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The P2X7 receptor is an upstream regulator of dynamic blebbing and a pluripotency marker in human embryonic stem cells

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ABSTRACT

New methods are needed to reduce dynamic blebbing which inhibits cell attachment and survival during passaging of pluripotent stem cells. We tested the hypothesis that activation of the P2X7 receptor by extracellular ATP during passaging initiates dynamic blebbing. The P2X7 receptor was present in human embryonic stem cells (hESC), but not in differentiating cells. Extracellular ATP concentrations were 14× higher in medium during passaging. Addition of ATP to culture medium prolonged dynamic blebbing and inhibited attachment. Inhibition of P2X7 by specific drugs or by siRNA significantly reduced dynamic blebbing and improved cell attachment. When cells were incubated in calcium chelators (EGTA or BAPTA), blebbing decreased and attachment improved. Calcium influx was observed using Fura-4 when ATP was added to culture medium and inhibited in the presence of the P2X7 inhibitor. Over-expressing activated Rac in hESC reduced blebbing and promoted cell attachment, while a Rac inhibitor prolonged blebbing and reduced attachment. These data identify a pathway involving P2X7 that initiates and prolongs dynamic blebbing during hESC passaging. This pathway provides new insight into factors that increase dynamic blebbing and identifies new targets, such as P2X7, that can be used to improve the culture of cells with therapeutic potential.

1. Introduction

In many cells, the plasma membrane and underlying actin filaments interact dynamically with each other to produce cell surface blebs, which may be either dynamic (non-apoptotic) or apoptotic (Weng, 2016; Charras, 2008a; Charras & Paluch, 2008b; Sahai & Marshall, 2003; Hickson et al., 2006; Paluch et al., 2013; Julian & Olson, 2015). Dynamic blebbing occurs in three phases: nucleation, expansion, and retraction (Charras, 2008a). When the actin cortex separates from the overlying plasma membrane, pressure within the cell pushes a bleb through the ruptured actin. The bleb expands to its full size until actin reassembles beneath the bleb membrane and with myosin eventually retracts the bleb into the cell.

Dynamic blebbing occurs in a variety of cell types including fibroblasts, endothelial and mesenchymal cells, cancer cells, immune cells, germ cells, amoeba, parasites, bacteria (Khajah & Lugmani, 2016), and human embryonic stem cells (Weng, 2016; Guan et al., 2015a; Guan et al., 2015b). Dynamic blebbing has become a topic of recent interest because of its wide spread distribution in cells and its role in physiological and disease-related processes. Dynamic blebbing occurs as a normal process during cytokinesis (Charras, 2008a; Boss, 1955; Porter et al., 1973; Fishkind et al., 1991; Boucrot & Kirchhausen, 2007), and in some cells, dynamic blebbing is the driving force that enables cell migration (Hickson et al., 2006; Paluch et al., 2013; Maekawa et al., 1999). For example, in amphibian and fish embryos, dynamic blebbing facilitates cell movement during embryonic development (Blaser et al., 2006; Trinkaus, 1973; Wourms, 1972; Trinkaus, 1996), and cells dissociated from amphibian embryos can migrate using bleb-like protrusions (Holtfreter, 1943; Kubota, 1981; Satoh et al., 1976). Inverse blebs were recently shown to play a role in lumen formation during angiogenesis (Gebala et al., 2016). In addition to the role of blebbing in normal cells, dynamic blebbing is a factor in some types of disease. For example, *Entamoeba histolytica* cells invade organs, such as the liver, using bleb-driven motility (Maugis et al., 2010) and blebbing has been reported to enhance the motility, migration, and invasion of breast cancer cells (Laser-Azogul et al., 2014).

Human embryonic stem cells (hESC) undergo vigorous dynamic blebbing when they are freshly plated on Matrigel-coated dishes (Weng, 2016; Guan et al., 2015a; Guan et al., 2015b). hESC bleb longer and produce more blebs than other cell types during passaging. Dynamic blebbing slows cell attachment, and cells that fail to attach eventually undergo apoptotic blebbing and die. We have previously shown that in freshly passaged hESC, dynamic and apoptotic blebbing are separated temporally and are mechanistically distinct (Weng, 2016). Because

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dynamic blebbing interferes with cell attachment, ROCK inhibitors (such as Y27632) or blebbistatin, a myosin II inhibitor, are often added to culture media to block activation of myosin II, which in turn stops dynamic blebbing and facilitates cell attachment and survival (Watanabe et al., 2007; Harb et al., 2008; Li et al., 2009; Ohgushi et al., 2010). However, these inhibitors are not entirely specific and seem to cause excess spreading and stressing of cells during treatment (Ikenouchi & Aoki, 2016). Potent inhibitors of the ROCK pathway may not be suitable for use in expanding and culturing hESC and iPSC in therapeutic applications. Ideally, dynamic blebbing could be controlled by alternative methods that are not stressful and which do not have off-target effects.

While the downstream effectors of dynamic blebbing have been characterized (Watanabe et al., 2007; Harb et al., 2008; Li et al., 2009; Ohgushi et al., 2010; Aoki et al., 2016; Weng et al., 2015), little is known about what initiates dynamic blebbing in hESC upstream of Rho. P2X7 receptors are ATP-gated ion channels that upon activation lead to an influx of extracellular calcium. Binding of ATP to P2X7 induces opening of a channel selective for small cations, and with time the pore becomes larger allowing molecules up to 900 kD to enter (Volonte et al., 2012). P2X7 activity can be found in a number of cell types where it mediates the influx of Ca^{2+} and Na^{+}. In 2007, dynamic blebbing was observed in calvarial cells from wild type, but not P2X7 knock-out mice, suggesting the P2X7 receptor might regulate dynamic blebbing in these cells (Panupinthu et al., 2007). P2X7 receptors were coupled to activation of phospholipase D and A_2, and inhibition of these phospholipases suppressed ATP induced dynamic blebbing in osteoblasts (Panupinthu et al., 2007). ATP induced dynamic blebbing in several other cell types, including hepatocytes (Nicotera et al., 1986), thymocytes (Zheng et al., 1991), and macrophage cell lines (Verhoef et al., 2003). These data prompted us to look for P2X7 receptors in undifferentiated hESC and to examine their role in dynamic blebbing.

The specific purpose of this study was to test the hypothesis that dynamic blebbing is induced during passaging of hESC by activation of P2X7 receptors that respond to increased extracellular ATP released during cell passaging. Activation of P2X7 enables calcium influx which in turn initiates dynamic blebbing. By characterizing the initiation phase of dynamic blebbing in hESC, we expect to identify new targets and strategies to facilitate cell attachment during passaging, to improve plating efficiency and methods of hESC culture, reduce stress during culture, and to better understand the behavior and motility of cells representative of the epiblast in early post-implantation embryos.

2. Materials and methods

2.1. Antibodies and chemicals

Mouse anti-P2X7 antibody was purchased from Proteintech Group (Chicago, IL). Rabbit Oct4 antibody was purchased from Abcam (Cambridge, MA). Goat anti-mouse antibody IgG secondary antibody conjugated to Alexa Fluor 594 was purchased from Sigma-Aldrich (St Louis, MO) and goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluor 488 was purchased from Invitrogen (Carlsbad, CA). Apyrase, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA), and ethylene glycol tetraacetic acid (EGTA) were purchased from Sigma-Aldrich (St. Louis, MO). The P2X7 inhibitors, AZ11645373 and KN-62, were purchased from Tocris Bioscience (Minneapolis, MN). Vectashield antifade mounting medium with 4′ 6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Burlingame, CA).

2.2. Cell culture

Experiments were done using H9 hESC purchased from WiCell (Madison, WI) as frozen vials then expanded and frozen down in our lab as described in detail previously (Lin & Talbot, 2011; Behar et al., 2012). Before setting up experiments, hESC were expanded by plating on Matrigel-coated 6-well plates (Thermo Fisher Scientific, Waltham, MA). Cultures were maintained in mTeSR medium (Stem Cell Technologies, Inc. Vancouver, Canada.) in 5% CO_2 at 37 °C. When colonies reached 70%–80% confluency (equal to about 1–1.5 million cells), hESC were used in experiments. For single cell experiments, hESC were detached by treatment with Accutase (Affymetrix eBioscience, San Diego, CA) for 3 min. A 1 ml pipette was used to rinse cells off the plate by pipetting the Accutase solution repeatedly inside the well. Once the cells detached from the plate, Accutase was neutralized using mTeSR medium. To ensure that cells were single, they were passed through an 18-gauge syringe needle (0.33 × 12.7 mm) (BD, Franklin Lakes, NJ) before plating on a new culture dish. Dynamically blebbing cells were studied immediately after plating on Matrigel-coated dishes, while apoptotically blebbing cells were studied after 1.5 h of incubation on non-coated 35 mm high dishes (Ibidi, Madison, WI), which do not enable attachment.

2.3. RT-PCR

Total RNA was extracted from cultured hESC using an RNeasy Mini Kit (Qiagen, Valenica, CA) and RNA was checked for purity and degradation using the Agilent 2100 Bioanalyzer as described previously (Bahl et al., 2016). Only samples with a RIN (RNA integrity number) of 7 or higher were used for experiments. cDNA was prepared using a Qiagen RT2 First Strand Kit (Qiagen, Valencia, CA) from 400 ng RNA. cDNA was amplified with PCR using GAPDH and actin primers to verify that the cDNA synthesis reaction worked. Qiagen HotStarTaq Master Mix (Qiagen, Valencia, CA) and the BioRad Thermal Cycler (BioRad, Hercules, CA) were used for the PCR reaction. Primers used in this paper were as follows: P2X7: 5′-TAT CCC TGG TGC AAG TGC TGT- (forward), 5′-AGC TGT GAG GTG GTG ATG CAG- (reverse), Actin: 5′-ATC TGG CAC CAC ACC TTC TAC-(forward), 5′CGT CAT ACT CCT GCT TGC TGA-(reverse). GAPDH: 5′-GGA GCC AAA AGG GTC ATC ATC-(forward). 5′-AGT GAT GGC ATG GAC TGT GGT-(reverse). Lonza DNA FlashGels were used to run the PCR products which were imaged using a Lonza FlashGel imaging system (Lonza, Walkersville, MD).

2.4. Immunostaining

hESC were plated in 6-well plates at 20% confluency. When cells reached 70% confluency, they were washed with PBS and fixed in 4% paraformaldehyde/PBS at room temperature for 15 min using procedures described in detail previously (Weng, 2016). After washing with PBS, cells were incubated with blocking solution (10% normal serum from the same species as the secondary antibody in PBS and 0.1% Triton X-100) for 30 min at room temperature. Cells were then washed with PBS and incubated with 1%BSA/PBS for 20 min at room temperature. Cells were incubated with mouse anti-P2X7 antibodies and rabbit anti-OCT4 antibodies at 4 °C overnight, then washed with PBS two times the next day, and incubated at room temperature for 1 h with goat anti-mouse secondary antibodies conjugated to Alexa 594 and goat anti-rabbit secondary antibodies conjugated to Alexa 488. Cells were washed with PBS and mounted with Vectashield containing 4′ 6-diamidino-2-phenylindole (DAPI) for nuclear staining, and examined with a Nikon Eclipse T1 inverted microscope equipped with Elements deconvolution software.

In some experiments, embryoid bodies that had begun to differentiate were labeled with the P2X7 antibody. Embryoid bodies were prepared by incubating small clumps of hESC in hESC medium (400 ml Knockout DMEM, 100 ml fetal bovine serum, 5 ml 10 mM non-essential amino acid, 5 ml 200 mM L-glutamine, and 5 ml 100× beta-mercaptoethanol) without bFGF on bacterial-grade Petri dishes. Embryoid bodies were formed in suspension and fed every 2–3 days depending upon density and size. After 5 days, embryoid bodies were plated on Matrigel-coated dishes for another 3–4 days and then labeled with the antibody to P2X7 and OCT4 as described above.