Controlled embryoid body formation via surface modification and avidin–biotin cross-linking

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Abstract Cell–cell interaction is an integral part of embryoid body (EB) formation controlling 3D aggregation. Manipulation of embryonic stem (ES) cell interactions could provide control over EB formation. Studies have shown a direct relationship between EB formation and ES cell differentiation. We have previously described a cell surface modification and cross-linking method for influencing cell–cell interaction and formation of multicellular constructs. Here we show further characterisation of this engineered aggregation. We demonstrate that engineering accelerates ES cell aggregation, forming larger, denser and more stable EBs than control samples, with no significant decrease in constituent ES cell viability. However, extended culture ≥ 5 days reveals significant core necrosis creating a layered EB structure. Accelerated aggregation through engineering circumvents this problem as EB formation time is reduced. We conclude that the proposed engineering method influences initial ES cell–ES cell interactions and EB formation. This methodology could be employed to

further our understanding of intrinsic EB properties and their effect on ES cell differentiation.

Keywords Aggregation · Avidin–biotin · Cell density · Cell interaction · Embryoid body · Embryonic stem cells

Introduction

An established method for initiating embryonic stem (ES) cell differentiation in vitro comprises of 3D culture to promote natural cell–cell interactions and embryoid body (EB) formation (Koike et al. 2007; Kurosawa 2007). The presence of all three germ layers demonstrates that EB culture is a crude but effective means of recapitulating early stages of embryogenesis in vitro (Bratt-Leal et al. 2009; Itskovitz-Eldor et al. 2000). Theoretically, embryonic stem cells can differentiate to any cell type and many examples of in vitro differentiation exist including generation of skeletal and brain tissue (Bielby et al. 2004; Parekkadan et al. 2008). Consequently, ES cells provide a renewable source of cellular material for tissue engineering and regenerative medicine (Ungrin et al. 2008). However, generation of specified homogeneous cultures for clinical application remains problematic (Carpenedo et al. 2009; Karlsson et al. 2008).

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Researchers employ a myriad of aggregation methods for EB formation which promote natural aggregation or artificially force cell–cell interactions, such as rotary mass suspension (Abilez et al. 2006; Niebruegge et al. 2008) or microwell centrifugation (BurrIDGE et al. 2007; Moeller et al. 2008), respectively. Many differences exist between methods regarding aggregation efficiency (Carpenedo et al. 2007). For example, rotary mass suspension enables large scale production of EBs but in heterogeneous populations which diminishes their suitability for clinical application (Rohani et al. 2008; Youn et al. 2006). Alternatively, microwell centrifugation allows for rapid formation of homogeneous EB populations but is laborious, time consuming and currently displays scalability and automation limitations (Kim et al. 2007). The high degree of variability between aggregation methods impedes our understanding of how EB formation influences ES cell differentiation (Mogi et al. 2009; Ng et al. 2005). Analysis of aggregation kinetics under defined and controlled parameters would help identify key aspects of EB formation that influence ES cell differentiation.

We have previously described a novel 3D culture system for controlled aggregation of ES cells and enhanced EB formation (De Bank et al. 2007). The method involves mild oxidation of surface sialic acid residues with sodium periodate to generate non-native reactive aldehyde groups which are then biotinylated with biotin hydrazide (De Bank et al. 2003). Biotin-avidin binding is then exploited to rapidly cross-link engineered ES cells in a controlled density-dependent manner upon avidin supplementation. It is envisaged that ES cell differentiation via EB formation could be standardized through identification of key features during aggregation and their relationship with the generation of specific cell types (Come et al. 2008).

Here we show optimization and further characterisation of engineered ES cell aggregation. Engineering enhances ES cell aggregation, rapidly forming EBs which are larger, denser and more stable than those in control samples, without detrimental effect to cell viability. This study also shows that EB formation is a complex interplay between cell–cell interactions, ES cell aggregation, proliferation and death, aggregation time and cluster agglomeration.

Methods and Materials

Cell Culture and Maintenance

Mouse ES cells (EK-CCE (embryo derived cell line, columnar epiblast epithelium) were a kind gift from Dr. Vasso Episkopou, Imperial College London, Robertson et al. 1986) were cultured on a feeder layer of mitotically inactivated mouse fibroblasts [cell line SNL, expressing leukemia inhibitory factor (LIF)] pretreated with 0.01 M Mitomycin C for 2 h. ES cells were maintained in standard culture medium (SCM) containing Dulbecco's modified Eagles medium (DMEM, Invitrogen, Paisley U.K.), 10% foetal calve serum (FCS, Sigma, Poole U.K.), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen), 2 mM L-glutamine (Sigma) and 500 µM β-mercaptoethanol (Sigma). SCM was supplemented with 500 U/mL LIF (Chemicon, Hampshire U.K.). Cultures were incubated at 37 °C and 5% CO₂ in a humidified atmosphere until confluent.

Cell Surface Engineering

ES cells were trypsinized from feeder layers and centrifuged at 1,000 rpm. Live cells were counted based on trypan blue exclusion after washing twice with 5 mL PBS. ES cells were centrifuged and suspended in 5 mL 1 mM sodium periodate (NaIO₄ in PBS, Sigma) for 10 min at 4 °C in the dark. Suspensions were then washed in 5 mL biotinylation buffer (PBS + 0.1% FCS, pH 6.5) and treated with 5 mL 5 mM biotin hydrazide in biotinylation buffer (Sigma) for 30 min at 37 °C under continuous agitation. Suspensions were centrifuged and washed in 5 mL avidin buffer (PBS + 0.1% FCS, pH 7.0).

Mass Suspension

Engineered ES cells were seeded into non-tissue culture treated 6 well plates in 2 mL SCM with only 1% FCS and supplemented with 10 µg/mL avidin (lyophilized avidin powder in PBS, Sigma) between 5×10^4 and 1×10^6 cells/mL. Minimal FCS was used to minimize both cell proliferation during initial aggregation (daughter cells may not express the surface modification) and possible interaction with either biotin groups and/or avidin. Cell suspensions

were incubated for 6 h under continuous rotation (0–30 rpm) on a gyrotwister 3D-shaker (Labnet International Inc., Dorset U.K.). Suspensions were supplemented with FCS to increase concentration from 1 to 10% to prevent starvation and allow proliferation, and incubated stationary for 9 days. Medium was changed every 2–3 days.

To analyze EB size and number variation, EB suspensions were analyzed with a Particle Sizing System (PSS; AccusizerTM 780A Autodiluter^{PAT}, PSS.NICOMP, California U.S.A.) using relevant PSS software (CW780md Version 1.55).

Quantification of Cell Aggregation

ES cell suspensions were imaged using a stereomicroscope (Nikon Eclipse TS100) at 10× magnification. A 0.25 cm square grid partitioned the suspension. All EBs within three diagonally arranged squares were counted and their diameters measured. Due to non-spherical configurations of initial cell clusters, EBs were defined as having a minimum diameter of 40 µm.

Analysis of Cell Death

ES cells/EBs were incubated for 1 h in Live/DeadTM solution (2 µM calcein AM and 4 µM ethidium homodimer-1 in SCM, Sigma). Suspensions were washed and suspended in PBS then imaged at excitation/emission 495/515 nm for calcein AM and 495/635 nm for ethidium homodimer-1 using fluorescence microscopy. To quantify EB core necrosis EBs were trypsinized before staining with Live/DeadTM solution, as detailed above. Dead cells were identified by red fluorescence and quantified by cell count.

Cell/DNA Quantification

ES cell pellets were suspended in 0.5–1 mL papain solution (1.06 mg/mL papain powder in papain buffer (0.1 M dibasic sodium chloride (Sigma), 5 mM cysteine hydrochloride (Sigma) and 5 mM EDTA, pH 6.5 with HCl)) and incubated overnight at 60 °C. Once digested, 34 µL of each sample was mixed with 0.5 mL Hoechst buffer [0.01 M Trizma base (Sigma), 0.01 M EDTA, 0.1 M sodium chloride (VWR International, Poole U.K.)] and 0.75 mL of Hoechst working solution (Hoechst stock solution (1 mg/mL bis-benzimide (Sigma) in SSC buffer) in Hoechst

buffer, 1:2,000). 300 µL of this mixture was transferred to a 96 well plate and fluorescence analyzed at excitation/emission 360 nm/460 nm.

Scanning Electron Microscopy

EB suspensions were washed in 5 mL PBS, allowed to settle and fixed overnight in 3 mL 3% glutaraldehyde (Sigma) at 4 °C. EBs were suspended in 1 mL 1% osmium tetroxide (TAAB, Berkshire U.K.) for 2 h then washed with 5 mL dH₂O and dehydrated through a series of ethanol washes each lasting 2–5 min. Once dehydrated, EBs were chemically dried with 5 mL hexa-methyldisilazane (HMDS; Sigma) twice, 5 min each. EBs were allowed to air dry overnight in an externally vented fume hood. Desiccated EBs were carefully transferred to carbon discs (Agar Scientific Ltd., Essex U.K.) mounted on aluminium stubs (Agar). EBs were gold-coated (Balzers Union Ltd., Liechtenstein) for 3 min in an argon atmosphere. Coated EBs were imaged using a JEOL 6060L scanning electron microscope (SEM; JEOL Ltd., Hertfordshire U.K.). Images were taken at 12–14 kV.

Haematoxylin and Eosin Stain

EBs were fixed in 3% glutaraldehyde (Sigma) at 4 °C overnight, suspended in 3% agarose gel (Roche, Hertfordshire U.K.) and embedded in paraffin wax. Embedded EBs were sectioned (4 µm thick), washed in xylene twice for 3 min to de-wax and rehydrated through a series of ethanol washes (100, 90, 70 and 50% in dH₂O) with a final rinse in dH₂O, each for 2–3 min. Once hydrated, sections were stained with Mayers haematoxylin (Sigma) and washed with dH₂O. Sections were washed with Scott's tap water (2% magnesium sulphate (Sigma) and 0.35% sodium bicarbonate (Sigma) in dH₂O) and partially dehydrated through 50, 70 and 90% ethanol in dH₂O washes, 2–3 min each. After being dipped in alcoholic 1% eosin (eosin in ethanol, Sigma), dehydration was completed with a 2–3 min wash of 100% ethanol and a final wash in xylene. Stained sections were left to air dry and mounted with DPX (Sigma).

Statistical Analysis

Non-parametric methods were utilized as data did not follow a Gaussian distribution, determined by a

Kolmogorov–Smirnov test. A Kruskal–Wallis test was employed to evaluate significant variation among medians within individual samples. A Dunns multiple comparison test was used to compare means of two individual data sets within the same sample. A Mann–Whitney test was employed to assess significance between two data sets from different samples. Significance is depicted by $P \leq 0.001$ ***, $P \leq 0.01$ **, $P \leq 0.05$ *.

Results

Optimization of Aggregation Parameters

The effect of increasing avidin concentration on engineered and control ES cell aggregation is shown in Fig. 1a. Suspensions with avidin supplementation generated significantly ($P \leq 0.001$) larger engineered EBs, and without avidin supplementation significantly ($P \leq 0.001$) smaller engineered EBs, both compared to control EBs. Increasing avidin concentration had no effect.

A second parameter affecting ES cell aggregation was rotation speed. Figure 1b shows the number of EBs in engineered, control 1 (non-engineered without avidin supplementation) and control 2 (non-engineered with avidin supplementation) samples quantified by PSS. Only samples rotated at 15 rpm exhibited elevated numbers of EBs. Engineered samples rotated at 15 rpm produced $\sim 4\times$ as many EBs as corresponding control samples.

A third parameter influencing aggregation is cell density. Figure 1c shows average EB diameters for suspensions seeded at 5×10^4 and 1×10^6 cells/mL over 3 days of aggregation. Engineered EBs were significantly ($P \leq 0.001$) larger than control samples at 5×10^4 cells/mL, but not at 1×10^6 cells/mL.

Aggregation Time and EB Formation

To assess a suitable time frame for EB formation, ES cell aggregation was investigated by light microscopy for the first 12 h and SEM over static culture for 5 days. Figure 2a depicts brightfield images of engineered aggregates after 4 h. Control 1 and 2 samples formed aggregates after 8 h. By 12 h both engineered and control samples had formed EB structures. Figure 2b shows SEM images of these EBs over

Fig. 1 Optimization of experimental parameters and effect on aggregation. Engineered and control ES cells were seeded at 5×10^4 cells/mL into SCM supplemented with varying concentrations of avidin, incubated for 24 h and average EB diameters measured (a). Engineered and non-engineered ES cells with (control 2) and without (control 1) 10 μg/mL avidin supplementation were seeded at 5×10^4 cells/mL into SCM and agitated at varying rotation speeds for the first 6 h of a 24 h incubation and analyzed by PSS (b). ES cells were seeded at 5×10^4 and 1×10^6 cells/mL into SCM with avidin supplementation, incubated for 3 days and average EB diameters measured (c). *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$. Error bars = SEM

5 days. Engineered and control samples formed disorganized aggregates exhibiting rough and uneven surface morphologies after 1 day. By day 5 these

Fig. 2 ES cell aggregation and EB formation. ES cells were seeded into suspension at 5×10^4 cells/mL, rotated at 15 rpm for the first 6 h of a 24 h static incubation and photographed at $10\times$ magnification every 4 h until visible EB formation (a). Surface morphologies of EBs were analyzed by SEM after 1, 3 and 5 days incubation (b)

structures became organized and compacted, exhibiting smooth and uniform surface morphologies.

Effect of Engineering on ES Cell Aggregation

The effect of engineering was investigated by EB diameter and number analysis over 9 days (Fig. 3). Engineered EBs were significantly ($P \leq 0.001$) larger than control EBs in suspensions seeded at 5×10^4 cells/mL (Fig. 3a). Only engineered EBs noticeably increased in diameter over time indicating cell proliferation and/or sustained aggregation. The number of EBs within all samples remained unchanged over 9 days (Fig. 3b).

Effect of Engineering on ES Cell Populations in Culture

The number of single ES cells in suspension significantly ($P \leq 0.001$) decreased over time in all samples seeded at 5×10^4 cells/mL, indicative of ES cell aggregation (Fig. 3c). There were significantly

($P \leq 0.001$) less single engineered ES cells in suspension over the first 5 days of culture compared to control ES cells. Engineered EBs constituted significantly ($P \leq 0.001$) more ES cells than control EBs in suspensions seeded at 5×10^4 cells/mL (Fig. 3d). All samples exhibited a decrease in constitutive cell number over time.

ES Cell Viability and EB Core Necrosis

ES cell viability within engineered (Fig. 4a) and control 1 (Fig. 4b) EBs was assessed by Live/DeadTM after 9 days. EB surfaces fluoresced green indicating live outermost ES cells. EB cores fluoresced red indicating core necrosis. To investigate further, the numbers of dead ES cells within EBs were quantified by cell counts following Live/DeadTM incubation. There was a significant ($P \leq 0.001$) increase in the percentage of dead cells within all samples between 3 and 5 days (Fig. 4c). The percentage of dead cells gradually increased from 5 to 9 days.

Internal EB Structure and ES Cell Density

EB structure was assessed by H&E staining of day 9 EB cross-sections. Sectioning revealed a layered internal structure comprising the surface, outer shell, inner shell and core, each exhibiting structural and density differences (Fig. 4d). Quantification of cell nuclei within cross-sections showed engineered EBs were significantly more dense than control EBs at both innermost ($P \leq 0.01$, Fig. 5a) and outermost ($P \leq 0.05$, Fig. 5b) regions. Average cell density within surface regions was significantly ($P \leq 0.05$) greater than that in core regions in both engineered (Fig. 5c) and control 1 (Fig. 5d) EBs. Internal EB density was not dependent on initial seeding density.

Discussion

By influencing ES cell aggregation via the proposed engineering method and furthering our understanding of EB formation, it may be possible to standardize EB-based protocols for efficient and directed ES cell differentiation (Mansergh et al. 2009). Here we characterize engineered ES cell aggregation and EB formation, identifying benefits of the novel 3D culture system.

Fig. 3 Analysis of aggregation dynamics. Engineered and control ES cells were seeded at 5×10^4 cells/mL into AM supplemented with $10 \mu\text{g/mL}$ avidin and rotated at 15 rpm for 6 h. Suspensions were then incubated in SCM under static conditions for a maximum of 9 days. Average EB diameters (a) and numbers (b) were measured every other day. The numbers of non-aggregated ES cells in suspension were

analyzed by Hoechst assay of flow through cells following suspension filtration with a $40 \mu\text{m}$ sieve to remove EBs (c). Whole samples without filtration were also analyzed by Hoechst assay. The numbers of ES cells constituting EBs were calculated by deducting flow through cell numbers from whole sample cell numbers (d). *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$. Error bars = SEM

Fig. 4 EB structure and constituent ES cell viability. Both engineered (a) and control EBs (b) were incubated with Live/Dead™ solution for 30–60 min to assess ES cell viability. The numbers of dead ES cells within engineered and control EBs seeded at 5×10^4 cells/mL (c) were analysed by cell counts. d shows an H&E stained cross-section from an engineered EB after 9 days static culture and diagrammatic representation of the internal structure. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$. Error bars = SEM

Fig. 5 Effect of engineering on ES cell density within the EB. Engineered and control EBs were fixed and sectioned after 9 days static culture in SCM. Sections were H&E stained and ES cell densities calculated via nuclei counts within a known area. ES cell densities were measured between engineered and control EBs at both the surface (a) and core (b). ES cell surface densities were compared to ES cell core densities in both engineered (c) and control 1 (d) EBs. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$. Error bars = SEM

We have previously demonstrated effective surface modification and biotinylation. After periodate treatment, cell viability was found to be at least 98% over a 24 h period assessed via trypan blue exclusion (De Bank et al. 2003). ES cell viability was also unaffected by periodate treatment and processing (data not shown). However, further parameters required adjustment including avidin concentration, rotation speed and seeding density. Engineered ES cells formed EBs without avidin supplementation showing that modification did not prevent natural ES cell aggregation. When engineered ES cells were cultured with avidin the resultant EBs were significantly ($P \leq 0.001$) larger than control EBs independent of increasing avidin concentration (Fig. 1a). Clearly engineering enhances cell–cell interaction, rapidly forming EBs in an avidin-dependent manner (De Bank et al. 2007). Keeping scalability and cost effectiveness of the whole system in mind, 10 $\mu\text{g}/\text{mL}$ avidin was chosen for all subsequent experiments. Rotating suspensions at 15 rpm generated the most EBs in all samples and was therefore employed in all further experiments (Fig. 1b). Finally, engineered samples only exhibited enhanced aggregation at low seeding density (Fig. 1c). Consequently, all ensuing experiments employed a low seeding density of 5×10^4 cells/mL.

Engineering allowed for accelerated aggregation via increasing permanent cell–cell adhesions upon chance collisions resulting in aggregate formation after only 4 h (Fig. 2a). Initial aggregation produced irregular, disorganized clusters which continued to accumulate ES cells, growing in size. These clusters were observed after 24 h exhibiting rough uneven surface morphologies (Fig. 2b; Song et al. 2003). After 3 days the clusters had formed stereotypical spherical EBs exhibiting smooth uniform surface morphologies. Surface morphologies resembled primitive endoderm, complimenting other studies (Fok and Zandstra 2005).

Engineered EBs were significantly ($P \leq 0.001$) larger than control EBs (Fig. 3a). The lack of significant diameter increase over time suggested two things, (1) available ES cells were exhausted after 24 h, and/or (2) proliferation of constituent ES cells contributed minimally to EB growth. The numbers of EBs between all samples were not significantly different (Fig. 3b), even though engineered EBs were significantly ($P \leq 0.001$) larger (Fig. 3a). Possible explanations include greater spacing between constituent ES cells, afforded by engineering. This may aid permeation of nutrients and gases across the engineered EB structure, addressing current problems with starvation and hypoxia within cultured tissues in vitro

(Bauwens et al. 2005; Hanjaya-Putra and Gerecht 2009). However, the discrepancy in diameter between engineered and control EBs may be explained by the fact that not all ES cells within control samples may have contributed to EB formation.

EB formation and growth depends on the availability of ES cells in surrounding suspension. As aggregation progresses, the initial number of available ES cells seeded into suspension would decrease. Engineered samples displayed a significant ($P \leq 0.001$) decrease in suspension cell number and an increase in cell number constituting EBs, demonstrating that EB size and growth were greatly dependent on constituent ES cell number and continued aggregation (Fig. 3c, d). Closer analysis of Fig. 3c and d show that 50% of engineered ES cells contributed to EB formation in the first 24 h and remained fairly constant over the 9 day period. Comparatively, only 25% of control ES cells contributed to EB formation after 24 h which then fell to 10% by day 9. Engineering clearly exhibits increased efficacy in EB formation. However, constituent ES cell number in both engineered and control EBs significantly ($P \leq 0.001$) decreased over time suggesting that cell number alone did not account for average EB diameter (Fig. 3d). Diminishing cell numbers indicated cell death and core necrosis. As EB diameter increased so too did the distance between innermost ES cells and the surrounding culture media. EB diameter was shown to increase over time even though core necrosis may have caused shrinkage through fragmentation and/or dissociation (Fig. 3a). This suggests that 3D conformation and intercellular orientation within the EB play major roles in maintaining structure during reorganisation (Enmon et al. 2001).

Cells were found to be thriving at the surface, but necrotic at the core (Fig. 4a, b). Further analysis revealed that by day 5, percentage of dead cells significantly ($P \leq 0.001$) increased (Fig. 4c). Engineered EBs exhibited similar levels of core necrosis to control EBs even though they were significantly larger, suggesting increase of cell viability.

EB structure can be broken into four distinct layers including the core, inner shell, outer shell and surface (Fig. 4d). The core composed necrotic ES cells and the surface composed proliferating ES cells. The surface was denser than the core, supporting the idea that the EB surface may have acted as a thick protective barrier that inadvertently restricted nutrient

and gaseous permeation (Choi et al. 2005; Sachlos and Auguste 2008). The outer and inner shells combine to represent the diffusion gradient of nutrient and gaseous exchange depicting transition between ES cell states at the surface and core (Jiang et al. 2005). Another factor affecting core viability may have been intercellular signalling promoting apoptosis as a result of surrounding environmental cues (Lee et al. 2005). Density analysis revealed engineered EBs to be significantly ($P \leq 0.05$) denser than control EBs (Fig. 5a, b). As a result, the lack of elevated necrosis within engineered EBs suggests a potential benefit of engineering on ES cell viability when developing large densely populated cultures. However, up to 80% of the whole EB became necrotic by day 9 in all samples. This demonstrates a serious problem with extended culture ≥ 5 days which is the current conventional culture period (Buttery et al. 2001; Yirme et al. 2008). A variety of alternative avenues could be explored to resolve this problem including vascularization of the EB structure (Feraud et al. 2001; Goodwin 2007).

Encouraging work within our group has investigated the importance of the proposed 3D culture system on ES cell differentiation and shown that engineering does indeed exhibit a degree of control. A recent publication shows that the engineered culture system enhances osteogenic differentiation when using the set parameters described above (Gothard et al. 2009). The hope now is that by altering and tweaking these parameters we could induce and control differentiation of multiple cell types.

Conclusions

In summary, engineering influenced ES cell-ES cell interaction, adhesion and subsequent EB formation. Engineered EBs were both significantly larger and more stable than control EBs. EBs exhibited a layered structure comprising a thriving surface, outer shell, inner shell and necrotic core. EB formation was found to be a complex relationship between ES cell aggregation, proliferation, death, aggregation time and cluster agglomeration. Engineering could provide means to manipulate EB formation affecting EB size, number and morphology, and constitutive ES cell number and viability, all of which have major impact

on downstream ES cell differentiation (Bauwens et al. 2008; Messana et al. 2008; Sachlos and Auguste 2008).

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