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CAR expression in human embryos and hESC illustrates its role in pluripotency and tight junctions

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Abstract

Coxsackie virus and adenovirus receptor, *CXADR* (*CAR*), is present during embryogenesis and is involved in tissue regeneration, cancer and intercellular adhesion. We investigated the expression of CAR in human preimplantation embryos and embryonic stem cells (hESC) to identify its role in early embryogenesis and differentiation. CAR protein was ubiquitously present during preimplantation development. It was localised in the nucleus of uncommitted cells, from the cleavage stage up to the precursor epiblast, and corresponded with the presence of soluble *CXADR3/7* splice variant. CAR was displayed on the membrane, involving in the formation of tight junction at compaction and blastocyst stages in both outer and inner cells, and CAR corresponded with the full-length CAR-containing transmembrane domain. In trophectodermal cells of hatched blastocysts, CAR was reduced in the membrane and concentrated in the nucleus, which correlated with the switch in RNA expression to the *CXADR4/7* and *CXADR2/7* splice variants. The cells in the outer layer of hESC colonies contained CAR on the membrane and all the cells of the colony had CAR in the nucleus, corresponding with the transmembrane *CXADR* and *CXADR4/7*. Upon differentiation of hESC into cells representing the three germ layers and trophoblast lineage, the expression of *CXADR* was downregulated. We concluded that *CXADR* is differentially expressed during human preimplantation development. We described various CAR expressions: i) soluble *CXADR* marking undifferentiated blastomeres; ii) transmembrane CAR related with epithelial-like cell types, such as the trophectoderm (TE) and the outer layer of hESC colonies; and iii) soluble CAR present in TE nuclei after hatching. The functions of these distinct forms remain to be elucidated.

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Introduction

Pluripotent human embryonic stem cells (hESC) can self-renew in an undifferentiated state while retaining the capacity to differentiate into cells representing the three embryonic germ layers. Understanding the mechanisms that establish and sustain this undifferentiated state has attracted significant attention during the last decade. Embryonic stem cells (ESC) and preimplantation embryos are two *in vitro* experimental models used to analyse the nature of pluripotency in the human. The inner cell mass (ICM) of blastocysts is used as the major source for hESC derivation (Thomson *et al*. 1998, Reubinoff *et al*. 2000). It is now generally accepted that hESC originate from precursor EPI cells (De Paepe *et al*. 2014). However, early cleavage stage blastomeres can give rise to hESC lines, indicating that they are pluripotent (Klimanskaya *et al*. 2007, Van de Velde *et al*. 2008, Geens *et al*. 2009). Previously, it has been described by us and other groups that the stemness markers defining hESC (*POU5F1*, *NANOG*, *SOX2* and *SALL4*) cannot be used to exclusively identify pluripotent cells in human preimplantation embryos (Cauffman *et al*. 2009, Niakan & Eggan 2013). This could be explained by differences in the processes establishing the undifferentiated state in these two experimental models. On the other hand, more information on other possible genes, which could specify this unique state, is required. This could also enrich our knowledge about the mechanisms underlying pluripotency.

During the analysis of recently published microarray data obtained on human preimplantation embryos and hESC (Dobson *et al*. 2004, Adjaye *et al*. 2005, Assou *et al*. 2009, Zhang *et al*. 2009, He *et al*. 2010), we observed an interesting abundance of coxsackie virus and adenovirus receptor, *CXADR* (*CAR*) RNA in these undifferentiated cells (confirmed by our unpublished data). We therefore hypothesised that CAR may be a potential marker for the undifferentiated state in human preimplantation embryos and hESC.

CAR is evolutionarily conserved in mammals and non-mammalian vertebrates. It belongs to the immunoglobulin superfamily of proteins and is known to process most adenoviral and group B coxsackieviral

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(CVB) infections (Bewley *et al*. 1999, Carson 2001, He *et al*. 2001, Freimuth *et al*. 2008). However, the presence of CAR is dispensable for CVB infection (Mena *et al*. 1999), which implies it may carry out a different function as well.

Indeed, before its involvement in viral infection, the role of CAR has been claimed to be important during embryonic development and regeneration. CAR is highly expressed during rodent embryogenesis, especially in the developing brain, but its expression significantly decreases after birth (Johansson *et al*. 1999, Nalbantoglu *et al*. 1999, Honda *et al*. 2000, Ito *et al*. 2000, Tomko *et al*. 2000, Carson 2001). In fact, CAR is found to be involved in neuronal stem cell niche formation via its function as a tight junction component (Hauwel *et al*. 2005). CAR has often been described in homotypic intercellular adhesion, particularly in tight junctions (Coyne *et al*. 2004, Philipson & Pettersson 2004, Coyne & Bergelson 2005). This fact is based on the ability of CAR to bind proteins, such as ZO1, β-catenin and MUPP1 (Cohen *et al*. 2001, Walters *et al*. 2002, Coyne *et al*. 2004). As a consequence of its connection to cellular adhesion, CAR has also been associated with carcinogenesis – exhibiting mainly tumour-suppressive activity (Kim *et al*. 2003, Pong *et al*. 2003, Fan *et al*. 2009, Wu *et al*. 2010, Majhen *et al*. 2011).

CAR-deficient mice are embryonically lethal due to insufficient heart function (Dorner *et al*. 2005), pointing out a critical role for CAR during cardiomyocyte development (Asher *et al*. 2005, Lisewski *et al*. 2008). Conditional knockout of CAR proved its importance in the formation of lymphatic endothelial intercellular junctions (Mirza *et al*. 2012). Recent work on human myocardial tissue has provided evidence for CAR being important for tissue restoration rather than the mediation of viral infection (Tatrai *et al*. 2011, Yasukawa *et al*. 2014). CAR expression levels are significantly increased in the regeneration of muscle fibres of the dystrophic mouse (Nalbantoglu *et al*. 1999) or in rats with autoimmune myocarditis (Ito *et al*. 2000). In humans, CAR is present in neonatal muscles, decreasing postnatally. This induction of CAR in self-renewing cells resembles the characteristics of stem cell markers.

The presence of CAR during normal embryonic development in neural, epithelial and muscle tissue suggests that it has a physiological function, which remains to be studied in detail. In this work, we investigated CAR expression in human preimplantation embryos and hESC.

Materials and methods

Human oocytes and preimplantation embryos

Human preimplantation embryos and oocytes were obtained for research at the Brussels' Centre for Reproductive Medicine with the approval of the Institutional Ethical Committee, the Federal Committee for Scientific Research on Embryos *in vitro*, and the couples' written informed consent. Female patients underwent controlled ovarian stimulation (Kolibianakis *et al*. 2004) and oocyte retrieval (Platteau *et al*. 2003). The oocytes were denuded from surrounding cumulus and corona cells (Van de Velde *et al*. 1997) and were matured *in vitro* from the germinal vesicle (GV) or the metaphase I (MI) stage until the metaphase II (MII) stage. Only oocytes matured at the moment of retrieval were injected (Devroey & Van Steirteghem 2004). All embryos were derived from normally fertilised oocytes (2PN) with a good morphology and normal developmental rate: G1 for the cleavage and compaction stages and blastulation with an A and B score for ICM and trophectoderm (TE) according to Alpha scientists (2011) (Embryology 2011). Days 2 and 3 embryos were created for research using donor oocytes and donor sperm with the permission of the Local and Federal Ethical Committees. Later stages (days 4–6) were mainly obtained from embryos diagnosed after preimplantation genetic diagnosis (PGD) as carrying genetic mutations; they were of good quality because human preimplantation embryos are known to develop normally after 1-cell biopsy for PGD and possess high implantation rates comparable with conventional ICSI embryos (Goossens *et al*. 2008). The embryos that were unsuitable for transfer and/or cryopreservation on day 3 but developed further normally were also used for the later stages, as well as some embryos that were created for research.

All the embryos were cultured individually in 25 µl droplets sequential M1 (EmbryoAssist) and M2 (BlastAssist) medium (Medicult) or Q1 (Quinn's Protein Plus Cleavage Medium) and Q2 (Quinn's Advantage Plus Blastocyst Medium) medium (Sage IVF) under oil (Vitrolife) (6% O_2, 5% CO_2 and 89% N_2).

Cell culture

Two fully characterised VUB hESC lines were used to execute this study (http://www.hESCreg.com) (VUB01 and VUB07) (Mateizel *et al*. 2006, 2010). They were derived from surplus IVF (IVF for VUB01 and ICSI for VUB07) embryos presumed to be genetically normal. Briefly, the cells were cultured in 20% O_2, 5% CO_2 at 37 °C in hESC medium: KnockOutTM-DMEM (KO-DMEM, Life Technologies) containing 20% KO-SR (KnockOutTM Serum Replacement; Life Technologies), 2 mM glutamine (Life Technologies), 1% nonessential amino acids (Life Technologies), 1 mM β-mercaptoethanol (Sigma–Aldrich), 4 ng/ml basic fibroblast growth factor – bFGF (Life Technologies), and 1% penicillin/streptomycin (Life Technologies)) on mytomycin-inactivated CF1 mouse embryonic fibroblasts (MEF) and were passaged by mechanical dissociation every 5–6 days. HESCs of both lines were used at early passages.

Differentiation of hESC

Osteogenic progenitor-like cells (OPLs) were generated and cultured in the EB medium (KO-DMEM (Life Technologies) containing 20% FCS (heat-inactivated FCS, GIBCO), 2 mM glutamine (Life Technologies), 1% nonessential amino acids (Life Technologies), 1 mM β-ME (Sigma–Aldrich) and 1% penicillin/streptomycin (Life Technologies)) as described previously (Mateizel *et al*. 2008).

Definitive endoderm was induced following a published protocol (Sui *et al*. 2012). HESC were cultured in RPMI 1640 – GlutaMAX (Life Technologies) supplemented with 0.5% B27 (Life Technologies), 100 ng/ml recombinant human Activin A (R&D systems, Minneapolis, MN, USA) and 3 μM CHIR99021 (Stemgent, Cambridge, MA, USA). After 1–2 days, CHIR99021 was removed from the culture medium and the cells were additionally cultured for 2 days.

Early neural ectoderm differentiation was achieved after 14 days of culture on Matrigel-coated dishes in the RPE medium (KO-DMEM (Life Technologies) containing 20% KO-SR (Life Technologies), 1% GlutaMAX (Life Technologies), 1% nonessential amino acids (Life Technologies) with 1 M nicotinamide (vitamin B3)) similar to the published protocol for retinal-pigmented epithelium cells induction (Idelson *et al*. 2009).

HESC were forced towards to the pure population of trophoblast epithelial as described previously (Marchand *et al*. 2011). The cells were initially cultured for 4–5 days in MEF-conditioned medium in the presence of bFGF (6 ng/ml, Life Technologies) and the following 10 days with recombinant human BMP-4 (100 ng/ml; R&D Systems), while omitting the bFGF.

Real-time RT-PCR

Total RNA from hESC, OPLs and hESC-derived trophoblast-like cells was extracted using the RNeasy Mini Kit (Qiagen). DNAse treatment (RNase-Free DNase Set, 18068015 (Invitrogen)) was carried out on all samples. High Capacity RNA-to-cDNA Synthesis Kit with no RT control (4390711, Life Technologies) was used to reverse-transcribe the isolated RNA. Transcripts in hESC were quantified by real-time RT-PCR (ABI Prism 7500 SDS; Applied Biosystems). We used TaqMan assays from Applied Biosystems to detect RNA levels for the following genes: *CXADR* (Hs00154661_m1); *SOX2* (Hs01053049_s1); *NANOG* (Hs02387400_g1); *COLL1* (Hs00164004_m1); *RUNX2* (Hs00231692_m1); *GAPDH* (Hs99999905_m1) and *RPS24* (Hs03006009_g1). Primers and probes for *UBC* (F: 5′-CGCAGCCGGGATTTG-3′; R: 5′-TCAAGTGACGATCACAGCGA-3′; probe TCGCAGTTCTTGTTTGTG) and *POU5F1*_iA (F: 5′-GGACACCTGGTCTGCGATTT-3′; R: 5′-CATCACCTCCACCACCTGG-3′; probe GCCTTCTCGCCCCC) were self-designed (Van Haute *et al*. 2009), while those for *KRT7* (F: 5′-GATGCTGCCTACATGAGCAA-3′; R: 5′-GATGATGCCGTCCAGGTC-3′ probe ACAGAGCTGCAGTCCCAGAT), *CGB* (F: 5′-CCGAGGTATAAAGCCAGGTACA-3′; R: 5′-TCCTTGGATGCCCATGTC-3′; probe GCACCAAGGATGGAGATGTT) were previously described (Marchand *et al*. 2011). *GAPDH*, *UBC* and *RPS24* were used as endogenous controls. We did not detect any signal in 'No RT' controls for our samples.

RT-PCR for CXADR *splice variants on human embryos*

RNA extraction from the embryos was executed as described previously (Cauffman *et al*. 2005). High-Capacity RNA-to-cDNA Synthesis Kit with no RT control (4390711, Life Technologies) was used to reverse-transcribe isolated RNA. To detect CAR expression, we carried out PCR using the same primers as reported by Dörner *et al*. (2004) (F: 5′-CATGGCGCTCCTGCTGTGCTTCGTGCTCCTGT-3′; R: 5′-AGGCTCTATACTATAGACCCAT-3′). PCR products were analysed by DNA electrophoresis under u.v. radiation.

Indirect immunocytochemistry

HESC colonies were plated on Matrigel-coated slides (177380, Lab-Tek/Nunc, Germany; Distributed by Thermo Fisher Scientific, Doornveld, Belgium) in the mTESR1 medium (Stem Cell Technologies, Grenoble, France) and cultured for several days to achieve flat expanded colonies.

Fixation of human embryos and hESC was performed with 3.7% formaldehyde (Merck; VWR International, Leuven, Belgium) for 10 min at room temperature. Human oocytes and embryos were individually manipulated in 50-μl droplets in a 96-well plate (Cellstar; GBO, Wemmel, Belgium). The samples were subsequently washed and permeabilised with 0.1% Triton X-100 (Sigma–Aldrich) for 20 min at room temperature. The samples were incubated overnight at 4 °C with polyclonal rabbit anti-CAR Abs (2 μg/ml; HPA030411, Atlas Antibodies/Sigma (Uhlén *et al*. 2012)), monoclonal mouse anti-Vimentin Abs (IgG1, clone V9; 1:30; V6389, Sigma–Aldrich (Ullmann *et al*. 2007)), monoclonal rat anti-SSEA3 Abs (IgM, 10 μg/ml; MAB4303, Millipore, Darmstadt, Germany), monoclonal mouse anti-ZO1 Abs (IgG1, 5 μg/ml; 339100, Invitrogen), monoclonal mouse anti-occludin Abs (IgG1, 6 μg/ml; 331500, Invitrogen), polyclonal goat anti-OTX2 Abs (IgG, 4 μg/ml; sc-30659, Santa Cruz Biotechnology, Heidelberg, Germany); monoclonal mouse anti-SOX17 Abs (2 μg/ml; SAB3300046, Sigma–Aldrich).

Control reactions for nonspecific binding were included in each experiment and carried out by replacing the primary Abs with a corresponding rabbit IgG (SC-2027, Santa Cruz Biotechnology, Heidelberg, Germany), mouse IgG1 (SC-2025, Santa Cruz Biotechnology, Heidelberg, Germany), or rat IgM (RGM00, Caltag, Buckingham, UK) at equal concentration as the corresponded primary Abs. The background signal for primary CAR antibodies in embryo staining was initially normalised using rabbit IgG (isotype control) (Supplementary Figure 1, see section on supplementary data given at the end of this article). The specificity of anti-CAR Abs was verified on embryos and hESC by blocking with peptide (EVHHDIREDVPPPKSRTSTARSYIGSNHSSLGSMSPSNMEGYSKTQYNQVPSEDFERTPQSPTLPPAKVAAPNLS) (20 μg/ml; provided by Atlas Antibodies, Stockholm, Sweden) (Supplementary Figures 2 and 3, see section on supplementary data given at the end of this article). Further verification of anti-CAR Abs provides the lack of the staining in *CXADR*-null CHO-K1 cells (You *et al*. 2001; Supplementary Figure 2C). Specificity of anti-OTX2 Abs has been verified by blocking with OTX2 peptide (P-15; sc-30659, Santa Cruz Biotechnology).

Alexa Fluor 488-conjugated F(ab′)2 fragment of goat anti-rabbit IgG (A11070, Invitrogen), Alexa Fluor 647-conjugated F(ab′)2 fragment of goat anti-mouse IgG (A21237, Invitrogen) and Alexa Fluor 647-conjugated F(ab′)2 fragment of chicken anti-goat IgG (A21469, Invitrogen) were used as secondary Abs at a concentration of 10 μg/ml for 1 h at room temperature in the dark. All primary and secondary Ab solutions were prepared in PBS supplemented with 2% BSA (Sigma–Aldrich). Extensive washing with PBS supplemented with 2% BSA was performed between all steps. After staining, samples were mounted using glass coverslips (24×3×50 mm) in SlowFade Gold antifade reagent with DAPI

(Molecular Probes). To prevent squeezing of the embryos, small round glass coverslips (Ø, 10 mm) were put between the coverslips. Confocal scanning microscopy with an Ar-HeNe laser (488/647) (IX71 Fluoview 300; Olympus, Aartselaar, Belgium) was performed to record the fluorescent images.

Results

Expression of CAR protein in human preimplantation embryos

We found that CAR protein is ubiquitously expressed during preimplantation development. We observed an equal distribution of CAR protein in the cytoplasm of mature oocytes (Fig. 1A). In 4-cell and 8-cell stage embryos, in addition to the cytoplasmic expression, CAR was also present in the nuclei (Fig. 1B and C). At compaction, the staining was still nuclear and cytoplasmic, while the protein started to concentrate in small patches on the membrane between the cells (Fig. 1D, arrow). At the early blastocyst stage, CAR protein strongly accumulated in distinct patches at the membrane of the outer cells, which still contained nuclear and weak cytoplasmic CAR. The inner cells of the early blastocyst displayed CAR in the cytoplasm, nucleus and in some cases on the membrane (Fig. 1E, white arrows). While the blastocyst matured, the earlier observed phenotype became more pronounced (Fig. 1F). In the expanded blastocyst, CAR was predominantly concentrated on the membrane of the TE cells. Yet, there were traces of perinuclear CAR inside TE cells. In addition, CAR protein was strongly distributed in the nuclei and the cytoplasm of the inner ICM cells (precursor epiblast, EPI). However, it is difficult to estimate the presence of CAR on the membrane of the precursor EPI cells, because the cells are tightly packed and expose to high levels of intracellular CAR. In addition, CAR was clearly observed in patches on the membrane between the ICM cells facing the cavity, known as the precursor primitive endoderm (PE) (Fig. 1F′, arrow). In brief, membrane-specific CAR expression appeared with compaction and was clearly observed on the TE and PE epithelial-like cell layers. In contrast, blastomeres of the early cleavage stages, ICM cells and precursor EPI cells were characterised by strong nuclear and cytoplasmic CAR expression, but we could not exclude the presence of the membrane CAR protein in those cells.

In total, 11 oocytes and 93 human preimplantation embryos (from which 25 were obtained after PGD) were used for this study. We did not detect a variation in the expression pattern of CAR protein between the two major groups of embryos – normal ICSI and PGD ICSI embryos. Negative controls are provided (Supplementary Figures 1 and 2).

CAR marks tight junctions in human embryos

We hypothesised that the accumulation of CAR protein into little aggregates on the membrane of compacting embryos may represent the formation of tight junctions. To determine *CXADR* as a marker for tight junction formation, we studied the expression of zonula occludens-1 (ZO1) protein in human preimplantation embryos. ZO1 normally localises on the membrane at intercellular tight junctions, also known as TJP1. Similar to CAR in the 8-cell stage embryo, ZO1 was absent from the membrane and concentrated within the cytoplasm, pointing to the absence of tight junctions at this stage (Fig. 1G). Later in development, e.g. in the full blastocyst, ZO1 was localised on the membrane of the TE and the ICM cells (Fig. 1H).

We further analysed colocalisation of CAR and ZO1 proteins at compaction and the blastocyst stages. CAR and ZO1 were predominantly aligned together on the membrane in these embryonic stages (Fig. 1I and J). These two proteins markedly gathered on the membrane of TE in tight junctions but also on the membrane of ICM cells (Fig. 1J, arrow). Similar results were obtained using another tight junction marker, occludin (OCLN), which also colocalised with CAR on the membrane of TE and ICM cells (Fig. 1K and L, arrow). In summary, CAR, ZO1 and OCLN, and thus the tight junctions, were not only present between the epithelial-like TE and precursor PE cells which are formed in the human embryo due to contact with the culture medium/environment but also inside the embryo (the ICM and precursor EPI).

CAR expression in undifferentiated hESC

We observed a similar phenomenon in hESC, another well-known example of undifferentiated cells. We utilised two normal hESC lines (VUB01 and VUB07) (Mateizel *et al*. 2006, 2010). The immunostaining carried out on undifferentiated hESC grown on Matrigel revealed CAR on the membrane of the outer cells contributing to the superficial epithelial-like layer of the colony (Fig. 2A and A′). There, it was colocalised with ZO1, implying the presence of tight junctions. Upon analysis of the inner cells of the hESCs colony, we discovered that CAR and ZO1 were absent from the membrane (Fig. 2D and D′). While there were traces of CAR in the cytoplasm, the protein was abundant in the nuclei of the superficial and inner cells of these colonies. This complete absence of CAR and ZO1 proteins from membrane suggests a lack of tight junctions between the cells inside the hESC colony. Negative controls for antibody specificity are provided (Supplementary Figure 3A and B).

To analyse whether the superficial cells of the hESC colonies, carrying membrane CAR, retain their undifferentiated state, we studied the expression of SSEA3, the cell-surface marker that disappears first upon the early - ate commitment in hESC (Enver *et al*. 2005). Apparently, superficial hESC cells also expressed SSEA3, confirming the presence of CAR-positive intercellular contacts in undifferentiated hESC (Fig. 2F and Supplementary Figure 3D).

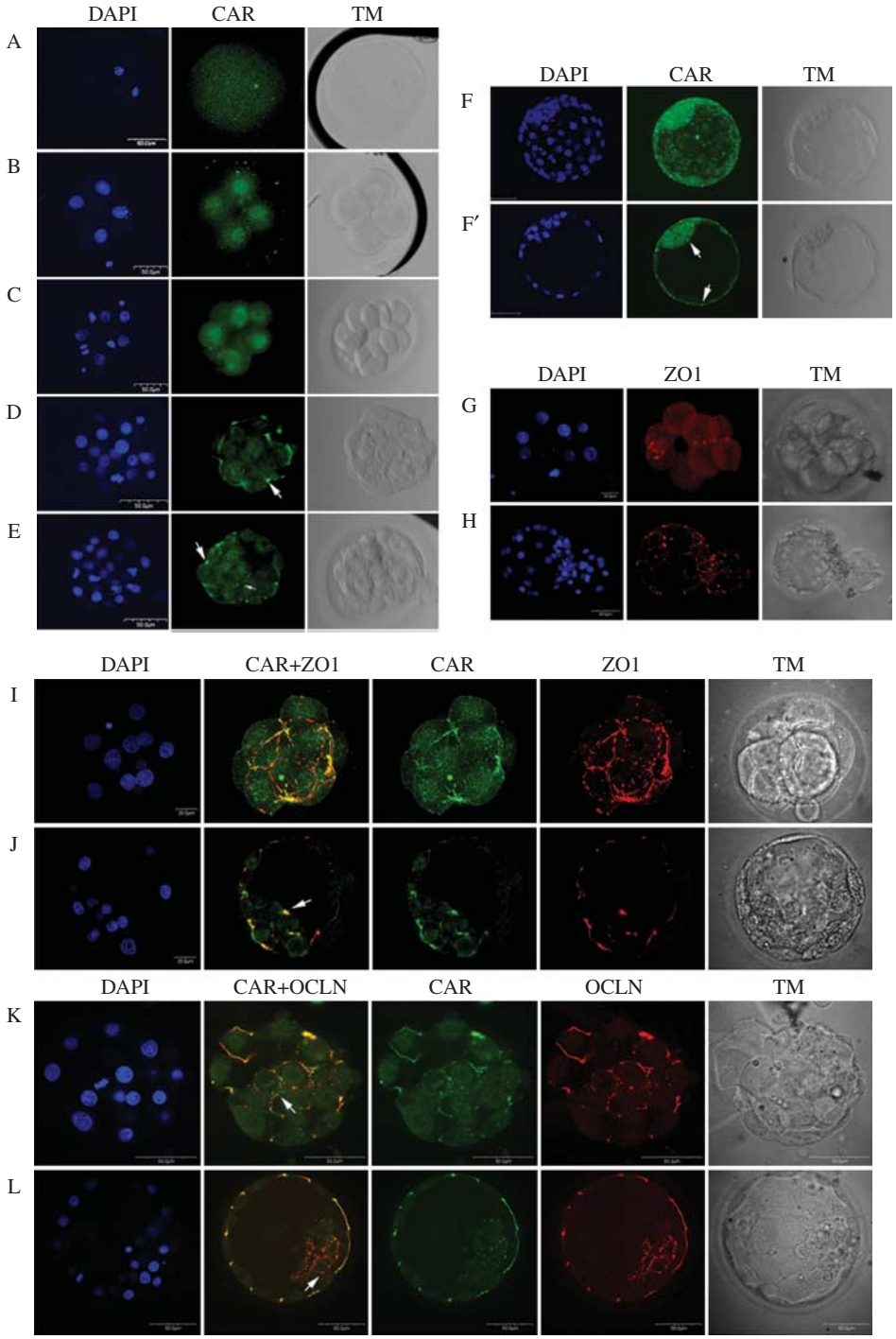

Figure 1 The expression pattern of CAR protein together with tight junction markers during human preimplantation development. CAR protein is expressed in the cytoplasm of mature oocytes (A; $n=9$; complete stack from the whole oocyte), while in 4-cell (B; $n=7$; complete stack from the whole embryo) and 8-cell (C; $n=7$; complete stack from the whole embryo) stage embryos it is also strongly present in the nuclei. From the compaction stage on, the protein is localised at the membrane of outer cells (D; $n=9$; one slide). CAR is concentrated in patches on the membrane at compaction and blastula stages (D, E and F′; white arrows). At the early blastocyst stage, the outer cells still contain CAR protein in the cytoplasm (E; $n=6$). At the expanding blastocyst stage, CAR is markedly more concentrated on the membrane than in the cytoplasm of TE cells, while in ICM cytoplasmic and nuclear CAR is predominant (F; $n=8$). CAR is also expressed on the membrane of the ICM cells facing the cavity (precursor primitive endoderm cells, white arrows, F′; (F′) is one slide and (F) is the summary stack of the same embryo). ZO1 protein is diffused in the cytoplasm of 8-cell stage embryos (G; $n=4$; complete stack from the whole embryo). At the blastocyst stage, ZO1 is localised on the membrane of TE cells marking tight junctions (H; $n=9$; complete stack from the whole embryo). CAR protein is predominantly colocalised with ZO1 on the membrane ($n=6$) at compaction (I; complete stack from the whole embryo) and blastocyst stages (J; one slide). Membrane CAR colocalised with another tight junction marker OCLN at the blastocyst stage (K and L; $n=4$); $n=$number of analysed embryos.

CAR expression in differentiated lineages of hESC

We noticed that in cells at the borders of the hESC colonies, upon spontaneous differentiation known as epithelial-to-mesenchymal transition (EMT) (Ullmann *et al.* 2007), CAR expression was lost. Coimmunostainings indeed confirmed that the mesenchymal-like vimentin-positive embryonic cells at the borders of the colonies were negative for CAR protein expression, both

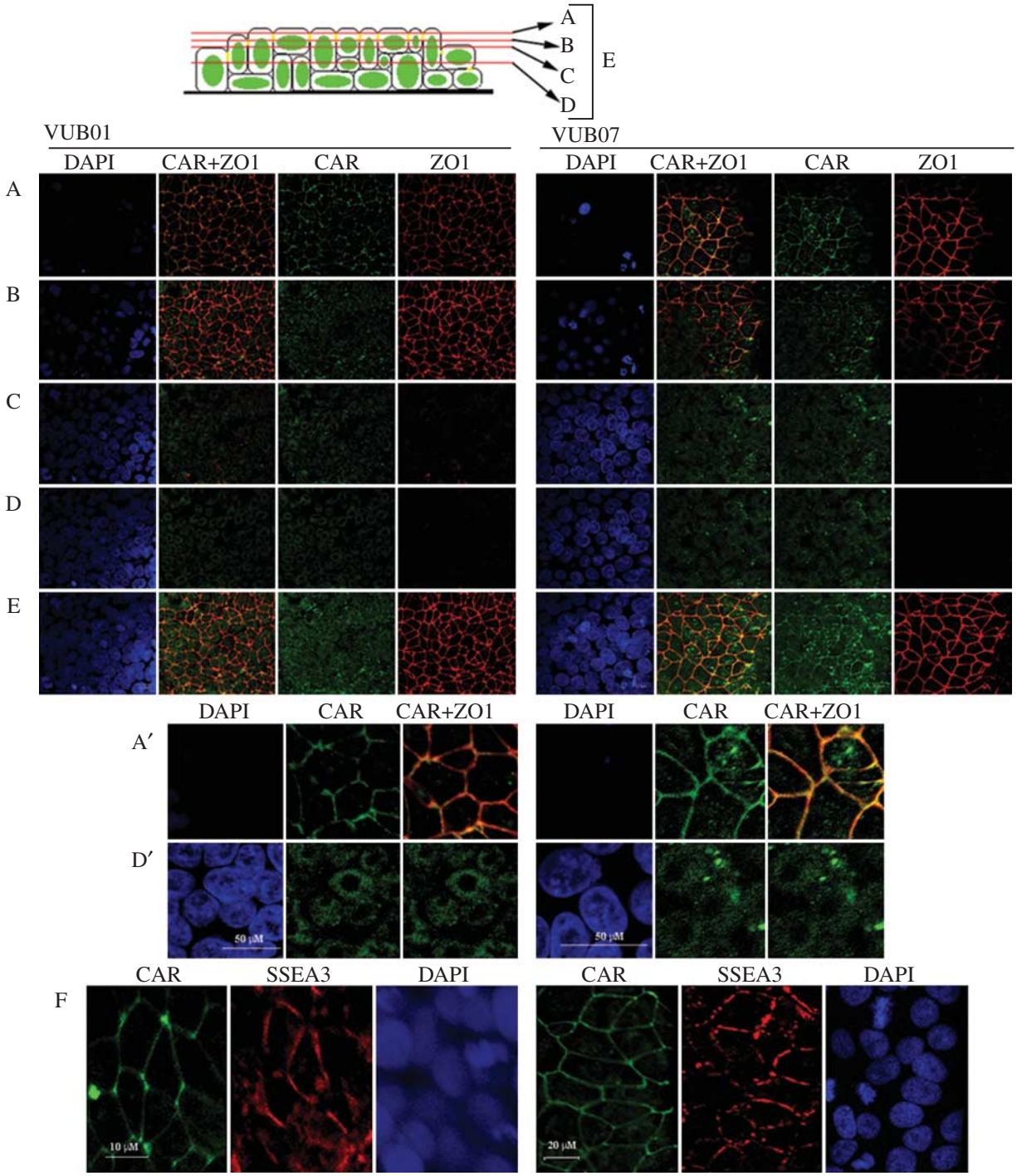

Figure 2 CAR protein expression in undifferentiated hESC. (A) The superficial layer of hESC VUB01 and VUB07 colonies. (A′) The superficial layer at higher magnification. The membrane CAR is detected colocalising with the ZO1-positive tight junctions between the cells of the outer layer. (B) In the cells deeper inside the colony traces of nuclear CAR can be detected. (C) and (D) represent sections through the inner part of a hESC colony. (D′) The inner part in higher magnification. CAR and ZO1 membrane staining disappear, while CAR can still be detected in the nuclei. (E) The merged image of all the sections (A), (B), (C) and (D). (F) The undifferentiated SSEA3-positive population of hESC expresses CAR on the membrane.

on the membrane and intracellularly (Fig. 3A, arrows and Supplementary Figure 3C).

To confirm the immunostaining results obtained on hESC cell lines, we carried out qRT-PCR analysis on a pure population of OPL cells derived as a result of spontaneous differentiation (Mateizel *et al*. 2008). *CXADR* expression was significantly suppressed in OPLs of VUB07 in comparison with undifferentiated hESC and was absent in OPLs of VUB01 (Fig. 3B). As a control, we analysed *NANOG*, *POU5F1_iA* and *SOX2*, which proved to be abundantly expressed in hESC and strongly downregulated in OPLs. The markers collagen type 1 (*COLL1*) and runt-related transcription factor 2 (*RUNX2*) were upregulated in OPLs, confirming differentiation into the osteogenic lineage (Aubin 1998). *COLL1* is one of the earliest markers specifying osteoblast progenitors (Liu *et al*. 2003). *RUNX2* (also known as CBFA1) is a transcriptional factor that has been described to play a crucial role in osteoblast lineage differentiation during embryogenesis (Ducy *et al*. 1997, Komori *et al*. 1997, Mundlos *et al*. 1997).

Because CAR was expressed by undifferentiated hESC and decreased in cells undergoing EMT, we further analysed the possibility of CAR protein to behave as a pluripotency marker in hESC. We observed that in hESC directed to differentiate into definitive endoderm, SOX17 was specifically expressed but CAR was completely absent (Fig. 3C). In addition, we obtained early-neural ectoderm cells positive for OTX2 at the first stages of directed hESC differentiation into retinal-pigmented epithelium. These cells still strongly expressed CAR on the membrane, while it was clearly excluded from the nucleus and cytoplasm (Fig. 3D). hESCs forced to undergo epithelial-like trophoblast differentiation, confirmed by expression of *KRT7* and *CGB*, showed significantly lower RNA levels of *CXADR* compared with undifferentiated hESC (Fig. 3F).

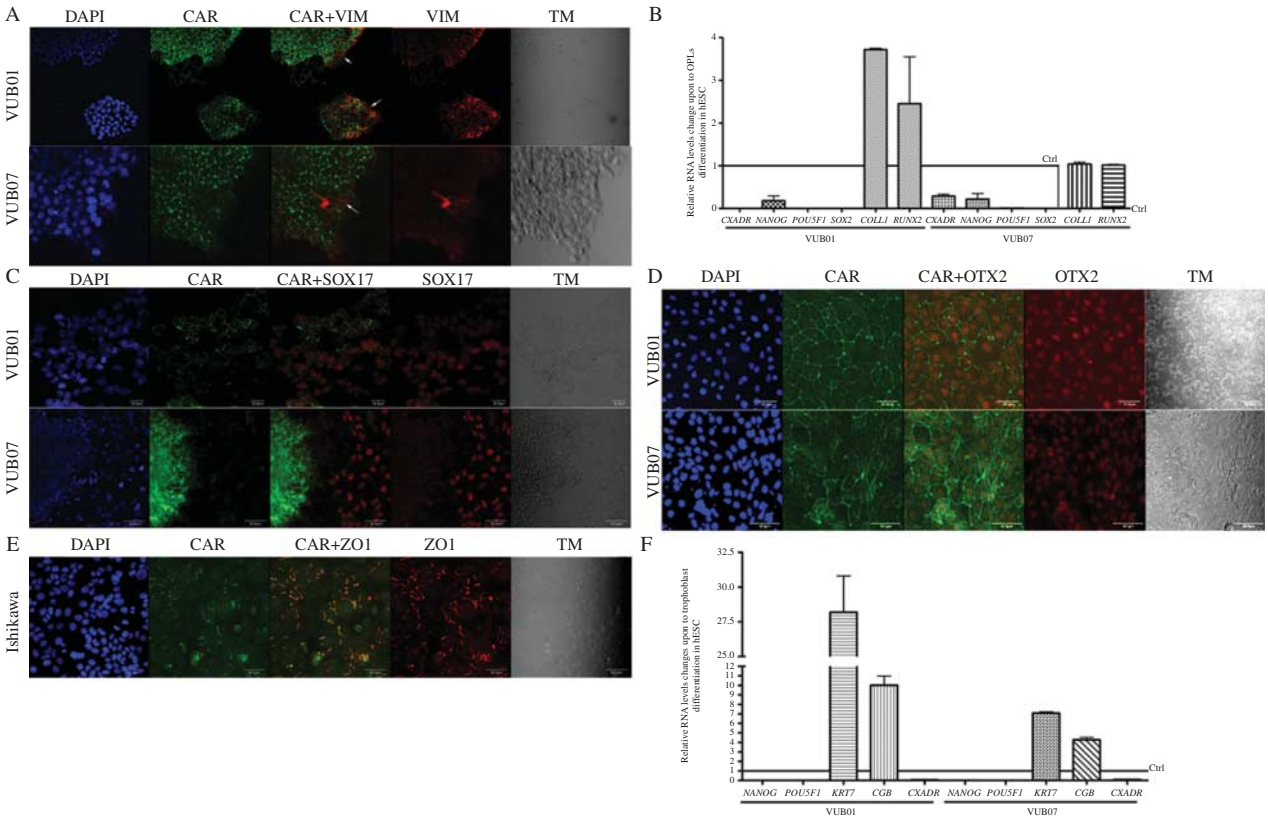

Figure 3 *CXADR* RNA and protein expression in differentiated hESC. (A) Vimentin-positive cells undergoing spontaneous EMT at the borders of the colonies (red) do not contain CAR protein (green) in VUB01 and VUB07 hESC lines (white arrows). (B) RNA levels of *CAR* were statistically significantly decreased in OPLs when compared with undifferentiated hESC (ctrl – control, equal to one) ($P=0.0286$ for VUB01 and $P=0.0022$ for VUB07). *GAPDH* and *UBC* were applied as endogenous controls. The control genes *COLL1* ($P=0.0286$) and *RUNX* ($P=0.0286$) are markers for the osteogenic lineage, while *NANOG* ($P=0.0022$ for VUB01 and $P=0.0152$ for VUB07), *POU5F1_iA* ($P=0.0286$ for VUB01 and $P=0.0022$ for VUB07), *SOX2* ($P=0.0286$) mark the undifferentiated state. *COLL1* and *RUNX2* were undetecteble in undifferentiated hESC (ctrl) VUB07, therefore, their levels equal 1 in VUB07 OPLs. (C) CAR (green) was absent in the endodermal SOX17-positive (red) differentiated hESC. (D) CAR (green) strongly remained on the membrane of the ectodermal OTX-2-positive differentiated hESC. (E) CAR (green) colocalised in the tight junctions with ZO1 (red) in epithelial endometrial adenocarcinoma cells. (F) *CXADR* expression was decreased upon trophoblast differentiation together with *NANOG* and *POU5F1*, while *KRT7* and *CGB* were strongly upregulated ($P=0.0286$). *GAPDH*, *UBC* and *RPS24* were used as endogenous controls; ctrl – control, undifferentiated hESC, equal to one. Statistical analysis was performed using the Mann–Whitney *U* test.

Considering the pattern expression of CAR in undifferentiated hESC, we assumed that its membrane form could be a marker for the epithelial cell type. Therefore, we decided to investigate CAR expression in endometrial adenocarcinoma (Ishikawa) cells, an epithelial cell line unrelated to human embryonic cells. We illustrated colocalisation of CAR protein with ZO1 on the membrane of Ishikawa cells (Fig. 3E). Intracellular CAR was not detected in this cell type.

Taking into account, the CAR expression patterns observed in hESC and epithelial cells, we conclude that nuclear CAR is specific for undifferentiated cells, while its membrane form is rather related with epithelial-like cell types.

Alternative expression of CAR

Surprisingly, analysis of expanded and already hatched blastocysts on day 6 showed that in approximately half of these embryos CAR protein was strongly concentrated in the nuclei with only minor traces on the membrane and in the cytoplasm of the TE, precursor EPI and PE cells. Tight junctions labelled by ZO1 could still be detected in those cells (Fig. 4A). However, in the other half of the hatched blastocyst, membrane CAR was still clearly present in TE cells (Fig. 4B).

As described earlier, *CXADR* RNA exists in different splice variants, which, in addition to the full-length transmembrane (TM) form, also encode soluble proteins lacking a TM domain (Dörner *et al.* 2004). We wanted to investigate whether alternative splicing of *CXADR* is present in expanded hatched blastocysts and could, possibly, correlate with highly dynamic pattern expression of CAR protein. The RT-PCR data illustrated a switch from the TM full-length *CXADR* to its splice variant *CXADR4/7* in some of the hatched blastocysts (Fig. 4C). We could predominantly detect the TM full-length membrane form of *CXADR* transcripts (1100 bp) in all the expanded blastocysts at day 6 (Fig. 4C, lines 1–5), together with a shorter splice variant *CXADR3/7* (∼500 bp). We assume that the former corresponds to the membrane CAR protein whereas the latter corresponds with the nuclear/cytoplasmic CAR protein. Some of the day 6 embryos that had spontaneously

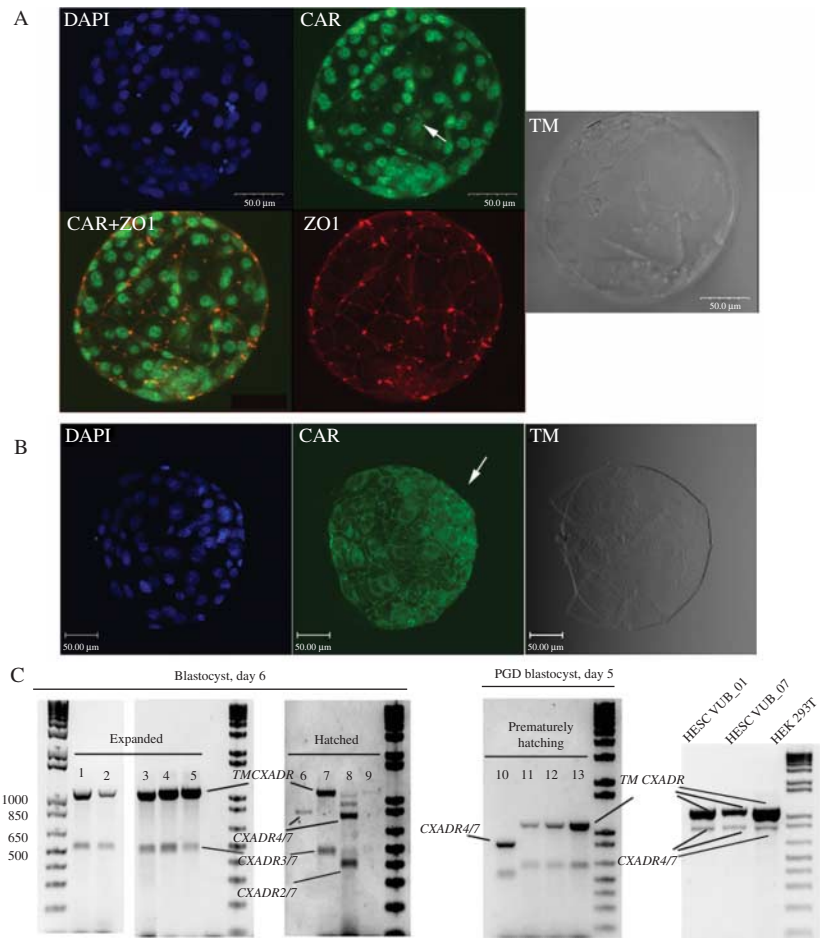

Figure 4 Alternative expression of CAR in expanded human blastocysts and hESC. (A) In hatched blastocysts, CAR strongly concentrates in the nuclei of TE cells ($n=5$ out of 8 analysed). ZO1 remains visible on the membrane of TE cells ($n=5$ out of 5 analysed). (B) While in the other three out of eight embryos, CAR is still present on the membrane and perinuclear of TE cells. Arrows indicate ICM positive for CAR. (C) Expanded, not hatched, blastocysts contain full-length (TM) *CXADR* RNA, while after hatching it disappears and *CXADR4/7* is produced. A similar effect was observed with shorter *CXADR* isoforms. PGD embryos prematurely hatching on developmental day 5 also show the switch of *CXADR* RNA expression. The single embryo PCR products were loaded on a gel: samples 1–5 – expanded blastocysts on day 6; 6–9 – hatched blastocysts on day 6; 10–13 – prematurely hatching PGD embryos on day 5. The studied hESC lines VUB_01, VUB_07 and HEK 293T simultaneously express full-length and *CXADR4/7*; shorter versions of *CXADR* are not detected in hESC.

hatched from the zona pellucida (ZP), however, stopped expressing TM *CXADR* and *CXADR3/7* mRNA and started to express *CXADR4/7* and *CXADR2/7* (Fig. 4C, lines 6–9). In one case, *CXADR* was almost undetectable (Fig. 4C, line 9). These findings supported the immunostaining data revealing the dominant membrane form of CAR in the expanded blastocyst before hatching, which was disappearing in half of the embryos after hatching (Fig. 1F). In PGD day 5 embryos, which hatched prematurely through the hole in the ZP from a biopsy, we observed a similar effect as that seen in the spontaneously hatched blastocysts at day 6 (Fig. 4C, lines 10–13). In hESC, we observed the simultaneous expression of the TM form and *CXADR4/7* RNA; we did not observe any of the short splice isoforms of *CXADR*. We detected the same isoforms of *CXADR* (TM and *CXADR4/7*) in a human embryonic kidney carcinoma cell line (HEK 293T), another epithelial cell type, known to contain CAR on the membrane (Fuxe *et al*. 2003).

Discussion

This study describes the expression pattern of CAR in human embryos and hESC. Our data obtained in these two different *in vitro* models are important for understanding the potential role of CAR during early embryonic development. We observed a variable expression of CAR protein and RNA in the diverse human embryonic cell types. Therefore, we suggest that distinct forms of CAR play different roles in the undifferentiated state and tight junction formation.

In parallel to the first differentiation in the human embryo (TE vs ICM), outer cells at compaction stage and later TE cells in the expanding blastocyst differ from the inner cells by the prominent accumulation of CAR on the membrane. Further investigation at the expanded blastocyst stage indicates the localisation of CAR protein at the tight junctions of the outer TE cells, as well as the inner cells (both PE and EPI) similar to ZO1 and OCLN (Bloor *et al*. 2002, Alikani 2005).

CAR and ZO1 colocalised only on the membrane of the superficial cells of the hESC colony. This finding supports previous observations by us and other groups (Ullmann *et al*. 2007, Boyd *et al*. 2009), indicating that an undifferentiated epithelial-like monolayer is formed on the surface of the hESC colony upon contact with the culture medium. Both this upper layer of the hESC colony and the TE of the early blastocyst represent epithelial-like cells exposed to the medium. However, TE is a monolayer that is at both sides (apical and basolateral) exposed to medium (culture medium and blastocoel fluid respectively), whereas in epithelium-like cells such as the upper layer of hESC and a monolayer of cell lines in cell culture, only the apical membrane is facing the culture medium. In turn, inner cells of the hESC colony resemble early cleavage stage blastomeres lacking membrane and carrying nuclear CAR protein.

The physiological function of CAR as a transmembrane component of tight junctions has been described previously (Cohen *et al*. 2001, Walters *et al*. 2002, Coyne & Bergelson 2005). Our observations are in agreement with these data and suggest an important role for CAR in the intercellular connections starting from the compaction stage in human preimplantation embryos. CAR protein has already been shown to contribute to the formation of tight junction and to have a barrier function in epithelial cells by forming a complex with ZO1 and recruiting it to homotypic cell–cell contacts (Cohen *et al*. 2001). Our co-stainings of CAR with ZO1 and OCLN confirm the connection of membrane CAR with tight junctions in human preimplantation embryos and add to previous findings on the timing and the character of cellular adhesion organisation during human preimplantation development (Gualtieri *et al*. 1992, Hardy *et al*. 1996, Bloor *et al*. 2002). In fact, the proper formation of the intercellular connections is critical for normal embryogenesis (Hardy *et al*. 1996, Alikani 2005). For this reason, by participating in the formation of tight junctions, CAR may play a crucial role in establishing the cellular integrity of the early human embryo. Previously, it was shown that intercellular junctions, in particular desmosomes, appear only between the outer cells before cavitation and are retained in the TE (epithelial cells) (Hardy *et al*. 1996). However, we found that the membrane form of CAR does not seem to be exclusively specific to the early epithelium in human embryos because we could clearly observe the presence of membrane-associated CAR, ZO1 and OCLN, and thus tight junctions between the inner cells at compaction and the blastocyst stage (non-epithelial cells). Interestingly, the presence of tight junction proteins in non-epithelial tissues has previously been described by other groups (Itoh *et al*. 1993, Howarth & Stevenson 1995).

We observed most of the CAR protein leaving the TE membrane in hatched blastocysts and becoming predominantly present in the TE nuclei, whereas ZO1 (and consequently tight junctions) were still present in the same place. This observation suggested that CAR might no longer be necessary for sustaining tight junctions further during development or TE cells may begin to loosen their intercellular connections. We assume that after hatching, the TE needs to become prepared for implantation (e.g. it has to undergo EMT), but the mechanism involved in this process has not yet been studied in humans. Even though a cytoplasmic localisation of CAR has previously been reported (Pickles *et al*. 1998, Toyofuku *et al*. 1998), we describe its nuclear localisation for the first time. In addition to the function of CAR on the membrane, intracellular CAR may presumably have a different role, possibly relevant for gene expression. Similar nuclear translocation of different types of receptors has already been shown in various cell types (Holt *et al*. 1994, Xia & Kemper 2005). In particular, several proteins of the apical junctional

complex (AJC) play a major role in cell–cell adhesion, paracellular barrier function and signalling. They form a complex of transmembrane and cytoplasmic proteins that are linked to the cytoskeleton and organise apical-basal polarity. Several proteins of the AJC can shuttle to the nucleus where they regulate the expression of transcription factors (e.g. β-catenin, YAP, PAR6) (McCrea *et al*. 2009, Spadaro *et al*. 2012). In addition to the fact that CAR was able to influence the nuclear cell cycle regulators of tumour cells *in vitro* (Okegawa *et al*. 2001), no other nuclear function has been reported so far. Thus, the exact meaning of this phenomenon of nuclear CAR remains unclear.

We also found nuclear CAR protein in pluripotent cells of preimplantation embryos, which we propose to be different from the CAR found in the TE of hatched blastocysts. It appeared in all the nuclei of the cleavage embryos starting from the 2-cell stage. During further development, we observed CAR in the nuclei of pluripotent ICM and precursor EPI cells but also in full blastocyst TE cells, supporting the hypothesis that those are not yet committed (De Paepe *et al*. 2013; Fig. 1L). It is possible, that nuclear CAR could be an important regulator of pluripotent cells in the human embryo, but its role and its relationship with the known pluripotency markers *POU5F1_iA*, *NANOG* and *SOX2* still needs to be investigated.

To pursue the idea of CAR being relevant for sustaining pluripotency, we investigated its behaviour in response to induced differentiation in pluripotent hESC lines. Importantly, CAR totally disappeared upon spontaneous EMT and as a result of the directed differentiation into definitive endoderm and trophoblast lineage. *CXADR* RNA levels were also downregulated in the OPLs of mesodermal origin obtained from hESC. Inhibition of CAR as a result of undergoing EMT has already been reported in cancer cells (Vincent *et al*. 2009). Another group also described a lineage differentiation-associated loss of CAR expression, but in mesenchymal stem cells (Hung *et al*. 2004). We found that nuclear CAR was absent in ectoderm-like early neural epithelium of differentiating hESC, while its membrane form remained. This was not particularly surprising because the differentiating cells represent epithelium but also because membrane CAR has already been suggested to be physiologically utilised in the morphogenesis of mouse immature nervous system (Hotta *et al*. 2003). Therefore, due to its disappearance upon differentiation, nuclear CAR is likely to be a marker for pluripotent cells resembling early cleavage stages of the preimplantation human embryo, while membrane CAR remains to mark the epithelium. Indeed, we found CAR on the membrane of epithelium-like cells obtained from hESC after differentiation and on endometrial adenocarcinoma epithelial type cells, supporting previously published observations in epithelial tissues and cell types exclusively displaying the membrane form and lacking nuclear forms of CAR (Cohen *et al*. 2001).

We aimed to confirm the immunocytochemistry data by RT-PCR. In addition to two isoforms (*CXADR7* and *CXADR8*) that are known to be localised at the membrane, there are three more (*CXADR4/7*, *CXADR3/7* and *CXADR2/7*) that do not contain a transmembrane domain and are meant to be accumulated in the cytoplasm or to be secreted (Tomko *et al*. 1997, Thoelen *et al*. 2001, Dörner *et al*. 2004, Excoffon *et al*. 2010). We showed that expanded blastocysts express transmembrane *CXADR* and *CXADR3/7*, which correspond with membrane CAR and intracellular CAR protein, respectively. Thus, we assumed that *CXADR3/7* is associated with pluripotency in human embryos. We also showed that after hatching, blastocysts expressed distinct forms *CXADR4/7* and *CXADR2/7* at the expense of TM *CXADR* and *CXADR3/7*. It is not possible to find out which isoform corresponds with the distinct nuclear proteins (precursor EPI or TE) at this moment because there are no antibodies available to discriminate between the distinct proteins (and there is no information on post-translational modifications). We suggest that there are two alternatives in hatched blastocysts: i) in TE cells, TM *CXADR* is spliced into *CXADR4/7*, while in precursor EPI, *CXADR3/7* is replaced by *CXADR2/7*; and ii) in TE cells, TM is substituted by *CXADR2/7*, while *CXADR3/7* is replaced by *CXADR4/7*. The switch in expression happens when the human embryo loses its protecting ZP as a result of normal and assisted hatching processes. Interestingly, in hESC we only found membrane and nuclear CAR corresponding with TM *CXADR* and *CXADR4/7* isoforms respectively. If hESC are derived from precursor EPI, we could speculate that the second option is happening after blastocyst hatching. However, it may well be that hESC do not represent precursor EPI but rather are an *in vitro* artefact after adaptation to long-term culture and passaging. Therefore, it is particularly remarkable that both hESC and HEK 293T cells produce the same isoforms TM *CXADR* and *CXADR4/7* and lack shorter splice variants. Perhaps *CXADR4/7* marks the tenancy of HEK 293T cells to their embryonic nature. Otherwise, the simultaneous expression of *CXADR* TM and *CXADR4/7* is the simple illustration of cell culture.

We speculate that exposure to the culture medium of human blastocysts *in vitro* reflects changes in CAR expression (an increase in splice isoforms and a decrease in full-length TM *CXADR*). It is likely that the phenomenon of activating a self-protective mechanism occurs *in vivo* in the uterus when the blastocyst escapes from the ZP before implantation and becomes exposed to pathogens for the first time (Zhao & Dean 2002, Koot *et al*. 2012). Hatched blastocysts might loosen the integrity of their TE exiting ZP (by removing CAR from the membrane, as we observed *in vitro*) and preparing to implant, therefore, become more accessible for viral infection. Cohen *et al*. (2001) described a critical role for membrane CAR in tight junctions in limiting infection

through the epithelium in human cell lines (Cohen *et al*. 2001). A viral attack can additionally selectively disrupt tight junction proteins (Kanmogne *et al*. 2005, Nakamuta *et al*. 2008). In fact, the destruction of tight junctions is critical for CAR-mediated coxsackievirus and adenovirus infection of polarised cells (Cohen *et al*. 2001). According to our data on *CXADRs*' splice isoforms, hatched human blastocysts may begin to protect themselves via the synthesis of soluble secreted CAR proteins lacking TM domain interfering with virus binding full-length CAR (Dörner *et al*. 2004). However, we did not prove the presence of soluble CAR in the culture medium of embryos or hESC.

Although this study is primarily describing the basic conformity between human embryos and ESCs, it was aimed to improve the clinical application of human embryos and hESC. The expanded knowledge on mechanisms regulating pluripotency will support studies of the direct differentiation of hESC lines. Research on human preimplantation embryos should improve artificial reproductive technologies. It is particularly important to expand the studies aimed to describing the effects of the culture conditions on the protein markers critical for normal human embryo development.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-14-0253.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

The authors have made the following declarations about their contributions: M Krivega: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; M Geens: data interpretation and manuscript editing; H Van de Velde: conception and design, financial support, provision of study material, data analysis and interpretation, manuscript writing, final approval of manuscript.

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