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Influence of Cell Confluency on the Expression of the α4 Integrin Subunit of Retinal Pigment Epithelial Cells

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Abstract

Integrins are a family of transmembrane glycoproteins that mediate cell-cell and cell-extracellular matrix interactions. The integrin α4 subunit is widely expressed by cells from the immune system and its expression by non-hematopoietic cells is scarce. In the present study, gene and protein expression of this integrin subunit was characterized in proliferating and quiescent human RPE cells. Immunofluorescent studies confirm that the α4 subunit is expressed *in vitro* by RPE cells, a result that has been validated by immunofluorescence and FACS analyses. The accumulation of the α4 integrin at cell-cell junctions in post-confluent RPE cell cultures negatively correlated with the level of expression of the mRNA transcript. Accordingly, transient transfection analyses reveal that the α4 promoter activity is considerably reduced when RPE cells form a confluent monolayer. Moreover, transfection of recombinant constructs bearing 5'-deletions of the α4 promoter segment allows the localization of strong negative regulatory elements on the −76 to −300 region of the α4 gene suggesting that its expression is intimately linked to the proliferative state of primary cultured RPE cells.

Keywords

Retinal Pigment Epithelium, Integrin Alpha 4 Subunit, Cell Culture, Confluency, Promoter

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1. Introduction

The retinal pigment epithelium (RPE) is a monolayer of polarized cells that separates the retina from the underlying vasculature. As part of the blood-ocular barrier, the RPE is responsible for the transport of nutrients from the choroidal blood vessels to the photoreceptors and, conversely, for the transport of waste products from the photoreceptors to the choroidal blood vessels [1]. In response to a traumatic retinal injury, RPE cells can become proliferative, leading to retinal disorders and vision loss [2]. A change in the expression of integrins has been observed when RPE cells become proliferative [3] [4].

Integrins form a family of transmembrane glycoproteins that regulate cell-cell and cell-extracellular matrix (ECM) interactions [5] [6]. They control many cellular processes, including adhesion, migration, proliferation, survival and differentiation [7] [8]. Integrins are heterodimeric molecules made-up of an α subunit, which confers most of the ECM ligand specificity, and a β subunit, that interacts with the intracellular cytoskeleton via adaptor proteins and determines the broad class of the receptor [9] [10]. To date, 8 β- and 18 α-subunits have been identified that can associate with each other to produce the 24 integrin heterodimers reported to date [11]-[15].

The α4 subunit can associate with either the β1 or the β7 subunit [8] [13] [14] [16]. Integrins $\alpha 4\beta 1$, also known as very late antigen-4 (VLA-4) [17], and $\alpha 4\beta 7$ mediate interaction with the immunoglobulin vascular cell adhesion molecule-1 (VCAM-1) as well as with the alternatively spliced CS-1 region of the ECM protein fibronectin (FN) [8] [13] [14] [16]. However, $\alpha 4\beta 7$ binds these two ligands with a lesser affinity than $\alpha 4\beta 1$ [18].

The integrin $\alpha 4\beta 1$ mediates both cell-cell and cell-ECM interactions and plays an important role in development and cell differentiation. This integrin is expressed by a variety of hematopoietic cells including lymphocytes, monocytes and eosinophils [19]-[22]. It plays an important role in the regulation of the immune response by promoting the recruitment of lymphocytes at inflammation sites [23]-[25] by its interaction with VCAM-1 on activated vascular endothelial cells, allowing for diapedesis [26].

Apart from the immune cells, the $\alpha 4\beta 1$ integrin has also been identified on dermal fibroblasts [27], in the epicardium [28] [29], on developing myocytes [30] [31], on neural crest cells [32] [33], on rhabdomyosarcomas [17], on melanoma [34]-[36] and on corneal epithelial cells [37]. Moreover, this integrin also plays an important role in development and cell differentiation. Indeed, interaction between $\alpha 4\beta 1$ and VCAM-1 is important in myogenic differentiation and α4 expression is induced during differentiation of C2C12 cells (a mouse myoblast cell line) and during muscle differentiation [30]. *In vivo*, antibodies that block either α4 or VCAM-1 inhibit the development of the sympathetic innervation of the heart [38]. Knockout mice lacking α4 die at embryonic day 11 due to cardiac and placental formation failure [29] [39].

In the embryonic retina, $\alpha 4\beta 1$ is expressed by retinal ganglion cells and undifferentiated retinal neuroblasts. It is proposed to function as a mediator for neurite outgrowth by its interaction with VCAM-1, FN and osteopontin in this developing tissue [40] and it is required for cell survival in the developing retina [41]. In the mature human retina, α4 expression is observed on cells from the outer nuclear layer, including rods and cones, cells from the inner nuclear layer, ganglion cells and RPE cells [3] [42] [43]. The present study was undertaken in order to evaluate whether expression of the integrin α4 subunit changes with increasing cell densities that replicate the proliferative states of primary cultured RPE cells (from sub-confluence (proliferating) to post-confluence (quiescent).

2. Materials and Methods

This study was conducted in accordance with our institution's guidelines and the Declaration of Helsinki. The protocols were also approved by our institution's ethics committee for the protection of human subjects.

2.1. Cell Culture

Primary cultures of RPE cells were established from human eyes rejected for transplantation and obtained from the Centre Universitaire d'Ophtalmologie (CUO) Eyebank Inc. (Québec, Qc, Canada) 24 h postmortem. Dissection was done according to a previously published method with some modifications [44]. Briefly, pieces of dissected RPE/choroid were incubated 45 minutes at 37˚C in 2.4% dispase (Roche Diagnostics, Laval, Qc, Canada). RPE sheets were gently detached by spraying growth medium against the tissue. RPE sheets were then cultured in Keratinocyte-SFM medium (Life Technologies, Burlington, Ont., Canada) supplemented with 5% bovine calf serum (Hyclone, Logan, UT) and various vitamins and proteins, as described [45]. For all experiments, cultures

were used at passage 2 and cells were plated at different densities as follows: sub-confluent cells: 2.5×10^4 cells/cm^2, 2 days in culture (which corresponded to approximately 80% coverage of the culture plate at the time the cells were harvested); confluent cells: 1.5×10^5 cells/cm^2, 2 days in culture (which corresponded to 100% coverage of the culture plate at the time the cells were harvested); post-confluent cells: 1.5×10^5 cells/cm^2, 2 weeks in culture (which corresponded to maintaining the cells at full confluency for 12 days prior to cell harvesting).

2.2. Immunostaining

The localization of the integrin α4 subunit at the cell membrane was monitored by immunohistochemistry on paraffin-embedded sections of human retina-choroid tissues and by immunofluorescence on *in vitro* human primary RPE cells cultured on glass coverslips.

For immunohistochemical *in situ* staining, human retina-choroid sections were fixed in 4% paraformaldehyde (Electron microscopy Sciences, Hatfield, PA), embedded in paraffin, and cut in 4 µm sections. After deparaffinisation and rehydration, samples were processed in a PT-Link (Dako, Mississauga, Ont., Canada) for antigen retrieval (Tris-EDTA, pH 9, 85˚C, 20 min). Immunohistochemical staining was performed manually using the EnVision Flex, High pH Kit (Dako) according to the manufacturer's protocol and using a rabbit monoclonal antibody against integrin α4 (clone EPR1355Y, Millipore, Billerica, MA). Nuclei were counterstained using Harris' hematoxylin. For negative controls, the primary antibody was replaced with normal rabbit serum.

Immunofluorescence was performed on cultured RPE cells grown on glass coverslips. A monoclonal antibody directed against the α4 integrin subunit (mouse anti-human integrin α4, clone HP2/1, Cedarlane, Burlington, ON, Canada) and the secondary antibody Alexa Fluor 488 goat anti-mouse IgG (Life Technologies) were used to identify the presence of this integrin subunit on RPE cells *in vitro*, as described previously [46]. For negative controls, the primary antibody was omitted.

2.3. FACS Analyses

RPE cells grown at different confluencies were harvested in PBS/EDTA 2 mM and fixed in 90% ethanol. Cells (2×10^5) were incubated for one hour with a mouse monoclonal antibody directed against the α4 integrin subunit (Cedarlane). As a negative control, this antibody was replaced by a mouse primary antibody isotype control (Dako). Cells were then incubated for 45 min with a goat anti-mouse phycoerythrin-conjugated secondary antibody (Cedarlane) and then analyzed by flow cytometry (Facscalibur, BD Biosciences, Mississauga, Ont., Canada).

2.4. Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction

Whole RNA extracts from RPE cells grown at various cell densities (sub-confluency, confluency and post-confluency) were reverse transcribed and PCR-amplified as described [45]. PCR products were in the linear range between 25 and 37 cycles for sub-confluent and confluent cells, and between 27 and 39 for post-confluent cells. The total number of cycles used for semi-quantitative RT-PCR was 32 and the experiment was done in triplicate using different populations of RPE cells derived from the retina of three different donors. The DNA sequence of the primers used for the amplification of the human α4 transcript were: 5'-TGGCGTGGTACAACTTGACTG-3' (forward primer), and 5'-CATGCGCAACATTTCATCCT-3' (reverse primer; 772-bp PCR product) and cycle parameters were 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec. The 18S primers (Ambion; Life Technologies) gave a PCR product of 489 bp. Bands were analyzed with the GelDoc2000 gel documentation system (Bio-Rad Laboratories, Mississauga, Ont., Canada) and the Quantity One 1-D image analysis software (Bio-Rad Laboratories).

2.5. Transient Transfections and CAT Assays

Plasmids containing the chloramphenicol acetyltransferase (CAT) reporter gene from the plasmid pSKCAT fused to various 5' deletions of the α4 gene (−1000α4CAT, −800α4CAT, −600α4CAT, −400α4CAT, −300α4CAT, −200α4CAT, −76α4CAT, −41α4CAT) were obtained from Dr. Glenn D. Rosen (Stanford University Medical Center, Stanford, CA). RPE cells were transfected using the calcium phosphate precipitation method as described [45]. Cells were harvested 48 h following transfection and CAT activities determined [47] and normalized to the amount of human growth hormone (hGH) encoded by the co-transfected plasmid pXGH5 and as-

sayed using a kit for quantitative measurement of hGH (Immunocorp, Montréal, Qc, Canada) as previously described [47]. The value presented for each individual test plasmid transfected corresponds to the mean of at least three separate transfections done with RPE cells from three different donors, each in triplicate. Student's t-test was performed for comparison of the groups. Differences were considered to be statistically significant at $P < 0.05$. All data are expressed as mean ± SD.

3. Results

3.1. The α4 Integrin Subunit Is Expressed on RPE Cells *in Situ* and *in Vitro*

The retinal pigment epithelium has a natural brown pigmentation, as seen in the *in situ* sections (**Figure 1(B)**, **Figure 1(D)**). Nevertheless, expression of the α4 protein was readily detected *in situ* in the RPE monolayer over their natural brown pigmentation (**Figure 1(A)**, **Figure 1(C)**), especially in the sections that were cut obliquely (**Figure 1(C)**). Meanwhile, other cells from the choroid also stained positive for this integrin. Expression of the α4 subunit was also monitored *in vitro* on primary human RPE cells cultured at varying cell densities. As shown on **Figure 2(A)**, a weak α4 signal could be observed on sub-confluent RPE cells, with a more intense staining at the cell edges. Cells that reached confluency had a stronger α4 staining at cell-cell contacts (**Figure 2(B)**). RPE cells that were left for 2 weeks at confluency (post-confluent cells) were tightly packed and also showed a positive α4 staining at cell-cell contacts (**Figure 2(C)**). The expression of the α4 integrin subunit by RPE cells has been further demonstrated by FACS analyses that clearly confirm the expression of this protein *in vitro* (**Figure 3**). The number of α4 positive cells reduced from 97.7% to 88.2% between the sub- and post-confluent states ($p = 0.007$; **Figure 3(a)**). The mean fluorescence intensity of α4 positive cells was similar in sub-confluent, confluent and post-confluent cultures (**Figure 3(b)**).

3.2. Transcription of the α4 Gene in RPE Cell Cultures

In order to evaluate the amount of α4 mRNA transcript present at different cell densities, total RNA from cul-

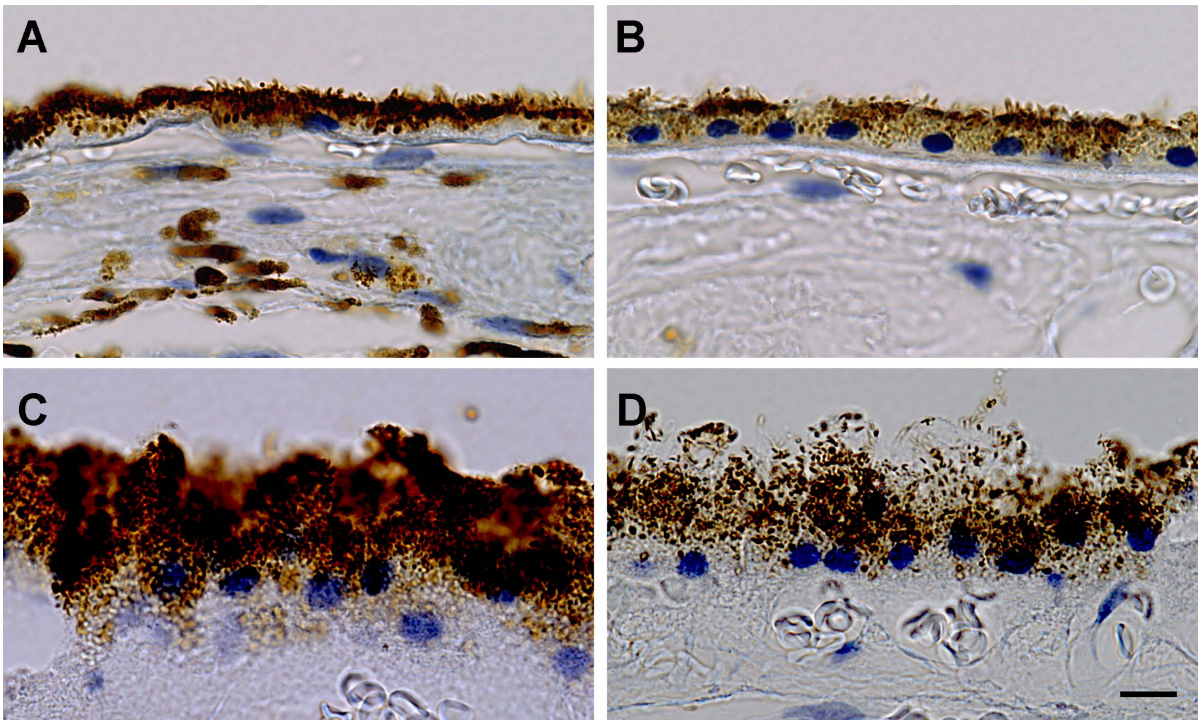


Figure 1. Immunohistochemical analysis of the α4 integrin subunit on paraffin sections of a human retina/choroid tissue. (A) (C) The α4 integrin subunit is detected diffusely throughout the RPE surface. (A) cross section, (C) oblique section; (B) (D) Negative controls using normal rabbit serum (B) cross section; (D) oblique section. Note that the retinal pigment epithelium possesses melanin granules that give a natural brown pigmentation to this monolayer. Scale bar represents 10 µm.

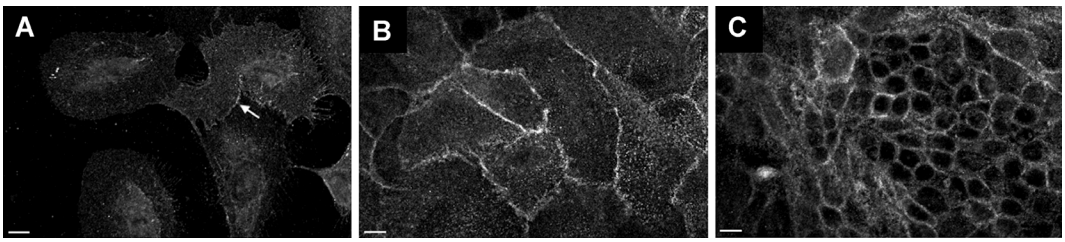


Figure 2. Immunofluorescence analysis of the α4 integrin subunit on cultured human RPE cells. (A) Sub-confluent cells are positively labeled for α4 at cell borders (arrow). The α4 staining is also present on confluent (B) and post-confluent (C) cells. Scale bar represents 10 µm and all pictures were taken at the same magnification.

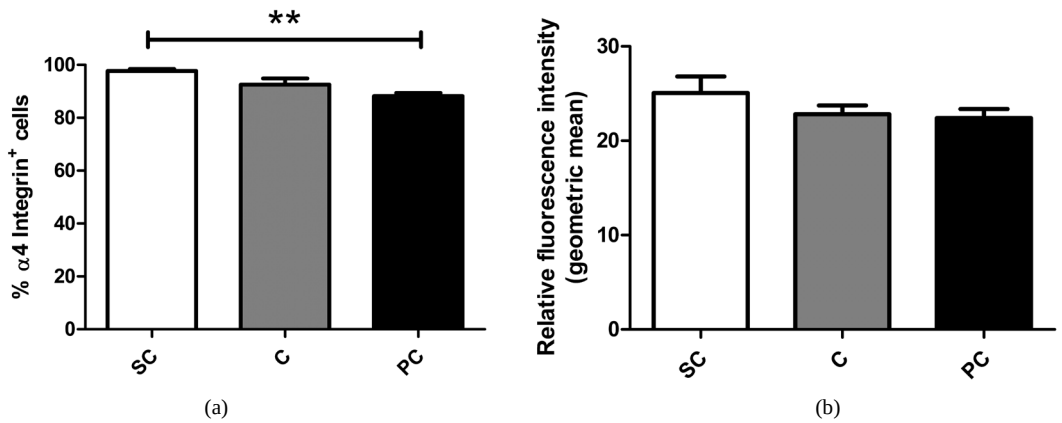
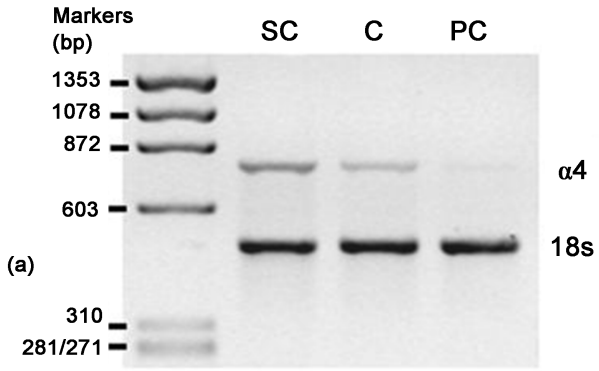


Figure 3. Expression of the integrin subunit α4 was monitored by flow cytometry in primary cultures of human RPE cells grown to sub-confluency (SC), confluency (C) and post-confluency (PC). (a) Percentage of α4 positive cells; (b) Relative fluorescence intensity, geometric means of the α4 positive cells. Mean ± SEM, n = 4, one-way ANOVA with a subsequent Bonferroni post-hoc test, $^{**}P < 0.01$.

tured RPE cells was isolated and used for semi-quantitative RT-PCR measurements. When normalized to the amount of transcripts encoded by the ribosomal 18S gene, a significant decrease (2.4 times) in the intensity of the PCR product corresponding to the α4 gene was observed between sub-confluent and confluent RPE cells (**Figure 4**). Transcription of α4 decreased further down (8.1 times) to a near undetectable level as RPE cells progressed from confluency to post-confluency. It is noteworthy that the intensity of the 18S PCR product remained almost unchanged (**Figure 4**).

Given that the level of α4 integrin subunit mRNA was influenced by cell confluency, we next determined whether the transcriptional activity normally driven by the α4 gene promoter was also similarly regulated upon transient transfection of α4 promoter/CAT recombinant constructs into sub-confluent, confluent and post-confluent RPE cells. As shown in **Figure 5(a)**, an 8-fold decrease in promoter activity was observed between sub-confluent and confluent cells when the −200α4CAT plasmid was used. The α4 promoter activity decreased further to a level 17-fold lower than that measured in sub-confluent cells when the −200α4CAT construct was transfected in 2 weeks post-confluent RPE cells. Therefore, the activity of the α4 promoter is repressed when RPE cells reach confluency. Furthermore, the −1000α4CAT plasmid, which bears the longest α4 promoter segment and is thus the most representative of the native gene, is highly repressed in cultured RPE cells, regardless of the state of confluency reached by the cells (**Figure 5(a)**). These results suggest that strong negative regulatory elements that repress α4 gene transcription when RPE cells reach confluency must be located on the 800 bp segment from the α4 promoter comprised between positions −200 and −1000.

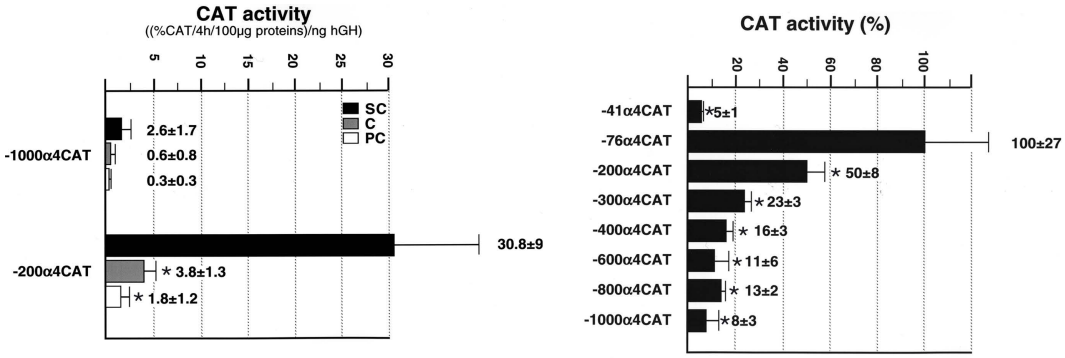
To more precisely delineate the position of these negative regulatory elements along the α4 promoter, CAT constructs bearing various deletions of the α4 promoter, ranging from −41 bp to −1000 bp relative to the α4 mRNA start site were transfected into sub-confluent cultures of RPE cells. The minimal α4 promoter sequences that directed maximal expression in RPE cells were found to be contained on the −76α4CAT construct (**Figure 5(b)**). Deleting further the α4 promoter down to position −41 resulted in a drastic 20-fold reduction in basal



(b)

	SC	C	PC
α4	193	80	10
18S	841	865	893

Figure 4. Expression of the *α*4 transcripts in primary cultured human RPE cells. (a) RT-PCR amplification products corresponding to the *α*4 integrin mRNA transcript were obtained from sub-confluent (SC), confluent (C) and post-confluent (PC) cultures of RPE cells and normalized to the 18S PCR product for semi-quantitative evaluation. The position of the PCR product corresponding to *α*4 (772 bp) is shown along with that corresponding to the 18S rRNA (489 bp); (b) Band density was calculated by the QuantityOne Image analysis software and reported as unit of intensity per millimeters.



(a) (b)

Figure 5. *α*4 promoter activity in primary cultured RPE cells. (a) Sub-confluent (black box), confluent (grey box) and post-confluent (white box) cultures of RPE cells (n = 6) were transfected with plasmids bearing fragments from the human *α*4 gene promoter extending either to 5' position −200 (in −200*α*4CAT) or −1000 (in −1000*α*4CAT) and fused to the CAT reporter gene. Cells were harvested 2 days following transfection and CAT activities determined and normalized to the amount of hGH secreted into the culture media. Asterisks (*) indicate CAT activities at both confluency and post-confluency that are statistically different from those measured in sub-confluent cells ($P < 0.05$; paired samples, t-test). S.D. is also provided; (b) *α*4/CAT recombinant plasmids bearing 5'-deletions of the *α*4 promoter (5' end-point ranging between positions −1000 and −41 relative to the *α*4 mRNA start site) were transfected into sub-confluent cultures of RPE cells (n = 6). Cells were harvested and CAT activities determined and normalized as in (a). *: indicates CAT activities from transfected RPE cells that are statistically different from those measured with the –76*α*4CAT plasmid ($P < 0.05$; paired samples, t-test). S.D. is also provided.

promoter function. Extending further the 5'-end of the *α*4 promoter resulted in a progressive reduction of *α*4 promoter activity that reached a level 13-times lower with plasmid −1000*α*4CAT relative to the activity directed by the −76*α*4CAT construct. However, most of this negative regulatory influence is primarily directed by the *α*4 promoter segment comprised between positions −76 to −300 as it accounted for nearly 80% of the repressive influence observed in RPE cells.

4. Discussion

When cultured RPE cells are left at post-confluency, they become quiescent and acquire morphological characteristics similar to those observed *in vivo*. Using RPE cells grown to the same cell densities as those reported in the present study, we previously established that sub-confluent and confluent RPE cells cultured *in vitro* are actively proliferating whereas post-confluent cells are quiescent [45]. In the present paper, we reported that a marked reduction in the transcription of the endogenous α4 gene as well as in the activity directed by the α4 promoter is observed when cultured RPE cells reach confluency, suggesting that α4 gene expression is intimately related to the proliferative state of RPE cells. In addition, we demonstrated that the quantity of α4 protein is similar between sub-confluent and confluent cells, and that the protein accumulates at the membrane level once cells reach confluency. This suggests that an immature form of the protein was present within sub-confluent cells, which increasingly translocated to the cell membrane during the maturation period of cell culture. The localisation at the cell junction upon reaching confluency is not unique to the α4 integrin subunit. Indeed, other cell adhesion molecules, such as N-Cadherin [48], have been shown to localise at the cell periphery when maintaining cultured cells at post-confluency for several weeks.

The stronger α4 staining at cell-cell junctions observed with confluent cells (as compared to sub-confluent cells) could seem contradictory with regards to the down-regulation of the mRNA observed in **Figure 4**. However, this apparent discrepancy can be explained by the accumulation of the protein at cell-cell contact leading to a negative feedback loop. Moreover, giving that the $\alpha 4\beta 1$ integrin has been reported to bind to itself [36] [49], the increase in cell-cell contacts, which are typical of confluent cultures, may facilitate detection of this integrin by promoting its clustering at the cell edges and could therefore explain the high fluorescence staining at this particular location in confluent cells.

Expression of the α4 integrin subunit in native RPE cells has been previously documented [42]. However, as RPE cells are mitotically inactive *in situ*, α4 expression in RPE cells is intriguing given its well-known role in the regulation of the immune response [24] [25], as well as in development, cell differentiation and migration [29] [30] [38] [39] [50]. In diseases such as proliferative vitreous retinopathy, the normally quiescent RPE cells dedifferentiate, proliferate and secrete extracellular matrix molecules that form fibrocellular membranes [51]. Fibronectin, a ligand of the $\alpha 4\beta 1$ integrin, is a major component of these epiretinal membranes [52]. The presence of a constitutive α4 expression in the native cells might facilitate RPE dedifferentiation, migration and proliferation and contribute to the progression of the disease. To that regard, blocking antibodies directed against the α4 subunit might prove a promising approach for the treatment of this fibrocellular disease.

The α4 promoter is more efficiently transcribed into sub-confluent cells and becomes heavily down-regulated as cells reach confluency. This pattern of expression is consistent with the data from the RT-PCR analyses (**Figure 4**) but yet differs from that previously observed with the α5 integrin promoter in RPE cells [45], as maximal level of α5 promoter activity was seen at confluency, and then decreased when cells reached post-confluency. Although constructs of similar lengths were used for the transfection studies, both the α4 and α5 promoters bear clearly different regulatory regions, suggesting that these genes are controlled by different sets of transcription factors. Most interestingly, our results also demonstrate the presence of one (or many) negative regulatory element(s) located between position −76 and −200 relative to the α4 mRNA start site. Of particular interest is the sequence −76/−200 which functions as a powerful transcriptional repressor in RPE cells [53]-[55]. This reduced promoter activity must be mediated by regulatory proteins other than the ZEB transcription factor since ZEB sites have only been located at positions −361 and −399 [56].

5. Conclusion

This study establishes that cell confluency generates a downregulation of the α4 gene expression that is dependent upon strong negative regulatory elements located on the −76 to −300 region of the promoter. Given that the expression of the α4 integrin subunit is scarce in non-haematopoietic cells and that it binds an alternatively spliced segment of FN that is normally expressed only during wound healing [57]-[59], its expression by RPE cells remains intriguing and deserves more studies.

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