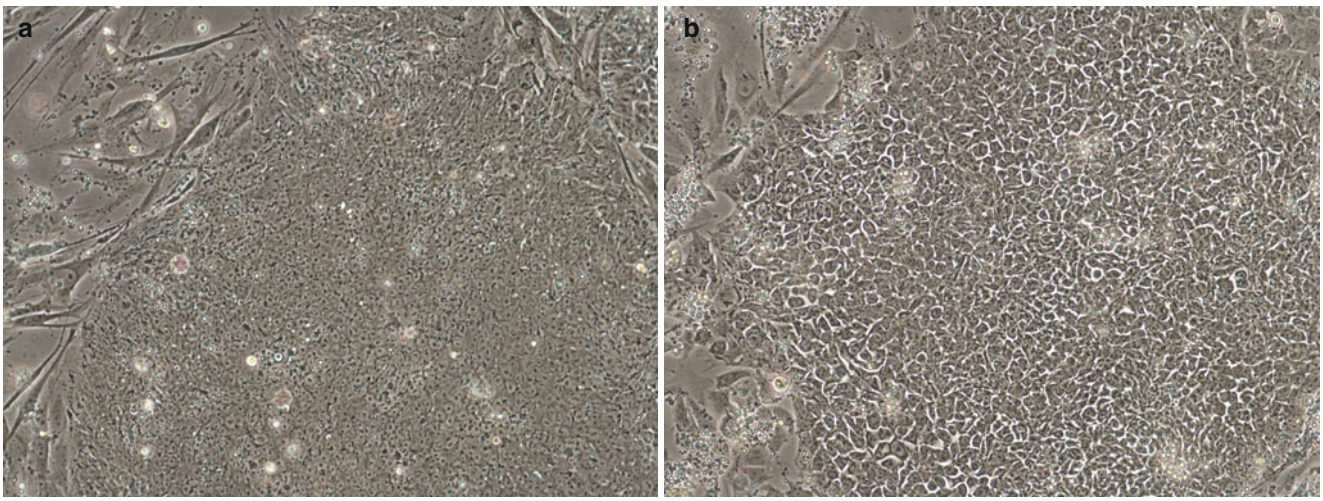

Fig. 5.12 Compacted colonies (**a**) and not-compacted colonies (**b**) observed in culture at the same time point. The colony in **a** is ready to be subcultured (both ×10 magnification)

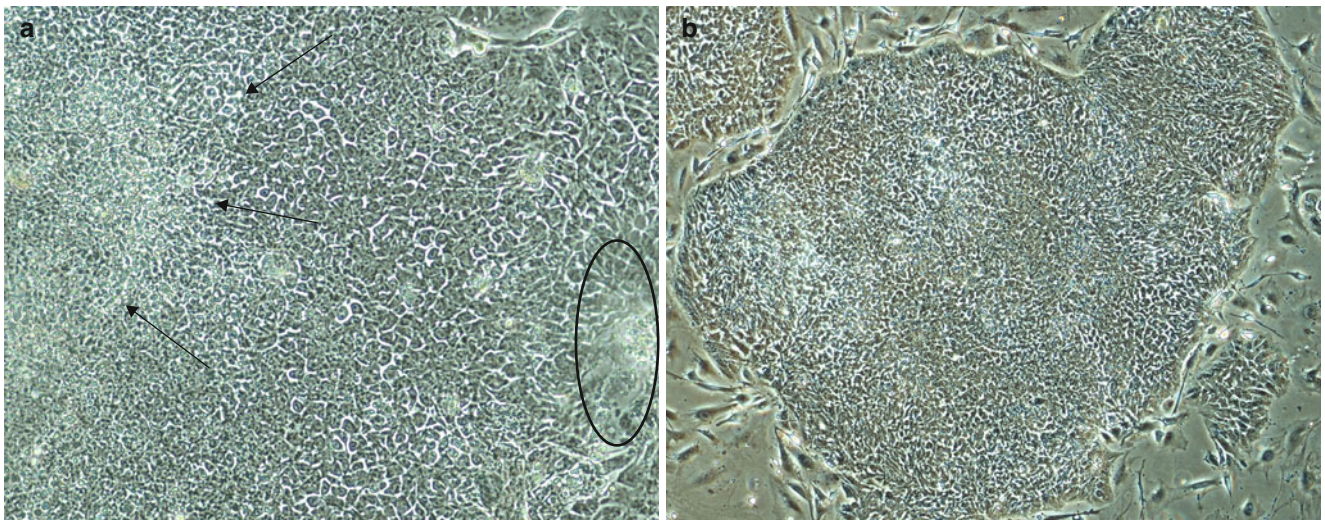

Fig. 5.13 Two images of iPSC colonies undergoing differentiation. (**a**) Areas showing the commencement of neuronal differentiation (*black arrows*), including the formation of a neuronal rosette (*black oval*). (**b**) Bumpy regions on the otherwise flat colony also indicate the initiation of neuronal differentiation. They will become rosettes 2–3 days after this type of morphology has been observed (**a**, ×10 magnification; **b**, ×4)

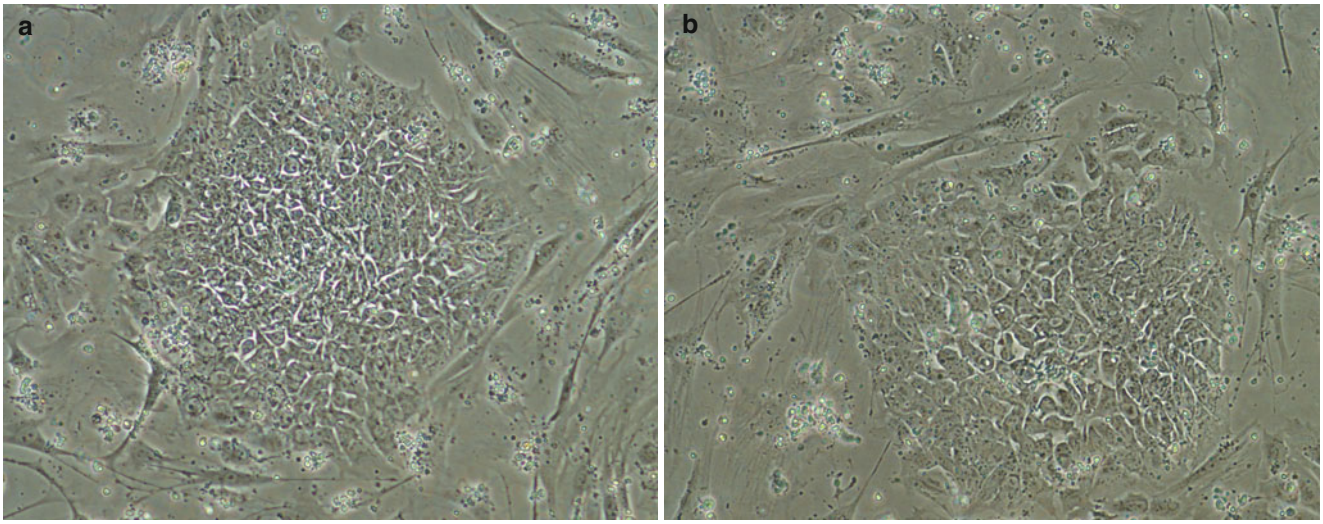

Fig. 5.14 (**a**, **b**) Small colonies 24 h after passaging, not compacted and without defined borders. At this stage they could remain undifferentiated, or they could differentiate. They need to be observed over the next 3 days (both ×10 magnification)

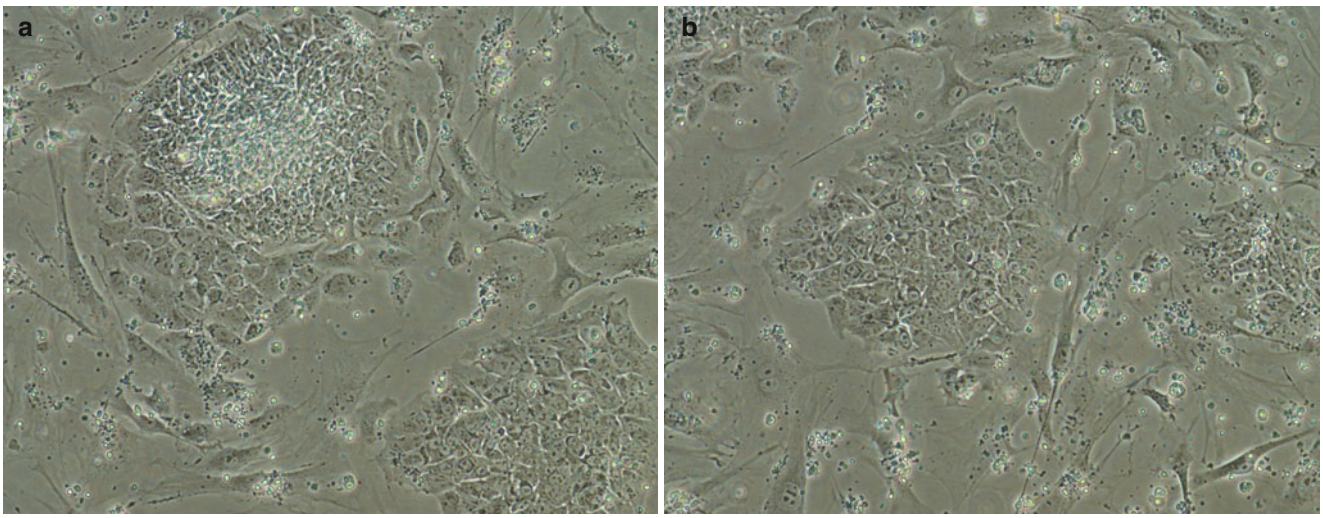

Fig. 5.15 Small colonies 24 h after passaging. (**a**) The top left colony has differentiated. (**b**) Small colonies that are not compacted and lack defined borders. As in Fig. 5.14, at this stage they could remain undifferentiated or could differentiate. They need to be observed over the next 3 days (both ×10 magnification)

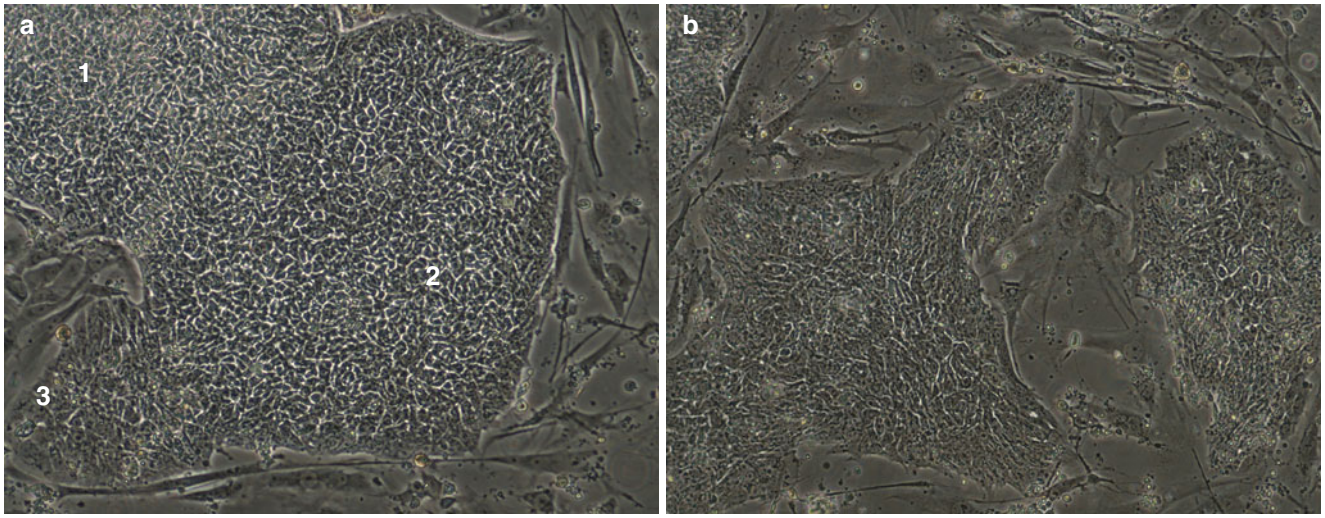

Fig. 5.16 (**a**) Two large colonies (*1* and *2*) and one small colony (*3*) merge. Colony *1*, on the *left*, is beginning to differentiate. Colonies *2* and *3* are not compacted, but are of relatively good quality. (**b**) These colonies lack defined borders, and all the cells within the colonies are elongated and irregular in shape. This is an indication of a transition in the cell towards a differentiated state (both ×10 magnification)

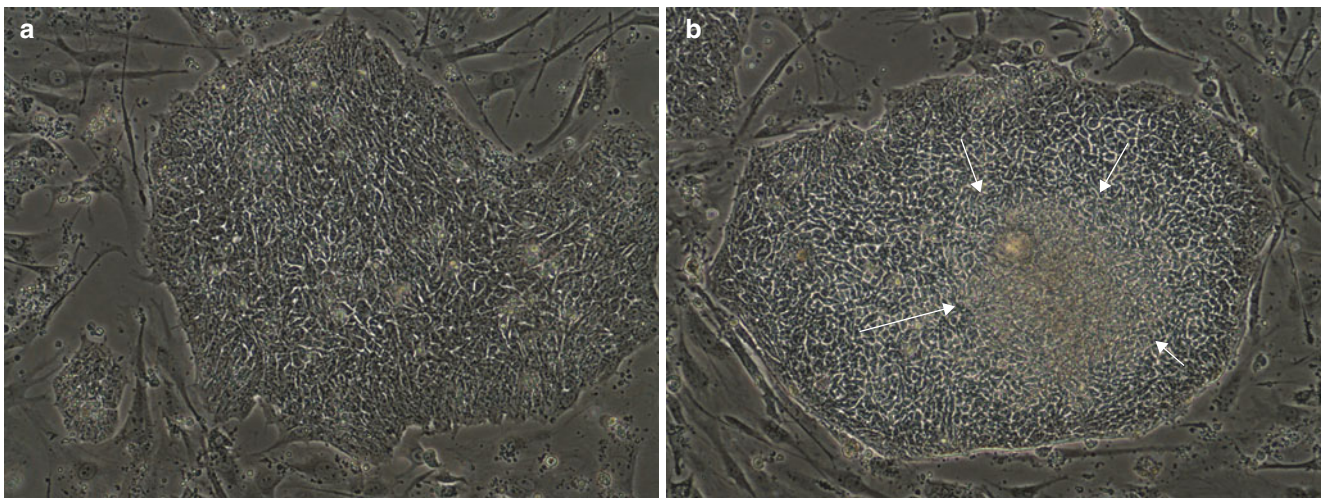

Fig. 5.17 (**a**) The morphology of these cells is disorganised and the cells are irregular in shape, with long cells aligning along the border. This arrangement may have arisen from the merging of two small colonies. (**b**) The border of the colony is well defined and the cells have compacted, but there is an area where the cells have overgrown in the colony (*arrows*), indicating transition of the cells towards a differentiated state (both ×10 magnification)

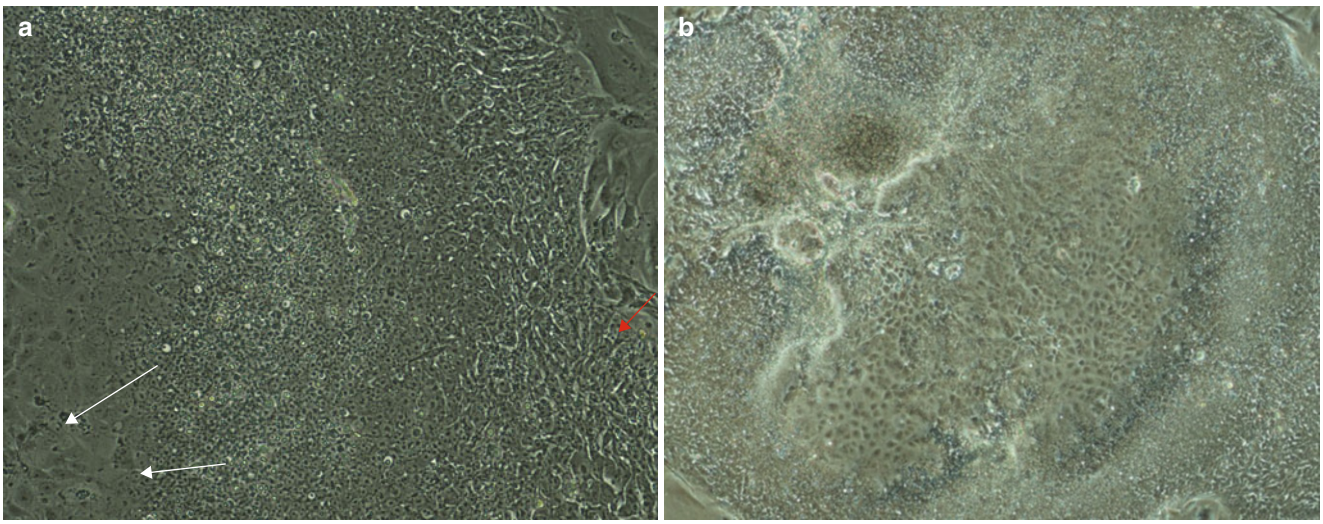

Fig. 5.18 Examples of differentiation in iPSC colonies. (**a**) An overtly differentiated area in the centre of the colony (*white arrows*). The region next to this area displays the morphology of compacted, undifferentiated cells, but the border of the colony is disorganised and contains differentiated elements (*red arrow*). Note that the differentiated cells could be removed, and the undifferentiated cells could then be passaged to rescue the culture. (**b**) This colony is more than 10 days old and it is completely differentiated, as evident from the changes in morphology seen in areas throughout the colony, coupled with the areas of overcrowding that are causing the cells to pile up (both ×10 magnification)

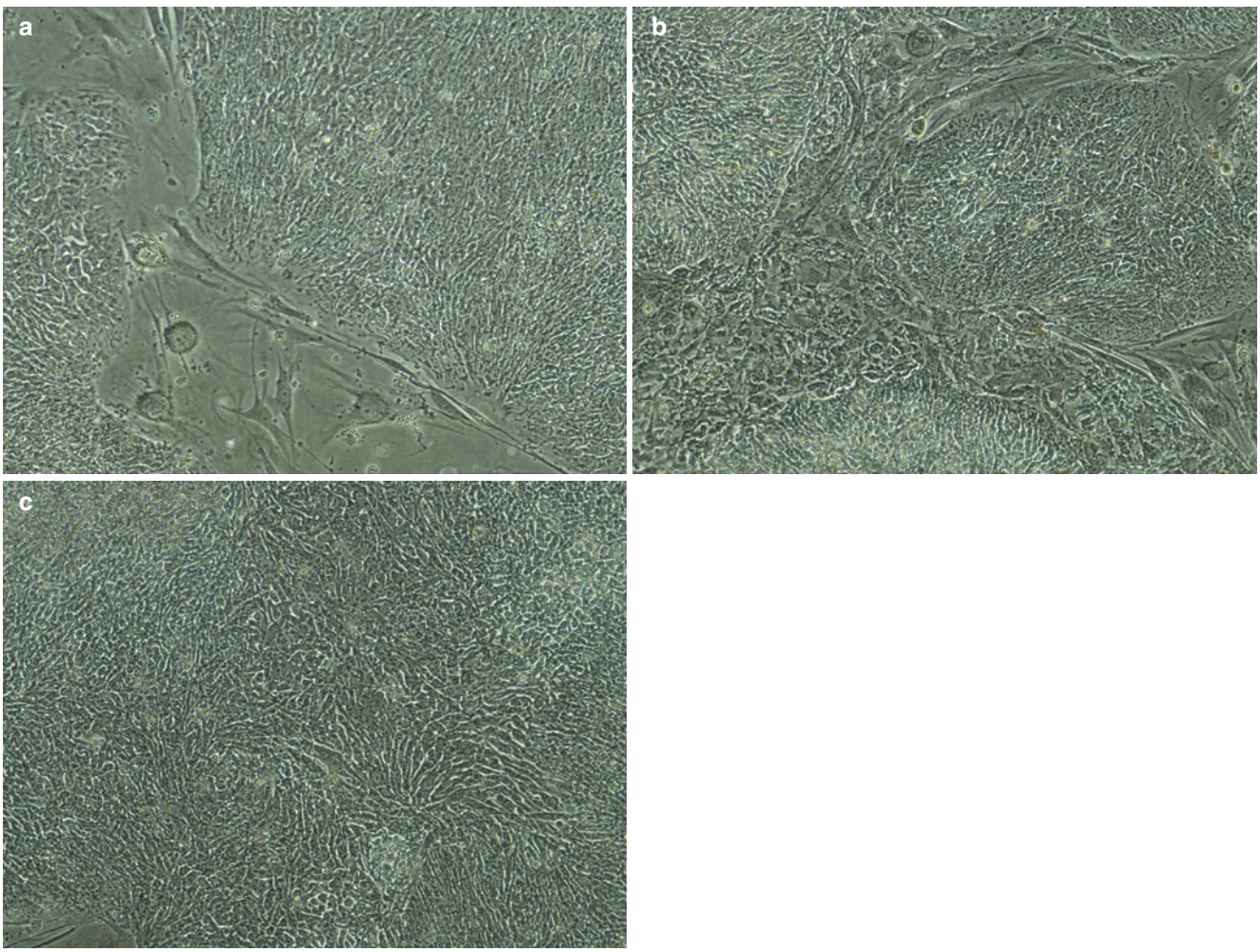

Fig. 5.19 (**a**–**c**) All these images display colonies with elongated cells suggesting a transition from the undifferentiated to the differentiated state, probably because the cells were passaged onto feeders at a density too low to support the cells in an undifferentiated state (all ×10 magnification)

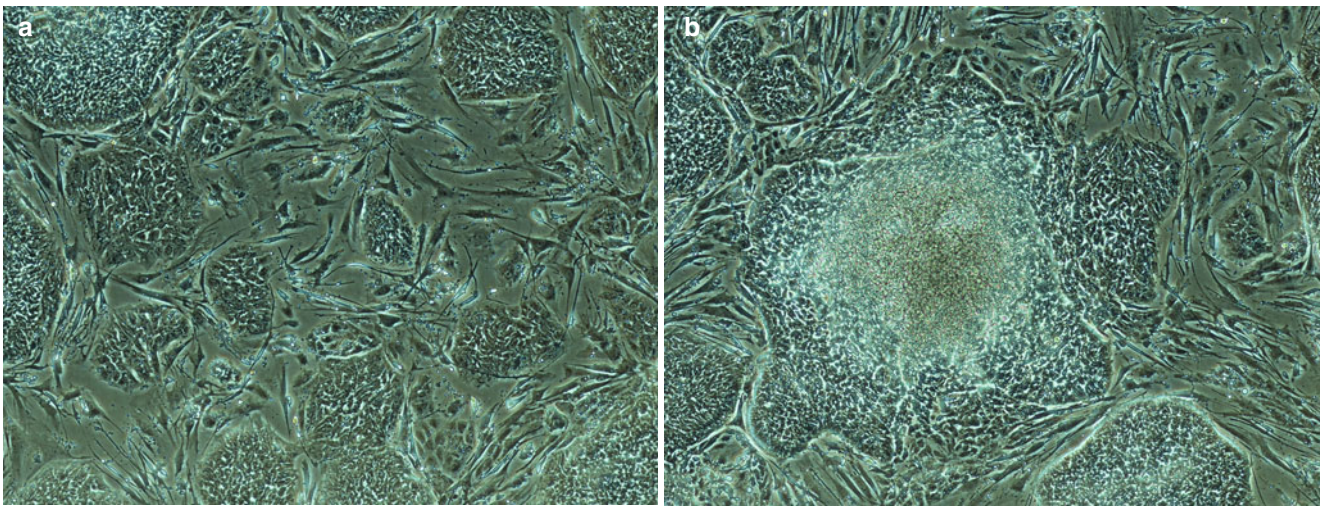

Fig. 5.20 (**a**) Small colonies at a high density 24 h after passaging. (**b**) Colonies from a 48-h culture of the same cell line. A multilayered colony can be seen, which has arisen from the passaging of a large clump that is not of an optimal size for passaging (both ×4 magnification)

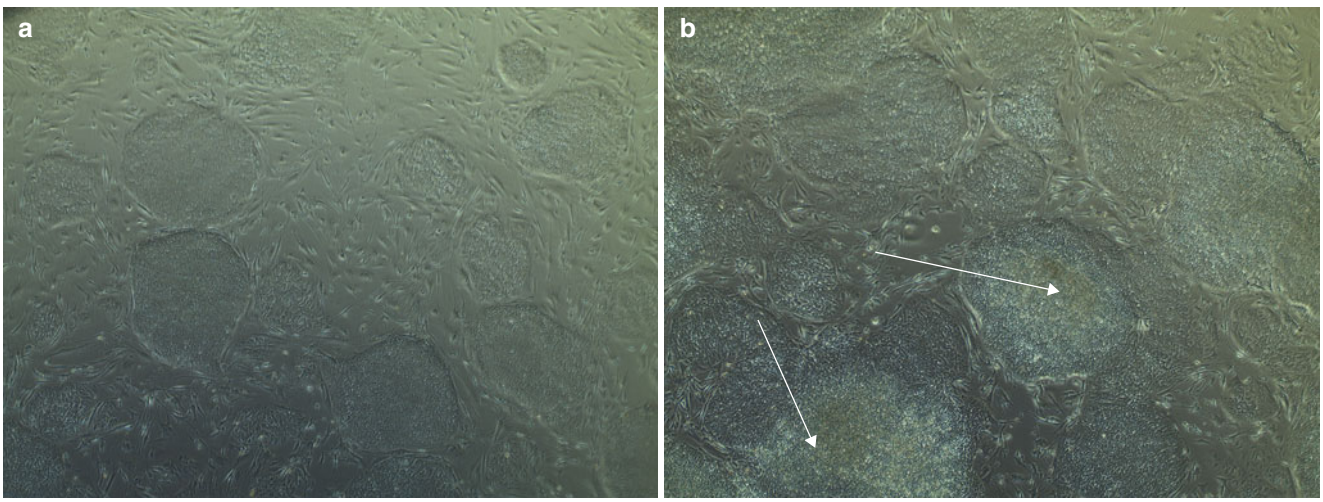

Fig. 5.21 High-density colonies 48 h after passaging. (**a**) Colonies with optimal morphology and density. (**b**) Multilayering (*white arrow*) of the colonies, which has probably resulted from the density of the cells being too high to be adequately supported in the culture system. This will lead to differentiation (both ×4 magnification.)

5.2 Human Induced Pluripotent Stem Cells on Human Feeders

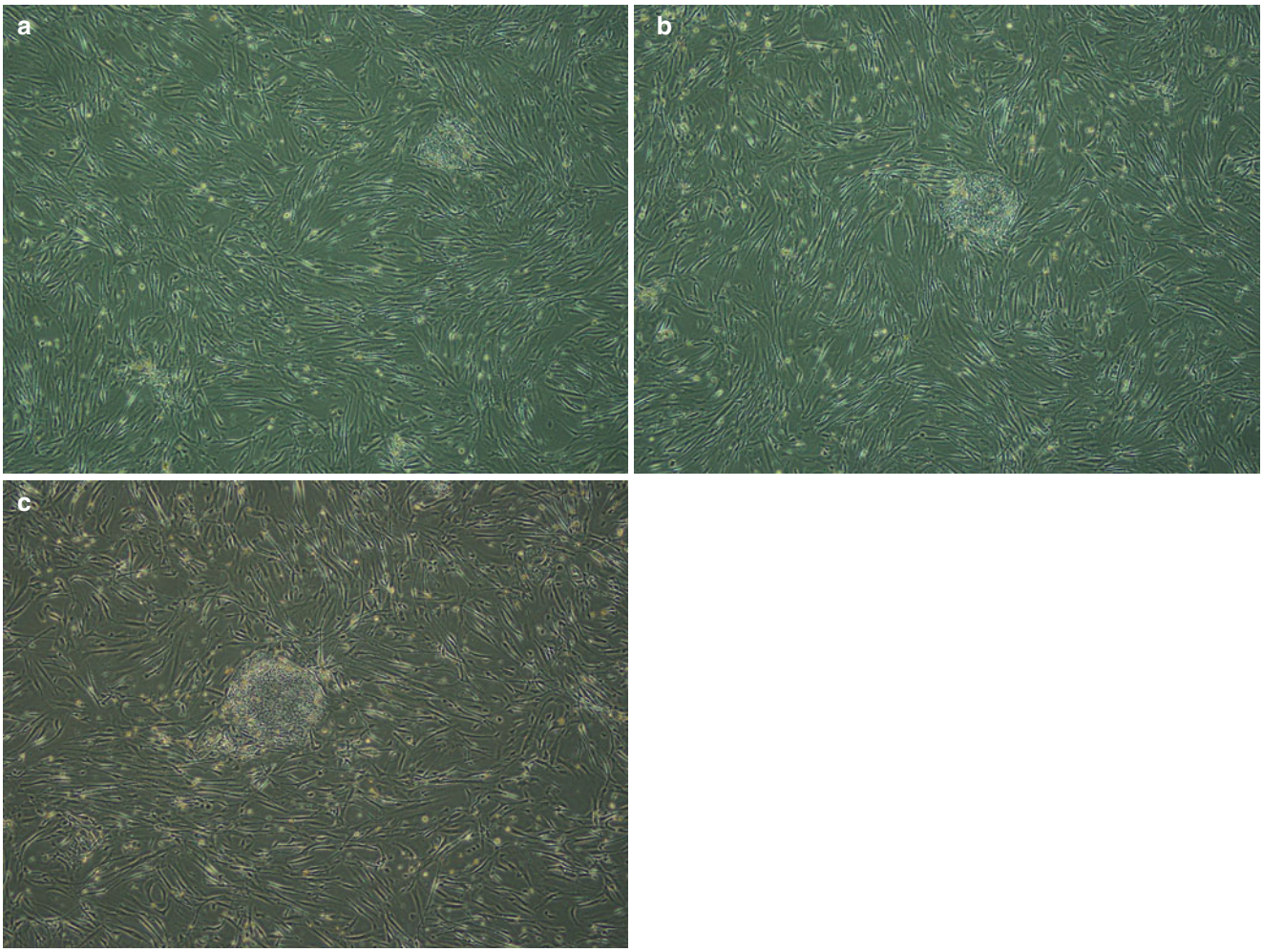

Fig. 5.22 (**a**–**c**) Colonies of human iPSCs on human feeders 24 h after manual passaging. The colonies are small, but the cells are compact (all ×4 magnification)

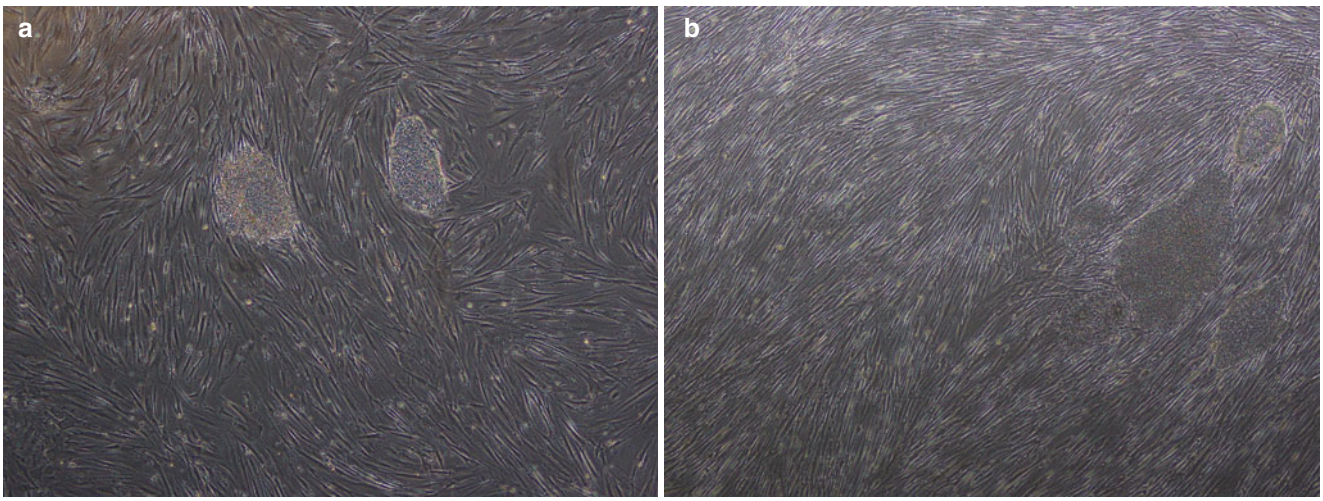

Fig. 5.23 Colonies of human iPSC on human feeders, passaged manually, shown 2 days after passaging (**a**) and 3 days after passaging (**b**). Again, the cells are compact (both ×4 magnification)

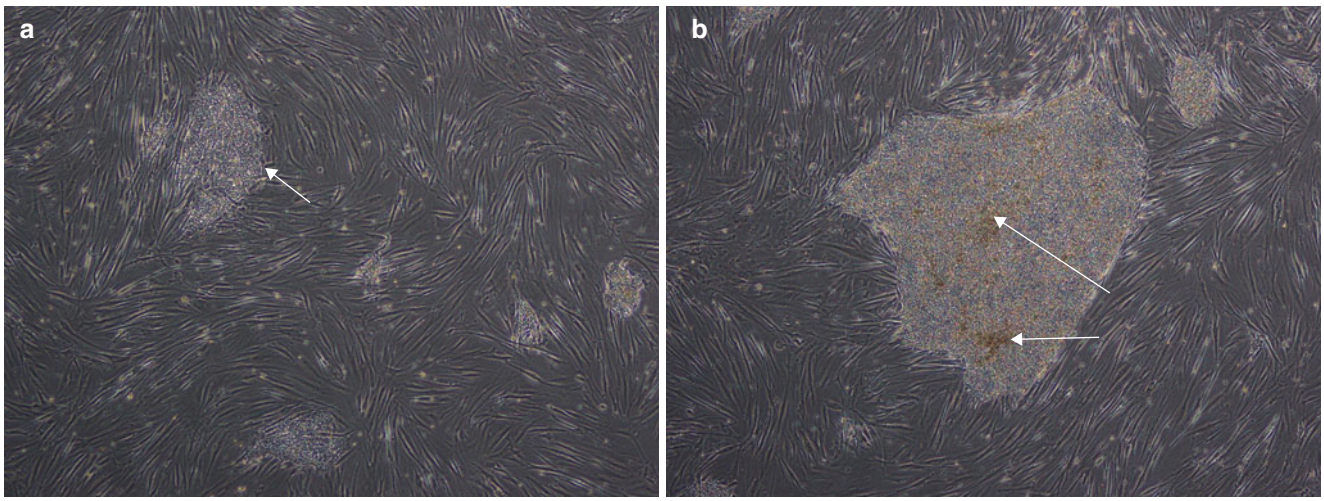

Fig. 5.24 (**a**) A culture 3 days after passaging. Note that defined geometric (sculptured) edges are forming (*white arrow*). This type of colony morphology is often seen early with manually passaged cells. (**b**) This 5-day-old large colony also shows the sculptured edges typical of manual passaging. Note the brown areas inside the colony (*white arrows*). These areas are not due to overcrowding but rather are often seen when feeder cells are carried over from the manual dissection of the colony and remain adhered to the hESCs. This colony is ready to be passaged (both ×4 magnification)

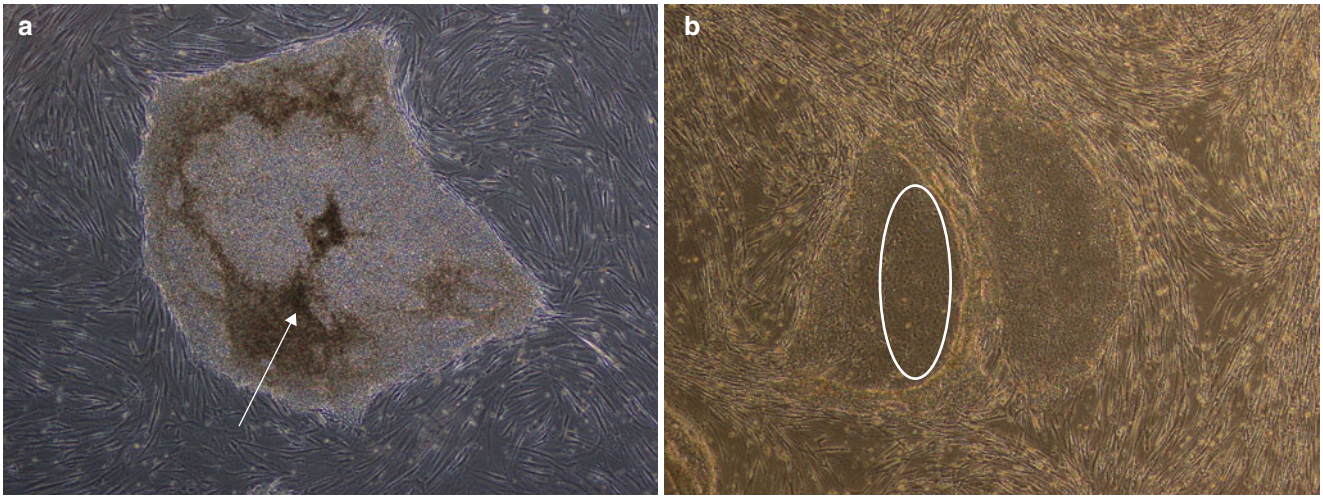

Fig. 5.25 (**a**) A colony in which feeder cells have been carried over (*white arrow*), similar to Fig. 5.24b. The general quality of this colony is good. (**b**) Two manually passaged colonies on Day 5. These colonies have well-defined edges and have almost fully compacted. An area of differentiation is present (*white oval*). These colonies are ready to be manually passaged (both ×4 magnification)

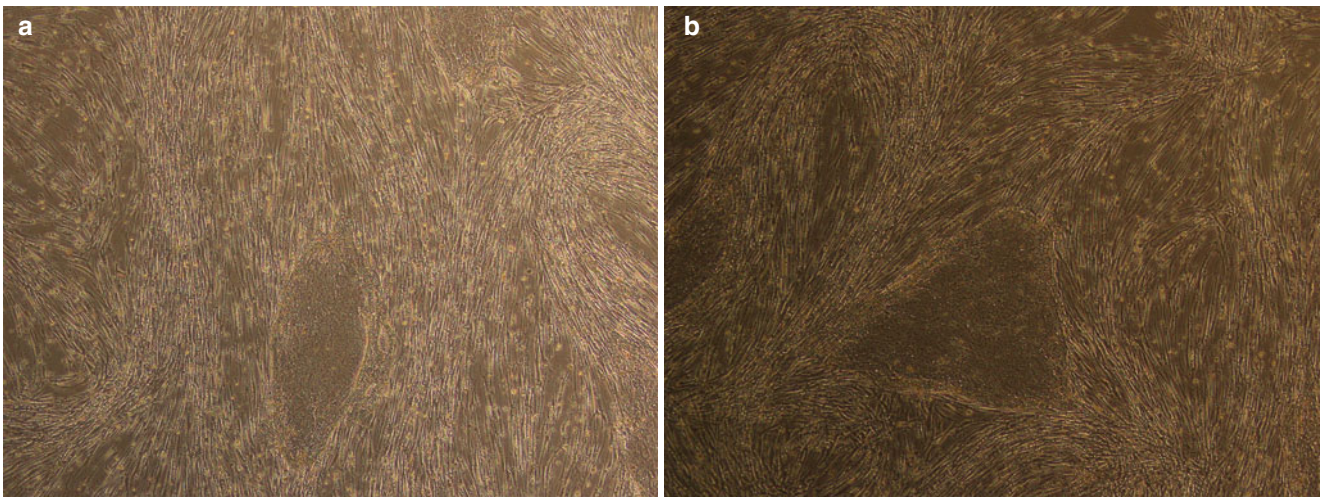

Fig. 5.26 (**a**, **b**) Colonies 8 days after passage. On both images, note the quality of the feeders, which are degenerating. These colonies should have been passaged earlier, but they still can be passaged if more optimal cultures are not available. This would not be the case for cells grown feeder-free; such cells from older colonies tend to detach from the plates once they are past their prime condition (both ×4 magnification)

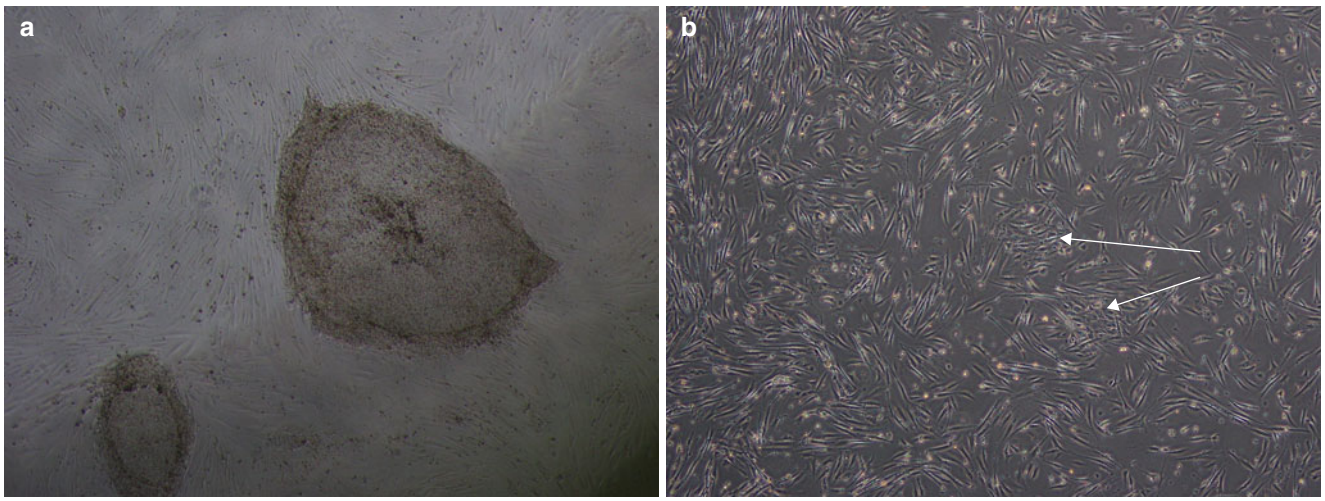

Fig. 5.27 (**a**) Colonies at the edges of the cell culture dish. Very good colonies often can be seen growing towards the edges of a cell culture dish and should be sought if colonies are not found in the middle portion of the dish. (**b**) This image shows human feeders onto which enzymatically treated iPSCs were passaged 24 h earlier. Small colonies (*white arrows*) can be seen growing on the feeders (both ×4 magnification)

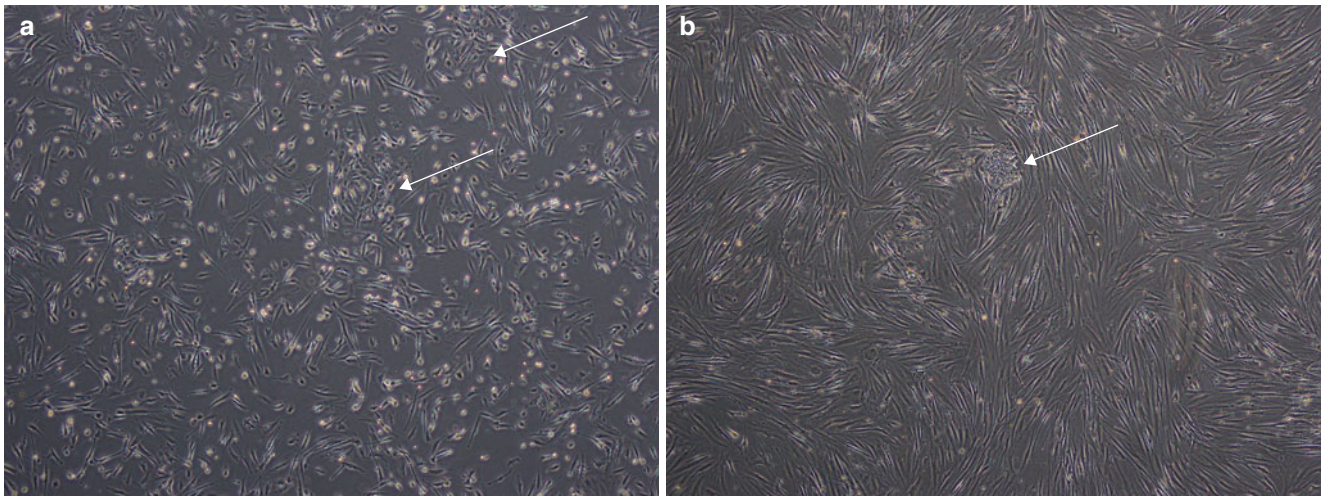

Fig. 5.28 (**a**) Emergence of colonies (*white arrows*) on human feeders 24 h after enzymatic passaging. (**b**) A colony of iPSCs in the same well as (**a**), 2 days after enzymatic treatment (*white arrow*). Note the density of the feeders. Human fibroblasts tend to be more resistant than mouse feeders to inactivation by mitomycin C, so human feeder density often increases with time (both ×4 magnification)

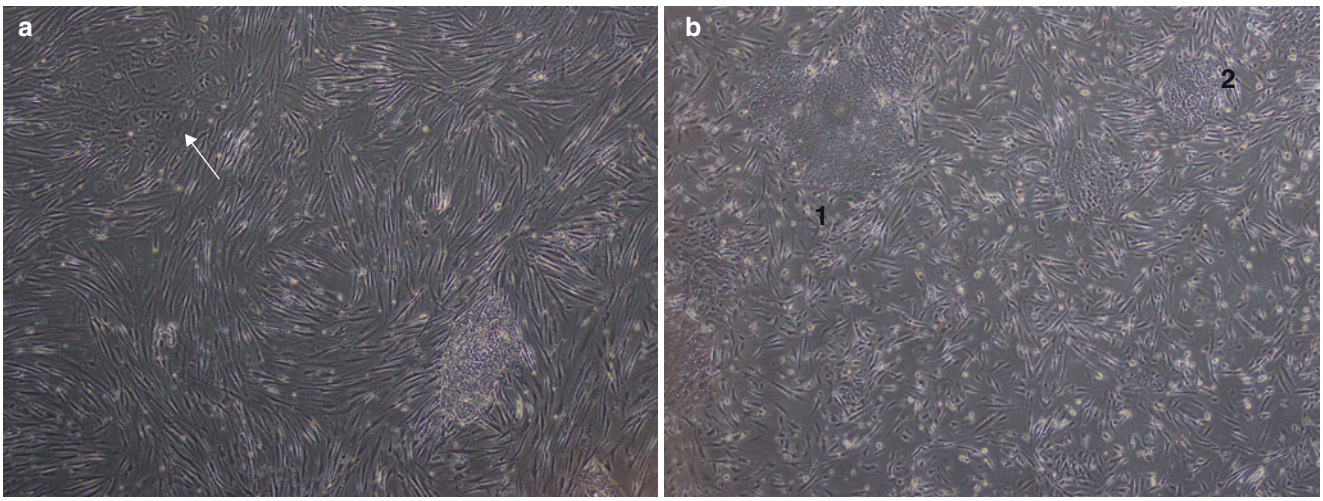

Fig. 5.29 (**a**) A compacting colony of iPSCs, 3 days after enzymatic passage onto human feeders. Note the differentiated colony (*white arrow*), with cells that are large and morphologically different from those seen in the iPSC colony. (**b**) A culture after 4 days. Colony *1* has compacted, and colony *2* is starting to compact (both ×4 magnification)

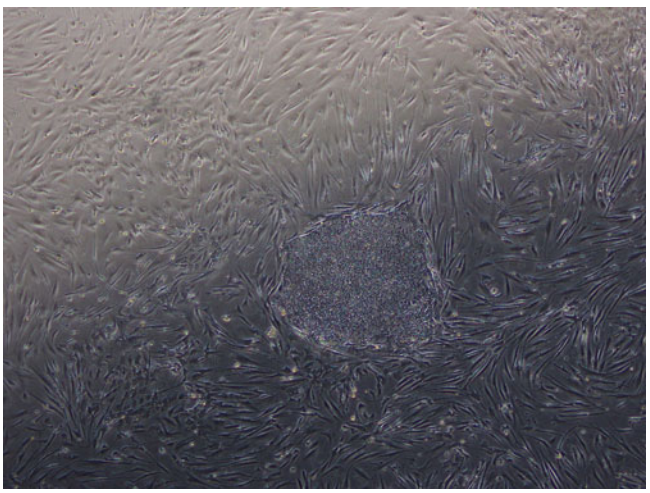

Fig. 5.30 The 5-day-old iPSC colony in this image is towards the edge of the six-well plate. It is compacted and undifferentiated, and is ready to passage enzymatically onto fresh human feeders (×4 magnification)

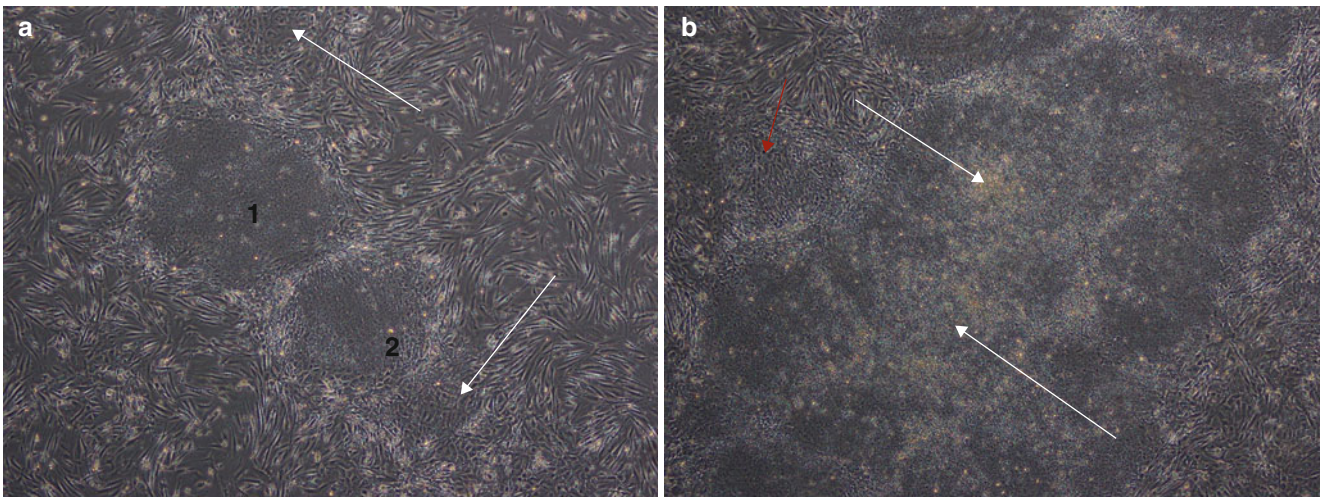

Fig. 5.31 (**a**) These iPSC colonies are 5 days old. They have been passaged enzymatically. The cells in colony *1* are undifferentiated and compacted, whereas those in colony *2* are stretched in the centre and poised to differentiate, and there are signs of differentiation around the edges of the colony. Two colonies have fully differentiated; these can be distinguished by their changed morphology (*white arrows*). (**b**) A number of compacted, undifferentiated, 10-day-old iPSC colonies on human feeders have fused together, and the cells are beginning to pile up on one another. This piling will promote differentiation and should be removed before enzymatic passaging. A differentiated colony (*red arrow*) on the edge of the fused colonies also should be removed prior to passaging. The occurrence of fused colonies in the centre of the plate is often due to a failure to distribute the dissociated cells properly after passaging, resulting in the pooling of cells in the centre of the six-well plate (*white arrows*) (both ×4 magnification)

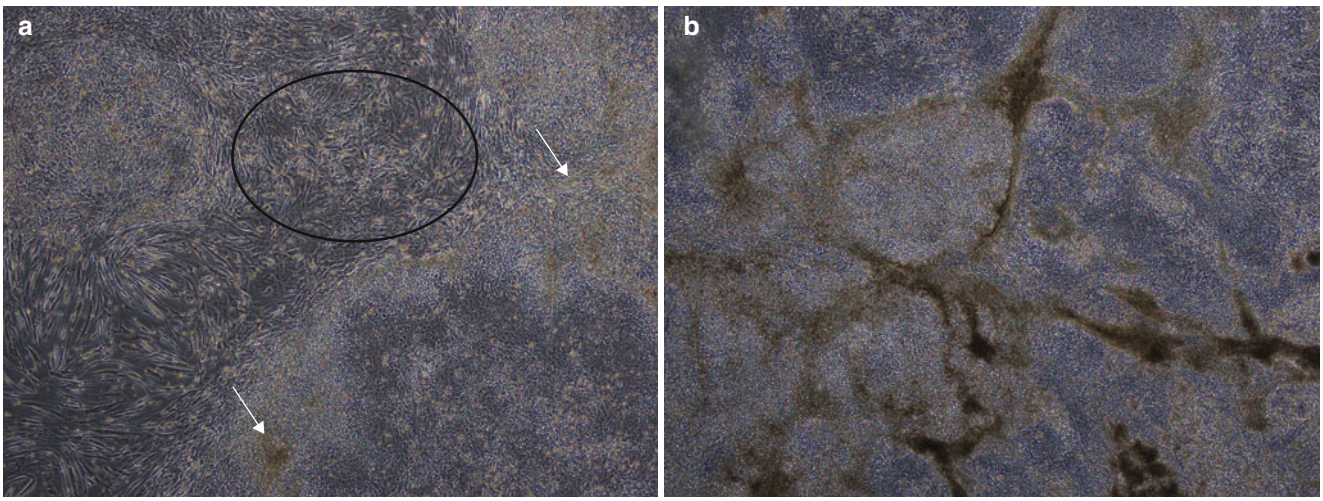

Fig. 5.32 (**a**) Two enzymatically passaged iPSC colonies that are 7 days old. Where the cells have piled up, areas of differentiation are seen (*white arrows*). It is also hard to distinguish human feeders from differentiated elements in some areas of the culture (*black oval*). (**b**) These enzymatically treated iPSC have been growing on human feeders for 10 days. The culture is very overgrown. Colonies have fused and cells have piled up along the borders of the colonies and within the colonies, promoting differentiation. Areas of undifferentiated cells remain scattered throughout the culture, however (both ×4 magnification)

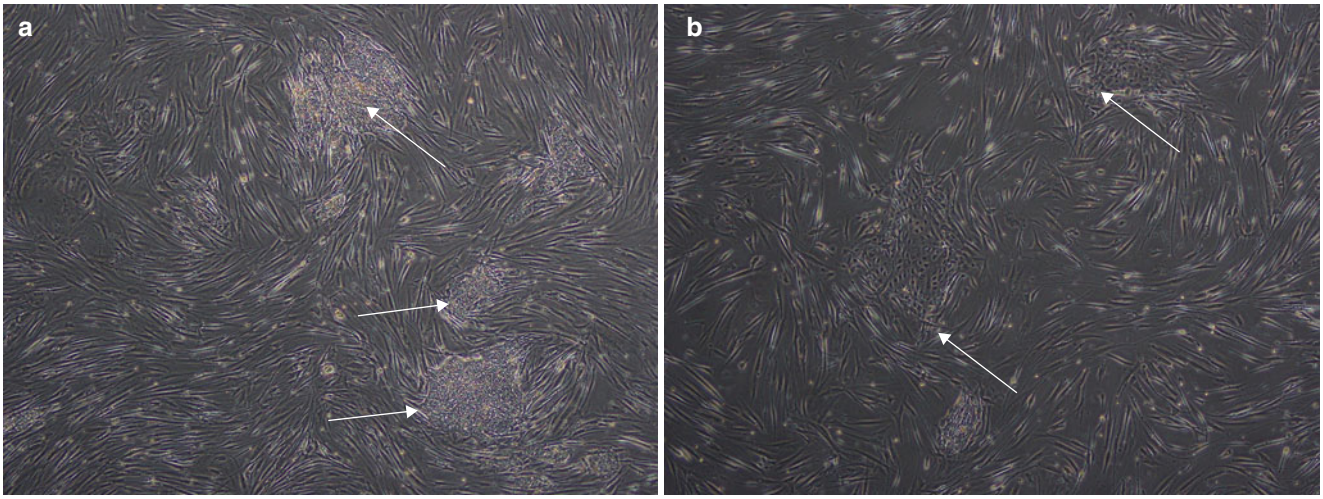

Fig. 5.33 (**a**) The colonies of iPSCs in this image are 3 days old (*white arrows*). Although the colonies are small, areas of cells piling up can be observed, and the colonies will begin to differentiate within a couple of days. (**b**) These 4-day-old iPSCs (*white arrows*) have differentiated after being passaged onto a lower density of human feeders than normally used to maintain them (both ×4 magnification)

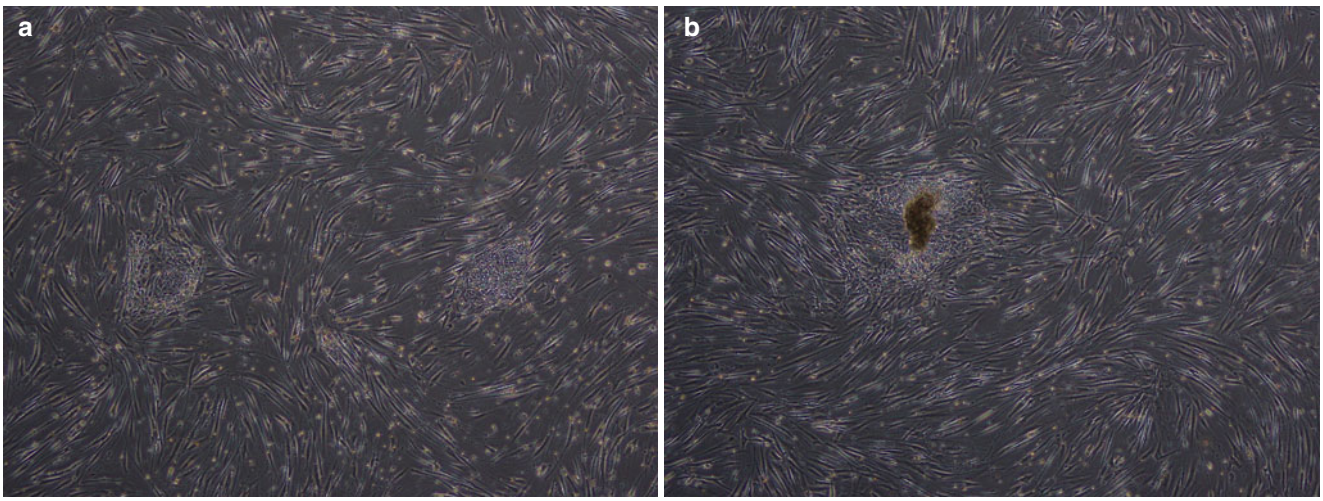

Fig. 5.34 (**a**) Two 3-day-old iPSC colonies growing on human feeders produced from a human embryonic fibroblast line. (**b**) This iPSC colony is from a manually passaged culture and shows a piling up of cells due to the carryover of feeders. The colony is disorganised, has started to differentiate along the edges and displays stretched cells that are poised to differentiate (both ×4 magnification)

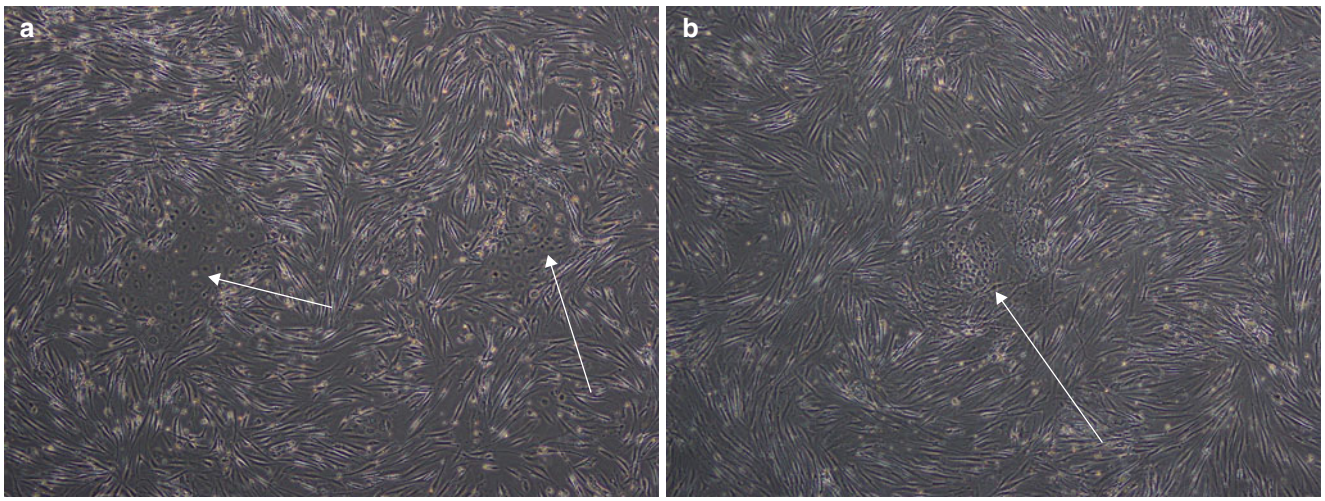

Fig. 5.35 (**a**) Enzymatically treated iPSCs that have been growing on human feeders for 5 days and have differentiated (*white arrows*). Note the morphology of the cells in this differentiated colony: they are large cells and no longer display large nucleoli. (**b**) This 4-day-old, enzymatically passaged colony is also differentiated (*white arrow*). Again, note how the morphology differs from the undifferentiated iPSCs (both ×4 magnification)

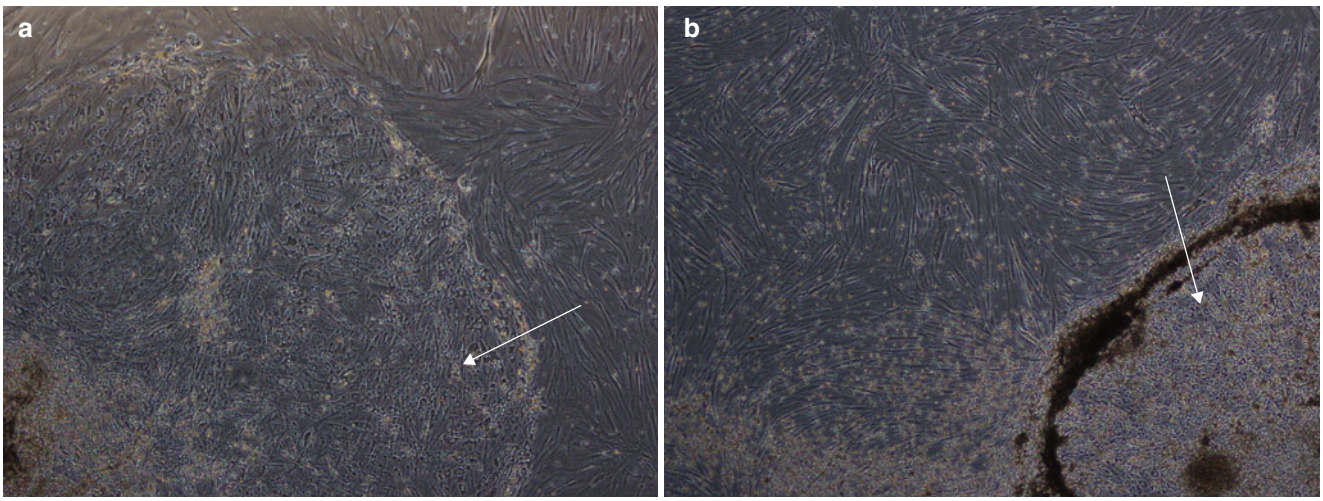

Fig. 5.36 (**a**, **b**) These two images show enzymatically treated iPSC colonies grown for 18 days on human feeders. Unlike similar colonies grown feeder-free, which tend to lift off the cell culture dish when left in culture after they have reached confluency, these cells grown on human feeders will stay attached to the feeders, maintaining the shape of the colony, with well-defined edges. However, these colonies can be seen to have differentiated, and areas of cells have become apoptotic (*white arrows*). If passaged onto fresh feeders, they will fail to adhere (both ×4 magnification)

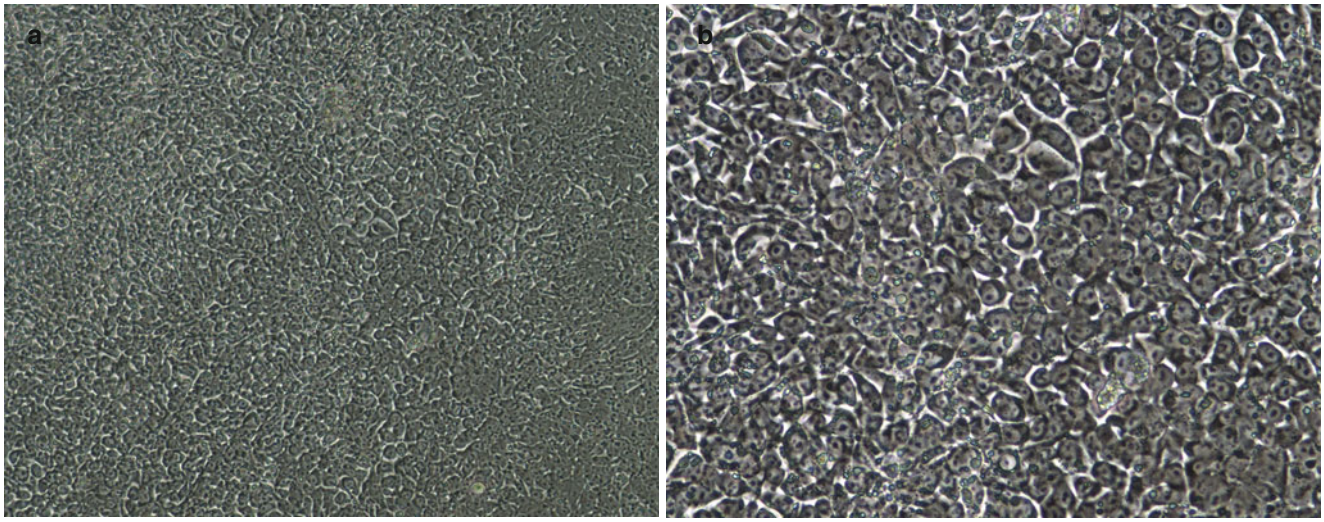

Fig. 5.37 Challenge: what can you say about this figures (**a**) and (**b**)? See page 199 for answer

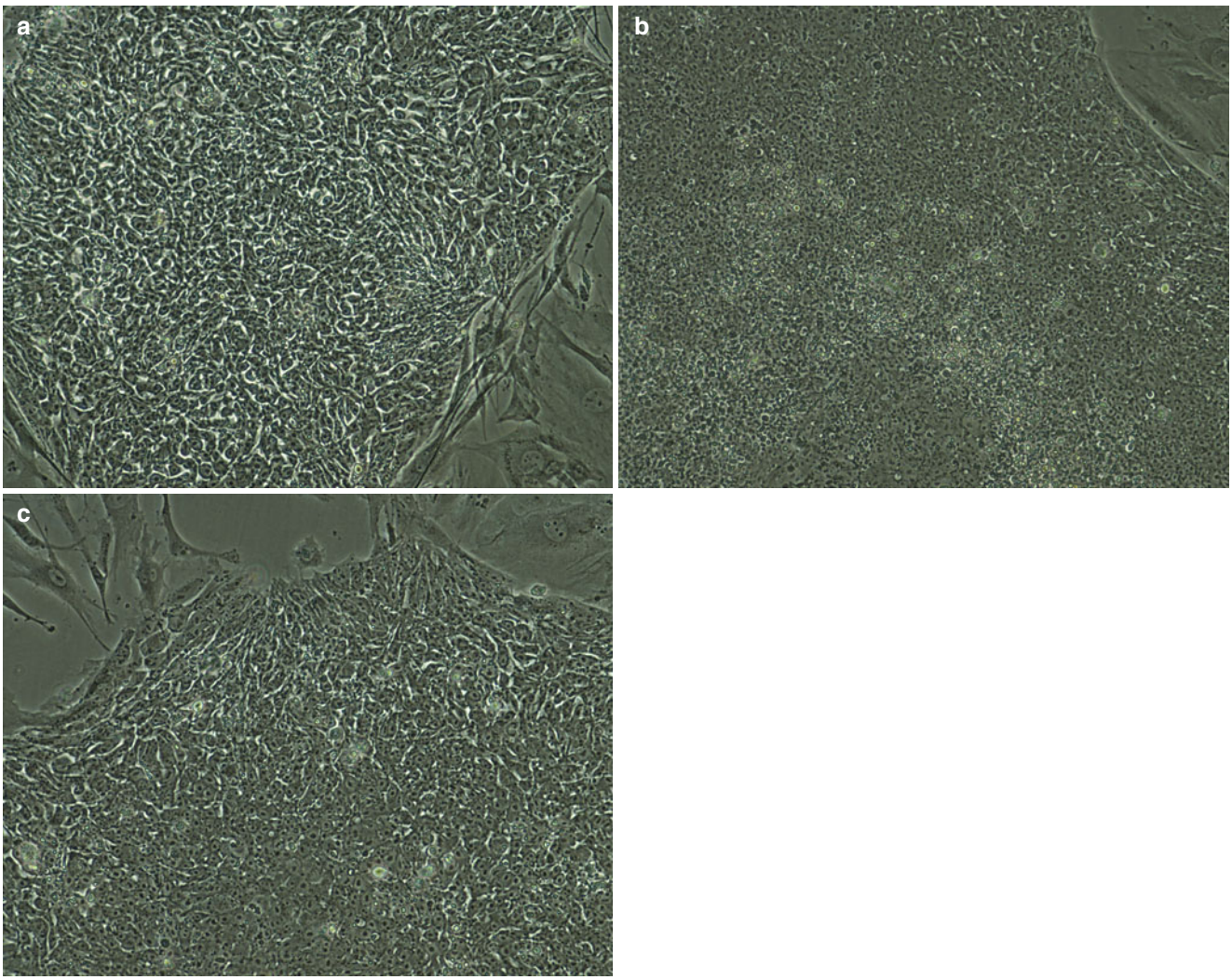

Fig. 5.38 Challenge: what can you say about figures (**a**), (**b**) and (**c**)? See page 199 for answer

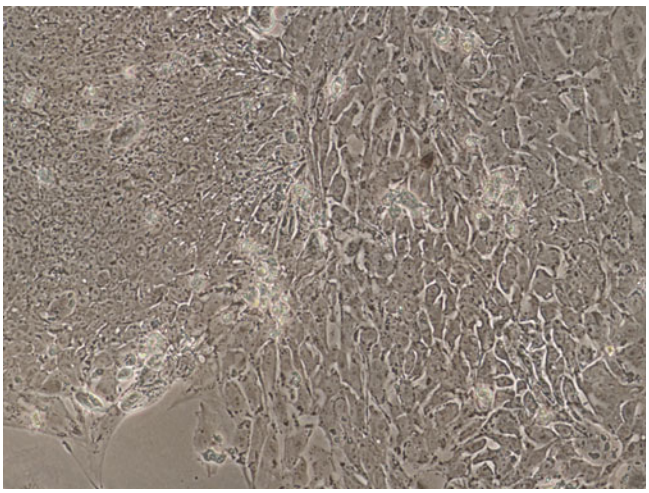

Fig. 5.39 Challenge: what can you say about this figure? See page 199 for answer

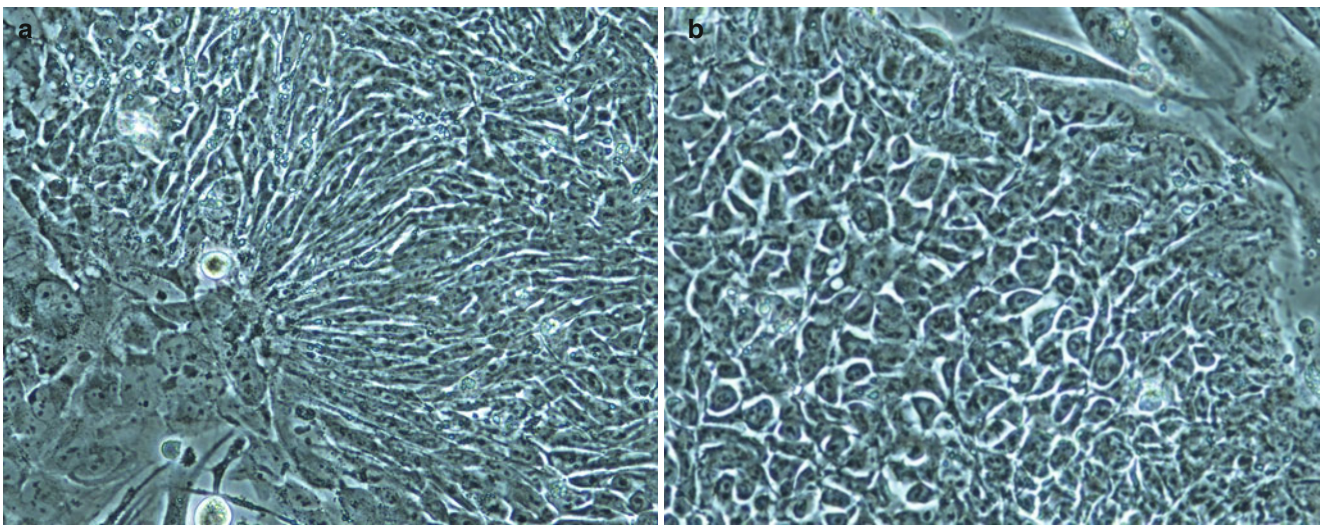

Fig. 5.40 Challenge: what can you say about figures (**a**) and (**b**)? See page 199 for answer

Suggested Reading

Amit M, Itskovitz-Eldor J. Morphology of human embryonic and induced pluripotent stem cell colonies cultured with feeders. In: Amit M, Itskovitz-Eldor J, editors. Atlas of human pluripotent stem cells: derivation and culturing, Stem cell biology and regenerative medicine. New York: Humana Press; 2012. p. 15–39.

Johkura K, Cui L, Asanuma K, Okouchi Y, Ogiwara N, Sasaki K. Cytochemical and ultrastructural characterization of growing colonies of human embryonic stem cells. J Anat. 2004;205: 247–55.

Sathananthan H, Pera M, Trounson A. The fine structure of human embryonic stem cells. Reprod Biomed Online. 2002;4:56–61.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131:861–72.

Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126:663–76.

Pluripotent Cell Lines Grown on Different Substrates and Surfaces

6

As stated in Chap. 2, the use of inactivated fibroblasts as feeders for the derivation, expansion and maintenance of pluripotent stem cells (PSCs) is still the predominant method used in most laboratories. PSCs hold great promise for clinical utility in the field of regenerative medicine and are useful tools in areas such as toxicology and the development of disease models. However, these applications require defined and refined culture systems, which are essential to ensure that processes and assay systems are reliable, reproducible, robust, efficacious and safe. A feeder-free system also facilitates activities such as the scale-up of processes and high-throughput screening. In the clinical setting, the ultimate goal is to produce a culture system that is xeno-free (free of animal products), in which the culture medium is chemically defined and the cell culture substrate is a defined synthetic matrix. Amongst the advantages of defined xeno-free culture systems is the amelioration of intrinsic batch-to-batch variation introduced into the system via the use of biological products, as well as a reduction in the risk of contamination with viruses associated with animal products.

Cell culture systems have been evolving to satisfy the needs and requirements of the scientific and clinical communities. Feeder-free systems are generally more expensive than the feeder-based systems, but this cost must be weighed against the purpose and utilisation of the culture system and should not compromise the quality or function of the cells grown in the culture system. A number of different culture systems are currently available. Some systems combine an undefined biological matrix, such as Matrigel™, with an undefined conditioned medium or defined culture medium. Other systems combine defined matrices such as fibronectin, vitronectin or laminin (in either a native or recombinant form) with defined or undefined media. Recently, synthetic coatings comprising polymers or other engineered substrates, usually presented on plastic surfaces, have been used with defined media.

As will be discussed in Chap. 10, when moving from one type of culture system to another, the cells need to be adapted. Briefly, this adaptation process will vary from cell line to cell line, and the change in colony morphology is a key indicator in the assessment of this adaptation process. This process may take a number of passages before the cells settle down to produce a morphology indicative of an optimised culture system, and as part of this adaptation process, a number of parameters must be addressed, including the method of passaging and the time between passages.

In feeder-free systems, defined media may vary in their composition and concentration of components such as growth factors and chemicals, and these changes will have a profound effect on the maintenance of a cell line. In general, commercial suppliers will have optimised media to be used in conjunction with defined substrates and enzymes, in order to maximise the performance of a system. Nevertheless, it should be stressed that changes in the parameters of a cell culture system must be assessed on a cell line-by-cell line basis.

The images in this chapter look at a number of PSC lines that have been adapted from feeder-based to feeder-free culture systems. It is easier to see the evolution and morphology of the colonies in feeder-free systems than in feeder-based systems, in which the feeders can interfere with the observation and assessment of the status of the colonies. In the feeder-free systems, colonies often appear larger and looser than in the presence of feeders, but as with the colonies in feeder-based systems, as the cells in the colonies divide, the individual cells compact and produce the typical PSC colony morphology (Figs. 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 6.10, 6.11, 6.12, 6.13, 6.14, 6.15, 6.16 and 6.17). These images showing two individual cell lines that have been adapted and grown for 20 passages on commercially available STEMPRO™ hESC SFM a fully-defined feeder free medium.

L. Healy, L. Ruban, *Atlas of Human Pluripotent Stem Cells in Culture*,
DOI 10.1007/978-1-4899-7507-2_6, © Springer Science+Business Media New York 2015

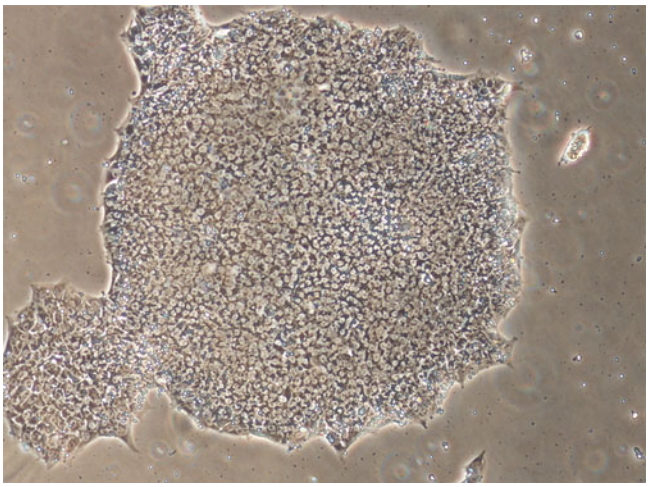

Fig. 6.1 Very good feeder-free culture of a human embryonic stem cell (hESC) line. Small and large colonies merge, and cells within the colony are compacted and roundish in shape, with large nuclei, notable nucleoli and spaces between cells on the edges (×4 magnification)

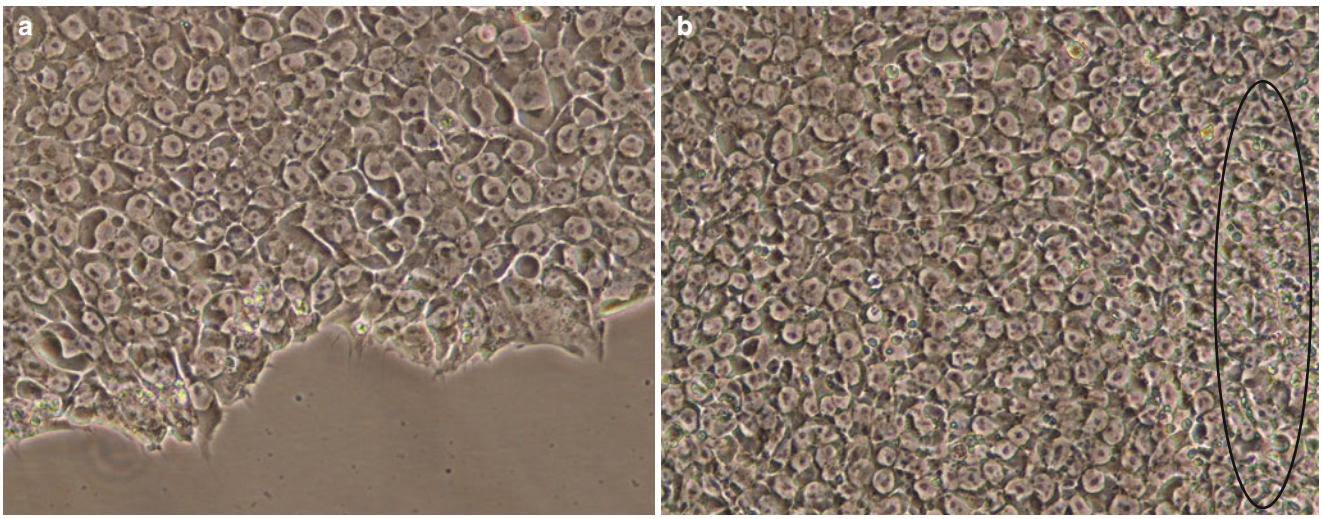

Fig. 6.2 (**a**) Undifferentiated cells at the edge of a colony with spaces between the cells. (**b**) Typical morphology of good-quality, undifferentiated stem cells within the centre of the same colony. Cells are overcompacted (*inside black circle*). This culture is ready for passage (both ×10 magnification)

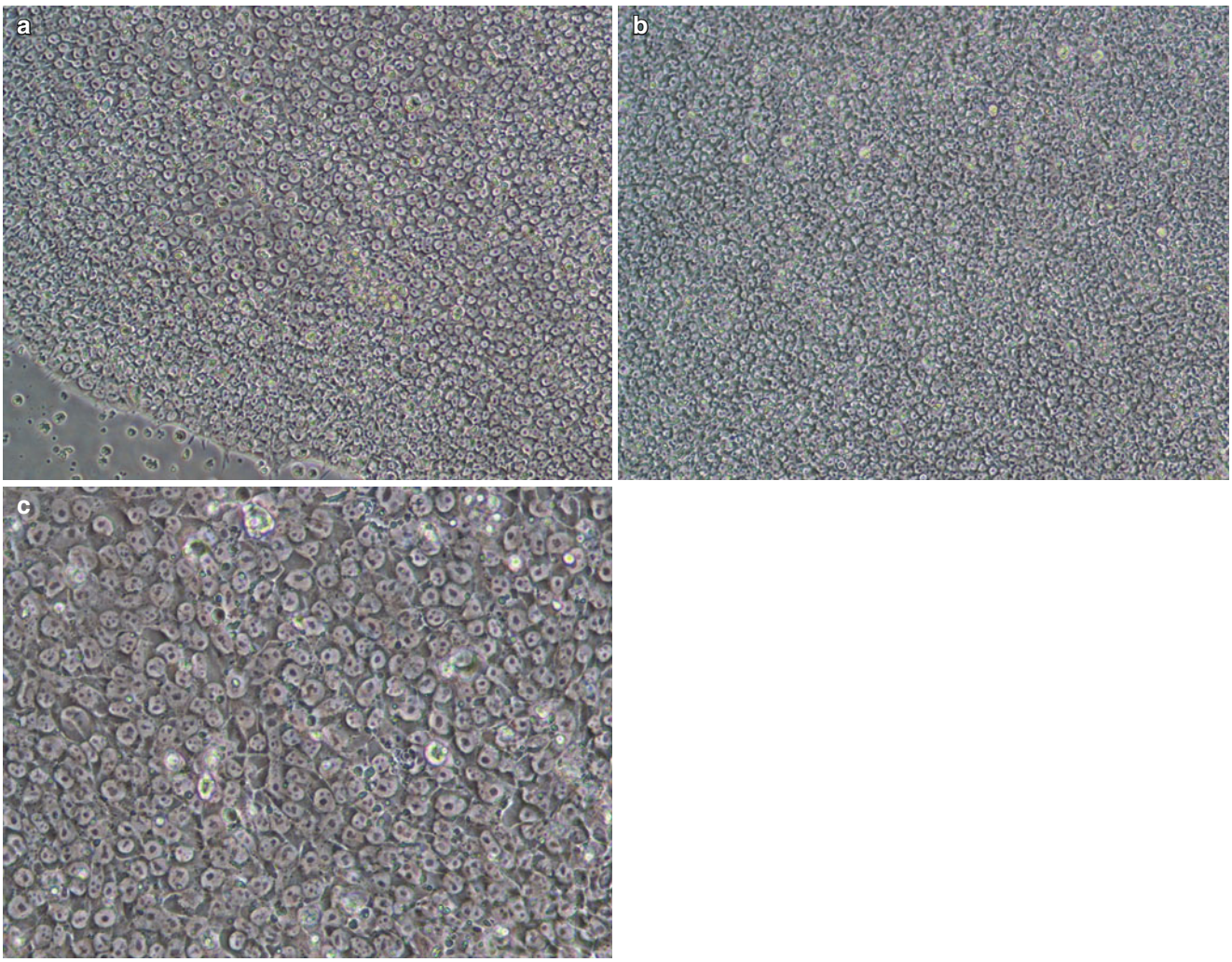

Fig. 6.3 Good-quality, undifferentiated cells on the edge of a colony (**a**) and in the middle of the colony (**b**, **c**) (**a**, **b**, ×10 magnification; **c**, ×20)

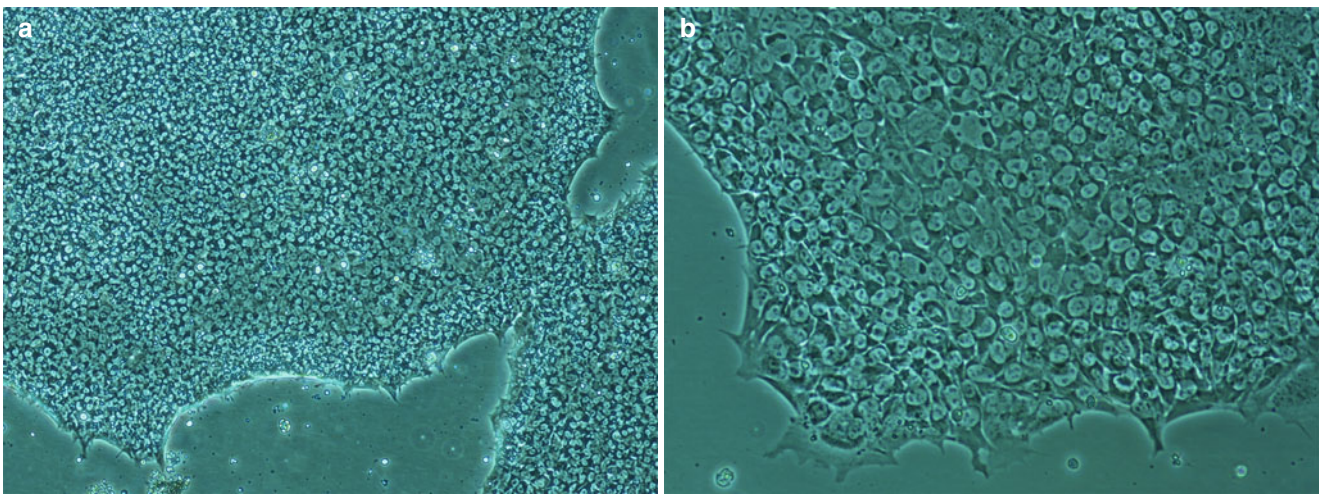

Fig. 6.4 (**a**) Two large colonies composed of compact cells, which are merging. The colony is ready to be passaged. (**b**) A compacted, undifferentiated colony (**a**, ×10 magnification; **b**, ×20)

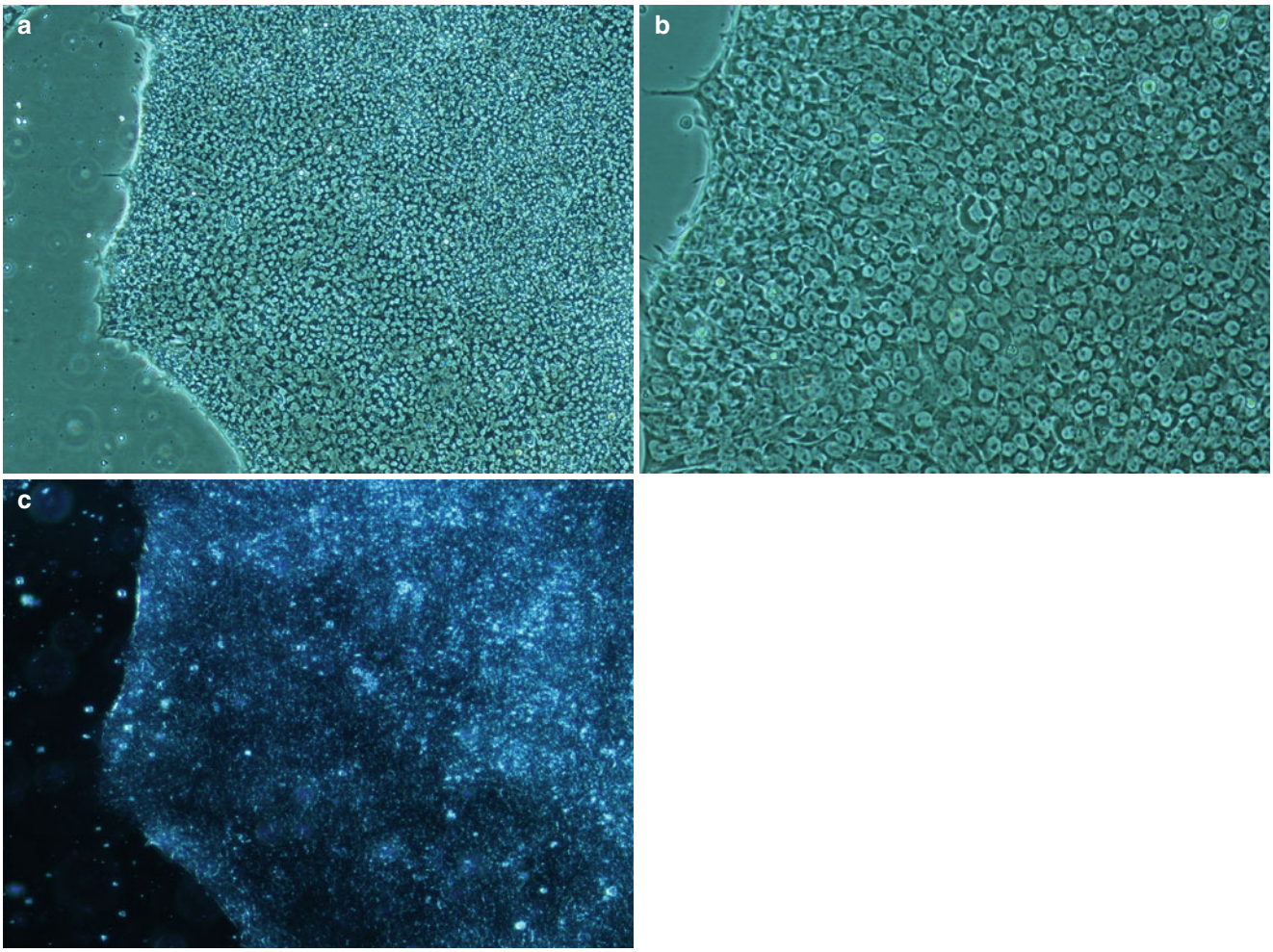

Fig. 6.5 (**a**–**c**) Another example of good-quality pluripotent stem cells (PSCs) in a feeder-free culture system. Comparing the brightfield and darkfield images (**a** *vs* **c**) makes it easy to see the overcompacted cells and multiple layers of cells in the centre of this colony, where differentiation will start to appear, probably within 24 h. Than the rest of the colony. This culture is ready to passage (**a**, ×10 magnification; **b**, ×20; **c**, darkfield image, ×10)

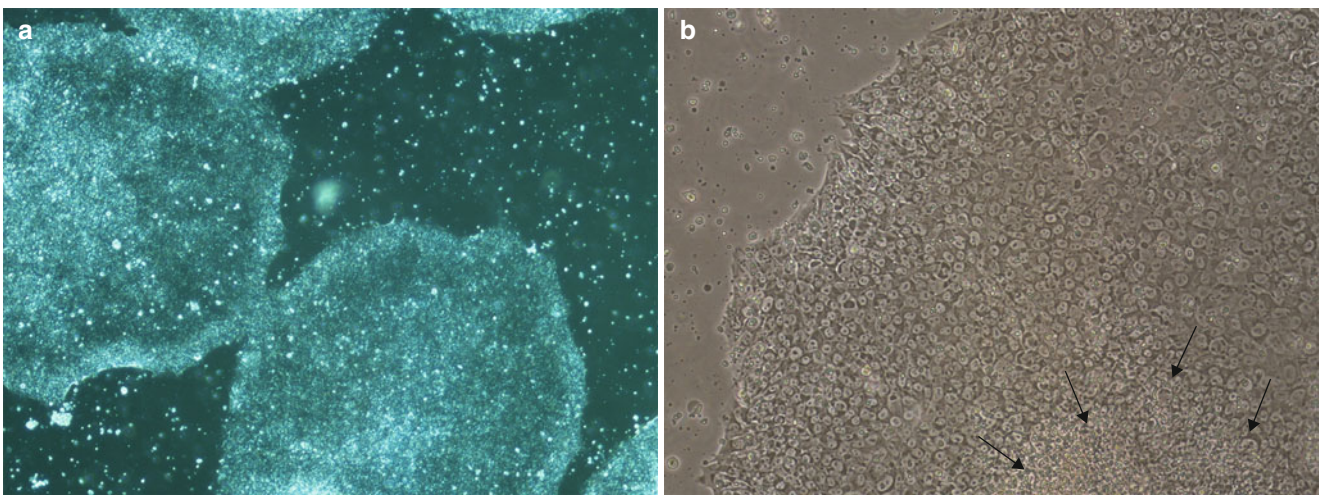

Fig. 6.6 (**a**) Another example of a good-quality, feeder-free culture. The centres of these colonies have overcompacted and look whiter than the rest of the colonies in this darkfield image because of the increased thickness as the cells start to multilayer. (**b**) In this image, the overcompacted centre of the colony (*black arrows*) can be observed (**a**, ×4 magnification; **b**, ×10)

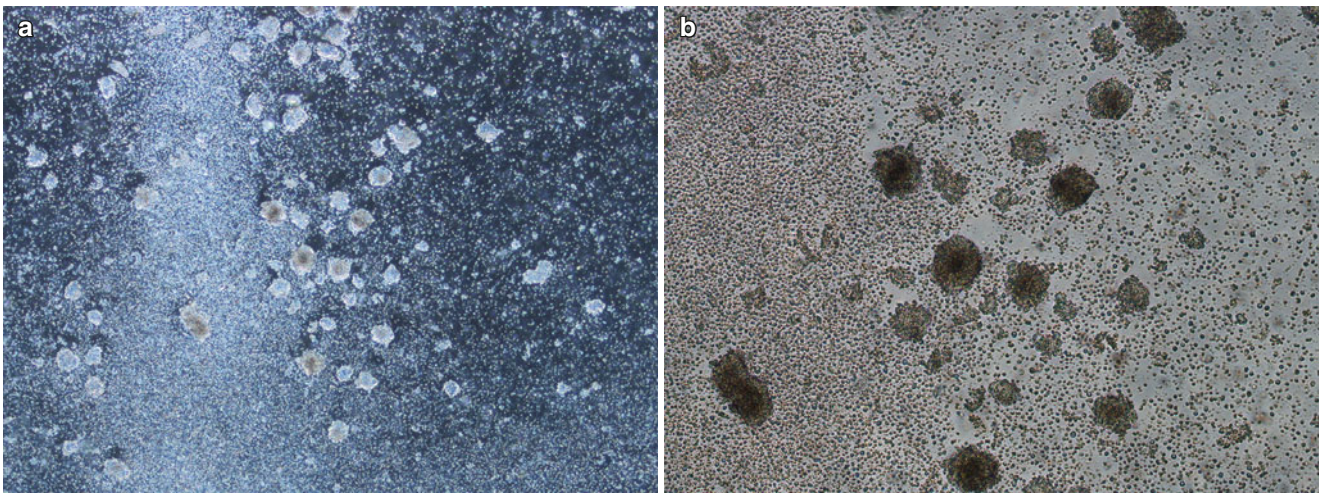

Fig. 6.7 (**a**, **b**) Images showing the size of clumps produced for passaging following mechanical scraping. The clumps are never uniform or similar in size. Avoid passaging very large or very small clumps, as these are prone to differentiate following attachment (**a**, ×4 magnification; **b**, ×10)

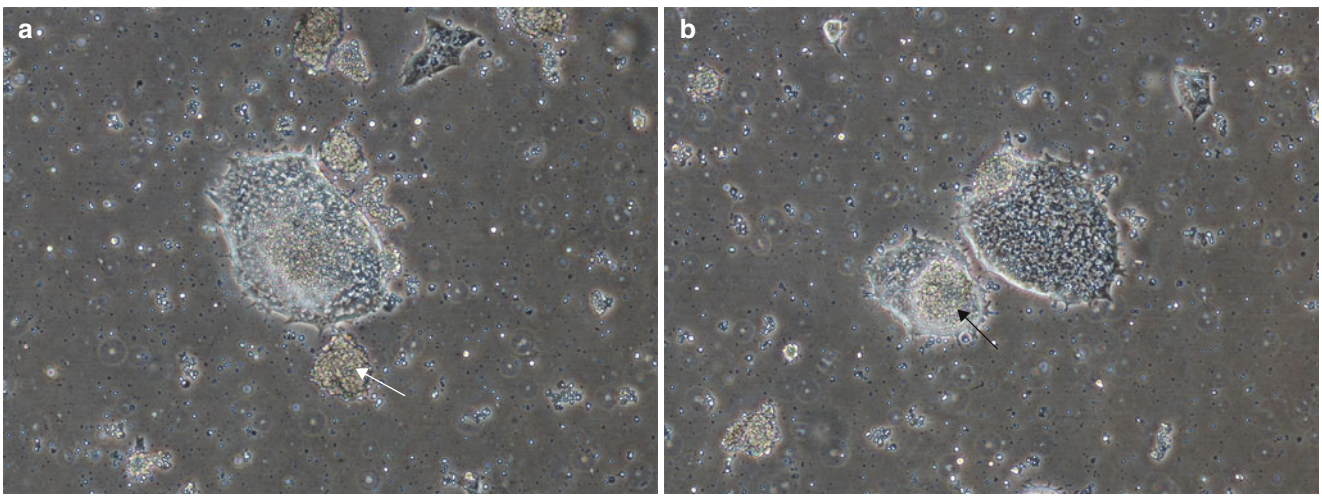

Fig. 6.8 Colonies of cells 24 h after passaging. The day after passaging, stem cells never look good, whether in feeder or feeder-free systems. (**a**) A big clump of cells has attached, but a smaller one (*white arrow*) has not attached and is floating. (**b**) Sometimes part of an attached clump resembles a sphere or an embryoid body (*black arrow*), but with time, they may spread and form very good colonies (both ×10 magnification)

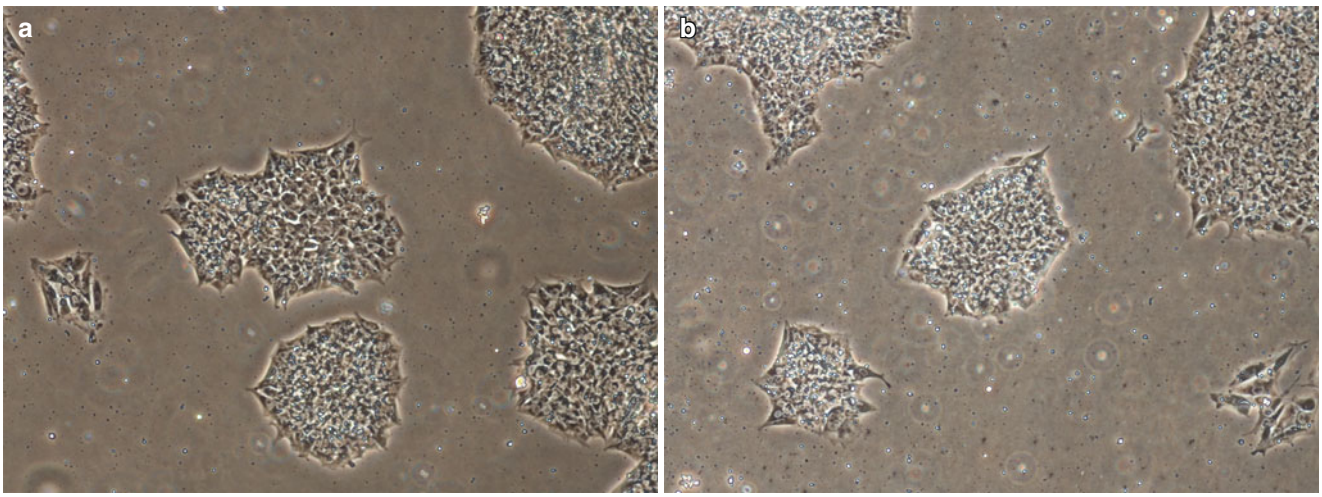

Fig. 6.9 (**a**, **b**) Examples of very good feeder-free cultures, 24 h after passaging. The colonies are small, and after adhesion have spread out (both ×10 magnification)

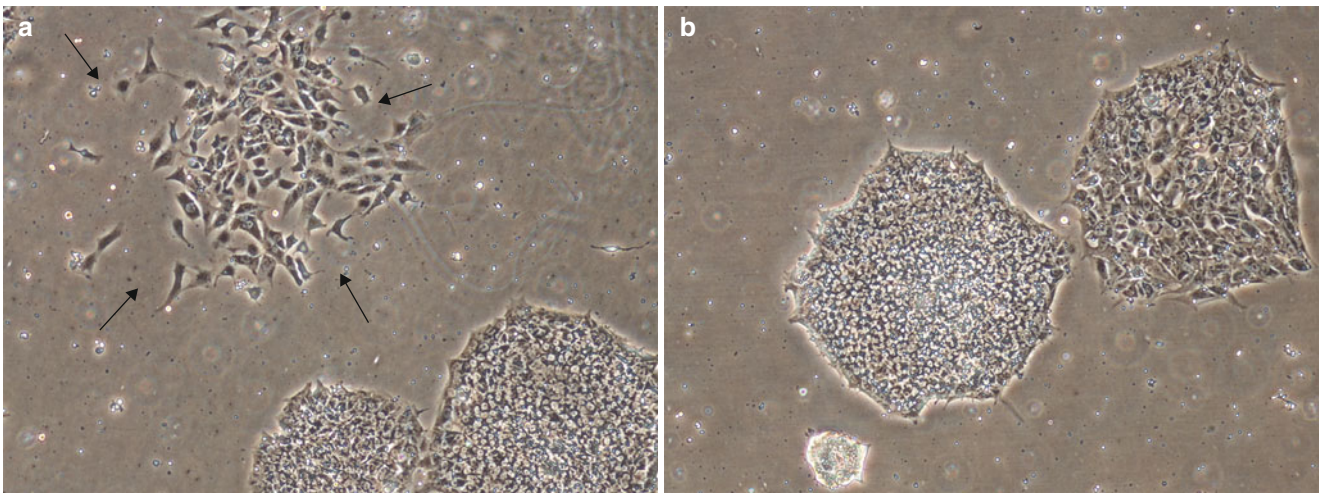

Fig. 6.10 (**a**) Two small, compacted colonies are merging in the bottom of this image. One group of cells has not compacted (*black arrows*) but in time will form a good, compacted colony. This non-compact colony would never survive on feeders because the cells are far away from each other and would differentiate almost immediately, but a good-quality matrix will keep these spread colonies in an undifferentiated state. (**b**) Two colonies: one compacted (*on the left*) and one yet to compact (*on the right*) (both ×10 magnification)

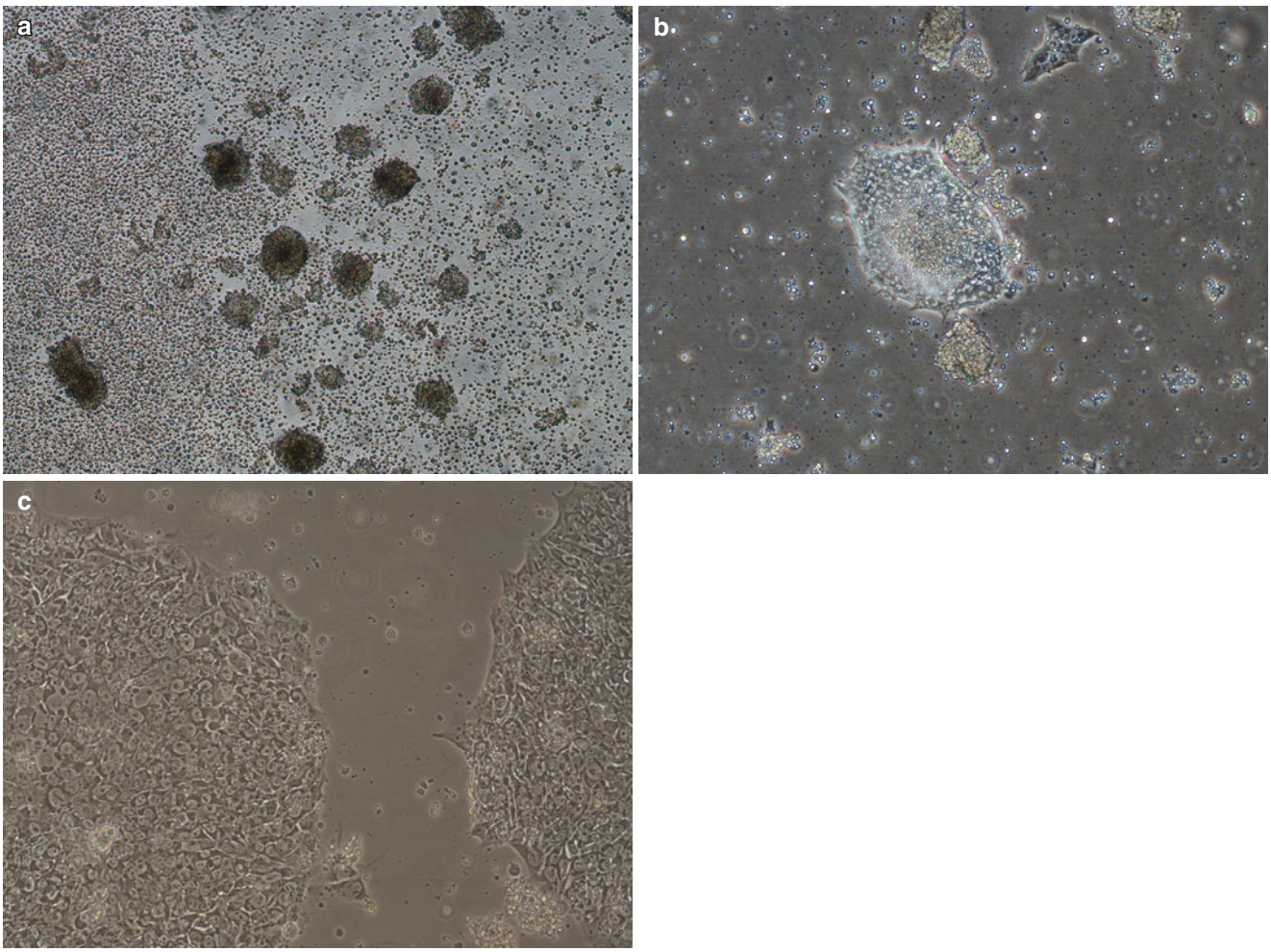

Fig. 6.11 A sequence of images: clumps after mechanical scraping and passaging (**a**), 24 h after passaging (**b**), and 48 h after passaging (**c**) (**a**, **b**, ×4 magnification; **c**, ×10)

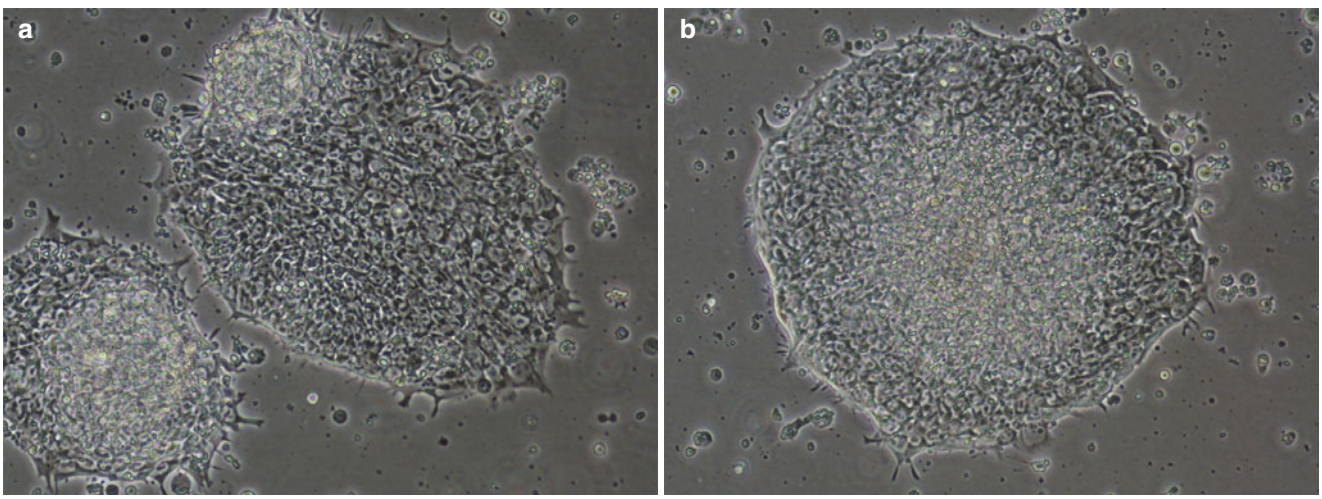

Fig. 6.12 Cells 24 h after passaging. Cells are growing on top of each other in a small colony (**a**) and in the centre of the colony (**b**). After 24 or 48 h, they will spread and form good colonies (both ×10 magnification)

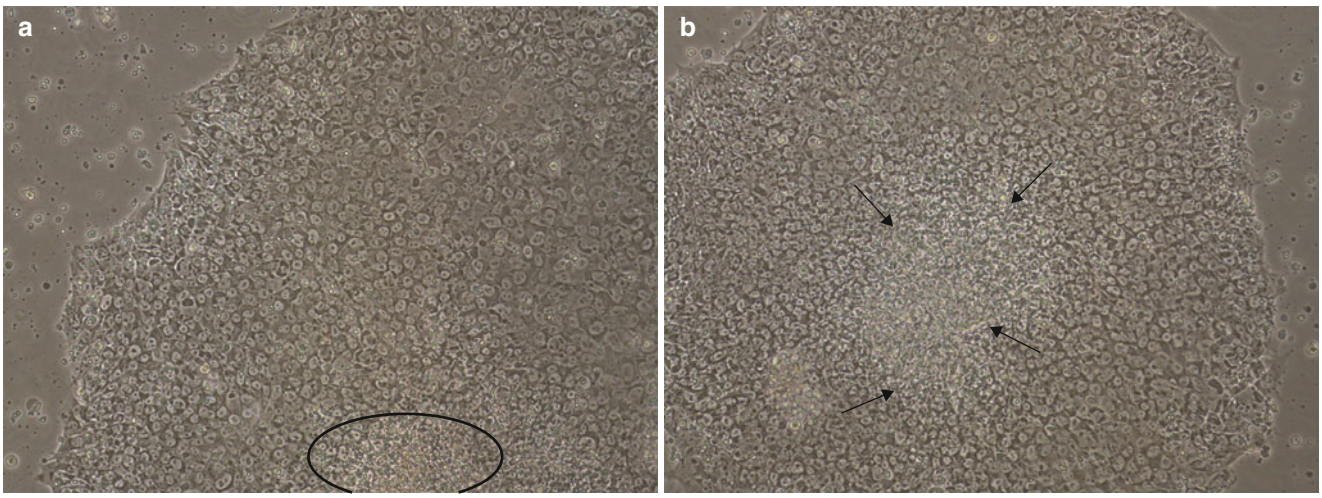

Fig. 6.13 (**a**, **b**) Images of the same colony showing an overcompacted area in the centre of the colony (*black circle* and *black arrows*) (both ×10 magnification)

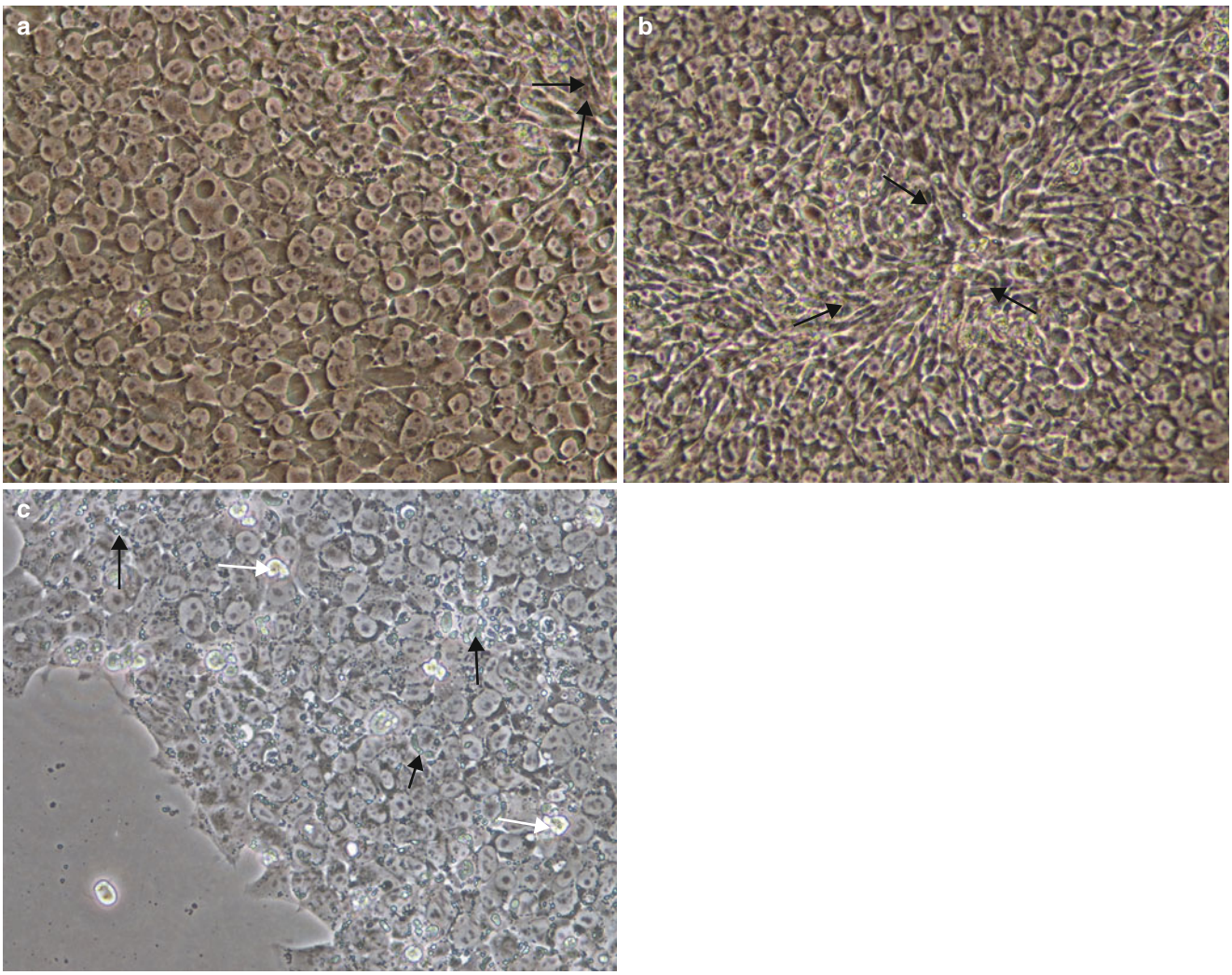

Fig. 6.14 (**a**, **b**) Areas in colonies demonstrating cells assembled into rosette-like structures (*black arrows*). (**c**) A slightly older colony exhibiting cells of different shapes: some are becoming apoptotic and popping out from the colony (*white arrows*), and others have developed lipid structures inside of cytoplasm (*black arrows*). These differences in morphology indicate cell stress (all ×20 magnification)

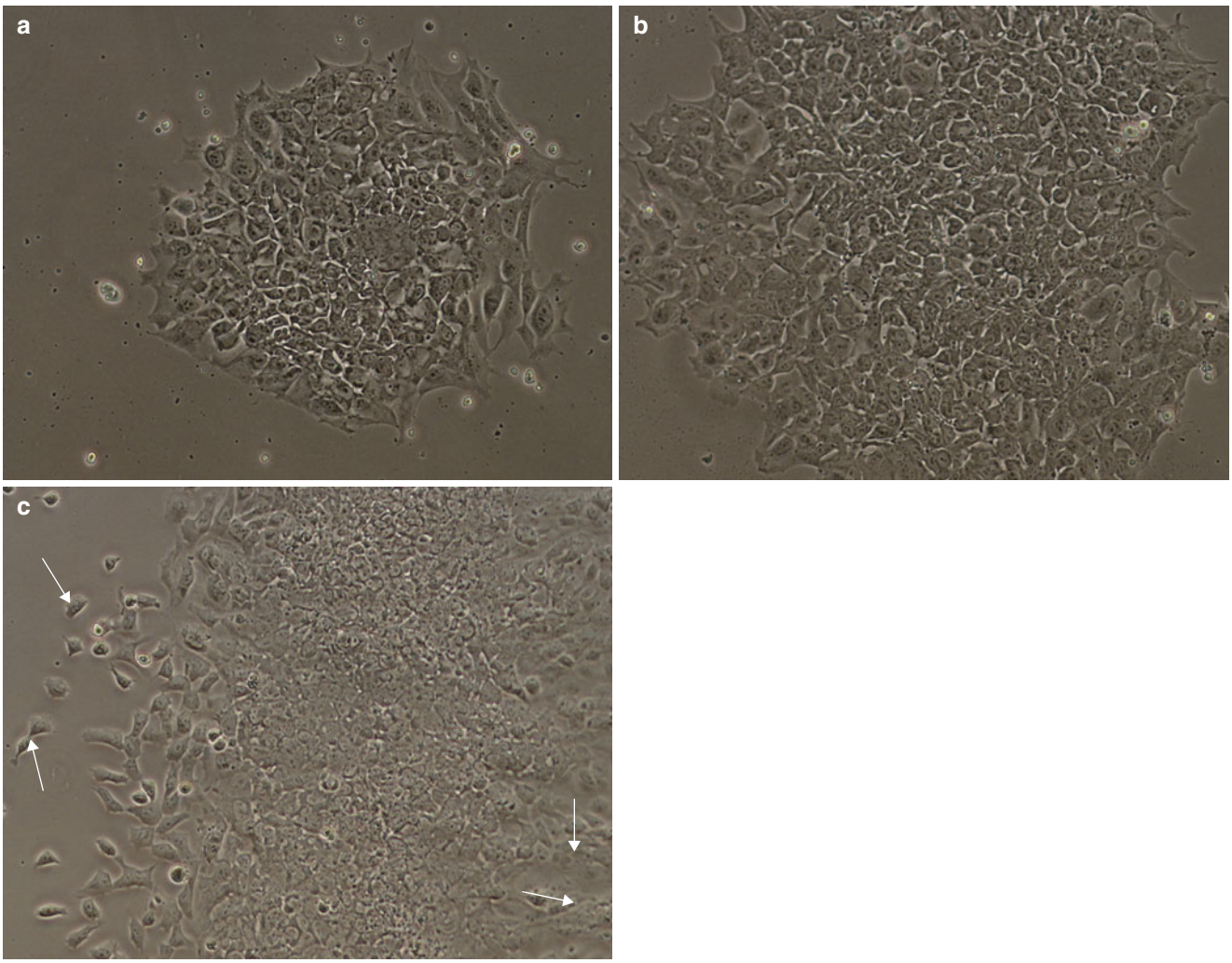

Fig. 6.15 (**a**) A small colony in which the cells are beginning to compact in the centre. (**b**) A colony in which the cells are not yet compacted and are actively proliferating. (**c**) An old colony with differentiated cells in the centre and on the edges (*white arrows*) (all ×10 magnification)

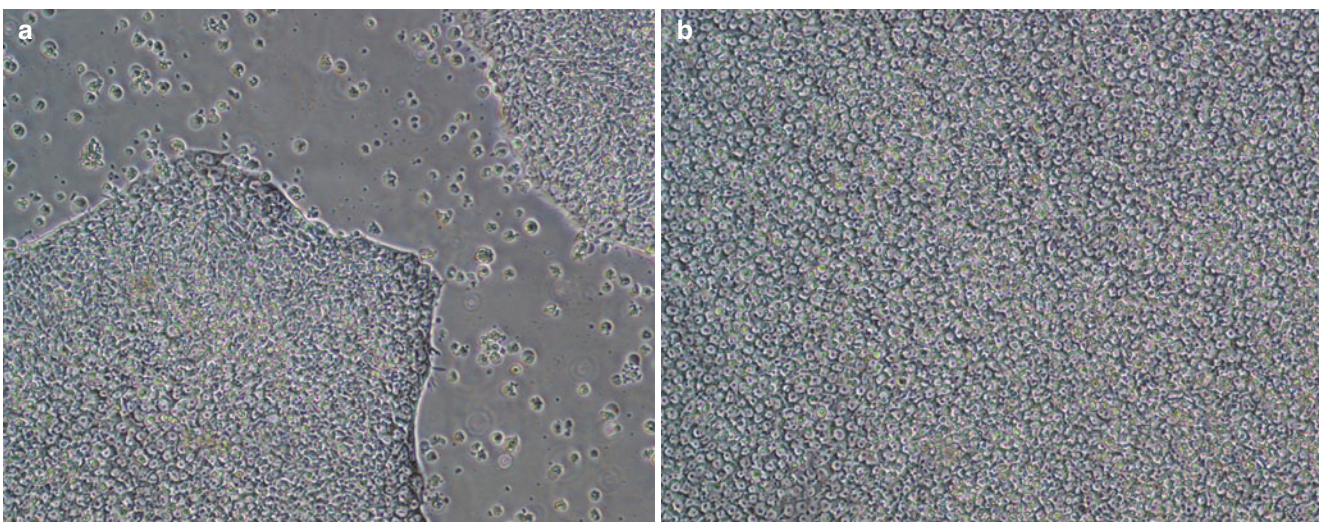

Fig. 6.16 (**a**, **b**) These images show a relatively good feeder-free culture. However, the colony has areas where the cells are overgrown and over-compacted. It would have been advisable to passage the cells 24 h before this image was captured (both ×10 magnification)

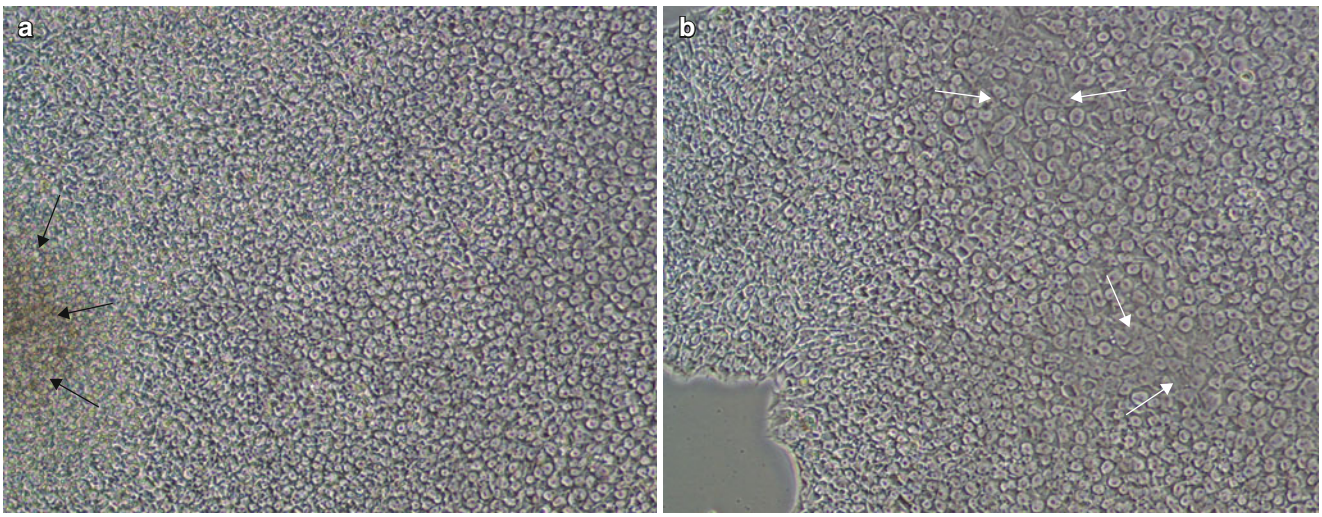

Fig. 6.17 (**a**) Part of a colony where cells are growing on top of each other (*black arrows*) but are still undifferentiated. This colony could be manually passaged by cutting the colony into small pieces and passaging the pieces onto a fresh matrix. (**b**) An example of a good, compacted colony, which should be passaged. If it is left for another 24 h, the part of the colony where cells are larger and more flattened (*white arrows*) would be prone to differentiation (both ×10 magnification)

The chapter also includes a number of images (Figs. 6.18, 6.19, 6.20 and 6.21) from an adapted "difficult-to-grow" cell line. In all cases, if the colonies are overconfluent or overcompacted, they will differentiate spontaneously into rosette-like structures. Obviously, the particular cell line shown in these images is very easy to direct into different neural cell types.

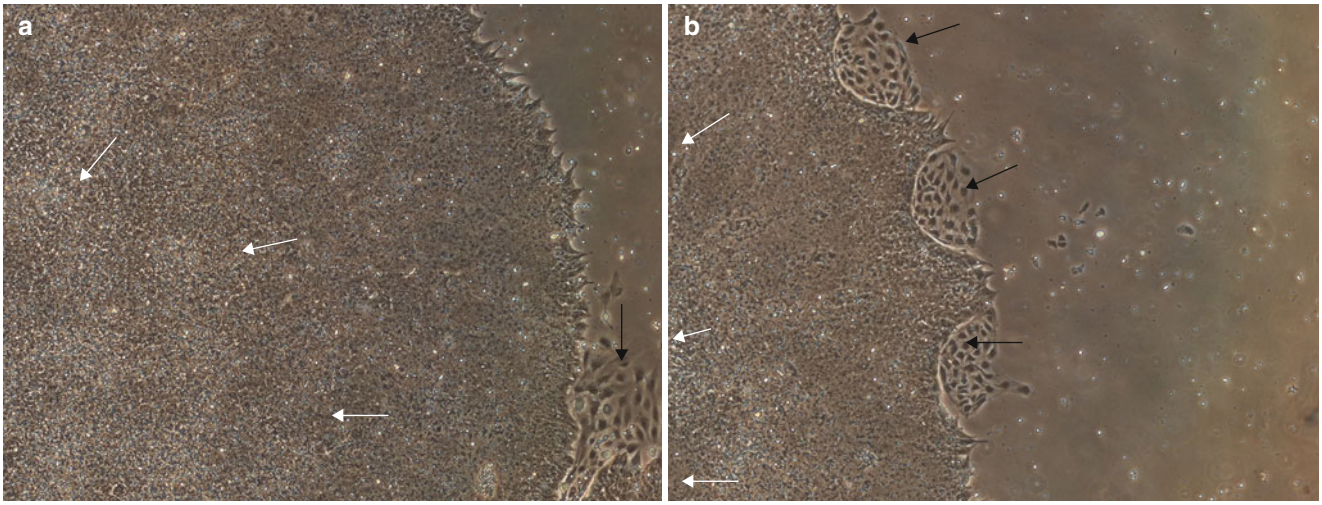

Fig. 6.18 (**a**) A large, overcompacted colony with slight differentiation in the right-hand corner (*black arrow*). The centre of this colony shows cells of an unidentifiable morphology, which might be neuronal progenitors (*white arrows*). (**b**) The centre of this colony is differentiated (*white arrows*). The cells around the edges of the colony are stem cells, differentiated into epithelial-like cells (*black arrows*) (both ×10 magnification)

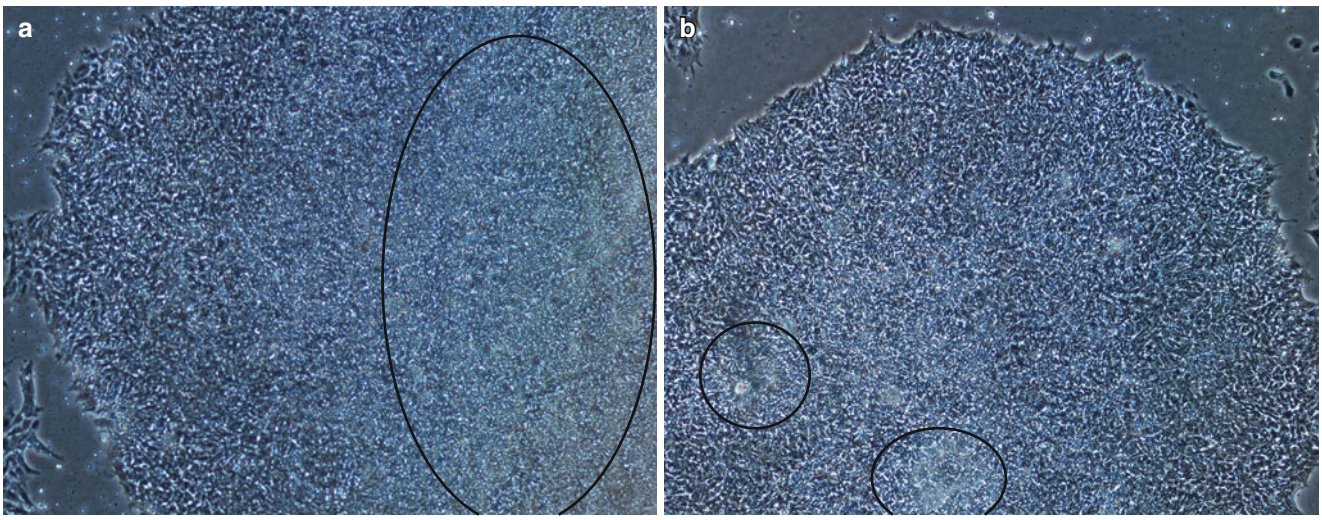

Fig. 6.19 (**a**) An old, overcompacted colony with cells beginning to differentiate on the right-hand side of the image (*black oval*). (**b**) Another old, overcompacted colony with differentiation beginning in the centre (*black circles*) (both ×10 magnification)

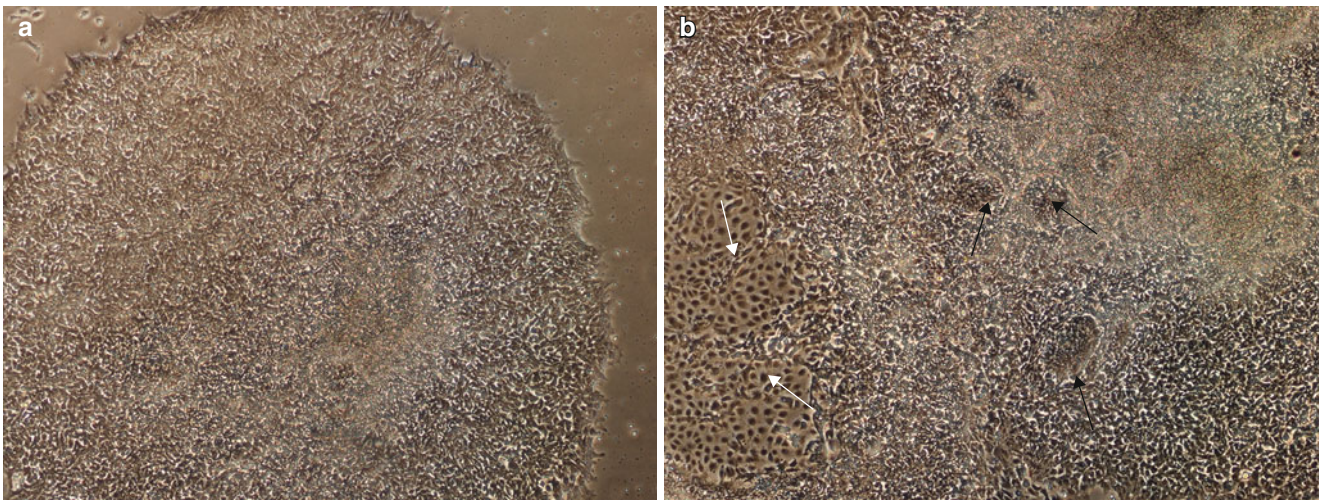

Fig. 6.20 (**a**) Typical morphology of spontaneous differentiation of a "difficult" cell line, with a "bumpy" mixture of monolayer and multilayer cells. (**b**) An overgrown colony with many neuronal rosettes (*black arrows*) and a monolayer area of neuronal progenitors (*white arrows*) (both ×10 magnification)

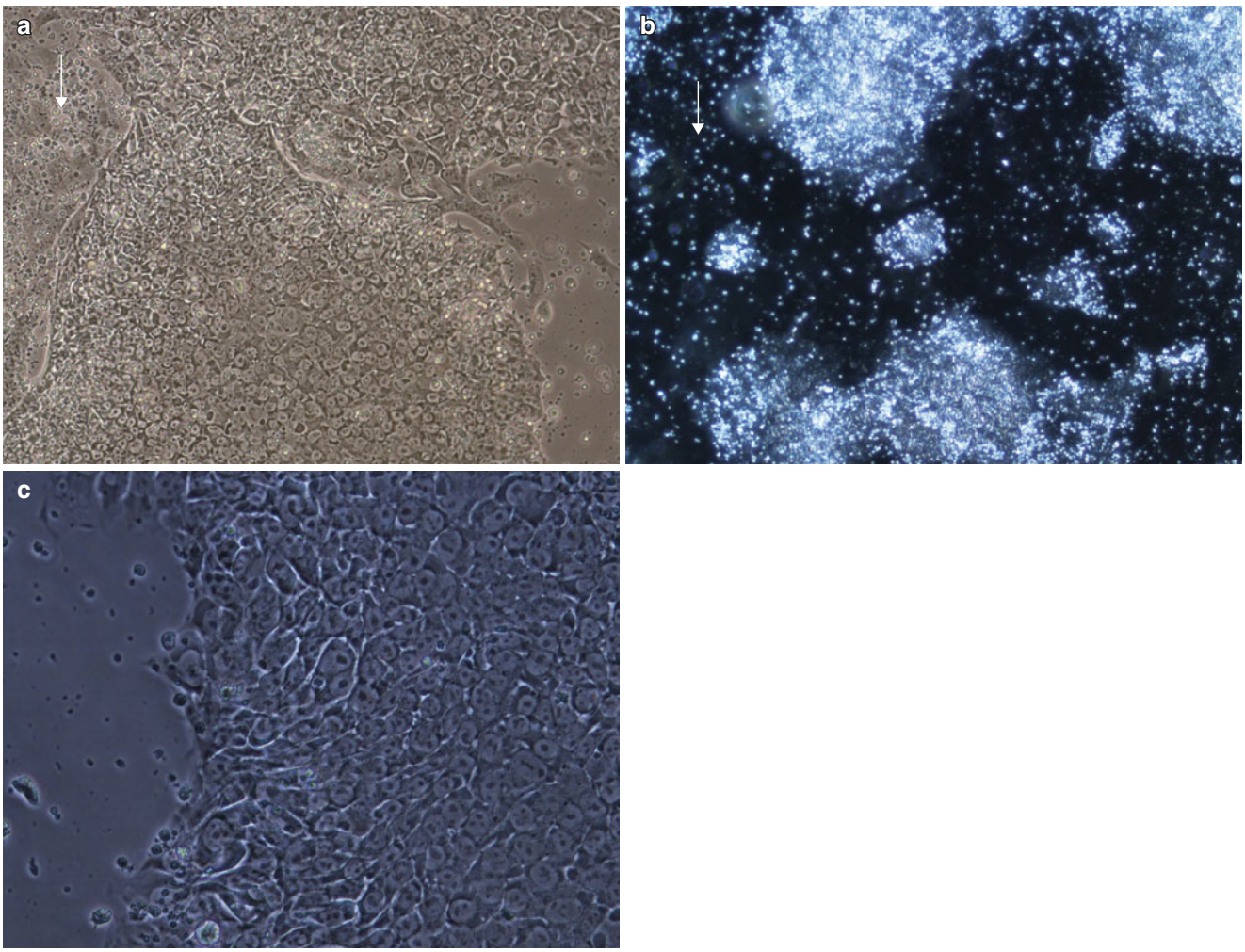

Fig. 6.21 (**a**–**c**) During the first few passages of adaptation from one cell culture system to another, cells usually do not form round colonies (as shown on **b**). Many floating, apoptotic cells (*white arrows* on **a**, **b**) can be seen. With an "easy" cell line, it can take no more than three to five passages to adapt to the new culture system. A cell line that is not easy to culture requires more care, and adaptation can be cell line specific, involving very careful observation of the morphology and rate of growth of the cells, adapting the passaging regimen accordingly. **c** shows good-quality morphology around the edge of the colony, with a typical stem cell morphology having large nuclei and spaces between the cells (**a**, ×10 magnification; **b**, ×4; **c**, ×20)

Another set of images (Figs. 6.22, 6.23, 6.24, 6.25, 6.26, 6.27, 6.28, 6.29, 6.30, 6.31, 6.32, 6.33 and 6.34), comprising a collection of six individual cell lines, demonstrates the different morphologies of hESCs and iPSCs that have been adapted from a feeder-based system to a feeder-free system and grown for three passages feeder free on different matrices in three different types of commercially available feeder-free culture media. As the growth characteristics are specific to each cell line, only general comments can be made about the status of the cells when using morphology alone. Two of the cell lines tested failed to survive in one feeder-free combination of matrix and medium. The hESC and iPSC lines grown on Matrigel™ in mTeSR™1 have been used as the control system to compare with the morphologies of the cells grown in the test media.

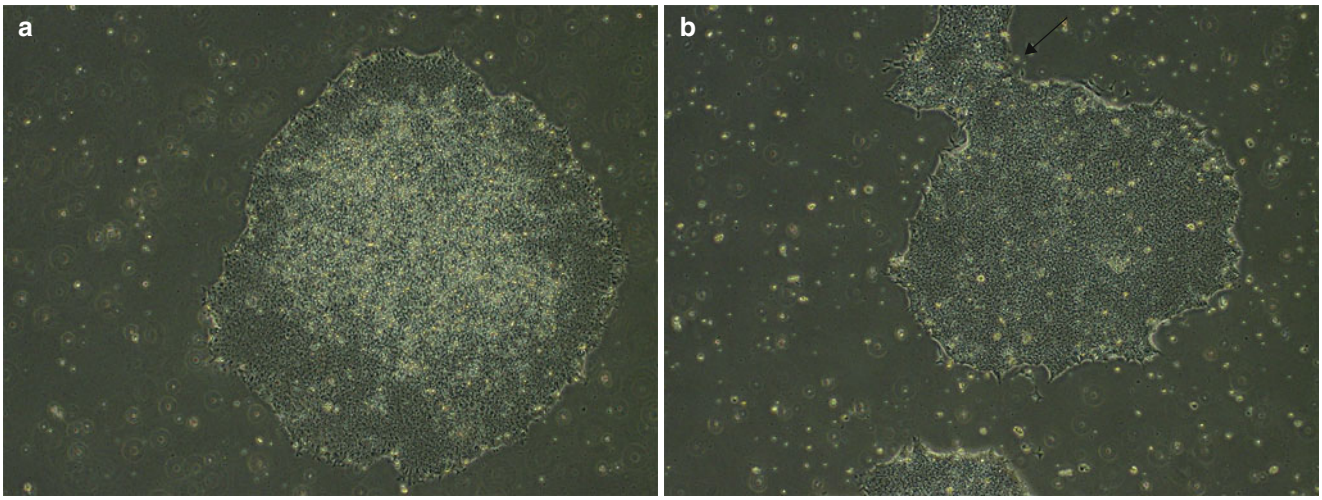

Fig. 6.22 These hESC lines were grown on Matrigel™ for 5 days in mTeSR™1. The cells have undergone three passages after transition from human feeder–dependent growth. (**a**) Cell line 1: The colony in this image exhibits a good morphology, with compact cells. The centre is beginning to overcompact. It would be advisable to passage the cells at this stage, as areas of differentiation will start to arise within the colony within 24 h. (**b**) Cell line 2: This colony also displays very good morphology, but from the shape of the colony, it appears that a large colony and a small colony have fused together (*black arrow*) (both ×4 magnification)

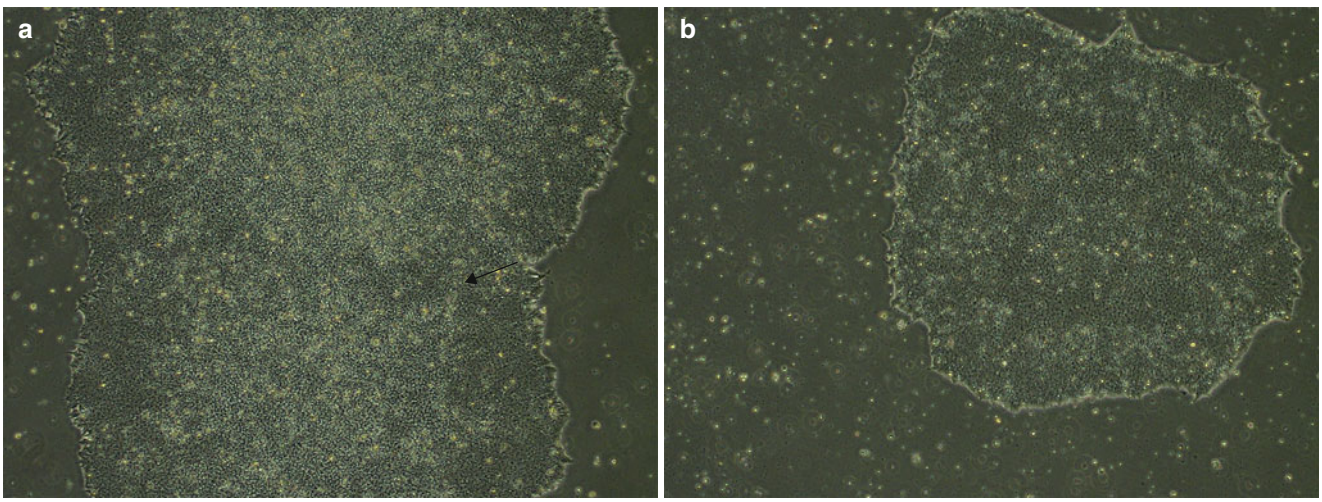

Fig. 6.23 These hESC lines were grown on Matrigel™ for 5 days in mTeSR™1. The cells have undergone three passages after transition from human feeder-dependent growth. (**a**) Cell line 3: The colony in this image exhibits a good morphology, with compact cells. As in Fig. 6.22a, the centre of the colony is beginning to overcompact, and as in Fig. 6.22b, it appears that two colonies have merged (*black arrow*). (**b**) Cell line 4: The colony in this image exhibits a good morphology, with compact cells (both ×4 magnification)

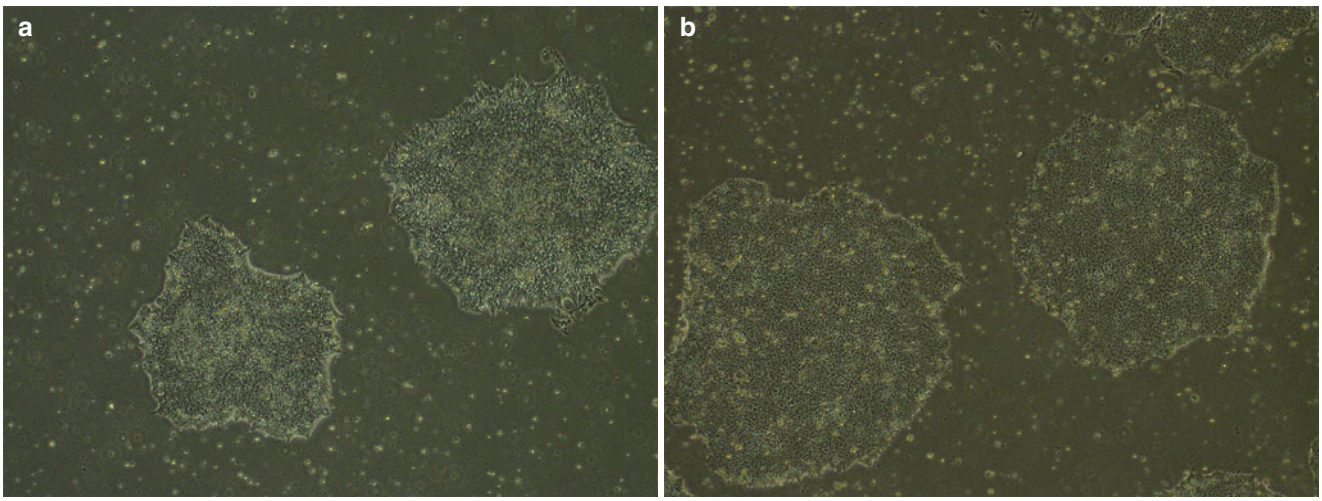

Fig. 6.24 These hESC lines were grown on Matrigel™ for 5 days in mTeSR™1. The cells have undergone three passages after transition from human feeder–dependent growth. (**a**) Cell line 5: The colony in this image exhibits a good morphology, with compact cells. The edges of the colony are slightly spiky, and the colonies appear to be smaller than those of the cell lines in the two previous figures, possibly indicating a slower proliferation rate for this line. (**b**) Cell line 6: good colony morphology, with compact cells (both ×4 magnification)

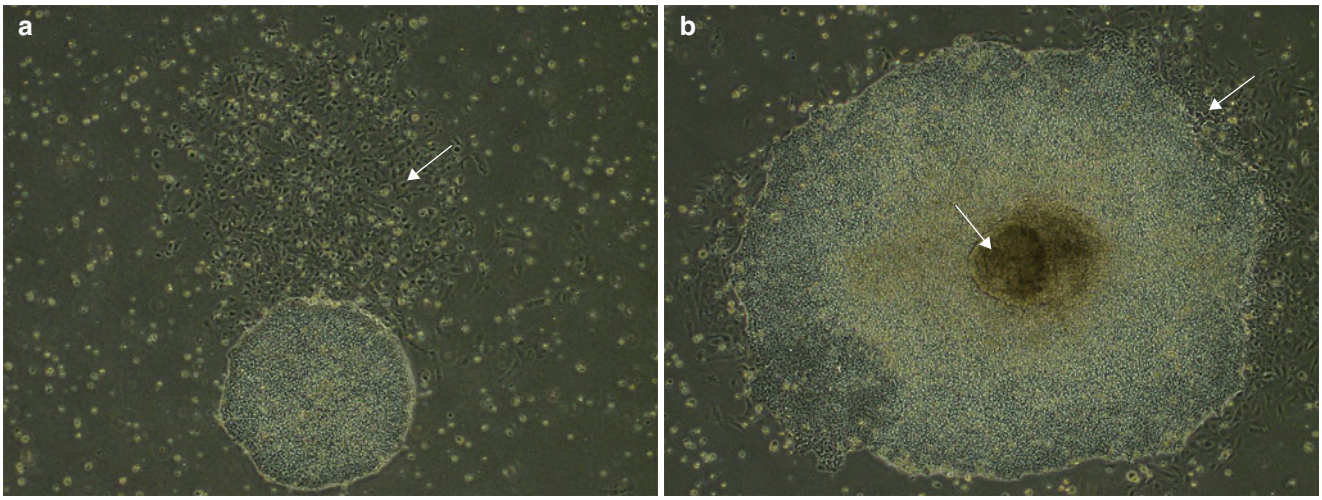

Fig. 6.25 These hESC lines were grown on a specially coated, commercially available matrix for 5 days in mTeSR™1. The cells have undergone three passages after transition from human feeder–dependent growth. (**a**) Cell line 1: colonies are of good morphological quality, although some areas of differentiation are seen (*white arrow*). The colonies for this cell line appear smaller than their equivalent on Matrigel™. (**b**) Cell line 2: the colonies show a good morphology, but areas of differentiation can be seen both within the colony and around the edges (*white arrows*) (both ×4 magnification)

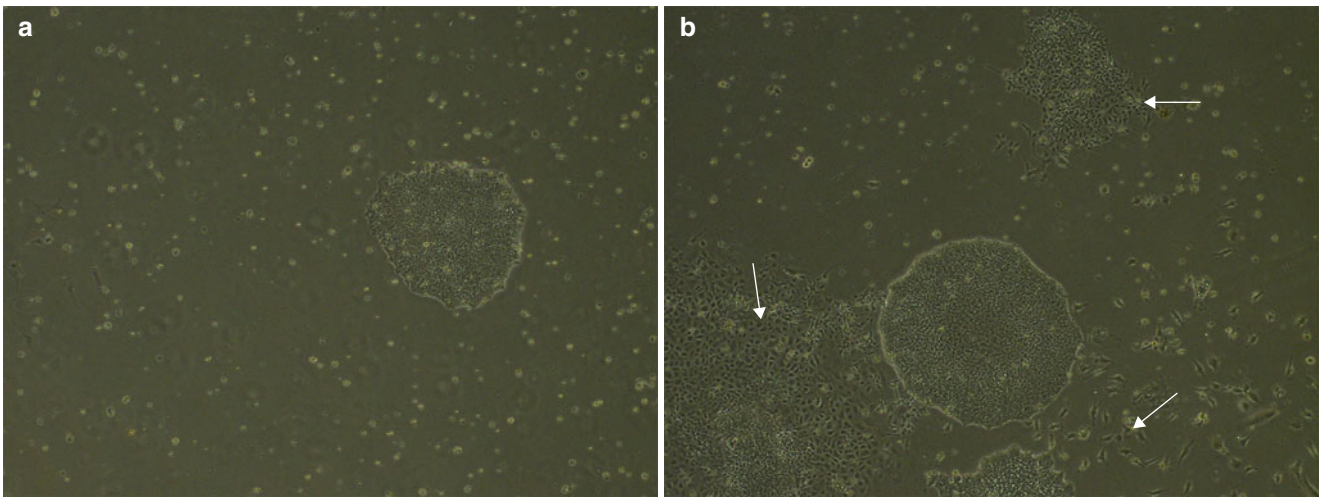

Fig. 6.26 These hESC lines were grown on a specially coated, commercially available matrix for 5 days in mTeSR™1. The cells have undergone three passages after transition from human feeder–dependent growth. (**a**) Cell line 3: the colony in this image exhibits a good morphology, with compact cells. (**b**) Cell line 4: the colony in this image exhibits a good morphology, but differentiated colonies are also observed in the culture dish (*white arrows*) (both ×4 magnification)

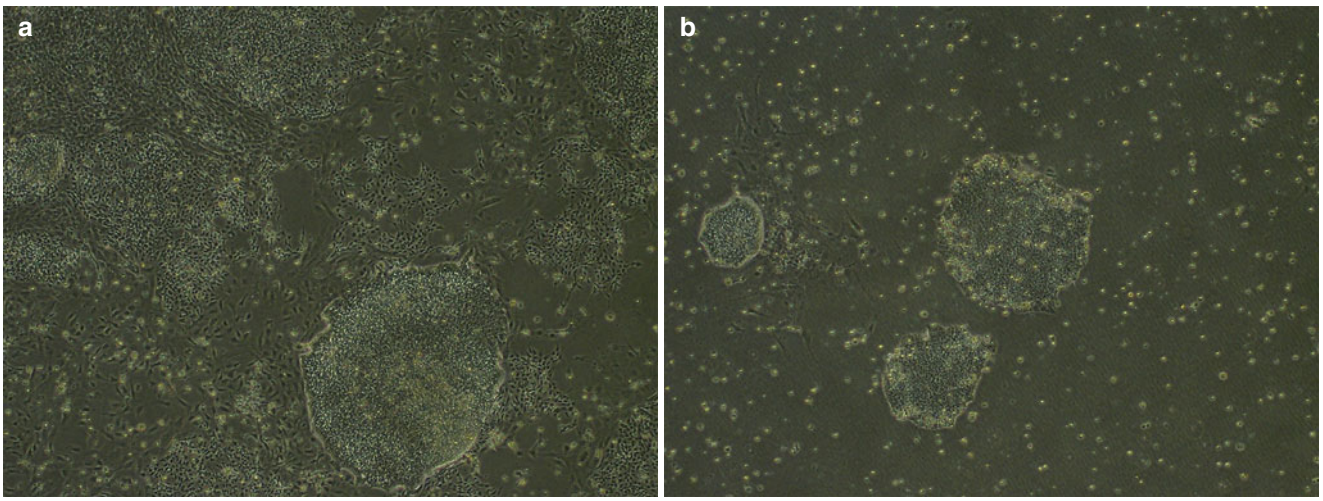

Fig. 6.27 These hESC lines were grown on a specially coated, commercially available matrix for 5 days in mTeSR™1. The cells have undergone three passages after transition from human feeder–dependent growth. (**a**) Cell line 5: although there is a discrete colony exhibiting a good morphology with compact uniform cells, numerous areas of differentiation are evident. (**b**) Cell line 6: the colonies, though small, display a compact, well-defined morphology. Very little differentiation is observed (both ×4 magnification)

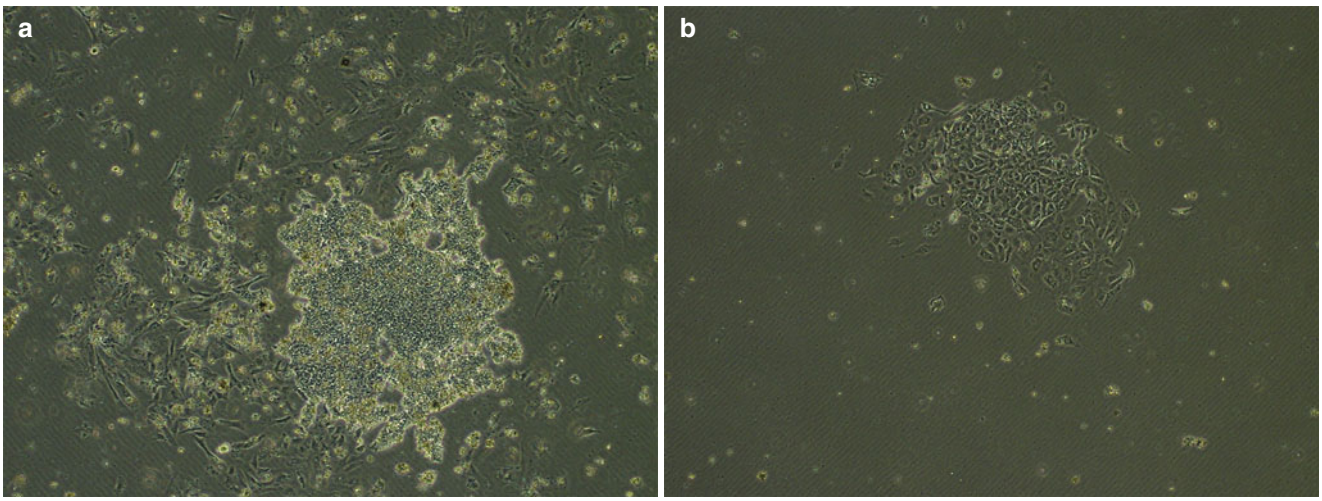

Fig. 6.28 These hESC lines were grown on a specially coated commercially available matrix for 5 days in a commercial medium 1. The cells have undergone three passages after transition from human feeder-dependent growth. (**a**) Cell line 1: the morphology is far from ideal. The colonies display large areas of differentiation, although undifferentiated colonies are also evident. (**b**) Cell line 2: the cells in the culture system are sparse and dispersed. The colonies show a loose aggregation of cells, which could compact or differentiate (both ×4 magnification)

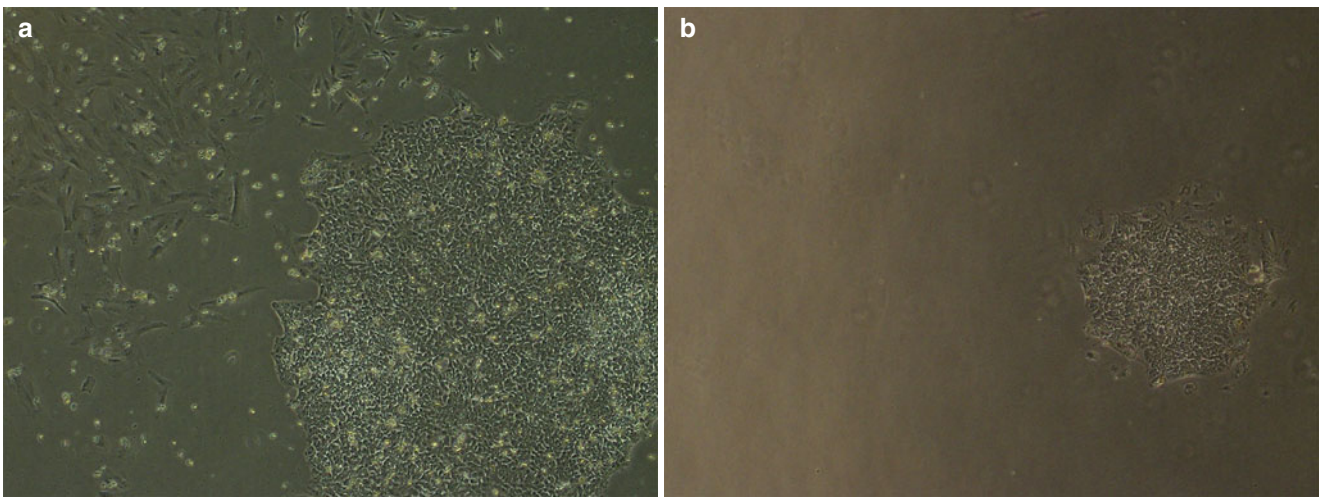

Fig. 6.29 These hESC lines were grown on a specially coated commercially available matrix for 5 days in a commercial medium 1. The cells have undergone three passages after transition from human feeder–dependent growth. (**a**) Cell line 3: colonies in this culture system are large and compact, but loose, differentiated colonies are also present. (**b**) Cell line 4: this cell line produces sparse, small, compact colonies with no areas of differentiation demonstrated (both ×4 magnification)

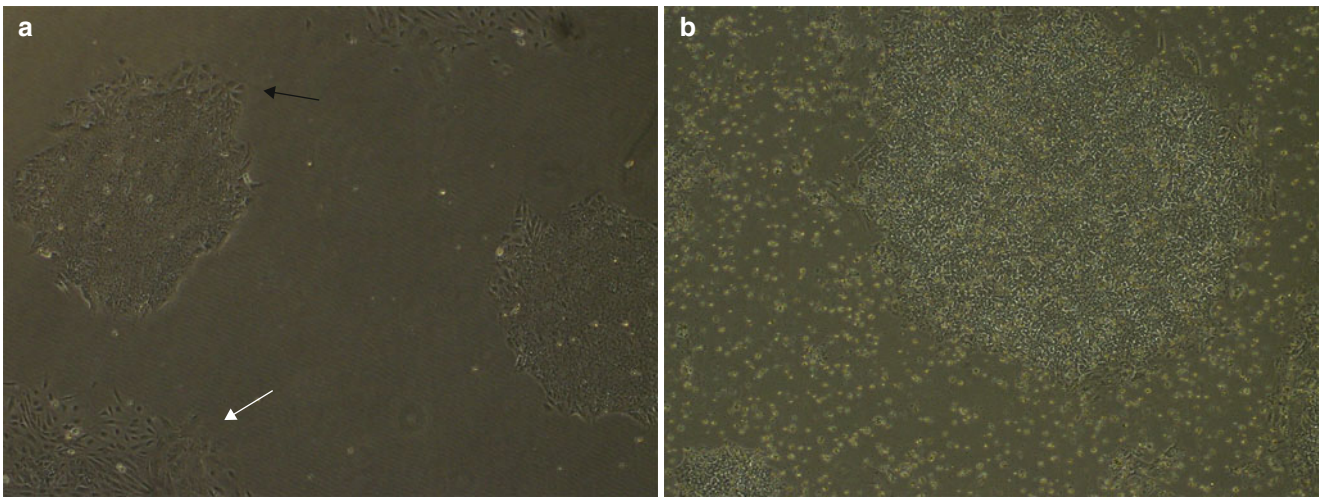

Fig. 6.30 These hESC lines were grown on a specially coated commercially available matrix for 5 days in a commercial medium 2. The cells have undergone three passages after transition from human feeder–dependent growth. (**a**) Cell line 1: colonies are large and compacted, with larger, less-compacted cells around the edges of the colonies (*black arrow*). The colonies also appear thin and fragile; they must be handled with care when changing media, as these colonies disintegrate easily. There is evidence of differentiation (*white arrow*). (**b**) Cell line 2: compacted colony with no differentiation but many floating apoptotic cells in the culture (both ×4 magnification)

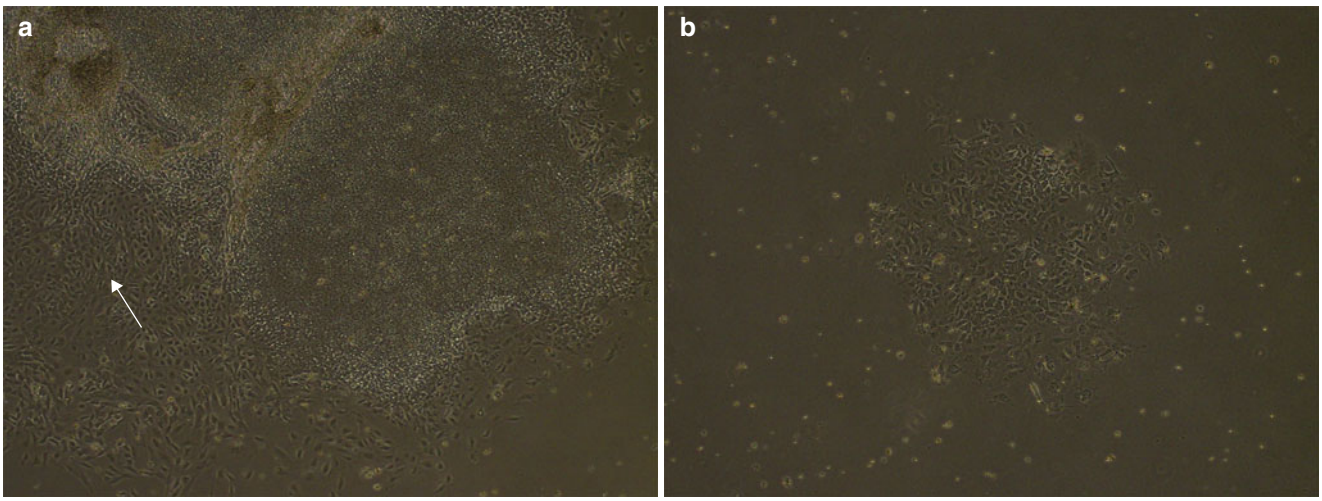

Fig. 6.31 These hESC lines were grown on a specially coated commercially available matrix for 5 days in a commercial medium 2. The cells have undergone three passages after transition from human feeder-dependent growth. (**a**) Cell line 3: the colonies comprise compacted cells with areas of differentiation outside their perimeters (*white arrow*). The colonies are thin and fragile. (**b**) Cell line 4: in this culture system, the colonies are small, sparse and loose; they do not compact (both ×4 magnification)

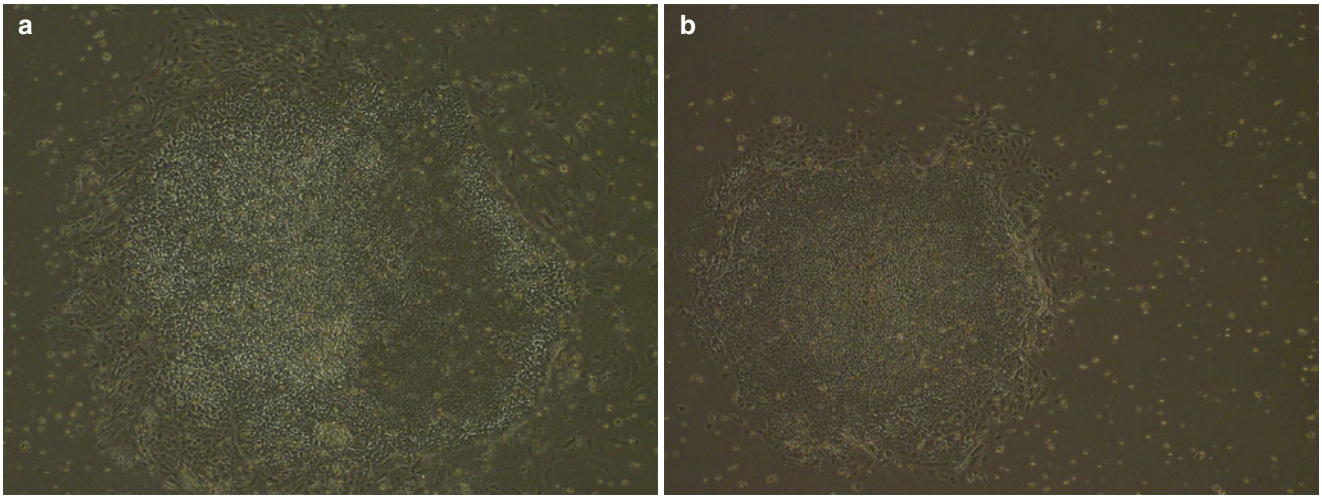

Fig. 6.32 These hESC lines were grown on a specially coated commercially available matrix for 5 days in a commercial medium 2. The cells have undergone three passages after transition from human feeder–dependent growth. (**a**) Cell line 5: colonies of compacted cells with differentiation around the edges of the colony. The colonies appear thin and fragile, and areas within them contain apoptotic cells. (**b**) Cell line 6: this colony of compacted cells shows differentiation around its perimeter. Again, the colonies in this culture system appear thin and fragile (both ×4 magnification)

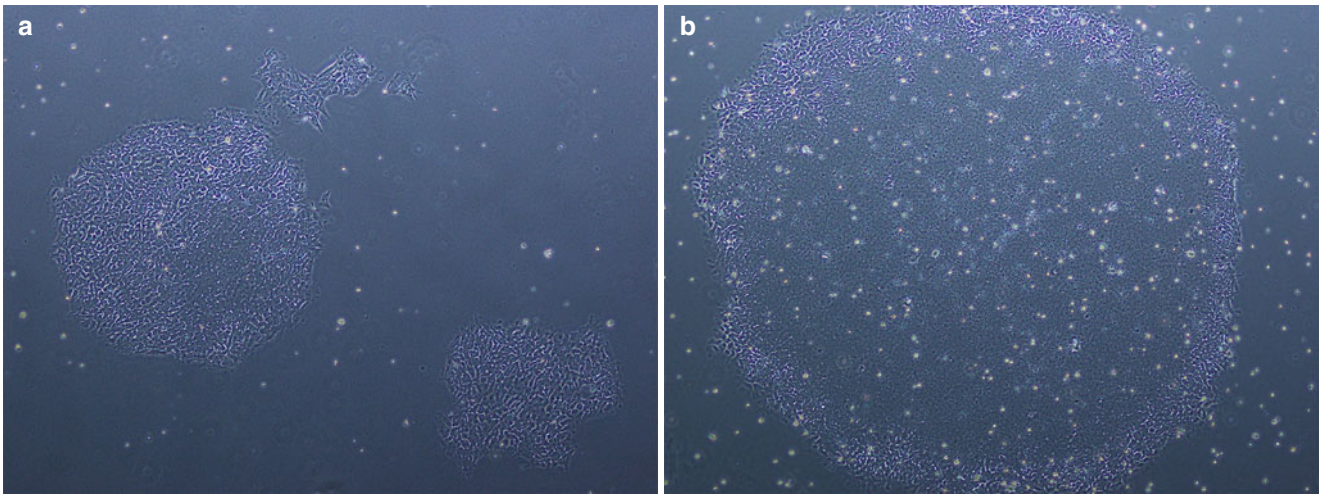

Fig. 6.33 Images of hiPSC lines grown on Matrigel™ in mTeSR™1. (**a**) On Day 3 post-passaging, the colonies are loose. (**b**) On Day 5 post-passaging, the colonies have compacted and display a good morphology. They are ready to passage and will overcompact if left for a further 24 h (both ×4 magnification)

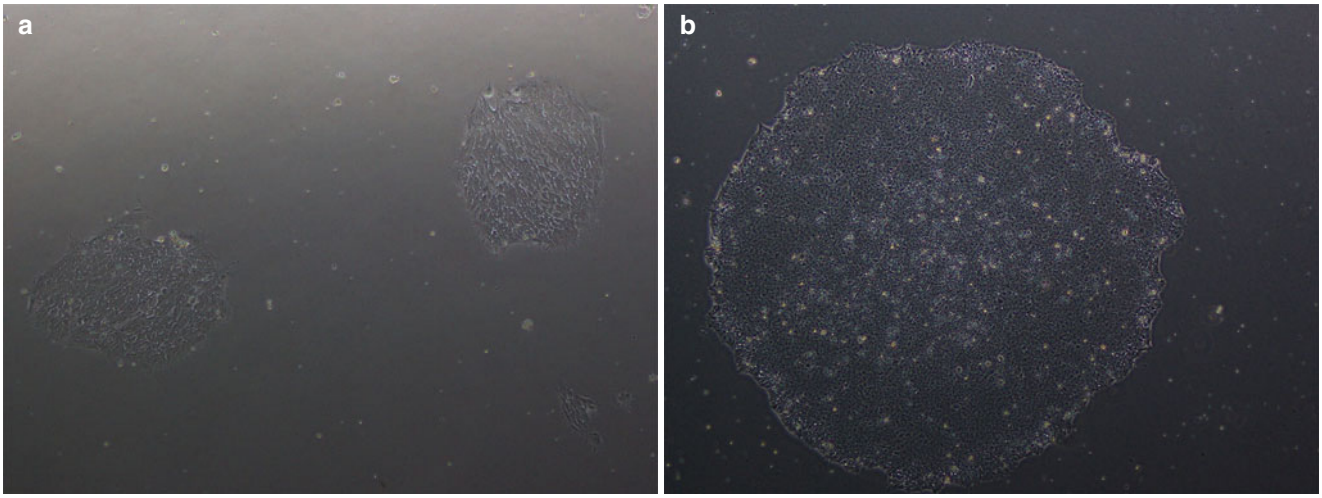

Fig. 6.34 Images of hiPSC lines grown on Matrigel™ in another commercial medium. (**a**) On Day 3 post-passaging, the colonies are loose and have not started to compact. (**b**) On Day 5 post-passaging, the colonies have compacted and display a good morphology with well-defined borders. These colonies are ready to passage and will overcompact if left for a further 24 h (both ×4 magnification)

The growth of PSCs in feeder-free systems brings with it many advantages, not least the removal of the lengthy process of fibroblast and feeder preparation and all the steps required in their quality control. Movement to feeder-free systems is becoming more common. From the point of view of clinical translation and other PSC modelling and testing applications, well-defined systems are a necessity, but these systems should be fit for purpose and require optimisation cell line by cell line.

Suggested Reading

Amit M, Itskovitz-Eldor J. Morphology of human embryonic and induced pluripotent stem cell colonies cultured in feeder layer–free conditions. In: Amit M, Itskovitz-Eldor J, editors. Atlas of human pluripotent stem cells: derivation and culturing, Stem cell biology and regenerative medicine. New York: Humana Press; 2012. p. 41–56.

Beers J, Gulbranson DR, George N, Siniscalchi LI, Jones J, Thomson JA, Chen G. Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions. Nat Protoc. 2012;7:2029–40.

Braam SR, Zeinstra L, Litjens S, Ward-van Oostwaard D, van den Brink S, van Laake L, et al. Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alphavbeta5 integrin. Stem Cells. 2008;26:2257–65.

Crocco MC, Fratnz N, Bos-Mikich A. Substrates and supplements for hESCs: a critical review. J Assist Reprod Genet. 2013;30:315–23.

Hernandez D, Ruban L, Mason C. Feeder-free culture of human embryonic stem cells for scalable expansion in a reproducible manner. Stem Cells Dev. 2011;20:1089–98.

International Stem Cell Initiative Consortium, Akopian V, Andrews PW, Beil S, Benvenisty N, Brehm J, et al. Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells. In Vitro Cell Dev Biol Anim. 2010;46:247–58.

Kleinman HK, Martin GR. Matrigel: basement membrane matrix with biological activity. Semin Cancer Biol. 2005;15:378–86.

Lambshead JW, Meagher L, O'Brien C, Laslett AL. Defining synthetic surfaces for human pluripotent stem cell culture. Cell Regen. 2013;2:7.

Ludwig TE, Bergendahl V, Levenstein ME, Yu J, Probasco MD, Thomson JA. Feeder-independent culture of human embryonic stem cells. Nat Methods. 2006;3:637–46.

Rodin S, Antonsson L, Niaudet C, Simonson OE, Salmela E, Hansson EM, et al. Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment. Nat Commun. 2014;5:3195.

Rodin S, Antonsson L, Hovatta O, Tryggvason K. Monolayer culturing and cloning of human pluripotent stem cells on laminin-521 -based matrices under xeno-free and chemically defined conditions Nature Protocols 2014;9:2354–68.

Saha K, Mei Y, Reisterer CM, Pyzocha NK, Yang J, Muffat J, et al. Surface-engineered substrates for improved human pluripotent stem cell culture under fully defined conditions. Proc Natl Acad Sci U S A. 2011;108:18714–9.

Sternberg H, Janus J, West MD. Defining cell-matrix combination products in the era of pluripotency. Biomatter. 2013;3:e24496.

Stover AE, Schwartz PH. Adaptation of human pluripotent stem cells to feeder-free conditions in chemically defined medium with enzymatic single-cell passaging. Methods Mol Biol. 2011;767:137–46.

Villa-Diaz LG, Ross AM, Lahann J, Krebsbach PH. Concise review: the evolution of human pluripotent stem cell culture: from feeder cells to synthetic coatings. Stem Cells. 2013;31:1–7.

Wang Y, Chou BK, Dowey S, He C, Gerecht S, Cheng L. Scalable expansion of human induced pluripotent stem cells in the defined xeno-free E8 medium under adherent and suspension culture conditions. Stem Cell Res. 2013;11:1103–16.

Passaging Pluripotent Stem Cells

7

The maintenance and expansion of undifferentiated pluripotent stem cells (PSCs) in cell culture systems requires the dissociation of cells and their transfer from culture vessel to culture vessel, in a process known as passaging. This chapter includes images acquired from different techniques used to passage PSCs. It is important to minimise the stress to the cells caused by the passaging activity, as this process can promote apoptosis, spontaneous differentiation and culture adaptation. Culture adaptation through selective pressure of cells in culture results in genetically abnormal karyotypes (Chap. 10).

In feeder-based culture systems, the method of choice is mechanical passaging, in which colonies displaying an optimal undifferentiated morphology are dissected under the microscope and the pieces are then transferred onto fresh feeder layers. This method is reported to maintain the genetic integrity of the cells over an extensive number of serial passages. However, this method is very time-consuming, especially when culturing large numbers of cells. Mechanical passaging can be combined with a mild enzymatic treatment, utilising enzymes such as collagenase IV, dispase and dilute trypsin, which aid in the detachment of the cells and result in the production of clumps of cells, which can then be transferred to cultures of fresh feeders. Again, this method is able to maintain the genetic stability of the cells over time. A number of laboratories have reported the use of enzymatic treatment alone to generate single cells that maintain a normal karyotype over a large number of passages in feeder-based systems, facilitating the expansion and bulking up of cells for processing purposes. It has been suggested that for feeder-based systems, a combination of manual passaging with a limited period of enzymatic passaging might be the optimum method to use to maintain the genetic stability of the PSCs over time (Figs. 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 7.10, 7.11, 7.12 and 7.13).

L. Healy, L. Ruban, *Atlas of Human Pluripotent Stem Cells in Culture*,
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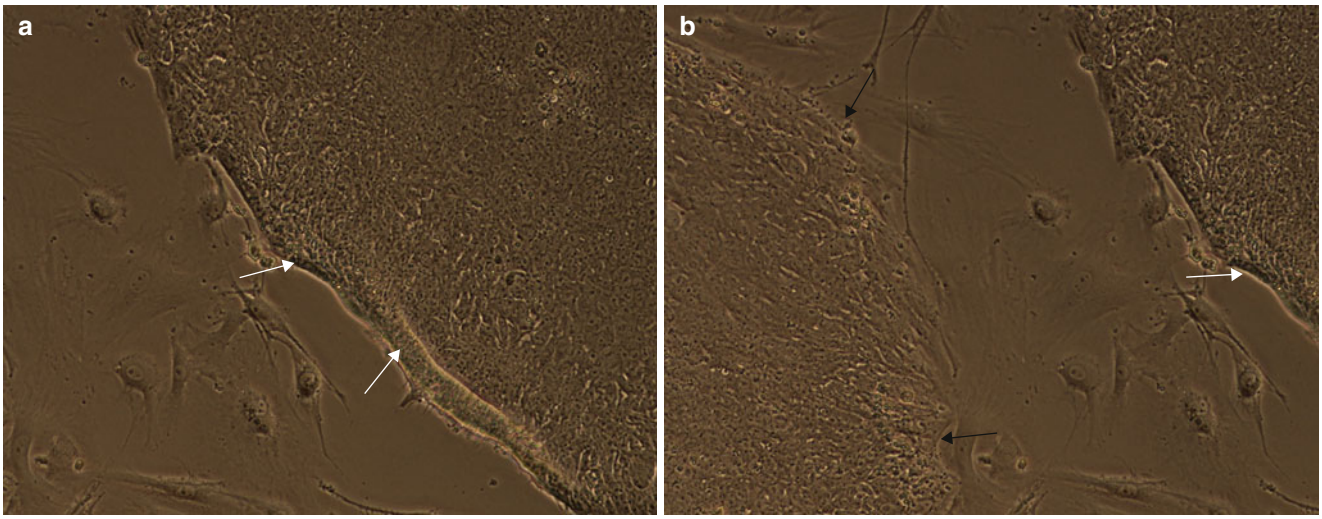

Fig. 7.1 (**a**, **b**) Traditional method of passaging human embryonic stem cell (hESC) colonies using collagenase type IV. In many protocols, hESCs are incubated for up to 30 min with collagenase. The authors normally incubate for about 5 min, to ease detachment of hESC colonies from feeder cells. In the case shown, the edges of the colonies curl up (*white arrows*). Not all thin monolayer colonies curl up (*black arrow* on **b**) (both ×10 magnification)

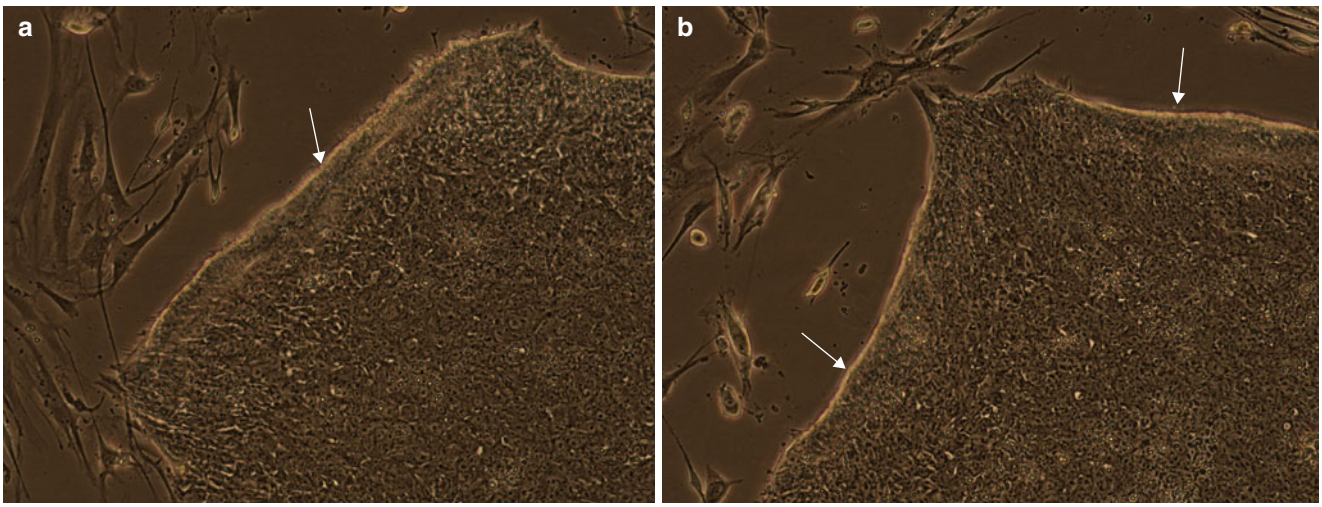

Fig. 7.2 (**a**, **b**) Following collagenase treatment, both colonies are starting to curl up (*white arrows*) (both ×10 magnification)

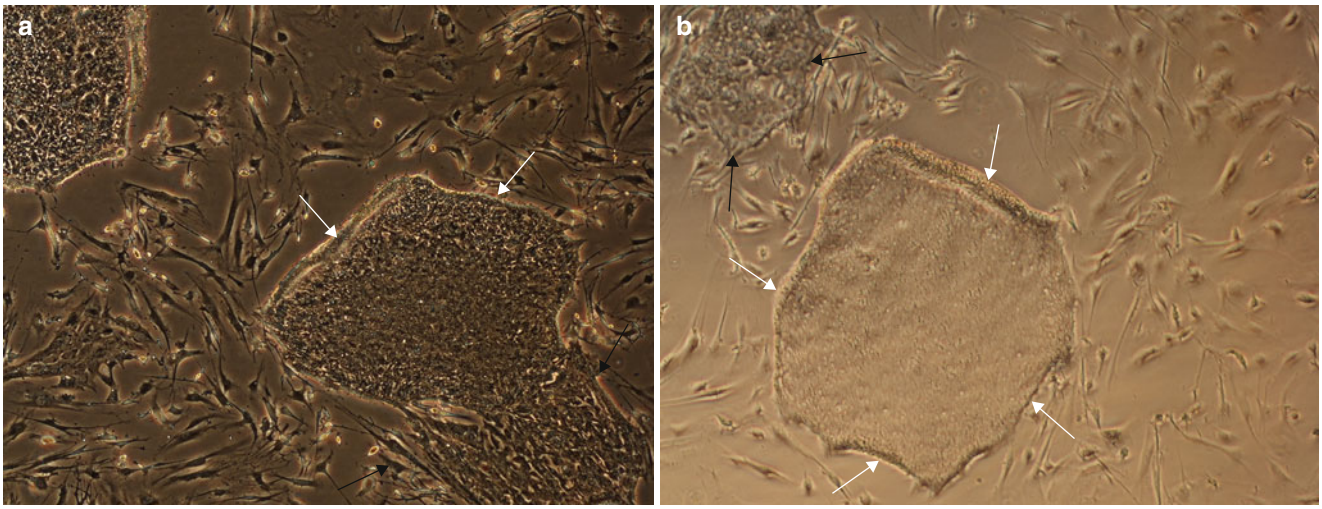

Fig. 7.3 (**a**, **b**) In both pictures, only the undifferentiated colonies are curled up along their edges, indicating detachment from the feeder layer of the cells (*white arrows*). The differentiated part of the colony is not curled up in **a**, and neither is the small differentiated colony in **b** (*black arrows*) (both ×4 magnification)

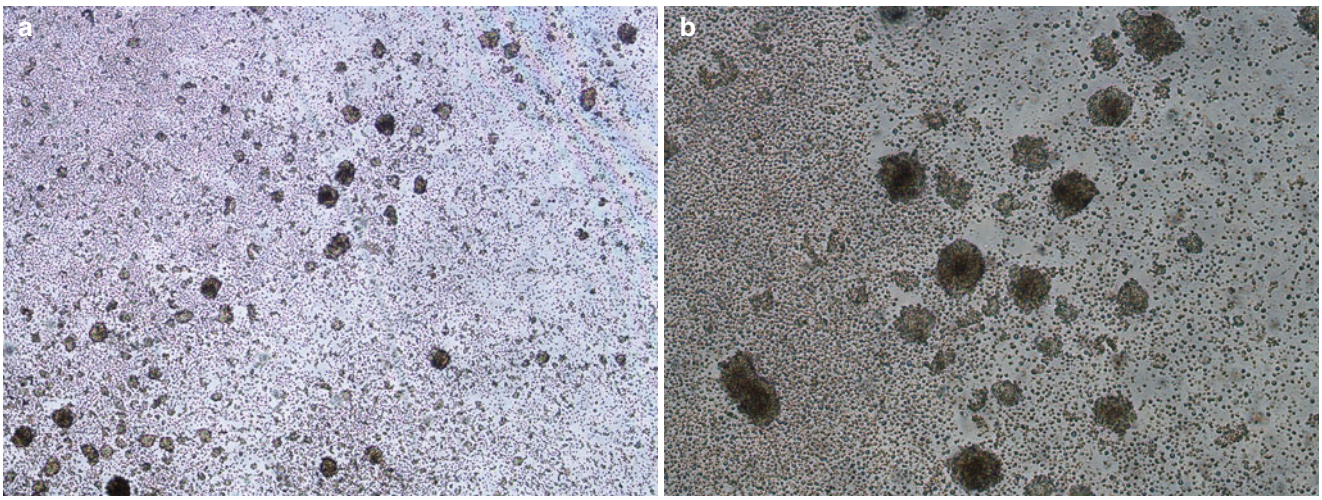

Fig. 7.4 (**a**, **b**) Mechanical cutting of colonies by hand. The images show the clumps or groups of cells produced following the combination of collagenase and mechanical scraping. These clumps vary in size (**a**, ×4 magnification; **b**, ×10)

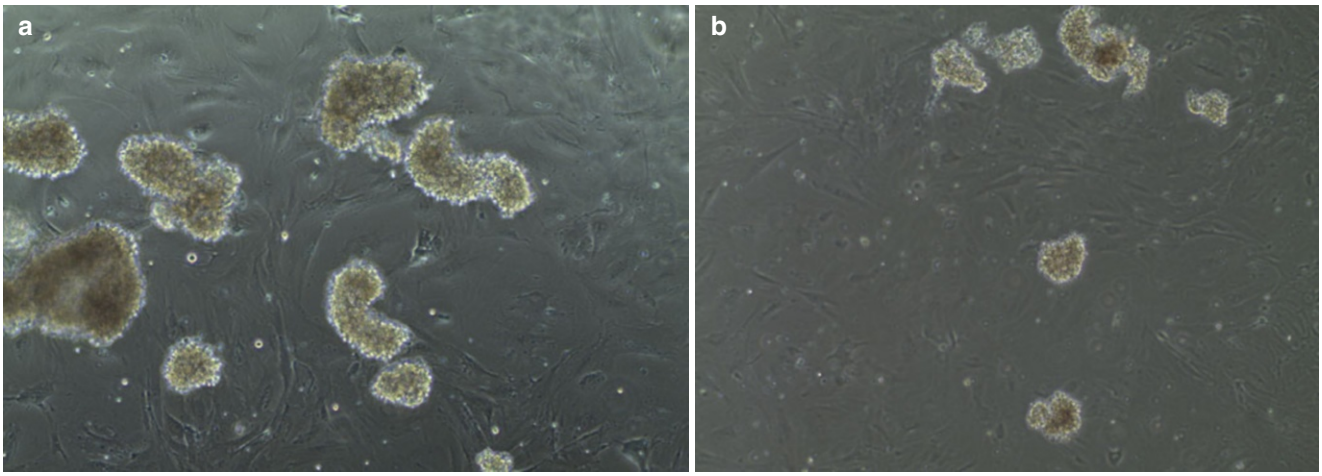

Fig. 7.5 (**a, b**) Mechanical cutting of colonies by hand. Some clumps are too large to settle down, attach to the feeders, spread out and form a colony with a good morphology. Judging clump size is subjective and comes with experience (×10 magnification)

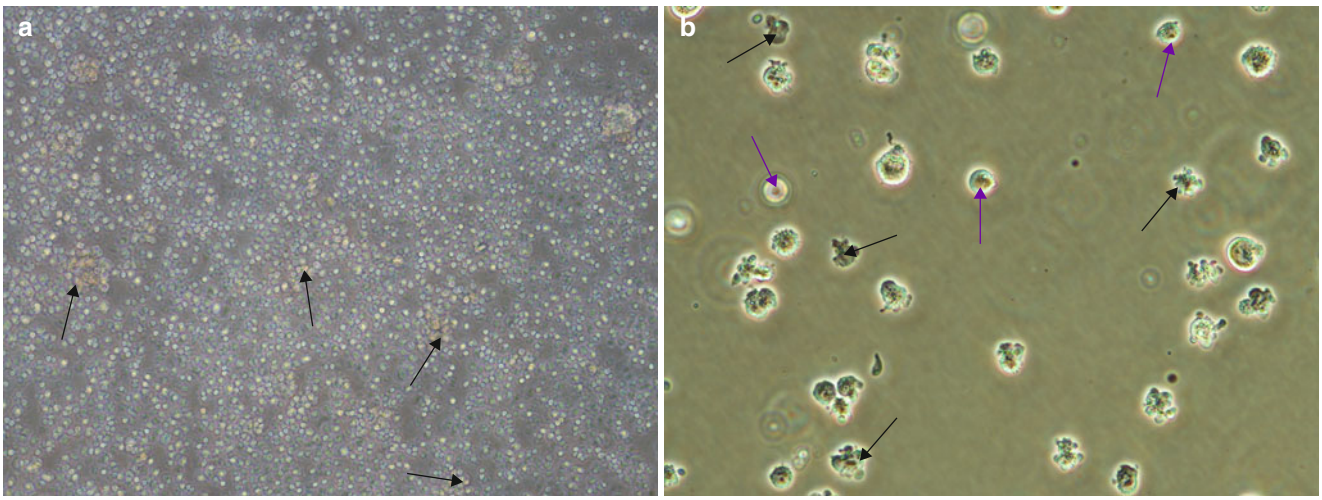

Fig. 7.6 (**a**) Single stem cells after enzymatic treatment. In this image, many single cells can be observed, but some of the clumps are still in small groups or clumps of three to four attached cells (*black arrows*). These small clumps normally give rise to good colonies. (**b**) The hESCs in this image are not optimal. Many of the cells are apoptotic (*black arrows*). Only a few are good; these can be observed as round, refractile cells with a visible nucleus (*purple arrows*) (**a**, ×4 magnification; **b**, ×20)

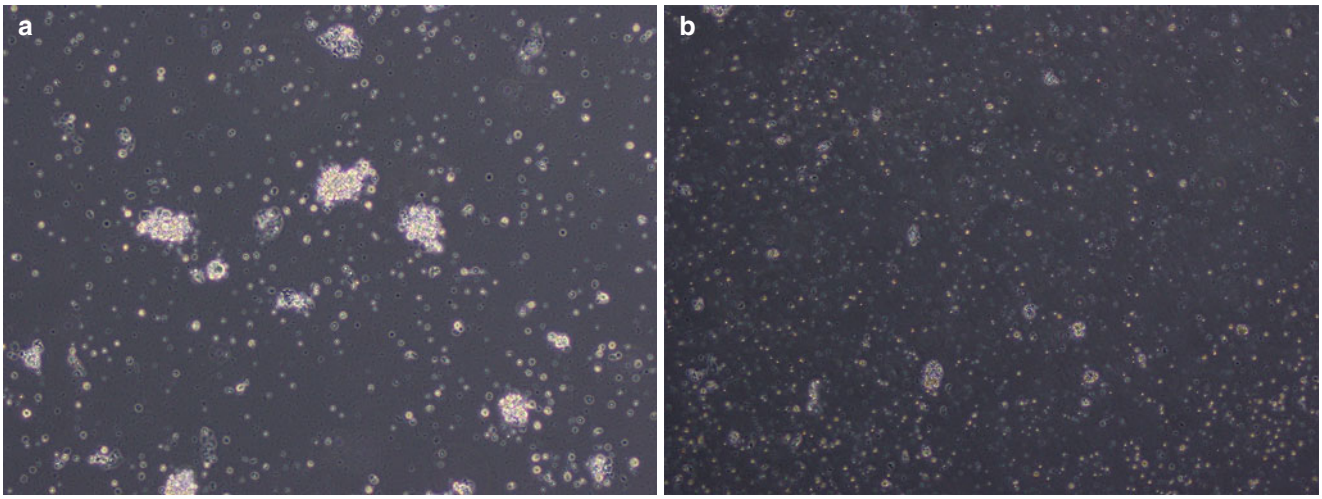

Fig. 7.7 (**a**) Cells treated enzymatically for 3 min to form small clusters of cells for passaging. With this particular enzyme, these clusters of 10–30 cells produce undifferentiated colonies of hPSCs when transferred to Matrigel™ or human or mouse feeders. If the cells are left in the presence of the enzyme for more than 5 min, mostly a high percentage of single cells are produced, and the colonies tend to differentiate spontaneously when passaged onto Matrigel™, although in co-culture with feeders, undifferentiated colonies can be produced from these single cells (**a**, ×10 magnification; **b**, ×4)

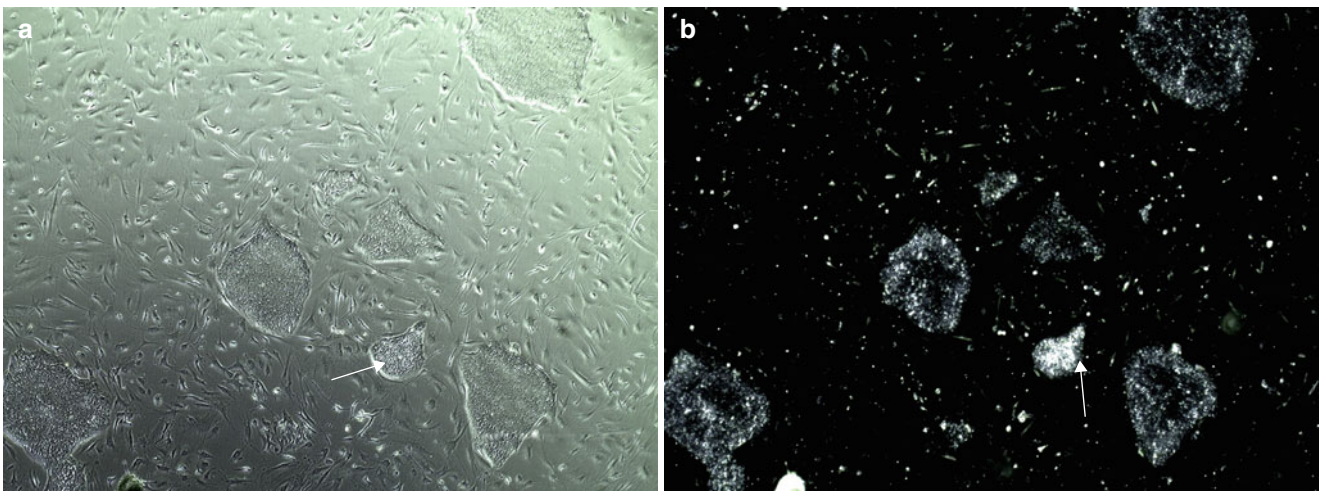

Fig. 7.8 (**a**) A brightfield phase contrast image showing a good-quality culture with exemplary morphology. Only one colony (*white arrow*) is potentially of less than optimal quality; this colony is multilayered, with cells lined up along the edges, and it displays an irregular shape, indicating that it is probably starting to differentiate (*see* Fig. 7.9). (**b**) A dark-phase contrast version of the image in (**a**). In general, the dark-phase contrast option is always used for mechanical passaging, as it gives an indication of the quality of the cells in the culture. Analysis confirms that the single multilayered colony with cells lined up along the edge and an irregular shape looks white under the microscope (*white arrows*), whereas all the other colonies look opaque. It is much easier to recognise the beginning of differentiation or extensive differentiation under dark-phase microscopy (both ×4 magnification)

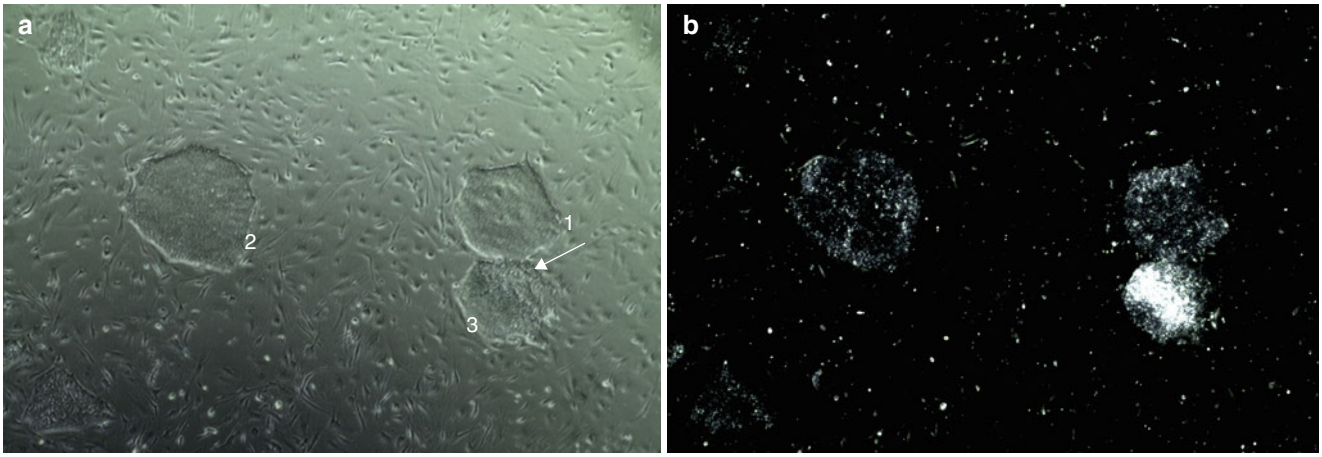

Fig. 7.9 (**a**) Image analysis of colony morphology using brightfield phase contrast reveals the following colony types (*1*) a good, undifferentiated colony; (*2*) a colony that is slightly uneven and bumpy in the centre, suggesting that it will have differentiated in 2 or 3 days and (*3*) a multilayered colony, of which the bottom half may still be undifferentiated. The other half of the colony (*white arrow*) has spontaneously differentiated. (**b**) Dark-phase contrast images of the same colonies reveal that the third colony is multilayered and dense in the middle, with no smooth edges on the right-hand side. This appearance usually indicates spontaneous differentiation (both ×4 magnification)

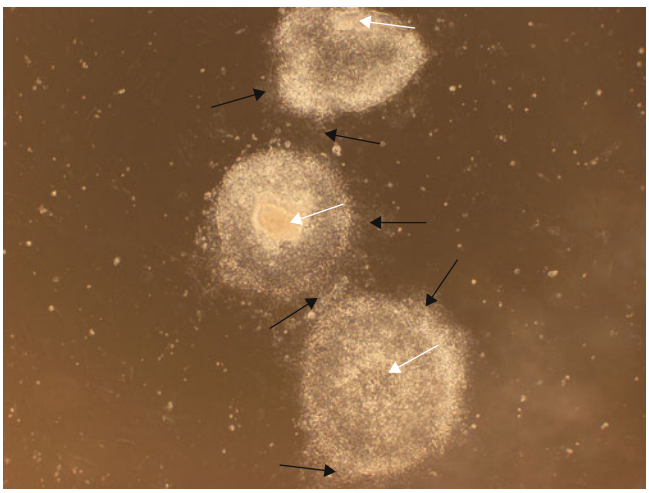

Fig. 7.10 An image analysis of a group of colonies reveals the following: The top colony displays areas of differentiation (*white arrow*) and edges that are not smooth; there is a skirt around the colony (*black arrows*). The middle colony is multi-layered in the centre (*white arrow*) and is probably differentiated around the edges (*black arrow*); it is not easy to distinguish this characteristic under the dissecting microscope. The bottom colony displays an area of differentiation in its centre (*white arrows*) and spontaneous differentiation around its perimeter (*black arrows*); the rest of this colony is undifferentiated (×4 magnification)

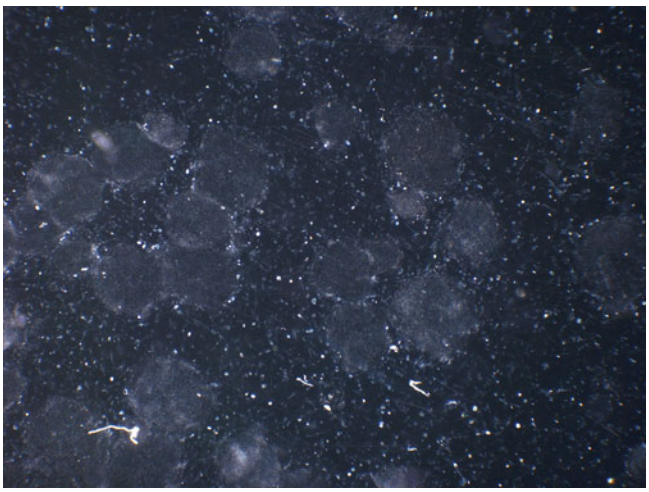

Fig. 7.11 Challenge: What can you say about this figure? See page 199 for answer

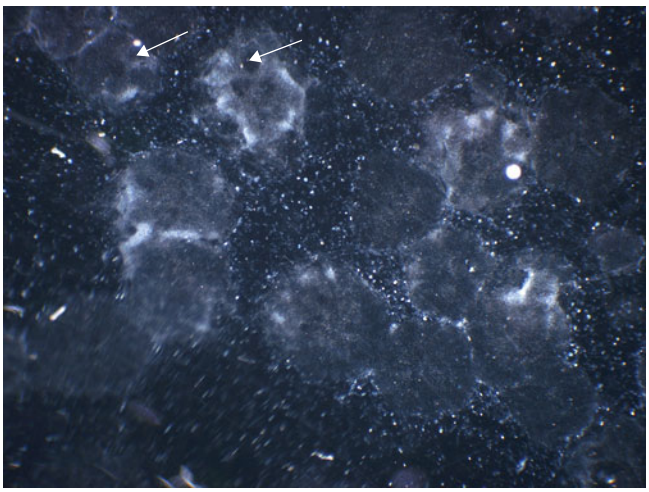

Fig. 7.12 Challenge: What can you say about this figure? See page 199 for answer

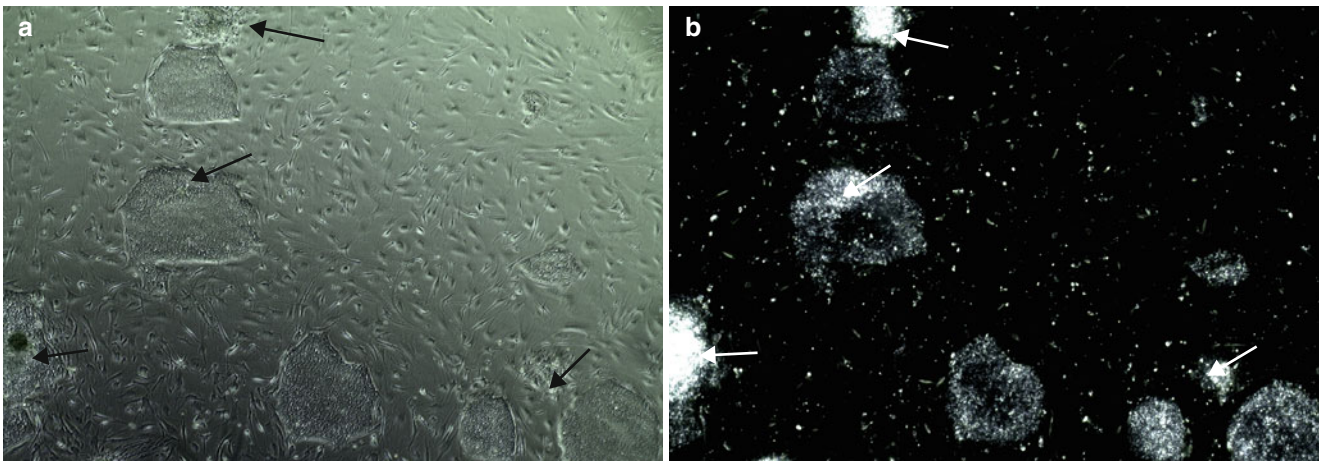

Fig. 7.13 Challenge: What can you say about this figures (**a**) and (**b**)? See page 199 for answer

In feeder-free culture systems, in which biological or synthetic matrices are combined with PSCs, the use of enzymes to produce clumps or single cells is much more common. These systems allow for ease of expansion of the PSCs, but the production of single cells for passaging in these culture systems has been reported to result in decreased cell survival and increased genetic instability in cultures.

As we move to the development of PSCs for cellular therapies, it is imperative that we establish robust, reliable, reproducible methods to expand cells undifferentiated in defined culture systems, whilst maintaining their optimal phenotypic, genotypic and functional characteristics. New, non-manual enzyme-free chemical cell dissociation technologies are being described that could have an impact on the high-throughput production of PSCs. These technologies may solve the problems posed by earlier passaging techniques and ameliorate a number of issues and concerns relating to the cell-therapy regulatory and manufacturing landscape.

Suggested Reading

Amit M, Itskovitz-Eldor J. Morphology of human embryonic and induced pluripotent stem cell colonies cultured with feeders. In: Amit M, Itskovitz-Eldor J, editors. Atlas of human pluripotent stem cells: derivation and culturing, stem cell biology and regenerative medicine. New York: Humana Press; 2012a. p. 15–39.

Amit M, Itskovitz-Eldor J. Morphology of human embryonic stem cells and induced pluripotent stem cell colonies cultured in feeder layer-free conditions. In: Amit M, Itskovitz-Eldor J, editors. Atlas of human pluripotent stem cells: derivation and culturing, Stem cell biology and regenerative medicine. New York: Humana Press; 2012b. p. 41–55.

Bajpai R, Lesperance J, Kim M, Terskikh AV. Efficient propagation of single cells Accutase-dissociated human embryonic stem cells. Mol Reprod Dev. 2008;75:818–27.

Beers J, Gulbranson DR, George N, Siniscalchi LI, Jones J, Thomson JA, Chen G. Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions. Nat Protoc. 2012;7:2029–40.

Costa M, Sourris K, Hatzistavrou T, Elefanty AG, Stanley EG. Expansion of human embryonic stem cells in vitro. Curr Protoc Stem Cell Biol. 2008;5:1C.1.1–1C.1.7.

Ellerström C, Hyllner J, Strehl R. Single cell enzymatic dissociation of human embryonic stem cells: a straightforward, robust, and standardized culture method. Methods Mol Biol. 2010;584:121–34.

Ellerström C, Strehl R, Noaksson K, Hyllner J, Semb H. Facilitated expansion of human embryonic stem cells by single-cell enzymatic dissociation. Stem Cells. 2007;25:1690–6.

Hartung O, Huo H, Daley GQ, Schlaeger TM. Clump passaging and expansion of human embryonic and induced pluripotent stem cells on mouse embryonic fibroblast feeder cells. Curr Protoc Stem Cell Biol. 2010;14:1C.10.1–15.

Heng BC, Liu H, Ge Z, Cao T. Mechanical dissociation of human embryonic stem cell colonies by manual scraping after collagenase treatment is much more detrimental to cellular viability than is trypsinization with gentle pipetting. Biotechnol Appl Biochem. 2007;47:33–7.

Leonardo TR, Schell JP, Nickey KS, Tran HT. Culturing human pluripotent stem cells on a feeder layer. In: Loring JF, Peterson SE, editors. Human stem cell manual. 2nd ed. London: Elsevier; 2012. p. 3–14.

Nie Y, Walsh P, Clarke DL, Rowley JA, Fellner T. Scalable passaging of adherent human pluripotent stem cells. PLoS One. 2014;9: e88012.

Oh SK, Kim HS, Park YB, Seol HW, Kim YY, Cho MS, et al. Methods for expansion of human embryonic stem cells. Stem Cells. 2005;23:605–9.

Ohnuma K, Fujiki A, Yanagihara K, Tachikawa S, Hayashi Y, Ito Y, et al. Enzyme-free passage of human pluripotent stem cells by controlling divalent cations. Sci Rep. 2014;4:4646.

Street CA, Bryan BA. Rho kinase proteins–pleiotropic modulators of cell survival and apoptosis. Anticancer Res. 2011;31: 3645–57.

T'joen V, Declercq H, Cornelissen M. Expansion of human embryonic stem cells: a comparative study. Cell Prolif. 2011;44:462–76.

8 Characteristic Staining Patterns of Undifferentiated and Differentiated Pluripotent Stem Cells

This chapter illustrates the patterns observed *in situ* in populations of pluripotent stem cells (PSCs) when they are stained with fluorescently tagged antibodies to detect antigens expressed on the cell surface or within the cells, thereby identifying a specific molecular marker. These patterns make it possible to judge the homogeneity of a population of cells. A core set of antibodies are used to determine the undifferentiated status of a PSC line. These include: The stage-specific embryonic antigens SSEA-1, SSEA-3, SSEA-4, the Trafalgar antigens TRA-1-60, and TRA-1-81 and Oct-4 (octamer-binding transcription factor 4). It is prudent to align the morphology of the colony with the staining pattern to demonstrate concordance.

The antibodies can be conjugated to a variety of different fluorochromes, enabling a number of molecular markers to be analysed simultaneously if required. Fluorescent dyes, such as DAPI (4′,6-diamidino-2-phenylindole), can also be used in this type of analysis. DAPI binds strongly to double-stranded DNA, producing a blue stain when viewed under a fluorescent microscope with the appropriate filter set. As a nuclear stain, DAPI can identify individual cells and is used in conjunction with the molecular marker-specific stains (Figs. 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 8.10, 8.11, 8.12, 8.13 and 8.14).

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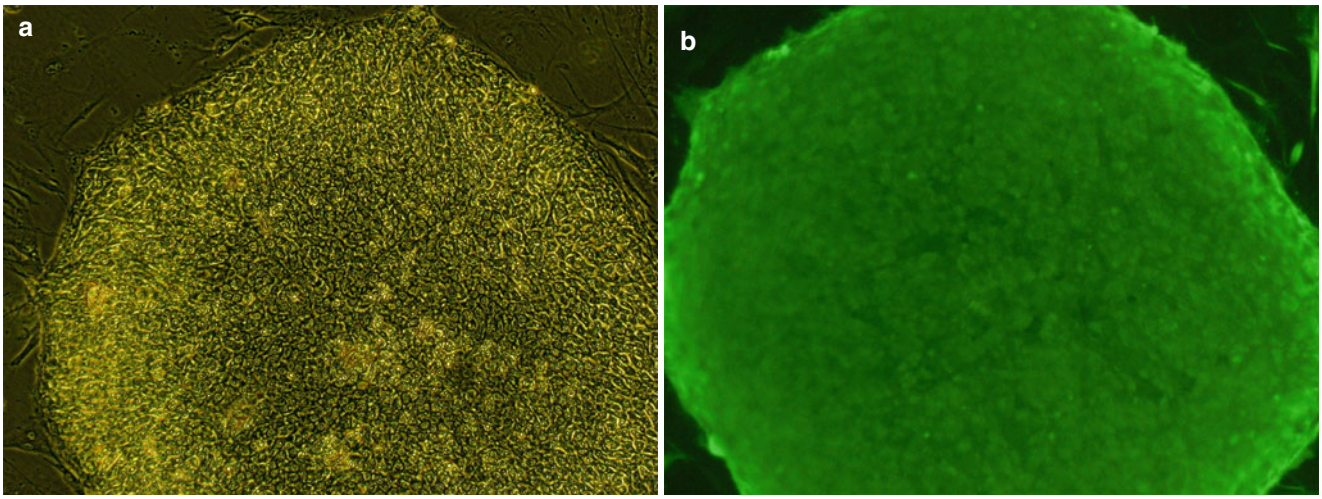

Fig. 8.1 A well-formed, compacted colony with defined borders. This colony shows the typical morphology of undifferentiated cells. (**a**) Image taken under phase contrast. (**b**) Staining with TRA-1-60, an antibody that recognises an epitope that is present on undifferentiated cells (both ×10 magnification)

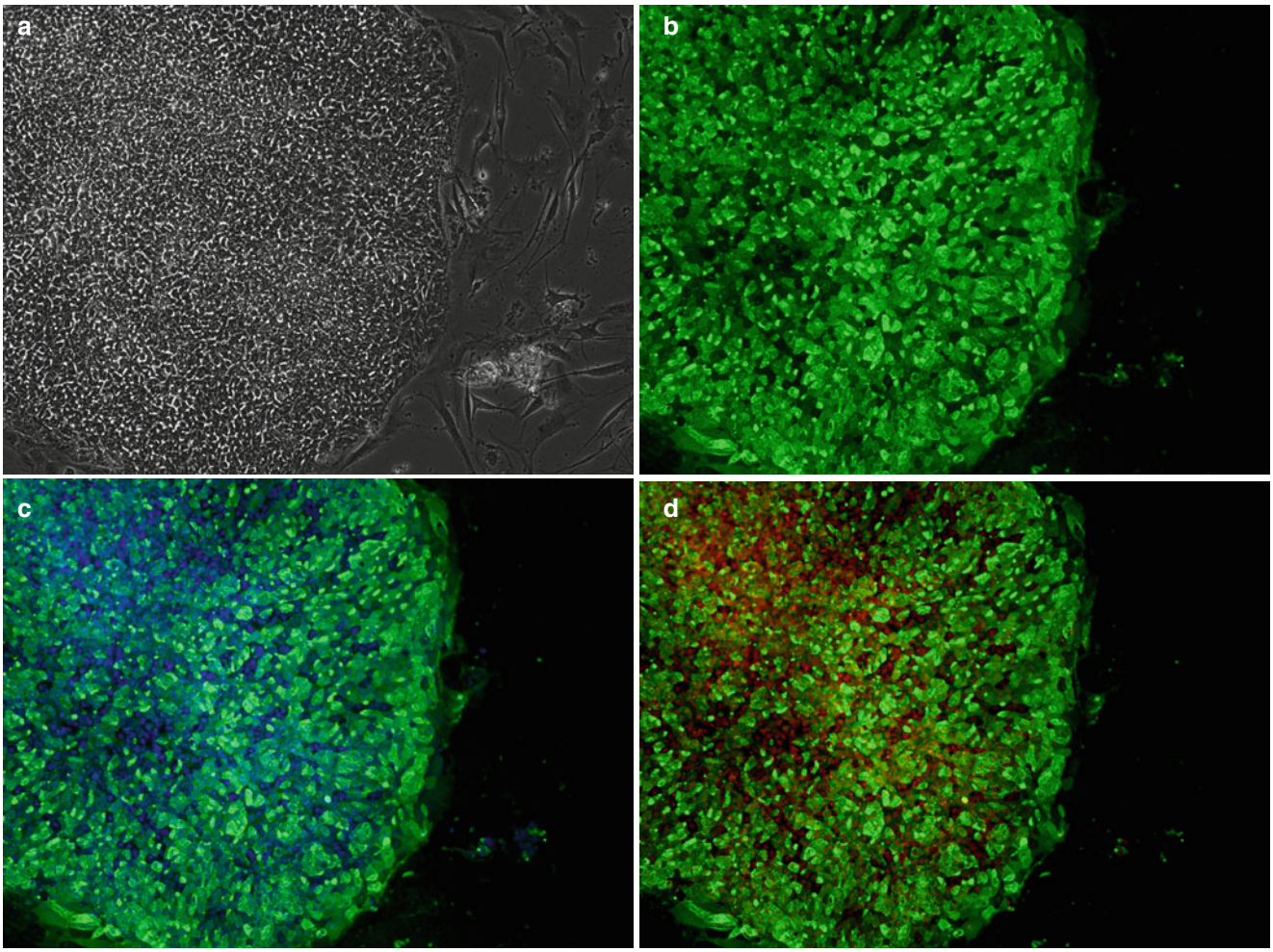

Fig. 8.2 An undifferentiated colony. (**a**) Under phase contrast microscopy. (**b**) TRA-1-81 staining alone. (**c**) TRA-1-81 merged with the nuclear stain, DAPI (4′,6-diamidino-2-phenylindole). (**d**) TRA-1-81 merged with Oct-4. Antibodies TRA-1-81 and Oct-4 recognise epitopes present on undifferentiated cells (all ×10 magnification)

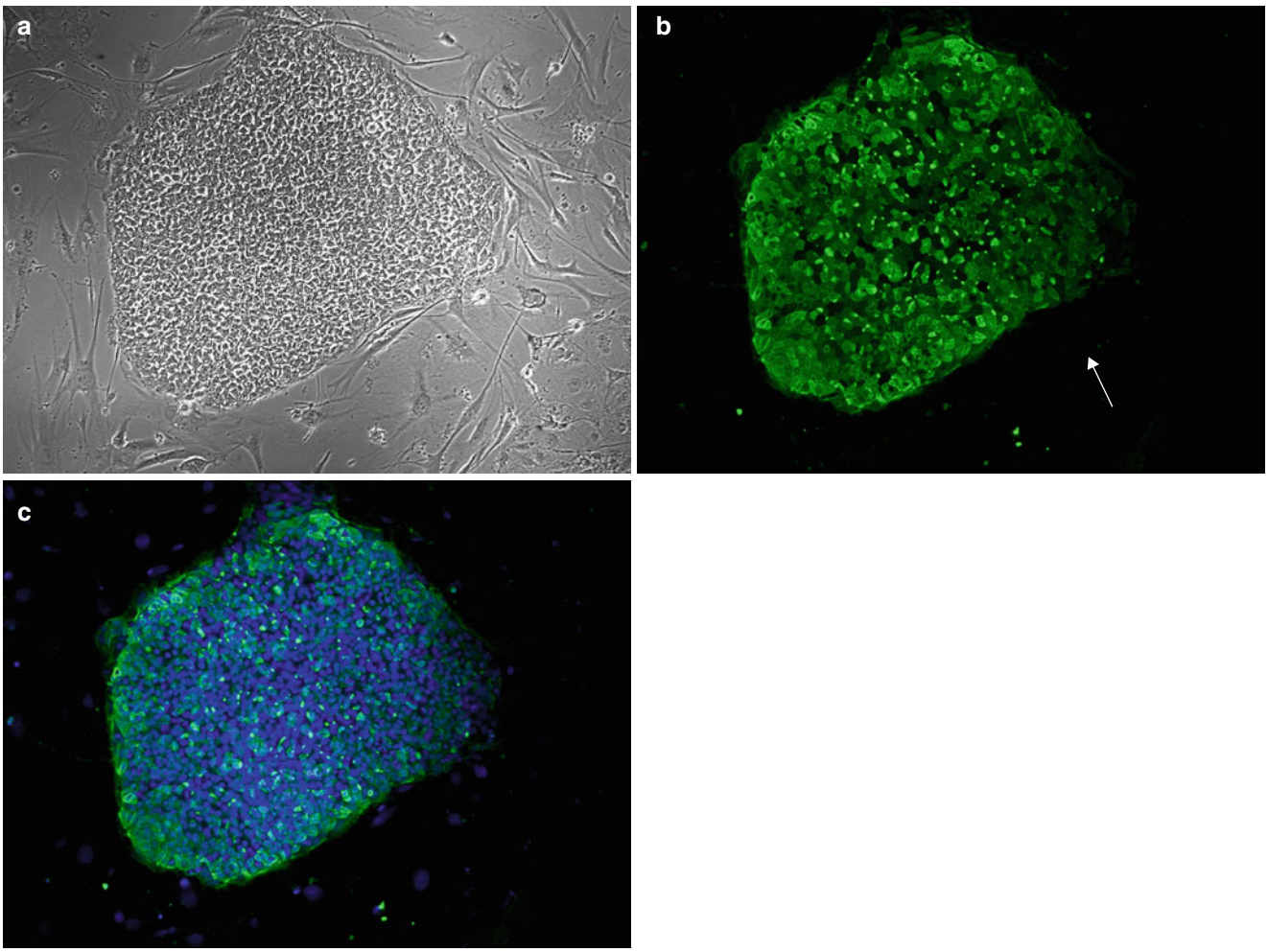

Fig. 8.3 Another example of a small, undifferentiated colony. (**a**) Under phase contrast microscopy. (**b**) TRA-1-60 alone. (**c**) TRA-1-60 merged with DAPI. This figure exhibits a good staining pattern: The feeders are negative (*white arrow*), and the nuclei of both the pluripotent stem cells (PSCs) and fibroblasts are stained with DAPI, whilst the TRA-1-60 staining pattern is evident on the cell surface (all ×10 magnification)

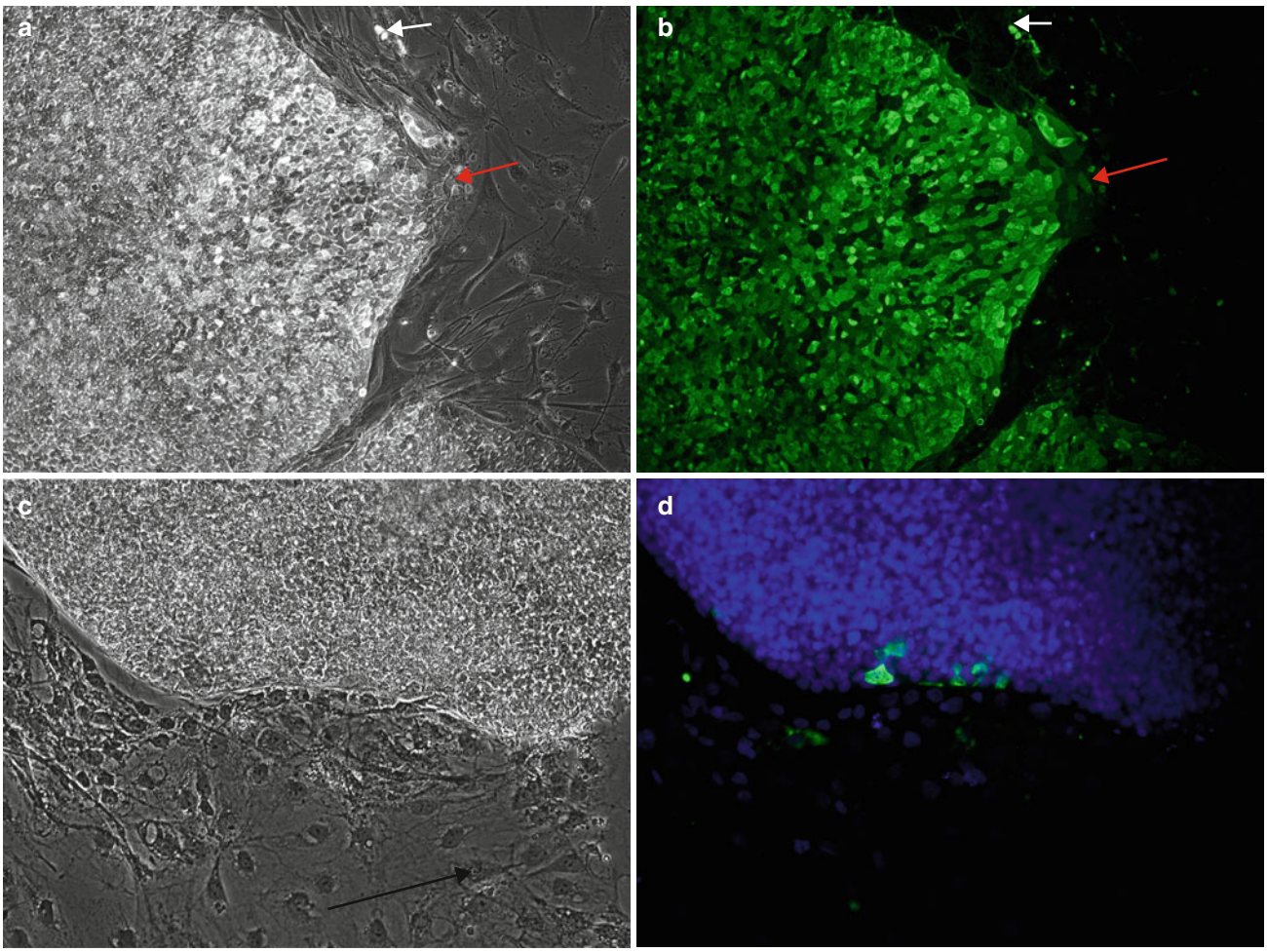

Fig. 8.4 This Day 10 culture is considered old, past the prime time for passaging. (**a**) The colony has become multilayered, but it is still very much positive for TRA-1-60. (**b**) Note that the feeders are negative for TRA-1-60. Apoptotic cells present in the culture display non-specific fluorescence (*white arrows in a and b*). A few cells are differentiating (*red arrow in a and b*) and display a fainter TRA-1-60 staining pattern than their undifferentiated counterparts. (**c**) An overconfluent, multilayered colony with differentiation outside the colony. From the morphology, this differentiation (*black arrows*) appears to be down the neural lineage. (**d**) SSEA-1 (stage-specific embryonic antigen 1) merged with DAPI stain. Only a few cells are stained with this marker. A positive SSEA-1 antibody stain is indicative of early differentiation, as this antibody recognises an epitope present on differentiating cells (all ×10 magnification)

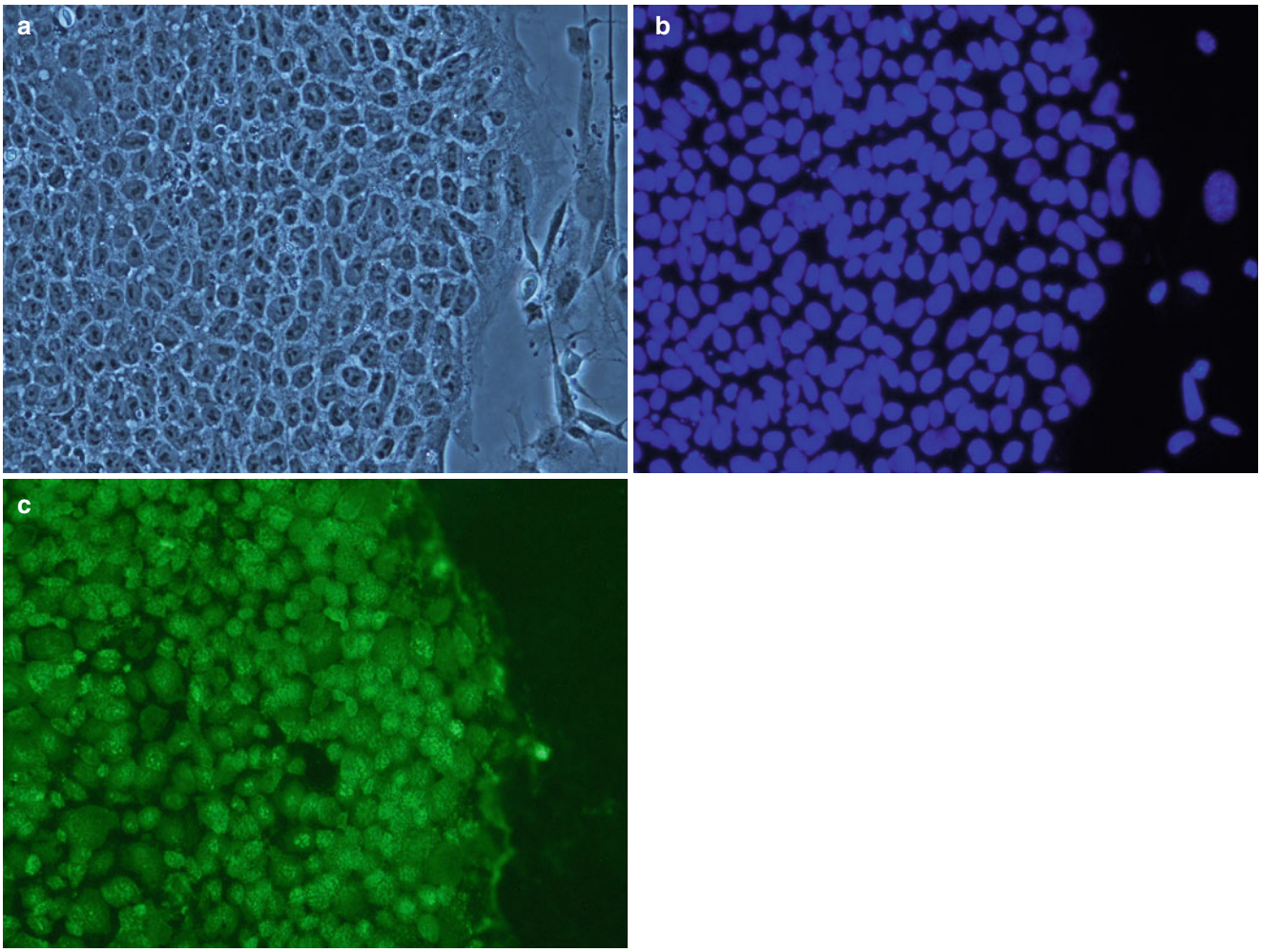

Fig. 8.5 An undifferentiated colony. (**a**) Phase contrast. (**b**) Nuclei stained with DAPI. (**c**) TRA-1-81 staining alone (both ×20 magnification)

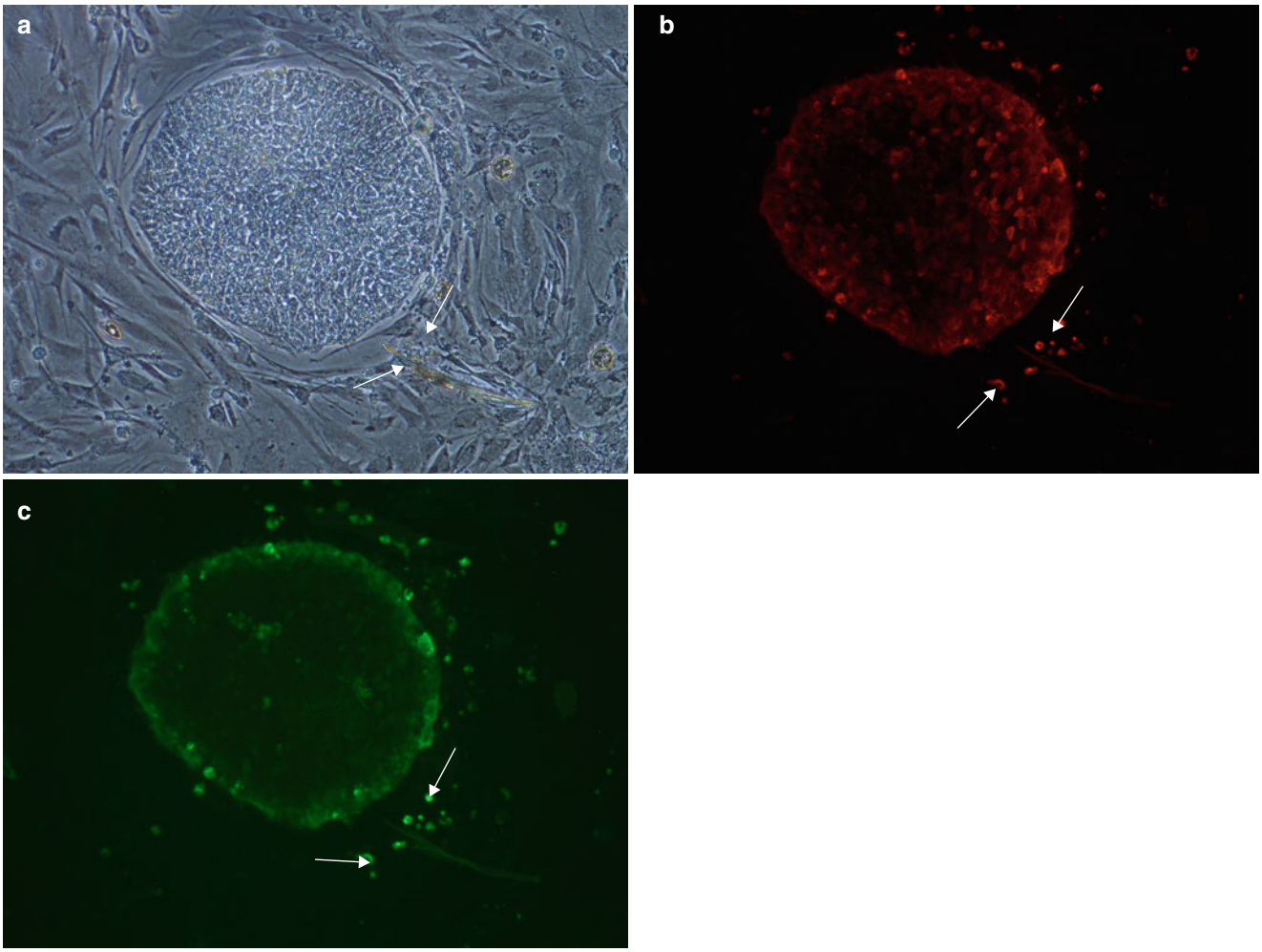

Fig. 8.6 (**a**) A small, undifferentiated colony growing on high-density feeders. These cells are stained with two markers that indicate that the cells are undifferentiated: Positive staining patterns can be seen for these antibodies in TRA-1-60 (**b**) and SSEA-3 (**c**). Apoptotic cells are present and display non-specific fluorescence (*white arrows*) (all ×10 magnification)

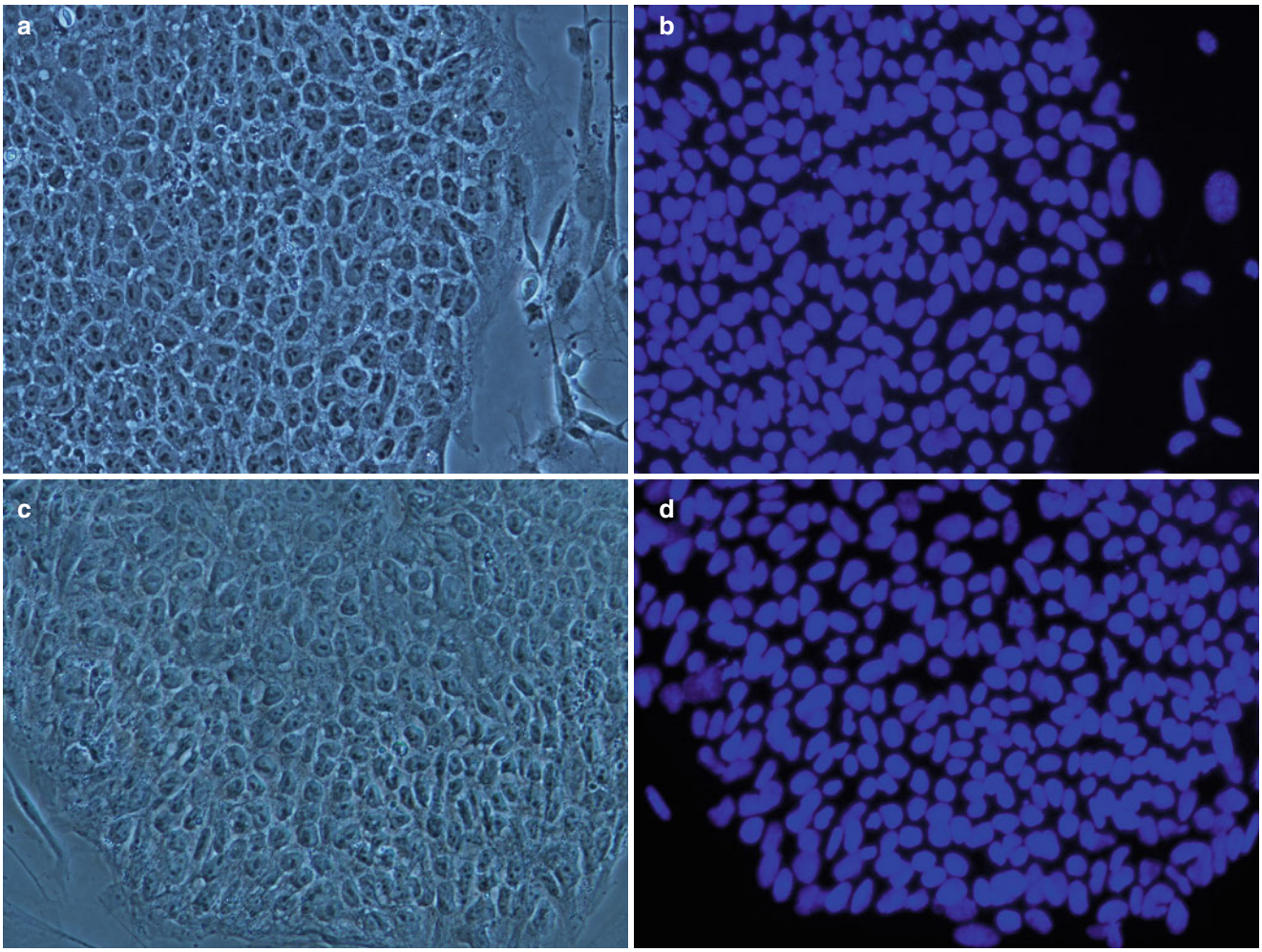

Fig. 8.7 (**a** and **c**) Undifferentiated colony of cells on mouse embryonic fibroblasts (MEFs). (**b** and **d**) The nuclei in these images are stained with DAPI. It is easy to recognise a good-quality colony morphology just from the DAPI staining pattern. In an undifferentiated colony, the nuclei are evenly spread, are close together, and are similar in size. The cells are also compacted. In the case of feeders, the nuclei are far away from each other and less round. They also appear in a less regular pattern (×20 magnification)

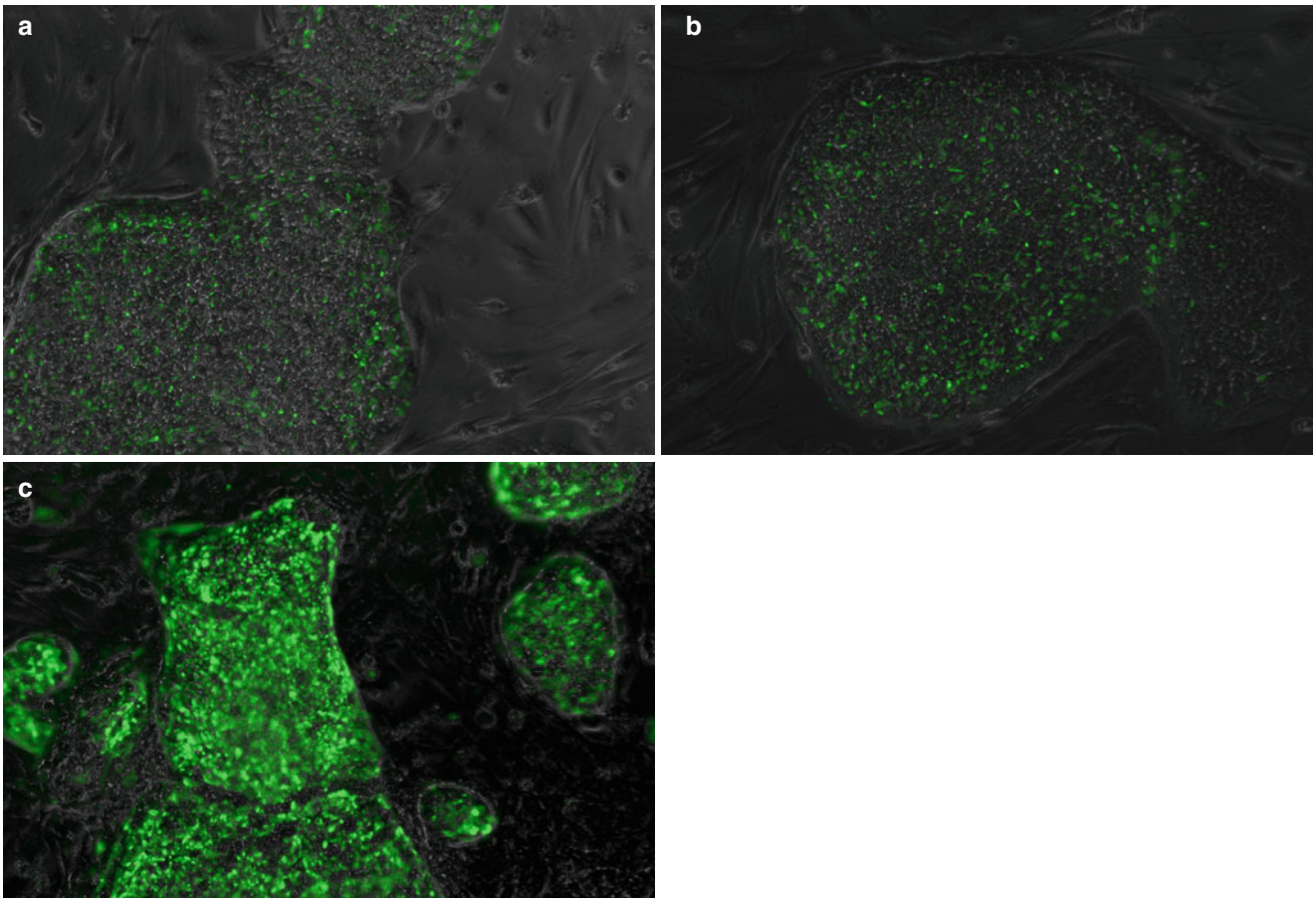

Fig. 8.8 (**a**–**c**) These images show live cells stained positive with the TRA-1-60 marker. The live stain intensity may vary depending on the light source (all ×10 magnification)

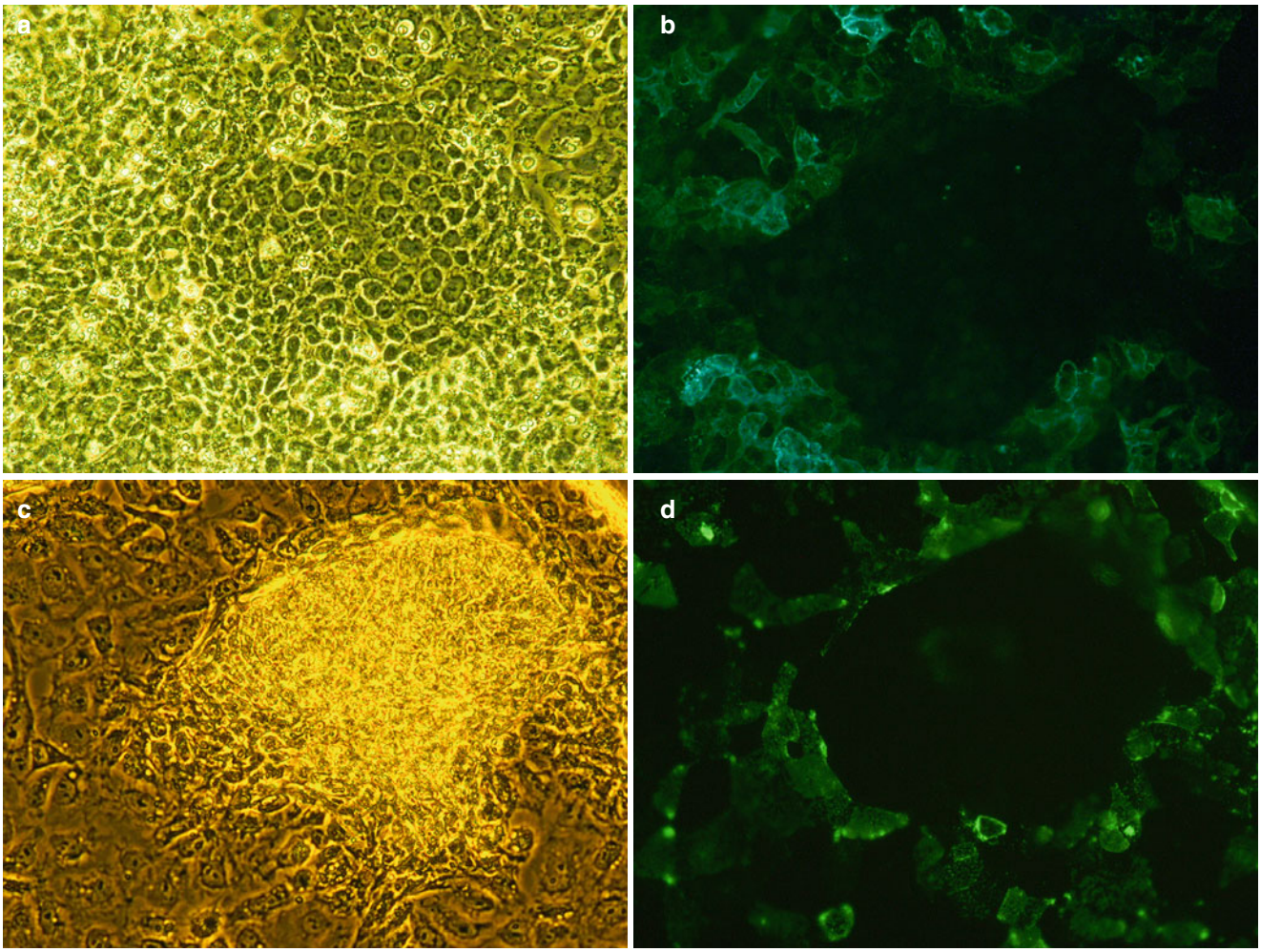

Fig. 8.9 (**a**–**d**) Day 10 colonies (**a**), This colony shows an area of differentiation in the middle, (**b**) stained negative with the marker TRA-1-60, indicating the undifferentiated state. This Day 10 colony (**c**) is still undifferentiated in the multi-layered centre and (**d**) stained negative with the early differentiation marker SSEA-1 (both ×20 magnification)

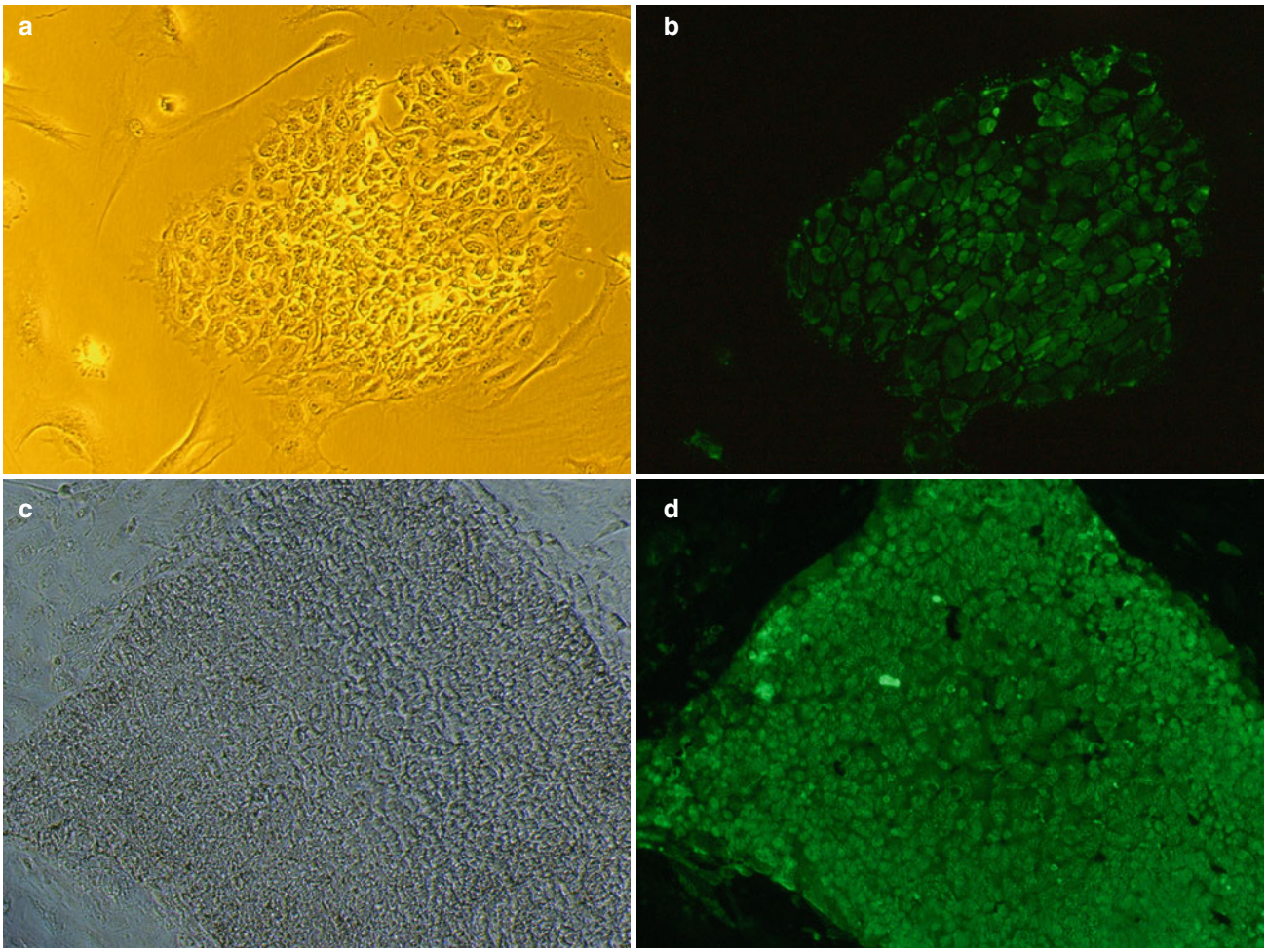

Fig. 8.10 A small, undifferentiated colony viewed under brightfield microscopy (**a**) and stained positive with another marker of the undifferentiated state, SSEA-4 (**b**). Another compacted, undifferentiated colony is seen under phase contrast (**c**) and stained positive with TRA-1-60 (**d**) (all ×10 magnification)

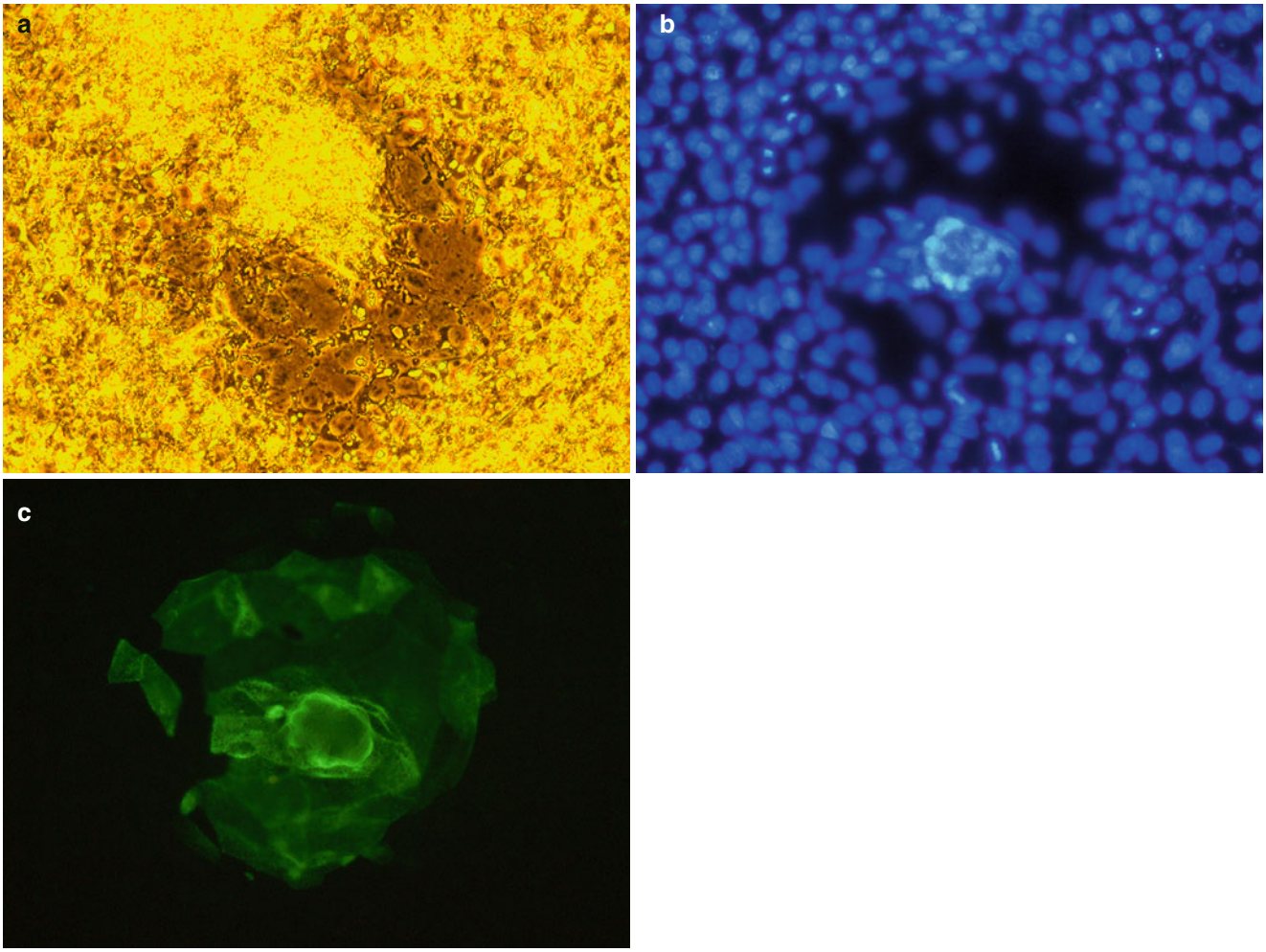

Fig. 8.11 A 14-day-old differentiated colony is seen under brightfield microscopy (**a**) and stained with DAPI (**b**) and SSEA-1 (**c**). Only the centre of the colony stains positive with SSEA-1, but it is also easy to recognise the differentiation in the middle of the colony from the DAPI staining pattern, which demonstrates that the nuclei are not evenly spread throughout the area, are not close together, are not of a similar size, and are not compacted. The SSEA-1 staining pattern supports this conclusion (all ×20 magnification)

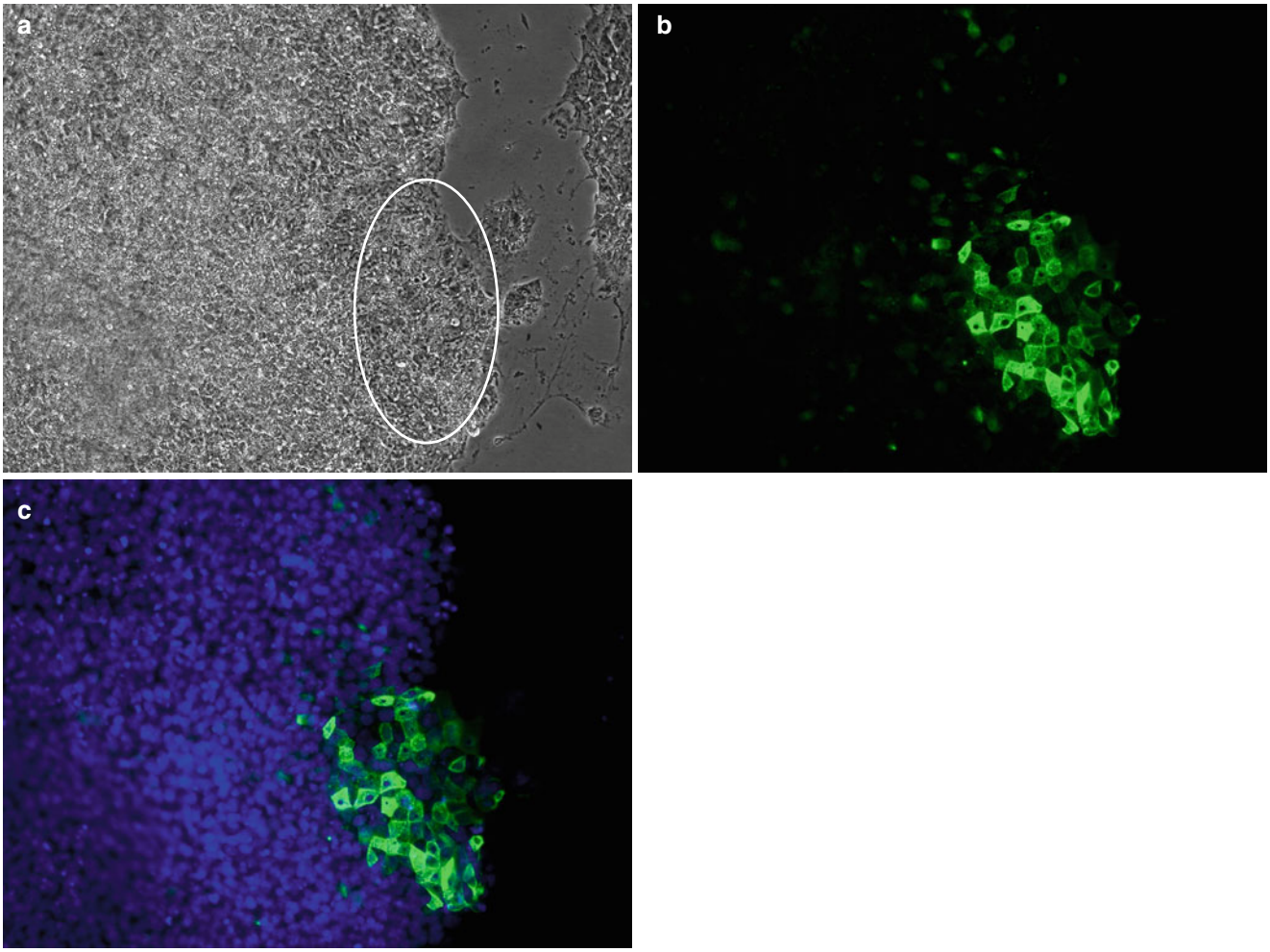

Fig. 8.12 (**a**) This example of an overconfluent, feeder-free, multilayered colony shows an area of differentiation (*white oval*) (**b**) stained positive with the SSEA-1 marker and (**c**) DAPI. Note that the nuclei stained with DAPI are very close together, owing to the multilayering of the cells. Interpreting the staining pattern with the SSEA-1 marker requires caution, as sometimes it appears in an unexpected part of the colony where the morphology of the cells is hard to distinguish (×10 magnification)

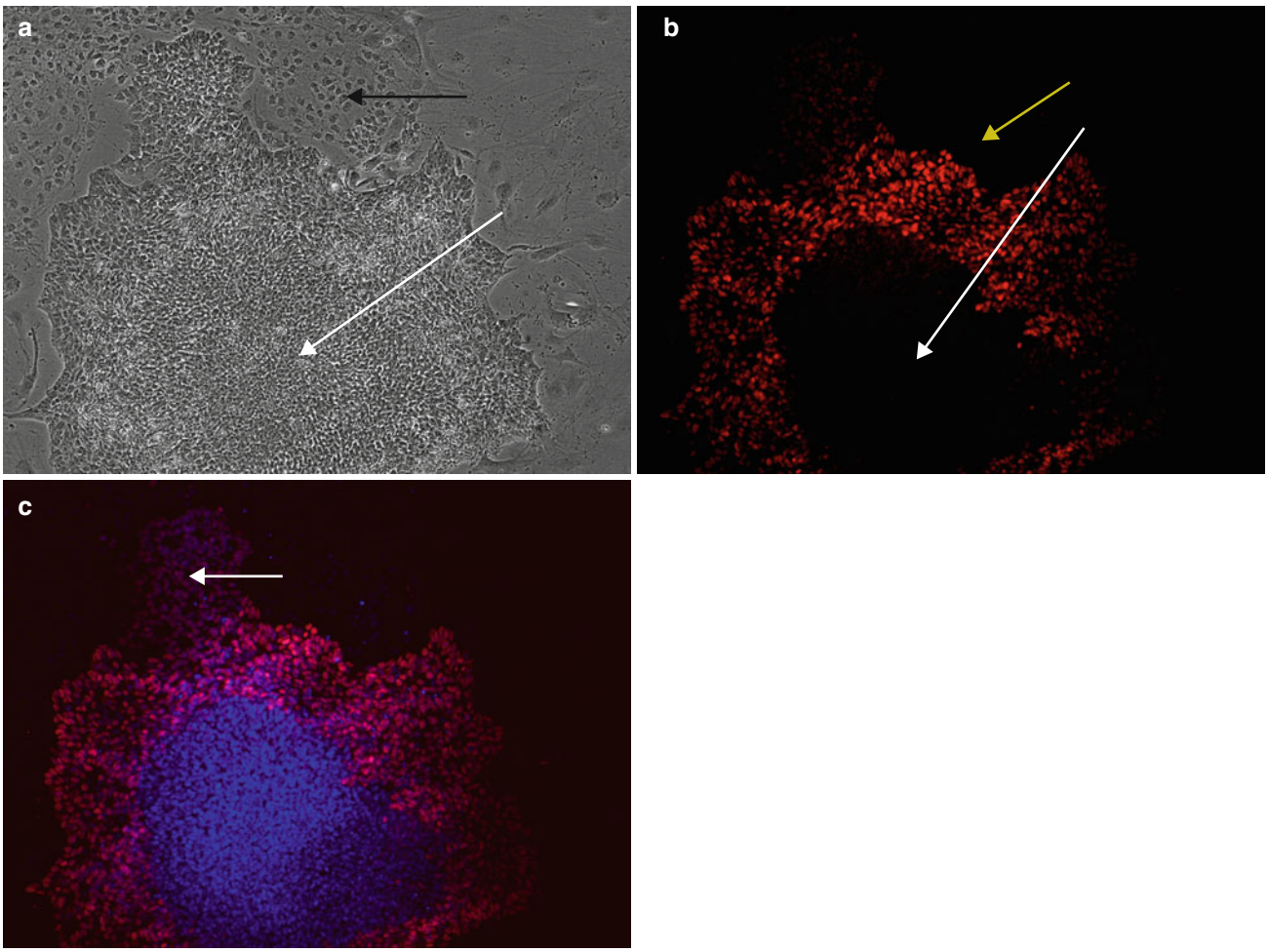

Fig. 8.13 (**a**) A good morphological example of a colony demonstrating undifferentiated and differentiated areas. There are Oct-4-positive cells within the partially differentiated colony. (**b**) In the centre of the colony, the cells are neural progenitors (*white arrow*); these stain negative for Oct-4. Outside the colony, there are cells that are differentiated into an unknown cell type (*yellow arrow*); these stain negative for Oct-4. (**c**) Oct-4 is merged with DAPI, clearly revealing the pattern of Oct-4 staining. Note the faint Oct-4 staining pattern in an area of the colony undergoing early differentiation (*white arrow*) (all ×10 magnification)

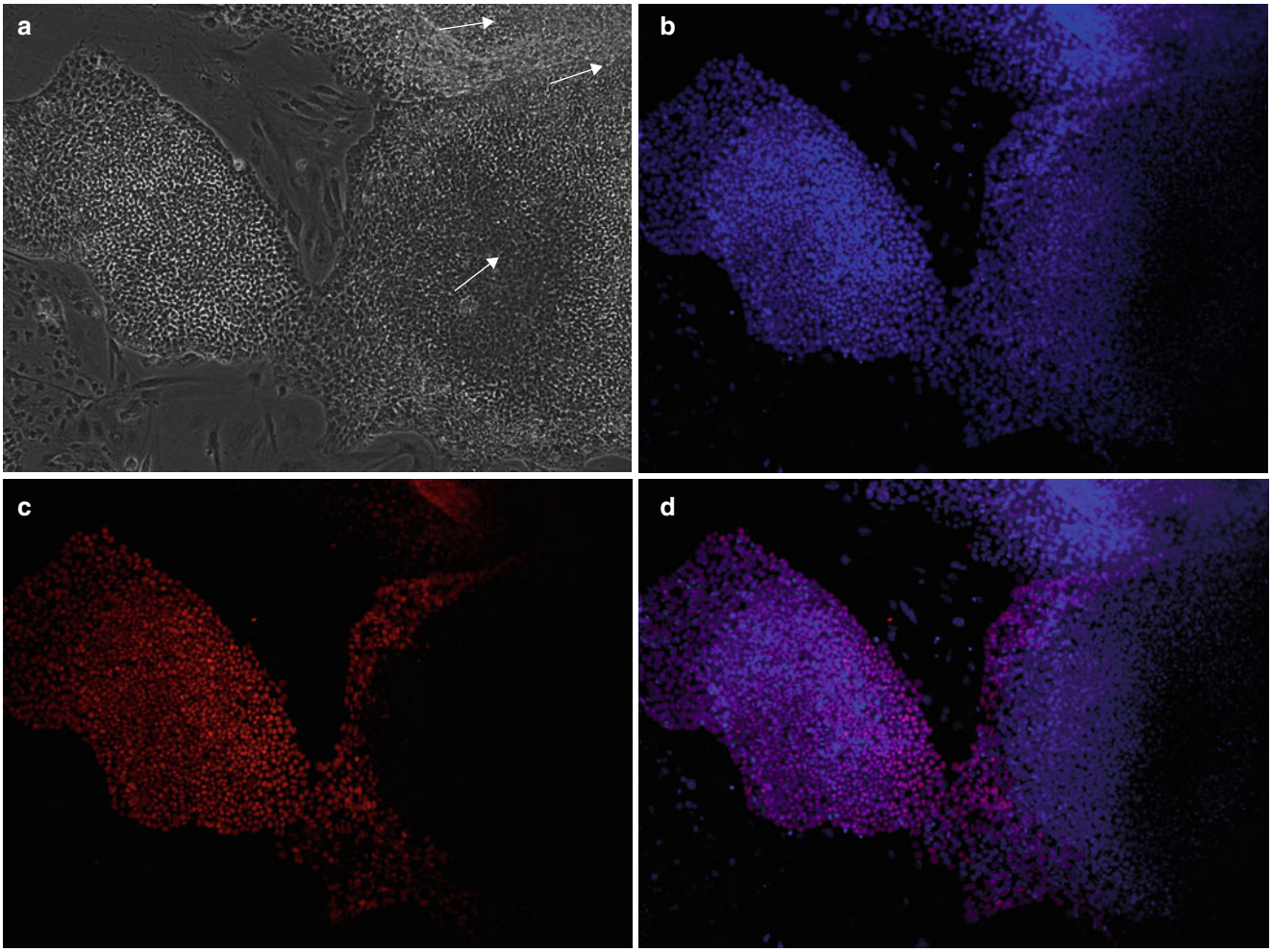

Fig. 8.14 Another example of cells with positive and negative Oct-4 staining within a 14-day-old colony that displays areas of differentiation. A (*white arrows*) (**a**) Brightfield. (**b**) DAPI. (**c**) Oct-4. (**d**) DAPI and Oct-4 merged (all ×10 magnification)

Fluorescently tagged antibodies can also be used in flow cytometry to monitor subsets of cells, but this technique, although quantitative, gives no indication of the spatial expression of the antigens on the cells within a colony or in a monolayer of cells. Each germ layer and cell lineage can also be characterised by a number of specific molecular markers, so that differentiation status can be assigned when required (Figs. 8.15, 8.16 and 8.17).

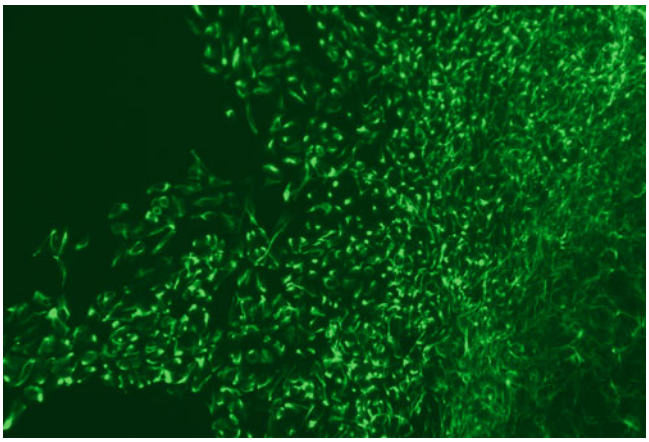

Fig. 8.15 Cells differentiating down the neural lineage, which show a positive antibody staining pattern for the early neural marker nestin (×10 magnification)

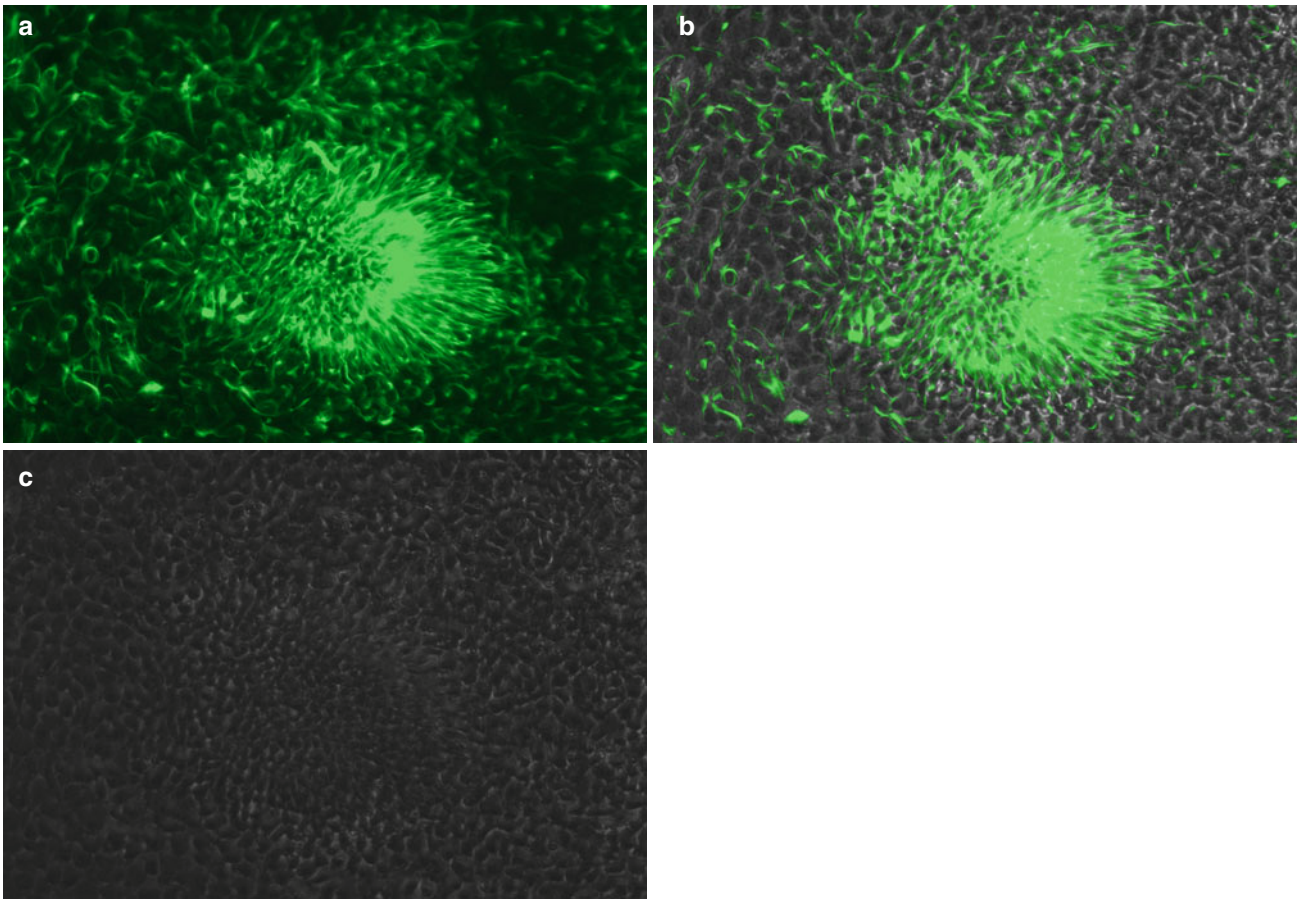

Fig. 8.16 Cells differentiating down the neural lineage. (**a**) Nestin-positive cells within a rosette in the middle of the image (**c**) A brightfield image showing a typical neural rosette. (**b**) A merged brightfield and fluorescent-stained image shows how the staining pattern coincides with the specific rosette morphology (**a**, **b**, **c**) ×10 magnification)

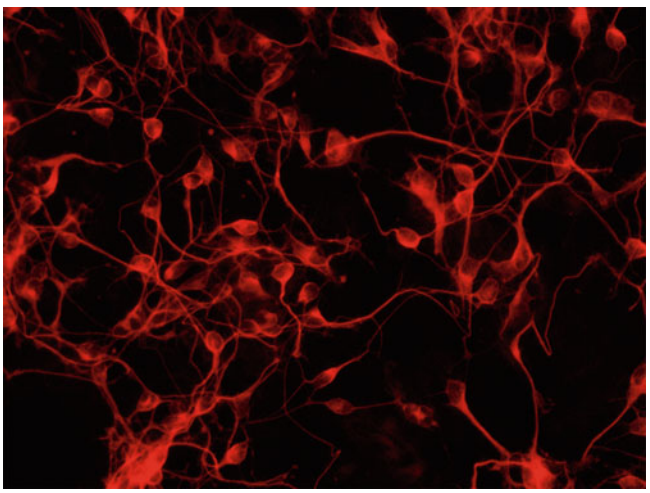

Fig. 8.17 Neural cells stained positive with β3 tubulin, a neural cell marker specific for mature cells of the neural lineage (×20 magnification)

The staining of cells in situ provides a rapid means to determine the quality of cells in culture, but unless live staining is coupled with aseptic technique, this technology is used as a surrogate assay to determine the status of the cell culture at a particular point in the in vitro processing.

Suggested Reading

Atlasi Y, Mowla SJ, Ziaee SA, Gokhale PJ, Andrews PW. OCT4 spliced variants are differentially expressed in human pluripotent and non-pluripotent cells. Stem Cells. 2008;26:3068–74.

Calloni R, Cordero EA, Henriques JA, Bonatto D. Reviewing and updating the major molecular markers for stem cells. Stem Cells Dev. 2013;22:1455–76.

Draper JS, Pigott C, Thomson JA, Andrews PW. Surface antigens of human embryonic stem cells: changes upon differentiation in culture. J Anat. 2002;200:249–58.

International Stem Cell Initiative, Adewumi O, Aflatoonian B, Ahrlund-Richter L, Amit M, Andrews PW, et al. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat Biotechnol. 2007;25:803–16.

Kannagi R, Cochran NA, Ishigami F, Hakomori S, Andrews PW, Knowles BB, Solter D. Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. EMBO J. 1983;2:2355–61.

Laevsky I. Immunostaining. In: Amit M, Itskovitz-Eldor J, editors. Atlas of human pluripotent stem cells: derivation and culturing, Stem cell biology and regenerative medicine. New York: Humana Press; 2012. p. 105–14.

Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell. 1998;95:379–91.

Nourse JL, Ziaeian B, Palmer T, Schwartz PH, Flanagan LA. Immunocytochemical analysis of human stem cells. In: Loring JF, Peterson SE, editors. Human stem cell manual. 2nd ed. London: Elsevier; 2012. p. 249–70.

Schopperle WM, DeWolf WC. The TRA-1-60 and TRA-1-81 human pluripotent stem cell markers are expressed on podocalyxin in embryonal carcinoma. Stem Cells. 2007;25:723–30.

Zhao W, Ji X, Zhang F, Li L, Ma L. Embryonic stem cell markers. Molecules. 2012;17:6196–236.

Derivation of Induced Pluripotent Stem Cells

9

Chapters 5 and 6 llooked at human induced pluripotent stem cell (hiPSC) lines growing in feeder-based and feeder-free systems. This chapter will review the derivation of hiPSCs using a non-viral delivery system. The various systems used to deliver the reprogramming factors, have pros and cons that must be taken into account when generating new iPSCs for specific purposes. Not all delivery systems work on all cell types. Some delivery systems have a residual effect on the host genome caused by the delivery system itself, as is the case for retroviral vectors. To date, the methods of choice are those that use agents that do not integrate into the host genome. These include Sendai viral vectors, episomal vectors, protein transduction, and RNA-based transfection.

It is the RNA-based systems that are described and illustrated in this chapter via daily inspection of the morphology of the cultures. Two systems are demonstrated; both produced reprogrammed cells. The first system used messenger RNA alone and was feeder based (Figs. 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 9.10, 9.11, 9.12, 9.13, 9.14, 9.15 and 9.16). This system requires the transfection of reprogramming factors daily for 17 days, with iPSCs being identified from day 18. Figures 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 9.10, 9.11, 9.12, 9.13, 9.14, 9.15 and 9.16 show the daily morphology of the cells that are being reprogrammed.

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DOI 10.1007/978-1-4899-7507-2_9, © Springer Science+Business Media New York 2015

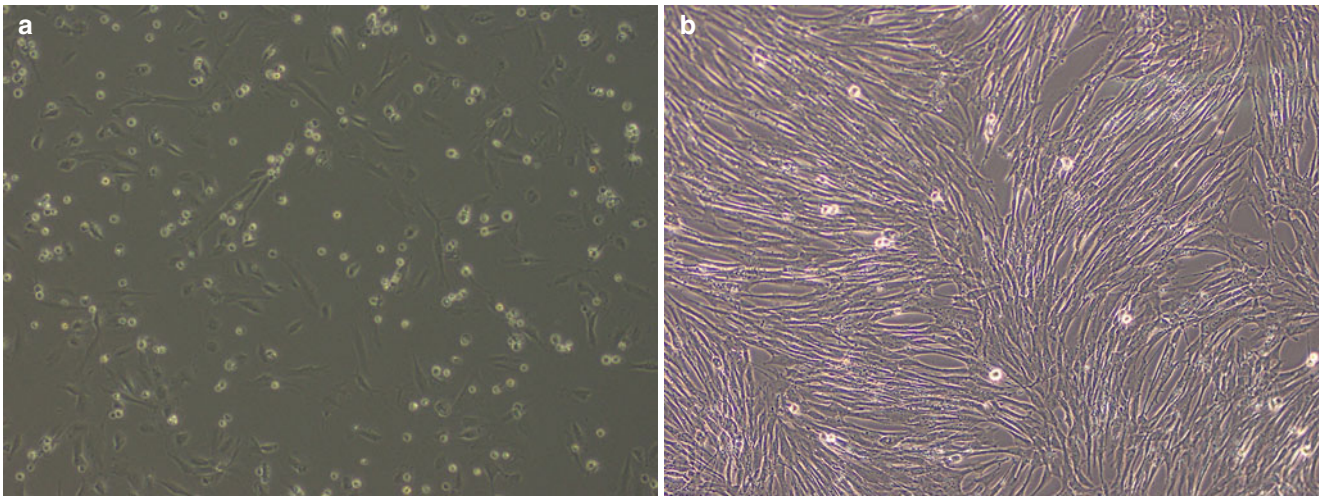

Fig. 9.1 (**a**) Cells at the stage Day -1 in the transfection process. The cells shown are inactivated newbornforeskin fibroblasts (NuFF), onto which the actively growing target fibroblasts will be plated. Because the feeder cells are inactivated, they are refractory to the transfection process, so only the actively dividing fibroblasts will take up the mRNA. (**b**) These cells are the actively growing fibroblasts MRC- 7,the target cells to be reprogrammed—at Day -1 prior to passaging. The target cells need to be actively proliferating in order to optimise the transfection with mRNA and increase the reprogramming efficiency (**a**, ×4 magnification; **b**, ×10)

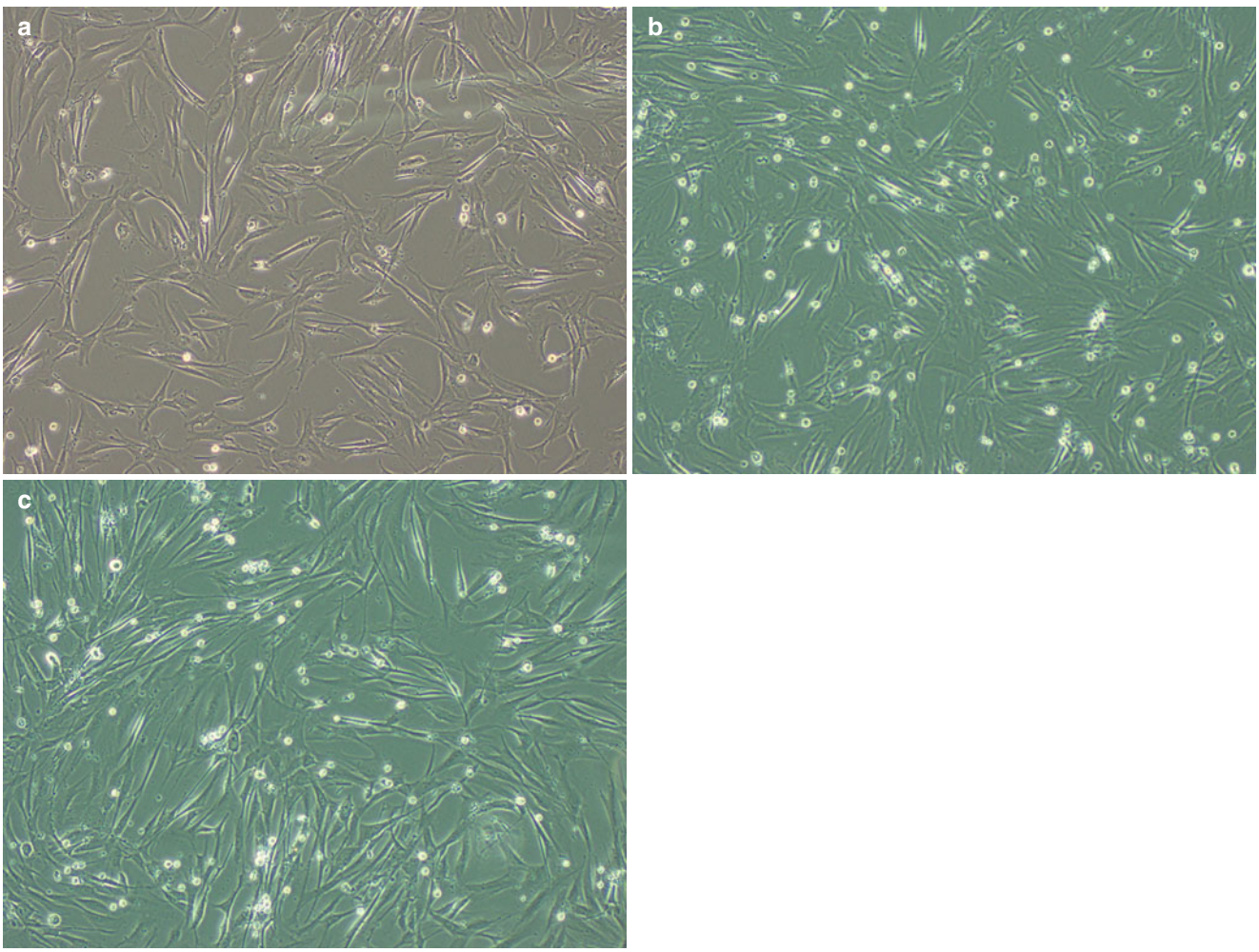

Fig. 9.2 MRC-7 Fibroblasts seeded at a concentration of 5000 cells per well of a six-well plate undergoing reprogramming. (**a**) Day 0. (**b**) Day 1. (**c**) Day 2 (all ×10 magnification)

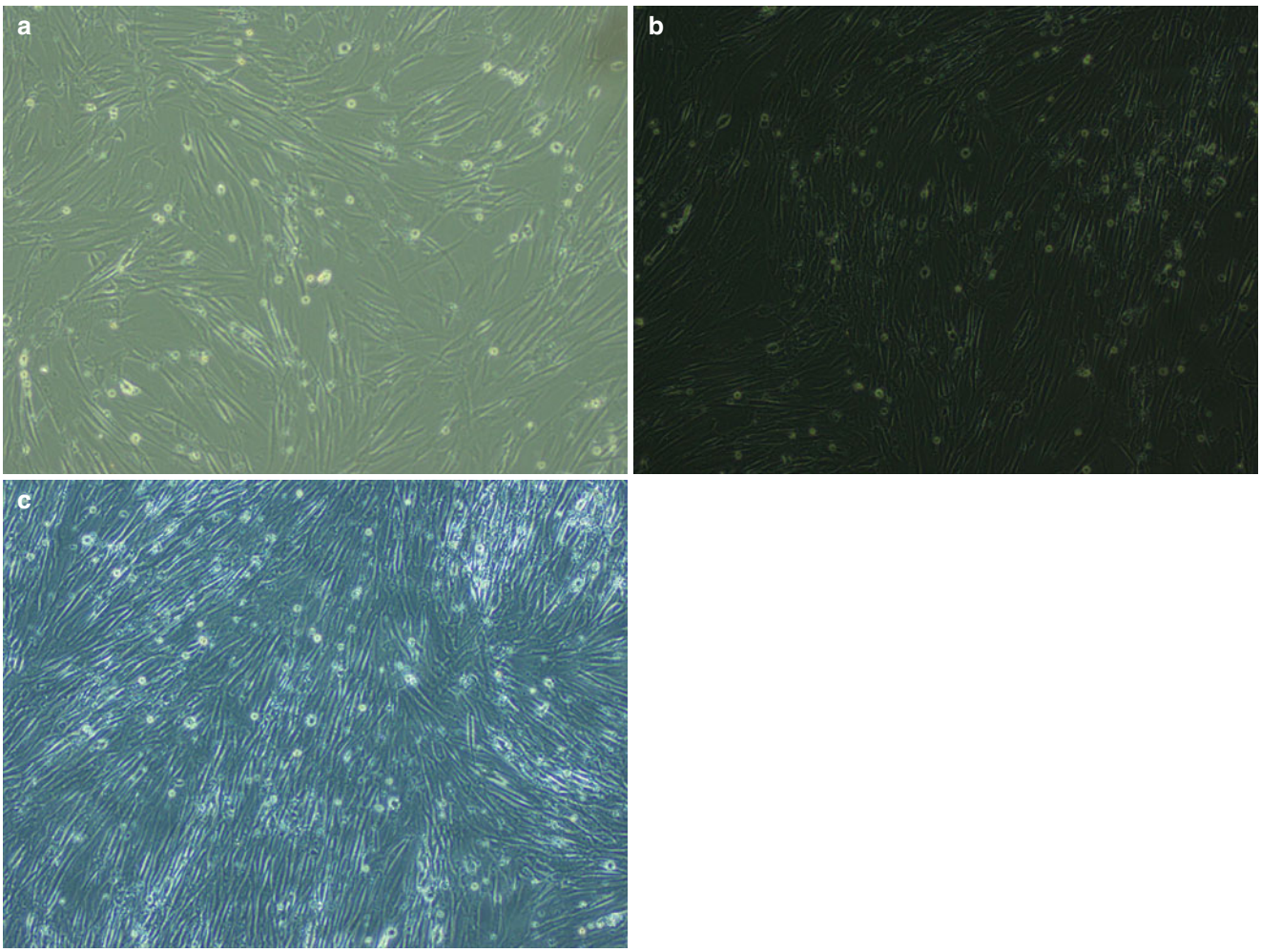

Fig. 9.3 The same fibroblasts. (**a**) Day 3. (**b**) Day 4. (**c**) Day 5 (all ×20 magnification)

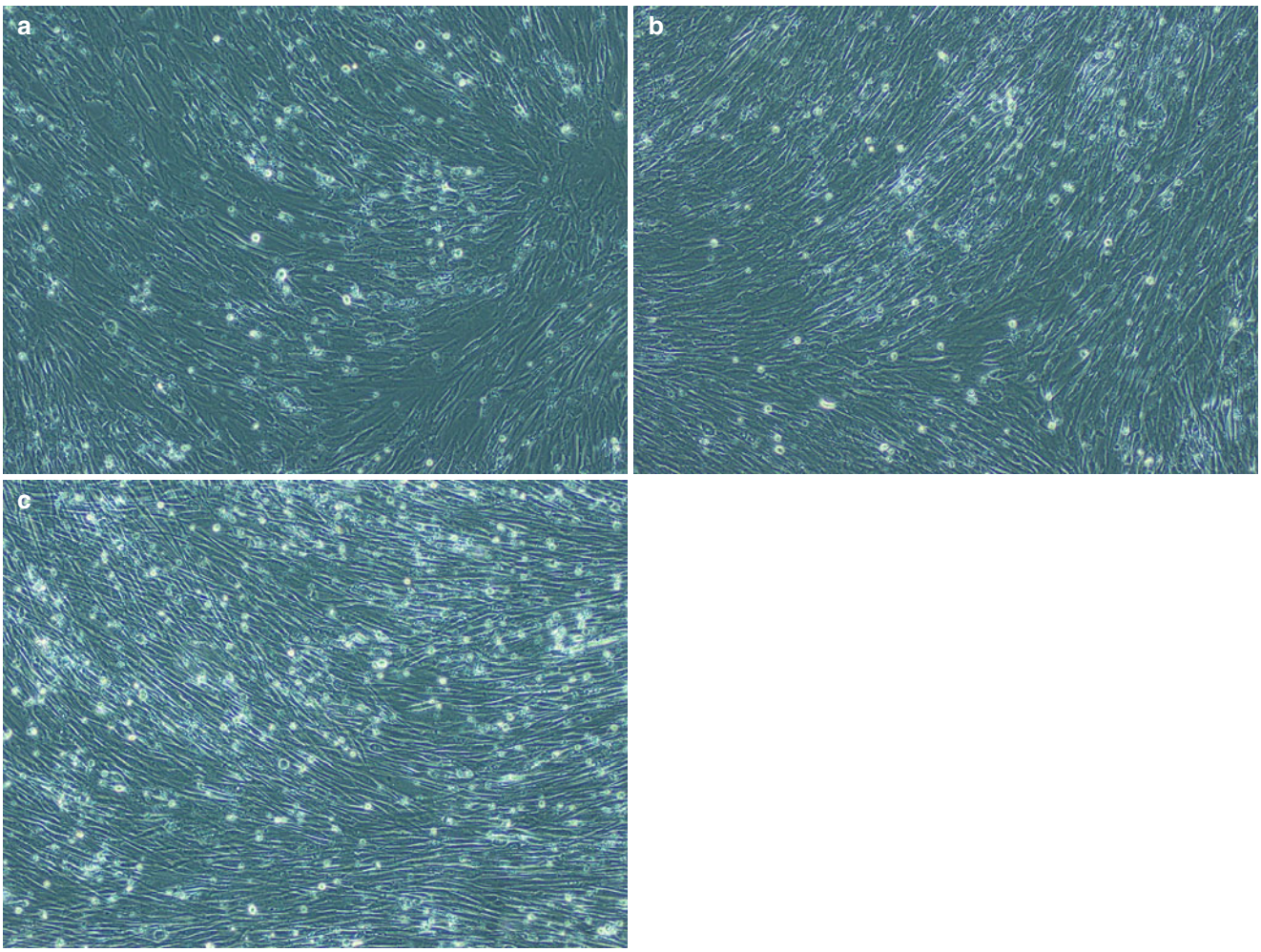

Fig. 9.4 The same fibroblasts. (**a**) Day 6. (**b**) Day 7. (**c**) Day 8 (all ×20 magnification)

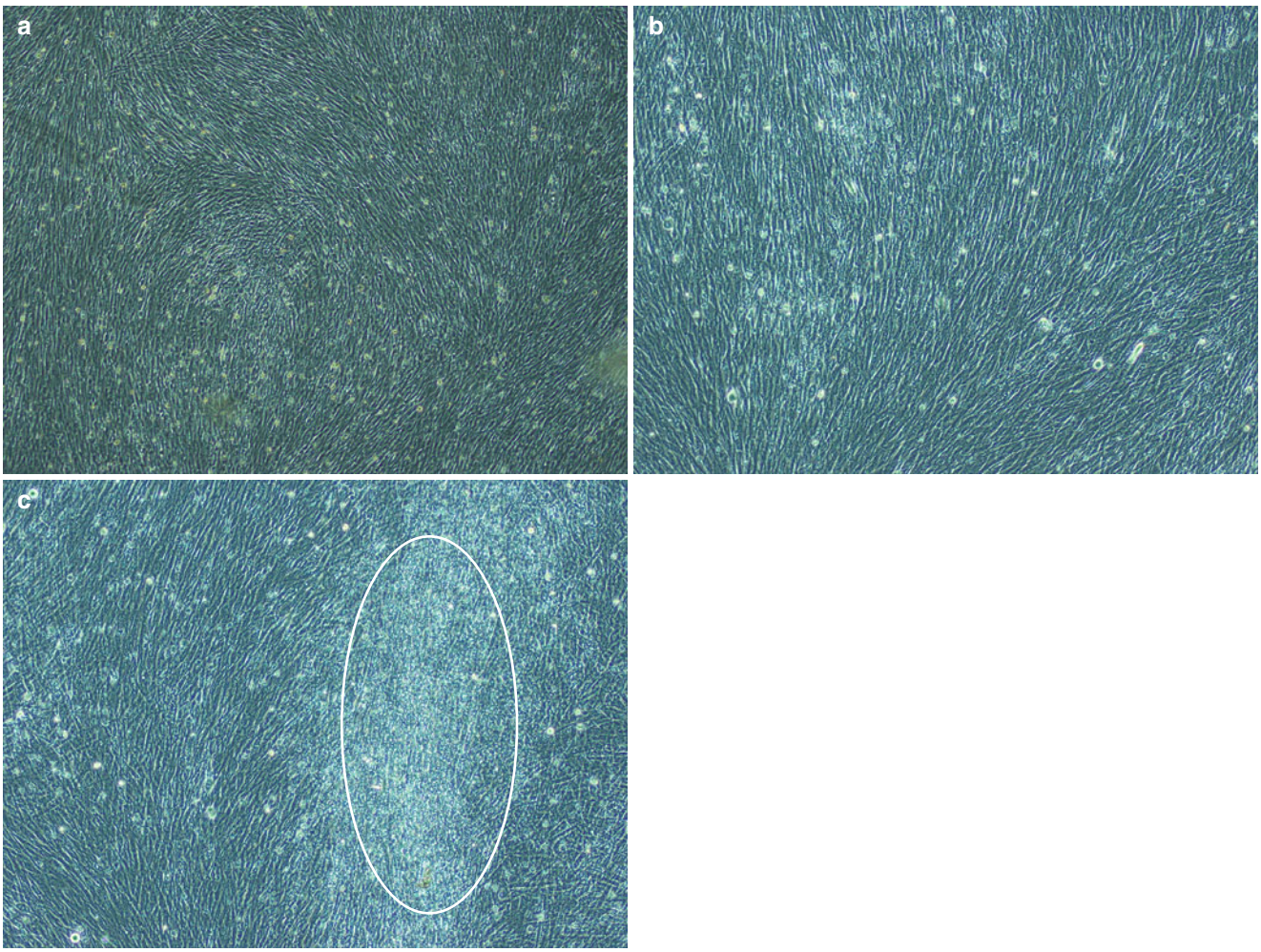

Fig. 9.5 MRC-7 fibroblasts being reprogrammed. (**a**) Day 9. (**b**) Day 10. (**c**) Day 11. On Day 11, note the area of overgrowth of cells in the culture (*white oval*), indicating a potential reprogramming of cells in the culture (all ×4 magnification)

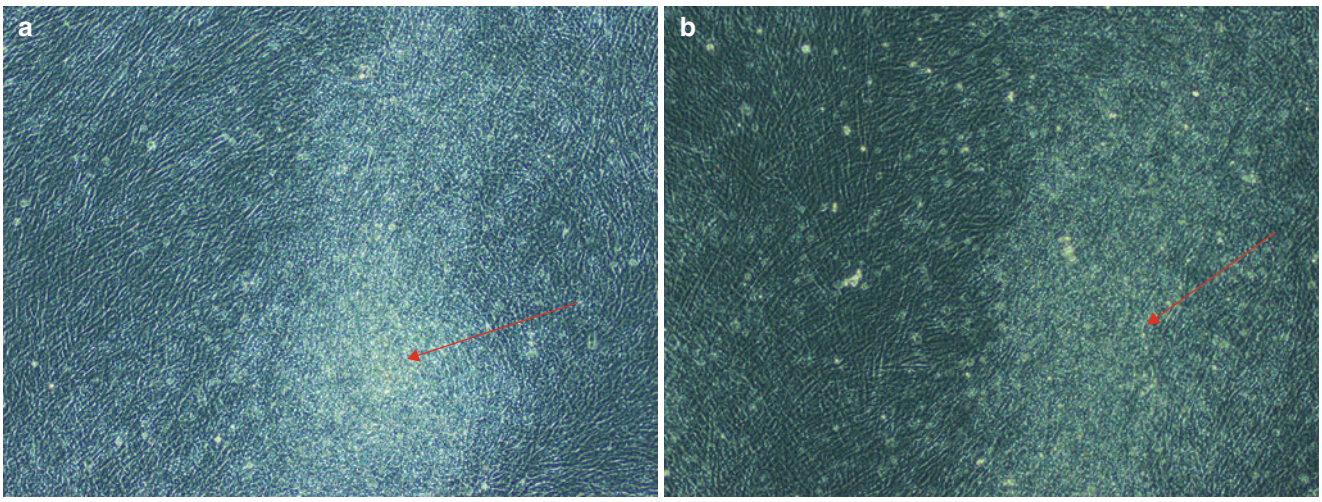

Fig. 9.6 MRC-7 fibroblasts seeded at a concentration of 5000 cells per well of a six-well plate and reprogrammed daily. (**a**) Day 12. (**b**) Day 13. As the days progress, the area of overgrowth is more evident and there is an indication of a change in morphology, from a fibroblast morphology to a disorganised patch of cells (*red arrows*). By day 13, it is obvious that the cells in the culture are too confluent and would prevent the outgrowth of reprogrammed cells. The reprogrammed cells would be outgrown by the fibroblasts that had not been reprogrammed (all ×4 magnification)

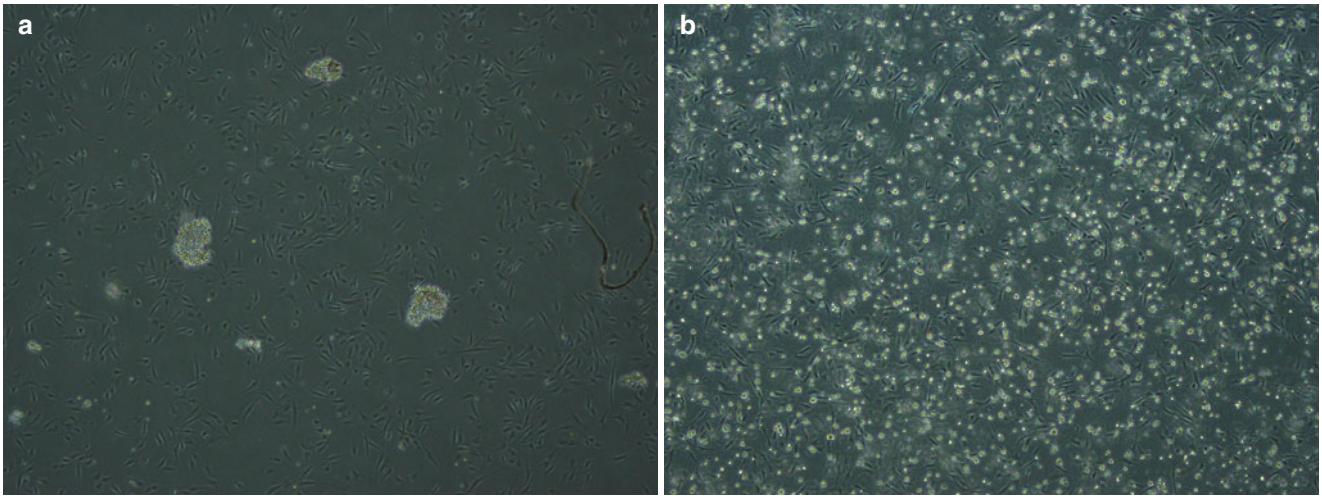

Fig. 9.7 On Day 14, the reprogrammed cells from two of the wells of the six-well plate were treated in two different ways and passaged onto fresh NuFF feeders. (**a**) The regions on the plate with morphological transformation of the fibroblasts were identified under a dissecting microscope, and the cells were passaged manually by cutting. The fragments were placed into a fresh NuFF feeder culture, to allow the reprogrammed cells to expand. (**b**) The cells from the well were treated with TrypLE™ Express (Life Technologies) and passaged onto fresh NuFF feeders (all ×4 magnification)

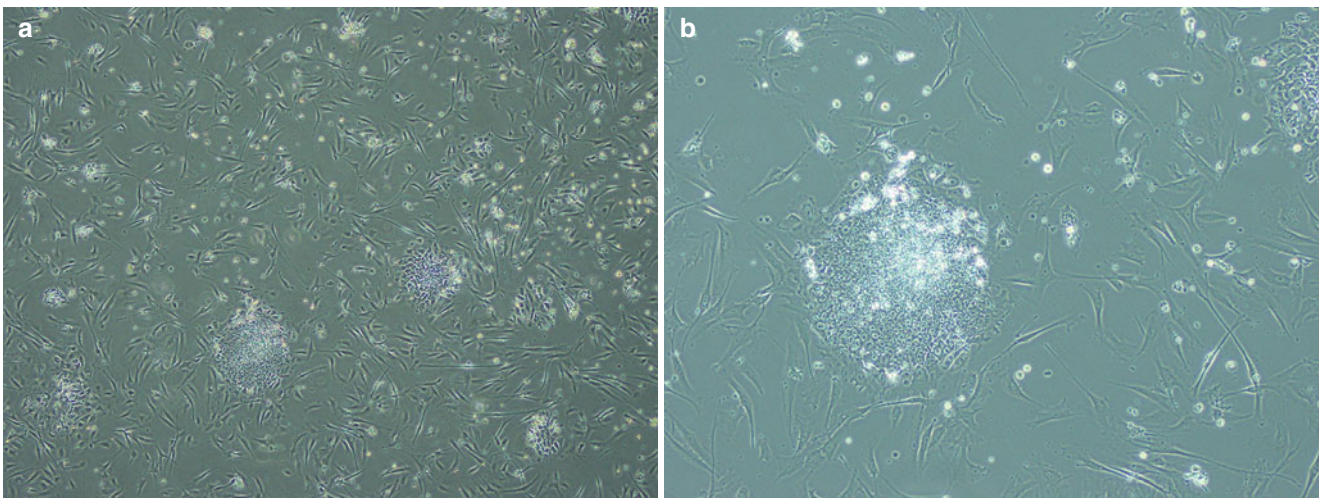

Fig. 9.8 (**a**) Day 15 MRC-7 areas of reprogrammed cells showing morphological transformation cut onto fresh NuFF feeders 1 day after transfer. (**b**) A higher magnification shows an iPSC colony containing cells with nucleoli characteristic of undifferentiated stem cells. (**a**, ×4 magnification; **b**, ×10)

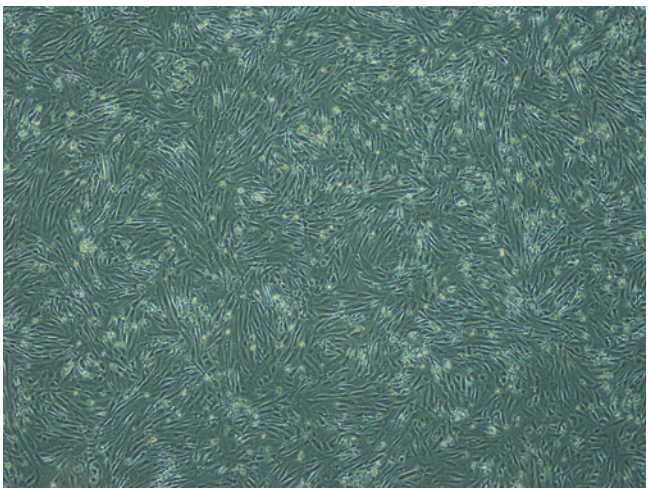

Fig. 9.9 Day 15 MRC-7 areas of reprogrammed cells showing morphological transformation following treatment with TrypLE™ Express and replated onto fresh NuFF feeders 1 day after transfer (×4 magnification)

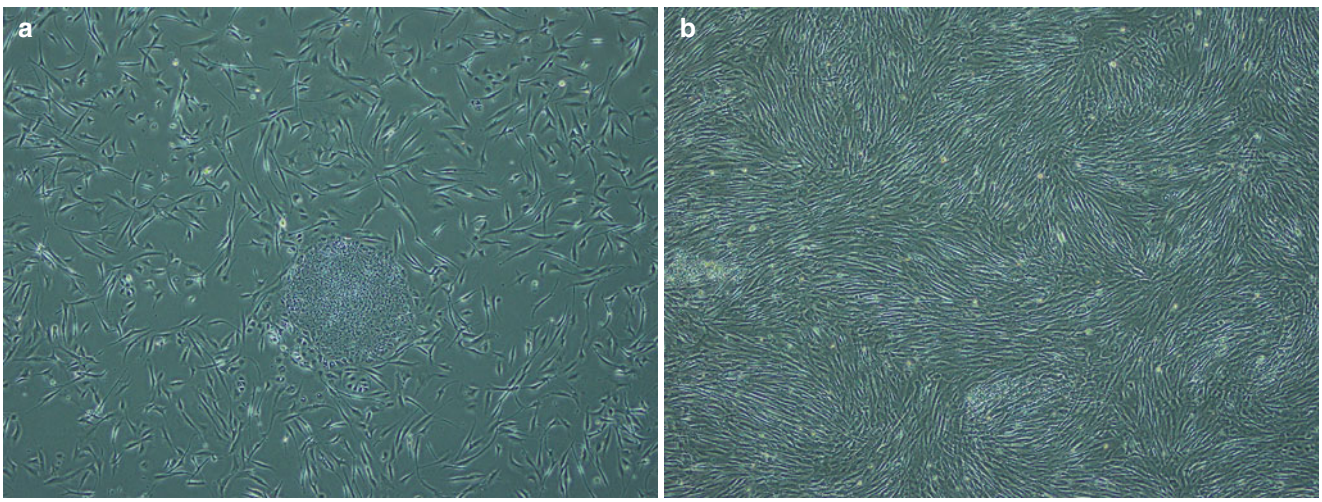

Fig. 9.10 (**a**) Day 16 MRC-7 reprogrammed cells cut onto fresh NuFF feeders 2 days after transfer. (**b**) Day 16 MRC-7 reprogrammed cells treated with TrypLE™ Express and transferred onto fresh NuFF feeders 2 days after transfer (**a**, ×4 magnification; **b**, ×10)

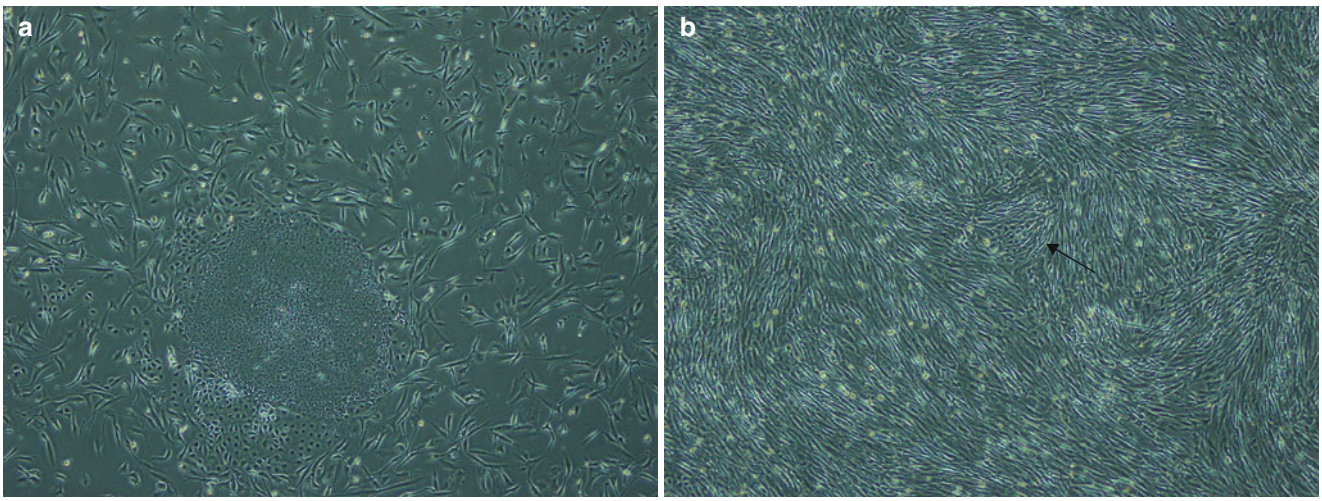

Fig. 9.11 (**a**) Day 17 MRC-7 reprogrammed cells cut onto fresh NuFF feeders 3 days after transfer. (**b**) Day 17 MRC-7 reprogrammed cells treated with TrypLE™ Express and transferred onto fresh NuFF feeders 3 days after transfer. A small area of reprogrammed cells can be seen emerging from the fibroblasts (*black arrows*) (both ×4 magnification)

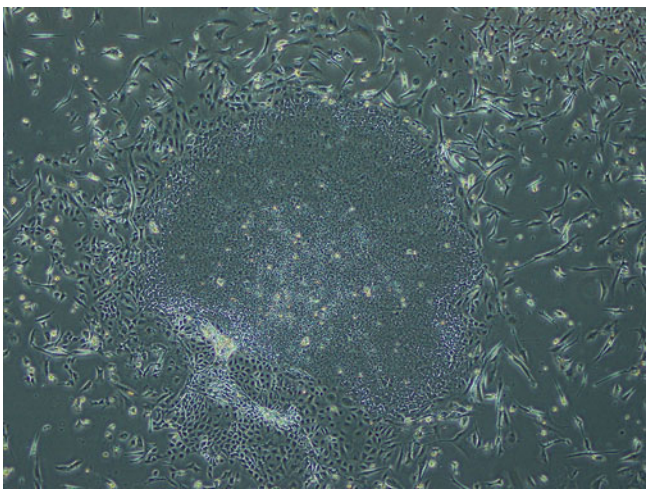

Fig. 9.12 Day 18 MRC-7 reprogrammed cells cut onto fresh NuFF feeders 4 days after transfer (×4 magnification)

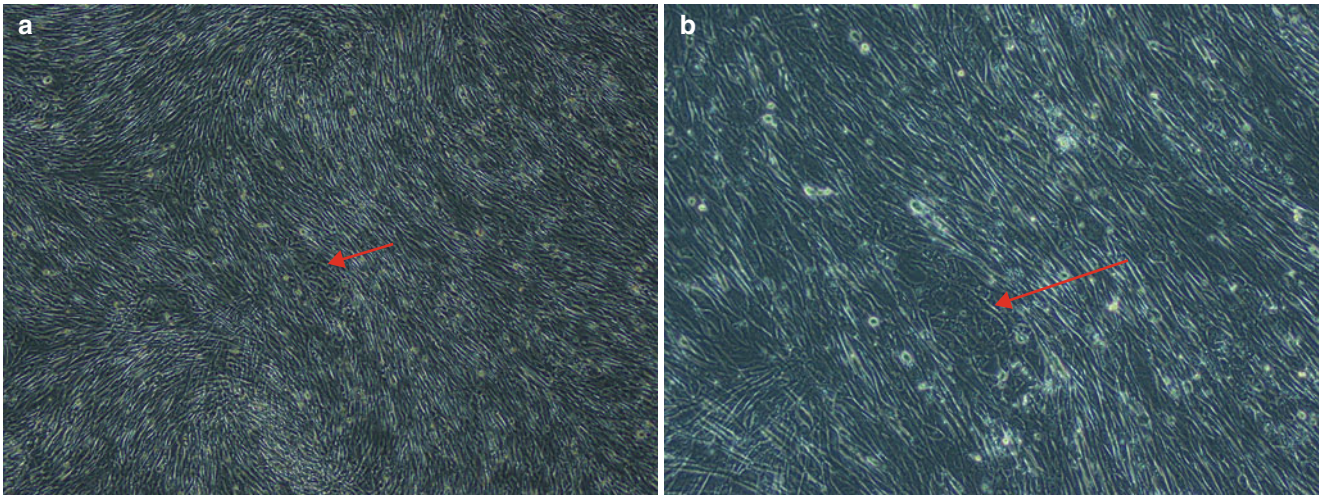

Fig. 9.13 (**a**) Day 18 MRC-7 reprogrammed cells treated with TrypLE™ Express and transferred onto fresh NuFF feeders 4 days after transfer. A colony of reprogrammed cells can be seen growing (*red arrow*). (**b**) The same image at a higher magnification, with the colony of growing iPSCs (*red arrow*) (**a**, ×4 magnification; **b**, ×10)

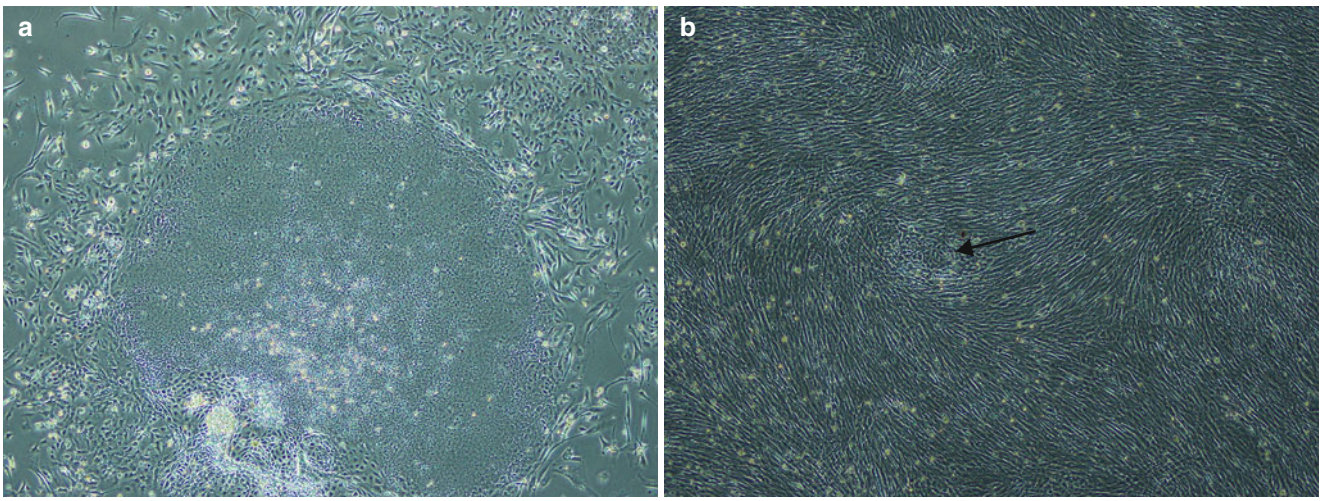

Fig. 9.14 (**a**) Day 19 MRC-7 iPSC colonies cut onto fresh NuFF feeders 5 days after transfer. (**b**) Day 19 MRC-7 iPSC colonies treated with TrypLE™ Express and transferred onto fresh NuFF feeders 5 days after transfer. A small colony of cells is growing among the fibroblasts (*black arrow*) (**a**, ×10 magnification; **b**, ×4)

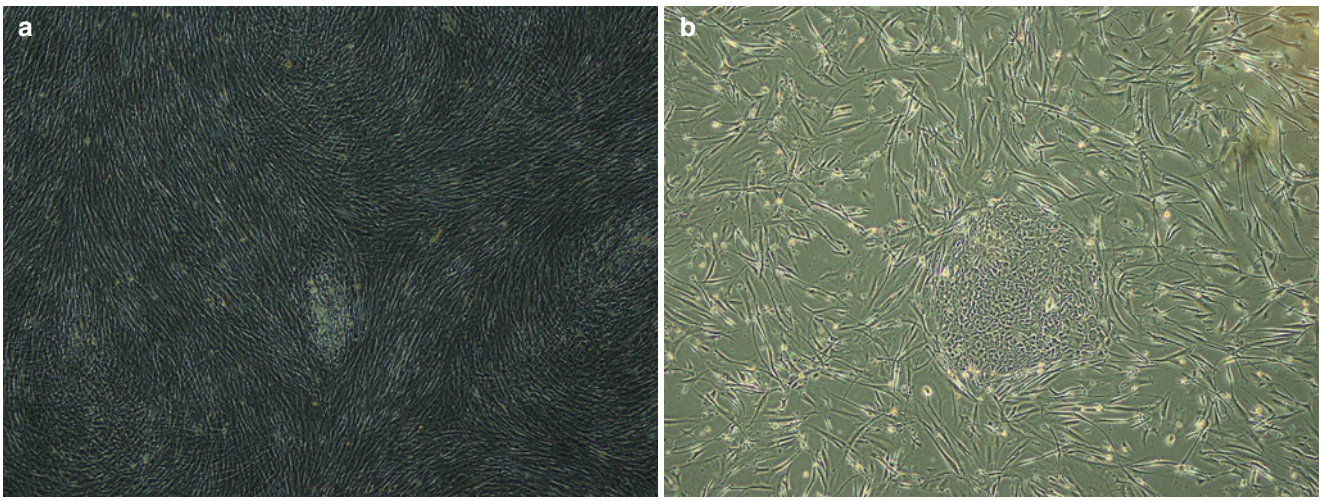

Fig. 9.15 (**a**) Day 20 MRC-7 iPSC colonies treated with TrypLE™ Express and transferred onto fresh NuFF feeders 6 days after transfer. (**b**) Day 23 MRC-7 iPSC colonies cut onto fresh NuFF feeders 5 days after transfer (both ×4 magnification)

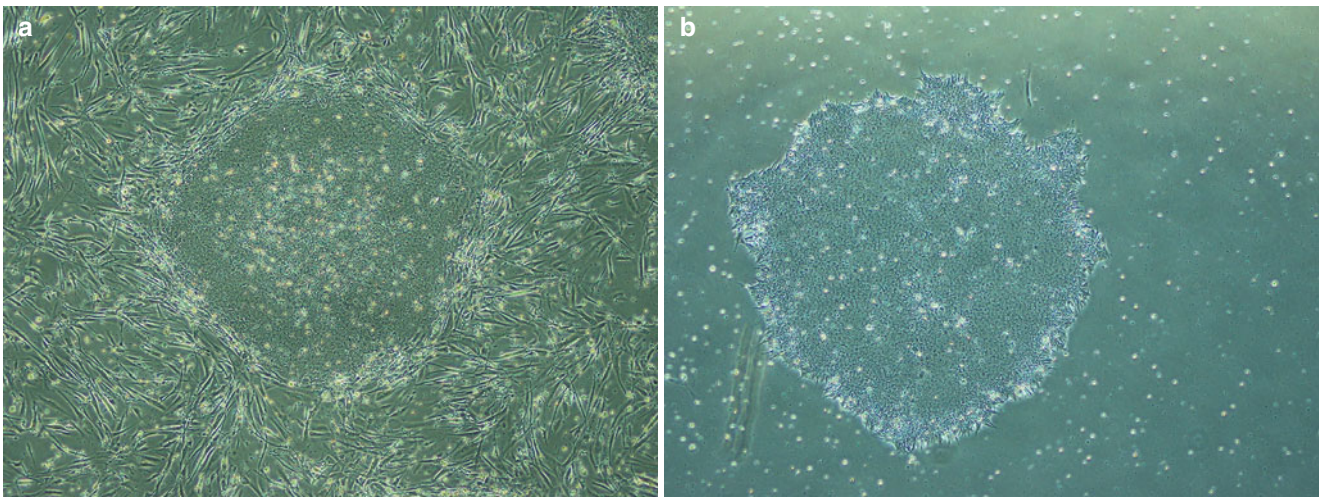

Fig. 9.16 (**a**) Day 27 MRC-7 iPSC colonies treated with TrypLE™ Express and transferred onto fresh NuFF feeders 6 days after transfer. A very large colony can be seen, but the morphology has changed from that of a fibroblast in several other areas, indicating the development of further iPSC colonies (*red arrows*). (**b**) A colony with good morphology, compact and undifferentiated, 12 weeks after the reprogramming process was initiated and transferred to feeder-free conditions (both ×4 magnification)

The second system which does not require feeders, used a combination of messenger RNA and microRNA; it was feeder-free (Figs. 9.17–9.28). In this system which requires 12 days of transfection, messenger RNA is used in combination with microRNA. Colonies of reprogrammed cells start to appear around day 8. Both reprogramming systems described, used the foetal fibroblast cell line MRC-7 as the somatic cell type to be reprogrammed.

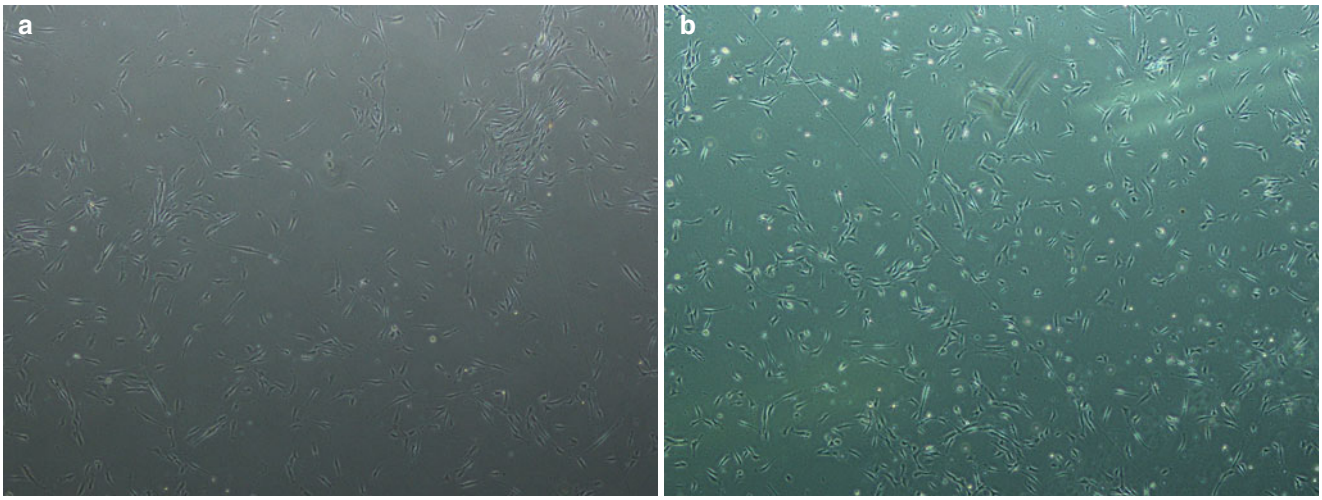

Fig. 9.17 (**a**) Day 0 MRC-7 fibroblasts at the start of the reprogramming process, plated on Matrigel™ at a density of 25,000 cells per well of a six-well plate. (**b**) the same fibroblasts being reprogrammed at Day 1, (both ×4 magnification)

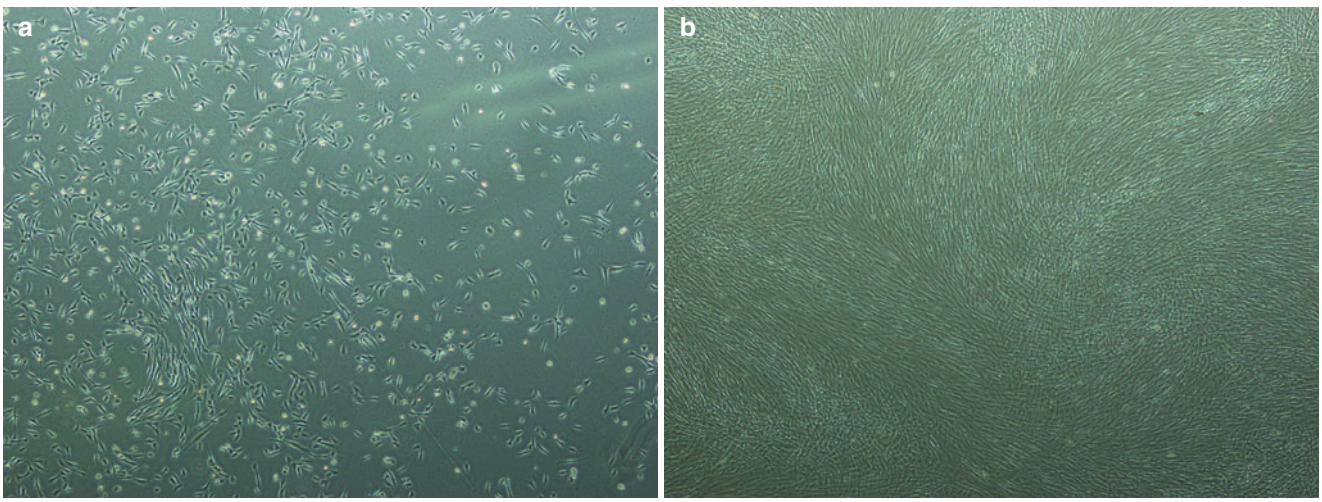

Fig. 9.18 (**a**) Day 2 fibroblasts and (**b**) Day 3 fibroblasts undergoing reprogramming (**b**) (both ×4 magnification)

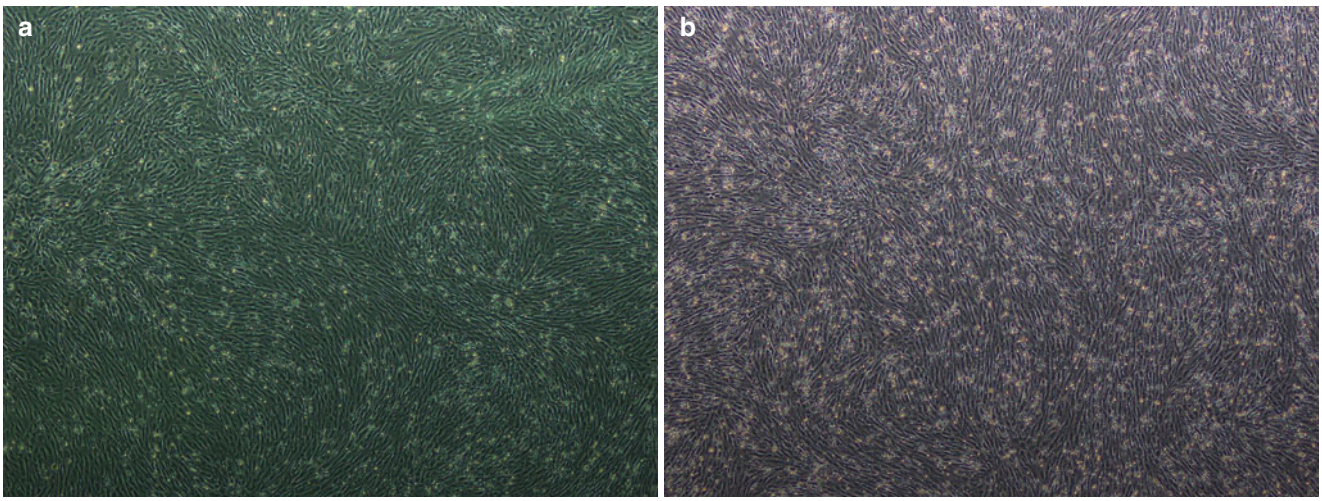

Fig. 9.19 (**a**) Day 4 fibroblasts and (**b**) Day 5 fibroblasts undergoing reprogramming (both ×4 magnification)

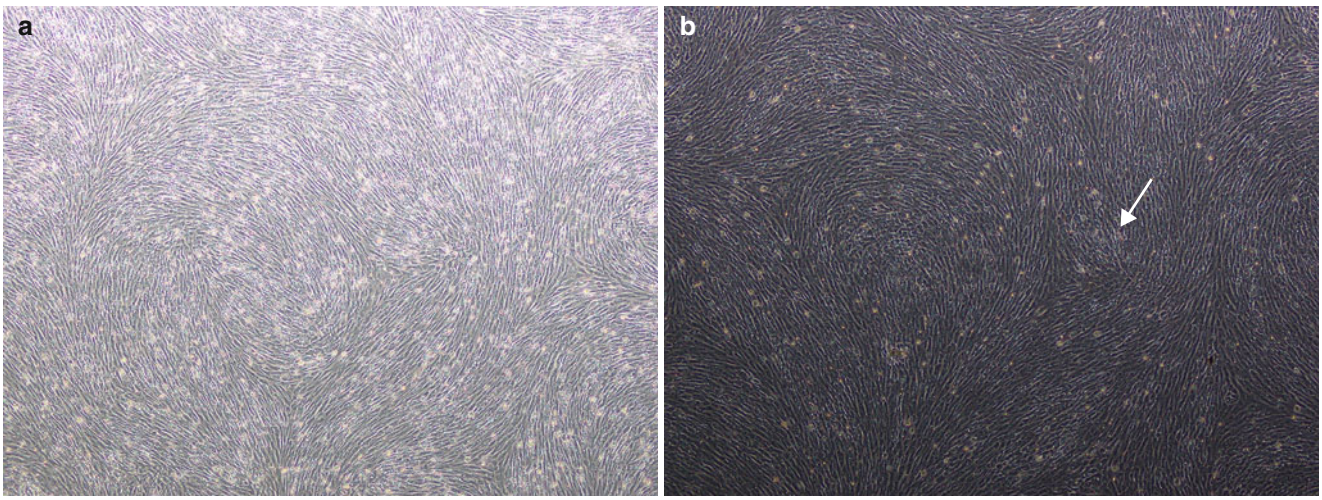

Fig. 9.20 (**a**) Day 6 fibroblasts and (**b**) Day 7 fibroblasts undergoing reprogramming note the change in morphology of the fibroblasts (**b**) (*white arrow*) (both ×4 magnification)

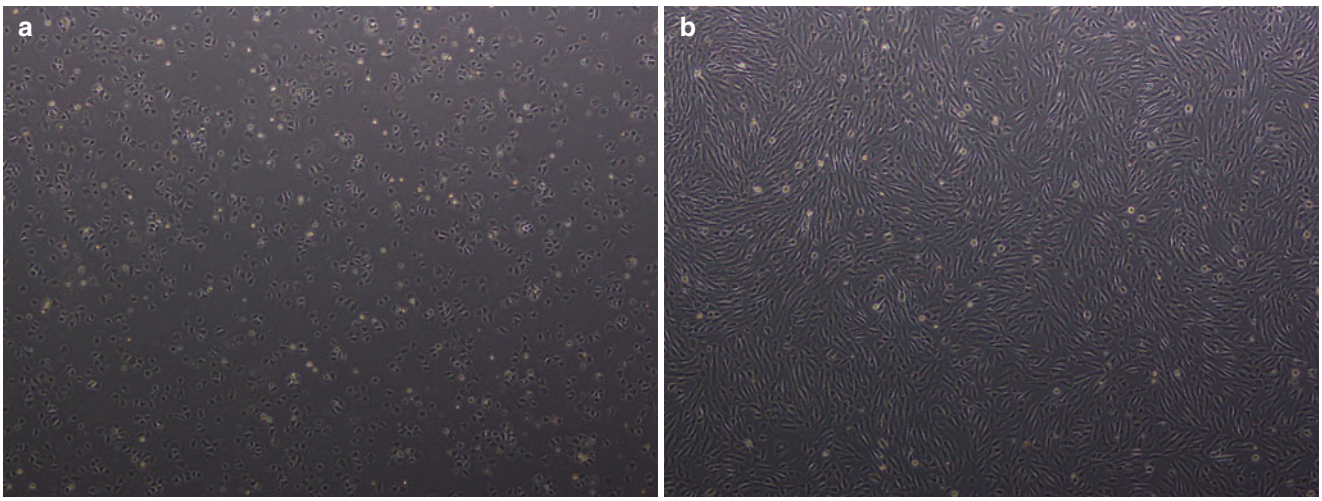

Fig. 9.21 (**a**) Day 7 fibroblasts undergoing reprogramming originally plated at a density of 25,000 cells per well of a six-well plate; dissociated using TrypLE™ Express and replated into a fresh plate coated with Matrigel™ (**b**) Day 8 fibroblasts undergoing reprogramming (originally plated at a density of 25,000 cells per well) and split on Day 7: 1 day post-splitting. (both ×4 magnification)

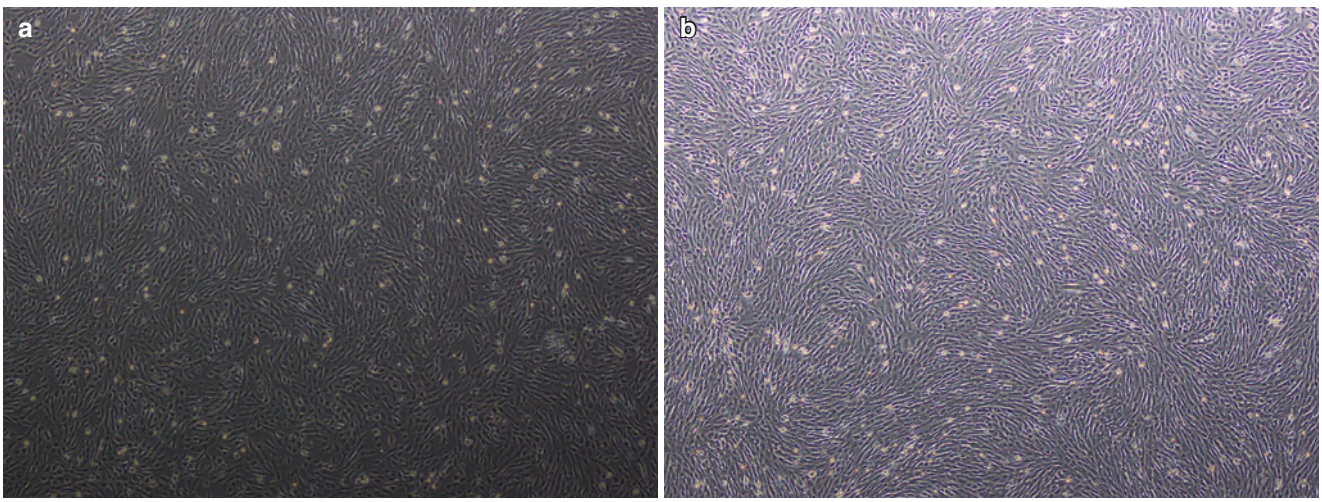

Fig. 9.22 (**a**) Day 9 fibroblasts undergoing reprogramming and split on Day 7: 2 days post-splitting. (**b**) Day 10 fibroblasts undergoing reprogramming split on Day 7: 3 days post-splitting (both ×4 magnification)

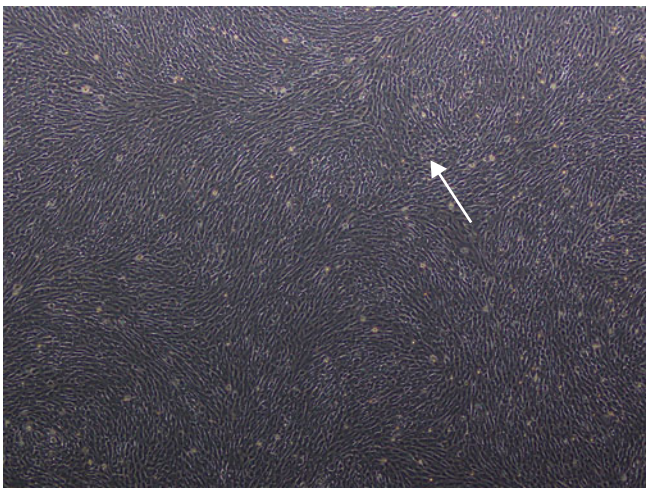

Fig. 9.23 Day 11 undergoing reprogramming and split on Day 7. Note the change in morphology of the fibroblasts 4 days after splitting (*black arrow*) (×4 magnification)

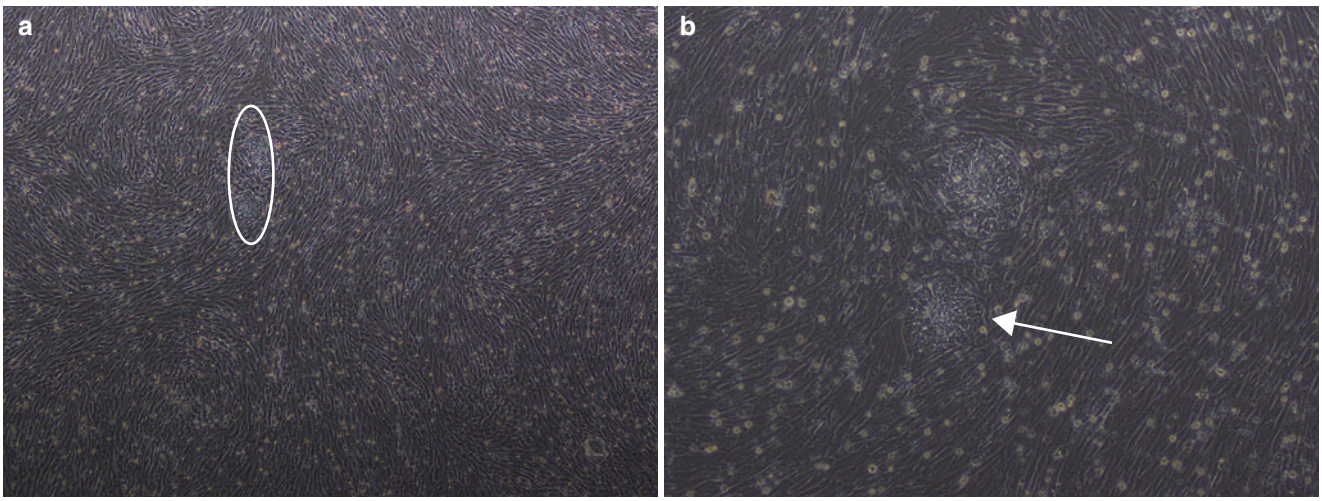

Fig. 9.24 (**a**) Day 12 undergoing reprogramming and split on Day 7. Five days after splitting, the formation of colonies from the reprogrammed fibroblasts is becoming evident (*white oval*). (**b**) At a higher magnification, the morphological change in the culture is clearly seen, revealing the presence of two colonies of cells (*white arrow*) (**a**, ×4 magnification; **b**, ×10)

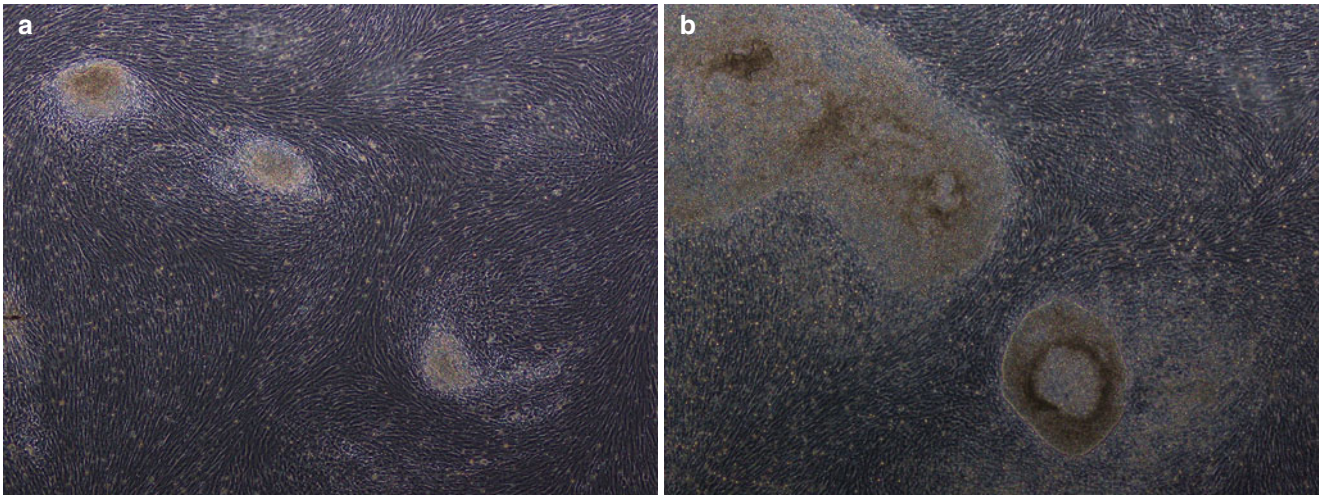

Fig. 9.25 (**a**) Day 14 fibroblasts reprogrammed split on Day 7. Seven days after splitting, a number of colonies of different sizes can be seen developing in the culture. (**b**) Day 18 fibroblasts reprogrammed and split on Day 7: 11 days post-splitting (both ×4 magnification)

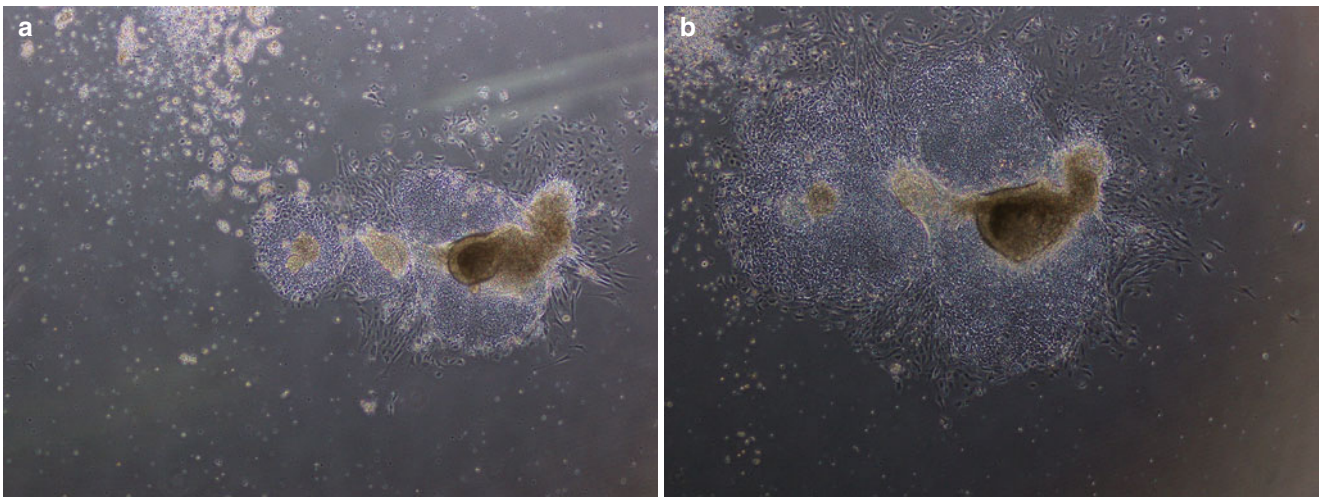

Fig. 9.26 (**a**) Day 19 fibroblasts reprogrammed and split on Day 7, with colonies cut and replated on Day 18 onto a Matrigel™ coated plate. This image is 1 day post-split. (**b**) The same colony at Day 20 (both ×4 magnification)

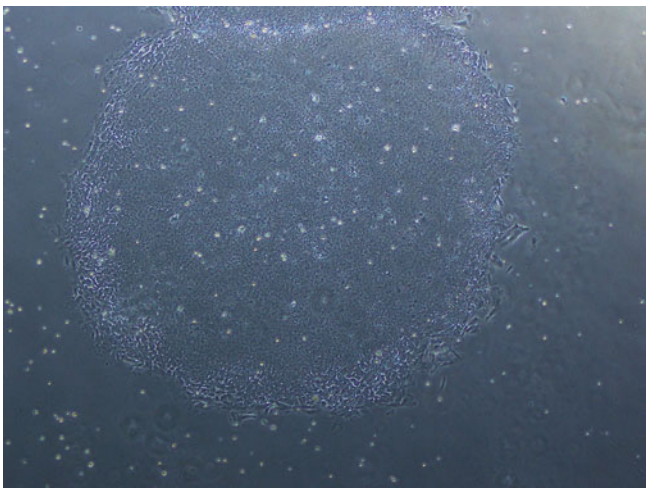

Fig. 9.27 Day 30 culture derived from a single colony maintained through manual passaging shown at passage 3

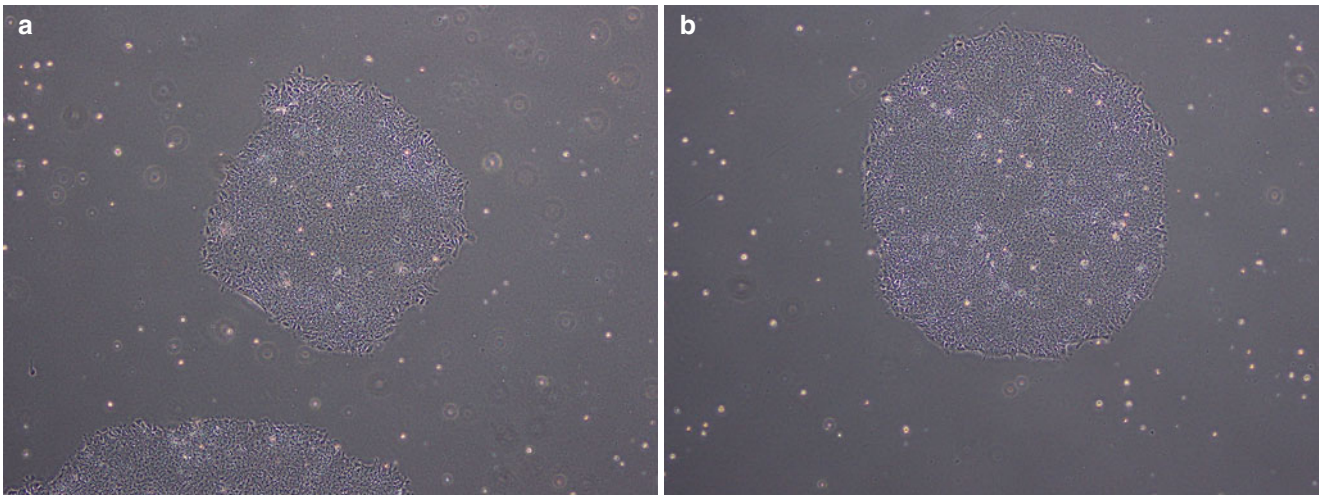

Fig. 9.28 Mechanical cutting of the colonies resulted in a fibroblast-free culture. The cells were maintained in two different media formulations: mTeSR™1 (StemCell Technologies) (**a**) and NutriStem™ XF/FF Culture Medium (Stemgent) (**b**) (both ×4 magnification)

Suggested Reading

Anokye-Danso F, Trivedi CM, Juhr D, Gupta M, Cui Z, Tian Y, et al. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. Cell Stem Cell. 2011;8: 376–88.

Chang CW, Lai YS, Pawlik KM, Liu K, Sun CW, Li C, et al. Polycistronic lentiviral vector for "hit and run" reprogramming of adult skin fibroblasts to induced pluripotent stem cells. Stem Cells. 2009;27:1042–9.

David L, Polo JM. Phases of reprogramming. Stem Cell Res. 2014;12: 754–61.

Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc Jpn Acad Ser B Phys Biol Sci. 2009;85:348–62.

Hu K. Vectorology and factor delivery in induced pluripotent stem cell reprogramming. Stem Cells Dev. 2014. doi:10.1089/scd.2013.0621.

Jacobs JP, Garrett AJ, Meron R. Characteristics of a serially propagated human diploid cell designated MRC-9. J Biol Stand. 1979;7: 113–22.

Kim D, Kim CH, Moon JI, Chung YG, Chang MY, Han BS, et al. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell. 2009;4:472–6.

Nakagawa M, Taniguchi Y, Senda S, Takizawa N, Ichisaka T, Asano K, et al. A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. Sci Rep. 2014;4:3594. doi:10.1038/srep03594.

Okita K, Yamakawa T, Matsumura Y, Sato Y, Amano N, Watanabe A, et al. An efficient nonviral method to generate integration-free human-induced pluripotent stem cells from cord blood and peripheral blood cells. Stem Cells. 2013;31:458–66.

Subramanyam D, Lamouille S, Judson RL, Liu JY, Bucay N, Derynck R, Blelloch R. Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. Nat Biotechnol. 2011;29:443–8.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131:861–72.

Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126:663–76.

Tavernier G, Mlody B, Demeester J, Adjaye J, De Smedt SC. Current methods for inducing pluripotency in somatic cells. Adv Mater. 2013;25:2765–71.

Warren L, Manos PD, Ahfeldt T, Loh YH, Li H, Lau F, et al. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell. 2010;7:618–30.

Warren L, Ni Y, Wang J, Guo X. Feeder-free derivation of human induced pluripotent stem cells with messenger RNA. Sci Rep. 2012;2:657. doi:10.1038/srep00657.

Warren L, Wang J. Feeder-free reprogramming of human fibroblasts with messenger RNA. Curr Protoc Stem Cell Biol. 2013;27:4A.6. 1–27. doi:10.1002/9780470151808.sc04a06s27.

Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, et al. Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell. 2009;4:381–4.

Zhou W, Freed CR. Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. Stem Cells. 2009;27:2667–74.

Zhou YY, Zeng F. Integration-free methods for generating induced pluripotent stem cells. Genomics Proteomics Bioinformatics. 2013;11: 284–7.

Culture Adaptation and Abnormal Cultures

10

When pluripotent stem cell (PSC) lines are moved from one cell culture condition to another, they need to adapt to the new system in order to regain homeostasis. This adaptation process has a direct effect on the biochemistry and physiology of the cell line and can produce changes in cellular metabolism, protein expression, gene expression, cell cycle and morphology. In addition, the stress of the adaptation process can cause the selection of cells with a growth advantage, often caused by changes in the epigenetics or genetic integrity of the cells.

The adaptation of a PSC line is cell line specific, so not all cell lines behave in the same way in identical situations. The cells require careful observation to monitor responses to a change in their environment; as stated throughout this atlas, morphological changes provide a good reflection of the status of the cells in a culture.

Cells can be changed directly from one culture system to another, or they can be changed in a stepwise manner by weaning the cells off one condition whilst introducing them gradually into the second condition. A number of situations arise when working with PSCs that are regarded as adaptation of cells; these include (but are not exclusive to) techniques that require adaptation to different types of passaging techniques, such as the change in a feeder-based system from mechanical clump passaging to enzymatic single-cell passaging and the change from mechanical passaging on feeders to enzymatic passaging on different feeder-free matrices. These changes will alter the morphology of the colonies of cells and the degree of differentiation seen within the colonies.

Depending on the cell line, it can take a number of passages before the cells return to their typical undifferentiated, pre-adaptation morphology—as few as three passages for some lines or as many as 10 for other lines. Morphology should always be assessed alongside other characterisation tests such as immunofluorescence, gene expression and differentiation. Adaptation may affect the functionality of cells, rendering them unable to differentiate. Indeed, if you see perfect, undifferentiated colonies passage after passage, with an increased proliferation rate and no sign of spontaneous differentiation, you should beware; this condition could suggest that the adaptation has affected the genetic integrity of the cells. Conversely, a slowing in the rate of proliferation and an increased rate of spontaneous differentiation in high-passage cell lines also can be indicative of the presence of chromosomal aberrations. These changes in the genetic integrity of cell lines are a well-reported phenomenon, and the same chromosomal changes in culture are seen consistently. It is wise to include regular karyotyping of cell lines in the testing regimen, with a suggested testing interval of every 10 passages.

Adaptation of PSC lines to different culture conditions is well practised. It facilitates the evolution of technologies using PSCs and is a necessity, but the cell cultures should be closely monitored. The images in this chapter show and describe changes in cell and colony morphology during the adaptation of a cell line from mechanical passaging to enzymatic passaging on feeders, over ten passages (Figs. 10.1, 10.2, 10.3, 10.4 and 10.5). The cryptic changes associated with changes in the genetic integrity of the cells in culture are also demonstrated (Figs. 10.6, 10.7 and 10.8).

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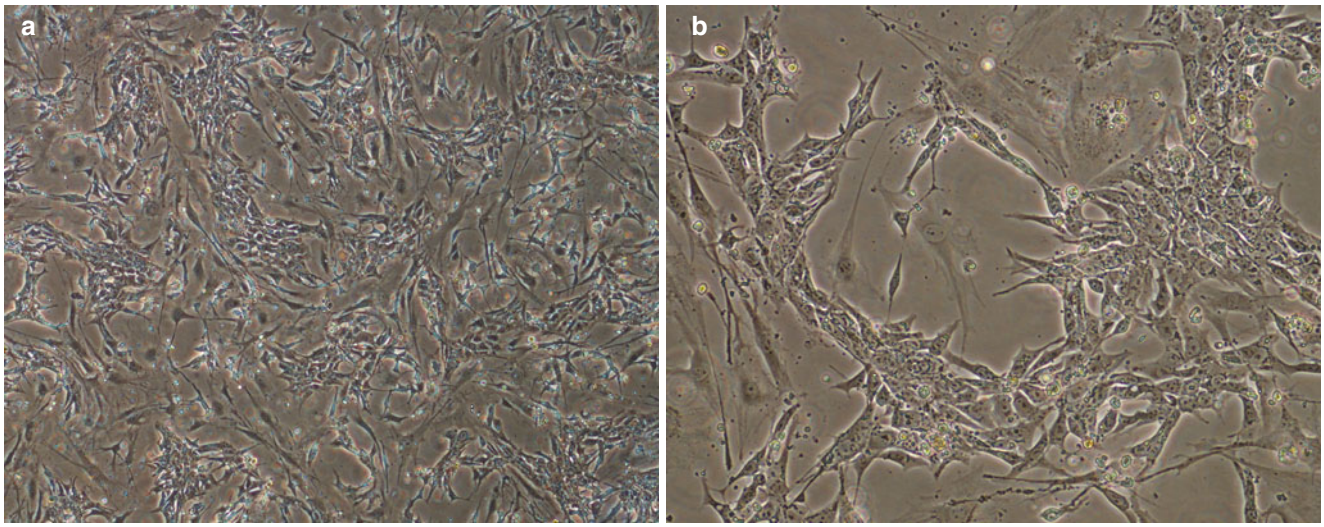

Fig. 10.1 (**a**, **b**) These images show a stage in the adaptation of a cell line from one culture system with mechanical passaging (where large clumps of cells are transferred between culture vessels onto fresh mouse feeders), to a single-cell dissociation method (where colonies are disaggregated enzymatically, producing either single cells or very small clumps of three to five cells). As with the mechanical passaging, these enzymatically treated cells are passaged onto feeders. This type of culture system adaptation can require a number of passages to achieve stability of the cells in the new system, and (as with many processes involving PSCs) this culture system adaptation is cell line specific. In (**a**, **b**), the morphology of the cells is very different from the morphology seen when cells are manually dissected. The morphology characteristic of the mechanical dissection method takes the form of discrete colonies exhibiting well-defined borders with compact cells within the colony. Enzymatic treatment of the cells initially produces a random morphology; with increasing numbers of passages, the morphology settles down to a monolayer in which the typical compact cell type with prominent nucleoli appears. The PSCs in this image are normal and have been adapting to the new culture system for a month. They have not yet acquired the robust morphological characteristics of the PSC (**a**, ×4 magnification; **b**, ×10.) It is worth remembering that if the cells take too long to adapt, the chances of their becoming genetically unstable could increase, conferring a growth advantage to the genetically abnormal cells present in the culture. This is exactly what happened to this cell line after about 10 passages, as captured in Figs. 10.3 and 10.4

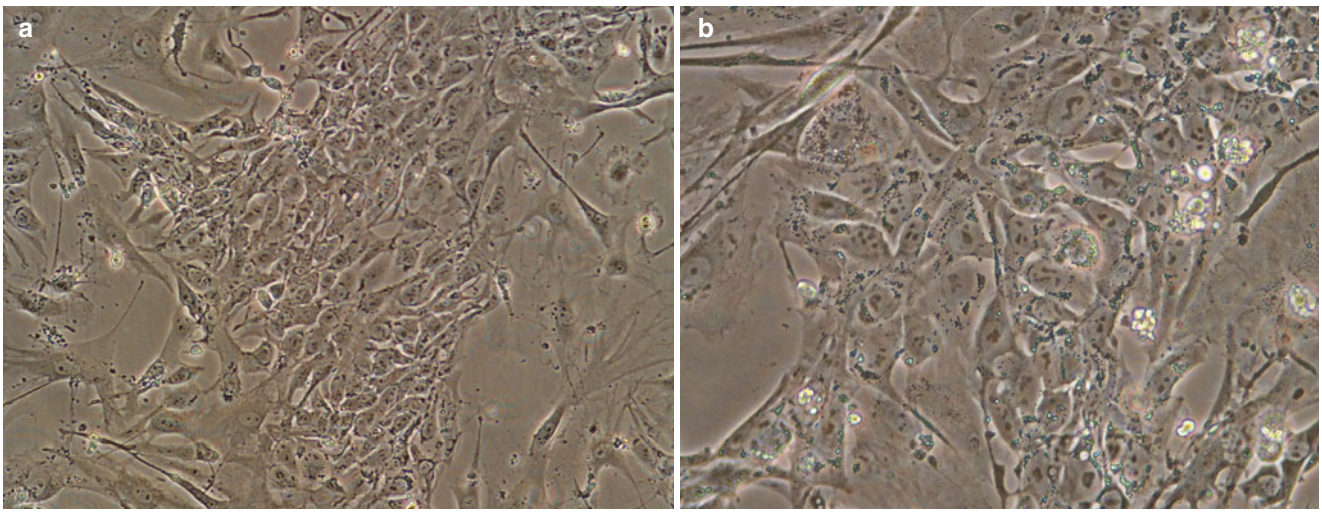

Fig. 10.2 (**a**, **b**) These images show the same cells as in Fig. 10.1 but use a higher magnification to see the fine detail of the typical nucleoli (**a**, ×10 magnification; **b**, ×20)

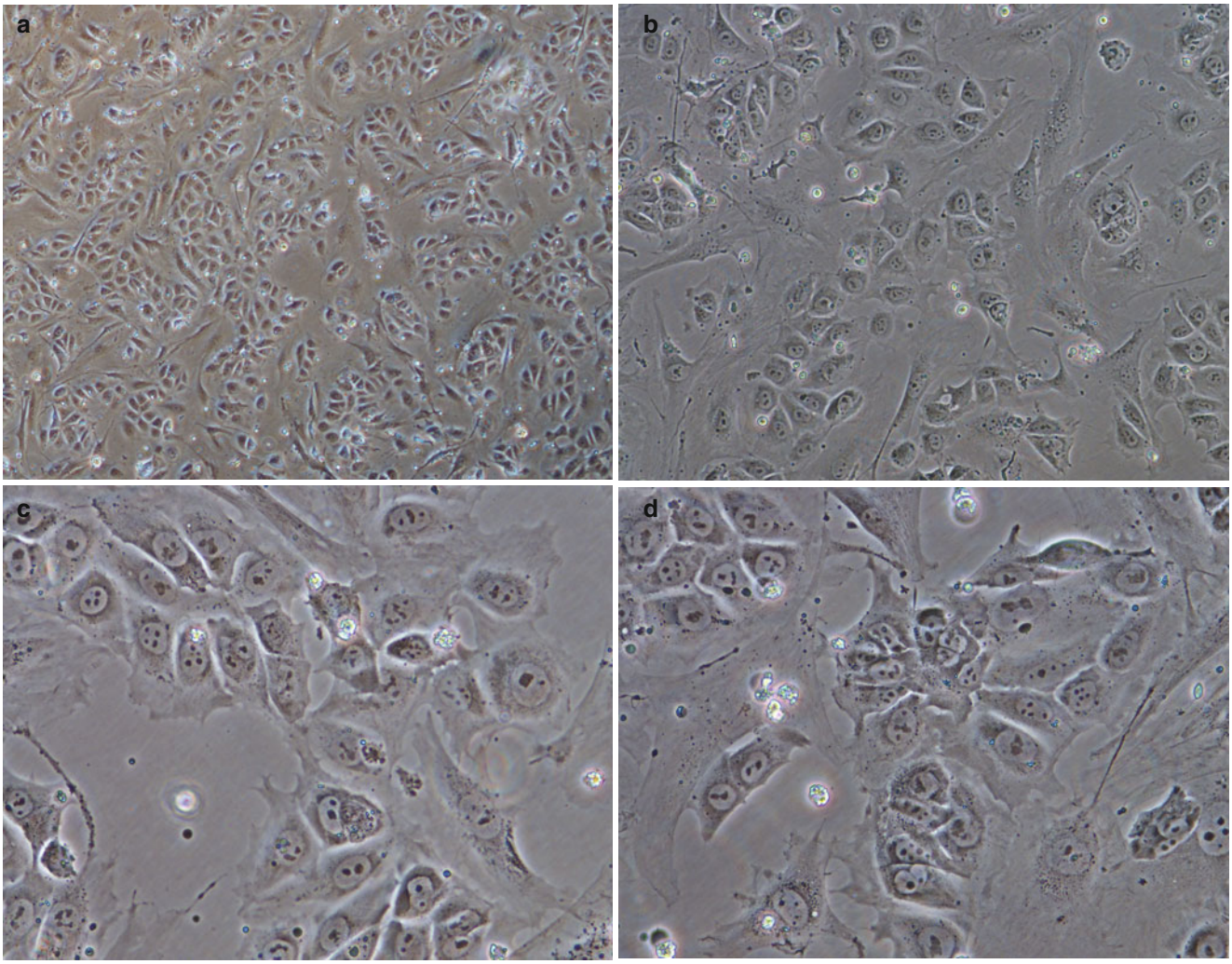

Fig. 10.3 (**a**–**d**) These images show the same culture as in Figs. 10.1 and 10.2, seven passages later. The cells exhibit a more organised morphology. However, these cells show an enhanced proliferative rate and could be split very hard (approximately 1:10). An apparent change in the proliferative rate of the cells in a culture or a trend towards no signs of spontaneous differentiation could indicate genetic instability of the cell line (**a** ×4 magnification; **b**, ×10; **c**, **d**, ×20)

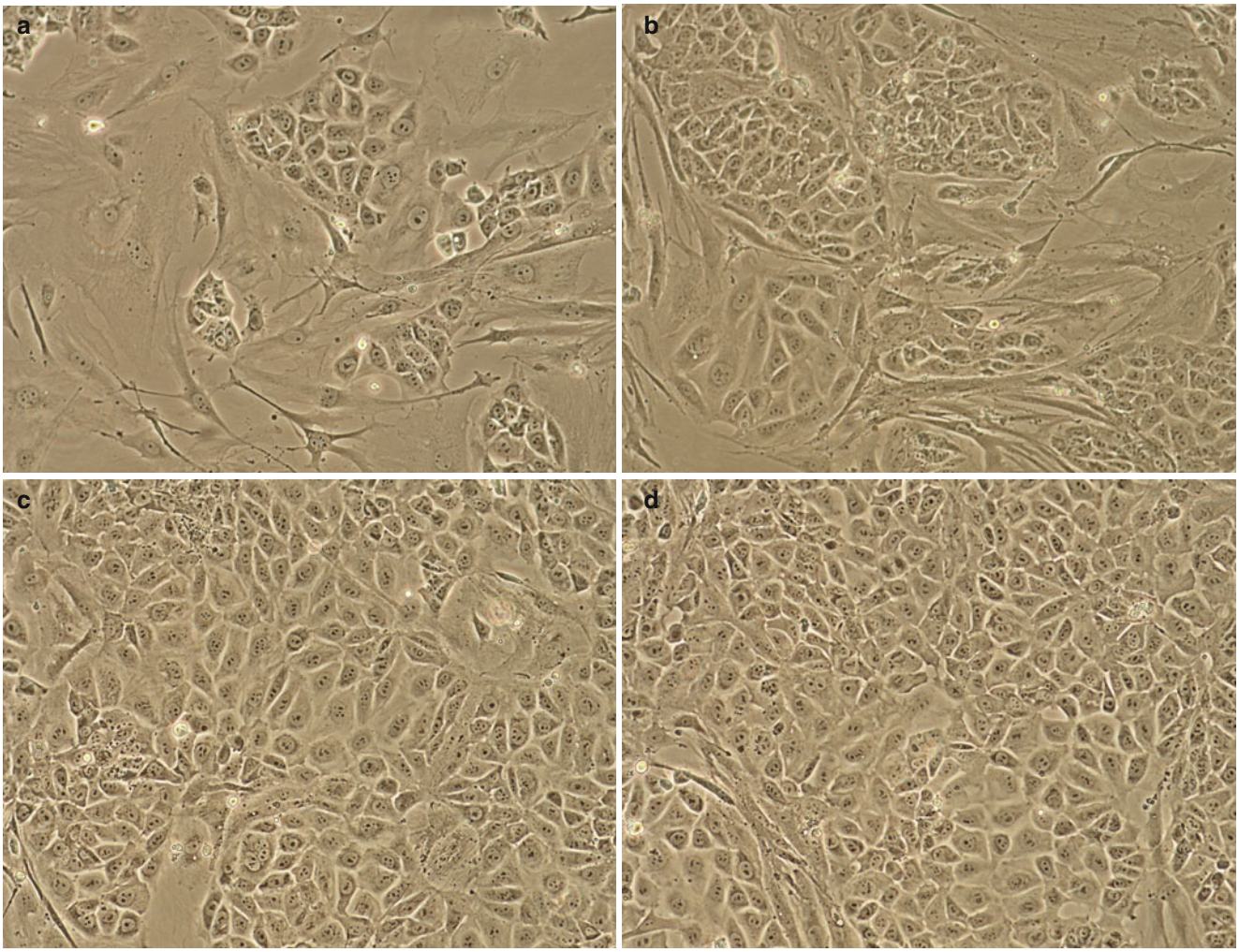

Fig. 10.4 (**a**–**d**) The same cells as in Fig. 10.1, ten passages later. Although individual small colonies can be identified, these merge into a monolayer of cells very rapidly. The morphology is more epithelial like before the culture becomes confluent. Upon confluency, the morphological phenotype becomes that of a monolayer. Following are two examples of abnormal karyotypes produced by the continual passaging of cells using enzymatic treatment (all ×10 magnification)

Karyotype: 48,XY,+i(17)(q10),+i(21)(q10)

Comment: A modal karyotype (in 6 cells) showed an abnormal male karyotype with a complement of 48 chromosomes including two additional structurally abnormal chromosomes, a long arm isochromosome 17 and a long arm isochromosome 21. Four copies of chromosome 17 long arm and chromosome 21 long arm were therefore present in these cells

Karyotype: 47,XX,+17[11]/46,X,der(X)t(X;17)(p11.1;q11.2)[8]/46,X,der(X)t(X;17)(p11.2;q11.2),del(9)(q12)[1]

Comment: Two abnormal female cell lines were detected in this culture. In 11 cells, a complement of 47 chromosomes, including three copies of chromosome 17 (TRISOMY 17) was seen. In 8 cells, a complement of 46 chromosomes was noted, including a structurally abnormal derivative X chromosome formed from an unbalanced translocation between the short arm of one X chromosome at breakpoint p11.2 and the long arm of one chromosome 17 at breakpoint q11.2. This rearrangement results in a loss of chromosome X material from p11.2 to pter and the gain of chromosome 17 material from q11.2 to qter

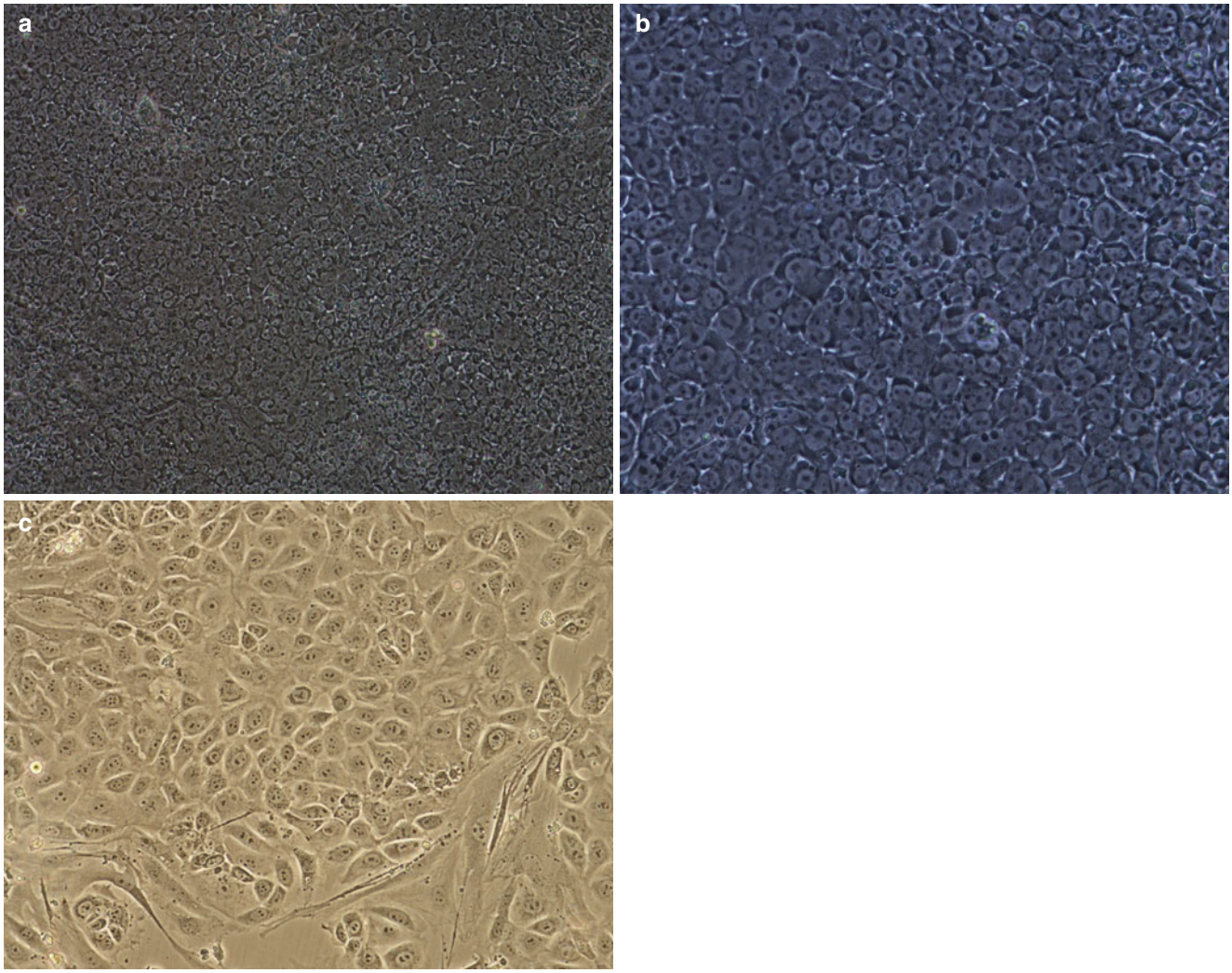

Fig. 10.5 These images show the cell line in Fig. 10.4 when it reaches confluency. (**a**) The compact morphology reminiscent of the good PSC colony is revealed. (**b**) At higher magnification, prominent nucleoli can be seen. (**c**) The non-confluent culture morphology is seen at higher magnification, demonstrating the presence of the prominent nucleoli typical of the undifferentiated PSC (**a**, ×10 magnification; **b**, **c**, ×20)

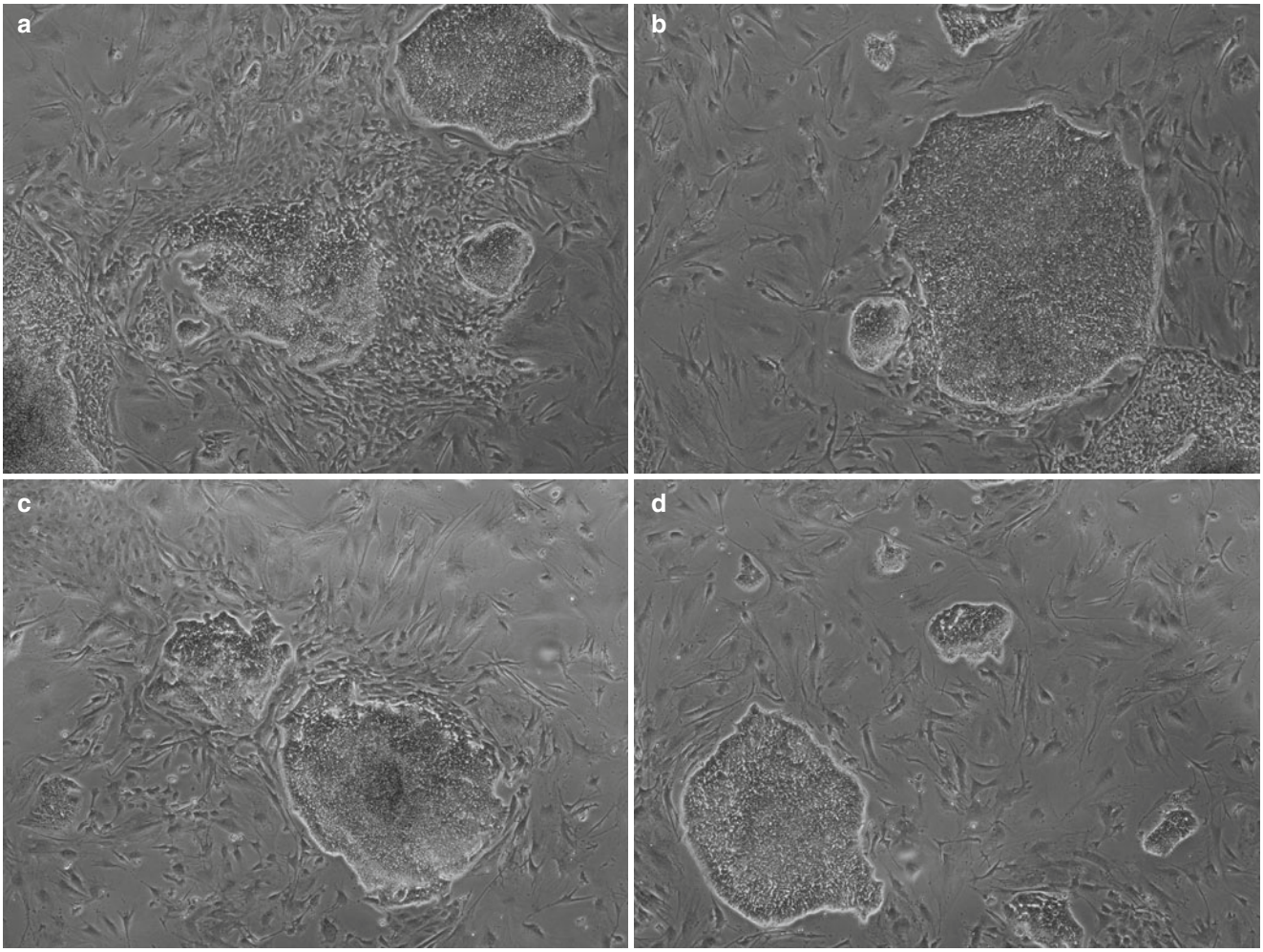

Fig. 10.6 (**a**–**d**) All four images depict the same cell line undergoing potential cryptic changes to the genome. Generally, over a period of up to five passages, the cells can exhibit gross changes in morphology, changing shape and developing a refractile appearance. Their growth rate slows, and they can increase their rate of spontaneous differentiation in a way that is atypical of that specific cell line. As with all of these changes in the characteristics of the cells, they are cell line specific and tend to arise in cells after more than 80 passages (all ×4 magnification)

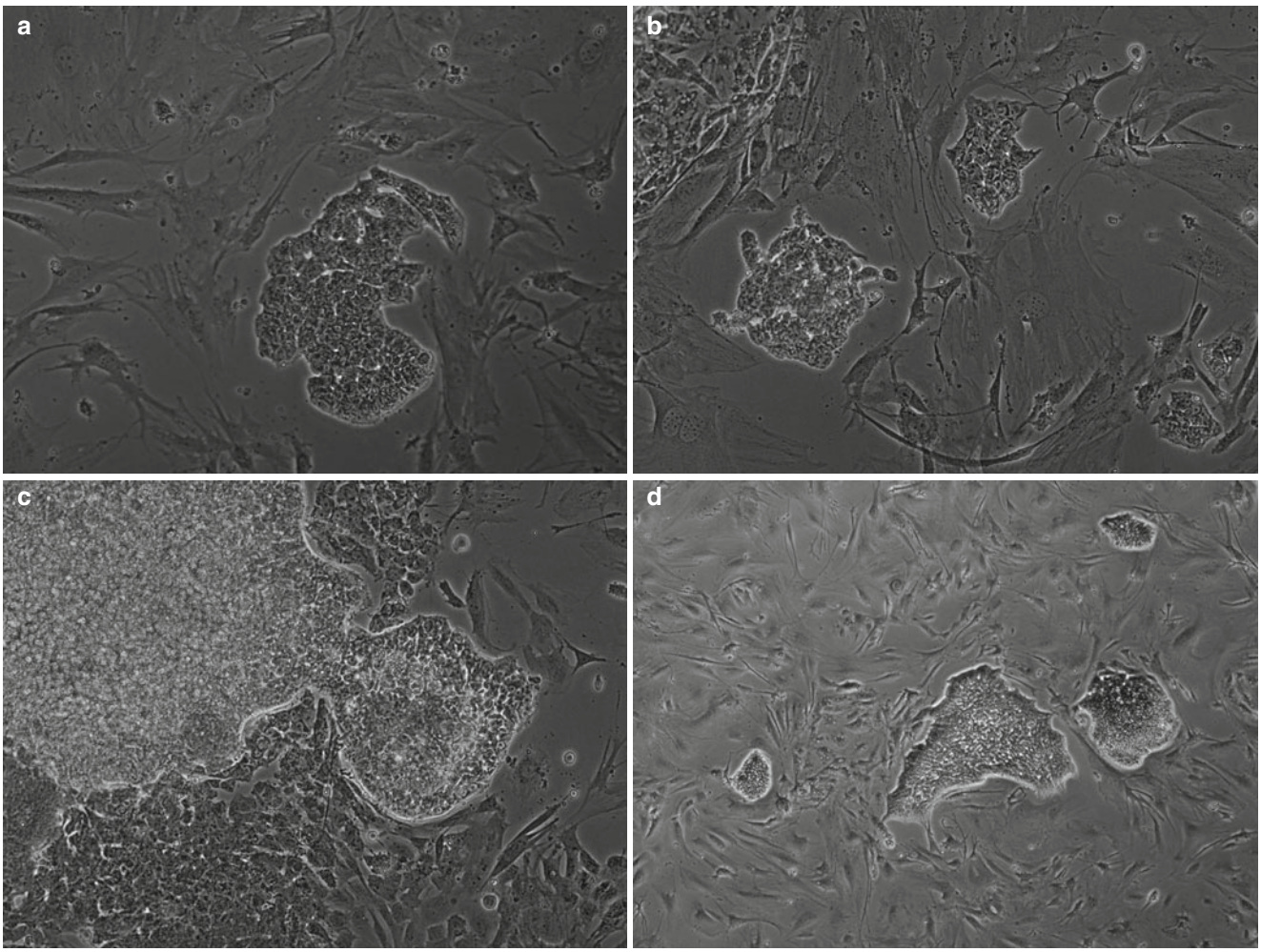

Fig. 10.7 (**a**–**d**) The cell line in this series of images slightly differs from the one in Fig. 10.6, but many of the phenotypic changes seen reflect those observed in that cell line (all ×4 magnification)

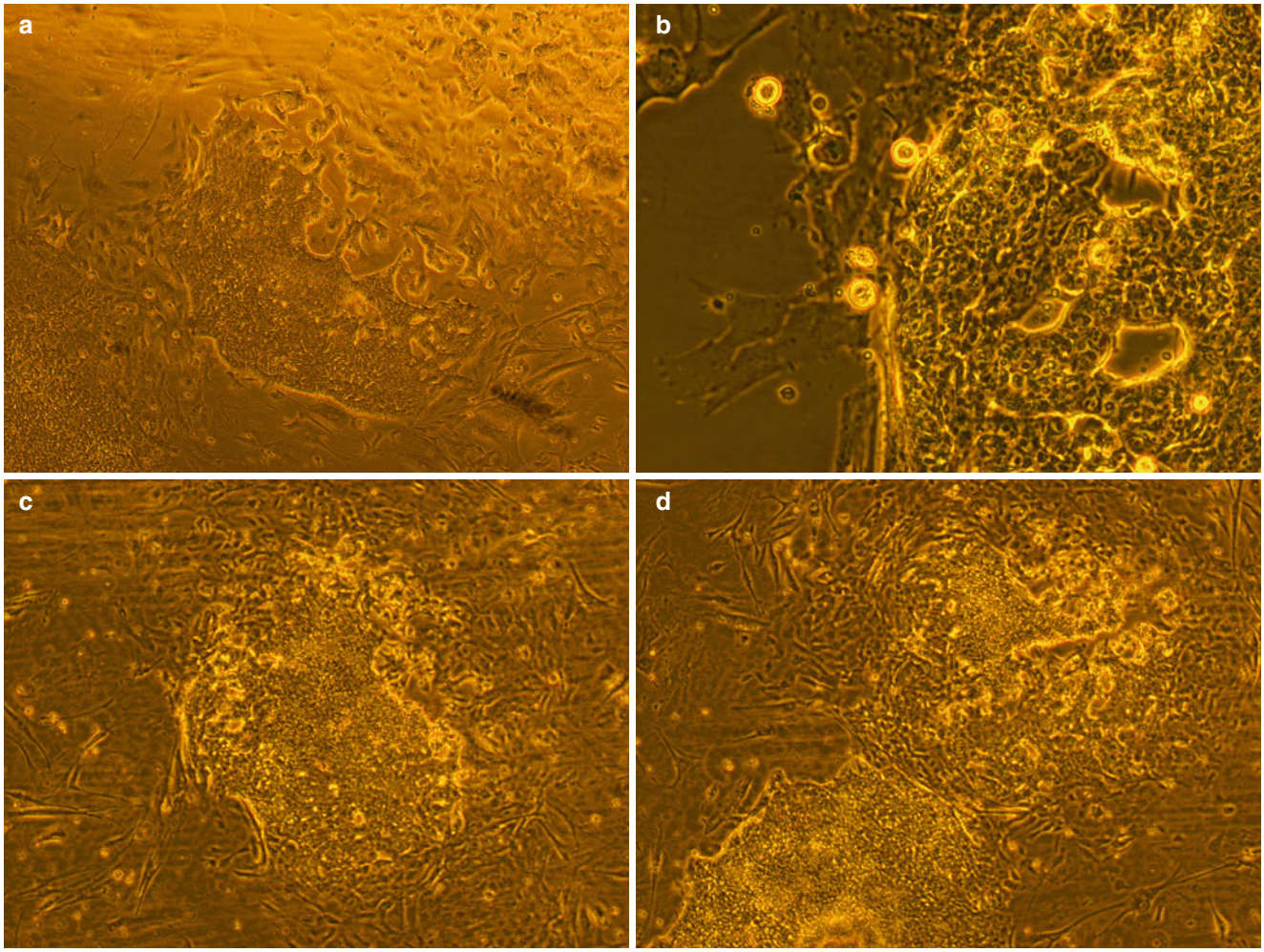

Fig. 10.8 (**a**–**d**) The cell line in these four images is going through the same cryptic genetic adaptation as the cells shown in Figs. 10.6 and 10.7. Their morphology is varied and disorganised. The morphology of all three of these cell lines returned to normal once they had gone through this period of genetic adaptation, and their rate of proliferation increased (**a**, **c**, **d**, ×4 magnification; **b**, ×10)

Suggested Reading

Abaci HE, Truitt R, Luong E, Drazer G, Gerecht S. Adaptation to oxygen deprivation in cultures of human pluripotent stem cells, endothelial progenitor cells, and umbilical vein endothelial cells. Am J Physiol Cell Physiol. 2010;298:C1527–37.

Amit M, Itskovitz-Eldor J. Morphology of human embryonic and induced pluripotent stem cell colonies cultured in feeder layer–free conditions. In: Amit M, Itskovitz-Eldor J, editors. Atlas of human pluripotent stem cells: derivation and culturing, Stem cell biology and regenerative medicine. New York: Humana Press; 2012. p. 41–56.

Baker DE, Harrison NJ, Maltby E, Smith K, Moore HD, Shaw PJ, et al. Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. Nat Biotechnol. 2007;25:207–15.

Barbaric I, Biga V, Gokhale PJ, Jones M, Stavish D, Glen A, Coca D, Andrews PW. Time-lapse analysis of human embryonic stem cells reveals multiple bottlenecks restricting colony formation and their relief upon culture adaptation. Stem Cell Reports. 2014; 3(1):142–55.

Ben-David, Arad G, Weissbein U, Mandefro B, Maimon A, Golan-Lev, Narwani K, Clark AT, Andrews PW, Benvenisty N, Carlos Biancotti J. Aneuploidy induces profound changes in gene expression, proliferation and tumorigenicity of human pluripotent stem cells. Nat Commun. 2014;5:4825. doi:10.1038/ncomms5825.

Bigdeli N, Andersson M, Strehl R, Emanuelsson K, Kilmare E, Hyllner J, Lindahl A. Adaptation of human embryonic stem cells to feeder-free and matrix-free culture conditions directly on plastic surfaces. J Biotechnol. 2008;133:146–53.

Catalina P, Montes R, Ligero G, Sanchez L, de la Cueva T, Bueno C, et al. Human ESCs predisposition to karyotypic instability: is a matter of culture adaptation or differential vulnerability among hESC lines due to inherent properties? Mol Cancer. 2008;7:76. doi:10.1186/1476-4598-7-76.

Chen KG, Mallon BS, Hamilton RS, Kozhich OA, Park K, Hoeppner DJ, Robey PG, McKay RD. Non-colony type monolayer culture of human embryonic stem cells. Stem Cell Res. 2012;9:237–48.

Harrison N, Baker D, Andrews PW. The significance of culture adaptation of embryonic stem cells for regenerative medicine. In: Baharvand H, Aghdami N, editors. Advances in stem cell research, Stem cell biology and regenerative medicine. New York: Humana Press; 2012. p. 17–27.

Johnson T. Promises and pitfalls of cell line adaptation: some basic protocols. BioProcess Int. 2006;3:S52–6.

Kunova M, Matulka K, Eiselleova L, Salykin A, Kubikova I, Kyrylenko S, Hampl A, Dvorak P. Adaptation to robust monolayer expansion produces human pluripotent stem cells with improved viability. Stem Cells Transl Med. 2013;2:246–54.

Na J, Baker D, Zhang J, Andrews PW, Barbaric I. Aneuploidy in pluripotent stem cells and implications for cancerous transformation. Protein Cell. 2014;5:569–79.

Närvä E, Autio R, Rahkonen N, Kong L, Harrison N, Kitsberg D, et al. High-resolution DNA analysis of human embryonic stem cell lines reveals culture-induced copy number changes and loss of heterozygosity. Nat Biotechnol. 2010;28:371–7.

Ramos-Mejia V, Bueno C, Roldan M, Sanchez L, Ligero G, Menendez P, Martin M. The adaptation of human embryonic stem cells to different feeder-free culture conditions is accompanied by a mitochondrial response. Stem Cells Dev. 2012;21:1145–55.

Stover AE, Schwartz PH. Adaptation of human pluripotent stem cells to feeder-free conditions in chemically defined medium with enzymatic single-cell passaging. Methods Mol Biol. 2011;767: 137–46.

Sun Y, Yang Y, Zeng S, Tan Y, Lu G, Lin G. Identification of proteins related to epigenetic regulation in the malignant transformation of aberrant karyotypic human embryonic stem cells by quantitative proteomics. PLoS One. 2014;9:e85823. doi:10.1371/journal.pone.0085823.

T'joen V, Declercq H, Cornelissen M. Expansion of human embryonic stem cells: a comparative study. Cell Prolif. 2011;44:462–76.

Tompkins JD, Hall C, Chen VC, Li AX, Wu X, Hsu D, et al. Epigenetic stability, adaptability, and reversibility in human embryonic stem cells. Proc Natl Acad Sci U S A. 2012;109:12544–9.

Zucchelli M, Ström S, Holm F, Malmgren H, Sahlén S, Religa P, et al. In vivo differentiated human embryonic stem cells can acquire chromosomal aberrations more frequently than in vitro during the same period. Stem Cells Dev. 2012;21:3363–71.

Infection

11

Cell culture contamination caused by microbial infection can occur at any time and can take many forms. Prevention is better than cure. Actively endorsing a policy of best practice in aseptic technique should help to lower the risk of microbial infection. When receiving a new stock of cell lines into the laboratory, it is advisable to quarantine the cells until they have been demonstrated to be free from contamination. Verifying freedom from contamination may involve observation under the microscope over a number of days to see if contamination is evident. Contaminated cells and cultures should be discarded unless you cannot replace the stocks of cells.

The culture of pluripotent stem cells (PSCs), as with other cell lines, is subject to contamination, and this chapter provides a number of images to demonstrate the types of contamination that may be encountered in the day-to-day culture of these lines (Figs. 11.1, 11.2, 11.3, 11.4, 11.5, 11.6, 11.7, 11.8, 11.9, 11.10, 11.11, 11.12, 11.13, 11.14, 11.15, 11.16 and 11.17). To date, we have not found a collection of such images in other PSC publications, so we believe they will be of interest.

Contamination can manifest itself as a change in the turbidity of the cell culture medium, a change in the colour of the medium, or as visible, macroscopic growth of an unwanted organism. This type of contamination is usually easy to detect and is typically due to bacteria or fungi. Other contamination that is harder to detect includes mycoplasma, viruses and cell line cross-contamination.

In keeping with best practice, PSC culture laboratories generally do not use antibiotics with established cell lines. This prevents the masking of an occult contamination with agents such as bacteria or fungi, thereby enabling their rapid detection in the cell culture system. Infection with viruses often manifests itself in a cell culture when the cells begin to lyse for no reason; this is known as a cytopathic effect, and this process helps to limit the survival of the virus in the culture. It should be remembered that viruses have very specific host-cell requirements, and this attribute is generally captured in the cell line risk assessment performed prior to the receipt of a cell line.

Mycoplasma, a Mollicute, is a very difficult contaminant to detect, as it can often have no visible effect on the growth of the cells in culture. It can have pleiotropic effects on the cells in culture, however, such as causing chromosomal instability, changes in metabolism and alteration in cell function. Mycoplasma is a common contaminant in cell cultures exchanged between laboratories and can easily be transferred between cell lines. Because it is not visible under the light microscope and has such wide-reaching effects on the host cell, it is important to test for this contamination in a manner that will detect a low-level infection robustly, reproducibly and reliably, using recommended quality-testing regimens. Cells should not be released from the quarantine process until the mycoplasma status has been established. Figures 11.1, 11.2, 11.3, 11.4, 11.5, 11.6, 11.7, 11.8, 11.9, 11.10, 11.11, 11.12, 11.13, 11.14, 11.15, 11.16 and 11.7 may be the first published and described images of PSC cultures infected with mycoplasma, which should aid in the identification of this type of infection in the laboratory setting.

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DOI 10.1007/978-1-4899-7507-2_11, © Springer Science+Business Media New York 2015

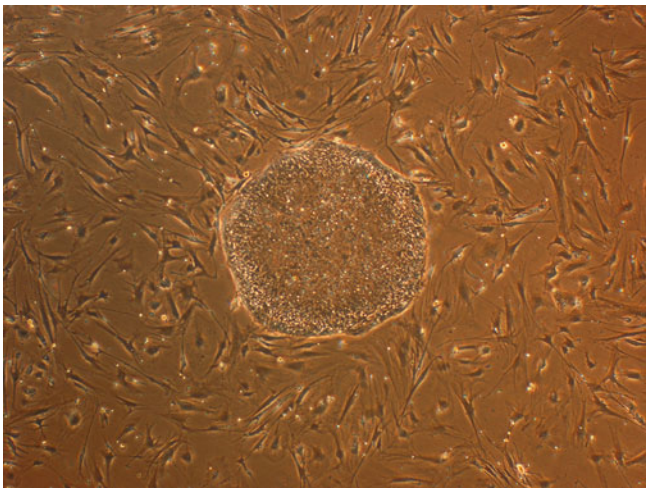

Fig. 11.1 Morphology of an uninfected cell line (×4 magnification)

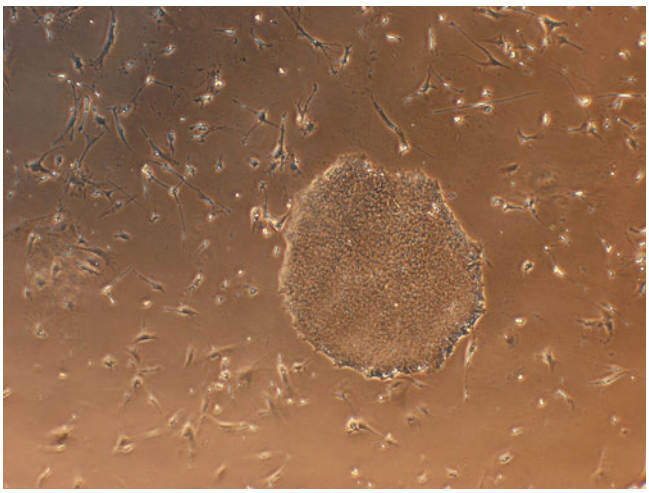

Fig. 11.2 A mycoplasma-infected cell line in comparison to the uninfected cell line in 11.1. The most important thing to note is the condition of the feeders, especially their morphology. The feeders in an infected culture are elongated and show signs of stress. (They are very spindly and stretched.) The feeder density is lower than expected, and many of the feeder cells are apoptotic, floating in suspension. However, the stem cell colony has a typical morphology and appears normal. The colony is compacted and undifferentiated and does not appear to be affected by the contamination (×4 magnification)

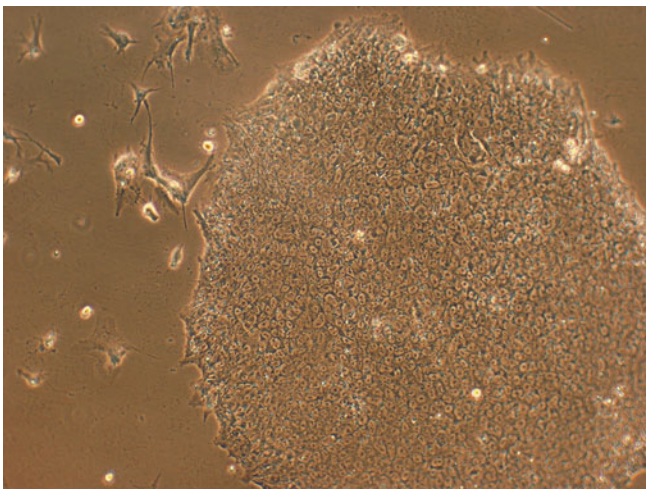

Fig. 11.3 The same colony as 11.2 at a high magnification (×10)

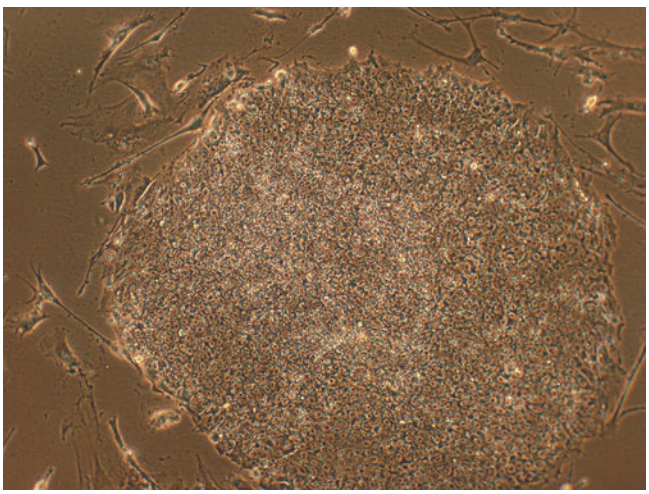

Fig. 11.4 Another mycoplasma-infected PSC colony, which has a less than perfect morphology but is still undifferentiated (×10 magnification). Colonies from mycoplasma-infected cultures will not stay undifferentiated, however. Within a couple of days after this image was captured, there would be no feeders left in the culture system to support the PSCs

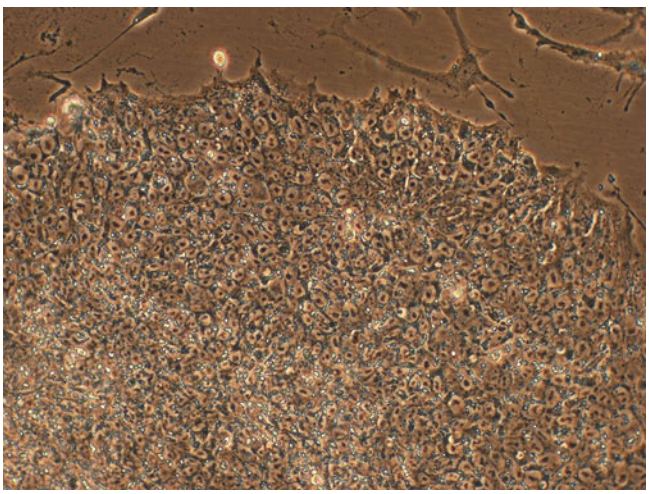

Fig. 11.5 The same as Fig. 11.4, at higher magnification (×20)

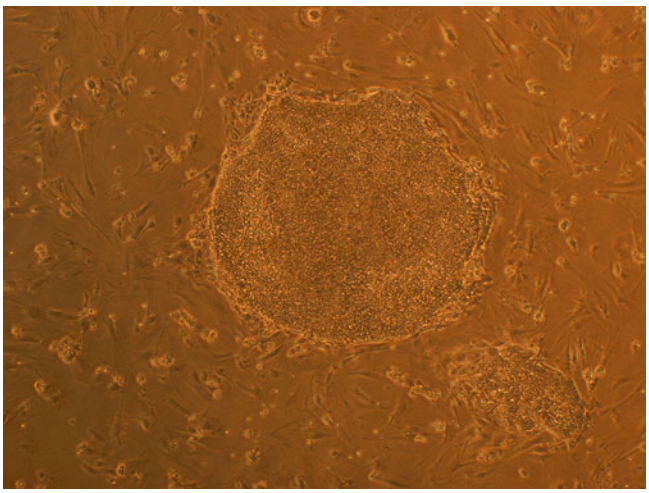

Fig. 11.6 Another example of mycoplasma-infected feeders. Many apoptotic cells are present in the culture. These cells often clump together, and a portion of the cells can remain attached to the bottom of the tissue culture flask (×4 magnification)

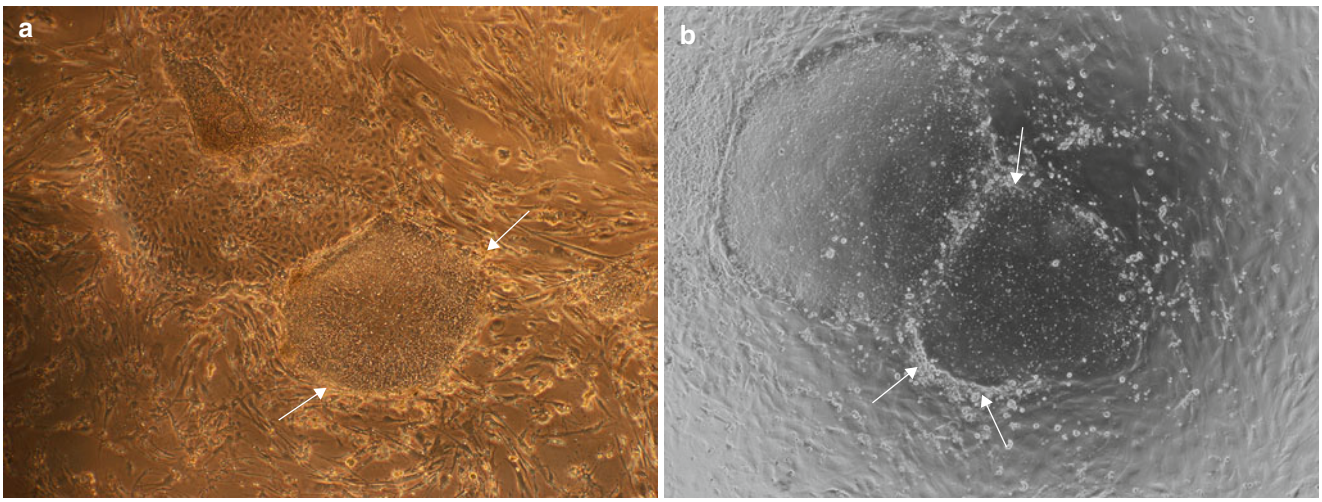

Fig. 11.7 (**a**, **b**) Further examples of cell cultures infected with mycoplasma. If these cultures are left undisturbed overnight, round cells will be visible around the edges of the stem cell colony (*white arrows*). If the tissue culture dish is shaken or lightly tapped, these cells detach and go into suspension (both ×4 magnification)

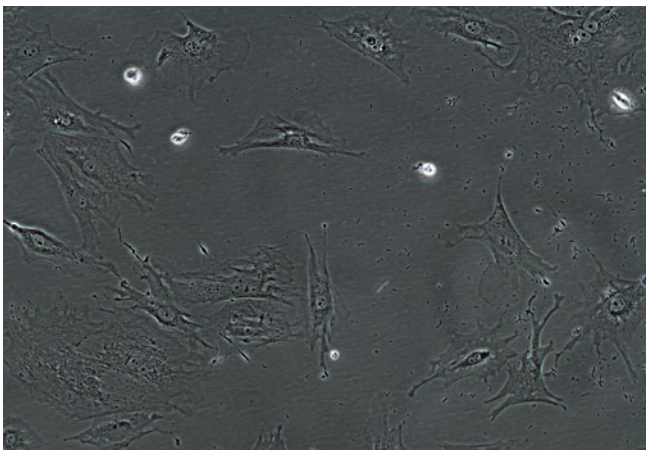

Fig. 11.8 A bacterial infection in culture (in this case, *Citrobacter freundii*) (×10 magnification). With this type of infection, the medium does not change colour and shows no turbidity; it remains clear, as in an uninfected culture. It is important to examine cultures very closely at different magnifications, and to be able to distinguish between Brownian motion and infection. With an infection, the number of bacteria present will increase with time

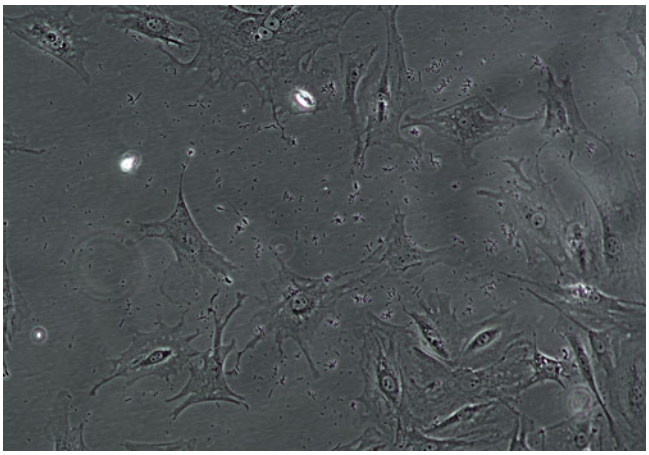

Fig. 11.9 The same culture as Fig. 11.8 at a higher magnification (×20). At high magnification, the bacteria can be seen moving across the culture with great speed

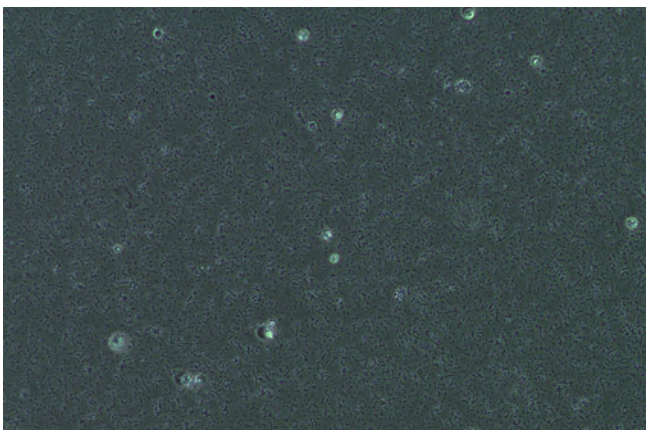

Fig. 11.10 The same culture as Fig. 11.8, 2 days later. The culture is now teeming with the bacteria. Note that the feeders have lifted off the tissue culture plastic (×20 magnification)

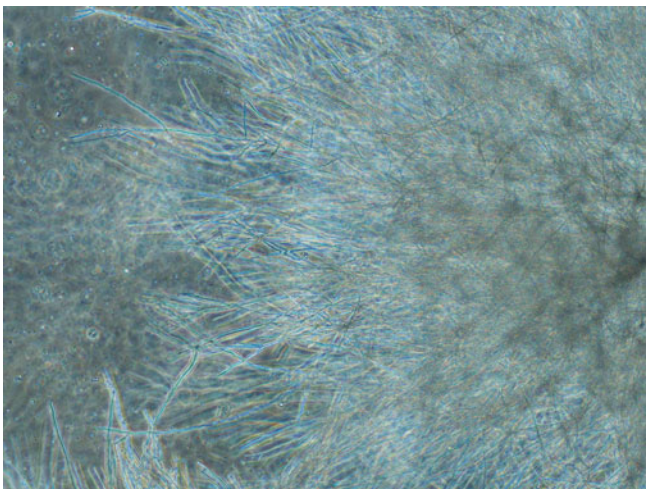

Fig. 11.11 Fungal contamination can look like cotton wool. The threads known as hyphae can be seen branching and knitting together to form the mycelium, characteristic of a fungal infection (×20 magnification)

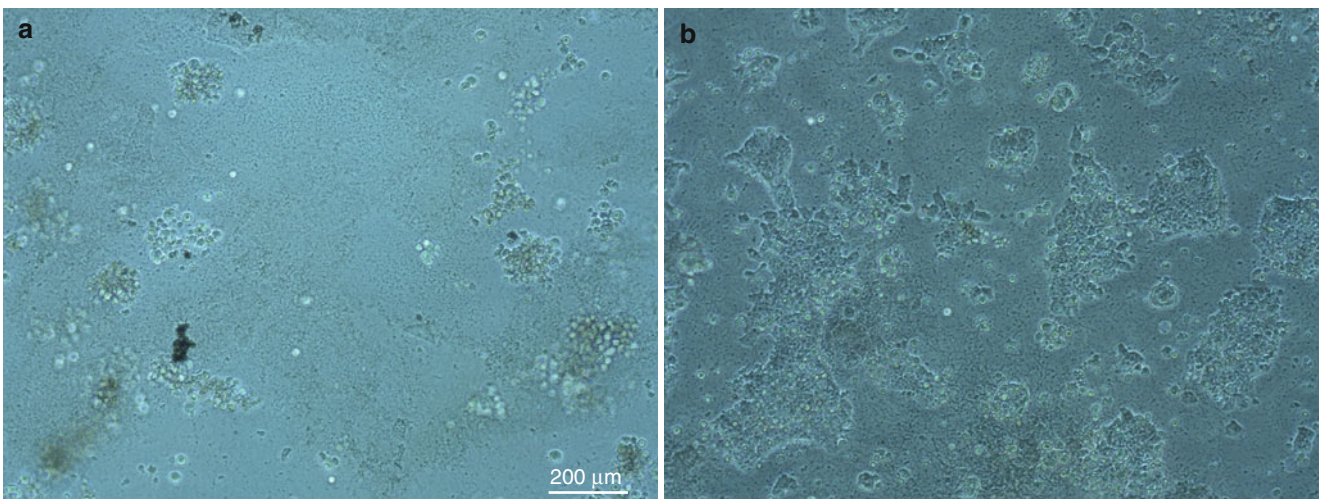

Fig. 11.12 (**a**, **b**) Typical bacterial infection, identified as *Staphylococcus epidermidis* (a skin commensal). With this type of infection, the medium changes colour overnight with respect to the phenol red indicator, from the normal pH (pink) to yellow (acidic). This change is accompanied by a change in the turbidity of the medium; it becomes cloudy (**a**, ×4 magnification; **b**, ×10)

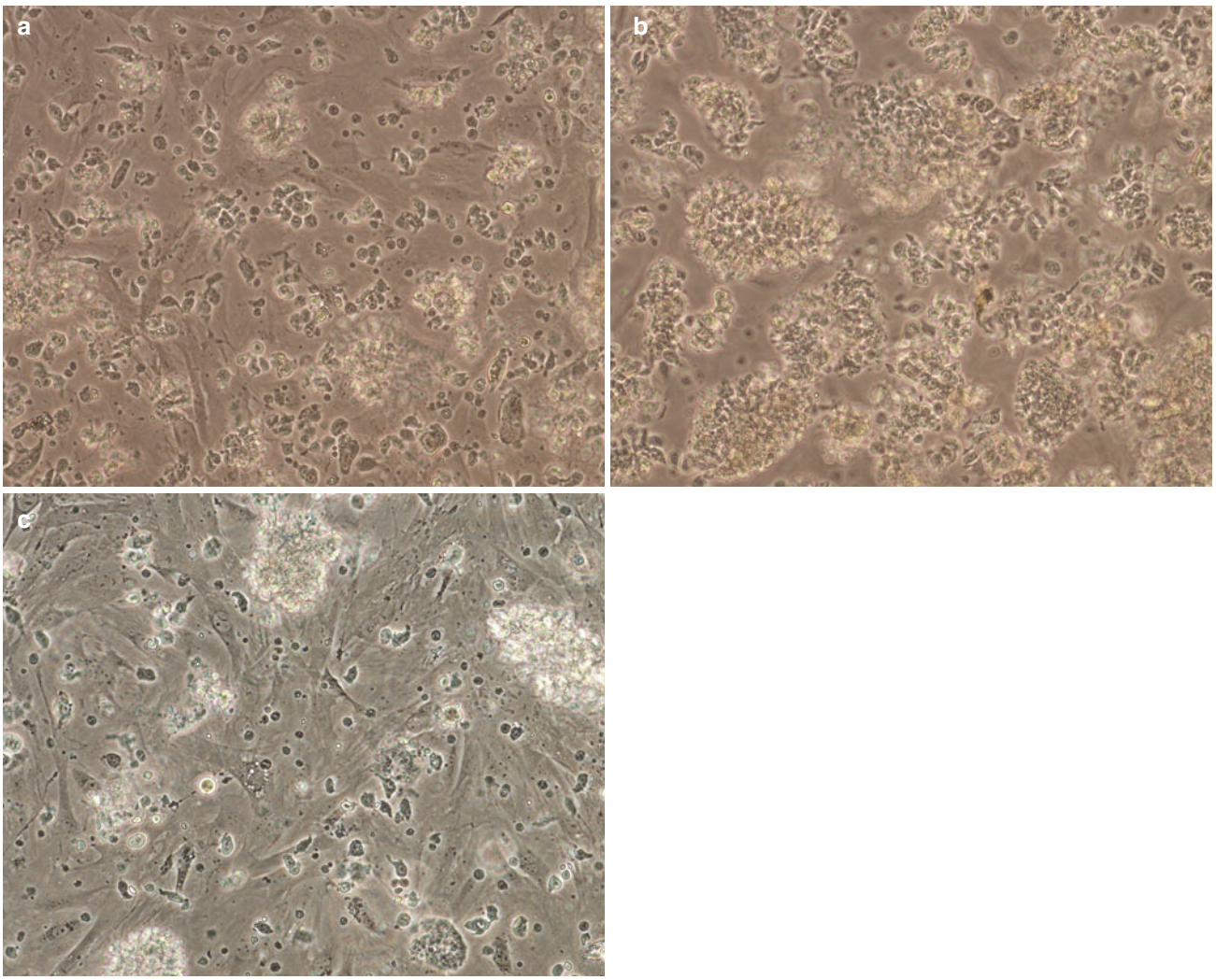

Fig. 11.13 (**a**–**c**) These images show an infection of a cell culture with yeast. Note the single cells and aggregates of yeast present in the culture. As with certain bacterial infections, the culture medium becomes yellow and turbid with time as the yeast multiply and overrun the culture. The feeders and PSC lift off the tissue culture plastic. If the culture has been left in the incubator for some time, it is possible to smell the yeast infection (all ×10 magnification)

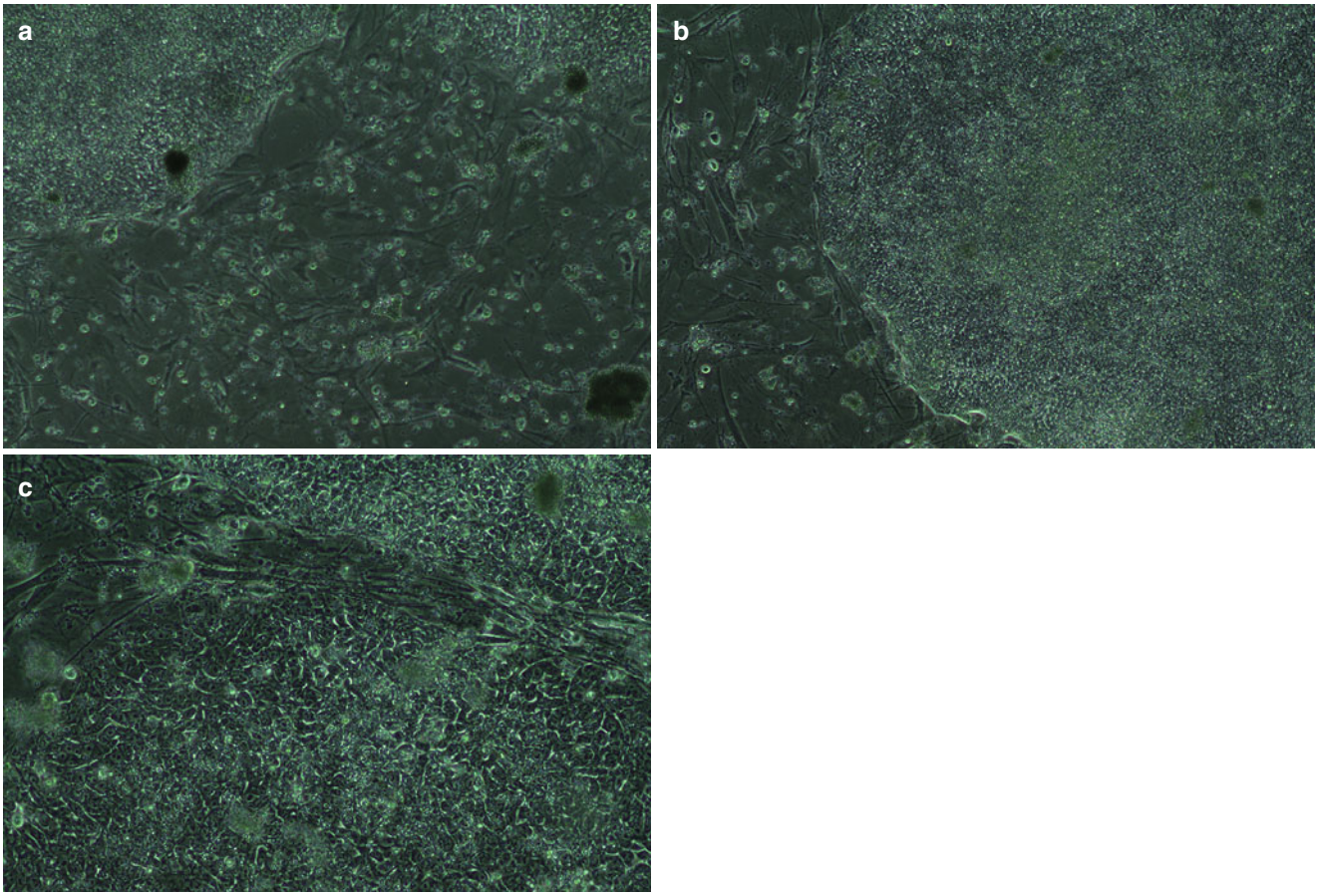

Fig. 11.14 (**a**–**c**) The microorganism shown in these three images is yeast, present as a low-level contamination. There are many types of yeast, exhibiting slightly different morphologies. The type of yeast causing the infection needs to be identified, because if yeast is becoming a recurrent problem in the cell culture, advice on how to eradicate it effectively should be sought (**a**–**b**, ×10 magnification; **c**, ×20)

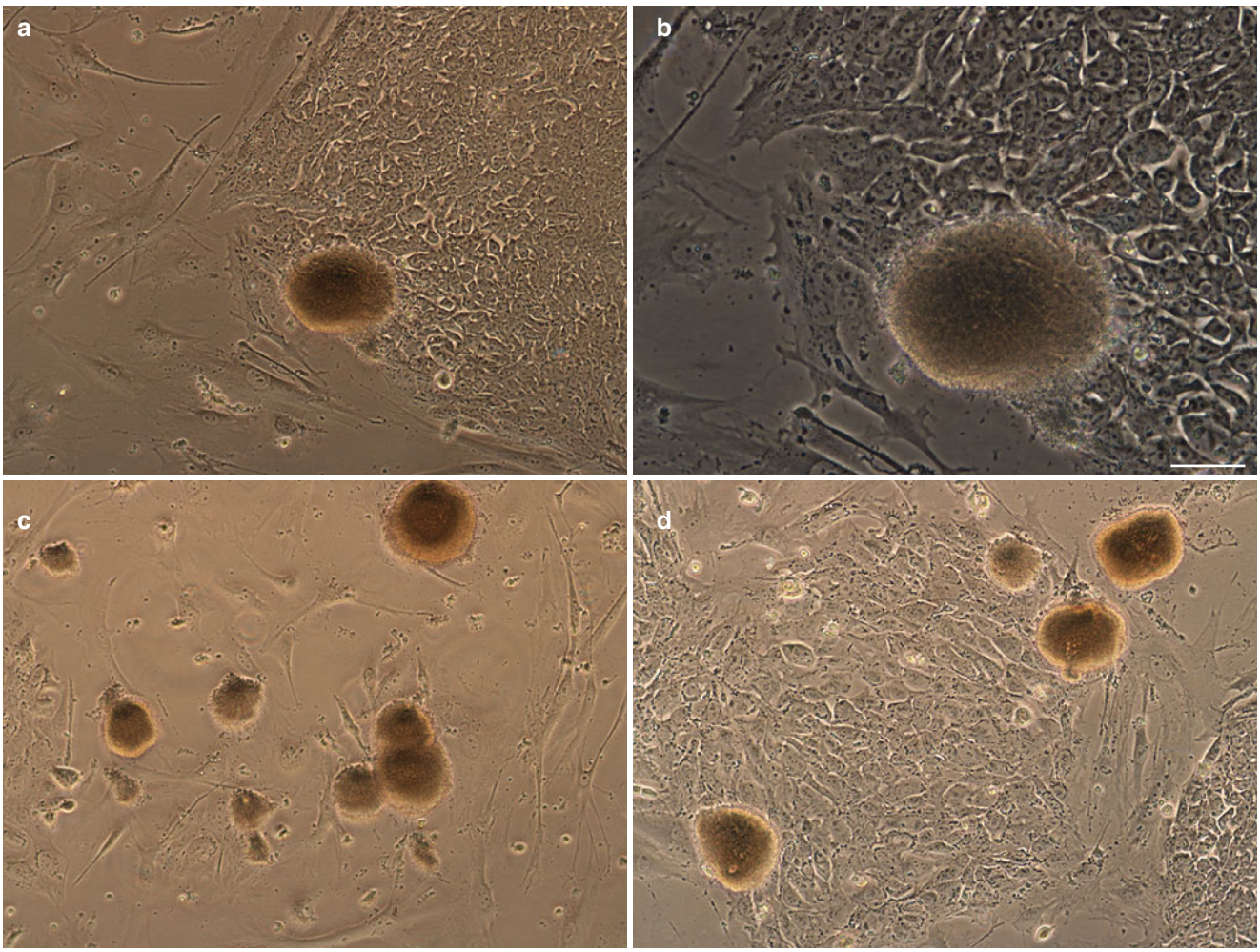

Fig. 11.15 (**a**–**d**) These four images display the same unidentified contamination. The cell culture medium does not change colour, since the microorganism does not grow quickly, and the culture medium does not display any turbidity (**a**, **b**, **d**, ×10 magnification; **b**, ×20)

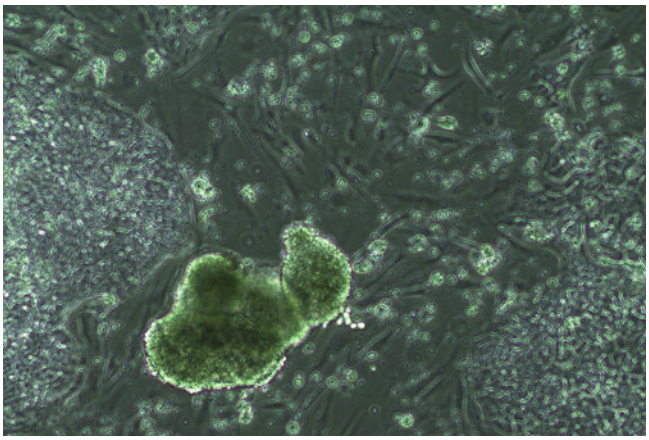

Fig. 11.16 Image of an unidentified contamination in cell culture (×10 magnification)

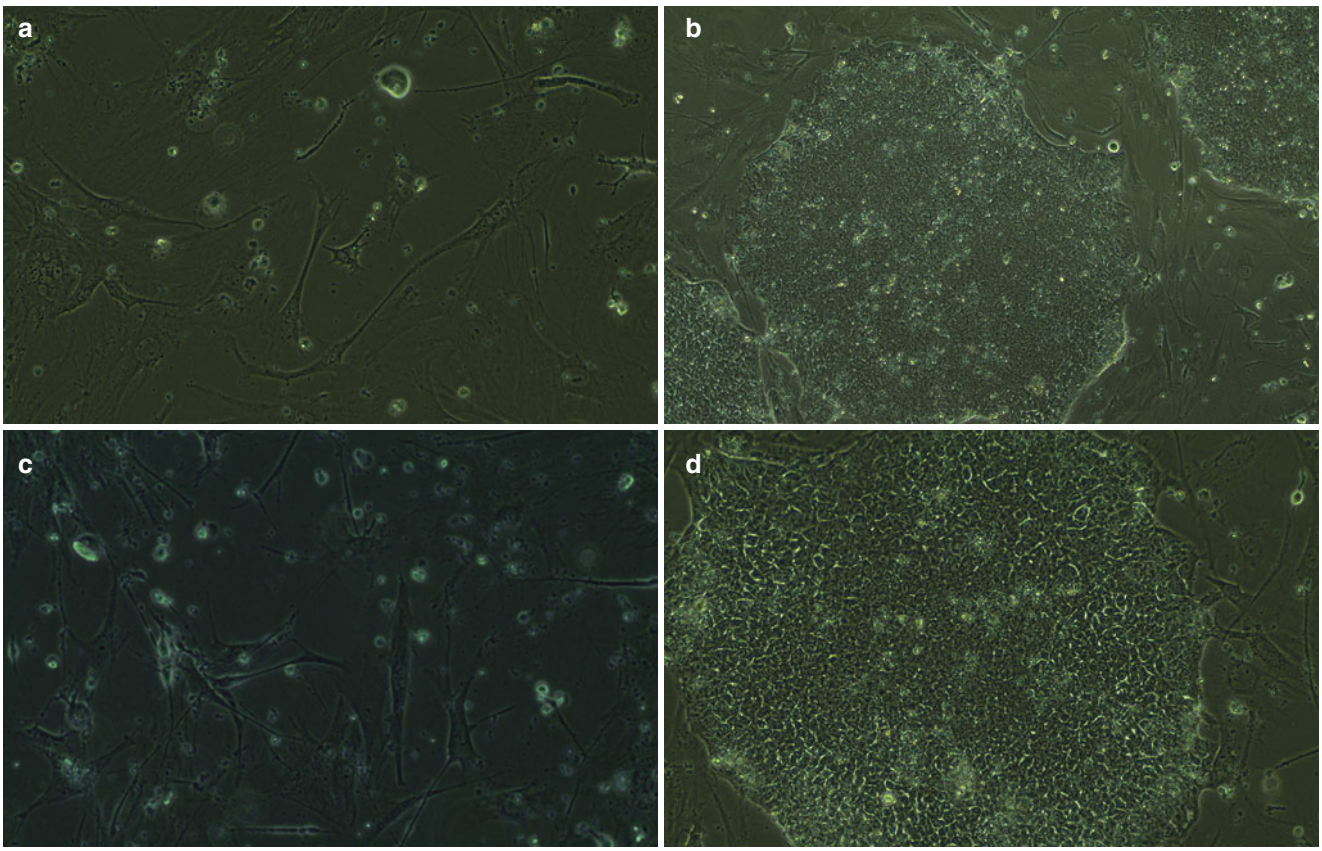

Fig. 11.17 (**a**–**d**) All four images show an unidentified contamination with the same microorganism in the cell culture (**a**, **c**, **d**, ×20 magnification; **b**, ×10)

Contamination is not restricted to microbial contamination. Cross-contamination of one cell line with another cell line will also have a devastating effect on the work of a laboratory, wasting both time and money. It is advisable to work with an authenticated cell line that has a known short tandem repeat profile and to demonstrate that this profile has been maintained before beginning a large work programme or the production of a peer-reviewed publication.

It is best practice to obtain cell lines from a reputable provider who can make available information on the provenance, history and testing of that cell line to demonstrate that it is free from specified contamination at the point of dispatch, and also can provide the same cell line again if required.

Suggested Reading

Freshney RI. Basic principles of cell culture. In: Vunjak-Novakovic G, Freshney RI, editors. Culture of cells for tissue engineering. Hoboken: Wiley; 2006. p. 3–22.

McLaren RS, Reid Y, Storts DR. Human cell line authentication: the critical first step in any project using human cell lines. Methods Mol Biol. 2013;963:341–53.

Slavin I, Schell JP. Contamination. In: Loring JF, Peterson SE, editors. Human stem cell manual. 2nd ed. London: Elsevier; 2012. p. 41–52.

Stacey GN. Cell culture contamination. Methods Mol Biol. 2011;731:79–91.

Stacey GN, Byrne E, Hawkins JR. DNA profiling and characterization of animal cell lines. Methods Mol Biol. 2014;1104:57–73.

Young L, Sung J, Stacey G, Masters JR. Detection of mycoplasma in cell cultures. Nat Protoc. 2010;5:929–34.

Miscellaneous Cell Types and Cell Lines of Interest

12

This final chapter serves to illustrate other companion cell types and cell lines that may be used in conjunction with pluripotent stem cells (PSCs), both in the setting of quality control and in the assessment of differentiated counterparts. They include mouse embryonic stem cell (mESC) lines, which are routinely grown without feeders (Figs. 12.1, 12.2, 12.3 and 12.4). These cells are easier to maintain than their human counterparts, but they are prone to spontaneous differentiation when grown as a monolayer of cells. In general, they require passaging every 2–3 days, depending on their original plating density. Human embryonal carcinoma cells (hECCs), in contrast, can be grown as a monolayer, but when overconfluent, they spontaneously differentiate (Figs. 12.5, 12.6 and 12.7). Both mESC lines and hECC lines are often used as control cell lines for the assessment of stem cell-specific antibodies and gene expression.

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DOI 10.1007/978-1-4899-7507-2_12, © Springer Science+Business Media New York 2015

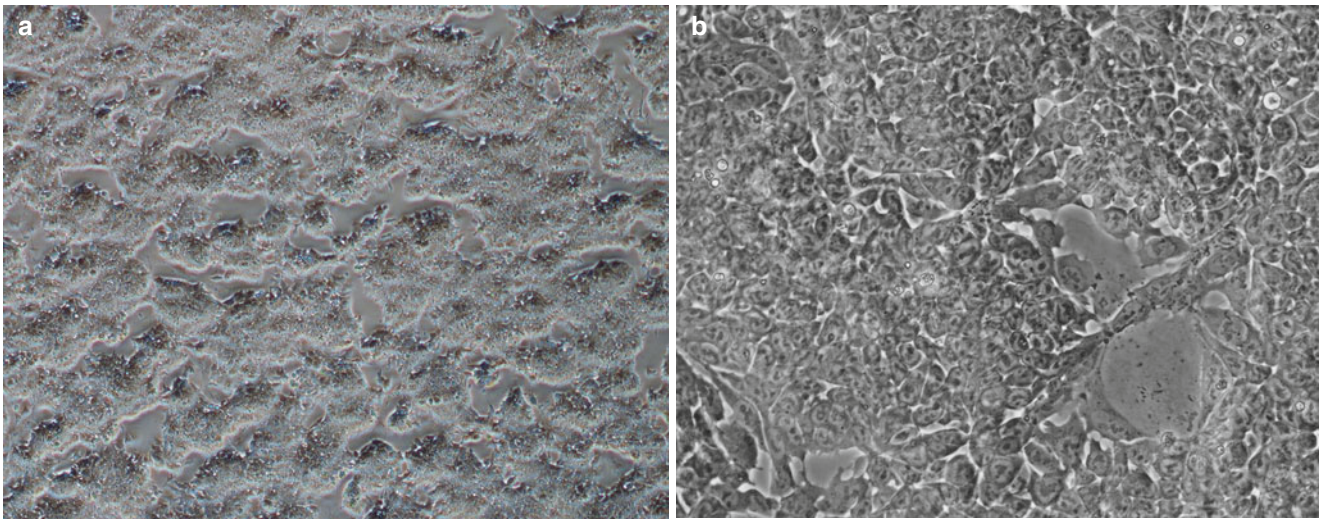

Fig. 12.1 Feeder-independent culture of a mouse embryonic stem cell (mESC) line at different magnifications (**a**, ×4 magnification; **b**, ×10)

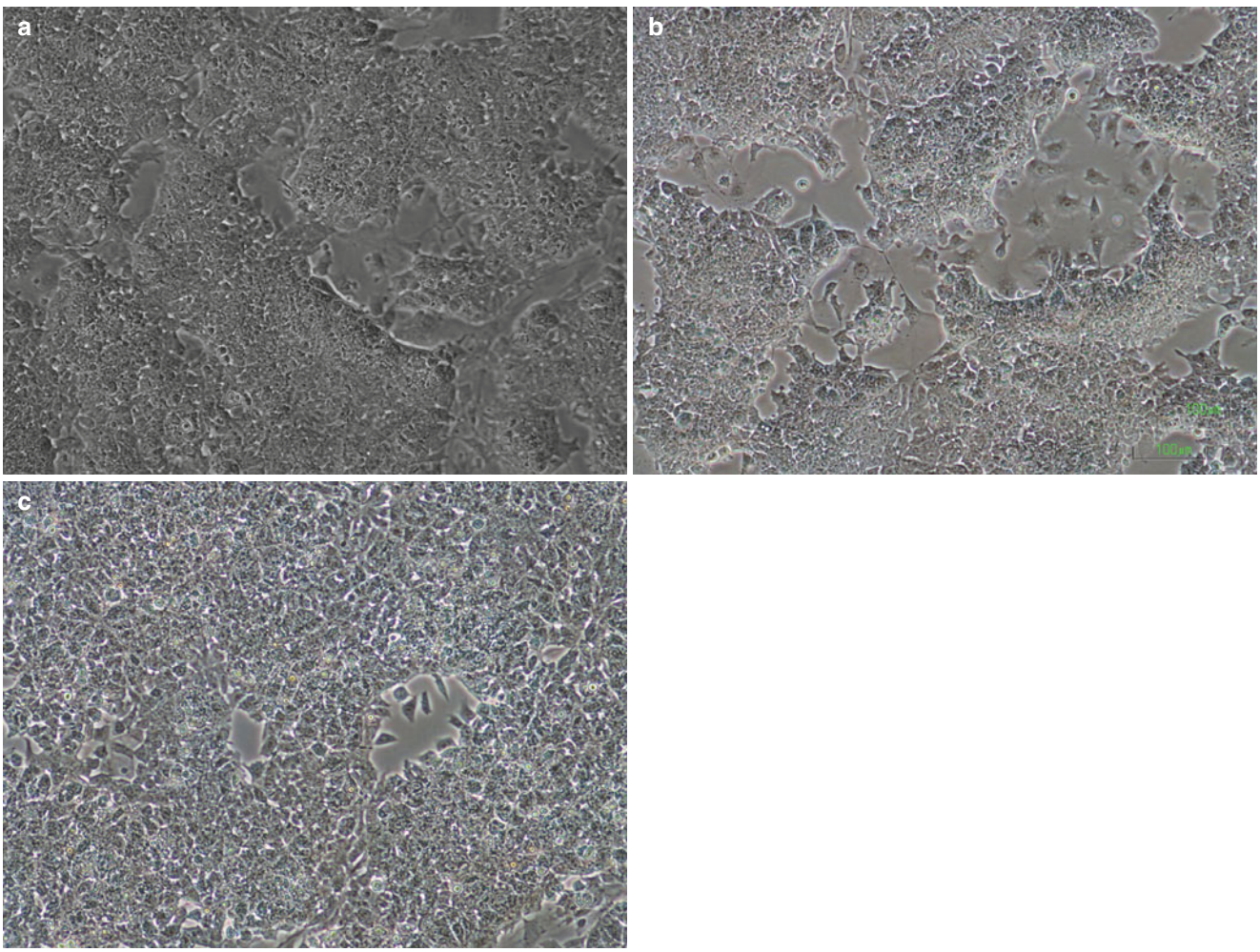

Fig. 12.2 (**a**, **b**) A good-quality, high-density culture of mouse embryonic stem cells (mESCs). In image (**a**), most of the cells grow within the colonies. It is very important not to let the culture of mESCs overgrow and form a monolayer, as shown in image (**c**). If the cells overgrow into a monolayer, they will differentiate. It is best to passage the cells every 2–3 days, when they look similar to the cells in culture shown in **a** and **b** (**a**, **b**, ×4 magnification; **c**, ×10)

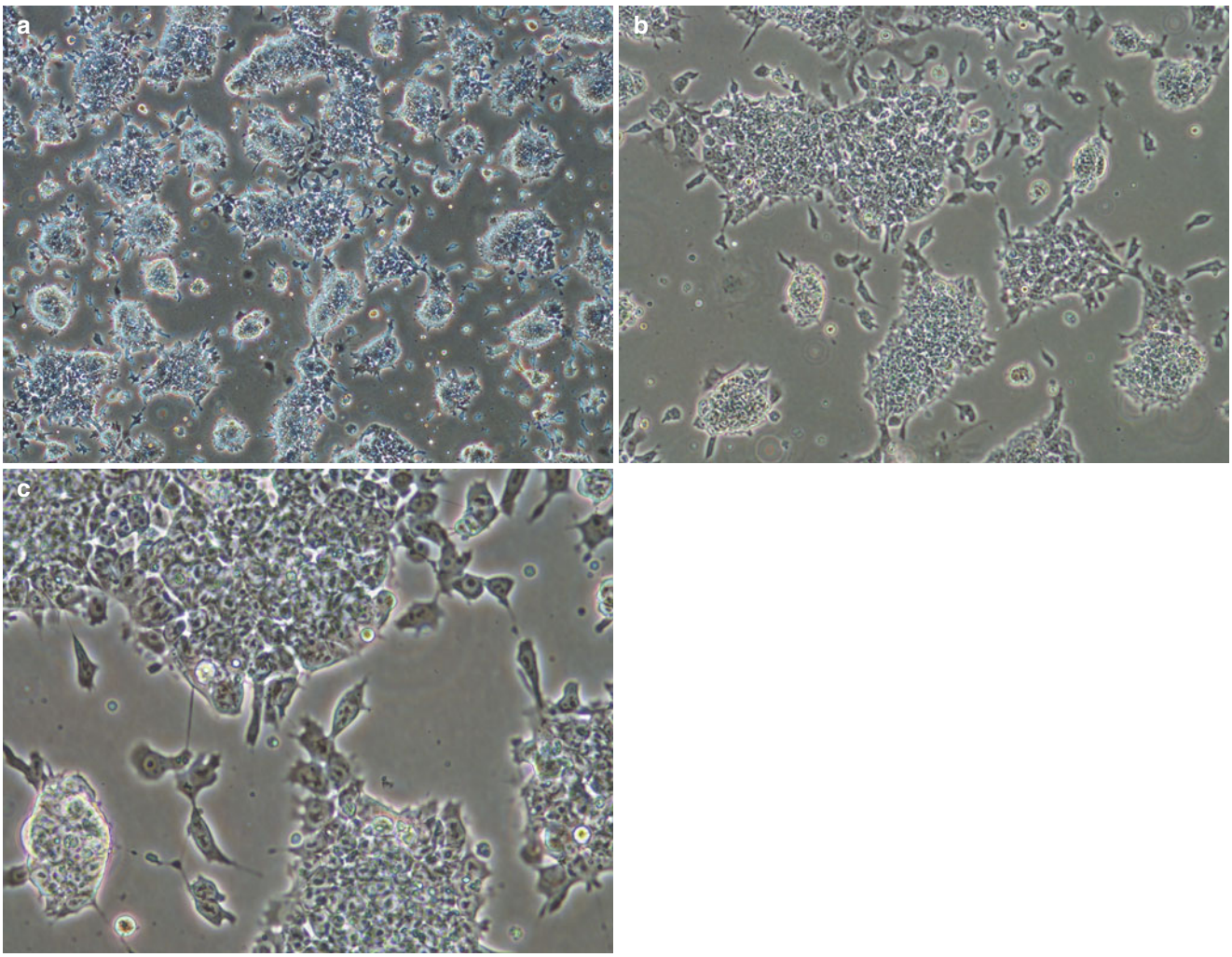

Fig. 12.3 (**a** and **b**, **c**) The mESCs within these colonies appear to display the morphology of stressed, cells in that although the cells have attached to the cell culture plate they have not started to spread out. There is also a number of apoptotic cells and cell debris present in the culture. This type of morphology in culture as is often seen during the first passage of the cells, after thawing, or after another biological insult. (**a**, ×4 magnification; **b**, ×10, **c** ×20)

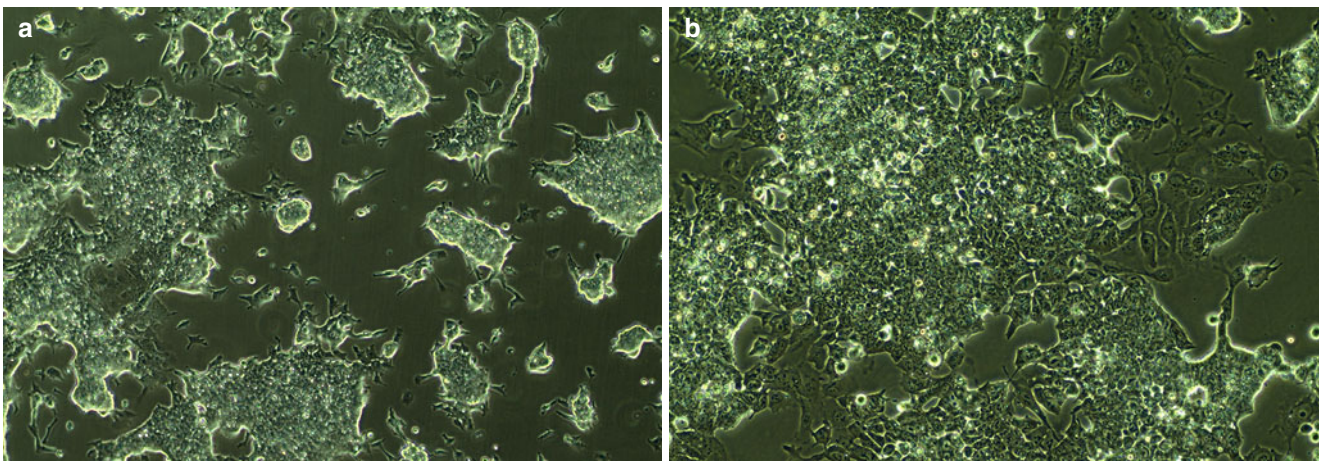

Fig. 12.4 These images illustrate more examples of mESCs under stress. (**a**) These cells appear bright and refractile (*bright ring* around the outside of the colonies). (**b**) This image shows an abundance of apoptotic cells (**a**, ×4 magnification; **b**, ×10)

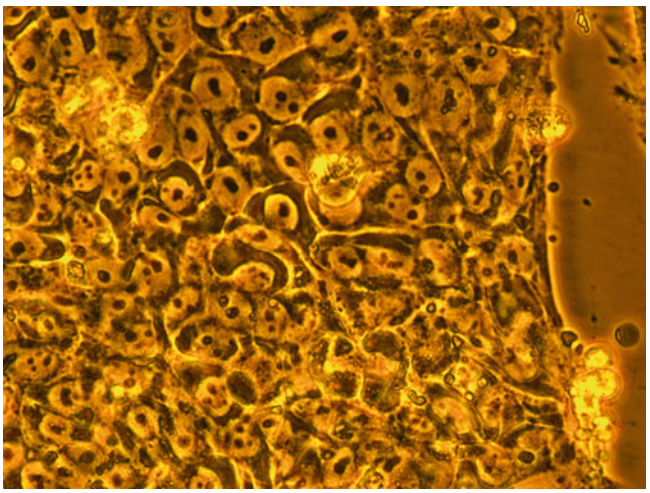

Fig. 12.5 Feeder-independent culture of a human embryonal carcinoma cell line (hECCs), showing typical embryonic stem cell morphology: compacted, small, round cells with prominent nucleoli and a large ratio of nucleus to cytoplasm (×40 magnification)

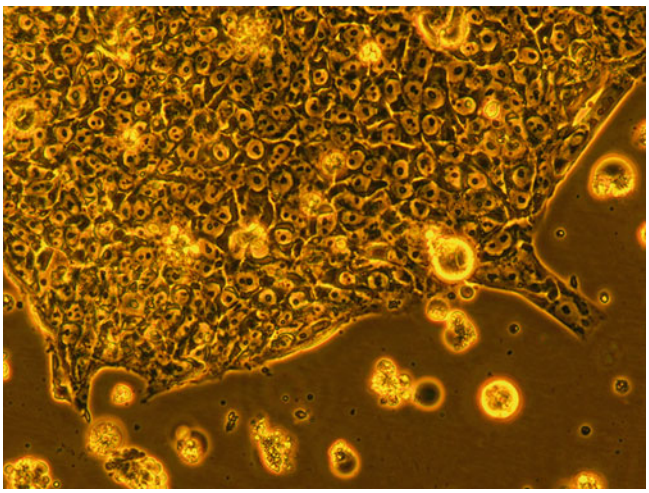

Fig. 12.6 A compacted colony of an hECC line with defined borders, spread out to produce a perfect monolayer. This appearance is characteristic of a good-quality colony. These cells grow on gelatin-coated plates, but when they become overconfluent, they tend to detach from the plate. Apoptotic cells are often seen in these cultures, but this is not an indication of stress (×10 magnification)

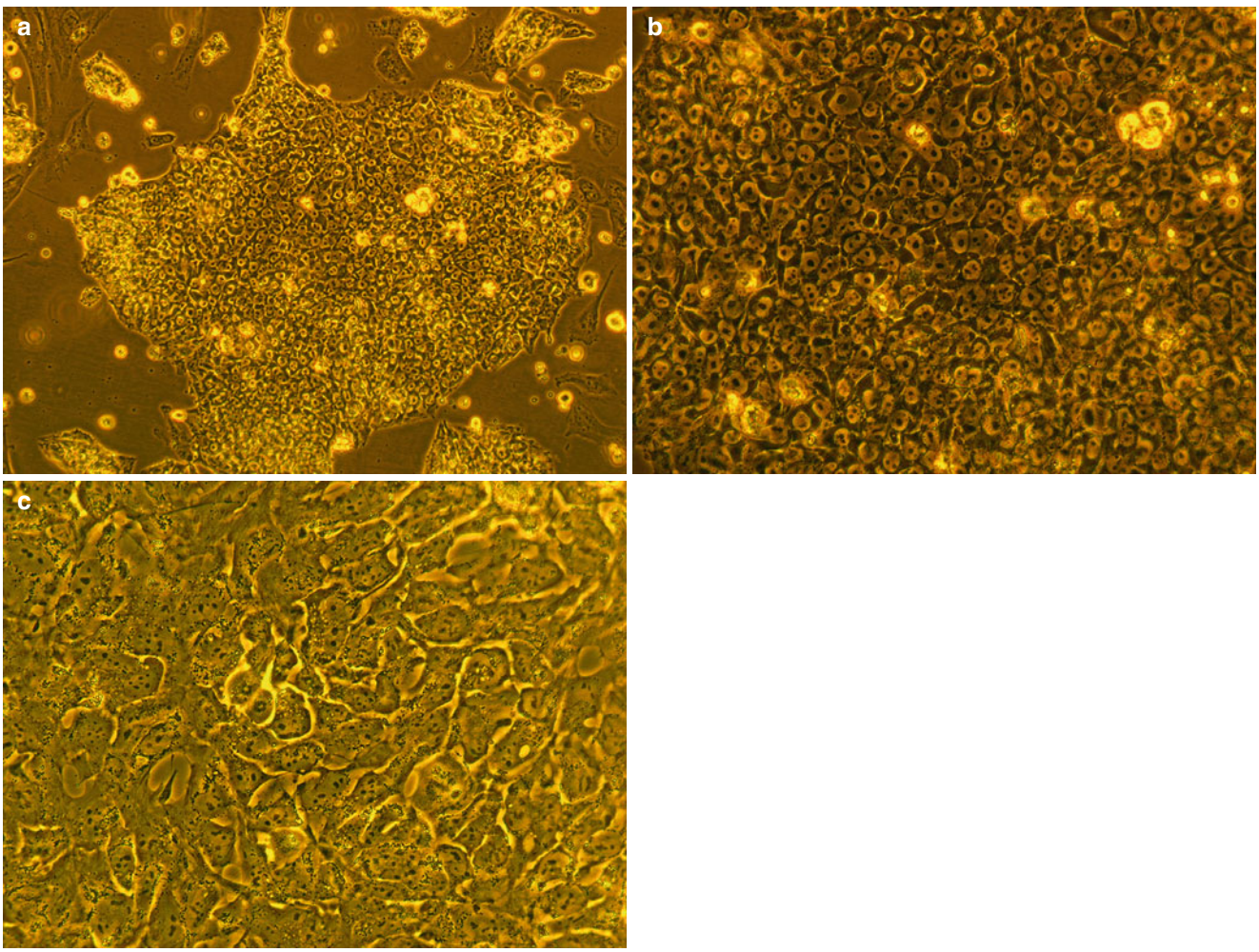

Fig. 12.7 (**a**–**c**) Feeder-independent culture of hECCs at different magnifications. This culture is of good quality, as every cell is easily distinguishable at each magnification (**a**, ×4 magnification; **b**, ×10; **c**, ×20)

The images in this chapter also include human and rat mesenchymal cells, used as companion cells in lineage-specific quality control (Figs. 12.8 and 12.9). Figures 12.10 and 12.11 show examples of human embryoid bodies, which are aggregates of cells grown in suspension, forming three-dimensional structures; these are used as tools for differentiation studies. Human neural cultures at different stages of differentiation serve to illustrate the changes in morphology as the cells mature and differentiate down a number of neural-related pathways (Figs. 12.12, 12.13, 12.14 and 12.15).

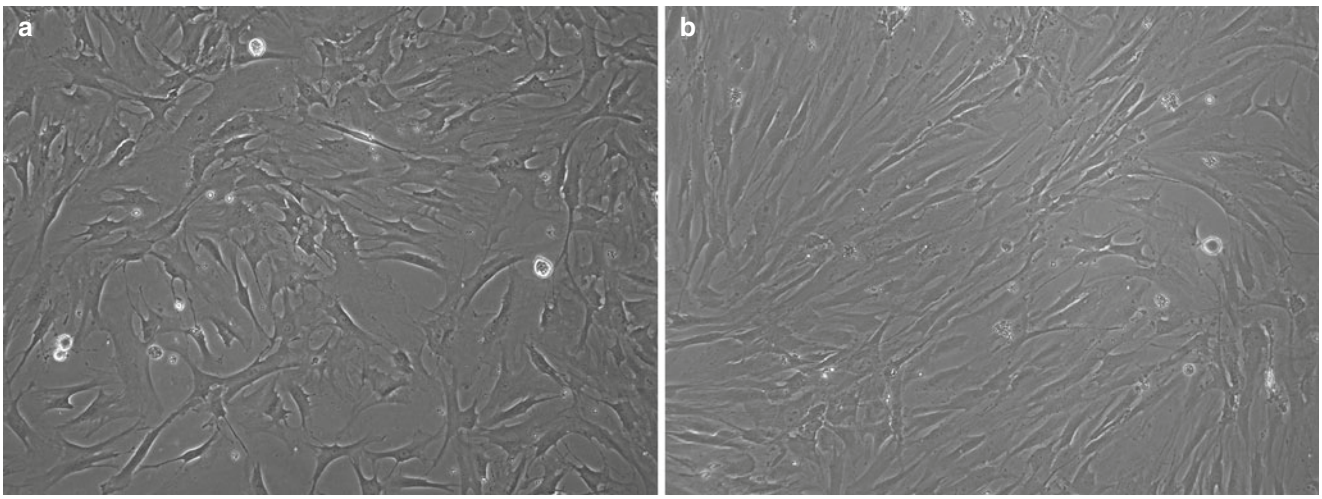

Fig. 12.8 Different confluency of human mesenchymal cells derived from adult human bone marrow (**a**, **b**, ×10 magnification)

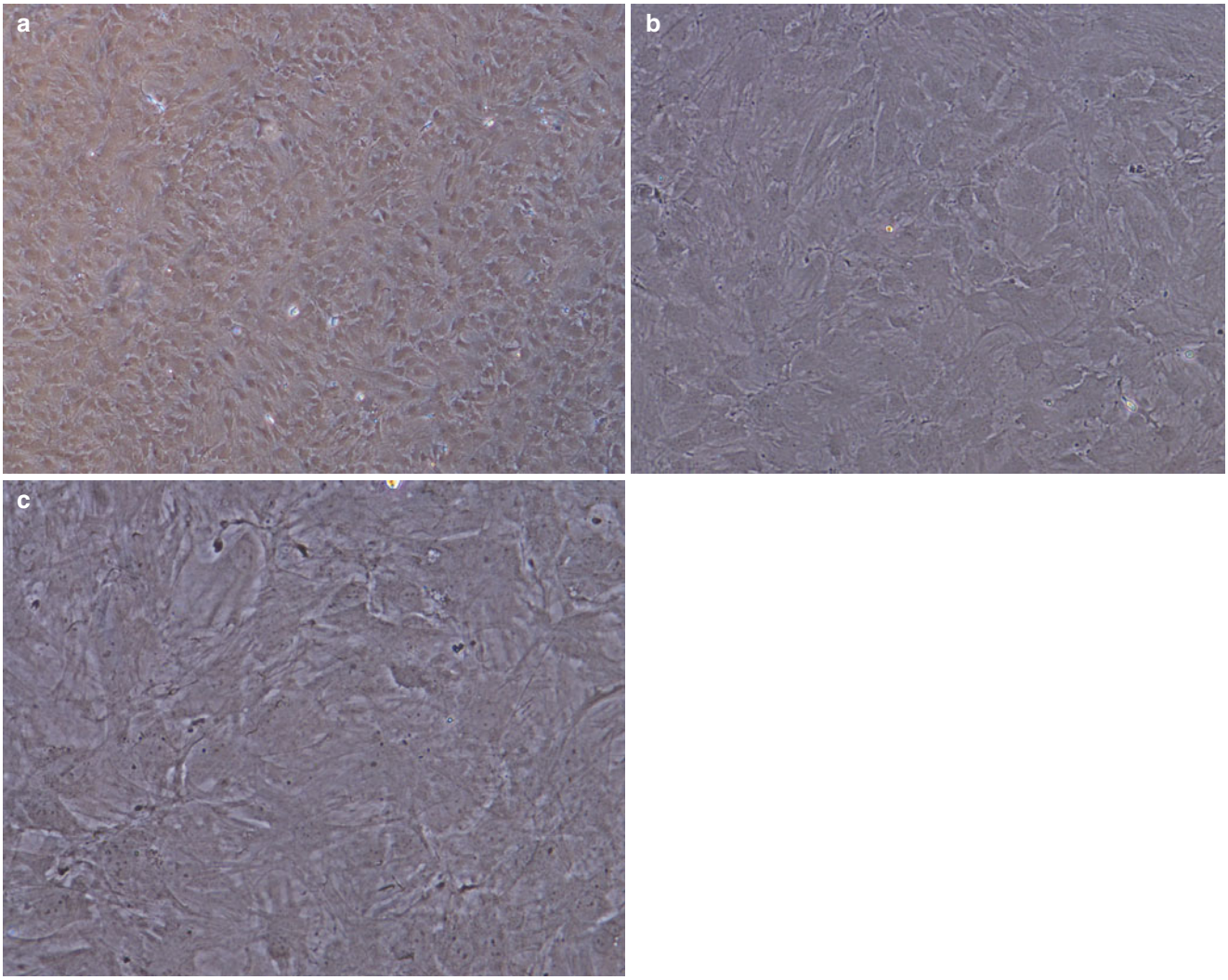

Fig. 12.9 (**a**–**c**) A confluent culture of rat mesenchymal cells derived from the bone marrow of an adult animal, shown at three different magnifications (**a**, ×4, **b**, ×10, **c** ×20)

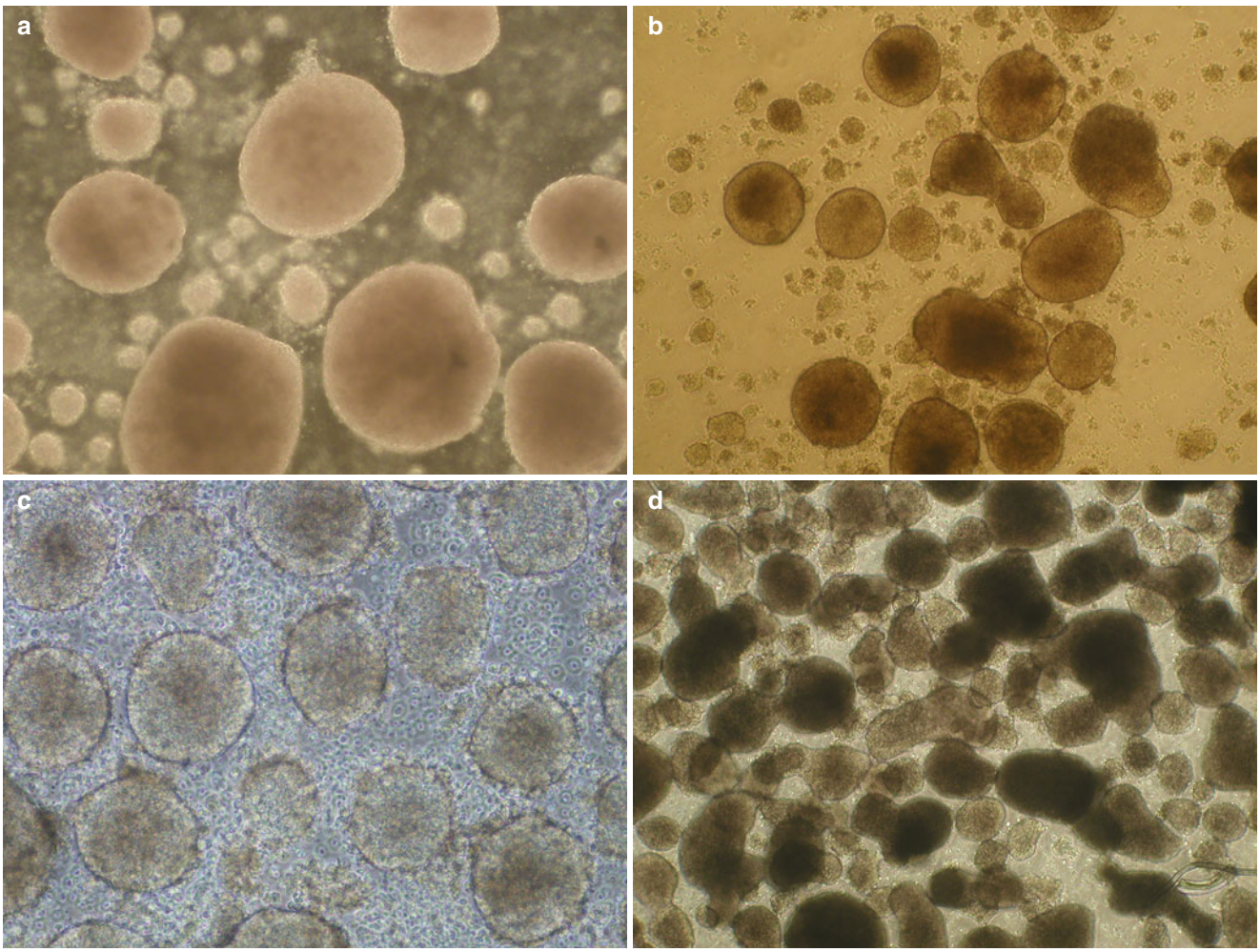

Fig. 12.10 (**a**–**c**) Images of different preparations of spontaneously formed embryoid bodies (EBs). Under normal circumstances, these are produced when cells are dissociated into single cells and allowed to aggregate on non–tissue culture treated plasticware. (**d**), These aggregates of cells spontaneously differentiate and can display elements of all three germ layers. Theses cells have been added to cell culture medium containing a cocktail of growth factors, which will play a pivotal role in the directed differentiation of these cells down a particular lineage. Note the heterogeneity in the size of these structures. It has been suggested that the size of the EB influences the choice commitment of the cells to a particular of germ layer during the differentiation process. In the presence of a cocktail of growth factors, EBs aggregate and appear to be very dark structures (**a** and **c**, ×20 magnification; **b** and **d**, ×10)

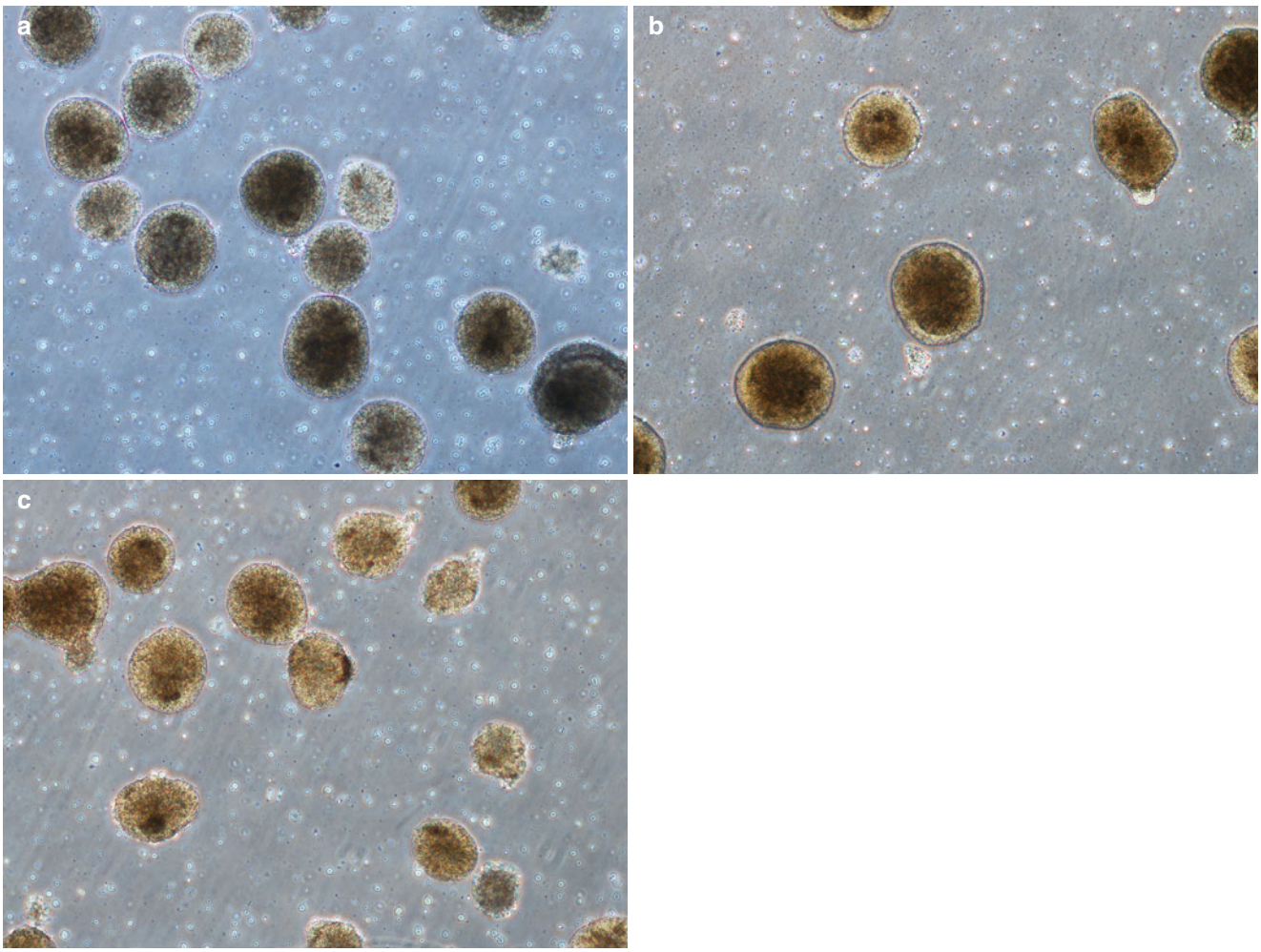

Fig. 12.11 (**a**–**c**) Another set of EBs produced using the AggreWell™ system. This system generates structures that are more uniform in size (**a**, **b**, **c** ×10 magnification)

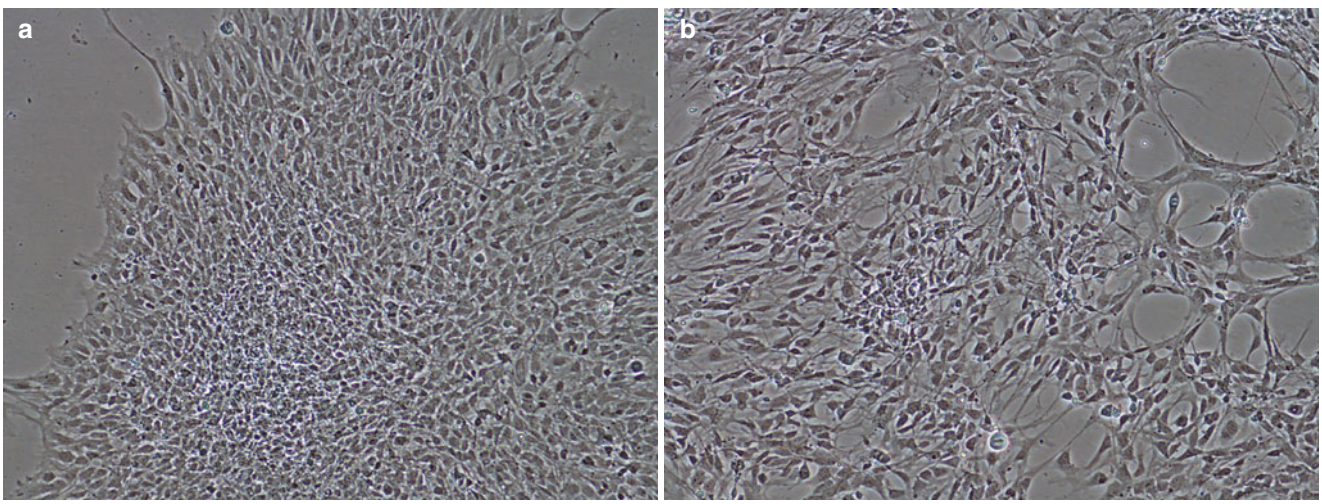

Fig. 12.12 (**a**, **b**) Neural progenitors growing as a monolayer displaying characteristic morphology (both ×10 magnification)

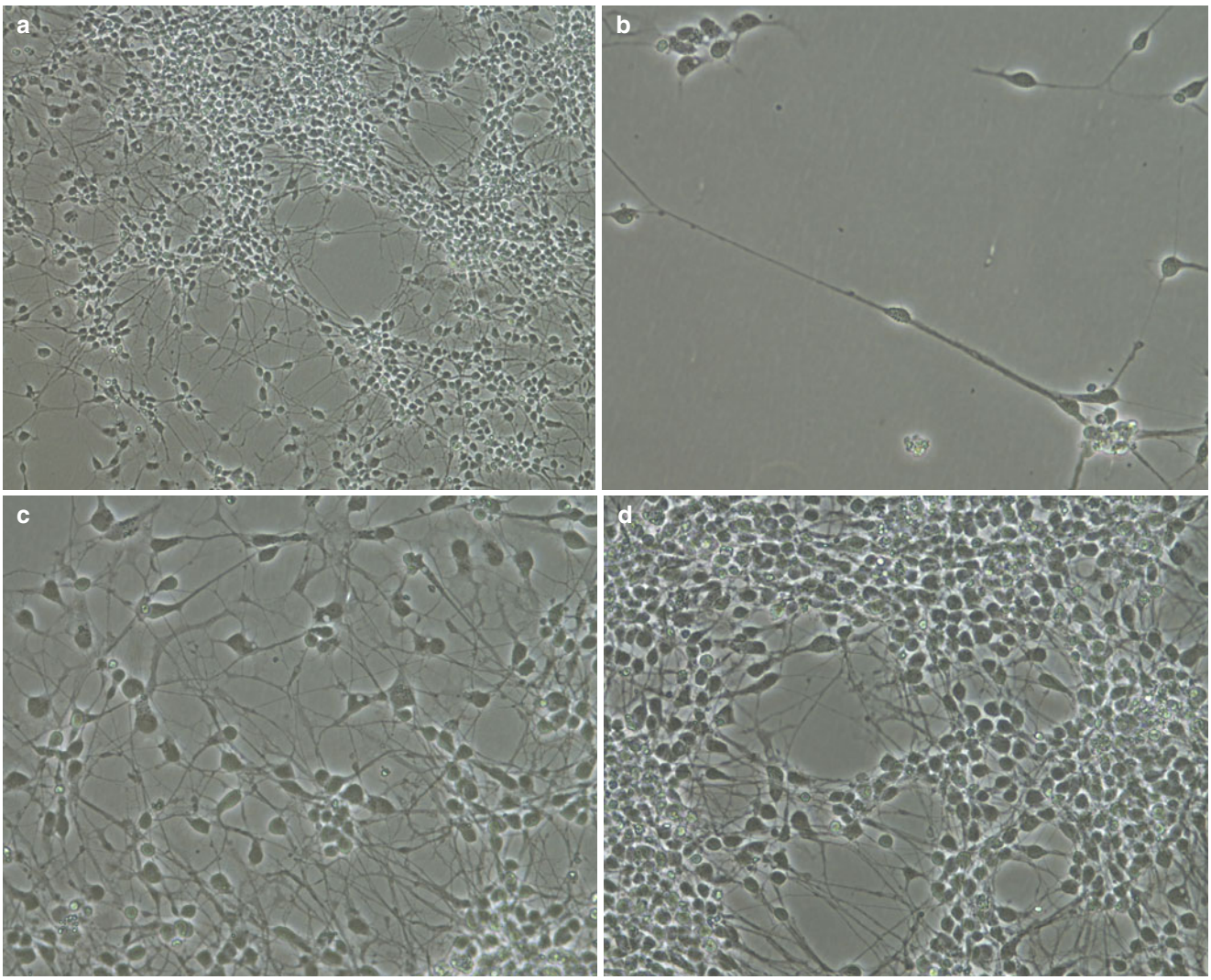

Fig. 12.13 (**a**–**d**) Later-stage neural progenitors, displaying an array of morphologies (**a**, ×4 magnification; **b**–**d**, ×10)

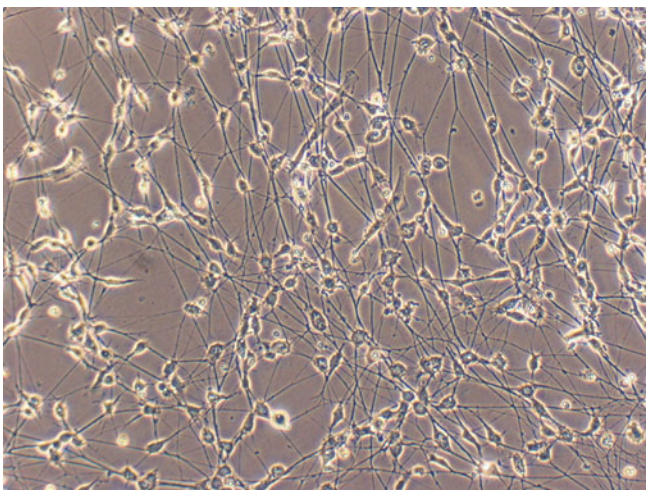

Fig. 12.14 Network of neural cells in culture (×10 magnification)

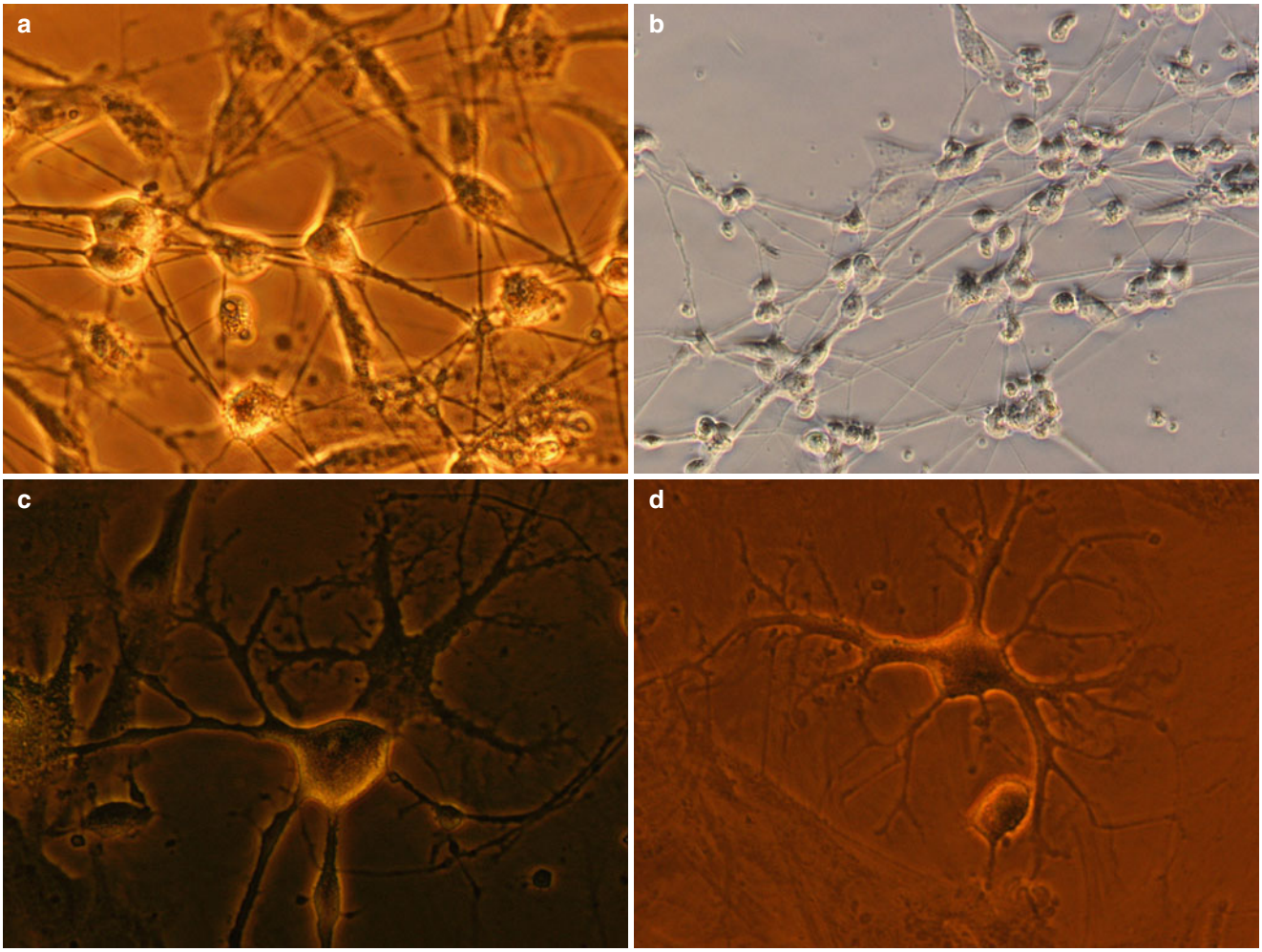

Fig. 12.15 (**a**, **b**) Neural cells with their characteristic cell bodies and axons. (**c**, **d**) These images display dendritic cells (**a**, **c**, **d**, ×20 magnification; **b**, ×10)

The chapter concludes with images of endovascular cells derived from a cytotrophoblast line originating from a human embryonic stem cell (hESC) line (Figs. 12.16 and 12.17).

Cells and tissues should be acquired from reputable repositories to ensure provenance, traceability, identity, safety and reproducibility. A list of several repositories follows the reading list.

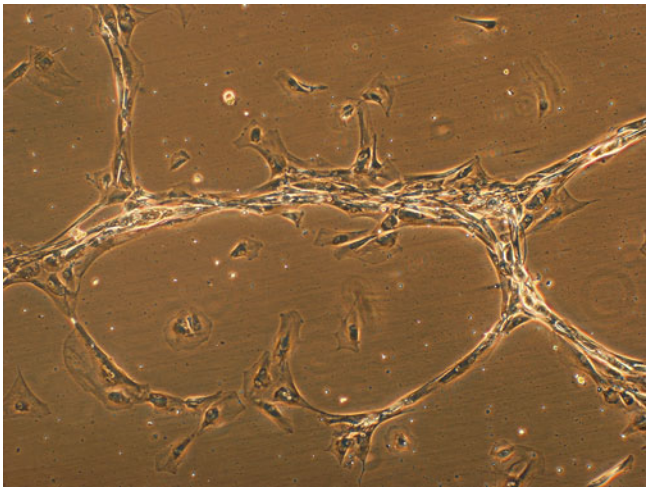

Fig. 12.16 Trophoblast-derived endothelial cells forming typical endothelial structures (×10 magnification)

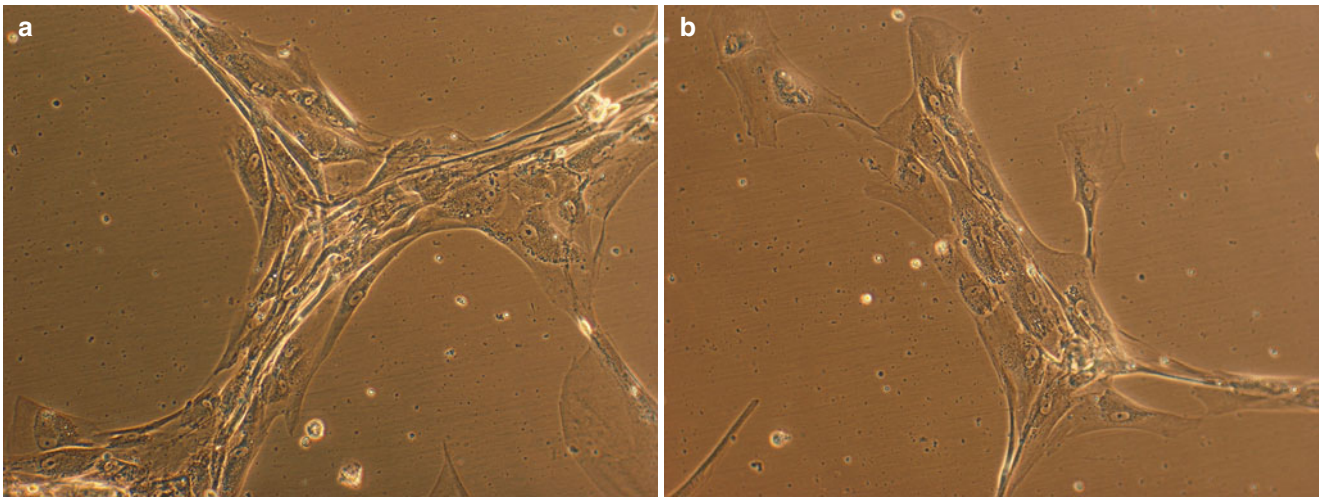

Fig. 12.17 (**a**, **b**) Trophoblast-derived endothelial cells forming typical endothelial structures, shown at a higher magnification (both ×20 magnification)

Suggested Readings

Andrews PW, Oosterhuis JW, Damjanov I. Cell lines from human germ cell tumours. In: Robertson EJ, editor. Teratocarcinomas and embryonic stem cells: a practical approach. Oxford: IRL Press; 1987. p. 207–48.

Aschner M, Suñol C, Bal-Price A, editors. Cell culture techniques, Neuromethods, vol. 56. New York: Humana Press; 2011.

Harun R, Ruban L, Matin M, Draper J, Jenkins NM, Liew GC, et al. Cytotrophoblast stem cell lines derived from human embryonic stem cells and their capacity to mimic invasive implantation events. Hum Reprod. 2001;21:1349–58.

Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, et al. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med. 2000;6:88–95.

Josephson R, Ording CJ, Liu Y, Shin S, Lakshmipathy U, Toumadje A, et al. Qualification of embryonal carcinoma 2102Ep as a reference for human embryonic stem cell research. Stem Cells. 2007; 25:437–46.

Kozhich OA, Hamilton RS, Mallon BS. Standardized generation and differentiation of neural precursor cells from human pluripotent stem cells. Stem Cell Rev. 2013;9:531–6.

Loring JF, Peterson SE, editors. Human stem cell manual. 2nd ed. London: Elsevier; 2012.

Mather JP, editor. Stem cell culture, Methods in cell biology, vol. 86. London: Academic Press (Elsevier); 2008.

Smith AG. Culture and differentiation of embryonic stem cells. J Tissue Cult Methods. 1991;13:89–94.

Tremml G, Singer M, Malavarca R. Culture of mouse embryonic stem cells. Curr Protoc Stem Cell Biol. 2008;5:1C.4.1–19.

Zhang SC, Wernig M, Duncan ID, Brüstle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol. 2001;19:1129–33.

Cell Line and Tissue Repositories

International Society for Biological and Environmental Repositories (ISBER). www.isber.org.

American Type Culture Collection (ATCC). http://www.atcc.org.

Coriell Cell Repositories. http://www.ccr.coriell.org.

Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures. www.dsmz.de.

European Collection of Cell Cultures (ECACC). www.phe-culturecollections.org.uk/collections/ecacc.aspx.

RIKEN Bioresource Center Cell Bank. https://en.brc.riken.jp/lab/cell/english/guide.shtml.

Answer to the Challenges

Fig. 4.26 Three-day-old hESC colonies with high density and very good quality (×4 magnification)

Fig. 4.27 The colonies are a little older (4–5 days) and have spontaneous differentiation on the edges of the colony (*black arrow*). The cells are starting to grow on top of each other (*white arrow*) because of a lack of space for the colonies to expand. Overall, this is still a good-quality culture (×4 magnification)

Fig. 4.31 The colony consists of small, round, compacted cells with large nucleoli. There are only a few apoptotic cells (*black arrow*) (×20 magnification)

Fig. 4.32 This colony is in total contrast to the previous colony. The cells are elongated and stretched, not round and compacted. This culture shows signs of stress, as many apoptotic cells are visible (×20 magnification)

Fig. 4.49 (**a**, **b**) Both images show good hESC cultures (both ×10 magnification)

Fig. 4.50 (**a**) Good, compacted colony. (**b**) A colony that has not compacted (both ×10 magnification)

Fig. 4.59 All cells within the colonies are elongated and irregular in shape, indicating a transition in the cell towards a differentiated state (×10 magnification)

Fig. 4.60 Typical hESC morphology seen under high magnification: small, roundish cells with prominent nucleoli (×10 magnification)

Fig. 4.61 (**a**) A relatively good, undifferentiated colony, with defined borders. The colony contains round, compacted cells with large nucleoli. (**b**) This colony shows slight differentiation. These areas of differentiation can be seen as white patches of cells and are typical of the beginning of the formation of neural rosettes. These will become evident if the cultures are left for a further 48 h. (**c**) Typical structures of neural rosette formation (**a**, **b**, ×10 magnification; **c**, ×20 magnification)

Fig. 4.62 Two small colonies on good-quality mouse feeders. All the cells within colony number *1* are elongated, lined up, and poised to differentiate. In comparison, colony number *2* displays a perfect, undifferentiated morphology (×4 magnification)

Fig. 5.37 (**a**, **b**) Images of a good-quality culture at two different magnifications. Note the typical morphology of the undifferentiated stem cells, with prominent nucleoli and spaces between the cells (**a**, ×10 magnification; **b**, ×20 magnification)

Fig. 5.38 Three images of colonies. (**a**) Disorganised cells that could initiate differentiation. (**b**, **c**) Good, compacted colonies ready to be passaged (all ×10 magnification)

Fig. 5.39 Two colonies that have merged. The colony on the right has differentiated and displays large, disorganised cells. The colony on the left is relatively undifferentiated (×10 magnification)

Fig. 5.40 (**a**, **b**) Two images of colonies at high magnification. (**a**) Long, stretched, differentiating cells forming a neural rosette. (**b**) Roundish cells with prominent nucleoli and spaces indicative of undifferentiated stem cells (both ×20 magnification)

Fig. 7.11 This dark-field image displays a large number of colonies that appear to be undifferentiated. They are at a high density and appear opaque rather than white. White colonies under bright field are often multilayered and therefore are prone to spontaneous differentiation (×4 magnification)

Fig. 7.12 This dark-field phase contrast image again shows a number of colonies. Although many areas are opaque, very white patches of multilayered cells are seen within and around a number of colonies. In addition, two colonies (*white arrows*) have translucent areas visible, where the cells have differentiated. It would be wise to remove the differentiated cells prior to manually passaging these colonies (×4 magnification)

Fig. 7.13 Bright-field (**a**) and dark-field (**b**) images showing colonies that have spontaneously differentiated (**a**) (*black arrows*) and (**b**) (*white arrows*). The rest of the colonies look relatively undifferentiated and appear to be in good shape, but their uneven surfaces indicate that all these colonies will differentiate within 36–48 h. One of the reasons could be that an earlier culture from which they were passaged contained a number of cells that were starting to differentiate (×4 magnification)

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DOI 10.1007/978-1-4899-7507-2, © Springer Science+Business Media New York 2015

Glossary

This atlas, in a visual context, describes a number of characteristics of cells in culture. For the purpose of this atlas, we need to define these visual characteristics because of the subjective nature of the cells. However, standard terminology used in cell culture and pluripotent stem cell biology and abbreviations will also be defined in line with a number of relevant glossaries.

Adherent A cell culture where the cells attach to a substrate, such as the surface of tissue culture plasticware, and the cells spread, forming a monolayer.

Apoptosis A process of programmed cell death.

Batch A defined quantity of cells produced in a single process producing a product with little to no heterogeneity.

Cell culture The growth, expansion, and maintenance of cells in vitro.

Cell expansion The proliferation of cells resulting in their increase in number.

Cell morphology The shape and structure of cells.

Cell stress Response to adverse extrinsic factors in cell culture, which have an effect on the regulatory pathways of that cell, leading to cell survival or cell death. These responses can also be revealed in the phenotype of the cell by changes in morphology.

Cell surface marker The expression on the surface of a cell of a biomolecule specific to that cell type. These markers can be used to classify different types of cells.

Co-culture In this context, the growth together in a single tissue culture vessel of inactivated fibroblasts and PSCs.

Colony A biological structure, containing a number of cells all derived from the division of a single cell. In the case of PSCs, colonies have well-defined edges containing cells ideally with a high nucleus–cytoplasm ratio and prominent nucleoli.

Colony morphology The phenotypic analysis of the shape and structure of a colony.

Compacted This refers to the morphology of the cells within a colony. When the large cells originally seen in the developing colony condense down in size as the cells proliferate to form densely packed cells.

Confluent When adherent cells in culture touch each other and there are no spaces seen in the culture vessel. In this state, normal cells will tend to exhibit contact inhibition and will stop proliferating.

Cryopreservation A low-temperature cryoprotective process preserving the viability of cells.

Culture adaptation A physical change in the cell culture process requiring the cells to adjust from one culture condition to another. This is exemplified in the process of taking cells from a feeder-based system to a feeder-free system that may result in changes in morphology, rate of cell proliferation, and/or rate of spontaneous differentiation.

Differentiation The process that gives rise to specialised cells through the lineage restriction of stem cells.

Enzymatic passaging The harvesting and propagation of PSCs using enzymes to produce either cell clumps or single cells that can be transferred to a new culture vessel and will give rise to colonies with a good quality cell morphology.

Fibroblast A cell type involved in shaping and maintaining the structure of the connective tissues. This cell type, in general, is the most prevalent cell type in connective tissues and has the capacity to produce extracellular matrix and collagen. Although these cells can display diverse morphologies, the typical morphology seen with the mouse and human fibroblasts in cell culture is that of spindle-shaped elongated cells. These cells tend to be large and flat with an oval nucleus contained in the cell body and have extended branched cell processes.

Flask A specially treated plastic culture vessel used to grow cells, with the growth area denoted by a number (e.g., T 75 is a flask with a growth area of 75 cm^2).

Freezing technique Cells are frozen or cryopreserved using different techniques and reagents, and this process in turn has an effect on their recovery from freezing, their survival, their level of spontaneous differentiation, and their ability to differentiate.

Genetic adaptation A change in the genetic integrity of a cell or population of cells in a cell culture, due to selective pressures in the cell culture, which may favour this

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change and result in a growth advantage of cells that harbour this genetic change.

Germ layers The three primary layers of cells called endoderm, mesoderm, and ectoderm that give rise to all the tissues in the body.

Good quality cell morphology This is described in the following paragraphs in context.

In the context of MEFs and HEFs A cell culture where the fibroblasts have adhered to the plasticware and have spread out to form a monolayer of actively dividing cells. Where the cells do not look long and spindly, are not detaching, do not look refractory, and are not in suspension. To maintain these cells in an actively dividing state, they should not become confluent.

In the context of inactivated mouse and human feeders A cell culture where the mitotically inactive fibroblasts have adhered to the plasticware and have spread out to form a monolayer of cells. Where the cells do not look long and spindly, are not detaching, do not look refractory, and are not in suspension. These cultures might be produced at different levels of confluence, which have been shown to be optimum for individual PSC lines.

In the context of hESC and iPSC A cell culture that comprises colonies or a monolayer of homogeneous cells with a high nucleus–cytoplasm ratio and prominent nucleoli. The cells in colonies are compact. To maintain good cell morphology in a colony or in a monolayer, cells should not be allowed to over-compact and start to form multilayers, since this will promote spontaneous differentiation.

Human embryonic feeder layer Human feeder cells are produced by the inactivation of proliferating HEFs. In general, this inactivation is achieved using either mitomycin C or by irradiation.

Human embryonic fibroblasts (HEFs) Fibroblasts derived from primary human tissue, often neonatal foreskin.

Human embryonic stem cell (hESC) A cell with the potential to form cells representative of lineages from all three germ layers except those related to extraembryonic tissues. These cells are derived from the inner cell mass of a human embryo.

Human-induced pluripotent stem cell (hiPSC) Induced pluripotent stem cells derived from human origin.

Homogeneous morphology The morphology of all cells is identical.

Immunocytochemistry A technique utilising antibodies to identify antigens on specific biomolecules either within a cell or on the surface of a cell. This technique is known as immunofluorescence when the antigens recognised by the antibodies are detected using fluorescently labelled antibodies.

Inactivation of fibroblasts Treatment of actively proliferating fibroblasts to produce cells that no longer proliferate.

Induced pluripotent stem cell (iPSC) Cells produced by the reprogramming of adult cells, using factors that confer properties on these cells that make them embryonic stem cell-like.

IVF dish A tissue culture format specially manufactured to promote the in vitro culture of the ova and embryos.

Matrigel™ A substrate composed of a mixture of extracellular matrix proteins derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells. Used in cell culture to support the growth of cells. In PSC, it is used to maintain the cells in an undifferentiated state.

Monolayer In the case of PSCs, this is a single layer of cells growing on a substrate in a tissue culture vessel, generated through the process of enzymatic passaging. The cell culture no longer consists of discrete colonies. However, on occasion, colonies can be discerned that have fused together to form a monolayer. It can be more difficult to spot areas of spontaneous differentiation in this type of cell culture system.

Manual/mechanical passaging (including scraping and cutting) The harvesting and propagation of colonies of cells usually performed under a dissecting microscope where areas of spontaneous differentiation are removed prior to the manual cutting of cell colonies into pieces that when transferred into a new culture vessel will give rise to colonies with a good quality cell morphology.

Matrix Biological material derived from cells or synthesised, on which cells are grown in culture.

Mouse embryonic fibroblasts (MEFs) Fibroblasts derived usually from day 13.5 mouse embryos.

Mouse embryonic fibroblast feeder layer Mouse feeder cells are produced by the inactivation of proliferating MEFs. In general, this inactivation is achieved using either mitomycin C or by irradiation.

Mycoplasma A bacterium that is a member of the phylum Mollicutes.

Neural cell A cell that is committed to the neural lineage, derived from the ectodermal germ layer.

Neural rosette A classic morphology seen in the differentiation of iPSC commitment to the neuroepithelial lineage. Morphologically in culture, they display a radial structure comprised of columnar cells. These cells can go on to produce neurons, astrocytes, and oligodendrocytes.

Nucleoli These are the organelles located in the nucleus where ribosomes are produced.

Optimal feeder density A density of feeder cells that supports the growth and expansion of undifferentiated PSCs.

Over-confluent When confluent cells have been maintained in a confluent state over a number of days.

Overcompacted This refers to the morphology of the cells within a colony where the cells transition from a being monolayer of cells to a becoming a multilayer of cells.

Passaging The sequential process of transferring cells from one cell culture vessel to another. Each time a cell line is passaged, the passage number assigned increases by a value of 1.

Plating The seeding of cells in tissue culture media in a tissue culture vessel. This is often done at a defined number of cells per volume to achieve a certain density of cells. A single layer of cells grown on a suitable surface or substrate for optimal culture and/or co-culture characteristics.

Pluripotent A cell capable of developing into cells of all lineages but unable to develop into cell lineages derived from extraembryonic tissues.

Pluripotent stem cell (PSC) A cell with the potential to form cells representative of lineages from all three germ layers except those related to extraembryonic tissues. Pluripotency can only truly be ascertained via demonstration that the cells can form functional cells of all lineages. In truth, a demonstration of pluripotent potential via spontaneous differentiation or directed differentiation is the norm, and this is through assessment of morphology, gene expression, protein expression, and functionality.

Post-passaging Relating to time following the passage of the cells.

Primary culture A culture of cells produced by isolating and directly growing cells derived from tissue isolated from the body. These cells, if normal, tend to have a limited lifespan. They are not cell lines but may become cell lines either spontaneously (i.e., through culture adaptation) or by immortalisation (i.e., using an agent such as SV40) or by the use of transcription factors to produce iPSCs.

Senescence Deterioration of a cell in culture usually linked to cellular ageing or extrinsic factors.

Six-well plate A common tissue culture plate format, which is usually individually wrapped and sterile, consisting of six wells, each with an identical area on a single plate.

Spontaneous differentiation Commitment to a programme of differentiation without the addition of extrinsic germ layer or lineage-specific factors such as growth factors or small molecules. Manifest by a change in the morphology of a colony, part of a colony, or cell.

Sterility Absence of microorganisms other than the PSCs themselves.

Subculture The propagation of a cell culture through the harvesting and plating of the cells at a split ratio that will provide and maintain an optimal cell culture.

Suboptimal feeder density A density of feeder cells that does not support the growth and expansion of undifferentiated PSCs.

Tissue culture plasticware Plasticware that is treated in different ways to facilitate and promote the growth of cells in vitro. This treated plasticware is usually rendered sterile by processes such as gamma-irradiation.

Undifferentiated A cell that has the capacity to self-renew and maintain a genotype and a phenotype that are not committed to a germ layer or lineage. The morphology of this cell displays a high nucleus–cytoplasm ratio with prominent nucleoli.

Cell Culture and Stem Cell Glossaries

British Standards Institution. PAS 84:2012. Cell Therapy and Regenerative Medicine—Glossary. Available at: http://shop.bsigroup.com/Browse-by-Sector/Healthcare/PAS-84

Harvard Stem Cell Institute glossary. Available at http://dev.www.hsci.harvard.edu/glossary

International Society of Stem Cell Research: Glossary of Stem Cell-related Terms. Available at http://www.isscr.org/visitor-types/public/stem-cell-glossary

NIH Stem Cell Information: Glossary. Available at http://stemcells.nih.gov/info/pages/glossary.aspx

Sigma-Aldrich: Induced Pluripotent Stem Cell Glossary and Terms. Available at http://www.sigmaaldrich.com/life-science/stem-cell-biology/ipsc/glossary.html

Worthington Tissue Culture Glossary. Available at http://www.worthington-biochem.com/tissuedissociation/glossary.html

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