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Short communication

The self-renewal of mouse embryonic stem cells is regulated by cell–substratum adhesion and cell spreading☆

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ABSTRACT

Mouse embryonic stem cells (mESCs) undergo self-renewal in the presence of the cytokine, leukaemia inhibitory factor (LIF). Following LIF withdrawal, mESCs differentiate, and this is accompanied by an increase in cell–substratum adhesion and cell spreading. The purpose of this study was to investigate the relationship between cell spreading and mESC differentiation. Using E14 and R1 mESC lines, we have restricted cell spreading in the absence of LIF by either culturing mESCs on chemically defined, weakly adhesive biomaterial substrates, or by manipulating the cytoskeleton. We demonstrate that by restricting the degree of spreading by either method, mESCs can be maintained in an undifferentiated and pluripotent state. Under these conditions, self-renewal occurs without the need for LIF and is independent of nuclear translocation of tyrosine-phosphorylated STAT3 or β-catenin, which have previously been implicated in self-renewal. We also demonstrate that the effect of restricted cell spreading on mESC self-renewal is not mediated by increased intercellular adhesion, as evidenced by the observations that inhibition of mESC adhesion using a function blocking anti E-cadherin antibody or siRNA do not promote differentiation. These results show that mESC spreading and differentiation are regulated both by LIF and by cell–substratum adhesion, consistent with the hypothesis that cell spreading is the common intermediate step in the regulation of mESC differentiation by either LIF or cell–substratum adhesion.
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1. Introduction

Stem cell fate is regulated by soluble factors and interactions involving cell–cell and cell–extracellular matrix (ECM) contacts (Fuchs et al., 2004), as well as by mechanical forces that can regulate stem cell fate through effects on cell shape and spreading (Costa et al., 2012). Numerous studies have demonstrated the important role of cell shape in controlling the differentiation of various types of somatic stem cells including mesenchymal stem cells and epidermal stem cells (McBeath et al., 2004; Gao et al., 2010; Connelly et al., 2010), but less is known about the influence of cell shape or spreading on the fate of embryonic stem cells. It is well-documented that the self-renewal of mouse embryonic stem cells (mESCs) is promoted by the cytokine leukaemia inhibitory factor (LIF) via the JAK-STAT3 signalling pathway (Burdon et al., 2002). Additionally, mechanisms involving the src-related kinase cYes (Anneren et al., 2004) and the Wnt/β-catenin signalling pathway (Sato et al., 2004; Hao et al., 2006) have been implicated in self-renewal, and it has been shown that inhibition of FGF and ERK signalling can promote mESC self-renewal in the absence of LIF (Ying et al., 2008). Prior to down-regulation of the ESC marker, Oct-4, mESCs undergo dramatic shape changes from being tightly packed and rounded, to flattened, spread cells, either following LIF withdrawal (Nichols et al., 2001) or after manipulation of the cYes or Wnt-signalling pathways (Anneren et al., 2004). Furthermore, small-molecule inhibitors that support self-renewal, such as FGF and ERK inhibitors, appear to prevent cell spreading and promote the formation of tightly packed colonies (Ying et al., 2008), which raises the possibility that self-renewal might be promoted by either inhibiting cell spreading and/or promoting cell–cell contact. In support of a role for cell–cell contact in promoting mESC self-renewal, two recent reports have shown that expression of the cell–cell adhesion molecule, E-cadherin, is required for the reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) (Chen et al., 2010; Redmer et al., 2011). However, the existence

Abbreviations: mESC, mouse embryonic stem cell; FAK, focal adhesion kinase; LIF, leukaemia inhibitory factor; ROCK, rho kinase; pPAA, plasma polymerised acrylic acid; PLGA, poly(lactic-co-glycolic acid).

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of E-cadherin-null mESCs that are unable to form tightly packed colonies but nevertheless self-renew, suggests that cell–cell contact is not an absolute requirement for the maintenance of mESC self-renewal (Larue et al., 1996; Soncin et al., 2009).

The importance of cell spreading in the regulation of mESC self-renewal, is highlighted by the fact that if mESCs are cultured on a strongly adhesive surface that forces them to spread, they down-regulate pluripotency markers even when cultured in the presence of LIF (Hayashi et al., 2007; Wells et al., 2009; Hunt et al., 2012). Similar results have been obtained following the induction of mESC cell spreading through the application of local force (Chowdhury et al., 2010; Uda et al., 2011).

The aim of the current study is therefore to investigate the relationship between cell spreading and LIF in the regulation of mESC self-renewal and differentiation, by investigating if differentiation could be inhibited in the absence of LIF by restricting the extent of cell spreading. To this end, cell spreading was regulated either by culturing mESCs on substrates with varying adhesivity, or by manipulating the cytoskeleton. The role of E-cadherin-mediated cell–cell contact, β-catenin localisation and STAT3 signalling in regulating mESC self-renewal under these different conditions was also investigated.

2. Materials and methods

2.1. Cell culture

E14 and R1 mESC lines were cultured on Nunc® tissue culture dishes coated with either 2.5% (v/v) ESC-tested foetal bovine serum (FBS) (weak adhesion) or 0.1% (w/v) gelatin and 10% (v/v) ESC-tested FBS (PAA laboratories, Yeovil, UK) in serum-free medium comprising Advanced® DMEM (Invitrogen UK Ltd., Paisley, UK) supplemented with 2 mM L-glutamine and 50 μM 2-mercaptoethanol, with or without 1000 U/ml LIF (Millipore, UK) or 10 μM ROCK inhibitor, Y-27632 (Calbiochem). Cells were passaged every 4 days. $LIFR^{-/-}$ cells were a gift from A. Smith and I. Chambers (University of Edinburgh, UK) and were maintained as above except that sIL6 receptor and IL6 were used in place of LIF (Robertson et al., 1993). To promote differentiation, embryoid bodies were produced from ESC single cell suspensions in medium containing 10% (v/v) FBS in non-adherent bacteriological plastic dishes for 4 days and then cultured for 7 days in chamber slides in the absence or presence of 1 μM retinoic acid to promote neural differentiation.

2.2. Synthetic culture substrates

RESOMER® polymer LR 708 (poly(lactic-co-glycolic acid) 70:30, Boehringer Ingelheim Bracknell, UK) was dissolved in dichloroethane at a concentration of 3% (w/w) and the polymer substratum fabricated by freeze-drying (Zhang et al., 2005). Prior to culture, the thin dry films were rinsed with PBS and sterilised by soaking in 100% ethanol overnight. Ethanol was removed by washing 3 times in sterile distilled water and once in sterile phosphate buffered saline (PBS). A negatively charged substratum with controlled surface density of carboxylic acid functionality was fabricated by Plasso Technology Ltd. (a spin-out company of The University of Sheffield, UK), via plasma polymerisation of acrylic acid with octadiene on 3.5 cm bacteriological Petri dishes (France et al., 1998). The number of carboxylic acid groups per one hundred carbon atoms was determined to be ∼12% by X-ray photoelectron spectroscopy.

2.3. Microscopy

Immunofluorescence was performed as previously described (Smyth et al., 1999). Fixed cells were incubated overnight at 4 °C with primary antibodies to Oct-4, β-catenin, pan-STAT3, pY-STAT3, pan-FAK (all from Santa Cruz, Heidelberg, Germany), Nanog (Abcam, Cambridge, UK), von Willebrand Factor, laminin-1 (all from Sigma, Poole, UK), TUJ1 (R&D Systems Europe, Ltd., Abingdon, UK), alpha-fetoprotein (ICN Biomedicals Ltd., Irvine, UK) or pFAK (Cell Signalling, Beverly, USA). Secondary antibodies were anti-rabbit-488 and -594, anti-mouse IgG2a-594, anti-mouse IgG2b-488 and -594, anti-mouse IgG1-488 (all from Invitrogen). F-actin was visualised using phalloidin labelled with Alexa fluor 594 (Invitrogen). Digital images were acquired using a Leitz DMRB microscope with 20× air and 40× oil objectives with a Sensys® camera and recorded with Metamorph® software. For sub-cellular localisation, samples were observed by confocal microscopy using a Leica TCS-SP2 microscope with 40× oil objective. Images were recorded digitally using Leica software. Quantitative image analysis used 3 × 3 pixel square masks placed at five random points over the membrane, cytoplasm and nucleus for the determination of the intensities. Images for reproduction were prepared with Adobe Photoshop and Illustrator CS-2.

2.4. siRNA and RT-PCR

siRNA duplexes (CCGAGAGAGTTACCCTACA) targeted to region 1135-53 of E-cadherin mRNA together with control siRNA duplexes were from Eurogentec Ltd. 50 nM siRNA was introduced using Lipofectamine™ (Invitrogen) according to manufacturer's instructions. RT-PCR or immunofluorescence analysis was performed 24–96 h following transfection. Real-time mRNA quantification was performed with a Rotagene 3000 (Corbett Research Pty., Australia) using Sybr Green JumpStart Taq ReadyMix (Sigma) as described (Murray et al., 2007).

3. Results and discussion

E14 mESCs cultured on FBS-coated substrates in the presence of LIF, maintained expression of the ESC marker, Oct-4, and grew as unspread, round cells in small colonies where cell–cell contact was maintained, whereas without LIF, the cells lost Oct-4 and started to spread on the substratum, leading to reduced colony formation (Fig. 1A). In the initial stages of LIF withdrawal, although the majority of cells were still Oct-4 positive, changes were observed in the actin cytoskeleton; thus, instead of being restricted to the outer membrane of cells at the periphery of ESC colonies, cortical F-actin was detected around the entire membrane of all cells after two days culture without LIF (Fig. 1A). Because the changes in cell morphology and actin redistribution occurred prior to loss of Oct-4 expression, we investigated if cell spreading itself affects self-renewal. To do this, we first reduced the adhesivity of tissue culture substrates by omitting gelatine and decreasing the FBS concentration used for coating the substratum from 10% to 2.5%. After 3 passages on the weakly adhesive substratum, E14 ESCs continued to express Oct-4 even in the absence of LIF, and cortical actin remained near the outer surfaces of cells located at the colony periphery. In contrast, Oct 4 could no longer be detected in cells cultured for the same time period on strongly adhesive substrates in the absence of LIF (Fig. 1B). Similarly, the R1 mESC line maintained expression of markers of self-renewal when cultured on the weakly adhesive substratum without LIF, indicating that this LIF-independent self-renewal is not restricted to one mESC line (Fig. 1C). Furthermore, following culture on weakly adhesive substrates for at least 12 passages in the absence of LIF, mESCs were still able to differentiate as cavitating embryoid bodies with well-defined ectodermal and endodermal epithelia, the cells of which went on to express markers for derivatives of all 3 germ layers (Fig. 1D). On the other hand, mESCs cultured on strongly adhesive

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