5. RESULTS

5.1 Surface antigen characterization of the panel of human cell lines

5.1.1 Introductory remarks

During cell biology experiments several human cell lines were used, comprising endothelial progenitor cells, primary endothelial cells and solid tumours-derived cells. In particular, the main characteristic of endothelial progenitor cells is their ability to differentiate into endothelial cells, thus participating in vessel growth [35]. Primary endothelial cells are known to express high levels of the developmental integrin α5β1 and of integrin αvβ3. Moreover, it is known that many solid tumours over-express integrin αvβ3 because it is useful for the cross-talk with the ECM, which drives both migration and homing toward distant sites.

A panel of human cell lines was characterized for the expression of surface antigens of interest, namely integrins αvβ3, αvβ5 and α5β1, by means of flow cytometry and immunofluorescence, in order to gain both quantitative and qualitative data. In particular, the cell lines undergoing the screening were EPCs, HUVECs, ECV-304, T98G, PC-3, Caki-1, H460 and MDA-MB-231 (see **paragraph 5.1.2** and **Table V** for details).

5.1.2 Flow cytometry

EPCs expressed four-fold amounts of integrin α5β1 in respect of integrin αvβ3. Moreover, FACS analysis highlighted that HUVECs expressed three-fold amounts of integrin α5β1 in respect of integrin αvβ3, even if integrin α5β1 amount was clearly lower than that of EPCs. This result was really intriguing. Given that integrin α5β1 is mainly involved in physiological angiogenesis, whereas integrin αvβ3 plays a pivotal role in pathological and post-natal angiogenesis [1], the pattern of expression of integrins α5β1 and αvβ3 in endothelial progenitors and primary endothelium seemed to confirm that the earlier the precursor was, the higher the expression of integrin α5β1 resulted. Again, the good and similar amounts of integrin αvβ3 displayed by both endothelial progenitors and primary endothelial cells suggested that this marker plays an important role in endothelial biology.

Multiforme glioblastoma and renal clear cell carcinoma cells diplayed results similar to those of HUVECs, with a three-fold expression of integrin α5β1 in respect of integrin αvβ3. Bladder carcinoma cells expressed approximately the same amounts of integrin α5β1 and αvβ3, suggesting that a comparison between these cells and multiforme glioblastoma cells (as a reference for highly expressing integrin α5β1 cell line) was a good way to discriminate the specificity of test compounds for integrins αvβ3 or α5β1, given that an established solid-phase receptor-binding assay for integrin α5β1 was not planned (see paragraph **5.2.1**). Both prostate cancer and non-small cell lung cancer cells expressed high levels of integrin α5β1, with very low amounts of integrin αvβ3. Finally, breast adenocarcinoma cells deserved a particular attention, because FACS analysis revealed that these cells expressed almost exclusively integrin α5β1. This result allowed me to consider MDA-MB-231 cells as a potential reference cell line to study cyclic RGD peptides selectively driven to integrin α5β1. Moreover, during this study breast adenocarcinoma cells were used as a negative control in cell biology screenings regarding compounds selectively driven to integrin αvβ3, such as fluorescein-conjugated cyclic RGD peptides and RGD-coated gold nanoparticles (see **paragraphs 5.5** and **5.6**, respectively).

Finally, flow cytometry demonstrated that integrin αvβ5 was very faintly expressed in all cell lines, excepted bladder carcinoma cells. The fact that these cells expressed good levels of all markers of interest (namely integrins αvβ3, αvβ5 and α5β1) did not render them an appealing single model to investigate the biological activity of cyclic RGD peptides. In fact, ECV-304 cells putatively bound every biologically active cyclic RGD molecule, without allowing me to discriminate if the compound was really selective.
Results obtained by FACS analysis on the human cell panel are outlined in **Table VII**I and **Figure 15**.

Cell line	Integrin αvβ3		Integrin αvβ5		Integrin α5β1	
	%	MFI	%	MFI	%	MFI
EPC	98.9	10575	53.2	1585	99.6	46351
HUVECs	99.4	11049	33.8	1315	100	30974
ECV-304	96.8	3322	98.2	1977	99	3867
T98G	85.5	4741	10.4	1663	98.9	13041
PC-3	29.5	1402	11.9	1200	98.4	8229
Caki-1	64.2	4536	46.7	2600	97	11900
H460	0.5	377	56.2	1215	99.8	5883
MDA-MB-231	6.4	592	16.4	795	97.3	4208

Table VIII. *Flow cytometry results. Integrin αvβ3, αvβ5 and α5β1 amounts are expressed as percentage and mean fluorescence intensity.*

5.1.3 Immunofluorescence

Immunofluorescence was performed on different substrates relating to the specificity of each marker, namely integrins αvβ3, αvβ5 and α5β1. Thus, cells were plated on vitronectin in order to characterize integrins αvβ3 and αvβ5 expression, or on fibronectin to characterize integrin α5β1 expression. Immunofluorescence on EPCs was not performed because of the discontinuous availability of cells.
On the whole (**Figure 16**), immunofluorescence confirmed the results obtained with flow cytometry. Integrin α5β1 was widely expressed by the whole cell panel, with a particular focus on HUVEC and T98G cells. Integrin αvβ3 displayed very good levels of expression on HUVEC, ECV-304 and T98G cells, with a staining fainter in H460 cells and mainly absent in MDA-MB-231 cells. Finally, integrin αvβ5 expression was really low in each cell line. However, it is noteworthy that the antibody used to stain integrin αvβ5 was hardly optimized and has not been extensively characterized in the literature.

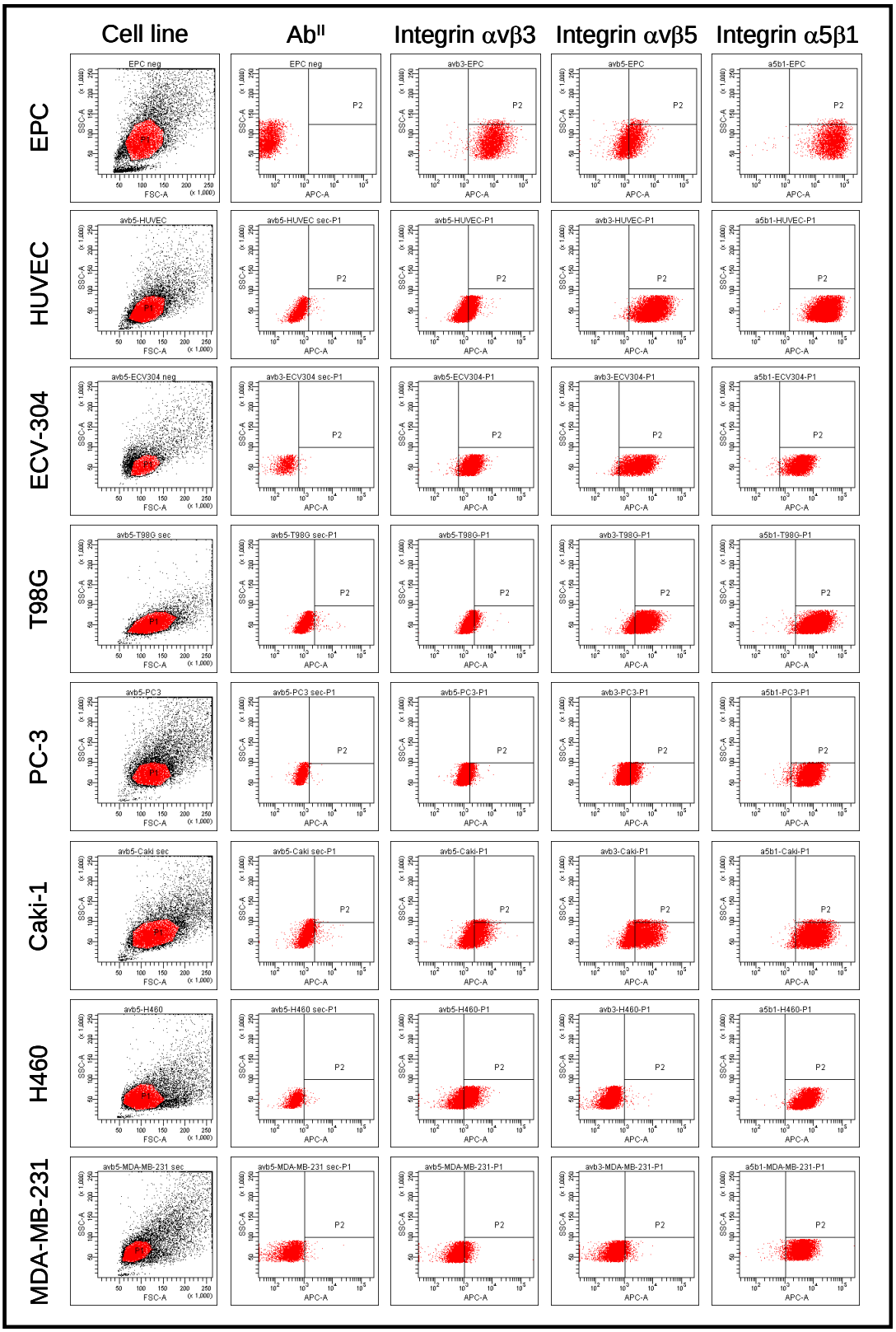

Figure 15. *FACS analysis performed on the panel of human cell lines. Cell line: scattergram, representing cell volume (FSC, Forward Scatter, x axis) and cell complexity (SSC, Side Scatter, y axis). Ab^{II}: secondary antibody staining, used as a negative control. P2: positive events (namely, cells positive for the marker of interest).*

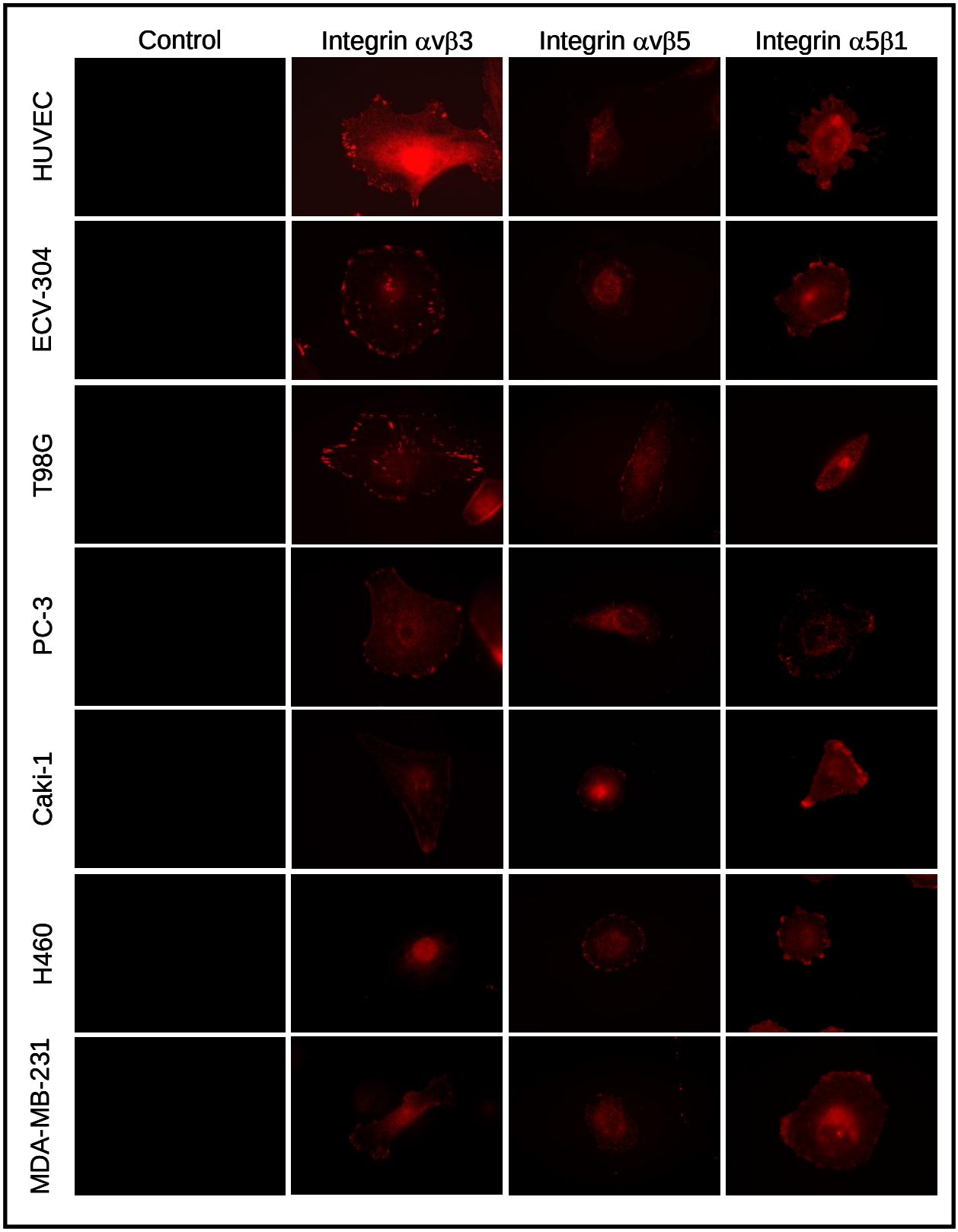

Figure 16. *Immunofluorescence performed on the panel of human cell lines. Red: integrin αvβ3, αvβ5 or α5β1, respectively. Original magnification: 63x.*