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Expression of connexins in human preimplantation embryos in vitro

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Abstract

Intercellular communication via gap junctions is required to coordinate developmental processes in the mammalian embryo. We have investigated if the connexin (Cx) isoforms known to form gap junctions in rodent preimplantation embryos are also expressed in human embryos, with the aim of identifying species differences in communication patterns in early development. Using a combination of polyA PCR and immunocytochemistry we have assessed the expression of Cx26, Cx31, Cx32, Cx40, Cx43 and Cx45 which are thought to be important in early rodent embryos. The results demonstrate that Cx31 and Cx43 are the main connexin isoforms expressed in human preimplantation embryos and that these isoforms are co-expressed in the blastocyst. Cx45 protein is expressed in the blastocyst but the protein may be translated from a generally low level of transcripts: which could only be detected in the PN to 4-cell embryos. Interestingly, Cx40, which is expressed by the extravillous trophoblast in the early human placenta, was not found to be expressed in the blastocyst trophectoderm from which this tissue develops. All of the connexin isoforms in human preimplantation embryos are also found in rodents pointing to a common regulation of these connexins in development of rodent and human early embryos and perhaps other species.

Background

An appropriate temporal and spatial pattern of intercellular junctions is needed for successful preimplantation development and implantation in human embryos. Experiments on rodent preimplantation embryos have shown that the onset of E-cadherin expression is essential for compaction [1] and expression of the tight junction protein complex is responsible for maintaining cellular polarity of the trophectoderm through positioning the basolateral Na^+/K^+-ATPase (for review see [2]).

Both human and rodent preimplantation embryos express an array of junctional proteins, including components of tight junctions, desmosomes and other cell adhesion molecules. While comparison of rodent and human preimplantation embryos has shown broad similarities between the two species there are also some notable differences. These include the lack of detectable β3 intergrin and later expression of ZO-2 at the transcript level in human embryos, as well as low expression of ZO1α+ transcripts and poor membrane assembly of junctional proteins [3,4]. In addition to these junctional complexes,

human preimplantation embryos, like rodent embryos [5-7] form gap junctions [8]. Gap junctions allow the direct exchange of ions, small metabolites, second messengers and nucleotides between the cytoplasm of neighbouring cells. Each gap junction channel is formed by docking of two hemi channels on adjacent cells and each hemi channel is composed of six connexin subunits surrounding a water filled pore. Twenty different connexin isoforms have been identified in the human and 19 in the mouse (for review see [9]). Variation in isoform composition of gap junctions allows diversity in the communication properties between cells of different tissues or even between cells within the same tissue. Connexin mutations have been identified in genetically inherited human diseases (for review see [10]) suggesting that these communication channels have fundamental functions. Targeted connexin gene deletion experiments have confirmed that isoform composition influences the specificity of gap junction function (for review see [9,10]) while evidence from, targeted insertion experiments has shown that channels are also able to share functions [11].

In human embryos Cx43 protein was shown to be expressed throughout preimplantation development while Cx26 and Cx32 were detected only occasionally in the trophectoderm of late blastocyst stage embryos. Evidence suggests that aberrant expression and distribution of the Cx43 channel proteins may affect the survival potential of human embryos [8]. In mouse and rat, transcription of 8 connexin isoforms was detected during preimplantation development (for review see [7,12]), with transcripts of, Cx43, Cx31, Cx31.1 and Cx45 also detected at the protein level. Unlike the mouse, Cx26 was found at both the mRNA and protein level in the rat blastocyst [7]. Of all the connexin isoforms, only Cx43 and Cx31 were abundantly expressed and both were identified in the trophectoderm as well as in the inner cell mass and were seen to co localise in the same gap junctional plaque [13,14]. Despite the expression of multiple connexin isoforms, the functional significance of heterogeneous connexin composition of plaques is still uncertain. Neither Cx43 nor Cx31 gene deficiency in mice resulted in impaired preimplantation development or inhibited implantation. This could indicate functional compensation for missing connexin isoforms. However, experiments with Cx43 knockout preimplantation embryos did not show up-regulation of other connexin isoforms while complete blocking of communication properties had no effect on the development or physiology of cultured mouse embryos [7]. Cx31, Cx43 and Cx45 rapidly become segregated to different tissues after implantation in mice: Cx31 is restricted to the trophectoderm lineage and is present in the spongiotrophoblast of the placenta whereas Cx43 and Cx45 are the connexins of the embryo proper [13,15]. For this reason it is speculated that the expression of multiple connexins allows the embryo to undergo rapid diversification into embryonic and extraembryonic tissues. However in the human, Cx31 has not been detected even very early in the first trimester placenta and primary trophoblast cells. Instead of Cx31, Cx40 is the characteristic connexin of the proximal cells of the extravillous trophoblast which can be functionally compared to the rodent's spongiotrophoblast [16,17].

In this study, we have extended our understanding of connexin expression in early human embryos by examining Cx26, Cx31, Cx32, Cx40, Cx43 and Cx45 at both transcript and protein level. We sought to provide further evidence for the hypothesis that multiple connexin isoforms are present in the preimplantation human embryo and identify similarities and differences from rodent connexin expression patterns with a view to understanding cell communication patterns required for blastocyst formation.

Materials and methods

All reagents were purchased from Sigma (Dorset, UK) unless otherwise stated.

Embryos

Embryos were donated with fully informed consent by patients being treated by *in vitro* fertilisation (IVF) at Leeds General Infirmary, Leeds, UK, St Mary's Hospital, Manchester, UK, and Manchester Fertility Services, Whalley Range, Manchester, UK. All research was carried out with the permission of local ethical committees, and in accordance with the licence conditions of the Human Fertilisation and Embryology Authority (HFEA: project licence R0026). cDNAs from 3 embryos at each stage of development were probed for connexin gene expression (as described by Bloor et al., [3]). Antibody staining was carried out on 5–6 embryos at the blastocyst stage for each connexin protein: a total 37 embryos were used including appropriate controls. Early cleavage stage embryos (pronucleate to 8-cell-stage) were considered to be of the highest possible quality since they were cultured from unselected frozen pronucleate stage embryos donated to the programme. They were not embryos discarded from replacement cycles. All the pronucleate, 2-cell and 4-cell-embryos were from pregnant cycles (i.e. had siblings which developed to term). One of the 8-cell-embryos was from a pregnant cycle, while the two other 8-cell-embryos were siblings from a cycle that did not result in pregnancy. However, the donating parents were fertile since they later achieved a spontaneous pregnancy. Embryos lysed at blastocyst stage had been transferred from Leeds General Infirmary to the University of York at early cleavage where they were cultured to blastocyst. These embryos had undergone some initial selection in that they had not been chosen for replacement or freezing. However, they were

clinically graded I or sometimes II and, at the time of lysis, all were assessed morphologically to be of high quality with >30 cells and clear evidence of an ICM. Polyspermic or multinucleated embryos were not used in this study.

Embryo thawing

Embryos were thawed using standard clinical protocols [18]. Briefly, they were transferred sequentially through thawing solutions, T1 (1 M 1,2 propanediol, 0.2 M sucrose in phosphate buffered saline (PBS)), T2 (0.5 M 1,2 propanediol, 0.2 M sucrose in PBS), T3 (0.2 M sucrose in PBS), and T4 (IVF Universal; MediCult, UK) with 5 minutes incubation at each stage. Embryos were judged to be viable if they survived thawing followed by 1 h in culture. The rate of survival was 85%.

Embryo culture

For culture of early cleavage stage embryos (up to 8-cell), immediately post-thaw, embryos were transferred to 200 μl drops of pre-equilibrated medium (IVF Universal; MediCult, UK) under oil and incubated at 37°C in 5% CO_2 in air under the standard conditions used for IVF at St Mary's hospital. For blastocyst stages, embryos were cultured to early cleavage stage in 70 μl drops of IVF medium under oil (BDH) at 37°C in 5% CO_2 in a humidified incubator; the standard embryo culture conditions at Leeds General Infirmary. Embryos were then transferred to the University of York where they were cultured individually in 4 μl drops as described previously [3,19]. Embryos were transferred to a fresh, pre-equilibrated 4 μl drop of embryo culture medium every 24 hours.

Lysis, 3' cDNA generation and 2° amplification (polyA PCR)

The technique was as reported in [3] and adapted from [20,21] and has been extensively validated [22-24]. A single embryo was transferred in a minimum volume of culture medium (< 0.5 μl) to 4.5 μl complete lysis buffer as in [3]. Briefly reverse transcription was carried out using 25 units (0.5 μl) of reverse transcriptase (Superscript RNAseH$^-$, Invitrogen, UK) at 37°C for 15 minutes, 65°C for 10 min followed by cooling on ice. Limitation of the reverse transcription step to 15 min resulted in the formation of cDNAs of 300–600 bp, reducing the possibility of size-dependent preferential amplification during subsequent PCR amplifications and this maintains representation [24]. One volume of tailing buffer (0.33 M potassium cacodylate, 6.7 mM $CoCl_2$, 0.67 mM DTT, 0.16 mM dATP, 0.45 units/μl rTdT (Invitrogen, UK)) was added and the reaction incubated at 37°C for 15 min, 65°C for 10 min. This step polyadenylates the 1st strand cDNA, allowing subsequent global amplification of cDNA using a single Not1dT_{24} oligonucleotide primer (Invitrogen).

PCR amplification of the polyA-tailed cDNA was carried out as previously described [3,25] by the addition of 2 volumes of primary PCR reaction mix to which were added Not1dT_{24} oligonucleotide primer and 0.16 units/μl Taq polymerase). The sequence of the Not1dT_{24} oligonucleotide primer is as follows: 5'CATCTCGAGCGGCCGCTTTTTTTTTTTTTTTTTTTTTT 3'. Amplification was carried out using the following cycling profile: initial denaturation at 94°C for 2 min, 25 cycles of 1 min at 94°C, 2 min at 42°C, 6 min at 72°C, linked to a further 25 cycles of 1 min at 94°C, 1 min at 42°C, 2 min at 72°C.

The 300–600 bp primary amplification products were subject to a further amplification as follows: 1 μl of primary PCR reaction was used as template in a 50 μl final reaction volume containing 10 mM Tris.HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTPs, 2 μM Not1dT_{24} oligonucleotide, 0.025 units/μl Taq polymerase. Amplification was carried out using the following cycling profile: initial denaturation at 94°C for 2 min followed by 50 cycles of 30 sec at 94°C, 30 sec at 54°C, 30 sec at 72°C.

Controls at each step included embryos lysed and subject to the amplification protocol without reverse transcriptase (RT negatives), confirming the absence of contaminating genomic DNA and no embryo material. Human RNA from a variety of tissues (Human total RNA master panel II, BD Biosciences, UK) was amplified using the same protocol to produce positive control cDNA. Negative and positive control samples were probed for the presence of target genes in tandem with test samples as below.

Normalisation of amplified cDNA

Serial dilutions of secondary amplification products were prepared and used as templates in a PCR reaction to amplify β actin as in [3] using the following cycling profile: initial denaturation at 94°C for 1 min followed by 23 cycles of 30 sec at 94°C, 30 sec at 62°C, 30 sec at 72°C. 10 μl of the resultant amplification products were visualised following electrophoresis on a 2% agarose gel stained with ethidium bromide. cDNA pools were discarded if no β actin signal was detected. To probe cDNAs for the presence of test genes, the samples were diluted in the ratio indicated from the β-actin serial dilution: normally a 10 fold concentration of the cDNA dilution at which β-actin was just detected being used. If product could not be detected gene-specific PCR was also attempted on undiluted samples but results were identical.

Gene specific PCR

Primers were designed to amplify target genes in the 500 base pairs immediately preceding the poly adenylation signal in the gene sequence. Primers were designed using PRIMER version 0.5 (copyright 1991, Whitehead Institute

Table 1: Gene specific amplification primers

Target Gene	Primer pair sequences (5' – 3')	Accession number	Position in sequence	Fragment Size (bp)	Annealing Temp (\r C)
β actin	GACAGCAGTCGGTTGGACC	M10277	3163–3179	387	62
	CAGGTAAGCCCTGGCTGC		3549–3532		
Cx26 (a)	GTTTAACGCATTGCCCAGTT	M86849	874–893	174	62
	TGTGGCATCTGGAGTTTCAC		1047–1028		
Cx26 (b)	AGGCCTGTCCAACACATCTC		1760–1779	244	64
	AGGGGGTAAGCCAAACAAAC		2003–1984		
Cx31	TGCAGTGGAGAGGAGGTCTT	BC012918	1454–1473	184	66
	CAGTTGTGGGGAGGAAGATG		1637–1618		
Cx32 (a)	GGGTACAAGAGATGGGATGC	BC039198	1200–1219	202	64
	GCCAGCAAGCACTATTCCTC		1401–1382		
Cx32 (b)	CCCTGGTTTTCTGGAGTCAC	NM_000166	1273–1292	206	62
	CCCTGCTCCAACTTATCTGC		1478–1459		
Cx40 (a)	TTGCAACCTTTCCTTCTGCT	BC013313	1878–1897	180	64
	GTGAACAGCCAAGGGAGAAA		2057–2038		
Cx40 (b)	CCCTGCTAGGGAGTCACTGT		1929–1948	201	62
	CTGGTCAGGGTTCGAGAGAG		2129–2110		
Cx43	CTGACATGCATGCAAGAAGAA	BC026329	2802–2782	217	64
	TCTTTTGGAGTGACCAGCAA		2586–2605		
Cx45	AGATCAGGATGGCTCAGGAA	U03493	1022–1041	155	64
	GTTCTTCCCATCCCCTGATT		1176–1157		

for Biomedical research). Primer pair sequences (Invitrogen) used in target gene amplification are shown in table 1. Where PCR products were not amplified from any cDNA sample in the panel, a second set of primers was designed and used to confirm the result. All amplification products were partially sequenced to verify identity using ABI Big dye technology (ABI, UK). For target gene amplification 1 μl of normalised cDNA was used as template in a 25 μl final reaction volume containing 10 mM Tris.HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTPs, 1 μM forward primer, 1 μM reverse primer, 0.025 units/μl Taq polymerase (Roche, Lewes, UK). To remove non-specific amplification of Cx43 product, amplification using Cx40 primers was carried out using HotMaster Taq (Eppendorf, Cambridge, UK) according to the manufacturer's instructions. Amplification was carried out using the following cycling profile: initial denaturation at 94°C for 1 min followed by 50 cycles of 30 sec at 94°C, 30 sec at appropriate annealing temperature (see Table 1), 30 sec at 72°C. Ten μl of the resultant amplification products were visualised following electrophoresis on a 2% agarose gel stained with ethidium bromide. All primers successfully amplified cDNA fragments of the appropriate size from control human cDNA

Immunocytochemistry

All embryos were double labelled to maximise the information gained from this scarce resource with a minimum of 5 embryos stained for each Cx isoform. Blastocysts for immunocytochemistry were obtained from the University of York where they were fixed in 1% paraformaldehyde in phosphate buffered saline (PBS) prior to transportation to the University of Manchester (in approx 14–24 h). Embryos were recovered, washed through PBS supplemented with 4 mg/ml IgG free BSA (PBS/BSA, Stratech, UK). The zona pellucida was removed by sequential incubation in pronase (2.5 u/ml in PBS/BSA) for 5 mins then acid Tyrode's for 1–2 mins. After extensive washing, embryos were permeabilised in 0.01% Triton X-100 in PBS/BSA for 3 mins. The embryos were washed and transferred to a 25 μl drop of primary antibody for 1 hour under oil (Table 2). Rabbit antibodies were pre-adsorbed with keratin prior to use to remove any anti-keratin antibodies [26]. Embryos were washed then incubated in 25 μl of a second primary antibody from a different species. After washing they were incubated in a mixture of appropriate secondary antibodies previously checked for cross reactivity (Molecular Probes, UK). Nuclei were stained using 0.25 μg/ml Hoechst 33342 in PBS/BSA for 30 min. After final washing, embryos were transferred to a 0.1 mm microslide (Camlab, Cambridge UK), which was sealed then mounted onto a glass slide prior to visualisation of staining by confocal microscopy. Microscopy was performed using a BioRad MRC 600 laser scanning attachment (BioRad Microscience, UK) linked to a 90 MHz Pentium Compaq personal computer running COMOS Version 6 control software and NiZeiss microsope or a BioRad MRC1024 MP confocal head mounted on a Nikon Eclipse TE300 fluorescence microscope (BioRad, UK). Images were processed using Biorad LaserSharp software. Controls were incubated sequentially with normal rabbit

Table 2: Antibodies used in immunocytochemistry

Antigen	Antibody Isotype	Dilution	Secondary Antibody	Dilution
Cx26	Rabbit polyclonal [34]	1:1000	Alexa fluor 546, goat anti rabbit IgG	1:125
Cx31	Rabbit polyclonal [35]	1:300	Alexa fluor 546, goat anti rabbit IgG	1:125
Cx40	Rabbit polyclonal [36]	1:100	Alexa fluor 546, goat anti rabbit IgG	1:125
Cx43	Mouse IgG (Zymed)	1:500	Alexa fluor 488, goat anti mouse IgG	1:125
Cx45	Rabbit polyclonal [35]	1:500	Alexa fluor 546, goat anti rabbit IgG	1: 125

Table 3: Transcript expression

	PN			2 cell			4 cell			8 cell			Blastocyst		
Gene	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
β actin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cx26	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Cx31	+	+	-	-	+	+	+	+	+	+	+	-	+	-	+
Cx32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cx40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cx43	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+
Cx45	-	+	-	+	-	-	-	+	-	-	-	-	-	-	-

Expression of β actin, connexins 26, 31, 32, 40, 43 and 45 in cDNAs amplified from three individual embryos (1,2,3) at the pronucleate (PN), 2-cell, 4-cell, 8-cell, and blastocyst stages of development. + denotes trancript detected, – denotes transcript not detected.

serum and mouse IgG in place of primary antibodies. All images for test antibodies and controls were collected using identical confocal settings and were manipulated identically after collection.

Results

Transcript expression

All embryos in the panel expressed at least one connexin isoform. Gene expression results are summarised in Table 3 and data are shown in Figure 1. Cx26 was detected in one 8-cell-embryo only and Cx45 was detected in a single pronucleate and a single 4-cell-embryo. Interestingly, no signal for Cx40 could be amplified despite use of two primer sets, both of which gave a positive signal with control cDNA. However, Cx31 transcripts were found in all developmental stages in most of the embryos. Cx31 was detected in 2/3 of the pronucleate embryos, 2/3 of the 2-cell-embryos, 3/3 of the 4-cell-embryos, 2/3 of the 8-cell-embryos and 2/3 of the blastocysts. Cx43 was detected in all pronucleate embryos, 2/3 of the 2-cell-embryos 1/3 of the 4-cell-embryos and all 8-cell and blastocyst stage embryos. No transcripts of Cx32 were found at any stage of preimplantation development.

Immunocytochemistry

Immunocytochemistry was used to investigate protein expression for Cxs26, 31, 40, 45 and 43 in fixed human blastocysts (Table 4) For Cx26, 5/6 embryos were positive for the protein, and in the same embryo cohort, 5/6 of these embryos also showed staining for Cx43. The same embryo was negative for both Cx26 and Cx43. Some correlation was seen between intensity of staining for Cx26 and 43. For Cx31, only 1/5 of the embryos were stained and in the same cohort 3/5 showed staining for Cx43. Again, there was some correlation between Cx31 and 43 staining, with 2/5 embryos negative for both proteins and the single Cx31 positive embryo showing the only bright junctional staining observed with Cx43. When Cx45 was examined, 4/6 embryos showed staining while 5/6 showed staining for Cx43. One of the embryos was negative for both Cx45 and Cx43. The bright punctuate staining of both Cx31 and Cx45 showed complete colocalisation with Cx43, and there was also approximately 50% colocalisation between Cx26 and Cx43 protein staining. No staining could be detected above background in 6 embryos stained for Cx40. Representative images of positively stained embryos are shown in Figure 2.

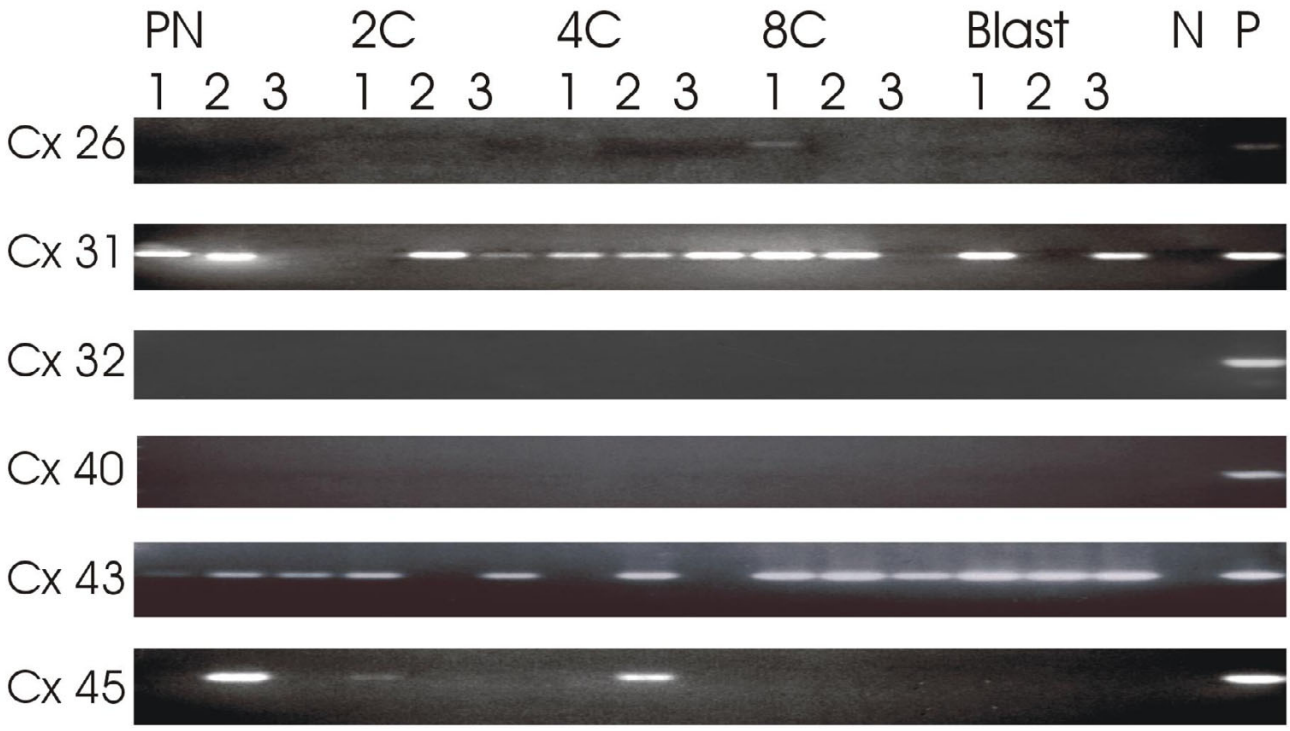

Figure 1
PCR amplification of Cx26, Cx31, Cx32, Cx40, Cx43 and Cx45 from cDNAs amplified from three individual embryos (1,2,3) at the pronucleate (PN), 2-cell (2C), 4-cell (4C), 8-cell (8C), and blastocyst stages of development. N denotes negative control; P denotes positive control.

Discussion

The present study revealed several connexin isoforms in developing preimplantation human embryos identified at the transcript and/or protein level, Cx26, Cx31, Cx43 and Cx45, whereas Cx32 and Cx40 were not detectable. This study extends the observations of Hardy et al. [8] that diverse connexin proteins are expressed in human preimplantation embryos in a similar pattern to that seen in rodents. In addition, the present study confirms that Cx43 is the predominant transcript as well as the predominantly expressed gap junction protein in human preimplantation embryos. Because of limited availability of human embryos we focused on those connexins identified as playing an important role in early embryonic and placental lineage development in rodents such as Cx43 and Cx45 for the embryo proper [13,15] and Cx31 for placental development [13,14]. Cx45 transcripts were detected in one third of PN and early cleavage stage embryos but not in later embryos, although the protein was expressed by blastocysts. This suggests that transcripts are generally at extremely low abundance and not detected in this study. However, it might also indicate that there is a delay between transcript synthesis and translation and protein assembly as has been found for junctional components in the mouse [27]. In contrast to the observation of Hardy and colleagues on Cx proteins [8], Cx32 transcript was not seen in our investigations. However, we did identify a weak band for Cx26 transcripts at the 8-cell-stage and immunoreactivity in the blastocyst stage. This may suggest that Cx26 transcripts are present at very low levels. It is notable that Cx32 and Cx26 proteins were only observed occasionally in late blastocysts by Hardy et al. [8]. This may suggest that these connexins appear prior to implantation and that the lack of Cx32 staining relates to differences in blastocyst developmental maturity. However these differences may be due to the response of human embryos to the different culture conditions used in the two studies [8]. Cx26 has been found in rat but not in mouse preimplantation embryos. The question arises as to the function of those weakly or inconsistently expressed genes.

In rodents, Cx31 represents a marker gene for the extraembryonic cell lineages but this has not been observed in

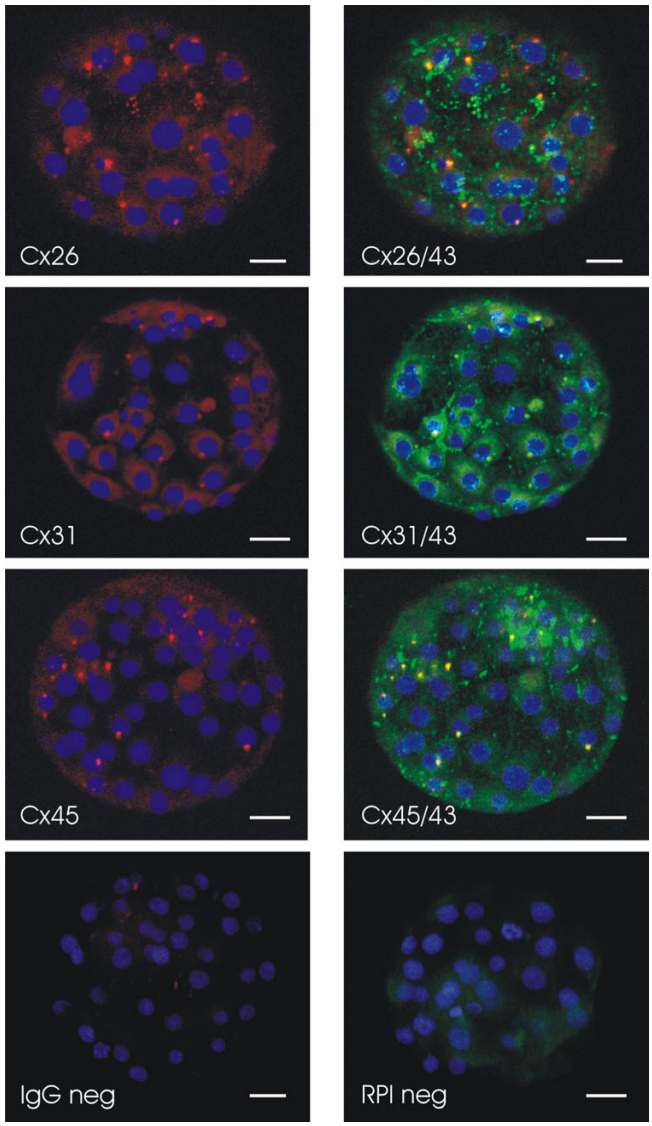

Figure 2
Confocal images of fixed human blastocysts showing protein localisation of Cx26, Cx31, and Cx45 (shown in red) and colocalisation (shown in yellow) with Cx43 (shown in green) (Cx26/43, Cx31/43, Cx45/43). Nuclei are shown in blue. Negative control images of blastocysts incubated with mouse IgG (mIgG) and rabbit pre-immune serum (RPI). Scale bars = 25 μm.

Table 4: Summary of staining human blastocysts with anti-connexin antibodies

Embryo	Cx43	Cx26	Cx31	Cx45	Cx40
1	+	+			
2	+	+			
3	+	+			
4	+	+			
5	-	-			
6	+	+			
7	+		-		
8	+		+		
9	-		-		
10	-		-		
11	+		-		
12	+			+	
13	+			+	
14	+			+	
15	+			+	
16	-			-	
17	+			-	
18	+				-
19	+				-
20	+				-
21	-				-
22	-				-
23	+				-

Individual human blastocysts were stained with antibodies to Cx 43,26, 31, 45 and 40 as indicated in materials and methods. Double staining with Cx43 and one other connexin was carried out in each case. +, specific staining detected; -, no staining detected.

humans. In this study, we therefore tried to elucidate the role of Cx40, which plays a major role in development of the human placenta [16,28,29]. Cx40 is found in the early human placenta in the extravillous trophoblast cell population which is functionally analogous to the spongiotrophoblast [16,30]. It appears to be produced instead of Cx31 which is expressed during mouse placental development in the spongiotrophoblast but is absent during human placental development. However, transcripts for Cx40 were not detected at any stage of human early development, nor was the protein observed at the blastocyst stage. This is in contrast to the trophoblast associated transcription factor Hand-1 which is expressed from the 4-cell stage and in blastocyst trophectoderm in human embryos [31]. Hand-1 is essential for differentiation of murine trophoblast giant cells after implantation and these cells have similar behaviour to human extravillous cytotrophoblast [32]. Interestingly, Cx31 expression was observed in human blastocysts. Transcripts were detected in all the 4-cell-stage embryos and 4/6 of the 8-cell embryos and blastocysts. However, except for one embryo, the corresponding protein was only detectable as a weak cytoplasmic fluorescence in the blastocyst suggesting that the protein only rarely forms functional gap junctions in the human embryo. It may be translated to only a limited extent in preimplantation human embryos or assembly may occur late in the blastocyst stage after hatching. This expression pattern differs from that seen in

rodent preimplantation embryos which express Cx31 and Cx43 in the same spatiotemporal pattern during preimplantation development. It is possible that the failure to detect significant Cx31 protein could reflect a loss of embryo viability as a result of extended culture [8].

Hardy et al. [8] showed that early human embryos express predominantly Cx43 proteins and that protein levels increase during preimplantation development up to the blastocyst stage. This observation is supported by our studies. Cx43 transcripts were detected in nearly all embryos and the protein was present in 74% of the blastocyst stage embryos observed. The Cx43 protein was, in most cases, arranged along the cell borders in the expected typical punctuate pattern. We have previously found that the uniform assembly of junctional components observed for mouse embryos, is often not seen in human embryos which show a more patchy distribution [3]. Double immunolabelling of this dominant protein with other connexin isoforms did not provide evidence for sorting of the connexins between the trophectoderm and inner cell mass compartments. Though staining for Cx26, Cx31 and Cx45 was lower than for Cx43, they exhibited mostly coexpression with Cx43, probably in the same gap junction plaque. These findings are again in accordance with observations in rodent blastocysts where Cx31 and Cx43 colocalize in the same gap junction plaque [13,14] although other data suggests these may not cooperate to form functional channels [33]. Indeed, staining patterns for all the connexin proteins investigated indicated lack of any obvious compartmentalisation between the two blastocyst cell populations. Though all embryos investigated were of good morphology, they exhibited variability in connexin pattern at the protein level. It was obvious from double staining that when Cx43 was missing or poorly expressed in an embryo then the other connexin isoform assessed was missing too. It remains to be seen if this is related to the viability of the embryos or to the effects of processing. Identification of the presence and location of intercellular junctional proteins including gap junction components could help characterize the quality of embryos in culture. For example, Ghassemifar et al. [4] investigated the tight junction complex in human embryos, including JAM and desmocollin and showed that the level of junctional transcript was positively correlated to the morphological grade. Gap junction components such as Cx31 and Cx43 seem to be additional marker molecules for orderly embryonic development in culture.

Conclusion

Cx43, Cx31 and Cx45 appear to be the main connexin isoforms expressed in human preimplantation embryos and these proteins are coexpressed in the blastocyst: a pattern which resembles that of rodent preimplantation embryos. Low or undetectable transcript levels for Cx26 together with weak protein expression in the blastocyst suggest that this protein is less critical to gap junction function while Cx45 protein may be assembled in the blastocyst from protein translated from a generally low level of transcripts. Cx40, the connexin characteristic of extravillous trophoblast in early human placentas, is not present in blastocyst trophectoderm. The similarities in connexin expression in the human blastocyst compared to the rodents suggest that in preimplantation development different species are using common mechanisms of intercellular communication for blastocyst formation.

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