

# A High Proliferation Rate is Critical for Reproducible and Standardized Embryoid Body Formation from Laminin-521-Based Human Pluripotent Stem Cell Cultures

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**Abstract** When aiming for homogenous embryoid body (EB) differentiation, the use of equal-sized EBs is required to avoid a size-induced differentiation bias. In this study we developed an efficient and standardized EB formation protocol for human pluripotent stem cells (hPSC) cultured in a laminin-521-based xeno-free system. As the cell proliferation rate of the cells growing on laminin-521 strongly affected the efficiency of aggregate formation, we found that recently passaged cells, as well as the addition of ROCK inhibitor, were essential for reproducible EB formation from hPSC single-cell suspensions. EBs could be obtained in a variety of differentiation media, in 96-well round-bottom plates and in hanging drops. Gene expression studies on differentially sized EBs from three individual human embryonic stem cell lines demonstrated that the medium used for differentiation influenced the differentiation outcome to a much greater extent than the number of cells used for the initial EB formation. Our findings give a new insight into factors that influence the EB formation and differentiation process. This optimized method allows us to easily manipulate EB formation and provide an excellent starting point for downstream EB-based differentiation protocols.

**Keywords** Human pluripotent stem cells · Laminin-521 · Embryoid body differentiation · Forced aggregation · Equal-sized embryoid bodies · Differentiation

## Introduction

Embryoid body (EB) formation is a standard approach to assess the differentiation potential of human pluripotent stem cells (hPSC) [1, 2], and is often a first step in direct differentiation protocols [3–5]. However, the size of individual EBs is highly dependent on the number of starting cells and this, in turn, has been reported to influence the differentiation into the three germ layers in general [6] and specifically into the hematopoietic [7] and cardiac lineage [8–10]. Thus, when comparing functional variability between individual hPSC lines, a standardized EB protocol yielding equal-sized EBs is required to avoid a size-induced differentiation bias.

Initially, human EBs were obtained from clumps of cells detached from a feeder layer and resuspended in a non-adherent Petri dish without disruption of cell-cell junctions [1]. This effective approach however generates EBs in which the size is difficult to control and varies between and within experiments [8, 11]. Equal-sized EBs can only be obtained from hPSC as a single-cell suspension, but hPSC as single cells undergo massive cell death [12, 13] due to the loss of E-cadherin mediated cell-cell contacts [14, 15]. This process can be blocked by Y-27632, a specific Rho-dependent protein kinase inhibitor (ROCKi) [16]. Efficient EB formation thus requires culture conditions that alleviate dissociation-induced cell death and stimulate single-cell aggregation.

Recently, there has been a significant shift from culturing hPSC on fibroblast feeders to clinically relevant, feeder-free and xeno-free culture conditions. One such system is the monolayer culture of hPSC on human recombinant laminin-

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521 (LN521), an extracellular matrix protein that effectively supports hPSC self-renewal [17, 18]. Human PSC on LN521 migrate to each other and form colonies without activation of dissociation-induced cell death, and therefore can be passaged as single cells without ROCKi addition. As the LN521-based hPSC culture differs significantly from other systems, and EB formation efficiency is highly dependent on the culture system used [8, 11, 19], we were not able to obtain single-cell based EBs from LN521-based cultures using previously published and commercially available protocols. Therefore, our aim was to develop an efficient protocol for equal-sized EB formation for this culture system and to uncover factors that influence this process.

## Materials and Methods

### Culture of hPSC

Human embryonic stem cell (hESC) lines VUB01, VUB02 and VUB06 were derived and characterized as previously described [20, 21]. All hESC lines are registered with the EU hPSC registry (<http://hpscereg.eu/>). Human induced pluripotent stem cell (hiPSC) lines VUBi002-D and VUBi004-D were derived from normal volunteer fibroblasts and characterized as described in the [Supplementary Methods](#). Human PSC were routinely cultured on dishes coated with 10 µg/ml LN521 (Biolamina) in NutriStem® hESC XF medium (NS medium; Biological Industries) with 100 U/ml penicillin/streptomycin (Pen/Strep; Thermo Fisher Scientific) and passaged as single cells in a 1:10 to 1:30 ratio using TrypLE™ Express (Thermo Fisher Scientific) when 70–80 % confluent. The cells were kept at 37 °C in 5 % CO<sub>2</sub>. All hPSC lines used in this study carried a balanced genetic content, which was determined by array-based comparative genomic hybridization (Human Genome CGH Microarray 4 × 44 K, Agilent Technologies) as previously described [22].

### Embryoid Body Formation

The influence of several factors on EB formation was tested: number of days in culture and the degree of confluence before EB formation, the method of single cell harvesting, the differentiation medium used and finally the recipient plate used to allow EB formation. The different combinations are shown in Table 1. All experiments were carried out on hESC, and the optimized protocol was validated with hiPSC.

#### Cell Culture Confluence

Human ESC were cultured in NS medium either for 5–7 days up to 100 % confluence, for 7–9 days with the last 2 days in differentiation medium up to over-confluence, or passaged

2 days before EB formation in a ratio leading to a final culture at 60–80 % confluence upon harvesting. For the set of experiments investigating the correlation between initial seeding density and number of days in culture before harvesting, hESC were seeded at different densities in NS medium ( $3 \times 10^4$ ,  $6 \times 10^4$  and  $10 \times 10^4$ ) and then harvested for EB formation after 2, 3, 4 or 5 days.

#### Cell Harvesting

Human ESCs were harvested using TrypLE™ Express and resuspended in the appropriate differentiation medium in the presence of 10 µM ROCKi (Stemcell Technologies). Alternatively, we tested 0.05 % Trypsin-EDTA, StemPro®Accutase® Cell Dissociation Reagent and enzyme-free Cell Dissociation Buffer (CDB; all from Thermo Fisher Scientific) for single-cell dissociation. We also detached cells using a Cell Scraper (BD Falcon) after a 5-min incubation in calcium and magnesium-free Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific).

#### Differentiation Media

We tested Knockout™-DMEM supplemented with 2 mM L-glutamine (Thermo Fisher Scientific), 1 % non-essential amino acids (Thermo Fisher Scientific), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 100 U/ml Pen/Strep and either 20 % Knockout™ Serum Replacement (SRdiff medium; Thermo Fisher Scientific) or 20 % heat inactivated fetal bovine serum (FBSdiff medium; Thermo Fisher Scientific). We also tested STEMdiff™ APEL™ Medium (APEL medium; Stemcell Technologies) with 100 U/ml Pen/Strep and in some modifications hESC were seeded in NS medium for EB formation. When specified, 5 % polyvinyl alcohol (PVA, Sigma-Aldrich) was added prior to aggregate formation.

#### Spin EBs

Cells were counted using the TALI® Image Cytometer (Thermo Fisher Scientific) and 500, 1000, 3000 or 5000 hES cells/well were seeded into round-bottom ultra-low attachment plates (Corning® #7007) or round-bottom non-treated 96-well plates (Corning® #3788). The plates were spun down at 600 g for 5 min. EB differentiation lasted for 21 days and the medium was changed every third day. Aggregate sizes were measured at day 7, 14 and 21 using the IX-81 fluorescent microscope (Olympus) with the Cell<sup>^</sup>F software (Olympus).

#### Hanging Drops

Human ESCs were passaged 2 days before EB formation leading to a final confluence of 60–80 %. Cells were harvested

**Table 1** Overview of the different EB formation conditions applied in this study

Cell culture conditions		EB formation conditions			
No. of days after passaging	Degree of confluence	Harvesting method	Differentiation medium	Recipient	
				Round- bottom 96-well plates	Hanging drops
5–7	~100 %	Trypsin-EDTA	FBSdiff	ULA and NTP	–
7–9	>100 %	TrypLE Trypsin-EDTA Accutase CDB DPBS	FBSdiff SRdiff	ULA	–
2	60–80 %	TrypLE CDB	SRdiff± PVA APEL NS± PVA	ULA and NTP	15 µl drops (only in APEL)

ULA ultra-low attachment, NTP non-treated plastic

with TrypLE™ Express and resuspended in APEL medium with 100 U/ml Pen/Strep and 10 µM ROCKi. Drops of 15 µl volume containing 1000, 3000 or 5000 cells were placed on the inside of the lid of 60-mm culture dishes filled with 6 ml PBS to avoid drying of the drops. After 1 day, EBs were transferred to Petri dishes and fresh medium was added.

#### Confirmation in hiPSC

For EB formation from hiPSC, cells were passaged 2 days before harvesting by TrypLE™ Express and resuspended in APEL medium with 10 µM ROCKi. Cells were either seeded at 5000 cells/well in the round-bottom non-treated 96-well plates or in hanging drops as described in the previous section.

#### Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA from 21-day VUB01, VUB02 and VUB06 EBs was extracted using the RNeasy Mini Kit (Qiagen) with on-column DNase digest. Reverse transcription was performed using the First-Strand cDNA Synthesis Kit (GE Healthcare). Quantitative RT-PCR was performed using the ViiA 7 thermocycler (Thermo Fisher Scientific) and ViiA 7 software v1.2 (Thermo Fisher Scientific). Cycling parameters were as follows: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Reactions were performed in a 20 µl final reaction volume, comprising 10 µl of qPCR MasterMix Plus Low ROX (Eurogentec), 40 ng of cDNA and either 1 µl of TaqMan gene expression assays for *ACTC1*, *CD34* (mesoderm differentiation), *GATA4*, *AFP* (endoderm), *TP63*, *PAX6* (ectoderm), *POU5F1* and *GUSB*, or 1.8 µM primer mix (IDT) and 250 nM probes (Thermo Fisher Scientific) for *NANOG* and *UBC*. *POU5F1* and *NANOG* were used as pluripotency markers, and *UBC* and *GUSB* as

endogenous controls. References for the assays and all primer and probe sequences can be found in Supplementary Tables 1 and 2.

Gene expression levels in EBs were also compared to positive controls for each embryonic germ layer. As a control for endoderm we used the commercially available hepatocarcinoma cell line HepG2 (kindly donated by Prof. Leo van Grunsven), for mesoderm we used hESC-derived cardiomyocyte-like cells (Supplementary Methods), and for ectoderm we used hESC-derived retinal pigmented epithelial (RPE)-like cells (Supplementary Methods). Quantitative RT-PCR for positive controls was performed as described above. *GATA4* and *AFP* were analyzed in HepG2 cells, *ACTA2* in cardiomyocytes (0.9 µM per primer from IDT and 250 nM probe #44 from Roche Universal Probe Library, cat. no. 04688040001), and *PAX6* and *TP63* in RPE cells. Quantitative RT-PCR for hiPSC EBs was performed as described for hiPSC characterisation in the Supplementary Methods. TaqMan gene expression assays for *CD34*, *GATA 4*, *TP63*, *GUSB*, or 1.8 µM primer mix and 250 nM probes for *NANOG*, *POU5F1*, *GAPDH* and *UBC* were used. Analysis was performed with *UBC*, *GAPDH* and *GUSB* as endogenous controls. All assays, primer and probe sequences can be found in Supplementary Tables 1 and 2.

#### Proliferation Assay

To compare the proliferation rate in different culture conditions, cells of VUB02 were either passaged 2 days before measurement and seeded at different densities in NS medium ( $3 \times 10^4$ ,  $6 \times 10^4$ ,  $8 \times 10^4$ ,  $10 \times 10^4$  and  $12 \times 10^4$  cells per cm<sup>2</sup>) or seeded 5 days before the measurement in NS/SRdiff medium at densities of  $4 \times 10^4$  and  $6 \times 10^4$  cells per cm<sup>2</sup>. After 2 or 5 days the cells were fixed in 3.7 % formaldehyde and the Click-iT®EdU Alexa Fluor® 647 Imaging Assay (Invitrogen)

was performed. The amount of EdU-positive cells was counted using an IX-81 fluorescent microscope (Olympus) with Cell<sup>F</sup> software (Olympus). We analysed three biological replicates and at least 400 cells per condition.

### Statistical Analysis

Statistical analysis was performed using the GraphPad Prism software (version 5.04; GraphPad Software, Inc.). For the proliferation assay, either an unpaired t-test or one-way ANOVA followed by Bonferroni correction was performed when comparing two or more conditions, respectively. The significance level was set at p-value <0.001. For the EB size at day 7, two-way Anova followed by Bonferroni correction was applied with p-value <0.05 as a significance level.

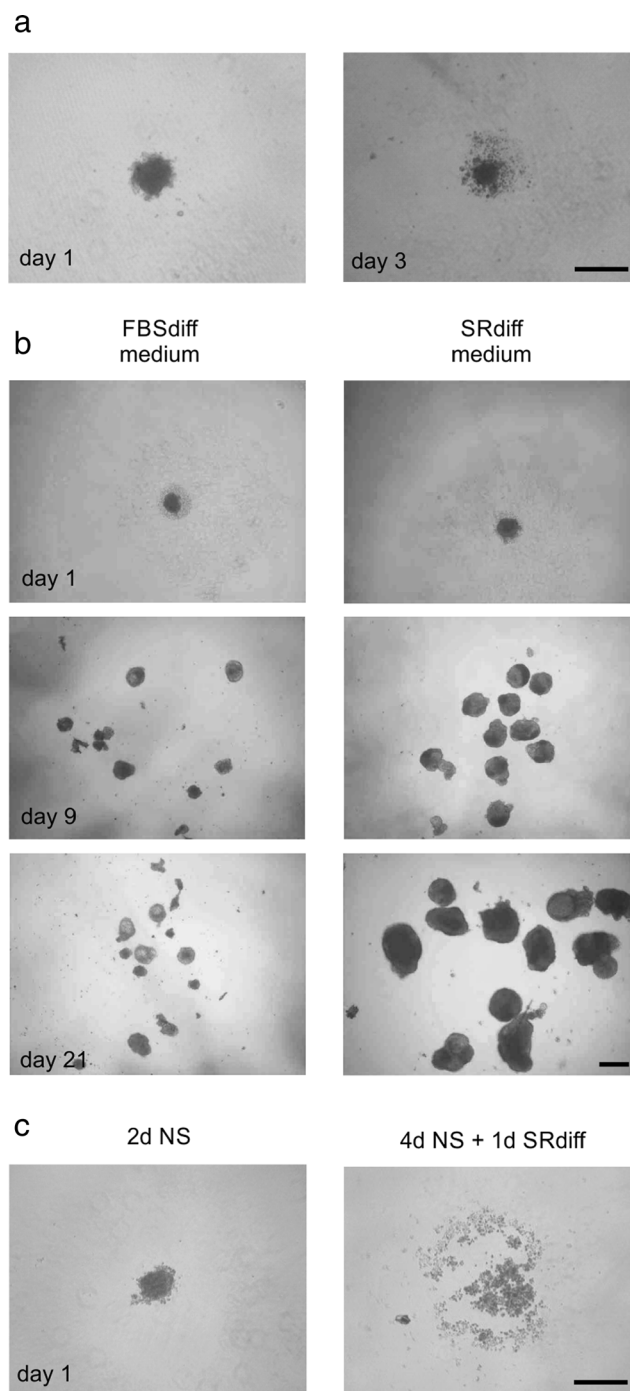
## Results

### Pre-differentiation Results in Highly Variable EB Yield

Initially, single hESC were harvested with Trypsin-EDTA, just before the cultures reached 100 % confluence, and spun down in round-bottom 96-well plates in FBSdiff medium with ROCKi. Use of non-treated 96-well plates did not result in EB formation, as cells attached to the bottom of the wells. When ultra-low attachment plates were used, aggregates were formed but failed to grow and often disintegrated after a few days (Fig. 1a). Next, we tried to pre-differentiate the cells in FBSdiff or SRdiff medium for 2 days before harvesting for EB formation. We obtained aggregates in both media, but those pre-differentiated in FBSdiff failed to grow resulting in much smaller EBs at day 9 and 21 than those pre-differentiated in SRdiff (Fig. 1b). Though we obtained aggregates using the pre-differentiation approach, the experimental outcome remained highly variable. Cells could either aggregate to form full EBs, partially aggregate resulting in several smaller spheroids or completely fail to form any aggregates. When comparing different cell harvest methods, we did not observe any difference in EB formation efficiency between enzymatic approaches (Trypsin-EDTA, TrypLE, Accutase) and the enzyme-free Cell Dissociation Buffer. However, EBs generated from DPBS-based single-cell suspensions were often much smaller. All aggregates cultured in FBSdiff were smaller than those in SRdiff (Supplementary Fig. 2). In further experiments we used TrypLE for cell harvesting and SRdiff medium for differentiation.

### The Growth Phase is a Crucial Factor for Successful EB Formation from Single Cells

Due to the high variability in EB formation efficiency in our initial attempts, we wanted to establish whether cell



**Fig. 1** Optimization of the equal-size EB formation protocol. Aggregates after EB formation (a) in FBSdiff medium from 100 % confluent culture and (b) in FBSdiff/SRdiff medium after the pre-differentiation step. (c) Images of culture wells after EB formation from the same passage of VUB02 cultured in different conditions. All scale bars represent 200 μm

proliferation rate, influenced by culture confluence and number of days since last passage, played an important role in EB formation. We generated EBs with cells from VUB02 that were cultured simultaneously for either 2 days in NS medium up to 60–80 % confluence or kept in culture for up to 6 days with the pre-differentiation step in SRdiff medium during the

last 2 days. While the first condition with recently passaged cells yielded well-formed spheroids, in the second one cells completely failed to aggregate (Fig. 1c). When repeating this EB formation approach using 2-day cultures in NS medium, we consistently obtained EBs between biological replicates and using different cell lines. Therefore, we concluded that cell passaging 2 days prior to aggregate formation is crucial for high reproducibility of the protocol. Importantly, also the use of ROCKi was essential, as spun down cells failed to form aggregates in the absence of ROCKi, even if they were freshly passaged.

### Efficient Human EB Formation in Different Systems

Starting EB formation from hESC cultured on LN521 using ROCKi and fresh cell passaging, we evaluated the influence of other variables. We replaced the more expensive round-bottom ultra-low attachment plates with round-bottom non-treated plates, in which freshly passaged hESC efficiently generated EBs without attachment to the wells. We also obtained EBs in commercially available xeno-free APEL™ medium and in NS medium. When compared to the aggregates generated in SRdiff, the shape of the EBs obtained in APEL and NS medium was rounder and more regular and the EBs grew larger during the 3-week differentiation (Supplementary Fig. 3A). APEL medium is a chemically defined medium containing PVA, a water-soluble synthetic polymer, which is said to be essential for aggregation of the cells [23–25]. However we efficiently obtained aggregates, both in SRdiff and NS medium, with and without the use of PVA. Moreover, addition of PVA did not affect the size or shape of EBs (Supplementary Fig. 3B). We also obtained EBs in APEL medium using the hanging drops technique, which to date is rarely achieved with hPSC (Supplementary Fig. 3C). Finally, we were able to successfully reproduce both the spin EBs and hanging drop EBs protocol, in APEL medium, on two iPSC lines from two different donors (Supplementary Fig. 3D).

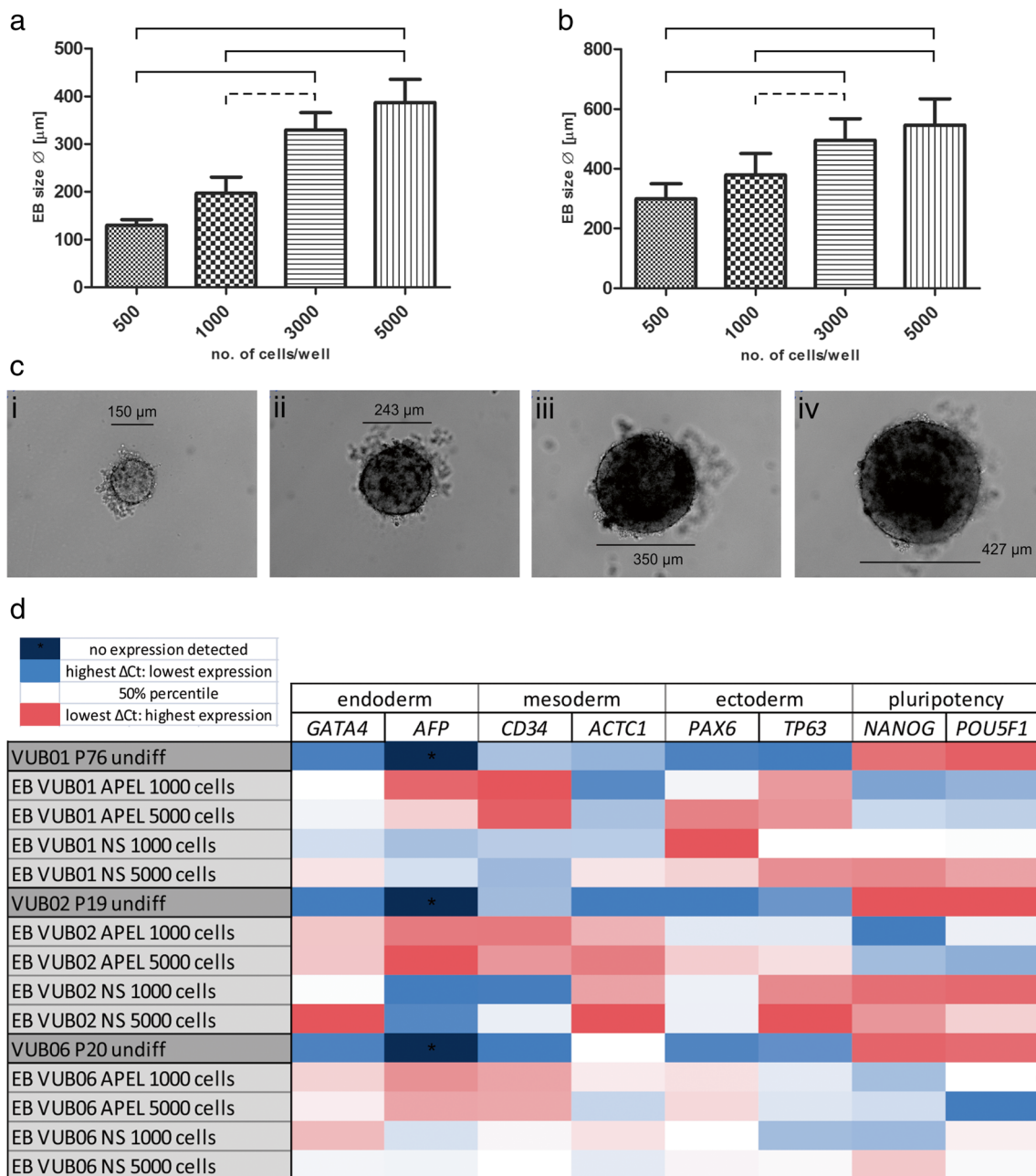
### Standardization of EB Size and its Effect on Differentiation

Using our standardized protocol, we investigated the relationship between the number of seeded cells and EB size. APEL and NS medium were chosen, as they result in the most regular-shaped EBs. A defined number of cells (500, 1000, 3000 and 5000 cells per well) from VUB01, VUB02 and VUB06 were seeded in round-bottom 96-well plates and the size of the EBs was monitored during a 3-week differentiation period. At day 7, we observed a clear correlation between the number of cells seeded and the diameter of the EBs obtained. In APEL medium, the diameter of the EBs ranged from 129

± 12 μm when 500 cells were seeded to 387 ± 48 μm after seeding 5000 cells. Aggregates cultured in NS medium were significantly larger, with diameters ranging from 298 ± 50 μm for 500 cells seeded to 546 ± 87 μm for 5000 cells seeded (Fig. 2). After 2 weeks of differentiation, the EBs grew much larger and lost their regular round shapes, precluding size determination.

To investigate the effect of EB size on the early differentiation outcome, we evaluated tri-lineage differentiation by gene expression studies in EBs obtained from 1000 and 5000 cells in APEL and NS medium after 3 weeks of differentiation (Fig. 2d). When EBs were differentiated in APEL medium, we observed a general downregulation of the pluripotency markers and upregulation of the differentiation markers. Only the mesoderm marker *ACTC1* showed a less dramatic increase upon differentiation, possibly due to an already more pronounced expression in undifferentiated cells. However, we did not observe any difference in differentiation propensity between the differently sized EBs. In fact, while the  $\Delta$ Cts varied only slightly between EBs of the same line obtained from 1000 and 5000 cells, the differences between the individual lines appeared more pronounced. Differentiation in NS medium resulted in more variable gene expression results. Although we could still detect the same trend of increased expression of differentiation markers and decreased pluripotency markers, the effect appeared less pronounced than after differentiation in APEL medium. Although in some cases we observed more apparent differences in gene expression between differentially sized EBs of individual lines, this trend was not confirmed when taking the three lines into consideration. In conclusion, while the number of cells used to generate an EB did not significantly affect the differentiation of the cells within, the differentiation medium, in contrast, seemed to have a dramatic impact on the general differentiation outcome.

Gene expression levels for EBs obtained from 5000 cells and differentiated for 21-days in APEL medium were also compared to positive controls for each embryonic germ layer. In general the results showed that the expression levels in EBs were more similar to the expression levels in positive controls than to hESC (Supplementary Fig. 4). Finally, we analysed tri-lineage differentiation in the EBs obtained from our hiPSC lines after the 21-day differentiation in APEL medium. We found upregulation of markers for all three germ layers and downregulation of pluripotency markers, confirming that aggregates from hiPSC also underwent early lineage specification (Supplementary Fig. 5). As the EB differentiation outcome was only evaluated by gene expression analysis, more work is needed to prove whether the 21-day differentiation in APEL medium allows for derivation of terminally differentiated cells within generated aggregates.



**Fig. 2** Correlation of EB size with the number of cells seeded. Average diameter of EBs obtained from different numbers of starting material (500, 1000, 3000 and 5000 cells seeded per well) after 7 days of culture (**a**) in APEL medium or (**b**) in NS medium. The experiment was performed on three hESC lines (VUB01, VUB02 and VUB06), error bars indicate the standard deviation, brackets indicate statistical significance between selected pairs (all brackets  $p < 0.001$ , except the

dotted bracket in A  $p < 0.01$  and the dotted bracket in B  $p < 0.05$ ). (**c**) Representative images of the 7-day cultured VUB06 EBs obtained in APEL medium from i) 500, ii) 1000, iii) 3000 and iv) 5000 cells seeded per well. (**d**) Gene expression of the 21-day VUB01, VUB02 and VUB06 EBs obtained from 1000 and 5000 cells in APEL and NS medium. Gene expression is presented as  $\Delta\text{Ct}$  ( $\Delta\text{Ct} = \text{Ct}_{\text{goi}} - \text{Ct}_{\text{ref}}$  where goi - gene of interest and ref - reference gene)

### Time in Culture Rather Than Culture Confluence Determines the Proliferative Capacity of hESC on LN521

To further investigate whether the number of days in culture significantly affects the cell proliferation rate and, as a consequence, EB formation efficiency, we compared EdU incorporation rates between different culture conditions. Defined

numbers of cells from VUB02 ( $3 \times 10^4$ ,  $6 \times 10^4$ ,  $8 \times 10^4$ ,  $10 \times 10^4$  and  $12 \times 10^4$  cells per  $\text{cm}^2$ ) were plated for 2 days in NS medium to reach a final culture confluence ranging from 40 % to 90 % before performing the proliferation assay, or plated for 5 days ( $4 \times 10^4$  and  $6 \times 10^4$  cells per  $\text{cm}^2$ ) to reach 100 % confluence. Cells that were passaged in NS medium 2 days before the analysis showed an average proliferation

rate of  $52 \pm 3$  %, whereas those that were kept in culture for 5 days had a significantly lower proliferation rate ( $37 \pm 2$  %,  $p < 0.001$ ) (Fig. 3a). Moreover, the cells cultured for 5 days also morphologically differed from the 2-day cultures showing large nuclei in contrast to the 5-day cultures with tightly packed nuclei and much cell debris (Fig. 3d). We hypothesized that the EdU incorporation drop might have been correlated to higher cell density. However, surprisingly, within the 2-day cultures EdU incorporation rates varied from 48 % to 58 %, but no correlation between the confluence of the cultures and the proliferation rate was observed (Fig. 3b). Similarly, no significant difference in proliferation rate between the initial seeding densities was detected after 5 days in culture (Fig. 3c). We also checked the EdU incorporation rate for cells that were cultured for 5 days with the last day in SRdiff medium, the pre-differentiation condition that we used in our initial EB formation protocol. As expected, the proliferation rate was significantly lower in comparison to the result from the 2d NS culture (Supplementary Fig. 6). Thus, we showed that recently passaged cells appear to proliferate at a higher rate when compared to the 5-day NS/SRdiff protocol and hypothesize that this high proliferation rate correlates with reproducible EB formation.

To confirm that the number of days in culture but not the initial seeding density influences the efficiency of equal-sized EB formation, we performed a series of aggregate formation experiments. Cells from VUB02 were seeded at  $3 \times 10^4$ ,  $6 \times 10^4$  and  $10 \times 10^4$  cells per  $\text{cm}^2$ . EB formation was initiated in APEL medium from 5000 cells after 2, 3, 4 and 5 days of culture. As in the previous experiments, cells that were cultured for 2 days reproducibly formed round EBs with only some dead cells around, a phenomenon that was always observed during EB formation. However, the longer hESC were kept in culture the more dead cells were present after EB formation. Though in this experiment we were still able to obtain aggregates from the 5-day cultured cells, these aggregates were much smaller and were surrounded by a thick layer of dead cells (Fig. 3e). Cells seeded at the three different initial seeding densities formed EBs equally well after 2 days in culture and equally bad after 5 days, which is in concordance with the results from the EdU incorporation experiment. In summary, this whole series of experiments confirms that EB formation efficiency is linked to the proliferation rate of hPSC cultured on LN521, which, in turn, is highly dependent of the days the cells are cultured.

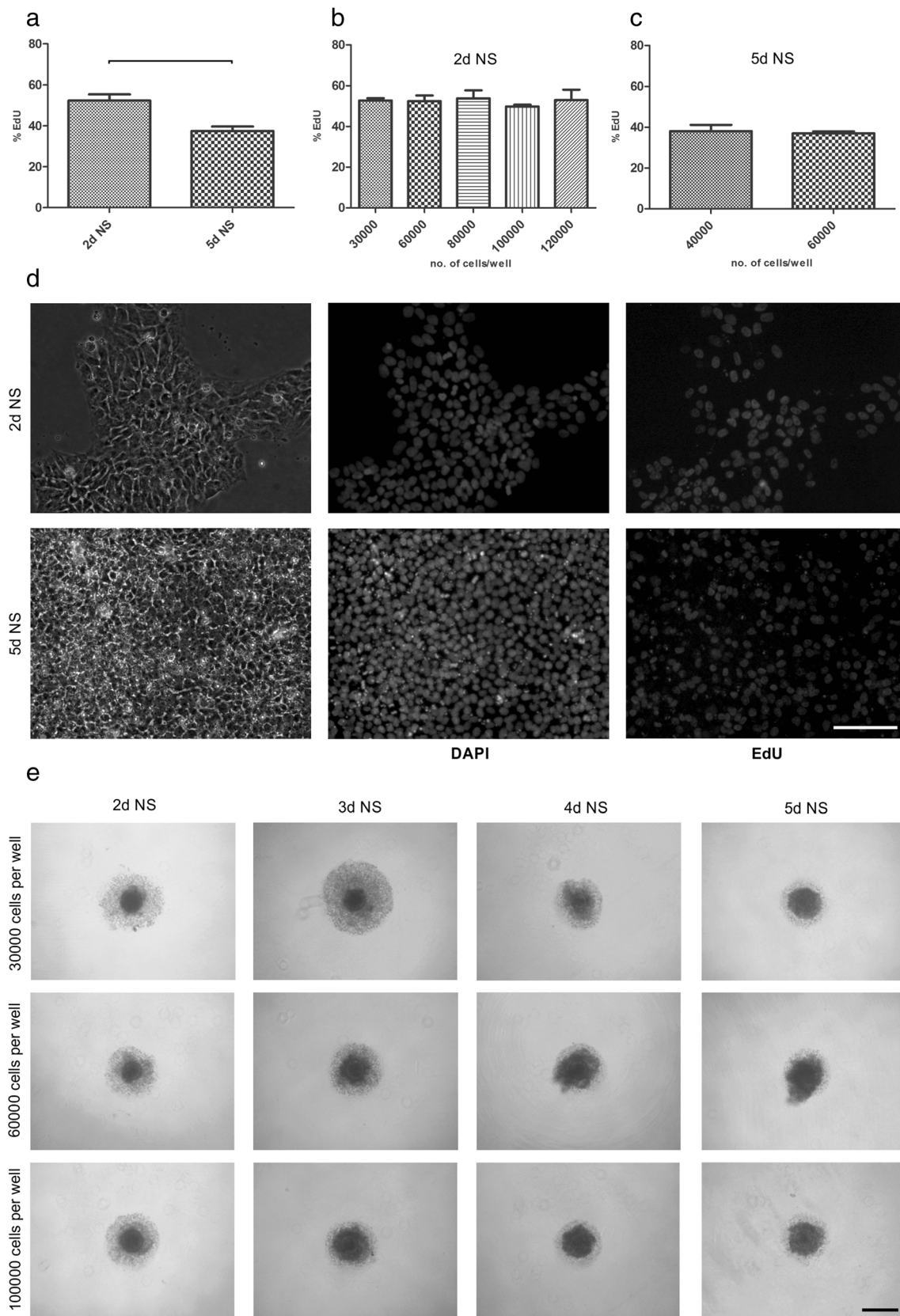
## Discussion

In this study, we developed an efficient and reproducible protocol for equal-sized EB formation for LN521-based hPSC cultures. Our systematic work allowed us to uncover factors essential for successful formation of aggregates using different

approaches. By fresh cell passaging and the addition of ROCKi, we obtained EBs from hESC and hiPSC lines in round-bottom non-treated and ultra-low attachment 96-well plates, in hanging drops and in different culture media. The developmental potential of the EBs produced under this method and subsequently differentiated for 21 days was confirmed by expression of early differentiation markers, proving their capability to differentiate into derivatives of all germ layers. More work is warranted to determine whether the differentiation step used in our study allows the generated aggregates to differentiate into fully committed somatic cells.

Enzymatic or non-enzymatic cell harvesting did not affect the aggregate formation efficiency in our system, although enzyme-free dissociation has been suggested to be less harmful for the hPSC survival after passaging [26]. This could be explained by the adaptation of LN521-cultured hPSC to enzymatic passaging. Therefore, cells cultured on LN521 would be expected to more easily form EBs from single cells [17, 18]. However, when cells were harvested after 1 week in culture, nearing 100 % confluence, the EB growth was either impaired or highly variable. Such low reproducibility was previously reported and linked to high expression levels of pluripotency marker *POU5F1* [19]. Human PSC cultured on LN521 show less spontaneous differentiation, compared to other culture systems [18]. A lack of partially differentiated cells among the undifferentiated hESC population could be a potential reason for the initial experimental failure. In a study by Ungrin 2008, a 72-h pre-differentiation step with serum-containing medium improved the reproducibility of the protocol. Nevertheless, they further demonstrated that serum induction was not essential, as the addition of ROCKi alone led to an even higher efficacy in EB formation [19]. In our hands, the pre-differentiation step decreased efficiency while the presence of ROCKi alone did not eliminate the high variability between biological replicates. However, ROCKi showed to be an essential addition to the harvesting medium. This chemical compound improves single-cell survival of hPSC but other potential downstream effects are still unknown. While there have been attempts to eliminate it from EB formation protocols, this only seems to be possible for some cell lines seeded at very high densities [27, 28], yielding large aggregates in which hypoxic areas can lead to cell necrosis.

The second determining factor to achieve a highly reproducible protocol was the growth rate of the cells. Interestingly, we found that the time in culture after passaging of the cells has a much more profound effect on the proliferation rate of cells on LN521 than the culture density. Cell passaging 2 to 3 days before aggregate formation creates an optimal cell population that is highly proliferative even at high densities, whereas cells that were kept in culture at higher densities for a longer period of time divide at a lower rate and contain more dead cells. Protocols with a similar pre-passaging step have already been described for EB formation in feeder-based [23] and other



feeder-free culture systems [24, 25]. High density culture provides suboptimal conditions for hPSC due to medium

acidification and shortage of nutrients. We hypothesize that cells which are exposed to this environment for 5 days have a



◀ **Fig. 3** Human ESC EdU incorporation rates and EBs generated in different culture conditions. (a) VUB02 cells seeded for 2 or 5 days in NS medium. The bracket indicates statistical significance between the selected pair ( $p < 0.001$ ). (b) VUB02 cells seeded for 2 days in NS medium at different initial seeding densities. (c) VUB02 cells seeded for 5 days in NS medium at different initial seeding densities. (d) Representative images of the VUB02 after 2-day culture and 5-day culture in NS medium. The scale bar represents 100  $\mu\text{m}$ . (e) Representative images of aggregates generated from VUB02 cells after 2 to 5 days in culture since last passage and from different initial seeding densities (30000, 60000 and 100000 cells per well). The scale bar represents 200  $\mu\text{m}$

lower proliferation rate than cells that were kept at high density for only 2 days. Our group recently demonstrated that hESC kept on LN521 at high density for 5 days show a decrease in proliferation, indicated by a decrease in the fraction of cells in S and G2/M phases, in comparison to cells that were kept at a very low density [29]. In our EB formation experiments the hPSC cultures always reached relatively high densities upon the day of harvesting. It is therefore very plausible that in a significantly lower density culture the proliferation rate of the cells would remain high, even after 5 days in culture. However, this condition is not relevant for EB formation as it does not provide enough material for the aggregate formation.

Our method was successfully reproduced in different culture systems and with both hESC and hiPSC, as well as in hanging drops. Furthermore, ultra-low attachment round-bottom plates could easily be substituted by a more economical version made of non-treated plastic. Multiwell plates allow for a good visualization and follow-up of the individual EBs whereas the hanging drop approach is less labour-intensive, but does not allow the culture of the aggregates in individual drops for a long period of time. Protocols for scalable mass EB production, necessary to obtain large amounts of differentiated cells for possible biomedical applications, are being developed [30, 31]. Although our protocol was developed in 96-well plates which require labour-intensive manipulations of individual EBs, the high reproducibility of our method will likely allow up-scaling into a high-throughput system.

Plating of a given number of hESC reproducibly resulted in EBs of equal and specific size, independently of the hESC line used, which is an important asset of our protocol. Moreover, EB sizes could easily be manipulated by changing EB culture medium. NS medium can be used for initial EB formation and, by boosting cell proliferation during the aggregation, results in larger EBs than in APEL medium, however it leads to less reproducible 21-day differentiation as evidenced by a more variable gene expression. While APEL medium induces spontaneous differentiation by depriving hESC of factors maintaining pluripotency, NS medium is designed to support the undifferentiated state and is therefore not appropriate to drive EB differentiation.

Although we were able to control EB size, our gene expression studies showed no significant effect of differentially sized

EBs on the differentiation propensity as previously reported [7–10]. In fact, we found that the gene expression between differently sized EBs from the same cell line was highly reproducible. The main factor influencing the final differentiation outcome was the differentiation medium. Since EBs grew very large and had irregular shapes at day 14 and 21, their size was only measured at day 7 of differentiation even though gene expression analyses were performed at day 21. It may well be that at the end of the differentiation period the difference in size of the EBs obtained from 1000 and 5000 cells is biologically insignificant, resulting in similar differentiation cues. Comparing our results to other reports is difficult, as every study used different methods to generate equal-sized EBs, different differentiation media, different cell numbers to obtain aggregates and checked the differentiation outcome at different time points.

In conclusion, we have shown that the crucial factors for EB formation are the cell proliferation rate and the presence of ROCKi. We have optimized a method that allows us to easily direct EB formation and size, which will be an excellent starting point for optimizing differentiation protocols directed towards a specific cell type.

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#### Compliance with Ethical Standards

**Conflicts of Interest** The authors declare no potential conflicts of interest.

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