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Gap junctions modulate apoptosis and colony growth of human embryonic stem cells maintained in a serum-free system ☆

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Abstract

We investigated the gap junctional properties of human embryonic stem cells (hESC) cultivated in a serum-free system using sphingosine-1-phosphate and platelet-derived growth factor (S1P/PDGF). We compared this condition to hESC grown on Matrigel in mouse embryonic fibroblast conditioned medium (MEF-CM) or unconditioned medium (UM). We show that in all culture systems, hESC express connexins 43 and 45. hESC maintained in S1P/PDGF conditions and hESC grown in presence of MEF-CM are coupled through gap junctions while hESC maintained on Matrigel in UM do not exhibit gap junctional intercellular communication. In this latter condition, coupling was retrieved by addition of noggin, suggesting that BMP-like activity in UM inhibits gap junctional communication. Last, our data indicate that the closure of gap junctions by the decoupling agent α-glycyrrhetinic acid increases cell apoptosis and inhibits hESC colony growth. Altogether, these results suggest that gap junctions play an important role in hESC maintenance.

Keywords: Human embryonic stem cells; Gap junction; Sphingosine-1-phosphate; Platelet-derived growth factor; Noggin; Bone morphogenetic proteins

Human embryonic stem cells (hESC) are pluripotent cells derived from the inner cell mass of in vitro fertilized human blastocysts [1,2]. hESC have generally been cultivated on a mouse embryonic feeder layer (MEF), in association with fetal calf serum, or knockout-serum replacement (KSR) plus bFGF. Xu et al. [3] developed a culture system where the feeder cell layer has been replaced by a combination of Matrigel and MEF-conditioned medium (MEF-CM) supplemented in KSR + bFGF. We recently described a serum-free culture system that combines sphingosine-1-phosphate (S1P) and platelet-derived growth factor AB (PDGF) to maintain hESC undifferentiated in presence of MEF [4]. This culture medium eliminates the need of the chemically undefined KSR. These different culture methods seem to be equivalent in maintenance of stem cells expressing specific stem cell markers and with the ability to form teratomas when injected into SCID-mice [1,3–5].

Gap junctions are intercellular channels consisting of two connexons, which are hemi-channels localized in the membrane of adjacent cells (for review, see [6]). Each connexon consists of six membrane proteins, termed connexins [6]. Gap junctional intercellular communication allows cell–cell exchange of inorganic salts and small metabolites of less than ~1 kDa [7]. Such intercellular coupling is implicated in various cellular processes including cell migration, proliferation, differentiation, and apoptosis [7–9]. We previously reported that hESC grown on MEF in presence of serum express connexin 43 and 45 mRNA and proteins, and possess functional gap junctions that

☆ *Abbreviations:* α-GA, α-glycyrrhetinic acid; BMP, bone morphogenetic protein; CM, conditioned medium; hESC, human embryonic stem cells; KSR, knockout-serum replacement; MEF, mouse embryonic fibroblasts; PDGF, platelet derived-growth factor; S1P, sphingosine-1-phosphate; UM, unconditioned medium.

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are regulated by protein kinase C [10]. Carpenter et al. [11] demonstrated the presence of connexin 43 and 45 mRNA and connexin 43 protein in hESC grown on Matrigel. In this system, the authors also described gap junctional communication between hESC [11]. However, the function of gap junctions in hESC remains unknown.

In this study, we aimed to study the intercellular communication of hESC grown in different culture conditions in order to assess whether the different current techniques used for the maintenance of hESC modify their intercellular communication. We thus compared the gap junctional properties of hESC maintained under two different published conditions: (1) in the presence of MEF and S1P/PDGF (as a serum replacement); (2) on a Matrigel matrix and in the presence of MEF-CM. Last we compared the above conditions to hESC grown on Matrigel in unconditioned KSR (UM). We also examined the role of gap junctional intercellular communication in hESC apoptosis and colony growth. This study is the first to address the function of gap junctional intercellular communication in hESC.

Materials and methods

Cell Culture. HES-3 cells were cultured as previously described [4,12]. Briefly, in S1P/PDGF experiments, hESC were grown in the presence of a mitotically inactivated MEF, in Dulbecco's modified Eagle's medium (DMEM, without sodium pyruvate, glucose 4500 mg/L), insulin/transferrin/selenium 1%, β-mercaptoethanol 0.1 mM, NEAA 1%, glutamine 2 mM, Hepes 25 mM, penicillin 25 U/ml, streptomycin 25 μg/ml (all from Invitrogen), S1P 10 μM (Biomol), and PDGF 20 ng/ml (Pepro Tech). In feeder-free experiments, hESC from bulk cultures [13] were plated onto Matrigel in a medium consisting of either 100% MEF-conditioned medium (MEF-CM) supplemented with bFGF 8 ng/ml as described in [14] or KSR 20% with bFGF 8 ng/ml (unconditioned medium, UM). In some experiments, noggin (500 ng/ml, R&D) was added to the UM. In serum experiments, hESC were grown in the presence of MEF, in DMEM (without sodium pyruvate, glucose 4500 mg/L), insulin/transferrin/selenium 1%, β-mercaptoethanol 0.1 mM, NEAA 1%, glutamine 2 mM, penicillin 25 U/ml, streptomycin 25 μg/ml, and FCS 20% (Hi-Clone). For each type of experiment, after attachment, medium was changed every second day (serum, S1P/PDGF) or every day (MEF-CM, UM). Extemporaneous dilution of S1P was made in 0.1% fatty acid-free bovine serum albumin (BSA, final concentration 0.01% BSA, Sigma). hESC were grown in a 5% CO_2 incubator at 37 °C. hESC grown in S1P/PDGF medium are referred to as S1P/PDGF-hESC. hESC grown in absence of MEF are referred to as Matrigel-hESC.

Immunocytochemistry. Cells were fixed in 100% ethanol for 10 min, air-dried, and immunostained as previously described [4,10] with either of the following antibodies: connexin 43 (Chemicon), connexin 45 (Chemicon), GCTM-2 (this laboratory), Oct-4 (Santa Cruz) or TG-30 (recognizes CD9, this laboratory). The secondary antibody used was conjugated with FITC (Dako). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma). Specificity was verified by the absence of any staining in the negative isotype controls (data not shown). Experiments were performed at least three times.

Scrape loading/dye transfer assay. Gap junctional intercellular communication was determined by the scrape loading/dye transfer assay as described in [15]. Briefly, the cells were washed three times in a pre-warmed $Ca^{2+}Mg^{2+}$–PBS buffer (140 mM NaCl, 5.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM Hepes, pH 7.35). In some experiments, cells were treated with phorbol 12-myristate 13-acetate (PMA, 1 μM, 60 min), or α-glycyrrhetinic acid (α-GA, 10 μM, 24 h, Sigma), or noggin (1 μg/ml, 30 min), and/or bone morphogenetic protein-4 (BMP-4, 10 ng/ml, 30 min, R&D). The colonies were scraped with a scalpel blade and incubated for 5 min with Lucifer yellow (1 mg/ml, Sigma) and rhodamine–dextran (1 mg/ml, Molecular Probes) diluted in the $Ca^{2+}Mg^{2+}$–PBS buffer. Due to its low molecular weight (522 Da), Lucifer yellow diffuses from cell to cell through functional gap junctions. On the other hand, rhodamine–dextran (10,000 Da) is too large to diffuse through gap junctions, and thus serves as a negative control in order to confirm that the Lucifer yellow transfer is solely due to gap junction coupling. The dye diffusion was observed by fluorescence. Before using this assay, we verified that the loading of Lucifer yellow by scraping a part of the colonies did not result in a loading of the dye into all cells of the colony (data not shown). Control colonies incubated with either marker in the absence of scraping demonstrated no uptake or dye transfer of Lucifer yellow or rhodamine–dextran (data not shown). Experiments were performed at least three times.

Apoptosis assay. Cells were grown in S1P/PDGF in presence or in absence of α-GA (10 μM) for 24 h. Apoptotic cells were quantified using In situ Cell Death Detection Kit (Roche). Briefly, the cells were fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton X-100 before incubation with the fluorescein-conjugated TdT enzyme. Samples were analysed on a flow cytometer (FC500, Beckman–Coulter).

Colony growth. Cells were grown in S1P/PDGF for 7 days in presence or absence of α-GA (10 μM). Phase contrast images were captured and HESC colony diameter was measured every day for 7 days. The colony diameter was recorded as the average of the shortest and longest diameters. Experiments were performed on 24 colonies per condition.

Statistical analysis. All experiments were performed at least three times. Significance of the differences was evaluated using two-way ANOVA followed by Bonferroni test (growth assays) or two-tailed *t* tests (apoptosis assays). Values of $p < 0.05$ were considered significant (*).

Results

As shown in Figs. 1 and 2, immunostaining of hESC revealed the presence of both connexin 43 (confirmed by Western blot, data not shown) and connexin 45 in cells grown in S1P/PDGF-supplemented medium and on Matrigel (with MEF-CM). As previously described, hESC cultivated in presence of MEF and serum communicate through functional gap junctions [10]. Thus, we examined gap junctional intercellular communication in cells grown in S1P/PDGF medium and on Matrigel using the dye transfer assay with Lucifer yellow and rhodamine–dextran (Fig. 3). When cells grown in S1P/PDGF medium were scraped and incubated in presence of these fluorescent dyes, we observed extensive Lucifer yellow diffusion through the colonies, whereas rhodamine–dextran remained at the site of the scrape injury (Figs. 3A–C). No Lucifer yellow diffusion was observed between hESC and the supportive feeder cells (Fig. 3). Lucifer yellow diffusion in S1P/PDGF-hESC was inhibited by acute treatment with PMA, a protein kinase C activator (Figs. 3D–F). When hESC grown on Matrigel in the presence of MEF-CM were scraped and incubated in presence of Lucifer yellow, we observed diffusion of the dye (Figs. 3G–I). This diffusion was also inhibited by PMA (Figs. 3J–L). Strikingly, hESC grown on Matrigel in UM did not exhibit Lucifer yellow diffusion (Figs. 4A–C). These cells, however, still express connexin 43, connexin 45, and the stem cell markers GCTM-2 and Oct–4 (Figs. 4D-G). These results suggest that MEF secrete molecules that regulate gap junctional intercellular communication in hESC.

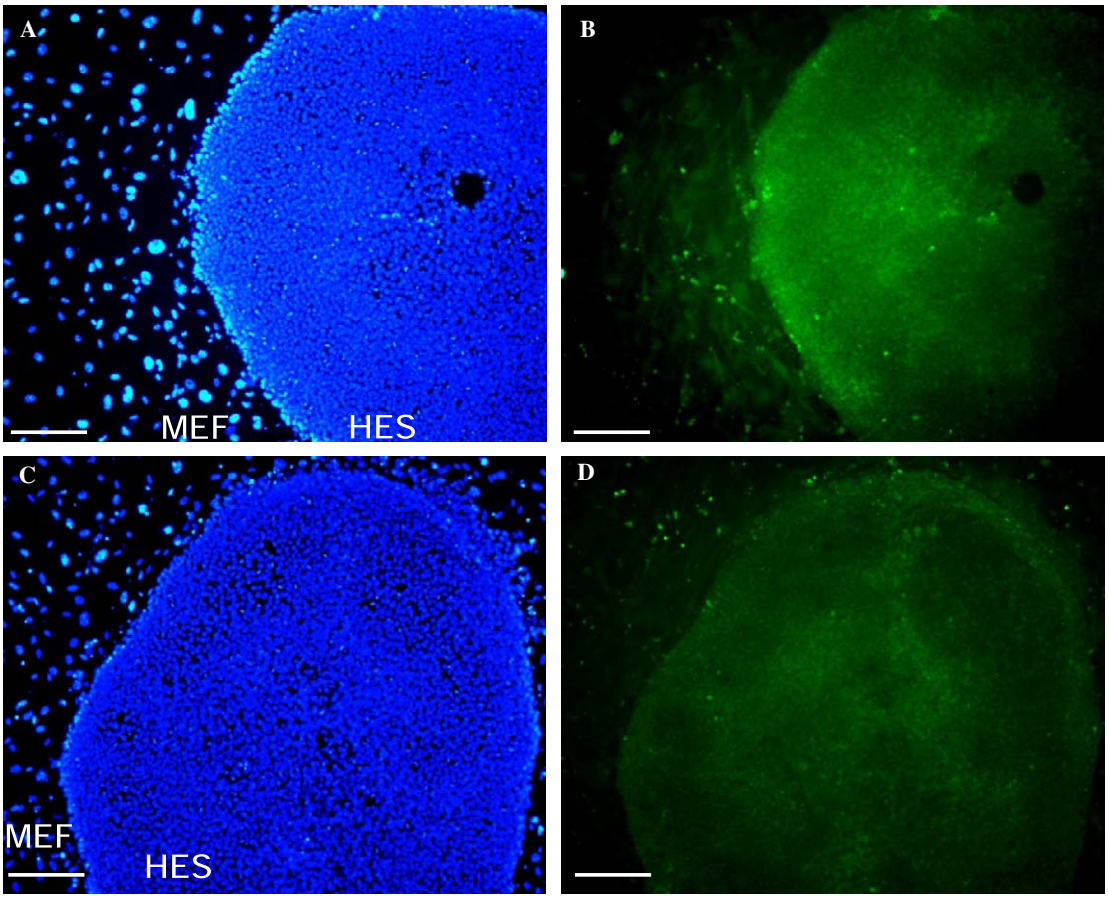

Fig. 1. Expression of connexin 43 and connexin 45 in hESC grown in S1P/PDGF medium. Immunostaining of HES-3 cells with DAPI (A,C) and connexin 43 (B) or connexin 45 (D). Scale bars: 50 μm.

A recent study by Xu et al. [16] showed that UM possesses BMP-like activity, and revealed that this activity was reduced after conditioning the medium with MEF. Xu et al. [16] also detected BMP antagonists, such as noggin in MEF-CM. We tested the hypothesis that BMP-like activity in UM inhibits gap junctional intercellular communication in hESC. Our results show that BMP-4 treatment (10 ng/ml, 30 min) inhibits Lucifer yellow diffusion in hESC grown in presence of serum (Figs. 4H–J). Furthermore, gap junctional intercellular communication was re-established by addition of noggin (1 μg/ml, 30 min) with BMP-4 (10 ng/ml, 30 min) (Figs. 4K–M). Finally, when noggin (500 ng/ml) was added to UM and hESC were cultured for 6 days on Matrigel, diffusion of Lucifer yellow was retrieved in hESC, indicating that hESC were coupled through gap junctions (Figs. 4N–P). Altogether, these data suggest that noggin/BMP are regulators of gap junctional intercellular communication in hESC.

We next considered whether the inhibition of gap junctional intercellular communication would influence hESC survival. We thus cultivated hESC on MEF in presence of S1P/PDGF with or without α-GA (10 μM), a reversible inhibitor of gap junctional intercellular communication, for 24 h (apoptosis assay) or 7 days (growth assay). The dose of α-GA that we used was sufficient to inhibit gap junctional intercellular communication (Figs. 5A–C). We then either: (a) measured the level of apoptosis, by performing TUNEL assay (Fig. 5D); or (b) assessed the growth of hESC colonies (Fig. 5E). As shown in Fig. 5D, treatment of hESC with α-GA for 24 h significantly induces apoptosis in $29.7 \pm 5.5\%$ of the cells ($n = 3$) as compared to only $16.7 \pm 3.6\%$ ($n = 3$) in the S1P/PDGF control ($p < 0.1$). Moreover, when hESC colonies were grown in presence of α-GA for 7 days, we observed a drastic diminution of their colony size (Fig. 5E). Indeed, while colonies cultivated in presence of S1P/PDGF reached a size of 1823.57 ± 59.26 μm ($n = 24$), the ones incubated in presence of α-GA grew to a size of 985.60 ± 173.95 μm ($n = 24$), representing $627 \pm 20\%$ and $306 \pm 54\%$ of their original size at day 0, respectively. This represents an inhibition of $51 \pm 9\%$ of colony growth ($p < 0.001$) by α-GA (Fig. 5E1). In the presence of α-GA, the remaining cells observed after 7 days appeared as a morphologically diverse array of cell types (Fig. 5E). These remaining cells did not express hESC markers Oct-4, GCTM-2, and TG-30 ($n = 3$, Supplementary data). As mentioned above, the measurement of apoptosis was performed after 24 h while the colony size was measured for a period of up to 7 days. This discrepancy in the timing of the experiment was solely due to the need of a sufficient number of cells

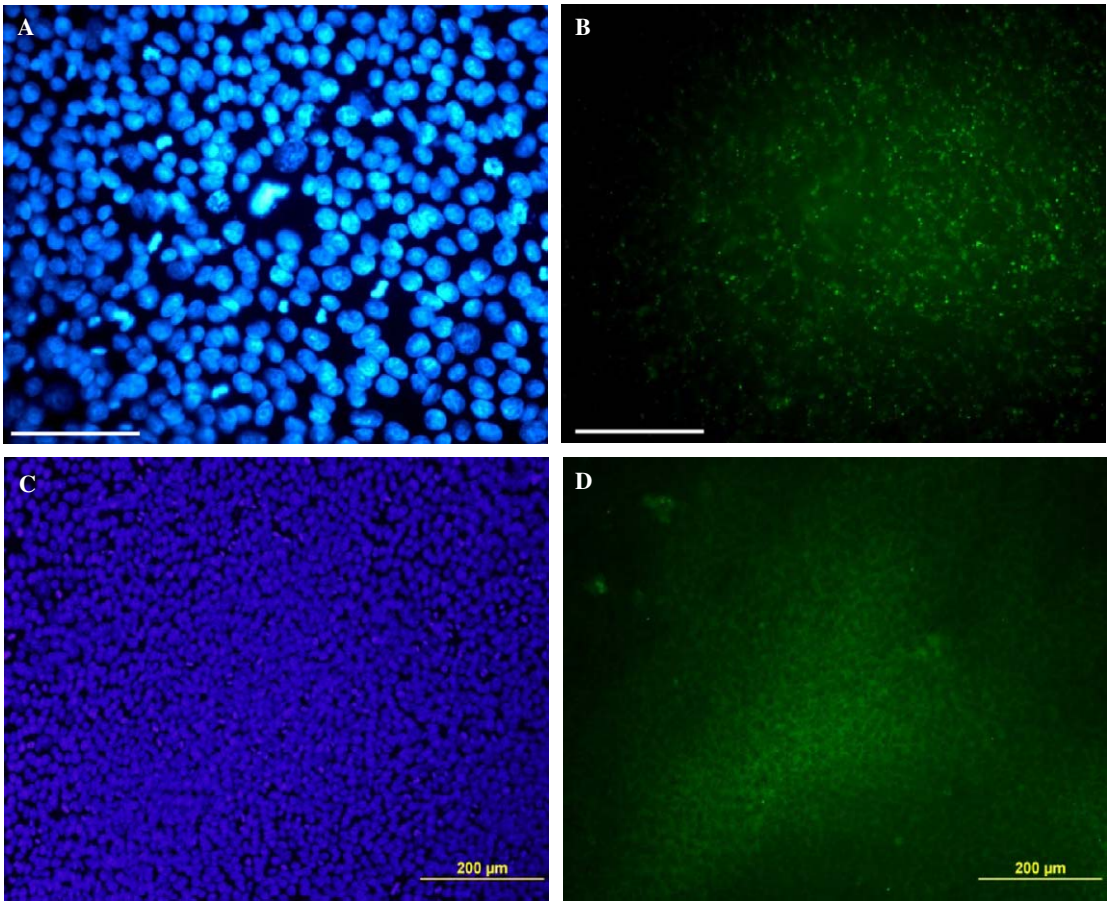

Fig. 2. Expression of connexin 43 and connexin 45 in hESC grown on Matrigel with MEF-CM. Immunostaining of HES-3 cells with DAPI (A,C) and connexin 43 (B) or connexin 45 (D). (A,B) Scale bars: 50 μm.

to perform FACS analysis. However, while after 24 h an increase in apoptosis was observed in the presence of α-GA, no statistically significant change was observed in regard of the colony size. We did not observe a statistically significant difference in apoptosis of hESC grown in serum-based medium by α-GA (data not shown). We hypothesize that in presence of serum, where the cells are in presence of a richer medium, the closure of gap junctions is not prerequested to hESC survival. However, in a minimal medium such as the S1P/PDGF medium, the intercellular gap junctional communication is necessary to hESC maintenance. Further work will be required to address this issue.

Discussion

Since a range of culture conditions have been described for hESC maintenance, we assessed whether different culture conditions would affect gap junctions in hESC. We and others previously reported that hESC, grown in presence of MEF and serum [10] or on Matrigel with MEF-CM [11,17], express connexin 43 and connexin 45. The results presented in this study confirm the results obtained by other groups on hESC grown on Matrigel and demonstrate that hESC grown in presence of S1P/PDGF also possess both connexin 43 and connexin 45. Ginis et al. [18], reported that hESC grown in presence of MEF and KSR express connexin 43 and connexin 45 while Bhattacharya et al. [19] demonstrated the presence of the two connexins in hESC cultivated in presence of MEF, serum, and KSR. Altogether, these results obtained in different culture conditions that maintain hESC undifferentiated suggest that the presence of both connexin 43 and connexin 45 is a characteristic of hESC.

Very little is known about gap junctional intercellular communication in stem cells. Many adult stem cells lack connexins (for review, see [20]). However, human mesenchymal stem cells have been shown to express connexins and form functional gap junctions [21,22]. Previous studies by this laboratory and other studies demonstrated the presence of gap junctional intercellular communication in hESC [10,11] as well as mouse ESC [23]. Similar to hESC cultured in serum, hESC grown in S1P/PDGF-supplemented medium and on Matrigel with MEF-CM are coupled through functional gap junctions, which are inhibited by protein kinase C activation. Furthermore, Carpenter et al. [11] described the presence of functional gap junctions in hESC grown on Matrigel. However, in their study, the observed Lucifer yellow diffusion was limited in range and required a longer time of incubation [11] than in our experimental conditions where Lucifer yellow diffusion was

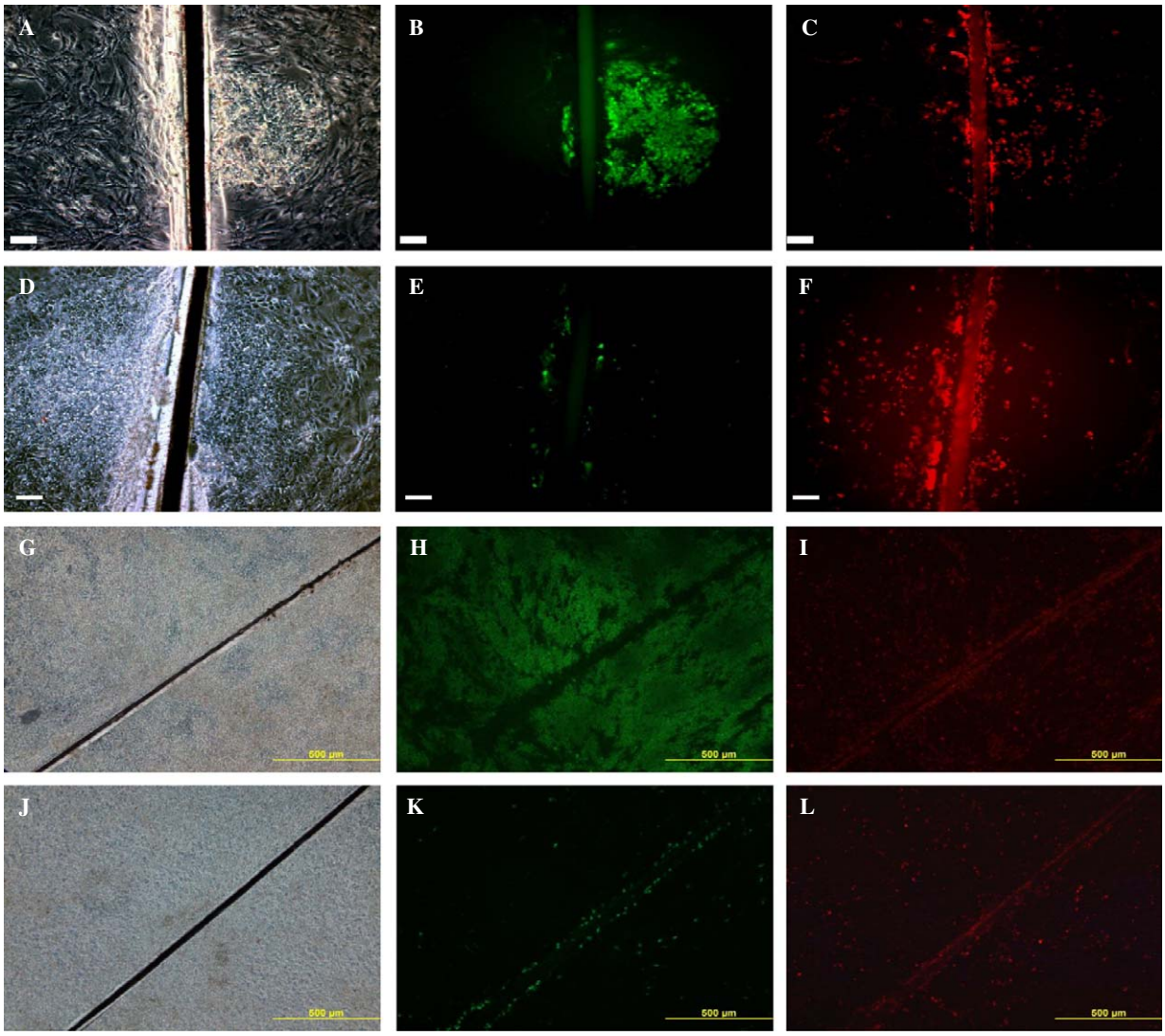

Fig. 3. Gap junctional intercellular communication in S1P/PDGF medium (A–F) and on Matrigel (G–L). Light (A,D,G, and J) and fluorescence micrographs with Lucifer yellow (B,E,H, and K) and rhodamine–dextran (C,F,I, and L) in HES-3 cells. Rhodamine–dextran was employed as a negative control. (D–F, J–L) Cells were incubated in presence of PMA (1 μM, 60 min). (A–F) Scale bars: 100 μm.

indeed rapid and extensive. This difference in dye diffusion could be attributed to the difference of techniques used to perform this test.

hESC cannot be maintained long term on Matrigel in UM. However, we observed that hESC still express some specific stem cell markers, Oct-4 and GCTM-2, after cultivation for a week under these conditions. In these conditions, although hESC still express connexin proteins, we observed no diffusion of Lucifer yellow, suggesting the absence of gap junctional communication between hESC. Further studies will be required in order to investigate if connexin 43 phosphorylation states are linked to gap junctional intercellular communication in hESC.

Our data indicate that gap junctional intercellular communication is inhibited in absence of MEF-CM, suggesting that a cross talk between MEF and hESC is involved in hESC-gap junctional intercellular communication. Candidates for such a cross talk are both BMPs and their antagonist noggin. BMPs have previously been described as inducers of hESC differentiation [24,25] while noggin, in combination with bFGF, has been shown to maintain hESC in the undifferentiated state [16]. Indeed, Xu et al. [16] demonstrated the presence of BMP-like activity in UM as well as the presence of noggin in MEF-CM. As shown in this study, while BMP-4 inhibits Lucifer yellow diffusion in hESC, gap junctional intercellular communication was retrieved with the addition of noggin. Furthermore, when noggin was added to UM, gap junctional coupling was re-established in hESC. Altogether, these data indicate that BMP-like activity in UM is likely to be inhibiting gap junctional intercellular communication between hESC.

Limited studies have addressed the role of gap junctional intercellular communication in stem cells and progenitor cells. In embryonic neural progenitors, the closure of gap junctions diminishes cell survival [26,27]. As shown in this

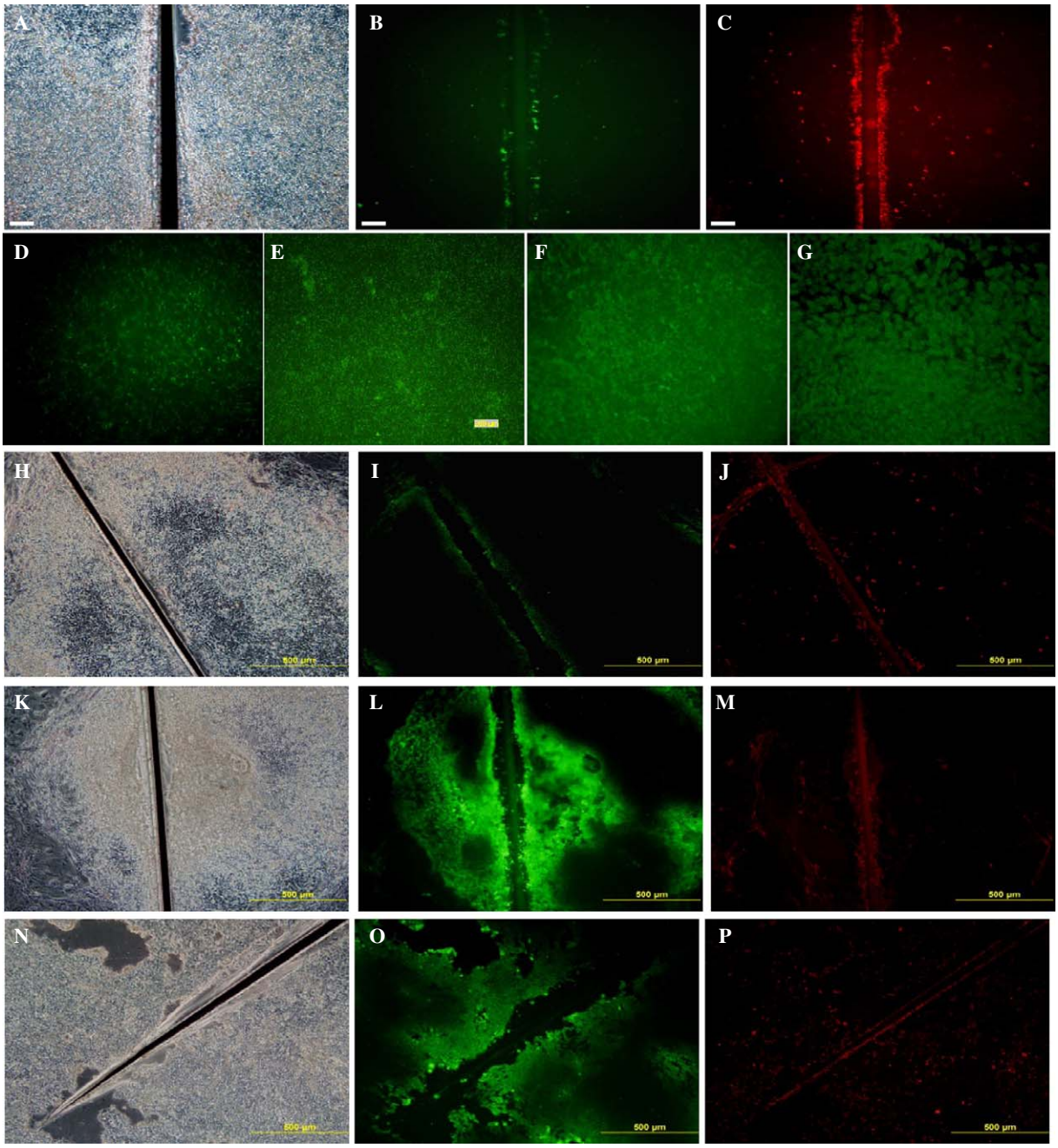

Fig. 4. Light (A,H,K, and N) and fluorescence micrographs with Lucifer yellow (B,I,L, and O) and rhodamine–dextran (C,J,M, and P) of either Matrigel-hESC grown in UM (A–C); or hESC grown in serum incubated with BMP-4 (10 ng/ml, 30 min) (H–J); or hESC grown in serum incubated with noggin (1 μg/ml, 30 min) and BMP-4 (10 ng/ml, 30 min) (K–M); or Matrigel-hESC grown in UM supplemented with noggin (500 ng/ml, 6 days) (N–P). Expression of connexin 43 (D), connexin 45 (E), GCTM-2 (F), Oct-4 (G) in Matrigel-hESC in UM. (A–D) Scale bars: 100 μm, (F–G) scale bars: 200 μm.

study, the closure of gap junctional intercellular communication by α-GA increases apoptosis and decreases colony growth of hESC cultivated in a serum-free medium.

As gap junctions allow intercellular communication between hESC, we hypothesize that molecules such as mitogens or survival factors diffuse within the hESC colonies through gap junctions. The nature of the molecules that can diffuse through gap junctions in hESC has not been established yet. Their identification will be an important step towards the elucidation of regulatory mechanisms of hESC survival, differentiation or death.

Acknowledgments

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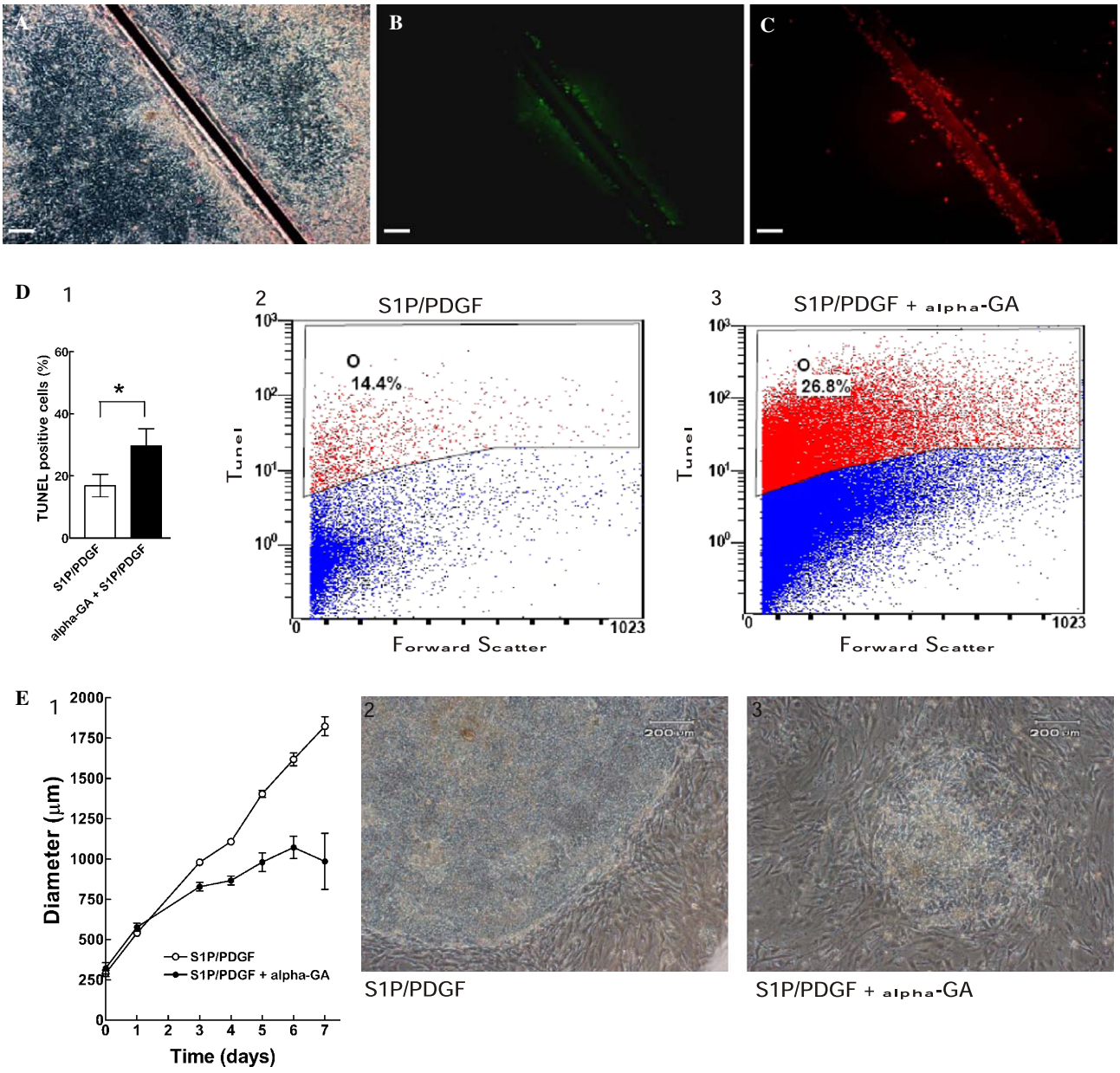

Fig. 5. Light (A) and fluorescence micrographs with Lucifer yellow (B) and rhodamine–dextran (C) of HES-3 cells incubated in presence of α-GA (10 μM, 24 h), scale bars: 100 μm. (D) FACS analysis of apoptotic cells in absence (S1P/PDGF) or presence of α-GA (10 μM, 24 h). (○) TUNEL positive cells, (○) TUNEL negative cells. (D1) Means ± SEM of three independent experiments. (D2–3) Representative data of at least three independent experiments, each gating was set according to its corresponding negative control. (E1) Time course of hESC colony growth, in absence (S1P/PDGF) or presence of α-GA (10 μM, 7 days). Means ± SEM of 24 samples per condition. (E2–3) hESC colonies grown for 7 days in absence (S1P/PDGF) (E2) or presence of α-GA (10 μM) (E3).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.03.127.

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