Epithelial-mesenchymal transition in rhesus monkey embryonic stem cell colonies: the role of culturing conditions

Birgit Maranca-Hüwel · Hans-Werner Denker

Received: 6 February 2009 / Accepted: 2 January 2010 / Published online: 5 February 2010 / Editor: J. Denry Sato © The Society for In Vitro Biology 2010

Abstract Colonies of rhesus monkey embryonic stem cells (rhESC; cell line R366.4) have been described before to show a spatially ordered process of epithelial–mesenchymal transition in vitro. In the present investigations, we have studied variables of culturing conditions which influence the reproducibility of the formation of crater-like ingression centers in the colonies. Critical parameters are found to be age and density of mouse embryonic fibroblast (MEF) feeder cell layers, the mode of mitotic inactivation of the MEFs (mitomycin C, or irradiation), and the mode of rhESC isolation during subculturing (enzymatic/mechanical cell cluster isolation; type of enzyme). The described culturing system appears to offer a reproducible in vitro model potentially useful for studies on cellular processes involved in gastrulation in the primate.

Keywords Embryonic stem cells · Epithelial–mesenchymal transition · Gastrulation · Ingression centers · Feeder cell layers

Introduction

Colonies of the rhesus monkey ES cell line (rhESC) R366.4 (WiCell Research Institute, Madison, WI) have recently been described to show a relatively ordered process of epithelial–mesenchymal transition (EMT) which may make them useful as an in vitro model for gastrulation in primates (Behr et al. 2005). This culture system uses flat ES cell

B. Maranca-Hüwel · H.-W. Denker (⊠) Institut für Anatomie, Universität Duisburg-Essen, Universitätsklinikum, Hufelandstr. 55, 45122 Essen, Germany e-mail: hans-werner.denker@uni-due.de colonies which imitate the topography of cell-cell interactions during primate gastrulation more closely than conventional embryoid bodies (EBs) would do (the latter being more appropriate as a model for mouse egg cylinders; discussed by Denker 2004). Formation of a central craterlike depression in these colonies (in which cell ingression, accompanied by EMT appeared to occur) was found when these cells were grown on mitotically inactived mouse embryonic fibroblasts (MEFs) used as feeder layers. Cells attaining a lower layer position via these ingression centers (ICs) were found to show typical signs of EMT, e.g., loss of the marker proteins E-cadherin and ZO-1, while the colonies upregulated the expression of the gene Snail2, a key regulator of EMT (Behr et al. 2005). Since this culture system may be of considerable interest as a model allowing to experimentally study cellular processes and genes involved in germ layer formation/gastrulation-associated events in primates, reproducibility of the ordered EMT process is crucial. In the present paper, we are describing the results of a series of experiments showing how the phenomenon is influenced by the age and density of MEF feeder cell layers, the mode of mitotic inactivation of MEFs, and the mode of rhESC isolation during subculturing.

Materials and Methods

General and experimental groups. The basic design of cell culture experiments was as described by Behr et al. (2005). In brief, rhESCs (cell line 366.4, originally obtained at passage 14 from WiCell Research Institute, http://www.wicell.org; Thomson et al. 1995) were cultured on mitotically arrested MEFs at 37°C and 5% carbon dioxide in air. RhESC medium consists of Knockout Dulbecco's modified Eagle's medium (Knockout-DMEM, Gibco-Life

Technology, Karlsruhe, Germany, http://www.invitrogen. com), supplemented with 20% Knockout serum replacement (Knockout-SR, Gibco), 0.1 mM β -mercaptoethanol (Serva, Heidelberg, Germany, http://www.serva.de), 1% nonessential amino acids (NEAS; Gibco), 1 mM glutamine (Gibco), and 2 ng/ml basic fibroblast growth factor (bFGF; Gibco). The medium was changed daily. Cell colonies composed of closely packed cells were incompletely dissociated every 3 to 4 d by incubation with either collagenase IV or trypsin (details see below) and pipetting before transfer onto freshly prepared feeder cells. In some experiments (see below), rhESCs were cultured on laminin instead of MEFs.

Cell culture dishes were purchased from Nunc (Langenselbold, Germany, http://nuncbrand.com). For immunohistochemical (IHC) labeling, cells were grown on glass coverslips (No. 1001, Assitent/Hecht, Sondheim, Germany, http://www.hecht-assistent.com) in 12-well plates (BD Falcon, San Jose, CA) for 4 d as described above. For morphology (resin sections), they were grown on plastic (Thermanox; Nunc) coverslips (see below).

Experimental groups:

- A RhESCs grown on MEFs
- A1 Mitotic inactivation of MEFs by mitomycin C vs. irradiation
- A2 Variation of feeder cell density
- A3 Variation of MEF preculture conditions
- A4 Dissociation of rhESCs at subculturing by collagenase IV vs. trypsin–EDTA
- B Feeder-free culture of rhESCs on laminin

MEF feeder cell layers. NMRI mouse embryos were obtained at day 14 postcoitum (day of vaginal plug= day 1); placenta, head, heart, liver, and intestinal tract were removed. The remaining parts of the embryos were dissociated in 15 ml 0.2% trypsin (Gibco) in Dulbecco phosphate-buffered salt solution without Ca⁺⁺ and Mg⁺⁺ (DPBS; Gibco), aided by mechanical mincing with a scalpel, followed by magnetic stirring with glass beads in an Erlenmeyer flask for 25 min at room temperature (RT). Cells and cell clusters were centrifuged at $110 \times g$ for 5 min at room temperature, then 35 ml MEF medium were added (DMEM (Gibco) with 15% fetal calf serum (FCS), Lot 40G6301F (Gibco), 0.1 mM β-mercaptoethanol, 50 IU/ml penicillin and 50 µg/ml streptomycin (Gibco), 1% NEAS, 2 mM glutamine). Cell pellets were resuspended and cultured in 10 cm Petri dishes from BD Falcon (http:// www.bdbiosciences.com; originally 3 to 4 embryo equivalents per dish, passage 0) at 37°C, 5% carbon dioxide in air. Every 2 to 3 d, cells were passaged by resuspending in 0.2% trypsin/DPBS (see above) at room temperature for 1 min and subcultured at a ratio of 1:3 or 1:5. Cells were used for experiments no earlier than passage 2 and no later than passage 5. In some cases, cells were frozen in liquid nitrogen at passage 0 to 1 (using 25% FCS and 10% dimethyl sulfoxide (DMSO), Merck, Darmstadt, Germany, http://www.merck.de); frozen cells were thawed quickly at 37°C, resuspended in MEF medium, centrifuged and cultured for 2-3 d. In order to be used for coculture with rhESCs, MEFs were mitotically arrested either with mitomycin C or by irradiation (see below).

Mitomycin C treatment of MEFs. In order to be used as feeder cells for rhesus embryonic stem cells, confluent MEFs (passages 2-5) were mitotically inactivated by treatment with 0.06 mg mitomycin C (Serva, http://www. serva.de) in 6 ml MEF medium for 2 h at 37°C and 5% carbon dioxide in air. Thereafter, the adherent mitomycin-C-treated mouse embryonic feeder cells (MCMEFs) were thoroughly washed three times with DPBS and released with 0.2% trypsin/DPBS. They were seeded onto 0.1% gelatin-coated plates (6 cm diameter) at a density of $5.5 \times$ 10^4 cells/cm² with MEF medium (the culture plates had previously been coated with 0.1% autoclaved gelatin (Fluka, Buchs, Switzerland, http://www.sigmaaldrich. com), dissolved in DPBS, at least for 2 h at 4°C, and washed once with DPBS). After 16-24 h, the MEF medium was replaced by rhESC medium. The next day, these MCMEF plates were ready to be used for coculturing with rhESCs. The culture on MCMEFs was used as the routine method for propagation of rhESCs unless stated otherwise.

Irradiation of MEFs. Two-day-old confluent MEFs (45 culture dishes at passage 4) were thoroughly washed with DPBS, released with 0.2% trypsin/DPBS (see above), suspended in MEF medium, collected in two vials (per 50 ml), and centrifuged at $110 \times g$ for 5 min. After discarding the supernatant, the cells were suspended in MEF medium and irradiated with 30 Gy on ice. Irradiated MEFs (IRMEFs) were centrifuged once more and resuspended in 45×1.8 ml freezing medium (= MEF medium+ 10% FCS and 10% DMSO) for cryoconservation. After thawing, the cells were seeded onto gelatin-coated plates at a density of 5.5×10^4 cells/cm² with MEF medium. Transfer to rhESC medium and coculture with rhESC was performed in the same way as with MCMEFs.

Cell number counting and mycoplasma testing. Cell densities were determined by counting in a Neubauer chamber. At thawing, cells were routinely tested for mycoplasma contamination by 4.6-diamidino-2-phenylindole (DAPI) staining (DAPI ×2 HCl, Serva, http://www.serva.de). Only mycoplasma-free cells were used.

Culture of rhESCs on MEFs. RhESCs (cell line 366.4. originally obtained at passage 14 from WiCell Research Institute, http://www.wicell.org; Thomson et al. 1995) were cocultured with mitotically arrested MEFs (MCMEFs or IRMEFs) on gelatin-coated plates in rhESC medium (see above). After 3 to 4 d, cultures were split by releasing cells from the culture plate with 0.1% collagenase IV (Gibco) for 15 min at 37°C or, alternatively, with 0.05% trypsin/ 0.53 mM EDTA (Gibco; experimental group A4) for 5 min at room temperature and by mechanically dissociating the colonies into small cell clumps by aspirating into a plastic pipette. It was important to avoid complete dissociation, i.e., small cell clumps must remain. Subsequently, the cells/clumps were washed, centrifuged twice with Knockout-DMEM at 250×g for 5 min, and seeded onto new MCMEFs or IRMEFs on gelatin-coated plates in a dilution of 1:3 or 1:4 (Amit et al. 2000). Every day, the culture medium was changed and living cells were morphologically checked with a Zeiss Axiovert 25 microscope (Zeiss, Göttingen, Germany, http://www.zeiss.de).

Culture of rhESCs on laminin. For feeder-free culturing, rhESCs (taken from routine propagation cultures on MCMEFs) were seeded onto laminin-coated dishes and were cultured in conditioned medium (see below). Dishes were coated with 2 μ g/cm² laminin (Tebu, Offenbach, Germany, http://www.tebu-bio.com) for 1 h at room temperature and washed once with DPBS. For subculturing, rhESCs were released with collagenase IV (see above) and were cultivated in conditioned rhESC medium (see below) with daily medium changes (Xu et al. 2001). No MEFs were observed among rhESCs already after few passages on laminin. In order to make sure to avoid any residual MEF contamination, rhESCs were passaged ten times on laminin, then frozen down for cryoconservation, and thawed for culturing when needed.

For obtaining conditioned medium, confluent MCMEFs were cultured in rhESC medium for 12 d. The medium was collected daily and was kept at 4°C, centrifuged at $6,200 \times g$ for 20 min at room temperature, and sterile-filtered with Gelman Vacucap 90PF filter unit 0.8/0.2 µm Supromed (PALL, Dreieich, Germany, http://www.pall.com). Now the conditioned medium was ready to be stored at -20° C; 0.4 µg/100 ml bFGF was added before using for cell culture.

Cryoconservation. For cryoconservation, rhESCs were suspended in medium supplemented with 10% DMSO and 10% Knockout-SR and were frozen for 1 d at -80° C before they were transferred to liquid nitrogen. For use, vials were quickly thawed at 37°C, and small volumes of rhESC medium were added as follows: 2×50 µl after 30 and 60 s, 2×100 µl after 2 and 3 min, 2×200 µl after 4 and 5 min, 2×300 µl after 6 and 7 min, 2×400 µl after 8 and 9 min, 2×500 µl after 10 and

11 min, $2 \times 600 \ \mu$ l after 12 and 13 min, $2 \times 700 \ \mu$ l after 14 and 15 min, $2 \times 800 \ \mu$ l after 16 and 17 min, and $2 \times 900 \ \mu$ l after 18 and 19 min. Subsequently, the cell suspension was spun at $250 \times g$ for 5 min and seeded on prepared MEF monolayers.

Immunohistochemistry and actin staining. For the IHC labeling of rhESCs, the tight junction-associated protein ZO-1 was selected (cf. Behr et al. 2005). Rabbit polyclonal antibody to human ZO-1 (61-7300) was obtained from Zymed WAK Chemie (Bad Homburg, Germany, http:// www.zymed.com) and used in 1:200 dilution in DPBS supplemented with 0.5% bovine serum albumin (BSA, Gibco). The secondary antibody, Alexa Fluor 633conjugated goat anti-rabbit immunoglobulin G (A-21071), was obtained from Molecular Probes (MoBiTec, Göttingen, Germany, http://www.mobitec.de) and used in 1:200 dilution in DPBS-BSA. Immunostaining was performed as follows: Samples were fixed in a 1:1 mixture of ethanol/ acetone for 10 min at RT. Nonspecific binding of the antibodies was blocked by incubation with DPBS-BSA (0.5%) for 15 min, and this was followed by incubation with the primary antibody (see above) over night at 4°C in a moist chamber. The primary antibody was omitted in control stainings. Thereafter, cells were rinsed in DPBS-BSA and incubated with the fluorescence-conjugated secondary antibody (see above) for 60 min at RT. For simultaneous F-actin staining, this latter solution was combined with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin (obtained from Sigma-Aldrich, Taufkirchen, Germany) at a concentration of 25 µg/ml. After rinsing with DPBS-BSA (and, briefly, with aqua dest), the samples were mounted in Vectashield (Vector, Burlingame, CA, http://www.vectorlabs.com).

Confocal laser-scanning microscopy (CLSM). Confocal microscopy was performed using a Zeiss Axiovert 100M microscope attached to a confocal laser-scanning unit (model LSM 510; Carl Zeiss) as described previously (Heneweer et al. 2002, 2003). Two helium-neon lasers with output at 543 and 633 nm were used as excitation sources. TRITC fluorescence was recorded with a 560-nm longpass filter, Alexa Fluor 633 fluorescence with a 650-nm longpass filter. Optical tomography was performed at 1 µm intervals using a 10-fold or a 20-fold objective, respectively, and a pinhole size corresponding to a value of 1.0 of the airy disk. Each slice was scanned eight times followed by averaging of the obtained images to improve the signal-to-noise ratio. Adobe Photoshop software (version 5.0; Adobe Systems Inc., San Jose, CA, http://www.adobe.com) was used for the arrangement of RGB color images.

Resin sections. Cells were grown on Thermanox coverslips as described above. For subsequent fixation and plastic

embedding, samples were rinsed once in Moscona's salt solution (Moscona 1952) and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, postfixed with 1% OsO_4 in cacodylate buffer, dehydrated with ethanol, and embedded in epoxy resin. The embedded cells were separated from the Thermanox coverslips by heating on a hot plate (coverslip down) at 100°C for 5 min followed by pealing the coverslip off. For light microscopy, 0.5-µm sections were mounted on glass coverslips and stained with a mixture of 1% methylene blue, 1% azur II, and 1% toluidine blue in 1% disodium tetraborate solution.

Results

- A Stem cells (rhESCs) grown on MEFs
- A1 Mitotic inactivation of MEFs by mitomycin C vs. irradiation

Culture on MCMEFs. When grown on MCMEFs (precultured for about 2 d with medium switch, see experimental group A3 below) for 4 d, the majority of rhESCs colonies developed crater-like ICs (mostly one such crater per colony) as described previously (Behr et al 2005). A cross (vertical) section of such a colony with IC is shown in Fig. 1A, B. In agreement with what has been described in more detail before (Behr et al. 2005), the rhESC colony was found to consist of two main layers: one densely packed layer of (epithelial type) cells on top of the MEF feeders and a more loosely packed layer below it. Both are interconnected in the central region where a deep depression, the crater-like IC, is found. In stacks of horizontal optical confocal laser-scanning microscopy sections (Fig. 2A-I), this depression appears as a dark hole in the upper sections of the stack and is filled with cells in the lowermost sections. In order to be able to distinguish between rhESCs and MEFs,

actin staining (labeling all cells) and ZO-1 IHC (labeling rhESCs, not MEFs) were employed. ZO-1 is an epithelial marker which at the same time allows monitoring phenotypic changes of rhESCs as occurring in the course of EMT. As Fig. 2*D*–*F* shows, cells in the middle and lower layers of the colony have downregulated ZO-1 expression which is in agreement with previous findings on EMT in these colonies (Behr et al. 2005).

Recommended method. Formation of ICs was observed regularly with the following protocol: MEFs are pretreated with MC as described above. Feeder cell density (as used at seeding) was 5.5×10^4 cells/cm². After 24 h of MCMEF culture, the MEF medium is switched to rhESC medium (see above). After further 24 h of MCMEF culture, the rhESCs are added for the start of coculture and colony formation. This is followed by standard culture of rhESCs on MCMEFs, with subculturing done every 3 to 4 d after collagenase treatment (see above) aided by mechanical dissociation into small clumps of cells (daily medium change).

Culture on IRMEFs. The method used for mitotic inactivation of MEFs turned out to be one critical factor for IC formation. When MEFs were mitotically inactivated by irradiation and used thereafter as feeder cells (IRMEFs) as described above, rhESCs formed dense multilayered colonies on these; however, no ICs were observed after the regular coculture period of 4 d (Fig. 3*A*–*L*), in contrast to culture on MCMEFs. RhESCs instead tended to pile up on top of the IRMEF layer. Cells in the lower layers of the colonies showed decreased ZO-1 staining indicating that a phenotypic change is occurring during piling up, in these colonies on IRMEFs, suggesting a diffuse EMT in contrast to formation of a localized IC (as on MCMEFs). The loss of IC formation capacity occurred progressively during re-



Figure 1. Vertical cross section of a rhESC colony growing on MCMEFs, showing morphology after resin embedding. (*A*) Semithin section. The IC is seen as a crater-like pit at whose margins rhESCs

extend under the MEF feeder cell layer and reach down to the substratum. *Scale bar*, 100 μ m. (*B*) Schematic.



Figure 2. Horizontal sections through a rhESC colony with IC, confocal laser microscopy. Representative sections selected from the stack of optical sections through a rhESC colony growing on MCMEFs (MEF preculture for 2 d with medium switch). Actin (labeling both ESCs and MEFs) is shown in *red (left column)*, ZO-1 (labeling only ESCs) in *green (middle column)*; *right column* composite. (*A*–*I*) Horizontal (*X*–*Y*) sections, (*J*–*L*) vertical (*X*–*Z*) sections. (*A*, *D*, *G*) upper, (*B*, *E*, *H*) middle, (*C*, *F*, *I*) lower level. The

positioning of the vertical sections (J-L) is indicated by a *white diagonal line* in (*H*). An IC is seen as a dark hole in the upper level section (*A*, *D*, *G*). The epithelial marker ZO-1 is strongly expressed in the ESCs of the upper layer of the colony (*D*) and in the cells lining the bottom of the IC (*center* of *E*) with accentuation of zonular tight junctions as typical for epithelial cells, and it is only weakly expressed and diffuse in most middle and lower layer cells (*E*, *outside of center*; *F*) indicating downregulation of the epithelial phenotype.

peated passaging on IRMEFs, whereas during the first culturing period on IRMEFs (after MCMEF), some colonies did still show ICs.

A2 Variation of feeder cell density. In a separate series of experiments, it was tested whether doubling the standard

feeder cell density would have any effect on IC formation in cultures on MCMEFs vs. IRMEFs. Both types of MEFs were seeded in a density of 5.5×10^4 or 11×10^4 cells per cm², and rhESCs were added for coculture as described. With both MEF densities, rhESC colonies formed ICs regularly on MCMEFs, whereas on IRMEFs no ICs were observed.



Figure 3. Horizontal sections through a rhESC colony lacking an IC, confocal laser microscopy. Representative sections selected from the stack of optical sections through a rhESC colony growing on IRMEFs (MEF preculture for 2 d with medium switch). Actin (labeling both ESCs and MEFs) is shown in *red (left column)*, ZO-1 (labeling only ESCs) in *green (middle column)*; *right column* composite. (*A*–*I*) horizontal (*X*–*Y*) sections, (*J*–*L*) vertical (*X*–*Z*) sections. (*A*, *D*, *G*) Upper, (*B*, *E*, *H*), middle, (*C*, *F*, *I*) lower level. The positioning of the vertical sections (*J*–*L*) is indicated by a *white vertical line* in (*H*). In

A3 Variation of MEF preculture conditions. Three protocols for MEF pretreatment were tested, as follows:

(a) Four-hour preculture in MEF medium

MEFs were only allowed to attach to the plate for 4 h but not precultured any further, then rhESCs were added for coculture in rhESC medium; colony morphology was checked after 4 d of culture. No ICs were observed, neither

contrast to rhESCs growing on MCMEFs, no IC is observed in any section (A, D, G). The epithelial marker ZO-1 is strongly expressed in the ESCs of the upper layer of the colony (D); margin of colony in E) with accentuation of zonular tight junctions as typical for epithelial cells, and it is only weakly expressed and diffuse in most middle and lower layer cells (*center* of E; F). This indicates that downregulation of the epithelial phenotype is occurring in deep layer cells even though no IC is formed in this case (in contrast to culture on MCMEFs, cf. Fig. 2).

on MCMEFs nor on IRMEFs. On MCMEFs, rhESCs grew in dense, exceedingly large plateau-like colonies that were overgrown by feeders, and tended to form wide ring-like elevations (Fig. 4A-D). On IRMEFs, rhESC colonies were also large (although not as exceedingly as on MCMEFs). However, they were not densely packed but more scattered; nevertheless, they were overgrown by the feeders also in this case. On both MCMEFs and IRMEFs, rhESC colonies

Figure 4. MEF pretreatment protocols which were found unsuitable for IC formation in rhESC colonies. Confocal laser microscopy; representative horizontal sections selected from the stacks of optical sections through two rhESC colonies. (A-D) MCMEFs, preculture for only 4 h in MEF medium (experimental group A3a); ZO-1 labeling (green). rhESCs form a wide ring wall towering the colony (upper sections of the stack (A, B) but no IC. (E-H)IRMEFs, preculture for 2 d without medium switch (experimental group A3b), composite of ZO-1 (green) and actin (red) labeling. The rhESCs (vellow/green) are loosely arranged in the lower sections (G, H) and intermingled with MEFs (red) even in the uppermost optical sections of the colony (E, F). No IC formed.



Figure 5. Comparison of dissociation by collagenase vs. trypsin-EDTA; confocal laser microscopy. Representative horizontal sections selected from the stacks of optical sections through two rhESC colonies growing on MCMEFs. RhESC colony formed after subculturing with dissociation by collagenase (A-C); rhESC colony after subculturing using trypsin–EDTA (D–F). Actin (labeling both ESCs and MEFs) is shown in red. Horizontal (X-Y) sections: (A, D) upper, (B, E) middle, (C, F) lower level. In contrast to rhESCs subcultured with collagenase dissociation (A-C), no IC is observed in the colony subcultured with trypsin-EDTA use (D-F).



penetrated through the feeder layers to the bottom of the dish.

(b) Two-day preculture in MEF medium without medium switch:

After seeding, MEFs were precultured in MEF medium for 2 d without medium switch. After addition of rhESCs, coculture was done in rhESC medium for 4 d. No ICs were observed, neither in MCMEF nor in IRMEF cocultures. As in (a), rhESC colonies were very large and dense on MCMEFs and somewhat smaller and less densely packed on IRMEFs (Fig. 4E-H). In both cases, the colonies penetrated the feeder cells layer and reached down to the substratum, but they were only incompletely covered by the feeders.

(c) Two-day preculture with switch from MEF medium to rhESC medium

After seeding, MEFs were precultured in MEF medium for 1 d which was exchanged for rhESC medium the next d. Again 1 d later, rhESCs were added and cocultured in rhESC medium for 4 d. On MCMEFs, multilayered rhESC colonies were formed showing typical ICs as described above (experimental group A1; Fig. 2), whereas on IRMEFs, no ICs were observed (Fig. 3). In both cases, the rhESC colonies were comparable insofar as they were compact and surmounted considerably the level of the feeder cells. As in (a) and (b), they reached down to the substratum, i.e., appeared to have pushed the feeder cells



Figure 6. Feeder-free culture of rhESCs on laminin; confocal laser microscopy. In contrast to rhESCs growing on MCMEFs, the cells form monolayers in this case but no IC is observed. Actin is shown in red (A), ZO-1 in green (B). (C) Composite.

aside (or MEFs had withdrawn) at least in the center of the colonies. The only difference between MCMEFs and IRMEFs was that a crater-like depression (IC, where cells showed signs of locally restricted EMT) was missing in cultures on IRMEFs.

A4 Dissociation of rhESCs at subculturing by collagenase IV vs. trypsin–EDTA. This series of experiments was done in order to check whether the type of proteinase used for dissociation of rhESCs (at subculturing) would influence IC formation. Only MCMEFs were studied in this case. Collagenase was compared with trypsin/EDTA treatment (concentrations and conditions see above). When collagenase was used, the described ICs were formed regularly, whereas no ICs were obtained after trypsin/EDTA use (Fig. 5A-F). It also turned out to be important for IC formation not to let the dissociation proceed too far, i.e., not until formation of a single cell suspension; instead, small clumps of rhESCs should always remain after digestion and mechanical dissociation.

B Feeder-free culture of rhESCs on laminin. When cultured on laminin (i.e., without MEFs), rhESCs formed monolayers, no multilayered colonies. No ICs were observed after 4 d (and up to 7 d) of culture (Fig. 6A-C).

Discussion

Cell biological processes and genes involved in the formation of germ layers (specifically mesoderm and endoderm) in cultures of ESCs are recently receiving considerable interest in ongoing research done by various groups, as illustrated by a number of recent papers focusing on EMT/gastrulation equivalent processes observed in ESC colonies (Behr et al. 2005; Gadue et al. 2006; ten Berge et al. 2008; Nakanishi et al. 2009). Remarkably, not only diffuse EMT events without any spatial order are being

found in ESC cultures but there is a recent interest in localized ICs observed in dense cultures, in particular in EBs, and these are now increasingly often addressed as centers of gastrulation events or even as primitive streaks (Gadue et al. 2006; ten Berge et al. 2008; Nakanishi et al. 2009). The obvious reason for this new focus of research is that if primitive streak-like structures appear in these various types of "EBs", any morphological order attained by these would greatly facilitate orientation of the investigator and would help with, e.g., focusing separately on endoderm formation (anterior primitive streak) vs. formation of the various parts of mesoderm (middle and posterior primitive streak; ten Berge et al. 2008; Nakanishi et al. 2009). This can facilitate the study of details of the regulation of involved gene activation cascades, e.g., Wnt and bone morphogenetic protein (BMP) signaling. In addition, using culturing conditions under which EMT occurs in a structured, localized manner like in a primitive streak may make these culture systems more valuable as a model for mammalian embryology.

The molecular regulation of EMT in ESC cultures is a very actual topic of ongoing research (Gadue et al. 2006; Eastham et al. 2007; ten Berge et al. 2008; Ullmann et al. 2008; Nakanishi et al. 2009). In coculture with feeder cells, regulatory/signaling molecules can of course partially be provided by the feeder cells, and consequently, the types of molecules released by feeder cells are being actively studied in a number of laboratories (Diecke et al. 2008; Eiselleova et al. 2008; Villa-Diaz et al. 2009). In addition to chemical signaling, physical factors provided by the three-dimensional architecture of cell aggregates and/or extracellular matrices have considerable influence on cell differentiation as observable in various in vitro culture systems (Hohn and Denker 1994). Whether this is to be seen as a direct effect on cell differentiation itself is a matter of debate. Even if cell differentiation primarily occurs stochastically (Losick and Desplan 2008), there is ample evidence that physical constraints largely modulate or even determine subsequent cell sorting and migration processes

and are of particular importance for pattern formation in embryology, specifically for basic body plan establishment (discussed by Denker 2004; Patwari and Lee 2008). In primates (including the human), the morphology of embryos at the primitive streak stage is considerably different from that of the mouse: flat embryonic disk vs. egg cylinder (with its "germ layer inversion"). Spherical EBs formed in vitro in suspension culture somewhat mimic the situation found in mouse egg cylinders (ten Berge et al. 2008). On contrast, flat cultures (which nevertheless still allow the formation of several cell layers) must be considered a better model for the situation in primates (with a presumable predominance of planar signaling; discussed by Denker 2004). The model used in the present investigation (Behr et al. 2005) allows to study EMT processes taking place as localized events, i.e., as ICs which are mostly found as single, crater-like formations more or less in the center of ESC colonies. These ICs are, however, not narrow like a real primitive streak would be in the embryo, and no anterior and posterior end can be discerned. It is well known from classical embryological studies (done, e.g., in the chick; for a discussion, see Denker 2004) that the location of induced primitive streaks largely depends on positioning effects of local sources of inductors. In addition, also the form and structure of primitive streaks is known to depend on physical parameters (cell density and number, aggregate size, matrix composition, and tension, etc.). As far as ESCs are concerned, it has already been shown that regulation of differentiation via the TGF β / activin/nodal pathway (which is involved in primitive streak formation/EMT) can be mimicked/modulated by mechanical strain (Saha et al. 2008). It appears probable that future investigations using the model studied here will show that the number, location, and form of ICs can be modulated by using local sources of primitive streak-relevant signals (e.g., nodal, BMPs, Wnts), or by modulating the physical properties of the feeder cell layer, for example by introducing gradients of density or tensegrity (Hohn and Denker 1994). This might be of much interest for the developmental biologist.

Not every individual ES cell line may necessarily have the same primitive streak formation potential under the same conditions. Thomson et al. (1996) have reported on the formation of very regularly structured embryonic disk anlagen (with primitive streak, but also extra-embryonic structures like amnion and yolk sac) in dense cultures of marmoset monkey ESCs. Such high degrees of order have not been described by any other author thereafter, with any other cell line (mouse, nonhuman primate, or human). It must be left open, therefore, whether in vitro conditions can be found which would allow to obtain such a high degree of order with any other ES cell line (which by the way would be a specific ethical problem in the human; Denker 2006). However, even without formation of a completely organized embryonic disk and primitive streak (i.e., with anterior–posterior axis), locally occurring EMT processes (i.e., ICs) can be an attractive model for researchers interested in basic processes involved in gastrulation-like processes in culture, in nonhuman species. To be suitable for this type of purpose, formation of these structures must be reliable and reproducible.

In the present investigations, we have studied the potential role of the following parameters:

(a) Feeder-free culture of rhESCs (on laminin) vs. culture on MEFs

No ICs were formed on laminin, only on MEFs. This is not surprising since ESCs form monolayers on laminin instead of multilayered colonies, and they take up a phenotype somewhat intermediary between epithelial and mesenchymal, which is consistent with observations made by other authors using various laminin isotypes (Domogatskaya et al. 2008; Miyazaki et al. 2008) or Matrigel (Ullmann et al. 2007, 2008; Van Hoof et al. 2008). In the latter case, a diffuse EMT without localized ICs was reported to occur at the margins of the colonies.

(b) Mode of mitotic inactivation of feeders (by MC or irradiation):

Only on MCMEFs, not on IRMEFs, was IC formation observed. The loss of IC forming capacity occurred progressively during passaging on IRMEFs, i.e., during the first culturing period on IRMEFs, some colonies still did show ICs. This should be of interest since IRMEFs are indeed used by some groups (McElroy and Reijo Pera 2008). The mechanism behind this unexpected difference in properties exhibited by the two groups of MEFs (differing effects of MC treatment and irradiation on cell differentiation and/or selection?) is at present unknown and could only be discovered by systematic investigation of cell biological properties and molecular composition of the cells as well as of the matrix they produce.

(c) Influence of feeder cell density

A density of MCMEFs of $5.5-11 \times 10^4$ cells/cm² at seeding was found suitable for IC formation. That the growth characteristics of ESC colonies are influenced by feeder cell density has also been observed by other authors although not studying IC formation (Heng et al. 2004; McElroy and Reijo Pera 2008). Villa-Diaz et al. (2009), on contrast, found feeder cell density not to be critical for ESC growth in vitro although, again, IC formation was not studied.

(d) Role of various preculture conditions of MCMEFs

Preculturing for 2 d with medium switch was found necessary for IC formation. This is consistent with the observations made by other authors that various growth characteristics of ESCs are strongly influenced by MEF preculture times (Villa-Diaz et al. 2009).

Schulz et al. (2003) observed that colonies of human ESCs on MEFs developed, under their culturing conditions, very large "craters" which may be related to the ICs described by Behr et al. (2005) and in the present communication. However, they interpreted this not as the result of an ingression and EMT event but as a special type of "differentiation" which appeared to be of relevance for the potential of the cells for subsequent neuronal differentiation. In the context of the present paper, it is of interest that those authors reported a strong effect of MEF preculture time: Crater formation was seen only with "fresh" MEFs, i.e., after a very short preculture time (6 h; plating density 1.2×10^6 cells per 35 mm dish).

(e) Type of enzyme used for dissociation of rhESCs at subculturing

It turned out to be critical for IC formation to use collagenase (and not trypsin-EDTA) for rhESC dissociation

and to avoid formation of a single cell suspension but to have small cell clumps remaining. This is in agreement with recommendations for culturing given by Amit et al. (2000; cf. also Heng et al. 2005; McElroy and Reijo Pera 2008; Villa-Diaz et al. 2009) although general cell growth and not IC formation have been the parameters in focus in those papers. That the aggregate size influences proliferation and/or differentiation of ES cells has been described by Bauwens et al. (2008) and Valamehr et al. (2008).

In summary (cf. Fig. 7), these investigations show that a previously described phenomenon, i.e., spatially restricted EMT in primate ESC colonies (ICs) can be obtained reproducibly in rhESC cultures if the described critical details of culturing conditions are observed, i.e., using MCMEFs (not IRMEFs), using a sufficient feeder cell density and specific MEF preculture time periods, and using collagenase IV plus incomplete mechanical dissociation (rather than trypsin–EDTA) for rhESC subculturing. For reproducible formation of ICs in rhESC colonies, we thus are recommending a protocol as detailed in the

rhESC

MCMEF



Trypsin/EDTA

Figure 7. Schematic summarizing protocols that were found suitable or unsuitable for IC formation in rhESC colonies. The recommended protocol is shown at the *bottom*.

"Results" section (experimental group A1, recommended method; cf. Fig. 7, lowermost panel). It is concluded that this in vitro model can be useful for the study of cellular processes involved in EMT, i.e., one major elementary process involved in the formation of germ layers (and their derivatives) in nonhuman primate ESCs. As developmental biology suggests, it can be expected that with further modifications of properties of the feeder cell substratum (or novel extracellular matrix replacements), the morphology of these ICs can possibly be modulated to mimic primitive streaks even more closely. This should hold true for all types of truly pluripotent cells (including induced pluripotent stem cells, iPSCs). No ethical problem is to be seen with the use of this model in nonhuman primate ESCs. On contrast, the situation is different in case of human ESCs and iPSCs where it cannot be ethically accepted to allow primitive streak equivalent formation in a research setting (Denker 2004, 2006, 2009) so that neither this model nor other EB formation models should be used with human pluripotent cells as long as they possess unrestricted gastrulation potential.

Acknowledgments The authors like to thank a number of colleagues for their help and collaboration in the course of these investigations: B. Gobs-Hevelke for active cooperation in cell culture, D. Schünke for electron microscopy, Dr. I. von Recklinghausen for contribution to the collection of primary data, Dr. E. Bruckmann for the establishment of laminin culture, Dr. C. Heneweer for help with CLSM and data processing, and Dr. U. Scheperjans for resin section microphotography.

Preliminary data have been presented at Stem Cell Network North Rhine Westphalia Interne Klausurtagung in Herne (Germany), 4–5 November 2005.

References

- Amit M.; Carpenter M. K.; Inokuma M. S.; Chiu C. P.; Harris C. P.; Waknitz M. A.; Itskovitz-Eldor J.; Thomson J. A. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biology* 227: 271–278; 2000.
- Bauwens C. L.; Peerani R.; Niebruegge S.; Woodhouse K. A.; Kumacheva E.; Husain M.; Zandstra P. W. Control of human embryonic stem cell colony and aggregate size heterogeneity influences differentiation trajectories. *Stem Cells* 26(9): 2300– 2310; 2008.
- Behr R.; Heneweer C.; Viebahn C.; Denker H. W.; Thie M. Epithelial– mesenchymal transition in colonies of rhesus monkey embryonic stem cells: A model for process involved in gastrulation. *Stem Cells* 23: 805–816; 2005.
- Denker H. W. Early human development: new data raise important embryological and ethical questions relevant for stem cell research. *Naturwissenschaften* 91: 1–21; 2004.
- Denker H. W. Potentiality of embryonic stem cells: an ethical problem even with alternative stem cell sources. J Med Ethics 32: 665– 671; 2006.
- Denker H. W. Induced pluripotent stem cells: how to deal with the developmental potential. *Reproductive BioMedicine Online* 19 (Suppl. 1): 34–37; 2009.

- Diecke S.; Quiroga-Negreira A.; Redmer T.; Besser D. FGF2 signaling in mouse embryonic fibroblasts is crucial for selfrenewal of embryonic stem cells. *Cells Tissue Organs* 188: 52– 61; 2008.
- Domogatskaya A.; Rodin S.; Boutaud A.; Tryggvason K. Laminin-511 but not -332, -111, or -411 enables mouse embryonic stem cell self-renewal in vitro. *Stem Cells* 26: 2800–2809; 2008.
- Eastham A. M.; Spencer H.; Soncin F.; Ritson S.; Merry C. L. R.; Stern P. L.; Ward C. M. Epithelial–mesenchymal transition events during human embryonic stem cell differentiation. *Cancer Res* 67: 11254–11262; 2007.
- Eiselleova L.; Peterkova I.; Neradil J.; Slaninova I.; Hampl A.; Dvorak P. Comparative study of mouse and human feeder cells for human embryonic stem cells. *Int. J. Dev. Biol* 52: 353–363; 2008.
- Gadue P.; Huber T. L.; Paddison P. J.; Keller G. M. Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. *Proc Natl Acad Sci USA* 103: 16806–16811; 2006.
- Heneweer C.; Adelmann H. G.; Kruse L. H.; Denker H. W.; Thie M. Human uterine epithelial RL95-2 cells reorganize their cytoplasmic architecture with respect to Rho protein and F-actin in response to trophoblast binding. *Cells Tissues Organs* 175: 1–8; 2003.
- Heneweer C.; Kruse L. H.; Kindhäuser F.; Schmidt M.; Jakobs K. H.; Denker H. W.; Thie M. Adhesiveness of human uterine epithelial RL95-2 cells to trophoblast: rho protein regulation. *Mol Hum Reprod* 8: 1014–1022; 2002.
- Heng B. C.; Liu H.; Cao T. Feeder cell density—a key parameter in human embryonic stem cell culture. *In Vitro Cell Dev Biol Anim* 40: 255–257; 2004.
- Heng B. C.; Liu H.; Cao T. Late-adhering human embryonic stem cell clumps during serial passage can yield morphologically 'normallooking' colonies. *Ann Clin Lab Sci* 35: 459–462; 2005.
- Hohn H. P.; Denker H. W. The role of cell shape for differentiation of choriocarcinoma cells on extracellular matrix. *Exp Cell Res* 215: 40–50; 1994.
- Losick R.; Desplan C. Stochasticity and cell fate. *Science* 320: 65–68; 2008.
- McElroy S. L.; Reijo Pera R. A. Culturing human embryonic stem cells with mouse embryonic fibroblast feeder cells. *Cold Spring Harb Protocols*; 2008. doi:10.1101/pdb.prot5042.
- Miyazaki T.; Futaki S.; Hasegawa K.; Kawasaki M.; Sanzen N.; Hayashi M.; Kawase E.; Sekiguchi K.; Nakatsuji N.; Suemori H. Recombinant human laminin isoforms can support the undifferentiated growth of human embryonic stem cells. *Biochem Biophys Res Commun* 375: 27–32; 2008.
- Moscona A. A. Cell suspensions from organ rudiments of chicken embryos. *Exp Cell Res* 3: 535–539; 1952.
- Nakanishi M.; Kurisaki A.; Hayashi Y.; Warashina M.; Ishiura S.; Kusuda-Furue M.; Asashima M. Directed induction of anterior and posterior primitive streak by Wnt from embryonic stem cells cultured in a chemically defined serum-free medium. *FASEB J.* 23: 114–122; 2009.
- Patwari P.; Lee R. T. Mechanical control of tissue morphogenesis. *Circ Res* 103: 234–243; 2008.
- Saha S.; Ji L.; de Pablo J. J.; Palecek S. P. TGFbeta/activin/nodal pathway in inhibition of human embryonic stem cell differentiation by mechanical strain. *Biophys J.* 94: 4123–4133; 2008.
- Schulz T. C.; Palmarini G. M.; Noggle S. A.; Weiler D. A.; Mitalipova M. M.; Condie B. G. Directed neuronal differentiation of human embryonic stem cells. *BMC Neurosci* 4: 27; 2003.
- ten Berge D.; Koole W.; Fuerer C.; Fish M.; Eroglu E.; Nusse R. Wnt signaling mediates self-organization and axis formation in embryoid bodies. *Cell Stem Cell* 3: 508–518; 2008.
- Thomson J. A.; Kalishman J.; Golos T. G.; Durning M.; Harris C. P.; Becker R. A.; Hearn J. P. Isolation of a primate embryonic stem cell line. *Proc Natl Acad Sci USA* 92: 7844–7848; 1995.

- Thomson J. A.; Kalishman J.; Golos T. G.; Durning M.; Harris C. P.; Hearn J. P. Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts. *Biol Reprod* 55: 254–259; 1996.
- Ullmann U.; In't Veld P.; Gilles C.; Sermon K.; De Rycke M.; Van de Velde H.; Van Steirteghem A.; Liebaers I. Epithelial–mesenchymal transition process in human embryonic stem cells cultured in feeder-free conditions. *Mol Hum Reprod* 13: 21–32; 2007.
- Ullmann U.; Gilles C.; De Rycke M.; Van de Velde H.; Sermon K.; Liebaers I. GSK-3-specific inhibitor-supplemented hESC medium prevents the epithelial-mesenchymal transition process and the upregulation of matrix metalloproteinases in hESCs cultured in feeder-free conditions. *Mol Hum Reprod* 14: 169–179; 2008.
- Valamehr B.; Jonas S. J.; Polleux J.; Qiao R.; Guo S.; Gschweng E. H.; Stiles B.; Kam K.; Luo T. J.; Witte O. N.; Liu X.; Dunn B.;

Wu H. Hydrophobic surfaces for enhanced differentiation of embryonic stem cell-derived embryoid bodies. *Proc Natl Acad Sci USA*. 105: 14459–14464; 2008.

- Van Hoof D.; Braam S. R.; Dormeyer W.; Ward-van Oostwaard D.; Heck A. J.; Krijgsveld J.; Mummery C. L. Feeder-free monolayer cultures of human embryonic stem cells express an epithelial plasma membrane protein profile. *Stem Cells* 26: 2777–2781; 2008.
- Villa-Diaz L. G.; Pacut C.; Slawny N. A.; Ding J.; O'Shea K. S.; Smith G. D. Analysis of the factors that limit the ability of feeder-cells to maintain the undifferentiated state of human embryonic stem cells. *Stem Cells Dev* 18: 641–651; 2009.
- Xu C.; Inokuma M. S.; Denham J.; Golds K.; Kundu P.; Gold J. D.; Carpenter M. K. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* 19: 971–974; 2001.