Chapter 2

Embryonic Stem Cells: Keeping Track of the Pluripotent Status

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Additional information is available at the end of the chapter

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Abstract

Embryonic stem cells are defined by their pluripotent status, which allows them to differentiate toward all cell types of an adult organism. This pluripotency can be characterized through many parameters, ranging from morphological traits, over certain enzymatic activities, to the expression of specific pluripotency factors, taken into account that these parameters may vary depending on the pluripotent stem cell type. As such, considerable differences are seen between human and mouse embryonic stem cell (ESC), or more generally stated, between primed and naïve pluripotent stem cells. This chapter offers an overview of the markers involved and the molecular biology techniques to monitor them during both ESC culture maintenance or differentiation experiments.

Keywords: Embryonic stem cells, Pluripotency, Pluripotency assessment, Noninvasive monitoring, RT-qPCR

1. Introduction

The hallmarks of embryonic stem cells (ESC) are their potential for self-renewal and their pluripotent status. The latter ensures that they can differentiate toward the three germ layers (endoderm, mesoderm, and ectoderm) and primordial germ cells, and eventually to all different cell types of an adult organism.

The first mouse ESC (mESC) were derived in 1981, independently by two different research groups [1, 2]. The first human ESC (hESC) in culture followed almost 20 years later [3]. Up to date, also for the derivatization of ESC from other species considerable research efforts have

been made [4]. As ESC are being derived from the inner cell mass of a blastocyst, they provide a good model to study fundamental processes in early development and cellular differentiation.

Equally important is that they also served as a template for the generation of induced pluripotent stem cells (iPSC) and contribute to a better (clinical) application of these cells in the future. As such, also in the field of induced pluripotency, tremendous progress has been made over the last decade. In 2006 and 2007, respectively, the research group led by Shinya Yamanaka developed a reprogramming cocktail for the establishment of mouse and human iPSC [5, 6].

Nevertheless, as the comparison of the different ESC and iPSC types teaches us, pluripotency comes in different intensities, instead of a single definition as was firstly assumed [7]. mESC are considered as 'true' pluripotent ESC and find themselves in a ground pluripotent state, as does the early preimplantation epiblast. hESC on the other hand, are primed pluripotent cells and resemble more to mouse epiblast stem cells (mEpiSC), which are derived from a postimplantation epiblast, in terms of culture requirements and molecular profile among other factors.

This chapter will give an overview on those different types of pluripotency and their according characteristics, and will then further elaborate on how these features can be monitored in order to keep track of the pluripotent status of ESC in culture.

2. Primed versus naïve pluripotency

hESC are considered to be primed, in that they are already predestined for one lineage or another, despite their remaining broad differentiation potential. In contrast, mESC are termed as 'naïve' pluripotent cells, while mEpiSC form the primed counterpart. It is clear that these different types possess different characteristics, which can be monitored via a range of different features and techniques, as is outlined below in more detail.

2.1. Pluripotent ESC morphology

A first distinctive feature is the difference in morphology. Naïve mESC form dome-shaped colonies, as do miPSC. However, primed hESC show a more flat morphology, with round individual cells having a high nucleus-to-cytoplasm ratio, as such resembling mEpiSC. These distinct appearances thus illustrate the different developmental states of the different cell types. The conversion of hiPSC to their naïve form changes the morphology from flat to domed [8]. It is remarkable how well these iPSCs resemble ESC in terms of morphology, even on an ultrastructural level [9, 10]. The latter reference describes the comparison of mESC, mouse embryonic fibroblasts (MEF), and iPSC derived thereof: after reprogramming, the accompanying morphological changes that the iPSC undergo make them virtually indistinguishable from ESC. Nevertheless, within the population of morphologically similar miPSC colonies, there still appears to be considerable variation in terms of molecular pluripotency, in contrast

to hiPSC, which show a much higher homogeneity among the colonies that are selected on analogous morphology [11]. Moreover, true hiPSC colonies have a typical hESC morphology that is very distinct from non-hiPSC colonies, hence morphology can very well serve as a criterion for identifying the real hiPSC colonies.

It is important to monitor the colonies' morphology in culture, as this will quickly visualize potential spontaneous differentiation. E.g. for hESC, if two or more colonies come into contact with one another, differentiation sets in at the contact area, and cells will start to pile up and acquire a more lengthened shape. Also in the center of a colony, cells might start to accumulate in multiple layers. As hESCs typically form flat colonies, timely passaging (at least on weekly basis, dependent on the culture system) is crucial to prevent overgrowing colonies and consequential spontaneous differentiation [12].

2.2. Pluripotency on a molecular level

2.2.1. Signalling pathways

The pluripotent state is regulated and maintained via several signaling pathways. As such, four major pathways involved in pluripotency can be distinguished for hESC: (1) the transforming growth factor β (TGFβ)-Activin-Nodal pathway, (2) the phosphatidylinositol 3-kinase (PI3K) pathway, (3) the Ras-Raf-mitogen activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK) (or MAPK/ERK) pathway, and (4) Wnt signaling [13–16].

TGFβ activates the TGFβ-Activin-Nodal pathway through the signal transducer SMAD2/3. The latter forms a complex with Smad4 and then translocates to the nucleus to trigger the expression of *NODAL* among other factors, which in turn stimulates self-renewal and inhibits differentiation. Also, the addition of activin A to hESC culture enables the activation of this pathway [14].

Addition of fibroblast growth factor (FGF; basic FGF; bFGF) on the other hand activates the PI3K pathway and the Ras-Raf-MEK-ERK pathway. In short, for the PI3K pathway, phosphatidylinositol 4,5-bisphosphate becomes phosphorylated by means of PI3K during activation. The resulting phosphatidylinositol 3,4,5-triphosphate subsequently binds with Akt (also known as protein kinase B). Activation of this pathway results in increased concentrations of Oct3/4, Nanog and Sox2 and thus in the maintenance of pluripotency [16, 17]. Additionally, activation of the Ras-Raf-MEK-ERK pathway leads to the activation of Ras (a GTPase), which in turn binds with Raf. This kinase phosphorylates another kinase, MEK, which then phosphorylates ERK (or MAPK). The latter translocates to the nucleus, leading to the phosphorylation of c-Myc, c-Jun, and c-Fos, factors involved in stem cell renewal [18, 19].

The Wnt signaling pathway can be activated by several Wnt ligands and is stimulated in culture e.g. by by 6-brominedirubin-3′-oxime. This agent inhibits glycogensynthase kinase-3 β which normally promotes the degradation of β-catenin in a complex with axin and adenomatous polyposis coli protein, by making it a target for the proteasome [19, 20]. As a consequence of this inhibition, β-catenin accumulates in the cytoplasm and a portion of this pool translocates to the cell nucleus and interacts with genes important for keeping hESC undifferentiated [15].

Importantly, reasonable differences exist between mESC and hESC in terms of signaling pathways. In hESC, endogenous BMP signals (interacting with the TGFβ-Activin-Nodal pathway) need to be suppressed [21], while on the contrary this pathway in mESC helps in maintaining the pluripotent state and BMP-4 can be added to their culture medium. Analogously, leukemia inhibitory factor (LIF) is added to mESC media, which promotes self-renewal by influencing Jak/Stat3 signaling, one of the downstream FGF/ERK pathways [13, 21]. hESC on the other hand, are not dependent on LIF supplementation.

2.2.2. Transcription factors

As was already pointed out in the previous paragraph, the transcription factors Oct3/4, Nanog, and Sox2 are part of the core pluripotency network. Oct3/4 (Octamer4 or Oct4, encoded by *POU5F1*) has been termed the gatekeeper at the start of mammalian development. This member of the Pit-Oct-Unc (POU) transcription factor family can activate its target genes' expression through binding on an octameric sequence motif (consensus sequence AGT-CAAAT) [22]. Although not being totally exclusive for ESC, it is nevertheless considered as one of the most important features to define a pluripotent cell state. In early embryos, loss of *POU5F1* expression causes the cells that were predisposed to form the ICM, to differentiate toward trophectoderm cells. Also, in ESC, lowering Oct3/4 levels to ≤50% or an increased expression above 150% leads to differentiation to trophectoderm or primitive endoderm cells, respectively [13]. Oct3/4 also appears to support the maintenance of mammalian germ cells, as apoptosis is induced when its expression is abrogated. Sox2 (*SOX2*) is a member of the SRY-related high-mobility group box-containing family and cooperatively functions with Oct3/4. However, its role goes further than just being a synergistic factor. Sox2-null embryos fail to give rise to ESC, but differentiate primarily to trophectoderm instead. Deletion of *SOX2* in hESC and mESC leads to differentiation, confirming Sox2 to be essential in itself to maintain the pluripotent state [23]. Nanog (*NANOG*) is another crucial pluripotency- and self-renewal-maintaining factor in both mESC and hESC. It was for instance shown that deletion of *NANOG* resulted in loss of pluripotency and induced differentiation, both in mouse ICM and ESC [24]. Its ability to maintain pluripotency is found to be independent of LIF supplementation to the cell culture. One of the possible mechanisms for self-renewal maintenance may be that Nanog represses the transcription of differentiation-promoting genes such as *GATA4* and *GATA6* [13, 24, 25].

Oct3/4, Nanog and Sox2 are involved in an intensive autoregulatory loop, as was for instance shown by the fact that knockdown of Sox2 led to a reduced expression of Oct3/4 and Nanog [23]. Additionally, they share a whole number of target genes, both in an activating or inhibitory manner. They stimulate genes involved in chromatin remodeling, histone post-translational modifications, TGFβ signaling, etc., and other ESC transcription factors, among which also themselves. They are able to inhibit the expression of many genes promoting ectoderm, mesoderm, or endoderm differentiation [26]. It was found recently that among other factors, the hypoxia inducible factor 2-α (HIF 2-α) is an upstream regulator of these key transcription factors, as it binds directly to predicted hypoxic response elements in the proximal promotor of *POU5F1*, *NANOG*, and *SOX2* [27].

This list of core pluripotency factors can be completed with Klf4, a member of the Krupple-like Factor family. Together with other members of its family, it helps in maintaining ESC self-renewal and regulating the expression of several other genes such as *NANOG*. It is also one of the components included for iPSC generation starting from human or mouse cells, in combination with *POU5F1*, *SOX2*, and *c-MYC*. Alternatively, a combination of *POU5F1*, *NANOG*, *LIN28*, and *SOX2* is applied for reprogramming.

Besides the aforementioned factors, there is a whole series of other transcription factors that are involved in pluripotency regulation and maintenance. For instance, for hESC, the expression of *REX1*, *FOXD3*, *GDF3*, *GABRB3*, *EBAF*, *POXDL*, *NODAL*, *ZFP42*, *LIN28*, *TCF3*, *EOMES*, and *SFPR2* is highly correlated to the expression of *NANOG* or are potential Nanog targets, as was elaborately described in [28]. Of note, there was also a number of genes found to be highly negatively correlated with *NANOG* expression, including *CDX2* and *CGB* (associated with trophectoderm differentiation), *GATA6* and *AFP* (extraembryonic endoderm), and *PAX6* and *NEUROD1* (neural lineage), once more confirming the role of Nanog in pluripotency regulation [28]. In mESC, the sustaining of the pluripotent status relies both on similar (e.g. *NANOG*, *POU5F1*, *SOX2*, *REX1*, and *FOXD3*) and different (e.g. *PECAM1* and *STELLA*) factors.

Yeo et al. [29] proposed the monitoring of DNA methylation patterns as a possible means to study the extent of hESC differentiation. DNA methylation is mostly associated with transcriptional repression, whether established via chromatin remodeling complexes or by direct blocking of transcription factor binding, and is as such involved in cellular programming events [30, 31]. During the hESC differentiation process toward embryoid bodies, the promotor regions of *POU5F1* and *NANOG* undergo substantial methylation, in contrast to those of *SOX2*, *REX1*, and *FOXD3* [29].

2.2.3. Pluripotency on RNA level

Besides the well-described transcription factors and cell surface markers, also long noncoding RNAs (lncRNAs) take up their role in pluripotency maintenance [32]. lncRNA molecules are transcripts from RNA polymerase II, that are over 200 nucleotides in size and that do not serve as a template for protein production. Instead, they prove to be involved in processes such as mRNA stability and translation modulation, and related epigenetic regulatory processes [33]. Both for mESC and hESC a characteristic set of lncRNAs has yet been identified, of which some are under the direct control of the core pluripotency transcription factor network [32]. The authors of the latter publication provide an elaborate overview of the different lncRNAs involved in the maintenance of ESC self-renewal and the preventing of differentiation. The interaction of lncRNAs with histone modifiers and other chromatin-associated proteins has been described in ESC, and they might serve as a scaffold to connect different chromatin modifying complexes.

2.2.4. Lamins

Also the expression of certain lamin proteins may serve as a marker for ESC differentiation [34]. Nuclear lamins are intermediate filament proteins within the nuclear lamina, that not only fulfill their role in the structural organization and support to the nuclear envelope but also participate in processes such as DNA replication and transcription. This family of proteins can be divided into 2 subgroups: lamins of the B-type (B1, B2, and B3) and of the A-type (A, AΔ10, C, and C2). Lamins B1 and B2 are expressed in both embryonic and adult cells, while A-type lamins are primarily found in differentiated cells, as was described for mouse and human cells. Nevertheless, when neuronal differentiation was induced in hESC, the expression of lamins A/C increased, as was also the case when differentiating toward cardiomyocytes. The authors hypothesize that the mechanism of action can be two-fold. A/C lamins might keep the differentiated state by directly influencing the nuclear structure and making it more rigid and thus less prone to chromatin remodeling, a process occurring during cellular differentiation. Alternatively or in complement, these lamins might also indirectly lock a specific gene expression pattern, by affecting the expression of other genes, as their interactions with several transcription factors have yet been described. In those hESC differentiation experiments, the authors could not point out a direct link with the expression of Oct3/4, as the expression of both markers overlapped in most cells, both in a high or low expression level or as one marker being more abundant than the other. The fact that A-type lamins are already present before total Oct3/4 decrease, makes these lamins a good marker for the indication of early differentiation. Of note, there was no overlapping expression found with TRA-1-60, TRA-1-81 or SSEA-4. For mESC, it has been described that they do show very low but yet detectable levels of Lamin A/C even when pluripotent, albeit in a much lower pattern than for differentiated cells [35].

2.2.5. Cell surface markers

Surface antigens are a valuable tool not only for the monitoring of the (non-)differentiation of stem cells but also for the isolation of a subset of cells. The function of most of these markers is not yet fully elucidated, a search that is further hampered because of their different expression patterns in different pluripotent cell types. Some antigens are associated with carbohydrate epitopes, linked with glycolipids (e.g. SSEA-3) or with glycoproteins (e.g. TRA-1-60), and it has been hypothesized that the core structures of these antigens are essential for the cellular function. For instance, the Lewis-X carbohydrate structure recognized as SSEA-1 may be important for compaction at the morula stage during mouse embryonic development [36].

One of the most well-known types of ESC surface markers is the class of stage-specific embryonic antigens (SSEA), which are of the glycosphingolipid-type. Undifferentiated hESC express SSEA-3 and -4 (globoseries structure), while SSEA-1 (lactoseries structure) is only expressed in low levels [36, 37]. Upon hESC differentiation, the expression of these markers is quickly downregulated, with SSEA-3 disappearing faster than SSEA-4. On top of that, there is a significant increase in SSEA-1 [38]. Contrastingly, the reversed pattern is seen in pluripotent mESC [36, 39]. Also another SSEA-molecule, namely, SSEA-5, has been identified on the

surface of hESC, which undergoes an even larger reduction upon differentiation than SSEA-3 or -4 [40]. Their expression depends on the combined actions of the different enzymes that are involved in their synthesis. Also the modified expression profile upon differentiation is primarily directed by an altered expression of the key glycosyltransferases (GT), with an upregulation of ganglio-related GT and simultaneous downregulation of globo- and lacto-series-related GT [41]. Of note, this list of glycosphingolipids is far from complete; hESC additionally express several other, less well-known markers of this type, of which the expression rapidly diminishes upon differentiation [38]. Although the SSEA have been challenged not to be essential for pluripotency maintenance [42], but to fulfill a role in cellular differentiation instead, their presence is nevertheless still considered as one of the criteria that must be fulfilled to categorize a cell as pluripotent.

TRA-1-60 and TRA-1-81 are surface carbohydrate antigens present on hESC [36, 37], but not in mESC [36, 39]. Retinoic acid-induced hESC differentiation significantly downregulates their expression [37]. They have been shown to interact with keratan sulphated proteoglycans, although the exact structural determinants of their epitopes remain unknown [43]. Their expression is related to that of podocalyxin, a transmembrane glycoprotein, which has yet been found to be highly expressed in both ESC and iPSC, and might serve as a carrier for the TRA-antigens [44]. Cell surface glycans on ESC might play a role in the modulation of multiple signaling pathways [43].

Besides the more frequently indicated SSEA- and TRA-markers, also several other categories of surface molecules have been described. Cluster of differentiation (CD) antigens can be subdivided into several classes such as integrins, adhesion molecules, glycoproteins, and receptors [36]. Some of them such as CD9, CD24, and CD133 are associated with mESC and hESC [36]. Of note, CD133 is also expressed in other cell lines like embryonic carcinoma cells and hematopoietic stem cells [36]. Integrins are important for keeping ESC undifferentiated. These cell surface receptors can bind several extracellular matrix proteins such as fibronectin, vitronectin, collagen, and laminin and provide in this way the cell-matrix interaction [36, 45].

Next to the pluripotency cell surface markers, a range of markers for differentiation has been described. Depending on the differentiation protocol applied, specific markers can be investigated. On top of the markers already mentioned under paragraph 2.2.2, SSEA-1 and Gata4 for example indicate hESC endoderm differentiation, and the possibilities are still expanding. For instance, Holtzinger *et al*. recently described new markers for hepatocyte differentiation from hESC and hiPSC [46].

2.2.6. Alkaline phosphatase

Tissue-nonspecific alkaline phosphatase (TNAP or AP) is a membrane-bound glycosylated enzyme which is highly expressed in pluripotent ESC [3, 47], and is rapidly upregulated upon the reprogramming of somatic cells into iPSC. Of note, the expression of AP is absent in mEpiSC [48]. AP expression significantly decreases during differentiation, making it a suitable marker for pluripotency assessment. One way to monitor AP in hESC is detection with TRA-2-29 and TRA-2-54 antibodies [36, 49]. Its specific function is not totally elucidated, but its importance for ESC is ascribed to its role in the metabolism of vitamin B6 and thus also of

the neurotransmitter γ-aminobutyric acid (GABA), a process that is considered to be imperative for ESC proliferation and self-renewal regulation. Additionally, it is very likely that because of the high proliferative rate of ESC, their need for substrate dephosphorylation is considerably higher than in somatic cells [48]. The activity of AP is highly correlated with its expression, which is mainly driven by the actual microenvironment of the cell, instead of via specific signaling pathways. MAPK p38 might play a role in AP's expression regulation, as deletion of p38 in ESC led to a decreased AP expression and activity, but the precise mechanism remains unknown.

2.3. Other pluripotency features

A high level of telomerase activity is also one of the typical ESC characteristics [3]. Telomerase is involved in maintaining telomere length and is able to add telomere repeats to chromosome ends. As such it plays an important role in sustaining the replicative time-span and self-renewal of ESC. Somatic cells do not display any telomerase activity, leading to shortened telomeres over time and entering senescence after a certain number of cell divisions. Even in adult stem cells only low levels of telomerase activity can be found, in contrast to ESC [50].

Pluripotent hESC also display an abbreviated cell cycle of about 16 h, in comparison to differentiated cells, due to a shortened G1 phase [51, 52]. Similarly, also mESC proliferate quickly and have a lengthening cell cycle upon differentiation. Because of their high proliferative pace, both mESC and hESC cultures need to be passaged very frequently, although the passaging technique is different between those two culture types: naïve cells are way more tolerant for single cell passaging than primed cells and thus allow better for bulk culture.

Besides confirming the presence or absence of certain characteristic traits, the most stringent way to show pluripotent potential is chimera formation. However, as hereby ESC are injected into a developing embryo, this is for obvious ethical reasons not possible for hESC. Additionally, pluripotency can be investigated through spontaneous ESC differentiation *in vivo* (teratoma formation) and/or *in vitro* (embryoid body formation). Alternatively, also the directed differentiation under stimulation by specific growth factors, small molecules, gene manipulation, etc. toward specific cell types can be assessed.

3. Pluripotency monitoring

3.1. Overview

The pluripotent state and cellular differentiation can be followed by means of multiple techniques, depending on the cell lines used, the experimental set-up, etc. As a start, microscopy is a very important asset in this monitoring process. Cell cultures' performance, colony, and individual cell morphology (whether or not during differentiation) can be checked by means of light microscopy. With the implementation of additional stainings with fluorescently labelled antibodies and nuclear stains (e.g. DAPI), a whole range of other parameters like nuclear morphology, the presence of core transcription factors, cell surface molecules, and

intracellular markers can be assessed with fluorescence microscopy. Often specific kits are available for a defined marker panel for pluripotency assessment of both ESC and iPSC lines, such as the Fluorescent hESC/hiPSC Characterization Kit provided by Millipore [53]. A noninvasive method for daily check-up of hESC cultures with microscopy is further described in Section 3.2. As an alternative, flow cytometry analysis after immunostaining can be applied for monitoring those markers. This technique is also applied for the analysis of cell proliferation rates and the distribution of a cell population across the different cell cycle phases.

A relatively quick way to obtain gene expression data of the described pluripotency factors, differentiation markers, lncRNAs, lamin proteins, etc., is the application of reverse transcription-quantitative PCR (RT-qPCR); one such protocol for relative quantification is described under paragraph 3.3. Larger transcriptomics experiments can be set up by means of microarray analysis and next-generation sequencing. To detect these markers' expression on a protein level, also multiple techniques are available such mass spectrometry, or 1D or 2D gel electrophoresis combined with Western blotting.

Enzymatic activities of e.g. alkaline phosphatase and telomerase can be determined with specific kits available for cells of both human and murine origin. Telomerase activity is most often checked by means of a TRAP assay, or telomeric repeat amplification protocol, in which the telomerase activity is (semi-)quantified with qPCR after an initial enzymatic incubation step. Also ELISA-based methods have been described, whether or not in combination with TRAP (elaborately reviewed in [54]). AP activity detection is often based on colorimetric assays, e.g. performed on the supernatant of cell cultures. Even live stains are available, that allow noninvasive monitoring with microscopy. Hereby a non-fluorescent substrate is added to the cell culture, that becomes fluorescent after dephosphorylation by AP. Such a cell-permeable and non-toxic fluorescent substrate does not accumulate in the cells but diffuses out of the cells after 2 h [49], and thus does not yield any problems for following analyses.

3.2. Noninvasive monitoring

3.2.1. Background

As described above, immunostaining and RT-qPCR are most often used methods for pluripotency and differentiation screening. Nevertheless, for those methods, ESC need to be harvested, which thus makes it impossible to monitor the same colony over and over again on a daily basis, as new samples have to be collected for each analysis. We present a method enabling pluripotency monitoring of the same specific colonies during a time-lapse experiment by using a reporter ESC line, as was published by Scheerlinck *et al.* [55].

3.2.2. Experimental aspects and workflow

A commercially available Oct3/4-eGFP Knock-In hESC line was used, in which the transcription of enhanced green fluorescent protein (eGFP) is coupled to the transcription of *POU5F1* [56]. After eGFP translation, a fluorescent signal (ex. 489 nm, em. 511 nm) is detected, which

can be measured using a flow cytometer (FC) or fluorescence microscope (FM) [57]. When using the latter, not only the pluripotency can be measured but also the morphology can be examined. FC can then be used to validate the FM results obtained and was thus used as a reference. However, FC was only used at the end of the experiments, because it requires cell harvesting and fresh samples, making the monitoring of the same specific colonies on a daily basis impossible.

This method can be applied both for feeder and feeder-free hESC culture conditions. Hence, a comparison was made between hESC cultured on MEF with regular DMEM/F-12 medium (with 4 ng/mL bFGF) on one hand and hESC on vitronectin-coated plates in combination with Essential 8 medium on the other. Both culture conditions were kept undifferentiated as for a regular ESC culture. Simultaneously, a differentiation experiment was set up, where differentiation was induced in case of MEF culture by omitting bFGF from the culture medium (spontaneous differentiation), whether or not combined with the addition of 2 μM retinoic acid (forced ectoderm differentiation). For feeder-free culture, only the latter condition with retinoic acid was applied. As such, there were in total three MEF conditions and two feeder-free conditions analyzed. The resolving power of the FM to compare differentiation status differences was determined by the comparison of those five conditions, of which only the ones containing bFGF are assumed to keep the hESC pluripotent. The resolving power of FC was determined by a complete 15-day differentiation of the Oct3/4 reporter cell line: the fluorescent signal quickly decreased during the first week and completely disappeared after 15 days in culture, falling back to the same level as UGENT2-cells, a non-reporter hESC line.

Importantly, possible auto-fluorescence of both the medium and the cells themselves needs to be investigated. Several amino acids (tryptophan, tyrosine, and phenylalanine) and vitamins (riboflavin) among other factors, all present in DMEM/F-12, are known to cause auto-fluorescence [58–60]. Subsequently, also non-reporter hESC (in our case the UGENT2 cell line) and MEF in case of feeder cultures need to be analyzed. When using FM, the signal-to-noise (S/N) ratio for each hESC colony can be determined by dividing the densitrometric mean of the colony (signal) by that one of its background (noise). For FC analysis of feeder cultures, it was not possible to distinguish the MEF population from the hESC in terms of FS/SS. Ideally, these MEF should be isolated by means of fluorescence-activated cell sorting after immunostaining for a specific marker such as vimentin or CD90 [61]. However, as FC is only used at the end of the experiment only a small contribution of the MEF (<10%) to the fluorescence histogram is expected, as the relative portion of the inactivated MEF gradually reduces over time compared to the growing hESC colonies.

3.2.3. Outcome

As stated above, the UGENT2 cell line and MEF were included in the analysis to determine the background auto-fluorescence. For FM, the MEF did not yield a detectable auto-fluorescence signal and did thus not impact the S/N ratio in comparison to feeder-free cultured hESC, although FC analysis revealed a low but present MEF auto-fluorescence intensity (**Figure 1A**).

	Experiment 1		Experiment 2		Experiment 3	
Rank	*Ref.*	*M value*	*Ref.*	*M value*	*Ref.*	*M value*
1	B2M	0,277	B2M	0,203	B2M	0,378
2	AluSq	0,284	AluSq	0,213	RPL13A	0,378
3	RPL13A	0,290	RPL13A	0,221	AluSq	0,386
4	HPRT1	0,472	GAPDH	0,267	PPIA	0,480
5	YWHAZ	0,593	SDHA	0,360	GAPDH	0,609
6	TBP	0,696	TBP	0,409	HPRT1	0,672
7	HMBS	0,886	YWHAZ	0,451	TBP	0,813
8	ACTB	1,151	ACTB	0,480	YWHAZ	0,903
9	GAPDH	1,350	HPRT1	0,506	HMBS	1,088
10	PPIA	1,476	PPIA	0,524	SDHA	1,216
11	SDHA	1,598	HMBS	0,550	ACTB	1,347
12	UBC	1,697	UBC	0,579	UBC	1,484

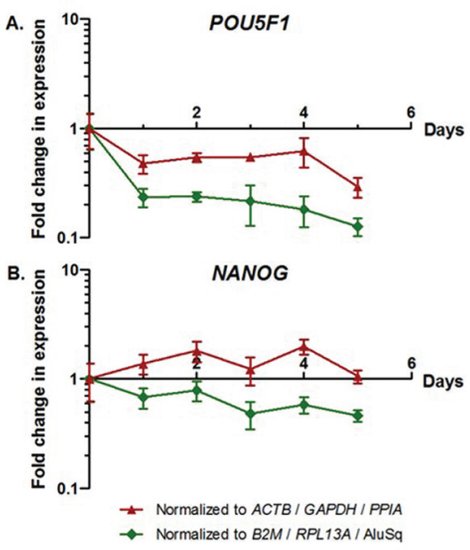

Figure 1. Noninvasive monitoring of hESC colonies. (A) Auto-fluorescence assessment by FC. (B) Results obtained by FM, expressed as S/N ratios. The differentiating conditions clearly show a decreased S/N ratio in comparison to the cells kept pluripotent. (C) FC results obtained at the end of the differentiation experiment, confirming the FM results. (D) Microscopic fluorescent images of a hESC colony in MEF culture conditions, under spontaneous (left) and retinoic acid-induced (right) differentiation.

For FM assessment, colony fluorescence was measured daily for 5 or 6 colonies per condition, during 6 days. The experiment was performed in triplicate and the obtained results are expressed as S/N ratios. As expected, addition of retinoic acid (directed differentiation) caused a significant decrease in S/N ratio over time both in feeder and feeder-free culture conditions, indicating a lowering expression of Oct3/4 in the course of the differentiation process. Also only omitting bFGF from the culture medium definitely leads to differentiation, as was confirmed by the decreasing S/N ratios, albeit in a slower rate than when also retinoic acid is added. No significant S/N ratio differences were found between feeder and feeder-free hESC cultures for both the non-differentiating and the differentiating conditions, confirming the low impact of MEF auto-fluorescence on FM measurements. Of note, for both undifferentiated culture conditions, an increase in fluorescence was seen toward the end of the experiment, which might be explained by an increase in eGFP/cell or more plausibly by the formation of multilayers (3D growth) resulting in an accumulation of fluorescent signal (**Figure 1B**).

The FM data were compared to the results of a FC hESC analysis (**Figure 1C**). As mentioned above, because of the destructive nature of this technique, this measurement is only performed at the end of the experiment. Both feeder-free and MEF grown hESC in the presence of bFGF retained their undifferentiated status. Of note, a small portion of cells in the latter population had a lower eGFP expression, most probably MEF as mentioned earlier (auto-fluorescence between 10^0 and 10^1, asterisk in **Figure 1C**). The finding that the eGFP signal/cell remained constant in the undifferentiated conditions indeed confirms that the daily increase in fluorescence as observed by FM is rather due to a multilayer effect resulting in an accumulated fluorescent signal. In the MEF condition without bFGF, most of the cells were still undifferentiated after 6-day culture (signal $>10^1$) but in comparison, in the condition with bFGF a

significantly higher number of cells with a 10^0 and 10^1 eGFP expression were observed. These results are in line with the FM measurements, in which it was shown that there is indeed a mix of differentiated (low fluorescence; S/N ratio = 2.26) and undifferentiated hESC (high fluorescence, S/N ratio = 13.75) on day 5 of spontaneous differentiation (**Figure 1D** left). The conditions with retinoic acid showed a clear drop in fluorescence toward the end of the experiment, as also confirmed by the FM results. Remarkably, FM images of retinoic acid differentiated hESC colonies on MEF revealed the existence of demarcated zones with highly accumulated fluorescence (S/N ratio = ca. 24) (**Figure 1D** right). This small population of high fluorescent 'islands' could not be discriminated using FC as these individual highly fluorescent cells were somewhat hidden in the tail of the fluorescence histogram.

In conclusion, it is important to bear in mind that for FM assessment, an increasing fluorescence intensity of a hESC colony does not correlate with an increased eGFP signal per cell, but with hESC growing in multiple layers. As such, only a decrease in signal can be directly interpreted as ongoing differentiation. A flat signal can be considered as an hESC culture with both pluripotent and differentiating cells [55].

Despite its usefulness, this method still has some limitations. One issue is the auto-fluorescence of the medium, necessitating the use of a different medium than that used for regular culture. In this experiment, hESC colonies were analyzed by using no medium at all, although this evokes stress to the cells. ThermoFisher has recently developed an auto-fluorescence-free medium called FluoroBrite DMEM, which can be used as basal medium for analysis during fluorescence [58]. It should nevertheless be further investigated whether this medium can also be used for hESC. Secondly, ideally the analysis should be done in the same conditions as during culture, meaning that a cell imaging system with regulated O_2 and CO_2 supply would be more appropriate. Different companies such as Zeiss could offer a solution in this regard. Nevertheless, even if a microscope as described in the experiments above is used, the fact that the cells are monitored in culture enables an immediate follow-up of their behavior.

3.2.4. Applications

The application of this method is definitely not restricted to an Oct3/4-eGFP ESC line but can be used for any reporter cell line, as long as potential auto-fluorescence is overcome, e.g. by the implementation of an auto-fluorescence-free cell culture medium. For instance, also Nanog-eGFP reporter hESC lines are available [62], as is an hRex-GFP hESC line [63]. Additionally, reporters for specific differentiation markers have been generated, with an increasing GFP expression during the course of the differentiation process, e.g. [64, 65]. The production of a Nanog-eGFP reporter to monitor fibroblast reprogramming has been published, too [66]. A reporter cell line including multiple markers clearly strengthens the use of the method described above. For example, Maass *et al.* created a reporter mESC line harboring Cntn2-eGFP and MHCα-mCherry, which will be upregulated during directed cardiac differentiation, as such allowing to monitor the development of cardiac progenitors [67]. However, to our knowledge, the development of a dual reporter ESC line combining two pluripotency markers has not been published. Furthermore, most reporter ESC lines described express (e)GFP, thus ruling out a possible combination with GFP-based alkaline phosphatase live stain [68].

However, ActivMotiv provides a CDy1 Dye (ex. 544 nm/em. 577 nm) to detect pluripotent stem cells live in culture, that can be combined with GFP expression [69, 70].

3.3. Quantitative PCR monitoring

3.3.1. Background

As mentioned above, one other efficient method to monitor the expression profile of multiple target genes (e.g. the core pluripotency transcription factors) is RT-qPCR. This technique allows to analyze samples in high throughput, at a relatively low cost. In this way, it can easily be applied for e.g. pluripotency monitoring of (long term) ESC cultures, as was recently elaborately described for hESC by Galán and Simón [71]. Besides culture monitoring, it can also be applied for the comparison of pluripotent and differentiating ESC. Importantly, as goes for all experimental work, also here an adequate set-up is of utmost importance. During RT-qPCR data analysis, a suitable normalization factor should be taken into account, to correct for potential technical variabilities that were included along the experimental process. To this end, multiple strategies have been used. Normalization to the number of cells might not be accurate and cell enumeration is particularly less easy when dealing with adherent cell cultures such as ESC [72]. Moreover, this normalization strategy does not take into account the variability that might have been included during sample preparation such as potentially insufficient enzymatic reaction efficiencies. Alternatively, correcting toward RNA mass quantity has been described, but also here the same issue applies, as potential technical variation from the complementary DNA (cDNA) preparation from messenger RNA (mRNA) is not considered. Ribosomal RNA (rRNA) makes up the major part of the total RNA pool and may be prone to regulation, which will cause a variable ratio between rRNA and mRNA. Hence, normalization for the total RNA content may not be representative for the amount of mRNA [72, 73]. The by far mostly favored method for data normalization is correcting to one or preferentially multiple reference or so-called housekeeping genes. This allows the researcher to correct for any variation between samples that might have been implemented along the protocol. These reference genes are expected to be expressed in a stable manner throughout all samples of a given experimental set-up.

3.3.2. Experimental aspects and workflow

To determine which references need to be included, different algorithms are available such as BestKeeper or Normfinder, but the most widely used is the geNorm algorithm, included in the qBase software (Biogazelle) [74]. We applied the latter to find suitable reference genes to be used for the comparison between pluripotent and differentiating hESC [75]. After putting in all RT-qPCR data, the software calculates a so-called stability value (designated 'M value') for each candidate, with a low M value indicating a higher gene expression stability throughout all samples. Afterwards, all reference candidates are ranked according to that value and the most adequate reference genes can be selected.

A differentiation experiment was set up, in which hESC samples from two different cell lines (UGENT1 and UGENT2) were collected daily during 8 days after retinoic acid-induction, plus

an extra sample on day 12 of differentiation (Experiment 1). This was repeated in two more experiments, albeit in a different time window: in Experiment 2 samples were collected daily during 6 days and in Experiment 3, cells were harvested every 4 h during day 3, 4, and 5 after onset of differentiation. After RNA isolation, quality control and cDNA preparation, RT-qPCR reactions were run in duplo, on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and a LightCycler 480 (Roche). Twelve reference loci candidates were included, mainly with SYBR Green detection (allowing melting curve analysis), except for *GAPDH* and *PPIA*, for which specific probes were used. All data were imported in geNorm and assessed for expression stability.

3.3.3. Outcome

According to the geNorm analysis performed for all three differentiation experiments, the most stable reference loci among the 12 included candidates were *B2M*, *RPL13A*, and Alu repeats. In contrast, the more classic reference genes such as *GAPDH* or *ACTB* did not perform so well (all M values listed in **Figure 2**) [75]. These findings were corroborated by the results obtained from the use of other algorithms for reference gene stability determination.

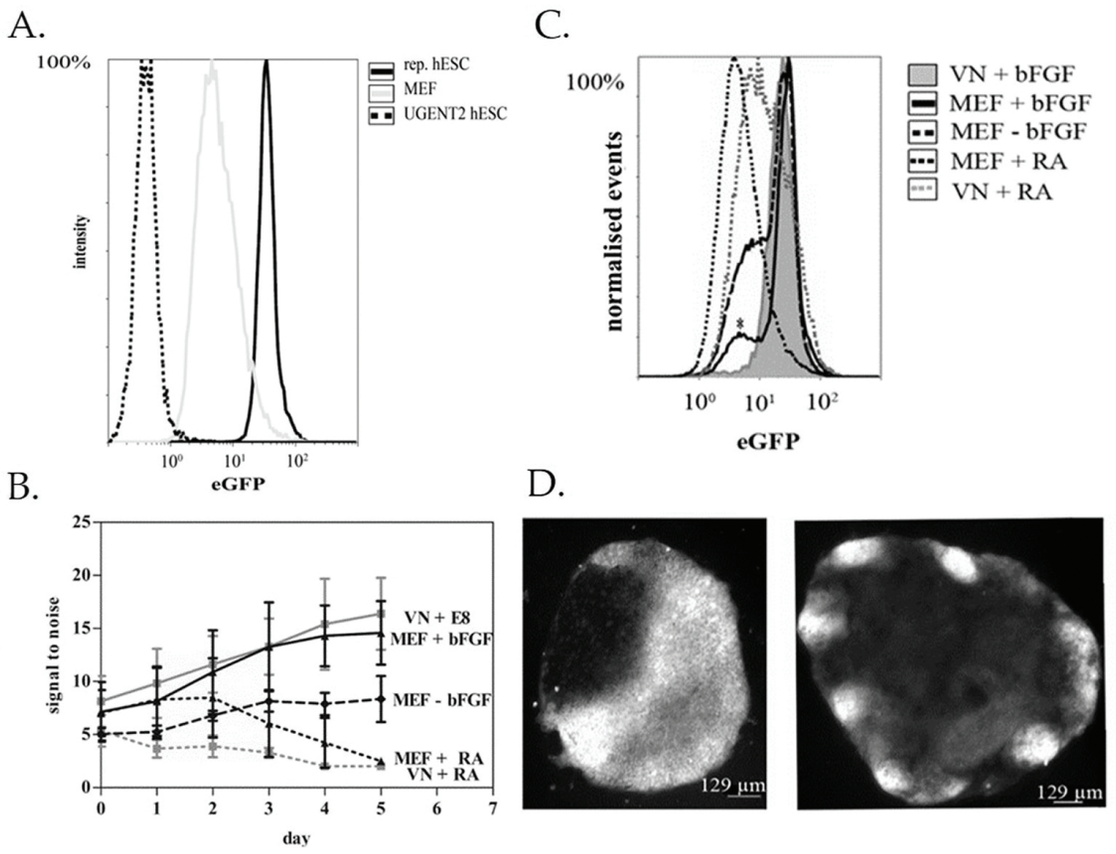

Figure 2. Reference genes selection and their application. A geNorm stability analysis was performed for 12 candidate reference genes; the table shows the resulting ranking. *B2M*, *RPL13A*, and Alu repeats appeared to be the most suitable references, performing significantly better than the more classic reference genes *GAPDH*, *ACTB*, and *PPIA* as is illustrated in the graph (fold change in expression over time during differentiation).

It is important to bear in mind that different experimental settings may require different references; e.g. one differentiation-inducing agent will not have the same influence on gene expression in general as another. Previously defined reference loci might thus not be blindly extrapolated to new experimental conditions. The difference in reference gene performance is illustrated by the comparison between to reference sets: *B2M*, *RPL13A*, and Alu repeats on one hand and *GAPDH*, *PPIA*, and *ACTB* on the other. These sets were used to normalize the expression of the pluripotency factors *POU5F1* and *NANOG*, which are supposed to decrease considerably after differentiation is induced. However, that decrease was significantly less pronounced (p-value = 1.30e-05) when applying the more classic reference genes, once more pointing out the importance of an adequate reference gene selection (graph **Figure 2**).

The fact that *B2M*, *RPL13A*, and Alu repeats are found to be the most stable genes is nevertheless not too surprising. *B2M* is expressed in every nucleated cell as it is a component of the major histocompatibility complex I. It is thus a very good candidate to apply as a normalization scalar for RT-qPCR analysis (e.g. [76]). Also *RPL13A*, involved in the protein translation process, has been widely used as a reference gene (e.g. [77]). The applicability of both genes was confirmed by our own data analysis. The Alu repeats are of particular interest, as their application provides a rather novel approach for data normalization [78]. These short interspersed elements are distributed genome-wide, which implies that a variation in the expression of a single gene will not substantially influence the total expression profile of these elements.

3.3.4. Applications

The setting described here specifies the monitoring of the pluripotency factors Oct3/4 and Nanog, but obviously any other marker of which the expression should be monitored and relatively quantified can be included. It is recommended to revaluate the reference genes' stability if an experimental set-up would be modified, but once the most suitable references have been established, any target gene can be implemented in this assay. As such, on top of the pluripotency markers, also the expression of specific differentiation markers can be monitored to follow development toward a certain cell lineage. This technique can as well be used for the assessment of noncoding RNA genes.

4. Conclusion

This chapter gives an overview on a number of typical characteristics which can be monitored to keep track of the pluripotent status of ESC cultures. Nevertheless, this list is far from complete and it is most likely that in time new biomarkers will be found while known features will be revaluated, as new culturing techniques are developed and the research field of induced pluripotency and naïve pluripotency further expands. Also from a technological point of view, novel methods will enhance these markers' detection and facilitate the discovery of new molecules.

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