TU/e technische universiteit eindhoven

The role of connexins 37, 40 and 43 in gap-junctional communication between progenitor and endothelial cells

Lenneke Prinzen
s483989
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Supervisors: dr. rer. nat. T. Gloe
dr. rer. biol. hum. P. Kameritsch
dr. J.L.V. Broers
Professors: prof. dr. med. U. Pohl
prof. dr. ir. F.P.T. Baaijens

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Introduction

Gap junctional communication has been shown to be an efficient way of communication between cells. Gap junctions allow passive diffusion of particles up to 1000 Da, as well as electrical coupling, between two cells. This type of communication allows a large number of cells to act as a functional unit, in several organs such as the endothelium. [De Wit, 2004; Sohl and Willecke, 2004].

Structure of gap junctions
Gap junctions are made up of connexins, proteins containing four transmembrane domains. Both terminals are located in the cytoplasm. Six connexins form a hemichannel, called connexons. Two connexons (one in each cellular membrane) form the gap junction (see figure 1). The connexins in a gap junction can differ from each other, either within a connexon or from their apposing connexin, giving rise to many different possible gap junction conformations. While gap junctions allow passive diffusion of particles, this variability makes some variation of conduction and regulation possible. [Sohl and Willecke, 2004].

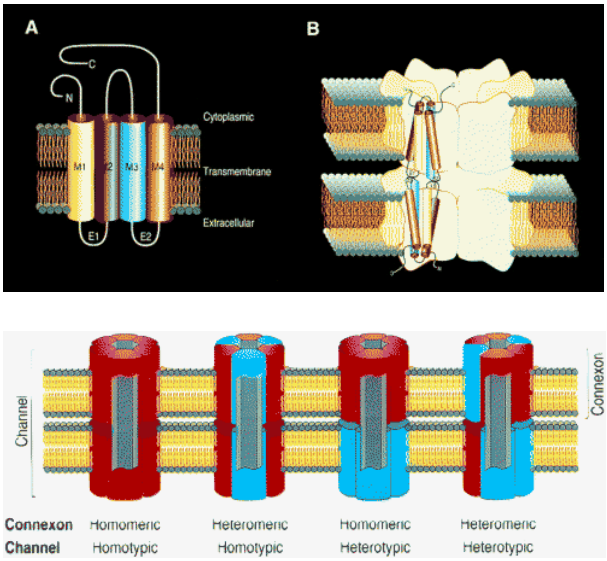


Figure 1: Top: (A) schematic drawing of a connexin, showing four transmembrane domains and the N and C – terminal, both on the cytoplasmic side of the cellular membrane. (B) Also the predicted conformation of two apposing connexins is depicted, thought to be accomplished by cysteine amino acids in the extracellular loops, making disulfide bonds possible. Bottom: Gap junctions are build up of two connexons, one in each cellular membrane; each consists of six connexins. Connexins can differ from each other either within a connexon or from their apposing connexin, as depicted. [Kumar and Gilula, 1996]

So far, 20 different connexin genes have been discovered in the mouse genome, and 21 in the human genome. Nineteen of these can be considered as orthologues pairs. The expression of connexins differs per cell type, and also the connexins seem to fulfill different functions in specific cell types. [Sohl and Willecke, 2004]

Gap junctional communication in the vascular system
Gap junctions are also found in vascular cells. Endothelial cells and smooth muscle cells express connexins 37, 40, 43 and 45 (the expression level depends on the type of vessel and localization in the vascular tree). This coupling has great impact on the vascular function, as the vascular tone (the organized dilation and constriction of arterioles) is influenced by gap junctional communication [De Wit, 2004; Haefliger *et al.*, 2004], and mice deficient in specific connexins show irregular responses [De Wit, 2004; Haefliger *et al.*, 2004; Sohl and

Willecke, 2004]. Intercellular coupling also seems to play a role in the transmigration of endothelial cells through the intima layer during angiogenesis, as the coordination of this process is thought to be accomplished by gap junctional communication between the endothelial cells [Haefliger *et al.*, 2004].

Endothelial progenitor cells

Endothelial progenitor cells (EPCs) are precursor cells originating in the bone marrow, destined to become different types of vascular cells, e.g. endothelial cells. Progenitor cells differ from stem cells by the fact that they are programmed to become a certain type of cell lineage whereas stem cells are undefined and can differentiate to any type of cell. EPCs are now mostly defined by the presence of CD43 and vascular endothelial growth factor receptor 2 (VEGFR-2), or by the presence of CD133 and VEGFR-2 to distinguish them from regular endothelial cells [Szmitko *et al.*, 2003]. EPCs were discovered in the human adult circulation only seven years ago [Asahara *et al.*, 1997]. Since then, much research has been done to their function in the human adult. One of their functions may be to aid in the maintenance of the endothelium and supporting angiogenesis. It has been shown that EPCs are incorporated in newly forming vessels and that, in general, EPCs increase the vessel density by increasing the number of endothelial cells [Patterson, 2003]. It has also been tested whether EPCs lead to enhanced angiogenesis after a myocardial infarction; this promises to be a beneficial approach of repairing the damaged heart [Szmitko *et al.*, 2003].
Interestingly, cardiomyocytes can trigger EPCs to differentiate to cardiomyocytes via gap junctional communication [Badorff *et al.*, 2003]. It seems that, depending on the type of cell the EPC communicates with, the EPC can be triggered to become that certain type of cell.

Goals

It is still unclear how EPCs are triggered to differentiate to endothelial cells. It is reasoned here that gap junctional communication, after the docking of EPCs to endothelial cells (for example a damaged site of the endothelium), could play a key role. The aim of this study was to determine the intercellular coupling between endothelial cells and EPCs, and whether this triggers the EPCs to become endothelial cells themselves. Therefore, the expression of connexins in EPCs and their ability to form functional gap junctions with endothelial cells was analyzed.
First, it was determined if gap junctional communication is possible for EPCs by testing if there are active gap junctions between two neighboring EPCs. This was done by patch clamping, by which an electrical voltage is applied to two neighboring cells and then determining if there is a current, and by dye injection, where one EPC is injected with a fluorescent dye, and the spreading of this dye to neighboring cells is monitored. The next goal of this study was to determine if EPCs contain connexins type 37, 40 and 43 to establish if it is theoretically possible for them to interact in gap junctional communication with endothelial cells. Next, it was investigated if active gap junctions are present between co-incubated endothelial cells and EPCs, by monitoring dye transfer from HUVECs to EPCs. Finally an attempt was made to determine the specific connexins responsible for gap junctional communication between EPCs and endothelial cells, by co-incubating EPCs with HeLa cell lines transfected with genes coding for one of the three connexins.

Materials and methods

EPC
Mouse embryonic EPCs (obtained from Hatzopoulos [Hatzopoulos *et al.*, 1998]) were cultured at 37°C with 5% CO_2, in Dulbecco's MEM containing 25 mM Hepes with 4500 mg/ml glucose, pyridoxine, and without Sodium Pyruvate (Gibco, Carlsbad, CA, USA). 200 mM L-glutamine (Gibco, 100x) was added, as well as Pen-Strep (Gibco), non-essential amino acids (Gibco), 20% fetal bovine serum (Gibco, heat inactivated at 55 °C for 30 minutes) and 7 µl/l β-mercaptoethanol (Applichem, Darmstadt, Germany). These cells were determined to contain RNA and proteins characteristic for mesodermal as well as for early endothelial cells, indicating a transition cell line between stem cells and endothelial cells. Also, these cells were shown to be able to differentiate to endothelial cells.

HUVEC
HUVECs were freshly isolated from umbilical cords and cultured at 37°C with 5% CO_2, in M199 (Gibco) with 20% newborn calf serum (NCS), 1.25% Pen-Strep and 20% endothelial cell growth medium (Promocell), according to Jaffe et al. [Jaffe *et al.*, 1973]. Cells were passaged once or twice before culturing them on glass cover slips, pre-coated with collagen. When they had reached confluence they were used for experiments.

HeLa wild-type, Cx37, Cx40 and Cx43
HeLa WT cells were cultured at 37°C with 5% CO_2, in DMEM containing 10% NCS and 1.25% Pen-Strep. Wild-type HeLa cells do not express detectable amounts of connexins. HeLa cell lines transfected with genes coding for either Cx37, 40 or 43 (a generous gift from prof. Willecke, Bonn, Germany) were cultured in the same medium, but also containing 1 µg/ml Puromycin to select the stably transfected cells.

Reverse transcribed polymerase chain reaction (RT-PCR)
RNA was isolated from EPCs and HeLa cell lines transfected with either one of the three connexins using the RNeasy mini kit and Qia-shredder (Qiagen, Hilden, Germany). Then the RT-PCR was prepared using the Titan One Tube RT-PCR system (Roche Applied Science, Mannheim, Germany) according to the protocol, and performed using either a PTC 100 cycler (MJ Research Inc, Reno, NV, USA). or a GeneAmp PCR System 2400 (Perkin Elmer, Boston, MA, USA).
The following primers were used: mouse-Cx37: - 5'GAA GAG CGG CTG GCG CAG AAG3', + 5'GCC ATT CTG AGG GGG TGG GT3', mouse-Cx40: - 5'GCT CTG GAC GAG GCT GGT GGG A3', + 5'GCC ATG CCA TGC ACA CTG TG3', rat-Cx43: - 5'CCC CAT NGG ATT TTG NTC TGC3', + 5'AGG GAA GGT GTG GCT GTC AGT3' (MWG Biotech AG, Ebersberg, Germany). To separate the resulting cDNA product a 1% agarose gel (Applichem) in TBE buffer was used (89 mM Tris(hydroxymethyl)-amino-methane (Tris), 89 mM Borate and 2 mM Ethylenediamine tetra acetic acid (EDTA), pH 8.5) with 5 µl ethidium bromide per 100 ml gel.

Western blotting
EPCs, HUVECs and the four different HeLa cell lines, grown to confluence on a 10 cm dish, were lysed using lysis buffer (containing 20 mM KH_2PO_4 (Merck, Darmstadt, Germany), 1 mM EDTA, 1 mM Pefabloc (Applichem), 1 µl Leupeptin (Applichem), 1 µl Pepstatin (Applichem), 50 mM NaF, 40 mM Na_4pyrophosphate, 2 mM Na_3VO_4 (activated) and 1% Triton X100, pH 7.3). The amount of proteins (determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA)) loaded on the gel was 80 µg per lane. A 12% acrylamid separation gel was used, as well as a 4% stacking gel. After protein separation the proteins were blotted on a nitrocellulose membrane (Peglab, Erlangen, Germany). The membrane was blocked using horse serum blocking buffer (200 mM NaCl, 50 mM Tris (pH

7.5), 3% BSA, 0.05% Tween 20 and 10% horse serum) for 30 minutes. The membrane was then incubated for two hours at 37 °C with the primary antibodies, either rabbit-anti-Cx37 or 40 (Alpha Diagnostics, San Antonio, TX, USA), or mouse-anti-Cx43 (BD Biosciences, San Diego, CA, USA), diluted 1:600 in horse serum blocking buffer. After extensive washing the secondary antibody (peroxidase goat-anti-rabbit or peroxidase goat-anti-mouse, Calbiochem), diluted 1:25,000 (anti-rabbit) or 1:10,000 (anti-mouse) in horse serum blocking buffer, was incubated at room temperature for one hour. After extensive washing horseradish peroxidase was added (electro-chemo-luminescence kit, Applichem) and films were developed.

Immunolabeling
Cells were fixed using 3% formaldehyde (in PBS^+) for 15 minutes. After washing with PBS^+ (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.4 mM $MgCl_2$, 8.1 mM Na_2HPO_4 and 0.91 mM $CaCl_2$), cells were permeabilized using 0.1% Triton X100 (in PBS^+) and incubated for 3 minutes. After another wash with PBS^+ the cells were incubated with 0.2% bovine serum albumin (BSA) in PBS^+ (PBSA). Next the cells were incubated with the primary antibody (same as for Western blotting), diluted 1:200 in PBSA for 60 minutes at room temperature. After washing with PBSA the secondary antibody (anti rabbit/ mouse, Molecular Probes, Eugene, OR, USA), diluted 1:200 in PBSA was incubated for 30 – 60 minutes in the dark at room temperature. Then the cells were washed with PBS or PBSA and put on cover slips in a drop of Confocal Matrix (Micro Tech Lab, Graz, Austria) on a microscope slide. Slides were analyzed using a confocal microscope (Zeiss, LSM 410 Microsystems, Oberkochen, Germany); single slices were recorded with a magnification of 40x.

Dye injection
This experiment was performed by dr. P. Kameritsch from the institute of Physiology at the Ludwig Maximilian University in Munich, Germany.
A fluorescent dye, which can only be passed on to neighboring cells via gap junctions (3.5 mM Alexa Fluor 488, Molecular Probes, Eugene, OR, USA), was injected in a single EPC using a micropipette. At certain time points (as indicated) after dye injection images were recorded using a digital camera, to monitor the spreading of the dye to adjacent cells. [Kameritsch, P *et al.* JCP, 2004, accepted].

Electrical coupling
This experiment was performed by prof. W. Nagel from the institute of Physiology at the Ludwig Maximilian University in Munich, Germany.
For whole cell patch-clamping a pair of adjacent EPCs was selected. The two micropipettes had an input resistance of 2-4 MΩ. The intercellular conductance was determined by applying pulses of 10 mV (from a holding potential of 0 mV) to one cell, while the second cell was kept at a constant voltage of 0 mV. The current measured in the second cell was plotted against time. A known gap junction inhibitor heptanol (2 mM) [Meda *et al.*, 1986] was added to determine if the measured current was specific for gap junctional conductance. [Kameritsch, P *et al.* JCP, 2004, accepted]

Co-incubation assay
The day before the experiment EPCs were PKH labeled (MINI26 from Sigma) to distinguish them from either the HUVECs or the HeLas (WT, 37, 40 or 43). At the same time either HUVECs or HeLas were plated on a 24 well plate so that these were confluent for the experiment. Right before the experiment the HUVECs or HeLas were stained with 2.5 or 5 µg/ml calcein in Hepes buffer (pH 7.3), incubated for 15 minutes. Next, the PKH stained EPCs were distributed on top of the HUVECs or HeLas, and centrifuged at 31g for 5 minutes so that the EPC are more likely to contact the HUVECs or HeLas. Then the cells were co-incubated for the times indicated at 37 °C. When indicated, heptanol (1:500) was added every ten minutes, to determine if the dye transfer was gap junction specific.

Results

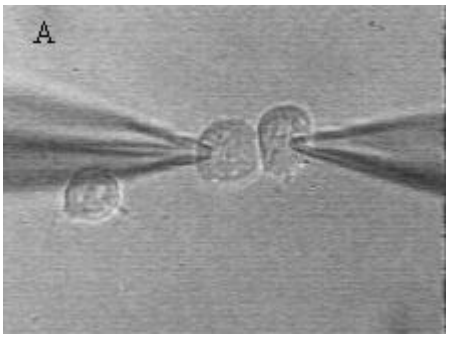
Detection of gap junctions between EPCs
First of all, it was determined if EPCs are able to compose active gap junctions with each other, to determine if they are in principle able of gap junctional communication.

Dye injection
With dye injection, one cell was loaded with a green fluorescent dye, Alexa Fluor 488 (a molecule of about 570 Da). This dye could only be passed to adjacent cells via gap junctions, by diffusion between the connected cytoplasms. The cells were monitored to determine the dye spreading to adjacent cells.

Figure 2: dye injection experiment with EPCs. Left: this cell is loaded with Alexa Fluor 488, a dye that can only be passed on to neighbor cells via gap junctions (time point 0). Center: after 80s about seven neighbor cells are fluorescent, indicating that dye is indeed passed on to neighbor cells via gap junctions. Right: after 320s even more cells are fluorescent.

A representative experiment is shown in figure 2, where cells adjacent to the initially loaded cell do acquire fluorescence in a matter that agrees with passive diffusion: the closer a cell is to the initially loaded cell, the more fluorescent it is. This indeed indicates that gap junctions are present between EPCs, and that they are capable to pass on Alexa Fluor 488 molecules to neighboring cells.

Patch-clamping
A second experiment to determine the presence of active gap junctions between EPCs was performed by patch-clamping a pair of EPCs. Results are shown in figure 3.



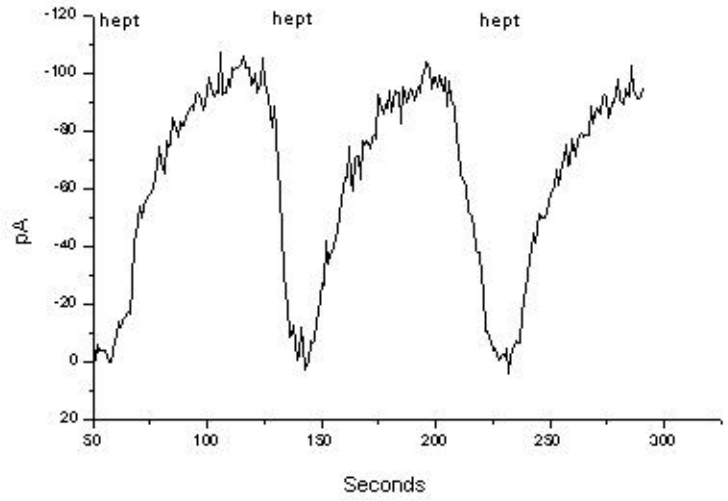


Figure 3: patch-clamping of two EPCs. A: image of the two patched cells, both coupled to electrodes. B: The electric current through the two cells. After ± 25, 125 and 200 seconds heptanol (hept) is added. This reduces the current to zero within 25 seconds. Because heptanol is a reversible gap junction inhibitor the current recovers, and reaches -100 pA about 50 seconds after addition of heptanol.

The electrical current sent by one electrode and detected by the other is reversibly blocked by heptanol, indicating the presence of gap junctions between the two cells.

Detection of connexins in EPCs

Transcription of connexin genes in EPCs

To determine whether connexins 37, 40 or 43 are transcribed in mouse embryonic EPCs, RT-PCR was performed using total RNA of these cells. As a positive control, HeLa cell lines, which do not contain any connexins in the wild type (WT) form, transfected with genes coding for one of the three connexins, were used. These are HeLa 37 (H37), HeLa 40 (H40) and HeLa 43 (H43). As a negative control, HeLa cells transfected with a non-corresponding connexin were used. The negative controls were indeed negative for all connexins, but only shown for Cx40 primers. The sizes of the amplified fragments of Cx37, 40 and 43 cDNA are about 1000, 500 and 900 basepairs, respectively.

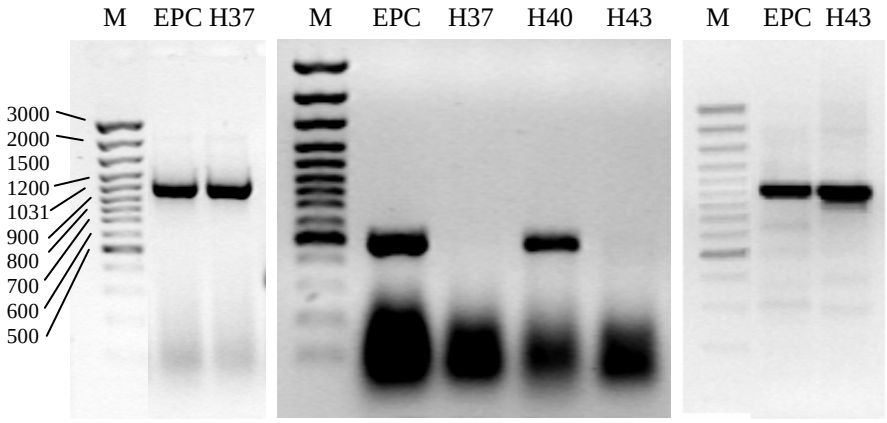


Figure 4: Agarose gels showing the results of the RT-PCR experiments. Left: EPC and HeLa 37 cDNA was primed for Cx37. Center: EPC, HeLa 37, Hela 40 and HeLa 43 cDNA was primed for Cx40. Right: EPC and HeLa 43 cDNA was primed for Cx43. M = marker, H37 = HeLa 37, H40 = HeLa 40, H43 = HeLa 43. The numbers on the left indicate the size of the markers in basepairs.

It turned out that mouse embryonic EPCs contain messenger RNA (mRNA) coding for all three connexins (see figure 4). This would suggest that also the three corresponding connexin proteins are present in mouse EPCs.

Connexin-protein detection in EPCs
In two different ways, it was determined if the three different connexins are expressed in EPCs: Western blotting and immunolabeling.

Western blotting

In figure 5 representive Western blot results are shown. As a positive control both the respective transfected HeLa cell line as well as human umbilical vein endothelial cells (HUVECs), which contain all three connexins [Van Rijen *et al.*, 1997], were used. As a negative control HeLa WT cells and a HeLa cell line transfected with a non-corresponding connexin-gene were used.
Cx37 and 40 were found to be negative in EPCs. HUVEC cells should contain all three connexins, however they also show negative on the Western blots for Cx37 and 40, even though the maximum amount of proteins was loaded on the gel and also after a detection time of an hour. This could indicate a low affinity of the respective antibodies. Since the transfected HeLa cell lines show overexpression of the respective connexins, they are clearly positive for either Cx37 or 40. Cx43 was found positive on the Western blot for EPCs, as well as one positive control (H43). However one negative control for Cx43 (HeLa WT) was found positive and one positive control (HUVEC) negative.
Due to these inconsistencies, the Western blots have to be optimized in further experiments.

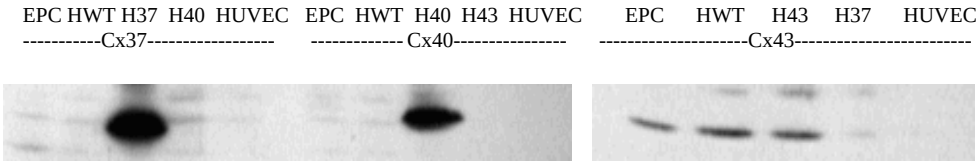


Figure 5: the Western blots analyzed for Cx37, 40 and 43 respectively. The analyzed lysates are indicated on top of the blots.

Immunofluorescence

A second experiment to determine whether mouse embryonic EPCs contain the three connexins, was done by immunolabeling of fixed EPCs, with antibodies specific for one of the three connexins. Representative results are shown in figure 6.

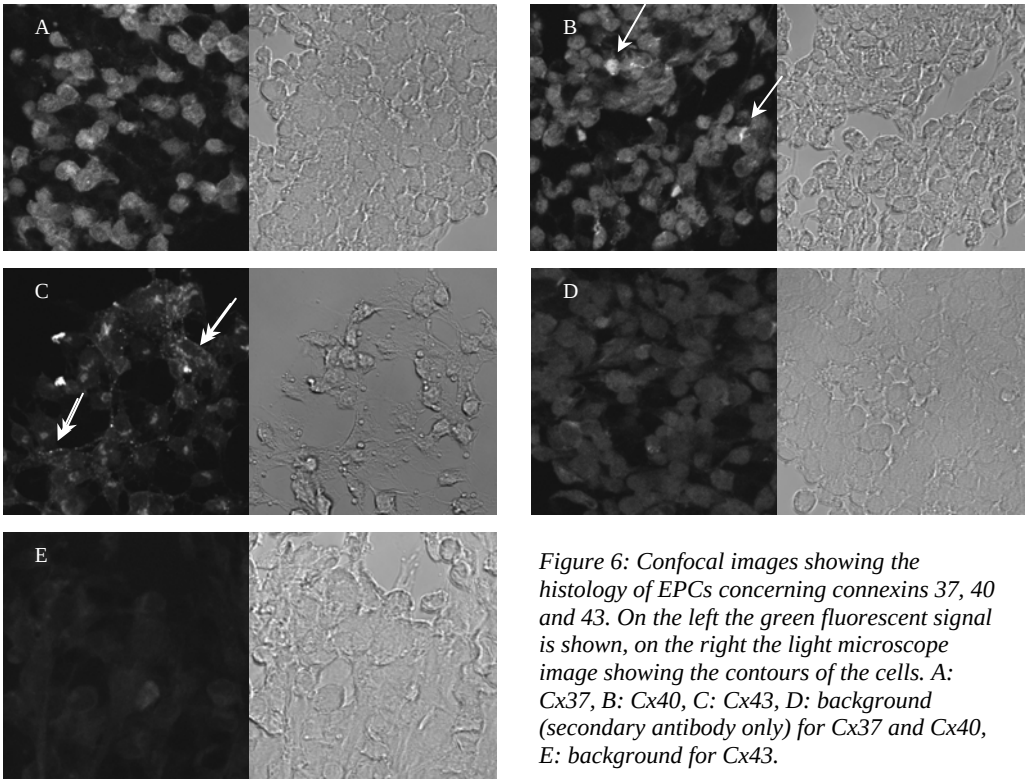


Figure 6: Confocal images showing the histology of EPCs concerning connexins 37, 40 and 43. On the left the green fluorescent signal is shown, on the right the light microscope image showing the contours of the cells. A: Cx37, B: Cx40, C: Cx43, D: background (secondary antibody only) for Cx37 and Cx40, E: background for Cx43.

As shown in figure 6, there appears to be a difference in the cellular distribution of the three connexins. Cx37 is only visible in about half of the cells. The cellular distribution is rather homogenous. For Cx40, more cells are fluorescent than for Cx37; about 80% of the cells show Cx40. The cellular distribution of Cx40 is similar to that of Cx37 for most fluorescent cells, but some are (partially) brighter stained (arrows in figure 6b), indicating higher concentrations of connexins. For Cx43, the cellular distribution is completely different. Again, not all cells are positive for Cx43, about 75% of the cells show fluorescence. The positive cells do not show the homogenous distribution, as do Cx37 and 40. Rather, bright fluorescent spots are visible along the cellular membrane (arrows in figure 6c). This indicates that in EPCs, Cx43 is present mostly in the cellular membrane in locally concentrated patches.

Detection of gap junctions between EPCs and HUVECs

To investigate whether gap junctions can be established between endothelial cells and EPCs, a co-incubation study was done with HUVECs, stained with green fluorescent calcein (a molecule that can only be passed on to adjacent cells via gap junctions), and EPCs, stained with red fluorescent membrane marker PKH26. The amount of calcein that is acquired by the EPCs after co-incubation of both cell populations is measured after certain time periods using flowcytometry. A schematic drawing of the experimental set up is depicted in figure 7.

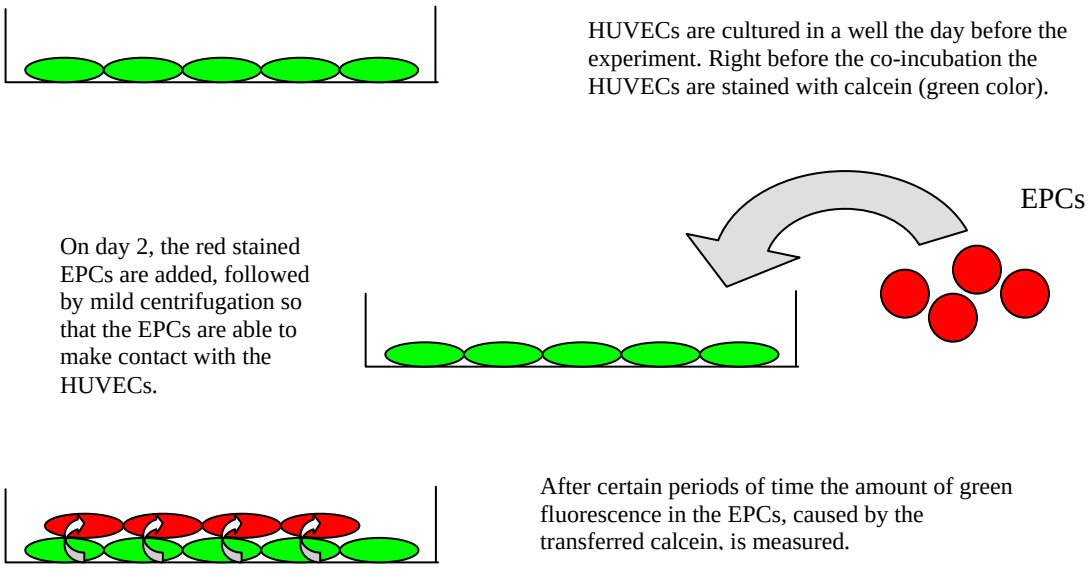


Figure 7: schematic depiction of the co-incubation studies.

Figure 8 shows the results for this co-incubation. As shown in three different ways, EPCs are able to acquire calcein after co-incubation. For every experiment 10,000 cells were analyzed. In figure 8a a dotplot shows the red (y-axis) and green (x-axis) fluorescence of the EPCs (every dot represents a cell). The vertical bar of the quadrant depicts the border between positive and negative fluorescence. The right shift of the cloud thus indicates the uptake of calcein by the EPCs. Figure 8b shows only the green fluorescence in a histogram. The results can be represented this way because the red fluorescence remains the same throughout the experiment. The right shift of the peak indicates the uptake of calcein by the EPCs. And figure 8c shows only the green fluorescence in a bargraph. Here, the means of the logarithmic values of the original data (an example of which is shown in the histogram in figure 8b) are plotted, together with the standard deviation (error bars). The logarithmic values are plotted here because in the logarithmic range, the histograms show a normal distribution. Therefore, the mean and standard deviation can be calculated for the logarithmic values of these data. When the error bars are not overlapping, these two populations can be considered as significantly different.
The uptake of calcein by EPCs through contact with HUVECs is time dependent. After five minutes, a significant amount of green fluorescence is detected in the EPCs; after twenty minutes this amount has increased even more (although not significantly different from the five-minute incubation).
Heptanol, a known nonspecific, reversible gap junction blocker, is able to decrease the mean fluorescence signal in EPCs after a twenty-minute incubation significantly, compared with the non-inhibited twenty-minute incubation, as shown in figures 8a and b by the shift to left and in figure 8c by the significant decrease in fluorescence mean.

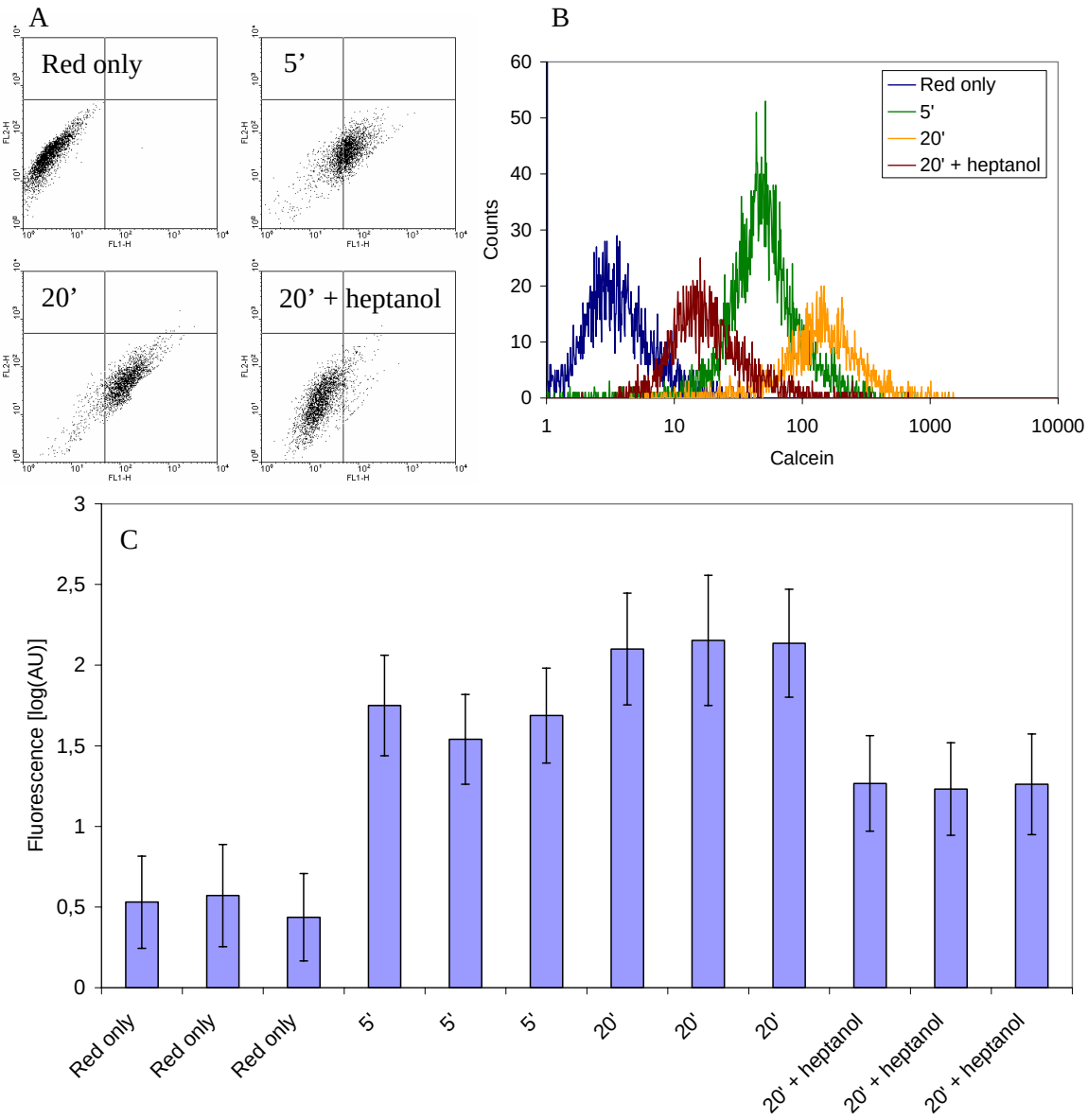


Figure 8: Results from the HUVEC-EPC co-incubation experiment, shown in three ways. A: Dotplot showing the green fluorescence channel on the x-axis and the red fluorescence channel on the y-axis. The vertical bar of the quadrant (shown in these figures) indicates the border between EPCs negative and positive for calcein. This was defined by the control, the only red EPCs (they were not co-incubated with HUVECs). All experiments were done in threefold, but only one is shown for each. B: Histograms of the green fluorescence channel for the four different experiments. All experiments were done in threefold, but for clarity only one is shown for each. C: Bargraph showing the mean logarithmic values of the green fluorescence channel for all experiments, in threefold (one of each is shown in A and B). Logarithmic values are plotted here because the distribution is normal in the logarithmic range. Therefore, the mean and the standard deviation (also plotted) could be determined. When the standard deviations of two bars are not overlapping, the means of the two bars can be considered significantly different.

Roles of the specific connexins in the formation of gap junctions

Next, it was investigated whether cell lines expressing only one specific connexin also were able to couple to EPCs via gap junctions, and allow the transfer of calcein. Therefore, HeLa cell lines transfected with a gene coding for either one of the three connexins (H37, H40 and H43, a generous gift from prof. Willecke, Bonn, Germany) were used. For control experiments, EPCs were co-incubated with HeLa WT (which do not express any connexins). Results are shown in figure 9. Co-incubation did not result in a significant dye transfer from HeLa cells to EPCs, independent of the HeLa cells used (WT, 37, 40 or 43).

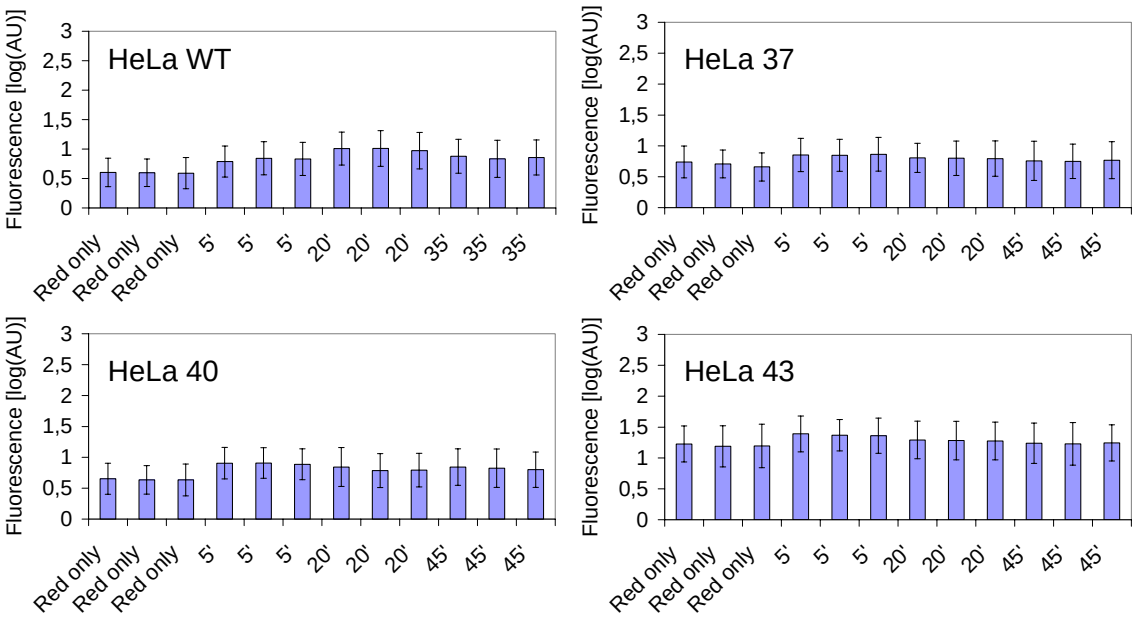


Figure 9: Results for the co-incubations with EPCs and either HeLa WT or one of the transfected HeLa cell lines. For none of the experiments significant differences were found between the controls and the co-incubated EPCs, indicating no significant calcein transfer to the EPCs.

Discussion

In this study, the goal was to determine if gap junctional communication is possible between (mouse embryonic) EPCs and (human umbilical cord) endothelial cells; first by determining if gap junctions can be present between EPCs themselves, and then by establishing which of the three connexins 37, 40 and 43 are expressed by EPCs. These three connexins are considered because endothelial cells also express these three connexins, and since EPCs are predestined to differentiate to endothelial cells these connexins are most likely to also be expressed by EPCs. Also co-incubation studies were done with EPCs and HUVECs in which the dye transfer from HUVECs to EPCs was measured after certain periods of time using flowcytometry. Finally, an attempt was made to establish the importance of the three different connexins in gap junctional communication with EPCs using HeLa cell lines (which naturally do not express any connexins) transfected with one of the three connexins.
The experiments in this study showed that EPCs contain mRNA coding for Cx37, 40 and 43, and are likely to contain mostly Cx43 proteins, and to a lesser extent also Cx37 and 40 proteins. Importantly, it was shown that active gap junctions can exist between HUVECs and EPCs by performing the dye transfer experiment. It could not be determined here which specific connexins were responsible for the dye transfer to EPCs.

EPCs contain connexins
EPCs appear to contain predominately connexin 43 (Cx43), but in addition they are also likely to contain connexins 37 and 40 (Cx37 and 40), only to a lesser extent. The fact that EPCs are likely to contain all three connexins is based on the RT-PCR results, showing positive for mRNA for all three connexins. The fact that Cx43 seems to predominate is based on the Western blot results as well as the histology results, both showing more positive for Cx43 than for Cx37 and 40. This indicates that gap junctions in EPCs are mostly build of Cx43. The histology of Cx37 and 40 showed a rather diffuse distribution, slightly more positive than the background, whereas Cx43 showed a bright, punctuate distribution. This punctuate distribution of connexins in the cellular membrane was also described in the literature [Yeh *et al.*, 2000], in this case in endothelial cells.
A remark should be made about the Western blot results. The reason that Cx37 and 40 were not found positive in the Western blot may be due either to low sensitivity of the antibodies, or to a low or heterogenous expression level of these connexins within a population. Therefore, it is possible that Cx37 and 40 are indeed present in EPCs, but under the detection level of the system. Also for Cx43 the Western blot results were not conclusive, since a positive control turned out negative and also a negative control positive. Supported by the immunofluorescence results, it may be concluded that all three connexins are present in EPCs, but predominantly Cx43.
The fact that EPCs contain Cx37, 40 as well as 43 is similar to the expression of connexins in endothelial cells; they also express these three connexins, in different expression levels depending on their location in the vascular tree and condition [Van Rijen *et al.*, 1997; Yeh *et al.*, 2000]. Of course, the expression of other than these three connexins cannot be excluded, since that was not investigated.

EPCs can form gap junctions with HUVECs
Based on the co-incubation studies, it was determined that active gap junctions can be present between EPCs and HUVECs. The fact that already after five minutes a significant increase in green fluorescence was found indicated that this coupling is relatively fast. This would be necessary in vivo, since EPCs are found in the adult blood circulation, and contact between EPCs and endothelial cells is only very brief in normal situations. When EPCs are needed to remain attached to the endothelium (for example, to aid in the repair of the endothelium), this has to be communicated fast. A five minute range in this case would be considered rather slow, but the fact that the amount of transferred calcein is already significant after this period

of time indicates that the transfer is indeed fast. Expecting that calcein is passively distributed after gap junctional coupling has been achieved, the initial transfer of calcein is the fastest. This is necessary for the fast communication needed in this case.
It cannot be determined here how exactly the relation is between time and calcein uptake, because only two time points were investigated. But when a passive diffusion is presumed (as is the case for substrates being exchanged by gap junctions), it can be expected that the increase in fluorescence signal (for the receiving cell) per time step will become smaller in time, and eventually become zero. These results fit this expectation.
Also the fact that EPCs have to be only loosely attached to the HUVECs to make gap junctional communication possible supports the theory that EPCs in the adult circulation are able to couple to endothelial cells by gap junctions: after five minutes the EPCs could still be removed from the HUVECs by simply removing the Hepes buffer. In other words, no firm attachment to EPCs is required for gap junctional communication.
An important note to this study is the fact that the EPCs used in this study were mouse embryonic EPCs, while the endothelial cells (HUVECs), as well as the HeLa cell lines, were human. It could be expected that these cell lines are not able to communicate via gap junctions, because the connexins do not match. However, it has been suggested that nineteen connexins in mouse and human can be considered as orthologues pairs, since the genes coding for the different connexins are preserved quite well throughout evolution. Especially the Cx37, 40 and 43 - genes can be considered as highly conserved in mouse and human with regard to their nucleotide- and protein similarity and transcriptional pattern. Therefore, one can compare functional aspects of these connexins in these two species [Sohl and Willecke, 2004]. In addition, the genes transfected into HeLa cells coding for Cx37 and 40 are mouse DNA sequences [Elfgang *et al.*, 1995; Hennemann *et al.*, 1992].
Furthermore, to investigate EPCs in adult circulation, HUVECs are not ideal, since they are isolated from umbilical cords. Therefore, because of the fact that mouse EPCs and umbilical cord endothelial cells were used in this study, this experimental set-up does not resemble natural gap junctional communication in human adult circulation.
Also, performing the co-incubation studies, only de novo formed gap junctions were analyzed and, in addition, only one-way communication was investigated as EPCs were only investigated to receive calcein. However, this investigated pathway does seem to be the most relevant one, since it is most likely that endothelial cells have to send signaling molecules to EPCs to trigger them to remain associated to the endothelium. Of course, this does not exclude the possibility that EPCs also communicate back to endothelial cells. This was however not investigated in this study.
And the last remark is that gap junctional communication in this study was analyzed using a dye molecule (either Alexa Fluor 488 or calcein). Of course, in actual gap junctional communication other molecules are transferred between cells, therefore the possibility of cells to exchange this dye molecule does not necessarily mean that this resembles the actual communication, since signaling molecules can differ in size, charge and shape. This way of analyzing is therefore only an approximation of the actual possibility of gap junctional communication between cells.

Functional gap junctions may contain more than one type of connexin
To determine the role of specific connexins in this communication, EPCs and connexin-transfected HeLa cell lines (H37, 40 and 43) were co-incubated. Surprisingly, in none of these experiments any significant dye transfer could be observed. One explanation for this would be that the expression of one of the three connexins only is not enough to form gap junctions with EPCs, in other words, that gap junctions consisting of at least two types of connexins are required. Another possible cause for these results could also be that surface proteins not present in HeLa cells are required to initiate gap junctional coupling. A third reason for the negative results could be that gap junctions between HeLa cells and EPCs establish much slower than between EPCs and HUVECs, so that the incubation time of 45 minutes was not enough to result in detectable dye transfer. The possibility of other surface proteins contributing to the docking of two cells was also suggested by Sohl and Willecke [Sohl and

Willecke, 2004]. Also, Hoffmann et al. [Hoffmann *et al.*, 2003] showed that significant dye transfer between HUVECs was already established after 15 minutes, whereas for transfected HeLa cell lines (in different combinations) at least 1.5 hours was needed to show an increase in dye transfer. Also, this increase was always (except for the H43-H43 combination) much smaller than for the HUVEC-HUVEC co-incubation. This indicates that gap junctional coupling with HeLa cells is indeed a much slower process than gap junctional coupling with HUVECs. A longer co-incubation time for EPCs with one of the HeLa cell lines should therefore be tested.

This study has shown that it is indeed possible for EPCs to couple to endothelial cells via gap junctions in a fast and efficient way. This strongly indicates that gap junctional coupling plays an important role in the communication between the two cell types. If this communication can indeed trigger EPCs to differentiate to endothelial cells and because of that can aid in either endothelium repair or angiogenesis remains to be elucidated.

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