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"Generation of a reporter system for monitorization of endothelial differentiation"

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Professor Doutor Ricardo Neves (Centro de Neurociências e Biologia Celular de Coimbra) e supervisão da FCTUC pelo Professor Doutor João Ramalho (Universidade de Coimbra)

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Contents

Abstract

Stem cell differentiation into endothelial cells has been an active field of research within the last years. The increased interest in this research field is a consequence of the huge clinical relevance of these cells and the associated future regenerative medicine treatments that could improve the recovery of pos-ischemic conditions and other angiogenic related states.

Despite this increase in research, nowadays the differentiation strategies into the endothelial lineage are very inefficient. This inefficiency is related both to the lack of information regarding the ideal cell source to the differentiation process and to the non-standardization of the *in vitro* conditions to induce the endothelial lineage commitment (growth factors, matrices, cytokines). Therefore strategies that permit a real time monitorization of the differentiation process, like viral transduction of tagged DNA, is a reliable and efficient way to evaluate these kinds of processes.

In this context, the main aim of this project was to generate a lentiviral tool specific for detecting the expression of an endothelial late marker (vascular endothelial-cadherin – VE-cadherin).

This objective was accomplished through the ligation of the VE-cadherin promoter and a mCherry fluorescent tag. After generating the reporter system, the validation of this tool was done by infecting human umbilical vein endothelial cells (HUVECs) and induced pluripotent stem cells (iPSCs). The results show that 5 days after infection HUVECs present an increased pattern of red fluorescence and no alteration was observed within the infected iPSCs. There is already on-going work to differentiate the infected iPSCs and monitor the red fluorescence levels.

Additionally, some information was generated regarding the internalization of organic nanoparticles (NPs) and adhesion and proliferation of iPSCs in possible endothelial inductive

substrates. The results show that the iPSCs are able to internalize nanoparticles even when the NPs are adhered to the substrate.

In conclusion, the results show the successful creation of a lentiviral construct that permits the direct monitorization of expression of mCherry associated with VE-cadherin promoter. In the future this might be a powerful tool either to be used in a stem cell context or to explore other cell sources and processes like transdifferentiation.

Keywords: lentivirus, vascular-endothelial cadherin, endothelial differentiation.

Resumo

O conhecimento na área da diferenciação de células estaminais na linhagem endotelial tem registado, nos últimos anos, um crescimento muito acentuado. Este fenómeno surge quer em consequência da relevância das células endoteliais na prática clínica, quer do potencial que, por esse motivo, representam enquanto fonte celular para uso em técnicas de medicina regenerativa, como por exemplo, na recuperação de condições como a isquémia.

No entanto, apesar da diferenciação endotelial constituir uma área activa de pesquisa, este processo continua a ser ineficiente). A baixa eficiência dos processos de diferenciação endotelial está associada com a falta de estandardização do processo, tanto quanto ao tipo de células a diferenciar, bem como as condições de cultura (factores de crescimento, citocinas, matrizes extracelulares). Com o intuito de desenvolver metodologias para colmatar esta lacuna, têm sido desenvolvidos sistemas virais que permitem a monitorização da expressão de genes de interesse.)

Neste contexto, o objectivo central deste projecto foi o desenvolvimento de uma ferramenta lentiviral que permitisse a monitorização da expressão da *vascular-endothelial cadherin* (*VE-cadherin*) (marcador endotelial tardio). Na construcção deste sistema lentiviral, o promotor da *VE-cadherin* foi associado a uma porção de DNA codificante para uma proteína fluorescente vermelha (*mCherry*).

A validação desta ferramenta foi efectuada com infecção de *human umbilical vein endothelial cells* (*HUVECs*) e em células estaminais de pluripotência induzida (*iPSCs*). Os resultados relativos a estas infecções mostram que, 5 dias após a infecção, as *HUVECs* apresentam uma alteração positiva a nível da fluorescência vermelha e nenhuma alteração foi registada a nível das *iPSCs* infectadas. Neste momento está em curso a diferenciação das *iPSCs* infectadas e os níveis de fluorescência estão a ser monitorizados.

Adicionalmente, foram gerados alguns dados relativos à internalização de nanopartículas orgânicas (NPs) e adesão e proliferação de *iPSCs* em matrizes extracelulares com potencial indutor para diferenciação endotelial. Resultados preliminares mostram que as *iPSCs* são capazes de internalizar as NPs mesmo quando estas se encontram aderidas ao substrato.

Sumariamente, os resultados mostram que a geração do sistema lentiviral desejado foi atingida com sucesso. Assim, futuramente, esta ferramenta poderá mostrar-se extremamente útil na monitorização da diferenciação endotelial utilizando diferentes contextos extracelulares (factores de crescimento, citocinas, matrizes) e utilizando diferentes fontes de células. Acresce que esta ferramenta poderá não abranger apenas o contexto de células estaminais mas também expandir-se para outras populações celulares e estudar nomeadamente processos de transdiferenciação.

Palavras-chave: lentivírus, diferenciação endotelial, *vascular-endothelial cadherin*

Abbreviations

BMPs – bone morphogenetic proteins

DNA - deoxyribonucleic acid

Dppa3 – stella

EB – embryoid body

ESCs – embryonic stem cells

FGF – fibroblast growth factor

Gbx2 - gastrulation brain homeobox 2T – histone acetyltransferase

HA – hyaluronic acid

hESCs – human embryonic stem cells

ICM – inner cell mass

IGFs – insuline-like growth factors

iPSCs – induced pluripotent stem cells

Klf4 - krueppel-like factor 4

miRNA – micro RNA

Oct4 – octamer-binding 4

Olig2 – oligodendrocyte transcription factor 2

PDGF – platelet derived growth factor

PI(3)K - phosphoinositide 3-kinase

PSC – pluripotent stem cell

Rex1/Zfp42 - reduced expression 1/zinc finger protein 42

RF – reprogramming factor

RNA - ribonucleic acid

RTK – receptor tyrosine kinase

SCs – stem cells

Sox2- sex determining region Y box 2

SSEAs – stage-specific embryonic antigens

TGF – transforming growth factor

VE-cadherin – vascular endothelial cadherin

VE-pr - vascular endothelial cadherin promoter

Chapter 1

Introduction

1. Introduction

Within the last years the knowledge regarding stem cells (SCs) has suffered an exponential growth. Processes like stem cell self-renewal, proliferation, differentiation and embryonic development are becoming more clear and constitute active research fields. Recently, some advances in the field of SCs enabled the artificial reprogramming of differentiated cells into a pluripotent state (induced pluripotent stem cells - iPSCs) and opened new ways for potential translation of stem cell technology into regenerative medicine.

This section introduces the general stem cell characteristics and also specifies some of the characteristics of embryonic stem cells (ESCs) and iPSCs.

1.1. Stem cells

Stem cells have two unique properties: the capacity of self-renewal and the differentiation potential (Mountford, 2008).

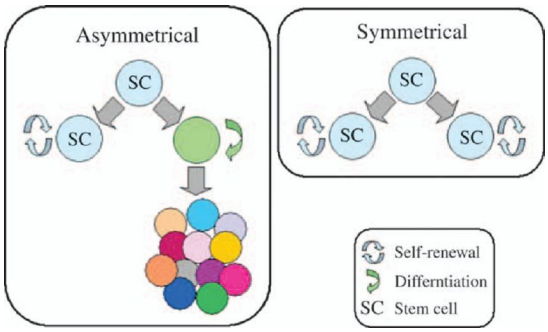


Figure 1 - Asymmetrical and symmetrical stem divisions (Mountford, 2008).

The self-renewal capacity is responsible for the replacement of stem cells and avoids their exhaustion. This mechanism can be accomplished by symmetric cell division when a stem cell gives rise to two daughter cells that maintain their stemness or by asymmetrical cell division when one stem cell originates a differentiated cell and another stem cell (Figure 1) (Mountford, 2008)..

The hierarchic classification of SCs takes into account their differentiation potential: from the most primitive cells to cells with a more restricted capacity of differentiation. Pluripotent stem cells are characterized as able of unlimited self-renewal and can give rise to every cells present in every tissue of the adult body (Eckfeldt, Mendenhall & Verfaillie, 2005).

The stem cell behaviour is regulated by intrinsic and extrinsic signals in a complex network of interactions. The regulation of stemness comprises environmental stimulus, transcriptional regulation, post transcriptional regulation, genetic and epigenetic control mechanisms that decide the fate of SCs (Figure 2) (Jaenisch & Young, 2008)..

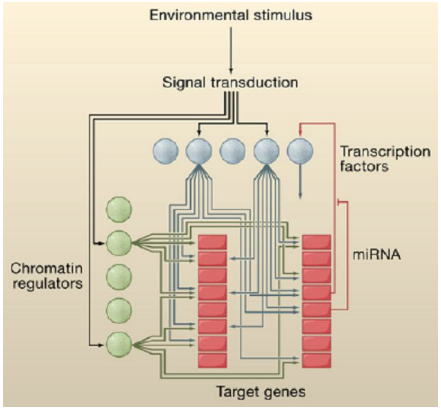


Figure 2 – Schematic representation of a transcriptional regulatory circuitry of pluripotency and possible connections between signal transduction pathways, transcription factors (blue circles), chromatin regulators (green circles) and their target genes (orange squares) (Jaenisch & Young, 2008).

The major regulators of self-renewal, pluripotency, differentiation and viability of human ESCs (hESCs) and pluripotent SCs (PSCs) involve signalling pathways like transforming growth factor β (TGF-β), receptor tyrosine kinase (RTK), wingless (Wnt), bone morphogenetic proteins (BMP) and others (Figure 3). The maintenance of pluripotency is associated with expression of genes such as octamer-binding 4 (Oct4), homeobox protein Nanog, sex determining region Y box 2 (Sox2), krueppel-like factor 4 (Klf4), stella (Dppa3), zinc finger protein 42 (Rex1) and gastrulation brain homeobox 2 (Gbx2). Furthermore hESCs have a characteristic pattern of surface markers that allow their recognition, like stage-specific

embryonic antigens (SSEAs) 3 and 4 (SSEA 1 negative), TRA-1-60, TRA-1-81, GTCM-2 and alkaline phosphatase (Pera & Tam, 2010). Additionally also extrinsic mechanisms have an important role in the fate of ESCs. In this way, the microenvironment where SCs are included named niche has also a central role in the SCs fate and control of self-renewal, survival and differentiation.

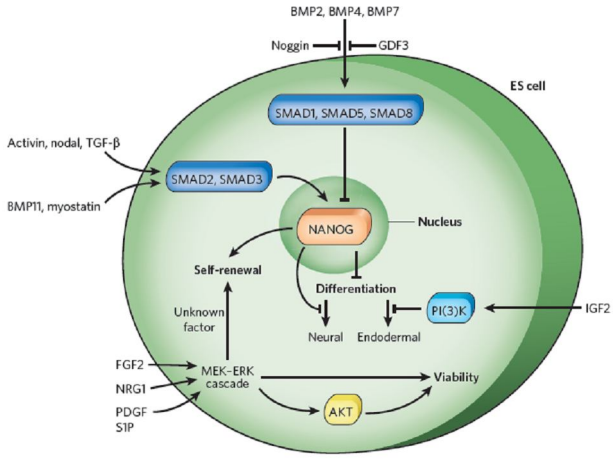


Figure 3 - Illustration of signalling cascades that regulate self-renewal, differentiation and viability (Pera & Tam, 2010).

Table I – Lineage specific differentiation of hESCs (Mountford, 2008)

Tissue type	Reference	Cell lineage	Disease application
Neural	Zeng *et al.* (2004)	Dopamine neuron	Parkinson's disease
	Nistor *et al.* (2005)	Oligodendrocyte	Spinal cord injury
	Li *et al.* (2005)	Motor neuron	Motor neuron disease, spinal cord injury
Liver	Hay *et al.* (2007)	Hepatocyte	Liver failure
Endocrine	D'Amour *et al.* (2006)	Islet β-cells	Diabetes
Cardiovascular	Mummery *et al.* (2002)	Cardiomyocyte	Myocardial infarction
	Wang *et al.* (2007)	Endothelial	Stroke, myocardial infarction, ischaemic limb disease
Eye	Haruta (2005)	Retinal epithelium	Retinal degeneration or injury
Skin	Ji *et al.* (2006)	Keratinocytes	Burns, traumatic skin loss
Musculoskeletal	Zheng *et al.* (2006)	Skeletal muscle	Muscular dystrophy
	Toh *et al.* (2007)	Chondrocytes	Cartilage loss
	Karner *et al.* (2007)	Bone	Loss from trauma or tumour, brittle bone disease
Blood	Vodyanik *et al.* (2006)	Lymphoid cells	Immune deficiencies
	Olivier *et al.* (2006)	Erythrocytes	Blood loss, sickle cell anaemia

In the stem cell field, one of the major steps resulted from research done by Evans, Kaufman and Martin that culminated in the isolation of embryonic stem cells in 1981 from the

inner cell mass (ICM) of mouse blastocyst (Katsumoto, Shiraki, Miki & Kume, 2010) and isolation and characterization of hESCs in the late 1990 by Thomson and colleagues (Eckfeldt, Mendenhall & Verfaillie, 2005).. Although the differentiation process is not yet a fully defined mechanism there are already several protocols that allow the successful differentiation of hESCs into specific cell lineages (table1).

Recently, Shinya Yamanaka and colleges achieved the reprogramming of differentiated human fibroblasts into iPSCs (Takahashi et al., 2007). Since then, several cell types have been generated from iPSCs, like human secreting insulin cells, human cardiomyocytes and human hematopoietic cells. iPSCs resemble ESCs in genomic, transcriptomic and epigenomic patterns and provide a way to study the mechanisms involved in reprogramming, lineage commitment and cellular differentiation (Hipp & Atala, 2008).. The reprogramming induction can be considered a two-stage process that initially embraces the inhibition of genes associated with the lineage specification and reversion of the epigenetic pattern associated with differentiated cells. While in the second stage, when the cell is partially reprogrammed, the exogenous reprogramming factors (RFs) will reactivate the transcriptional network responsible for pluripotency. The induced reprogramming of somatic cells into pluripotent cells involves a complex transcriptional network that may include octamer-binding 4 (Oct4) (Amabile & Meissner, 2009;), sex determining region Y box 2 (Sox-2), krueppel-like factor 4 (Klf4) (Spagnoli & Hemmati-Brivanlou, 2006) and c-Myc (Hipp & Atala, 2008; Spagnoli & Hemmati-Brivanlou, 2006; Wang & Steinbeisser, 2009).

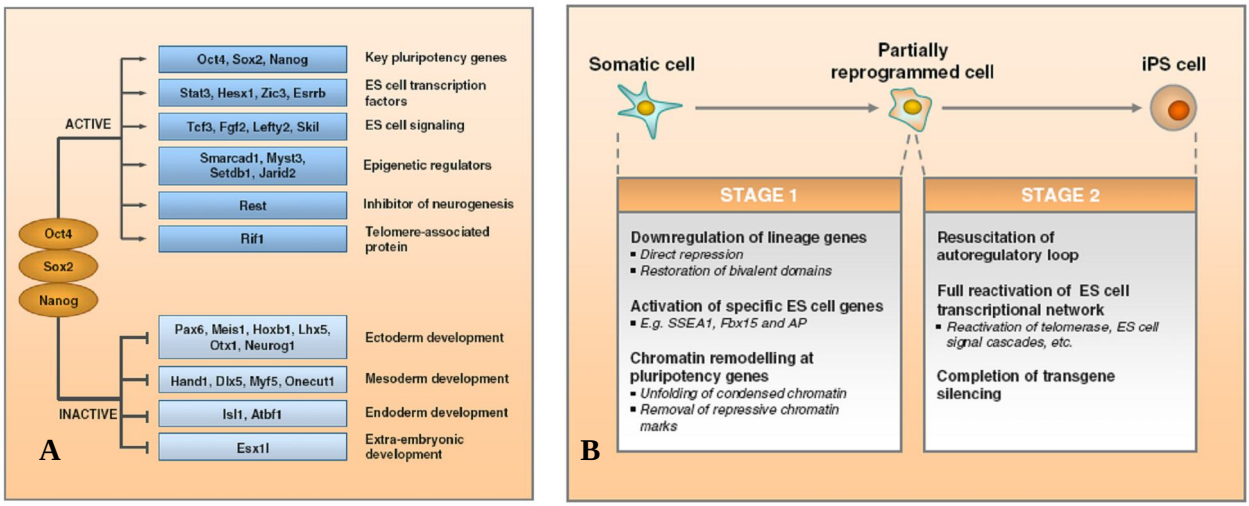


Figure 4 - A) Oct4, Sox2 and Nanog target genes and respective functions.; **B**) **T**he reprogramming mechanism as a two-stage process (Adapted from Schemer & Copra, 2009).

1.2. Differentiation

A better understanding of cellular differentiation and respective regulatory mechanisms is essential for the advance of the cell-based therapy of a broad spectrum of diseases. The SC manipulation and specially iPSCs can be the mean to generate this knowledge and contribute to a future improvement of the utilization of SCs in regenerative medicine (Spagnoli & Hemmati-Brivanlou, 2006).

In this section, the differentiation processes that occur during the embryonic development and also the strategies used to induce differentiation of SCs into the vascular lineage will be pointed out.

1.3. Mechanisms of differentiation in embryonic development

During embryogenesis the initial fertilized egg gives rise to the morula, where the first event of differentiation takes place when the trophectoderm lineage is established with

associated expression of trophectoderm markers, such as caudal-related homeobox 2 (Cdx2). Posterior divisions originate the blastocyst stage, where the second developmental decision occurs in the inner cell mass (ICM), within the blastocyst. The ICM differentiates into the epiblast (also known as the primitive ectoderm) and the primitive endoderm, where is thought that Nanog may have a central role by induction of epiblast markers expression (e.g. FGF5) and downregulation of markers of both the primitive endoderm (e.g.GATA4 and GATA6) and the ICM (e.g. Rex1 and Gbx2) (Spagnoli & Hemmati-Brivanlou, 2006).

The next embryonic developmental stage is the gastrulation and during this period several changes occur in cell motility, cell polarity, cell shape and cell adhesion (Wang & Steinbeisser, 2009). During this stage the pluripotent epiblast cells are allocated to the three primary germ layers, ectoderm, mesoderm and endoderm. These three germ layers will give rise to all types of cells that integrate the different adult tissues (Figure 5).

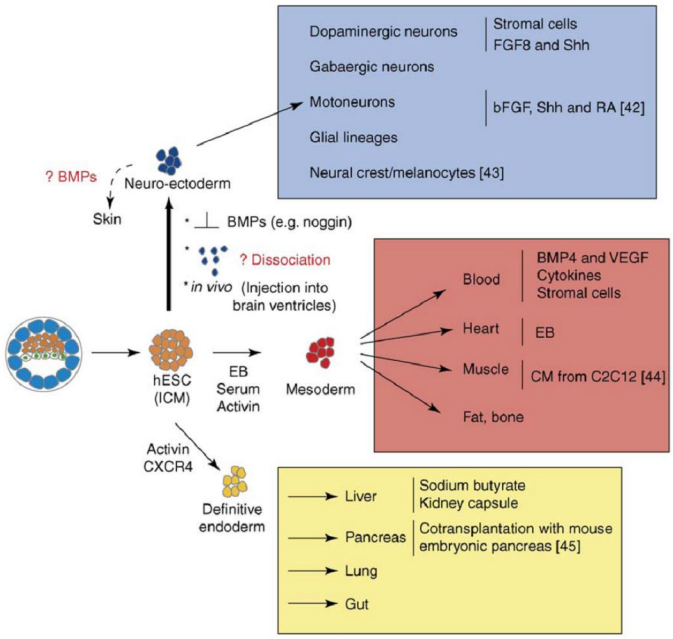


Figure 5 - Lineage differentiation within the inner cell mass during the normal embryogenesis (Spagnoli & Hemmati-Brivanlou, 2006).

Within the mesodermal differentiation, the endothelial progenitor cells (EPCs) share a common precursor cell with hematopoietic cells, the hemangioblasts. EPCs can also be derived from vascular progenitor cells (VPCs) and further differentiation processes from EPCs and VPCs into endothelial cells (ECs) depends on location in the embryo, access to specific factors (VEGF, Wnts, BMP and others) and paracrine signaling from the neighbouring stem/progenitor or even differentiated cells.(Figure 6) (Luo et al., 2011; Bai & Wang, 2008).

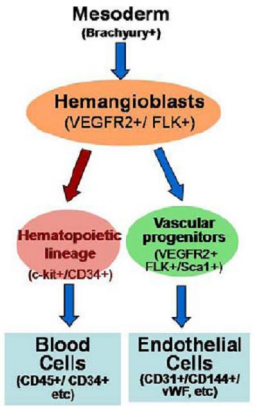


Figure 6 -.Progenitor cells driving endothelial cells (Luo et al., 2011)

1.4. Vascular lineage differentiation

The vessel formation is a complex mechanism that occurs during embryogenesis (Yancopoulos et al., 2000), tumoral angiogenesis, wound healing and after ischemic situations. The vessel formation comprises interactions between endothelial cells (ECs) and the mural cells (MCs) (pericytes and vascular smooth muscle cells -VSMCs) (Kitagawa & Era, 2010).

The vessel formation includes various processes and signalling molecules and studies in this area may give some clues for optimization of vascular *in vitro* differentiation protocols. The main pathways involved in differentiation of vascular cells involve vascular endothelial growth factor (VEGF), angiopoietin, TGF, Wnt, Notch and ephrin families (Yancopoulos, et al., 2000; Otrock et al., 2007). In addition to these factors there are other features that regulate the

angiogenic process such as soluble factors (e.g. TNF-α, HIF-1, angiogenin, angiotropin), membrane-bound factors (e.g. αvβ3-Integrin, vascular endothelial-cadherin) and the plasmin and metalloproteinase systems (Eble & Niland, 2009).

1.4.1. Endothelial cells (ECs)

In vitro generation of endothelial cells from PSCs can be achieved by either culturing as embryoid bodies (EB), by co-culturing with other cell lines or, as most recently described, by culturing SCs in defined chemical conditions followed by various culture manipulations including the addition of growth factors and cytokines, defined adhesion substrates, mechanical stress, and vascular progenitor cell isolation and culturing.

Within the endothelial differentiation process there are several signalling pathways like VEGF, Wnt and Notch, TGF-β family, together with ROS signalling and microRNAs that play an important role in the differentiation process (Figure 7).

Regarding the VEGF family it is composed by 5 members, VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PIGF) and several alternatively spliced isoforms. VEGF-A is the most potent pro-angiogenic protein and is involved in proliferation, sprouting and formation of endothelial cell's tube, it is a crucial mediator of early vascular formation and has also an essential regulatory role in post-natal angiogenesis. This molecule interacts with both specific tyrosine kinase receptors, $VEGFR_1$, also known as Fms-like tyrosine kinase-1 (Flt-1) and VEGF-R_2 also known as Kinase insert domain receptor (KDR). Additionally, VEGF-A also binds to the neuropilin-1 receptor (NP1), in a complex which promotes the binding to VEGF-R_2. VEGF-R_2 is the major mediator of VEGF induced responses (Luo et al., 2011).

The VEGF/VEGF-R_2 axis mediated EC differentiation, proliferation and migration involves several signaling pathways (Figure 7). After ligand binding, VEGF-R_2 induces receptor phosphorylation and activation of the classical extracellular signal-regulated kinases (Erk) pathway, via a protein kinase C (PKC)-dependent pathway involving activation of

phospholipase C-γ (PLC-γ), activation of PI3K and subsequently activation of AKT and HDAC3 (Figure 7). There are also some reports involving Shb as a downstream molecule of the VEGF/VEGF-R_2 axis, and as an amplifier of the VEGF-R_2 signal.

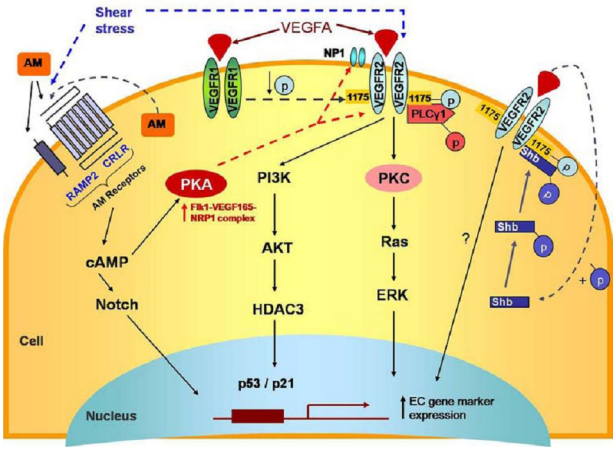


Figure 7 – Signaling pathways involved in the endothelial differentiation process (Luo et al., 2011).

Another important growth factor family involved in EC differentiation is the TGF-β family that includes TFG- β, inhibins, activin, nodal, anti-müllerian hormone, BMP-2, 4, 6 and 7, decapentaplegic and Vg-1. It has been demonstrated that TFG-β promotes the generation of ESC-derived EC progenitor cells through transcription factor snail and additionally a very recent report has demonstrated that inhibition of TGF-β signaling in the late phase of endothelial differentiation resulted in the up-regulation of Id1 that consequently increases the yield of ECs from hESCs. Interestingly, this study also found that TGF-β inhibition helped to consolidate the endothelial identity of the isolated ECs and also maintained proliferation for an extended period. On the other hand, in early endothelial cell commitment TGF-β plays a role and without this factor there is no vascular commitment (James, et al., 2010).

Also the Wnt large family of cysteine-rich secreted proteins is involved in an array of diverse processes such as embryonic growth, migration, and differentiation. Wnt signaling

controls cell proliferation, stemness maintenance, stem cell fate decisions, organized cell movements and the establishment of tissue polarity. The relevance of this pathway in the embryonic vascular development was shown by knockout mice studies reporting that β-catenin or Wnt co-receptors deficient mice fail to develop the mesoderm layer (Luo et al., 2011).

In addition to growth factors, there are also other factors like ROS and microRNAs that have an important role in the EC differentiation. ROS are important determinants of vascular function, acting not only as activators of cell growth and differentiation but also as modulators in pathological processes. As intracellular second messengers, ROS can activate several signaling molecules and pathways (G proteins; Src, Ras, JAK2, Pyk2, PI3K, and MAPK pathways), inhibit protein phosphatases, modify the activity of phospholipases, alter intracellular cation concentrations, and regulate expression and function of transcription factors such as NFκB, activator protein-1, and hypoxia inducible factor-1. Along with these angiogenic regulators, miRNAs and specially a family of angiogenic miRNA (Fish & Srivastava, 2009) can also modulate the expression of key angiogenic related molecules.

1.5. Strategies to induce in vitro differentiation of stem cells into endothelial cells

The *in vitro* differentiation may be achieved with different protocols for SCs according to the culture method (Kitagawa & Era, 2010) embryoid body (EB) formation, culture on feeder cells, chemical defined culture mediums (growth factors, cytokines), and more recently the use of nanotechnology and biomaterials are fields of expanding knowledge regarding manipulation of SC fate and differentiation.

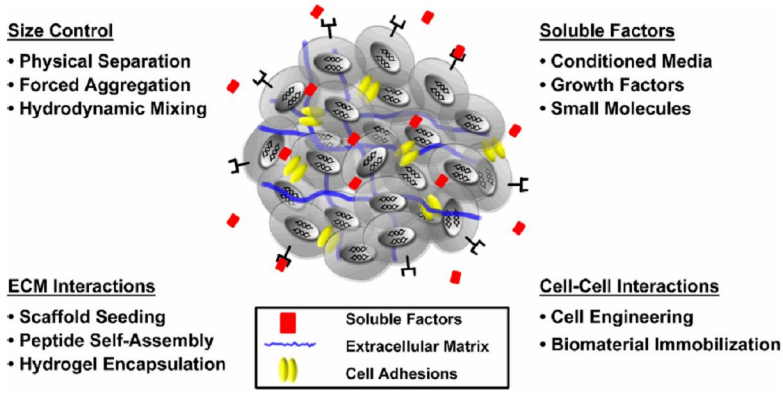


Figure 8 - Factors that control the differentiation processes within the embryoid bodies (Bratt-Leal, Carpenedo et al. 2009).

Although presently there is no standardized protocol to generate stem/ progenitor cell-derived endothelial cells (Kane et al., 2011), there are two major approaches in order to obtain ECs: the 3D embryoid body (EB) differentiation or 2D monolayer directed differentiation, associated with different serum and growth factor and cytokine differentiation media and different cellular sub-populations enrichment (either by FACS or MACS -). More recently the use of nanotechnology and biomaterials are also active research areas that are trying to contribute to improve the efficiency of the endothelial differentiation protocols (Ferreira et al., 2007a).

EB formation is a method widely used to examine the differentiation potential of SCs and offers the opportunity to study early three-dimensional assembly of pluripotent cells. The cells are cultured using different methods for embryoid body formation such as hanging drop method, static suspension cultures, entrapment systems like hydrogels (methylcellulose, fibrin or hyaluronic acid) and use of multi-well and microfabrication technologies (e.g. microwells, microfluidic chambers) (Ng et al., 2005). The differentiation of EBs is coordinated by factors like the size and three-dimensional organization of EBs, the microenvironment, cell-cell interactions and extracellular matrix interactions (Figure 13). Nevertheless, the formation of EBs triggers spontaneous differentiation of all cell types, it is an inefficient method with low endothelial differentiation yields (ranging from 1 to 3% for ECs) (Levenberg et al., 2002; Cho et

al., 2007; Li et al., 2008 ; ; Kane et al., 2011) because controlling the microenvironment within the EB is still a very difficult task (Blancas, Lauer & McCloskey, 2007). In order to circumvent the disadvantages and make the most of the advantages of EB based differentiation several groups reported mixed strategies that combine EB technology with other endothelial induction methods. Improved differentiation efficiencies were obtained with supplementation with angiogenic factors like VEGF-A (Blancas, Lauer & McCloskey, 2007; Ferreira et al., 2007; Nourse et al., 2010), amplification of EB derived homogeneous sub-populations with endothelial differentiation potential (PECAM-1, CD34, KDR) (Ferreira et al., 2007) (Levenberg et al., 2002; Ferreira, et al., 2007; Kane et al., 2011), hypoxia (Prado-Lopez et al., 2010) and suppression of the TGF-β pathway (James et al., 2010; Kane et al., 2011).. Also the use of different cell sources for endothelial differentiation has been an important field of study. The use of some progenitor cells (like endothelial progenitor cells EPCs) might constitute a way to improve the endothelial differentiation process by exploring the susceptibility of endothelial differentiation of specific stem cell populations (Eggermann et al., 2003; Dimmeler et al., 2001).

Recently the use of micropatterned extracellular matrix islands was shown to provide a way to reduce in some degree the heterogeneity of the differentiation process based in EB culture (Bauwens et al., 2008). In this study (Bauwens et al., 2008) EBs (with different sizes) were generated from different sized hESCs colonies and a connection was found between hESCs colony size, EBs size and propensity to mesodermal commitment. Larger EBs derived from hESCs small colonies were associated to higher mesodermal induction (Bauwens et al., 2008)

Regarding the 2D differentiation methods, these include co-culture with feeder layers like: mouse stromal cells (Vodyanik et al., 2005), mouse bone marrow stroma cells and mouse ECs (Vodyanik et al., 2005; Kaufman et al., 2001), prior to isolation and sub-culture. This type of differentiation strategies is associated with slightly higher efficiency in generating ECs

(around 10%) capable of forming durable vessels *in vivo* (Wang et al., 2007) but requires more laboratorial handling of the cells (separation from feeder-cells) and has a higher risk of contamination by non-human products. Recently a fast initiation method was described that employed a combination of matrix culture of collagen IV and a differentiation medium containing stem cell factor (SCF), VEGF-A and β-FGF (Lagarkova et al., 2008). The ECs generated in this study (Lagarkova et al., 2008) expressed immunological markers (vWF, CD105), endothelial specific genes (vascular endothelial-cadherin, KDR, endothelial nitric oxide synthetase), and formed endothelial characteristic networks on collagen matrix and in Matrigel assay but still, *in vivo* functional data was not reported (Lagarkova et al., 2008).

Very recently, a fully scalable feeder and serum-free method for the derivation of functional ECs without EB requirement was reported (Kane et al., 2010). The endothelial differentiation medium was supplemented with hydrocortisone, human epidermal growth factor, basic fibroblast growth factor and heparin (Kane et al., 2010). This study showed a rapid downregulation of pluripotency factors, concomitant with induction of vascular endothelial markers at the mRNA, miRNA, and protein levels. Moreover, the derived ECs respond to an NO stimulator, migrate, and spontaneously produce tube-like structures in monolayer cultures and when cultured in 3D matrices (Matrigel). The *in vitro* differentiated cells (10 days) were efficient at the induction of therapeutic neovascularisation and were incorporated into the blood perfused vasculature of recipient mice (Kane et al., 2010).

Biomaterial engineering as also contributed to the development of new means to achieve *in vitro* generation of endothelial cells and highlighted the importance of integration of biological and physicochemical sciences. These biological modalities in scaffold design fall into three major categories: those that mimic the original tissue architecture and strength, those that enable cell adhesion, and those that induce or contribute to the maintenance of cell phenotype (Krenning et al., 2008). Some of the elements from the extracellular microenvironment that may be incorporated into biomaterials to achieve their biological activity may include insoluble

extracellular matrix (ECM) macromolecules, diffusible molecules, and cell–cell receptors (Shekaran & Garcia, 2011). The current challenge of biomaterial engineering is to achieve the combination the all of these biological moieties and therefore mimic the native 3D complex biological microenvironments. The use of this kind of strategy will contribute to minimize adverse effects that biomaterials may have on the differentiating cells (Krenning et al., 2008). These bioinspired approaches have been applied in the development of biomaterials capable of directing specific cell functions (Shin, Jo & Mikos, 2003; Mann et al., 2001) and controlled degradation in a 3-D matrix (Lutolf et al., 2003; Raeber, Lutolf & Hubbell, 2005; Lutolf & Hubbell, 2005).

The screening of biomaterials and cell interactions is a time consuming process but to circumvent this hurdle new screening approaches based on microarray techniques and combinatorial libraries of polymeric biomaterials have arisen. (Bailey, Sabatini & Stockwell, 2004; Anderson, Burdick & Langer, 2004; Meredith et al., 2003). Some groups used robotics technology and produced a polymer microarray featuring three blocks of 1152 polymers (Anderson et al., 2005) , a similar technique was used to generate micro arrays for 576 different combinations of acrylate-derived monomers, and their interactions were investigated for embryonic stem cell (hESCs) growth and differentiation (Anderson, Levenberg & Langer, 2004). A microarray platform was developed for the culture of patterned cells on top of combinatorial matrix mixtures, enabling the study of differentiation in diverse microenvironment situations in parallel. The fabrication process used a standard robotic DNA spotter to form the cellular microarray that provides a 32 extracellular matrix combination of collagen I, III, IV, laminin, and fibronectin. The effect of these combinations was evaluated in terms of cellular differentiation in two contexts: maintenance of primary rat hepatocyte phenotype and differentiation of mouse embryonic stem (ES) cells toward an early hepatic fate (Flaim, Chien & Bhatia, 2005). One of the main advantages of this platform is that it can be easily adapted to other applications like cell differentiation towards other lineages, like the

endothelial. Another microarray-based screening system was developed for testing the effects of small molecules entrapped within the disc polymer were the cells are then seeded (Bailey, Sabatini & Stockwell, 2004).

While some cell types retain tissue-specific features on 2-D surface, it has become increasingly apparent that a 3-D physical environment will be required for others (Albrecht et al., 2005). Therefore, micro-fabrication techniques have been employed to fabricate 3-D microwells on a glass surface (Revzin et al., 2001) and also the combination of micro-patterning with the use of hydrogels to retain cells within a 3-D microenvironment (Liu & Bhatia, 2002; Albrecht et al., 2005). With these 3-D microarrays, multiple cell types were encapsulated within a confined 3-D geometry that maintained them in a viable and proliferative state for a few days (Liu & Bhatia, 2002; Albrecht et al., 2005). Although the approach is still in infancy, a 3-D microarray may be a next generation platform for the high throughput analysis of cellular responses to microenvironment components that resemble in vivo characteristics (Shin, 2007).

1.6. iPS derived endothelial cells

With respect to hiPSC-differentiation into ECs, some studies have shown that iPSCs have the potential to originate this type of cells by assessing the expression of lineage specific markers, capacity of tube formation and other specific characteristics of ECs (Choi et al., 2009a). However, the differentiation potential of iPSCs is still not yet fully characterized and it is not yet clear whether iPSCs undergo a series of cellular changes similar to hESCs following differentiation to specific lineages. iPSCs derived ECs exhibited a cobblestone-like appearance on culture dishes, were positive for eNOS, and showed to be positive for CD31 and VE-cadherin markers (Homma et al., 2010). Additionally, some hiPSC lines are capable of differentiation

into blood and endothelial cells with a differentiation pattern very similar to that observed with hESCs when co-cultured with mouse bone marrow stromal cell line OP9 (Choi et al., 2009b). Moreover, when applying the same differentiation method to iPSCs and hESCs, derived ECs showed a expression of endothelial-related genes like VE-cadherin, CD31, von Willebrand factor (vWF), and CD34 at levels similar to those seen in adult ECs (Taura et al., 2009).

1.7. Objectives

The main aim of our project comprised the generation of a tool of molecular and cellular biology that allows an efficient and specific high throughput screening method for endothelial differentiation. Additionally, other goal of this project embraced the characterization of possible means (nanoparticle internalization and culture in different substrates) to improve the efficiency of the endothelial differentiation process.

Chapter 2

Material and Methods

2. Material and Methods

2.1. Cell culture

2.1.1. iPS cell culture

The iPSCs were gently given by Ulrich Martin (reprogrammed from cord blood cells) (Haase, et al., 2009) These cells were cultured in a first phase in inactivated mouse embryonic fibroblasts (MEFs) and then cultured in a feeder-free system as described below.

The inactivation of MEFs was preceded by MEFs expansion where these cells were grown in Dulbecco's Modified Eagle's Medium (DMEM - Sigma) and 50% fetal bovine serum (FBS) (Invitrogen) and penstrep (Invitrogen). After 2 passages the MEFs were inactivated with 7mL of mitomycin C (8 microg/ mL Invitrogen) per T75 flask during 2 hours and 30 minutes.

The iPSCs were cultured in DMEM KO (Gibco), 20% KO serum replacement (Gibco), 5mL glutamine (Invitrogen), 1mL mercaptoethanol (Sigma), 250µL β-FGF (Prepotech), 5 mL non-essential aminoacids (Invitrogen) penstrep (Invitrogen) (iPS medium) when co-cultured with MEFs.

To transfer the iPSCs from the feeder system to a feeder-free system we used a magnetic assorted cell sorting (MACS) with CD326 antibodies (Milteny) (as schematized in

Figure 9). After detaching the iPSCs with trypsin (Invitrogen), the iPSCs were ressuspended in iPSCs medium with ROCK inhibitor (10µL/mL) to avoid apoptosis during the sorting process; the beads were added and mixed well. This mixture was incubated 15 minutes at 4ºC and washed with 2mL of iPS medium with ROCKi (10µL/mL). After washing the MACS column 3 times with 500µL of iPS medium the cell suspension was applied to the column and washed 3 times with 500µL of iPS medium. In the final step the iPSCs were collected in 1mL of iPS medium with ROCKi (Cayman) (10µL/mL) and plated in matrigel (BD) with iPS medium

previously conditioned by inactivated MEFs (supplemented with ROCKi 10µM and βFGF 10µM).

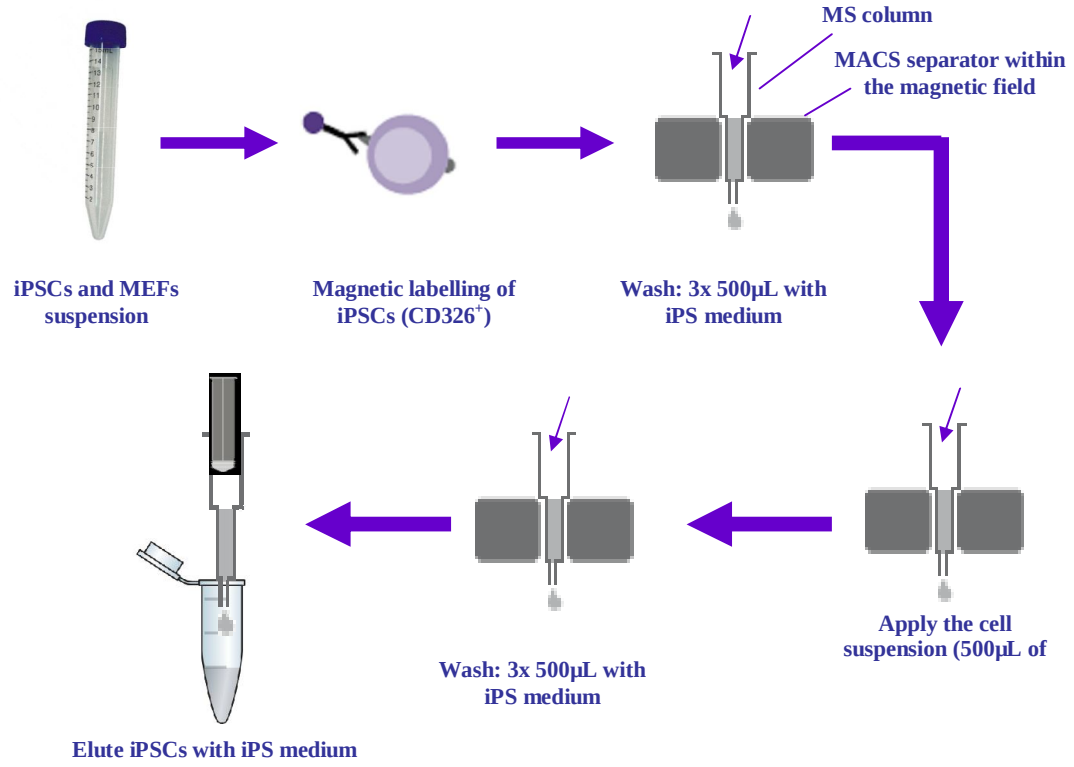


Figure 9 - Schematic representation of the MACS separation protocol (adapted from MACS handbook)

2.1.2. iPSCs characterization

2.1.2.1. Immunostaining

Immunofluorescent labelling of the iPSCs was performed to characterize the expression of pluripotency markers such SSEA-4, TRA-1-60 and TRA-1-80 antibodies (Cell Technologies). The cells were fixed with 4% paraformaldehyde (PFA) at room temperature during 15 minutes in glass coverslips coated with matrigel (BD), washed 1 time with phosphate buffered saline (PBS) followed by a blocking step with PBS with 5% bovine serum albumin (BSA - Sigma) and 2% FBS for 30 minutes at room temperature. The incubation with the

primary antibody (dilution factor 1:100) at room temperature during 60 minutes was followed by 3 washes with PBS and incubation with the secondary antibody (dilution factor 1:200) for other 60 minutes at room temperature. After the secondary antibody incubation the cells were washed 3 times with PBS, fixed with 4% PFA for 5 minutes at room temperature. The coverslips containing the cells were then stained with DAPI and mounted with mounting medium (Dako)

2.1.2.2. Nanoparticle internalization in iPSCs and adhesion to different substrates

After sterilization of the glass coverslips with ethanol 70% and UV light during 30 minutes the different coating protocols were performed (Table IITable II –). Some coatings were performed using a layer-by-layer method and in some cases the hyaluronic acid (HA) was integrated within other substrates (gelatine and matrigel).

Regarding the matrigel coating, the matrigel (BD) was thawed overnight at 4ºC and then diluted (1:50) in DMEM (Gibco) with ROCK inhibitor (Cayman 10µM). The coverslips were coated with matrigel solution during 30 minutes at 37ºC, and washed with PBS (Sigma).

Concerning the hyaluronic acid (HA) coating, 500µL of HA solution (5mg/mL in 0,15M NaCl 20mM HEPES buffer - pH 7,4) were added to each condition and incubated overnight at 37ºC. In some cases the HA was incorporated within other substrates (gelatine, matrigel) and not assembled by layer deposition. The excess of HA was aspirated and rinsed 1 time with PBS (Sigma)

Regarding the protamine sulfate (PS) coating, 500µL of PS solution (10mg/mL in PBS) were added to each condition and incubated during 20 min at 37ºC in order to make a layer-by-layer coating when other substrates were present. The excess of PS was aspirated and washed 1 time with PBS (Sigma).

Regarding the poly-dopamine coating , the coverslips were washed in 20 mL of methanol:water (1:1) for 10 minutes and then the coverslips were transferred to acetone for 10

minutes. The coverslips were dried and immersed into a dopamine.HCl solution (2mg/mL of 10mM Tris - pH 8,5) in a 24 well plate at 25ºC during 12-18 hours with agitation. The coverslips were then rinsed with deionised water and dried.

Regarding the methylcellulose coating, were added 500µL of a 5% solution of methylcellulose and incubated at 37ºC for 20 minutes. The excess of this solution was aspirated and rinsed 1 time with PBS (Sigma).

In the conditions with nanoparticles (NPs) adhered to the platforms a NP solution of 500µg/mL was used; 1mL of NP solution was added to the platform and centrifuged for 10 minutes at 4000rpm, followed by 2 washes with 500µL of PBS (Sigma) .In the conditions with nanoparticles in suspension a NP solution of 5mg/mL was used and 1mL of solution was added to each condition 2 hours after platting the cells.

Table II – Generation of different substrates for iPSCs culture

First layer substrate	**Second layer substrate**	**Nanoparticles**	
	Mixed substrate	**NP^+**	**NP^-**
Glass coverslips	None	----------	----------
	None	adhered	adhered
	Protamine Sulfate	adhered suspension	----------
	Protamine Sulfate Hyaluronic Acid	----------	adhered suspension
Matrigel	None	----------	----------
	None	adhered suspension	adhered suspension

	Hyaluronic Acid	adhered suspension	adhered suspension
Poly-dopamine	None	----------	----------
	None	adhered suspension	adhered suspension
	Hyaluronic Acid	----------	adhered suspension
	Protamine Sulfate	adhered suspension	----------
	Protamine Sulfate Hyaluronic Acid	----------	adhered suspension
Gelatine (1%) in PBS (pH=7,4) (Gelatin1)	None	----------	----------
	Protamine Sulfate	adhered suspension	----------
	Protamine Sulfate Hyaluronic Acid	----------	adhered suspension
Gelatine (1%) in NaCL HEPES buffer (pH=4,6) (Gelatin2)	None	----------	----------
	Hyaluronic Acid	----------	adhered suspension
	Hyaluronic Acid incorporated (inc)	----------	adhered suspension
Gelatine (1%) in PBS (pH=4,6) (Gelatin3)	None	----------	----------
	Hyaluronic Acid	----------	adhered suspension
	Hyaluronic Acid	----------	adhered

	incorporated (inc)		suspension
	Protamine Sulfate Hyaluronic Acid	----------	adhered suspension
Methylcelulose (5%)	Protamine Sulfate	adhered suspension	adhered suspension

2.1.3. HUVEC cell culture

The human umbilical vein endothelial cells (ATCC) were cultured in EGM_2 (Lonza).

2.1.3.1. HUVECs characterization

2.1.3.2. Immunostaining

Immunofluorescent labelling of HUVECs was performed to characterize the expression of endothelial characteristic markers such CD31 () and VE-cadherin (). The cells were fixed with 4% paraformaldehyde (PFA) at room temperature during 15 minutes in glass coverslips coated with gelatine (0,1%), washed 1 time with phosphate buffered saline (PBS) followed by a blocking step with PBS with 5% bovine serum albumin (BSA - Sigma) and 2% FBS for 30 minutes at room temperature. The incubation with the primary antibody (dilution factor 1:100 – VE-cadherin – Santa Cruz and 1:50 CD31 - Sigma) at room temperature during 60 minutes was followed by 3 washes with PBS and incubation with the secondary antibody (dilution factor 1:200) for other 60 minutes at room temperature. After the secondary antibody incubation the cells were washed 3 times with PBS, fixed with 4% PFA for 5 minutes at room temperature. The

coverslips containing the cells were then stained with DAPI and mounted with mounting medium (Dako).

2.1.3.3. Blasticidin selection

To perform the blasticidin selection cell culture medium was supplemented with different amounts (5µg/mL; 10µg/mL; 25µg/mL for HUVECs and 5µg/mL; 8µg/mL; 10µg/mL; 20µg/mL for iPSC) of blasticidin (Invitrogen).

2.1.4. 293T cell culture

The human embryonic kidney cell-line (293T - ATCC) was grown in DMEM (Gibco) with 10% fetal bovine serum (Sigma) and penicillin, streptomycin sulfate, and amphotericin B (Invitrogen).

2.2. Cloning experiments

To generate the construct of interest with a vascular endothelial cadherin promoter tagged with a fluorescent protein (mCherry) and with a blasticidin resistance gene we used three clones with the respective sequences in annex I.

2.2.1. Generation of the VE-cadherin promoter cherry lentiviral plasmid

The three clones listed below were used to generate the VE-cadherin promoter cherry fusion construct. The cloning strategy is described in detail below.

2.2.1.1. Plasmids used to create the final construct

- pCR TOPO II mcherry: 4071bp
- plenti6 ires egfp: 8274bp
- VPr-GFP construct: 8712bp

2.2.1.2. Transformation of DH5α and XL2 blue competent cells

To transform the competent cells (DH5α competent cells - Invitrogen or XL2 blue ultracompetent cells – Aggilient Technologies), with different target DNA sequences, the competent cells were thawed on ice and aliquoted into 50µL in a 1,5mL microcentrifuge tube (on wet ice). The DNA of interest was added to the cells (1µL- approximately 1ng) and incubated on ice during 30 minutes. The cells were heat shocked for 40 seconds at 42ºC and placed on ice during 2 minutes. For the recovery of the competent cells 450µL of Luria Bertani (LB) medium was added to each tube and incubated at 37ºC for 1 hour. The cell suspension was then plated on ampicilin selection plates and incubated at 37ºC overnight.

2.2.1.3. Restriction cloning strategy

2.2.1.3.1. First step of the cloning process

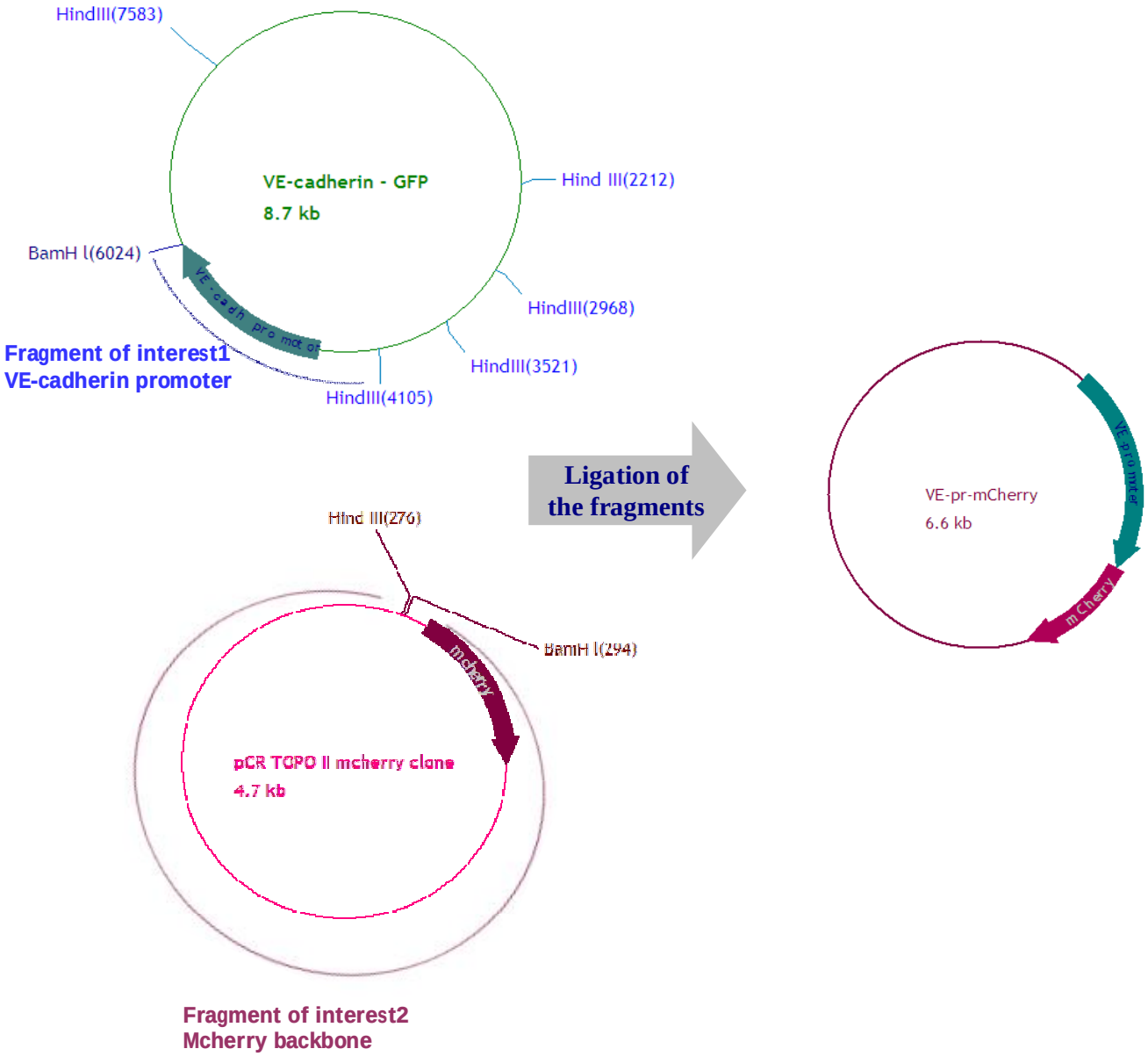


Figure 10 – Schematic representation of the first cloning step**.**

2.2.1.3.1.1 Confirmation of the orientation of the fragment of interest (VE-promoter)

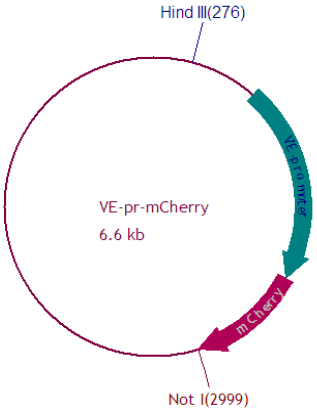


Figure 11 – Schematic representation of the strategy used to confirm the integration of the VE-promoter.

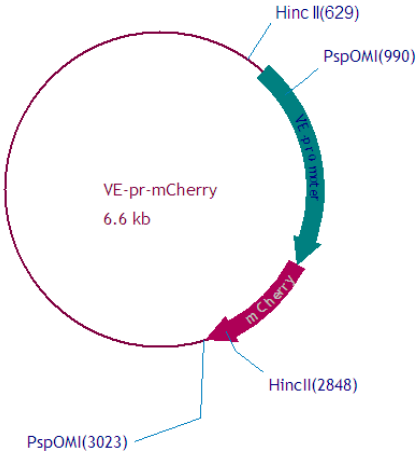


Figure 12 – Schematic representation of the strategy used to confirm the orientation of the integration of the VE-promoter and mCherry.

2.2.1.3.1.2 Second step of the cloning process

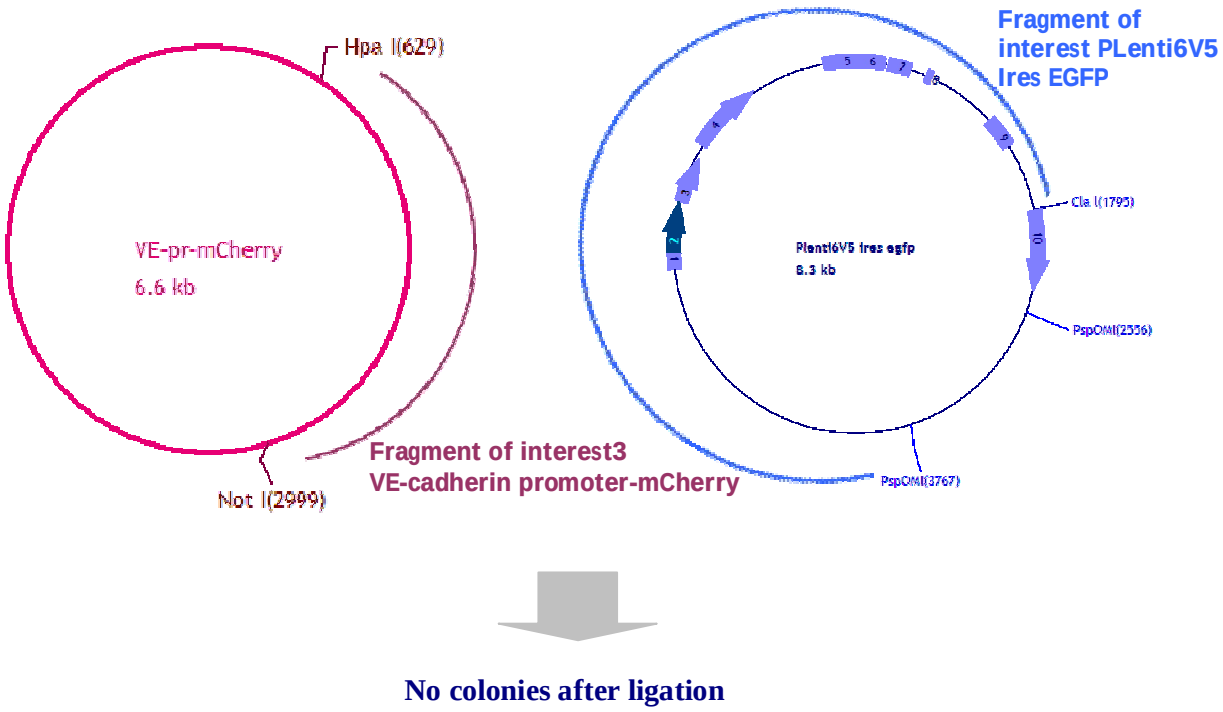


Figure 13 – Schematic representation of the second cloning step (first strategy)**.**

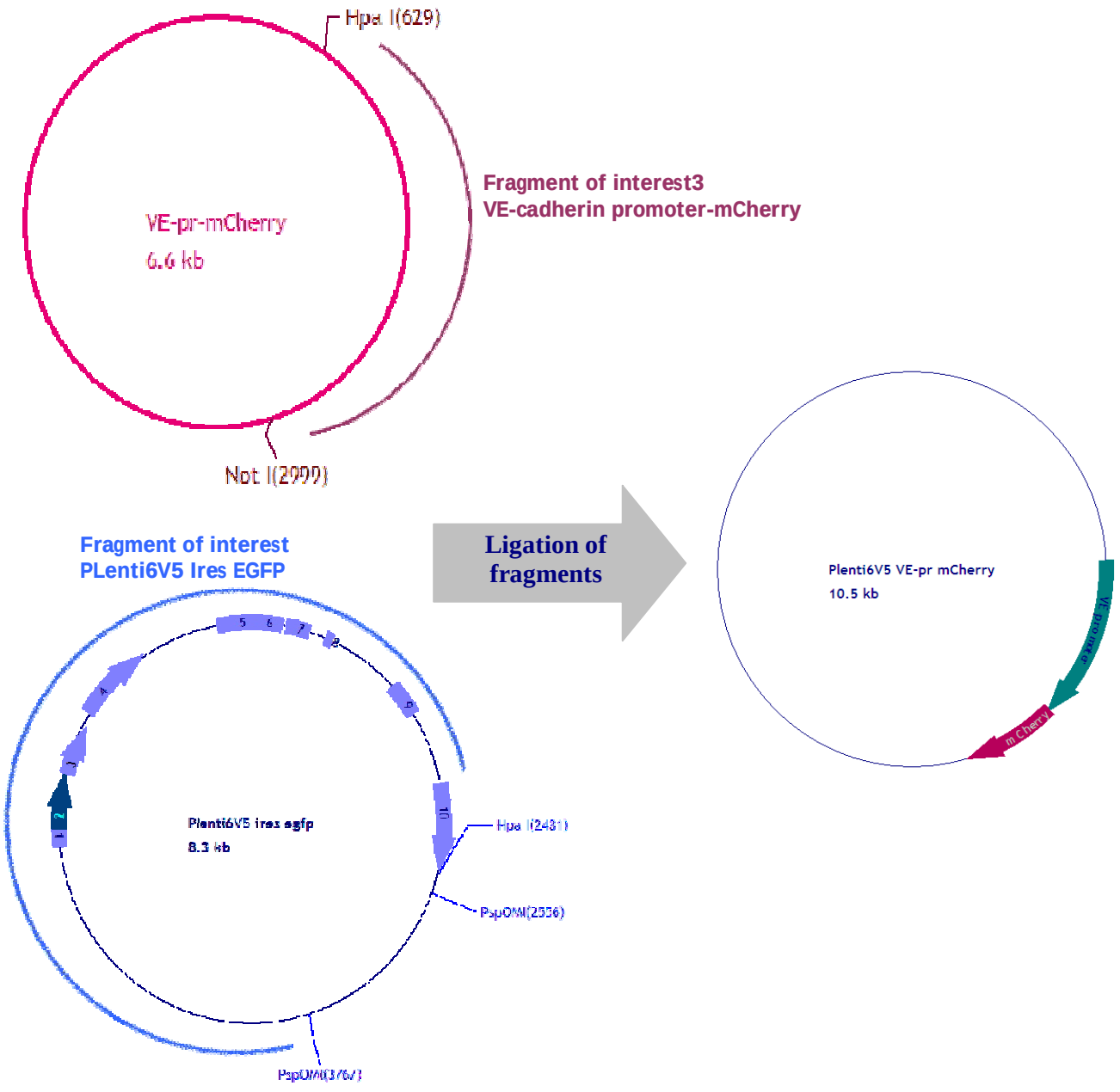


Figure 14 - Schematic representation of the second cloning step (definitive strategy)

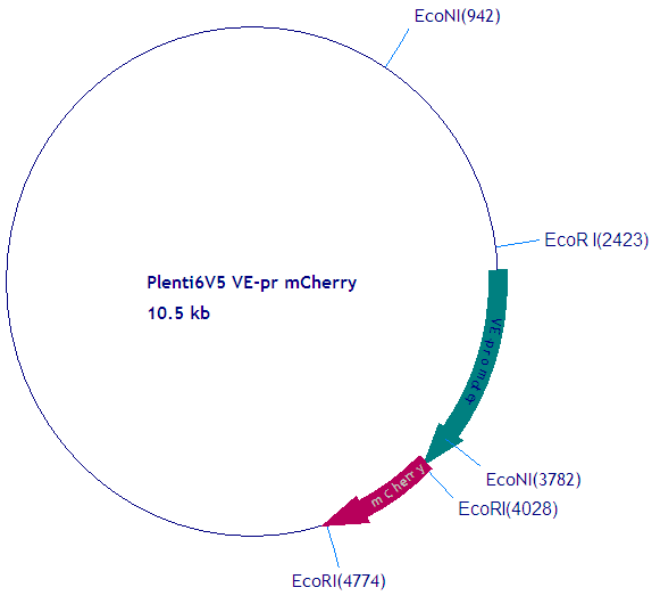


Figure 16 – Schematic representation of the strategy used to confirm the integration of the VE-promoter and mCherry fragments.

2.2.1.3.2. Restriction enzyme assays

The restriction enzyme reactions were performed in the buffers suggested by the company (New England Biolabs) for each enzyme used as shown in Table III.

Table III – Conditions used to the different restriction digestions.

Enzyme	**NEB buffer**	**Temperature of reaction**	**Time of reaction**
HindIII	Buffer 2	37ºC	4 hours
BamHI	Bam/Sal buffer	37ºC	4 hours
NotI	Buffer 2	37ºC	Overnight
EcoRI	Ssp buffer	37ºC	3 hours
EcoNI	Buffer 4	37ºC	3 hours
HpaI	Buffer 4	37ºC	4 hours
ClaI	Buffer 4	37ºC	4 hours
PspOMI	Buffer 4	37ºC	4 hours
T4 DNA Ligase	T4 DNA ligase	Room temperature	1 hour

To blunt cohesive ends we used Klenow large fragment polimerase in 120µL reactions with 10µL dNTPs (10mM) and 1µL of klenow large fragment.

2.2.1.4. Desalting the DNA digest

The QIAEX II Gel extraction kit from QIAGEN was used to desalt the DNA digest products from one restriction digestion so that a different restriction enzyme and buffer could be used in the next restriction steps. According to the size of the DNA fragments, 3 volumes of solution of buffer QX1 (solubilisation and binding buffer) (≤4 kb) or 3 volumes of buffer QX1 plus 2 volumes of H2O (4–10 kb) were added to the DNA digestion products. After ressuspending the silica particles (QiAEXII), by vortexing, 10µL of QIAEX II were incubated at room temperature 10 minutes with the previous mixture. This suspension was then centrifuged 30 seconds at 13000rpm and the pellet washed twice with 500µL of buffer PE (wash buffer). The pellet was then air dried for 10-15 minutes. The DNA elution was done in 20 µL of H2O by incubation during 5 minutes at room temperature (for DNA fragment ≤4 kb) or 50ºC (for DNA fragments with 4–10 kb), followed by a centrifugation of 30 seconds at 13000rpm and posterior recovery of the supernatant into a clean tube. An aliquot of the final DNA solution was run in a 1,2% agarose gel to ensure that the DNA was properly purified. The schematic version of the protocol is presented in Figure 17.

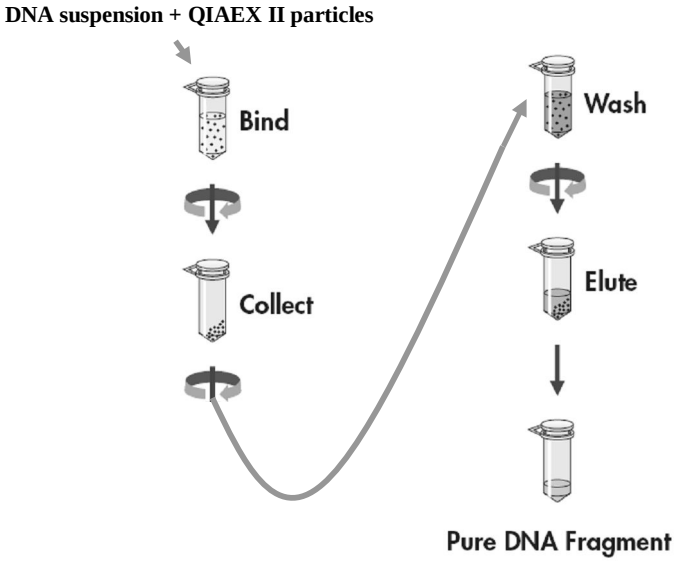


Figure 17 - Schematic representation of the strategy used to purify DNA digests (adapted from QIAEX II Gel extraction kit QIAGEN handbook)

2.2.1.5. DNA purification from starter cultures

To start a maxiculture a single colony was picked and incubated in 2mL of LB medium with ampicilin overnight at 37ºC with an agitation of 220rpm.

To purify DNA from bacterial starter cultures the QIAGEN QIA mini spin kit was used. After an overnight incubation, 1,5mL of the bacterial cultures were centrifuged (1,5mL microcentrifuge tube) at 13000 for 1 minute. The pellets were ressuspend in 250µL of buffer P1 (ressuspension buffer) by vortexing and 250µL of buffer P2 (lysis buffer) were added and mixed. After 5 minutes of incubation 350µL of buffer N3 (neutralization buffer) were added and mixed by inverting the tube. This suspension was centrifuged during 10 minutes at 13000rpm and the supernatant was transferred to a QIAprep spin column. This suspension was centrifuged during 1 minute at 13000rpm and the flow-through was discarded. The QIAprep spin column was washed by centrifugation (1minute 13000rpm) with 500µL buffer PB (wash buffer) and the flow-through was discarded. The QIAprep spin column was washed by centrifugation (1minute 13000rpm) with 750µL buffer PE (wash buffer). Another centrifugation was done (1 minute at 13000rpm) to eliminate residues of buffer PE. The QIAprep spin column was placed in a

microcentrifuge tube and 50µL of buffer EB (elution buffer) were added to the column. After 1 minute of incubation at room temperature this suspension was centrifuged for 1 minute at 13000rpm to elute the DNA. The schematic version of the protocol is presented in Figure 18.

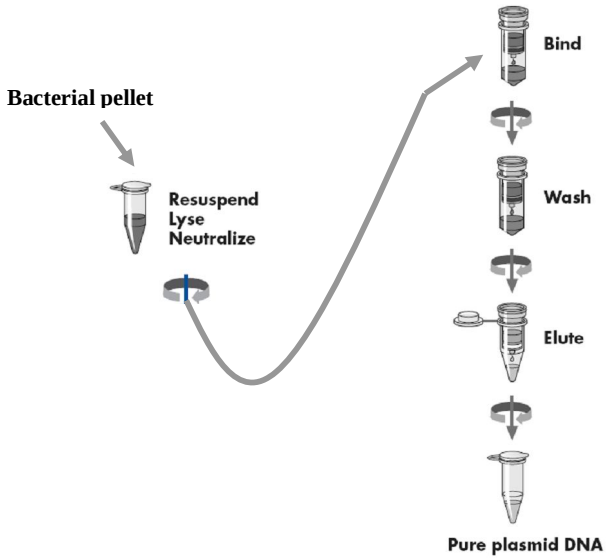


Figure 18 - Schematic representation of the strategy used to purify DNA from bacterial starter culture (adapted from QIA mini spin kit QIAGEN handbook)

2.2.1.6. DNA purification from maxicultures

After selecting the starter cultures of interest, bacterial maxicultures were established by incubating of 500µL from the starter culture into 100mL of LB medium containing ampicilin and incubated at 37ºC overnight with a rotation speed of 220rpm. The DNA purification from bacterial maxicultures was done with QIAGEN HiSpeed Plasmid Midi Kit from QIAGEN. The bacterial cells were then harvested by centrifugation (4000rpm 5 minutes; 4ºC) and the bacterial pellet was ressuspend in 6mL of Buffer P1 (ressuspention buffer). The ressuspension step was followed by the addition of 6mL of buffer P2 (lysis buffer - mix by inverting 4-6 times) and

incubation for 5 minutes at room temperature. The inactivation of the bacterial lysis was achieved by addition and mix of 6mL of chilled buffer P3 (neutralization buffer). The bacterial lysate was then poured into the barrel of the screwed QIAfilter Cartridge and incubated during 15-20 minutes at room temperature. The bacterial lysate was filtrated to a previously equilibrated (4 mL of buffer QBT) Hispeed Tip and the column was emptied by gravity flow. The column was washed with 20mL of buffer QC (wash buffer). To elute the DNA 5mL of buffer QF were pippeted into the column and the eluate was collected into a 15mL falcon. The DNA was precipitated by addition of 0,7 volumes of isopropanol. The eluate/isopropanol mixture was filtered with a 20mL syringe attached to a QIA precipitator and washed with 2mL of 70% of ethanol. The DNA collection was done in buffer TE. The simplified version of the protocol is presented in Figure 19.

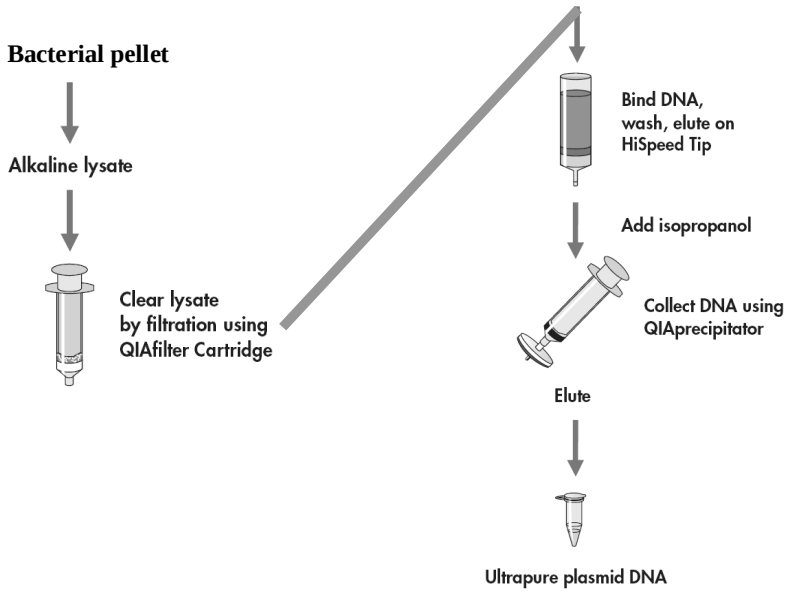


Figure 19 - Schematic representation of the strategy used to purify DNA from bacterial maxiculture (adapted from HiSpeed Plasmid Midi Kit QIAGEN handbook).

2.2.1.7. DNA quantification

The DNA quantification was done with nanodrop according to the manufacture good practices.

2.2.1.8. DNA gel extraction

For DNA gel extraction, after cutting the DNA band of interest from the agarose gel with a clean blade the QIAEX II Gel Extraction Kit from QIAGEN was used. The protocol used is the same described above and schematized in Figure 17 with an incubation of 10 minutes at 50ºC (solubilise of agarose) preceding the first step and an additional wash with buffer QX1 (to remove traces of agarose) after the first centrifugation.

2.2.1.9. Genomic DNA extraction

To extract genomic DNA from HUVECs and iPSCs the Flexigene DNA kit from QIAGEN was used. The number of cells never exceeded $2x10^6$ per reaction. After collecting the cells, the cells were spined down for 5 minutes at 1200rpm. The cell pellets were ressuspended in 300µL of buffer FG1 (lysis buffer) and 300µL of buffer FG2 (denaturation buffer) with QIAGEN protease (3µL per 300µL of buffer FG2) was added after homogeneous ressuspension. The solution was mixed by inverting the tube 3 times and 10 minutes incubation at 65ºC was done. After this incubation period, 600µL of isopropanol were used to generate DNA visible precipitates. The DNA precipitates was then centrifuged at 13.000rpm for 3 minutes and washed with 70% ethanol. The clean DNA precipitate was then air dried, ressuspended in buffer FG3 (hydration buffer) and dissolved during 30 minute at 65ºC.(Figure 20)

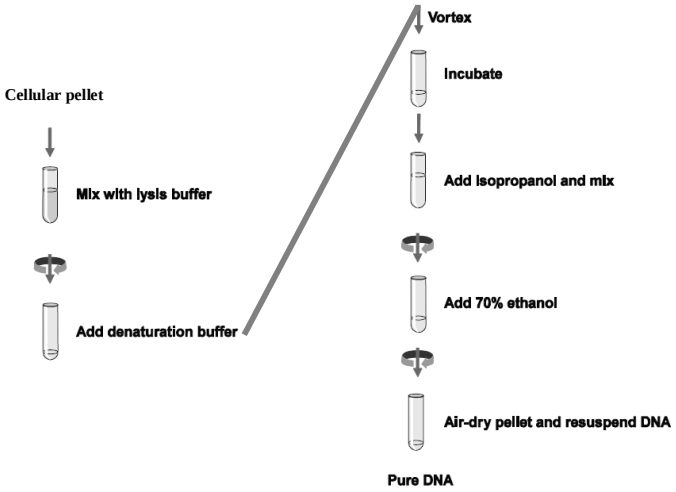


Figure 20 - Schematic representation of the strategy used to purify DNA from bacterial maxiculture (adapted from Flexigene DNA kit QIAGEN handbook).

2.2.2. Viral work

Lentiviruses are vectors based on the life cycle of retroviruses, as HIV, and for safety reasons a great majority of the viral proteins are not present (as tat protein).

In this work HIV-1 based pantropic lentivirus and a third generation packaging were used. In this type of packaging systems the cis-acting viral elements are inserted within the construct of interest and the transacting elements are within three individual packaging plasmids. This lentiviral packaging system comprises a plasmid for gag, that codes for the virion main structural proteins and pol, coding for the retrovirus-specific enzymes (pMDLg/pRRE). In a separate packaging plasmid there is the rev element, which is responsible for expression of a post-transcriptional regulator necessary for efficient gag and pol expression (pRSVrev). The third packaging plasmid is responsible for the formation of viral envelope (pMD2-G/VSV-G) (Spirin, Vilgelm & Prassolov, 2008; Dull et al., 1998).

The lentiviral genome consists of two identical single stranded RNAs, which are packaged in a virus capsid. The capsid includes the structural proteins encoded by gag and the products of pol, virus-specific reverse transcriptase, protease, and integrase (Figure 21).

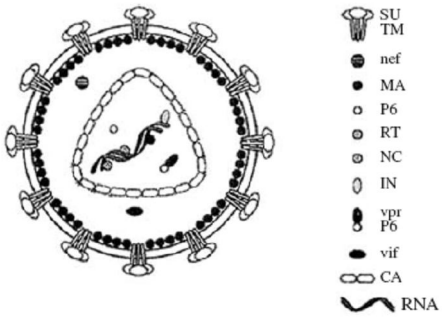


Figure 21 - Scheme of a virus particle (**Dull et al., 1998**)

2.2.2.1. Viral packaging

To achieve the viral packaging of the generated construct (named PLenti-VEpr-Cherry), pMDLg/pRRE, pRSVrev and pMD2-G (VSV-G) packaging plasmids were used (Figure 22).

The viral packaging was performed in 293T (ATCC) with the respective amounts of transfection agent (FuGene - Roche) and plasmids in the Table IV.The FuGene was mixed with DMEM without serum and incubated at room temperature for 10 minutes. After the incubation period plasmids were added and maintained at room temperature for 40 minutes. Lastly, this mixture was added to a 70% 293T culture flask (T75). The 293T medium was changed by fresh 293T medium after 16 h of incubation (at 37ºC).

Table IV – Conditions used to perform the viral packaging.

Component	**Amount (per T75 flask)**
FuGene	23,1 µL
pMDLg/pRRE	1,3 µg
pRSVrev	1,3 µg
pMD2-G (VSV-G)	1,3 µg
PLenti-VEpr-Cherry	1,95 µg

2.2.2.2. Concentrating the virus

Three days after the transfection of the 293T described in Table IV the viral supernatants were collected into 50mL tubes and spin down at 1500rpm during 5 minutes to pellet the cellular debris. After this centrifugation the supernatants were filtered (0,4µM filter – Appleton Woods) into Oak Ridge centrifuge tubes (Nalgene) and centrifuged at 22000rpm for 4 hours. The pellets were then ressuspended in 200µL of PBS and stored at -80ºC.

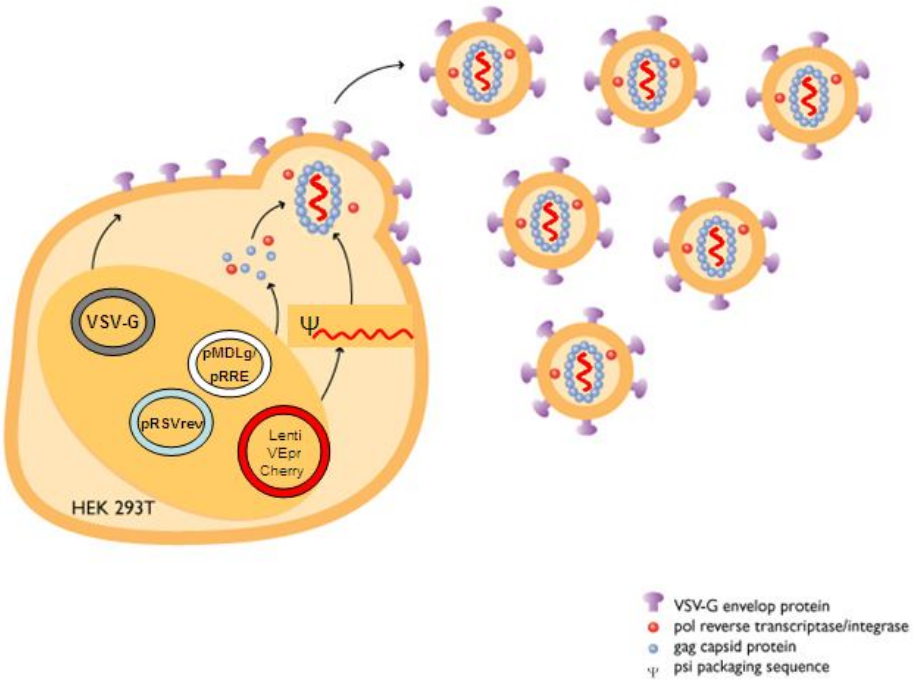


Figure 22 – Schematic representation of the viral packaging strategy (adapted from http://www.invivogen.com/docs/Insight_201004.pdf)

2.2.2.3. Viral titration

In order to titrate the virus, HUVECs were infected with different amounts of viral particles and 5 days after the infection the fluorescent signal in the HUVECs was quantified by fluorescent cell cytometry (Gallios -Beckman Coulter).

2.2.2.4. Viral infection

The viral infections (Figure 23) were performed either in suspension (for iPS) or with cells in adherent culture (for iPS and HUVECs). The incubation with the viral particles was done in normal medium used to grow the specific type of cell and during approximately 16-18 hours. After this period the cells were rinsed 1 time with PBS and the culture medium was changed with new medium.

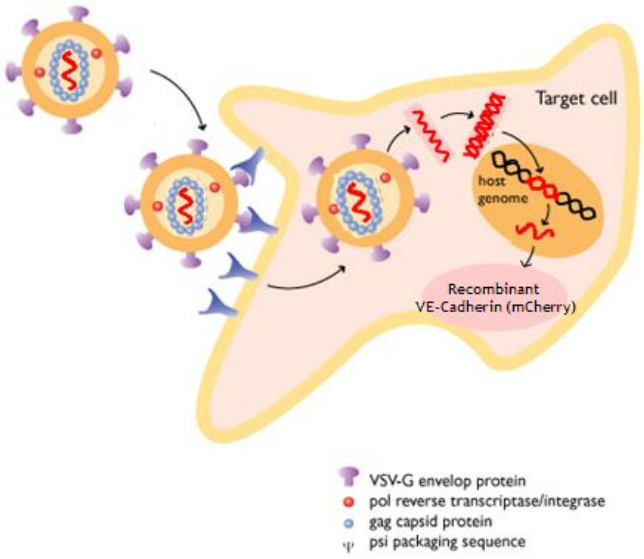


Figure 23 – Schematic representation of the viral infection strategy (adapted from http://www.invivogen.com/docs/Insight_201004.pdf).

After infecting the cells (HUVECs and iPSCs) the viral integration was accessed by polymerase chain reaction (PCR) with specific primers (as showed in Table V) to the mCherry fragment. After adjusting the PCR conditions the amplification of the fragment of interest was observed (Figure 60).

Table V – Conditions used to perform the PCR.

	Amount (μL) per PCR reaction
Hot start Taq	0,5
PCR buffer 10x	1,5
dNTPs	1,2
Primers (forward and reverse)	3
H_2O	8,8
DNA	1 (dilution factor 1:10)
PCR cycle conditions	
1 hold → 95ºC 15 minutes 30 cycles → 94ºC 20 seconds + 62,5ºC 20 seconds + 72ºC 20 seconds 2 holds → 72,0ºC	

Chapter 3

Results and Discussion

3. Results and discussion

3.1. Cell culture

3.1.1. Characterization of iPSCs

After establishing a feeder free iPS culture with selection of $CD326^+$ cells and culture in matrigel with conditioned medium, the iPSCs tend to acquire a different morphology (Figure 24) compared with the iPSCs cultured with MEFs (Figure 25). The MEFs secrete important growth factors that maintain the iPSCs in a pluripotent state and when plated without MEFs the iPSCs present a different morphology already described and must be cultured with iPS medium conditioned by MEFs (Wagner & Welch, 2010).

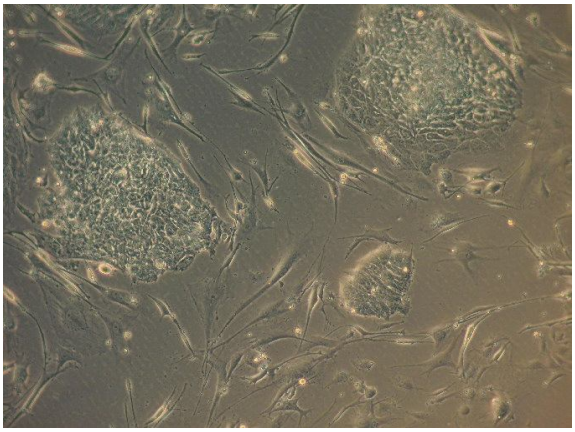


Figure 24- iPSCs cultured in MEFs.

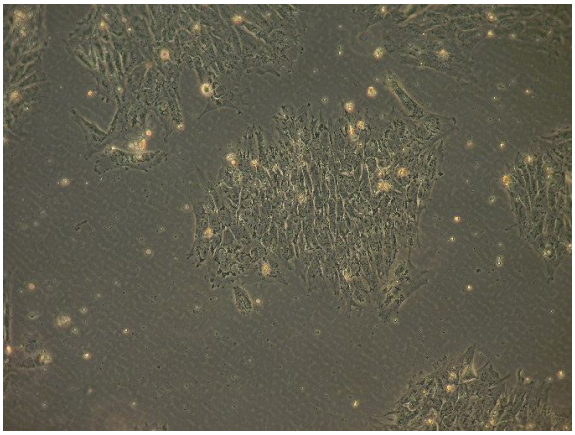


Figure 25- iPSCs cultured in matrigel in feeder-free conditions.

The characterization of iPSCs revealed that these cells express pluripotency markers (ref) like TRA1-60 (Figure 26), SSEA-4 (Figure 27), and TRA1-80 (Figure 28) and do not express endothelial markers like CD31 and vascular endothelial cadherin.

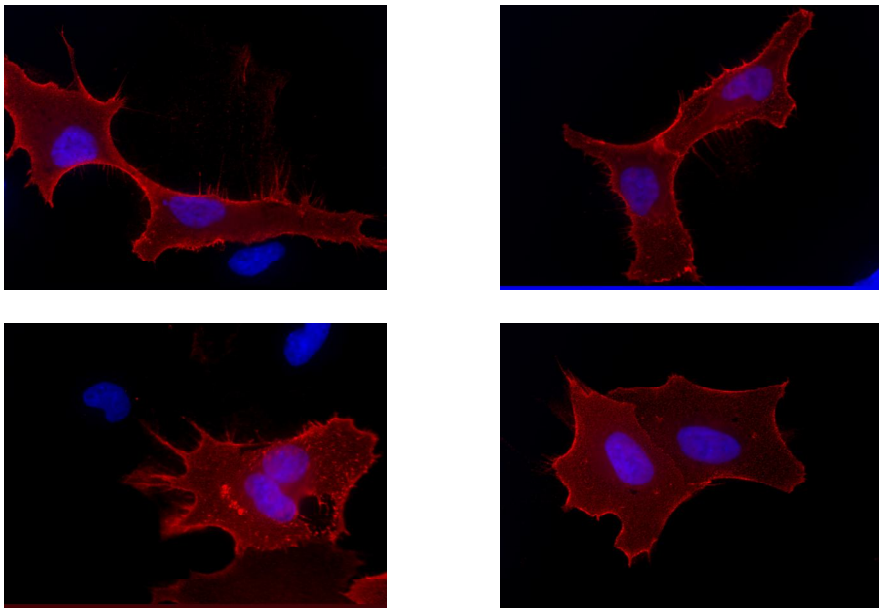


Figure 26- iPSCs immunolabeling with TRA-1-60.

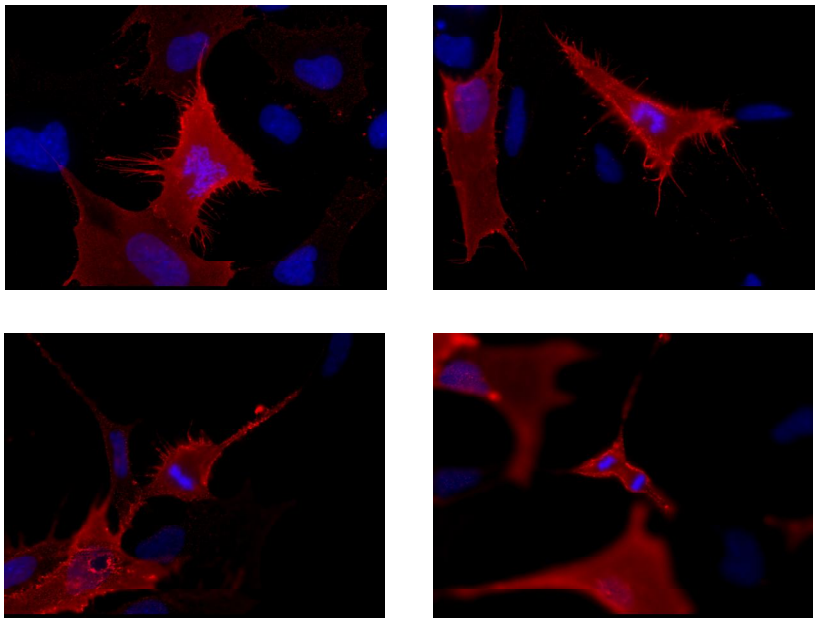


Figure 27- iPSCs immunolabeling with SSEA-4

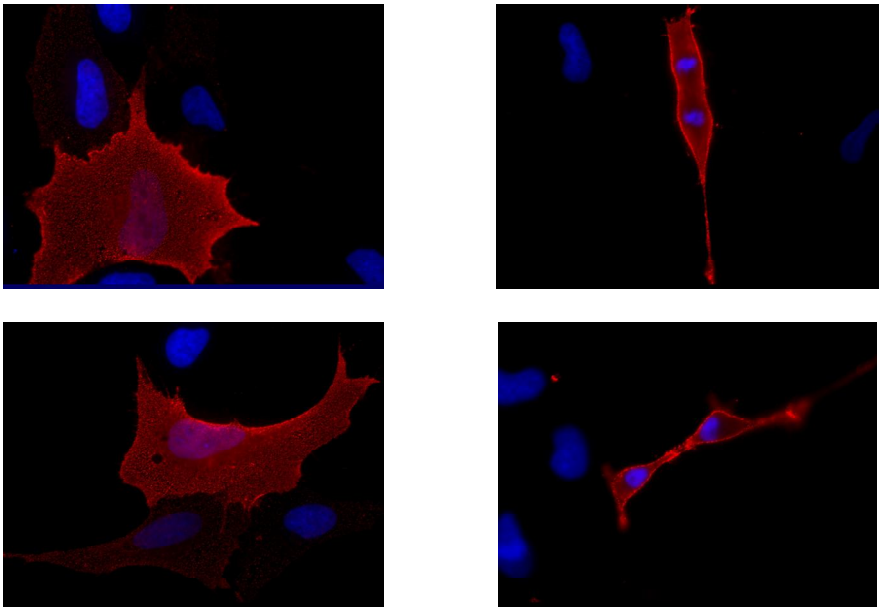


Figure 28- iPSCs immunolabeling with TRA-1-80.

3.1.1.1. Nanoparticle internalization by iPSCs

In terms of nanoparticle internalization these cells are able to internalize either organic nanoparticles (with positive - NP^+ or negative - NP^- surfaces) that are adhered to different substrates or nanoparticles in suspension. These nanoparticles can be modified with different cargo, therefore these nanocarriers can also be used in future work to deliver differentiation factors to iPSCs at different time points of the differentiation process. These internalization tests were done with several substrates to study both the adhesion and proliferation (Table VI) of the cells but also to try to assess the "tug-of-war" established between the cells and the different substrates for nanoparticle capture. A right balance of electrostatic interactions between nanoparticles and the substrate has to be found that enables the internalization of these nanoparticles by the cells. In the future these platforms could be tested to differentiate iPSCs into endothelial cells, combining the ability to promote cell adhesion, nanoparticle adhesion and incorporation of endothelial driving factors (hyaluronic acid and others.)

The preliminary results regarding these experiments show really different results regarding cell adhesion and proliferation and the platforms that provide a better result within these parameters are highlighted in Table VI. Although these results can not be used to generate statistical significant data there are some trends that seem to be patent. The presence of hyaluronic acid in the surface that contacts with cells seems to decrease the adhesion of cells (Table VI, Figure 31 and Figure 33). And in some way the nanoparticles seem to in some cases increase the adhesion of cells but in other situations appear to have the opposite effect (Table VI).

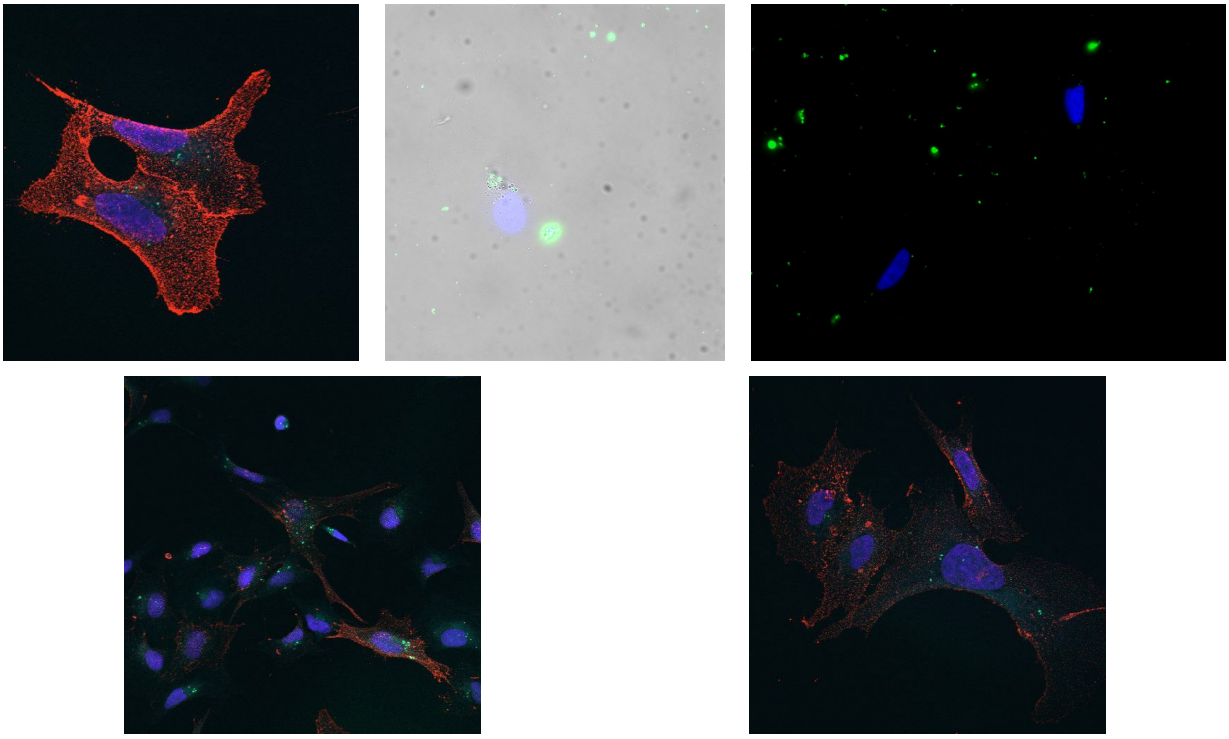


Figure 29 - iPSCs immunolabeling with SSEA-4 (red labelling), and NP^{+} pattern (green fluorescence) plated in a matrigel with NP^{+} adhered substrate.

Table VI – Number of cells per substrate after different substrate

Conditions	1 h	2 h	4 h	6 h	24 h	48 h	72 h	144 h	168 h
coverslip	2	2	3	2	2	0	0	0	0
coverslip+NP^+	0	1	2	2	0	0	0	0	0
coverslip+NP^-	2	3	3	4	0	0	0	0	0
coverslip+PS+NP^-	1	1	1	1	3	0	0	3	0
coverslip+PS+HA	0	2	1	1	0	0	0	0	0
coverslip+HA	0	1	1	1	1	0	0	1	0
coverslip+PS+HA+NP^+	0	2	1	1	0	0	0	0	0
coverslip+HA+NP^+	0	0	0	0	0	0	0	0	0
coverslip+matrigel	6	5	5	7	8	10	11	15	29
coverslip+matrigel+NP^+	4	4	8	6	8	2	1	0	0
coverslip+matrigel+NP^-	2	3	5	5	6	4	8	15	23
Poly-Dopa	7	8	7	6	6	5	22	30	104
Poly-Dopa+NP^+	4	4	6	5	4	0	0	0	0
Poly-Dopa+NP^-	5	6	6	10	13	10	12	83	187
Poly-Dopa+HA+NP^-PS	0	0	0	0	0	0	0	0	0
Poly-Dopa+HA	0	3	3	5	6	1	0	0	0
Gelatin1	4	8	5	7	4	3	4	2	2
Gelatin1+PS	2	8	7	5	2	0	0	0	0
Gelatin1+PS+NP^-PS	5	8	9	4	7	1	1	1	0
Gelatin1+PS+HÁ	0	0	0	0	0	0	0	0	0
Gelatin1+PS+HÁ+NP^-PS	0	0	0	0	0	0	0	0	0
Gelatin2	4	4	4	4	4	6	15	79	171
Gelatin2+HA	6	8	6	10	8	5	17	24	54
Gelatin2+HA+NP^-PS	4	3	5	5	4	4	9	65	75
Gelatin2 HA inc	3	5	6	5	5	4	7	11	0
Gelatin2+HA inc+NP^+	2	6	4	7	1	0	2	7	7
Matri HA inc	7	7	11	10	12	35	53	143	198
Matri HA inc +NP^+	2	3	4	6	9	10	14	92	179
Gelatin3	7	10	9	11	6	8	17	41	62
Gelatin3+HA	5	6	10	10	11	13	10	15	128
Gelatin3+HA+NP^-PS	4	6	7	6	6	4	3	11	29
Gelatin3 HA inc	9	8	7	6	11	14	12	24	20
Gelatin3 HA inc+NP^-PS	3	8	7	7	4	12	10	57	102
Poly-Dopa+PS+HA	0	3	2	2	1	0	0	0	0
Poly-Dopa+PS+HA+NP^+	0	5	12	15	16	12	11	131	176
Poly-Dopa+PS	4	6	5	3	2	2	0	0	0
Poly-Dopa+PS+NP^-	5	6	11	10	9	20	36	102	213
Methylc+PS+NP^-	1	3	3	3	1	1	1	1	0
Methylc+PS	1	2	3	3	1	0	0	0	0

The figures presented (Figure 29, Figure 30, Figure 31, Figure 32, Figure 33) show that iPSCs are able to internalize the two sets of nanoparticles associated with the different

substrates. Future work will be performed to further clarify the interaction of the different nanoparticles with the different substrates and to generate quantitative and statistical significant data.

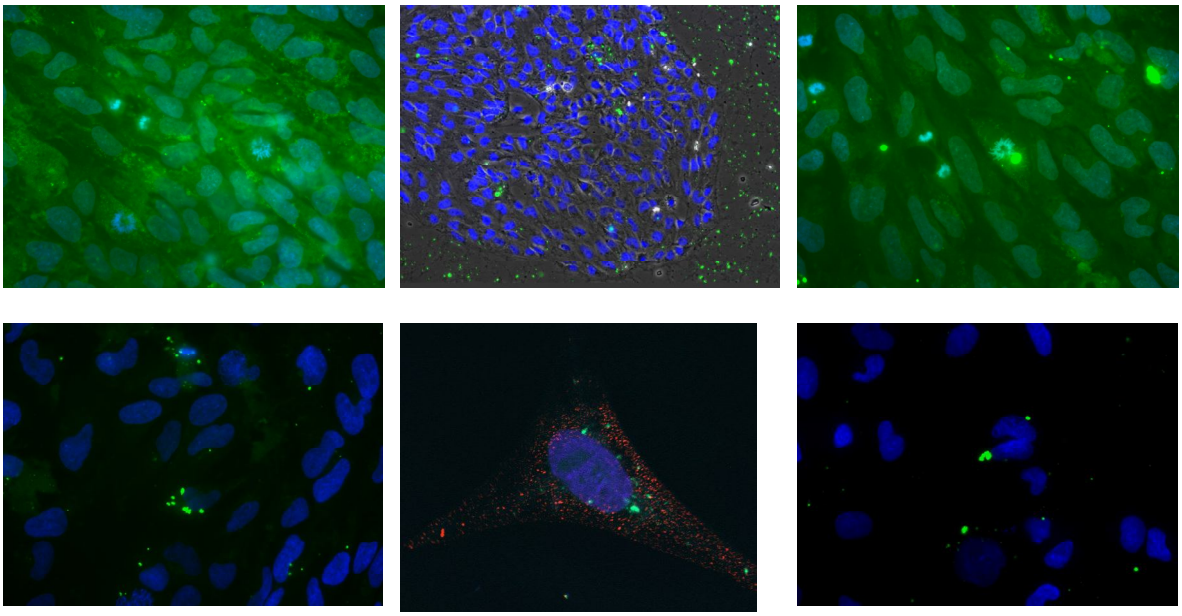


Figure 30 - iPSCs cultured in a matrigel with HA incorporated substrate with adhered NP^+; NP^+ pattern (green fluorescence); red labelling (imunostaining SSEA-4).

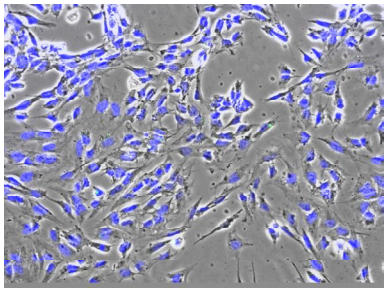


Figure 31 - iPSCs cultured in Poly-dopamine, PS, HA, NP^+substrate; NP^+ pattern (green fluorescence).

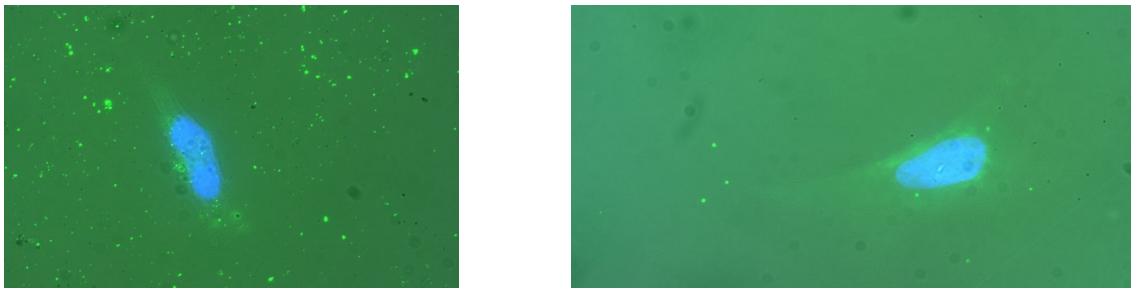


Figure 32 - iPSCs cultured in Poly-dopamine NP^+ (green fluorescence left image) substrate ; Poly-dopamine NP^- (green fluorescence left image)substrate.

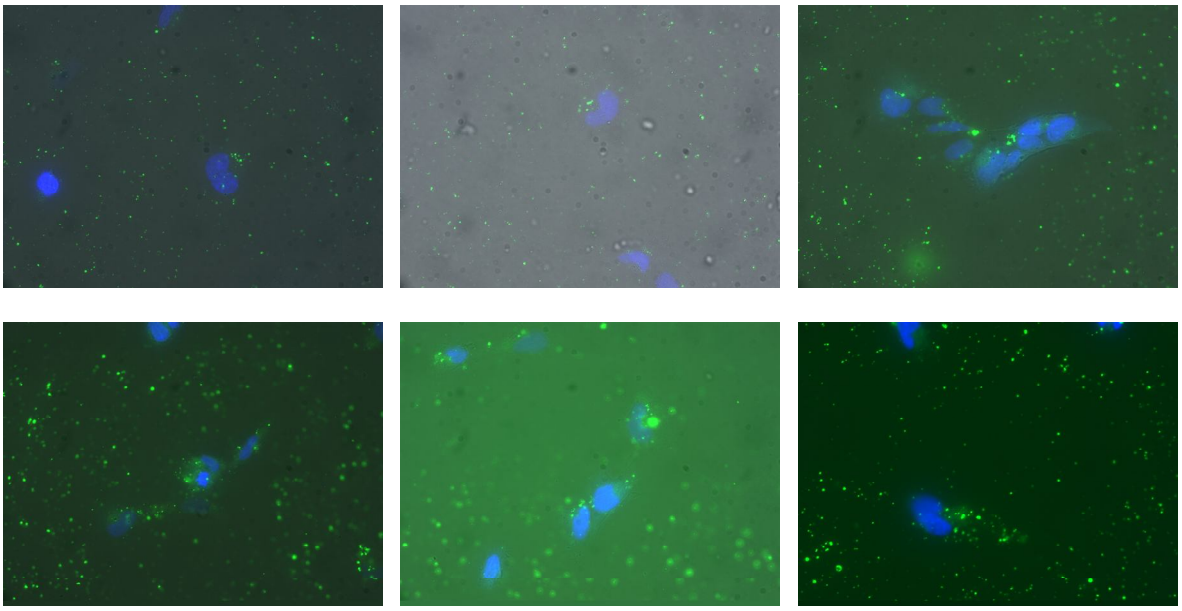


Figure 33 - iPSCs cultured in Gelatine 2 and HA substrate with NP^+ (green fluorescence left image)

3.1.2. Characterization of HUVECs

HUVECs showed regular expression of endothelial markers such as CD31 and VE-cadherin (Figure 34).

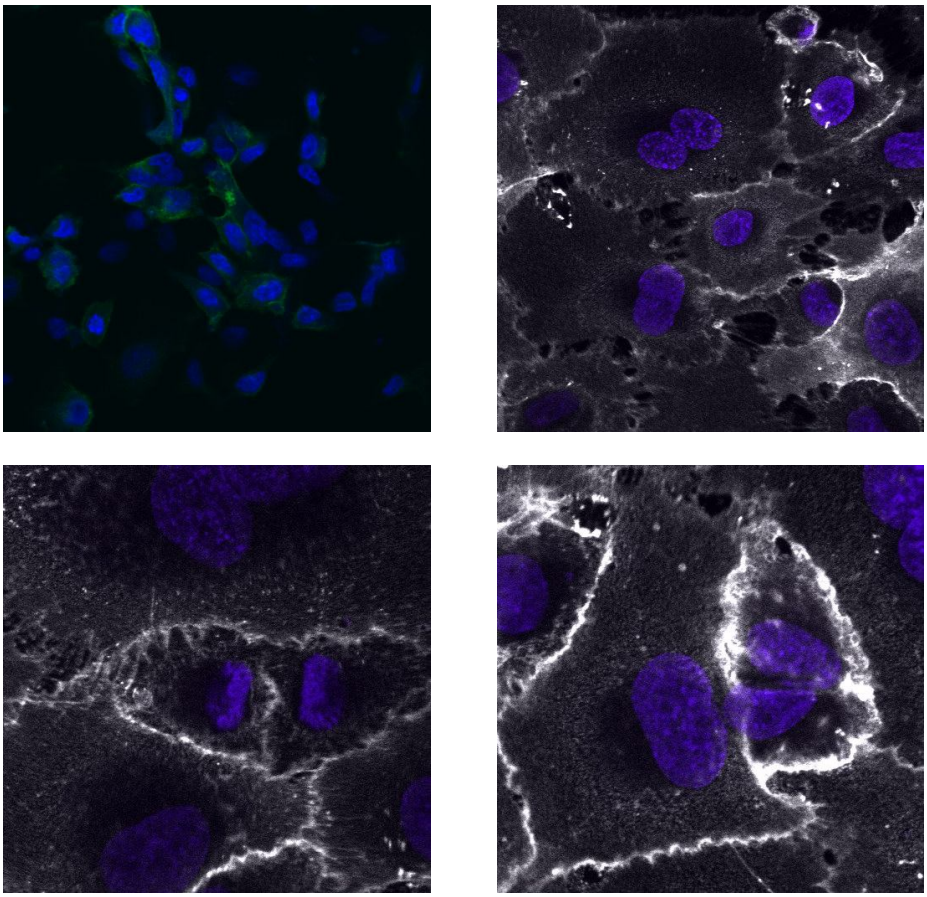


Figure 34- Imunolabeling of HUVECs with CD31 (white labelling) and VE-cadherin (green labelling)

3.2. Cloning experiments

3.2.1. Generation of the VE-cadherin promoter cherry lentiviral vector

In order to obtain the fragments of interest from the VEpr-GFP and mCherry clones illustrated on Figure 10 a restriction strategy that included HindIII and BamHI restriction enzymes was used. After transformation of competent cells, DNA amount was quantified (Table VII) in each clone and digestions were performed as briefly described below.

Table VII – DNA amount correspondent to each clone used

Vector/clone	DNA amount (ng/µL)
VEpr-GFP	444,2
Pcr-mCherry	576,3
PLenti6V5	280

The digestion of the 2 clones with Hind III was done during according to Table VIII.

Table VIII – Conditions used for HindIII digestion of VEpr-GFP and Pcr mCherry clones

Vector/clone	Water (µL)	DNA (µL)	NEB buffer 2 10x (µL)	HindIII (µL)
VEpr-GFP (insert)	77,74	11,26	10	1
Pcr-mCherry (vector1)	80,23	8,68	10	1

Figure 35 shows the digestion products resulting from the digestion of HindIII. To avoid alteration of BamHI activity in Hind III buffer (NEB buffer 2), after HindIII digestion the DNA products were purified (QIAEX II kit from QIAGEM) and then the BamHI digestion was performed as shown in Table IX.

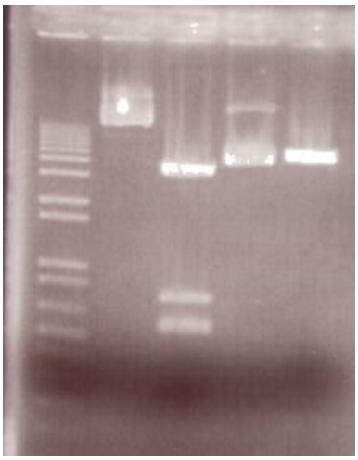


Figure 35 - HindIII digestion products. 1kb plus ladder; VEpr-GFP uncuted; VEpr-GFP after HindIII digestion; Pcr mCherry uncuted; Pcr mCherry after HindIII digestion (from left side to right side lanes)

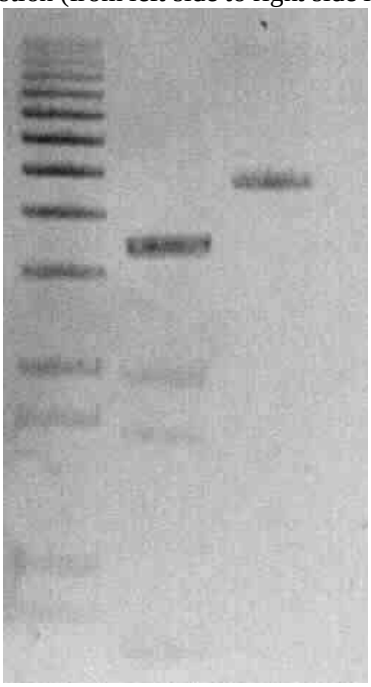


Figure 36- HindIII + BamHI digestion products. 1kb plus ladder; VEpr-GFP cuted; Pcr mCherry cutted (from left side to right side lanes)

Table IX - Conditions used for BamHI digestion of VEpr-GFP and Pcr mCherry clones

Vector/clone	Water (µL)	DNA (µL) after cutting with Hind II and desalted	Bam/Sal NEB buffer 10x (µL)	BamHI (µL)
VEpr-GFP	49	40	10	1
Pcr-mCherry	49	40	10	1

After BamHI digestion (Figure 36) in the case of the mCherry clone a dephosphorilation step (with AP – calph intestitinal phosphatase NEB) was done to avoid the re-annealing of the mCherry backbone.

Both digestion products were then gel purified and ligated. The ligation products (Figure 37) after transformation into competent cells did not originate any bacterial colony.

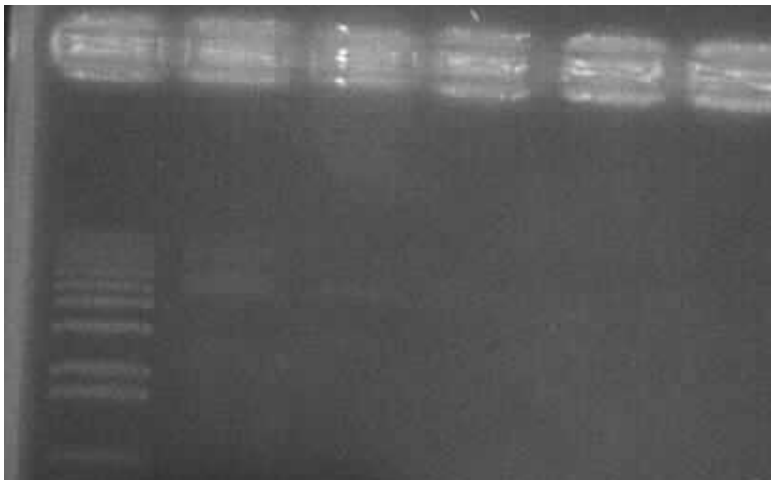


Figure 37 - HindIII + BamHI digestion products ligation. 1kb plus ladder; Pcr mCherry fragment ligated the VEpr-GFP fragment; Pcr mCherry fragment ligated with no VEpr-GFP fragment, Pcr mCherry fragment non ligated (from left side to right side lanes).

These results might have been due to excessive dephosphorilation of the mCherry backbone but also might reflect some DNA damage due to UV exposure during gel extraction. Therefore repetition of the digestions of the mCherry clone was done with the same enzymes but using a lower concentration of AP and not performing gel purification (A) (Figure 38, Figure 39, Figure 40, Figure 41) or without using AP and doing only gel purification of the fragments of interest (B) (Figure 38, Figure 39, Figure 40, Figure 41).

The digestion of the mCherry clone with Hind III was done as described in Table X.

Table X - Conditions used for BamHI digestion of Pcr mCherry clone

Vector/clone	Water (µL)	DNA (µL)	NEB buffer 2 10x (µL)	HindIII (µL)
Pcr-mCherry (vector1)	85,66	4,34	10	1

Figure 38 shows the digestion products resulting from the digestion of HindIII. The same purification step mentioned before was done and then the BamHI digestion was performed as shown in Table XI.

Figure 38- HindIII digestion products. 1kb plus ladder; Pcr mCherry uncuted; Pcr mCherry after HindIII digestion A; Pcr mCherry after HindIII digestion B (from left side to right side lanes)

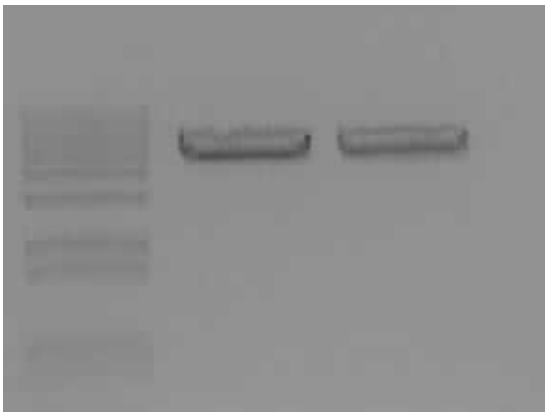


Figure 39- HindIII purified digestion products. 1kb plus ladder; Pcr mCherry uncuted; Pcr mCherry after HindIII digestion A; Pcr mCherry after HindIII digestion B (from left side to right side lanes)

Table XI - Conditions used for BamHI digestion of VEpr-GFP and Pcr mCherry clone

Vector/clone	Water (µL)	DNA (µL) after cutting with Hind II and desalted	Bam/Sal NEB buffer 10x (µL)	BamHI (µL)
Pcr-mCherry	49	40	10	1

Figure 40 - HindIII + BamHI digestion products.
Left side image: 1kb plus ladder; Pcr mCherry uncuted; Pcr mCherry cutted B (gel extraction); empty; 1kb plus ladder; Pcr mCherry cutted A (from left side to right side lanes).
Right side image: 1kb plus ladder; Pcr mCherry uncutted; purified Pcr mCherry cuted A; purified Pcr mCherry cuted B (from left side to right side lanes).

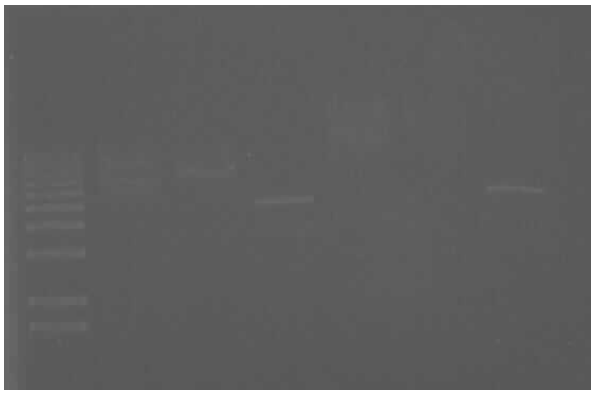


Figure 41- HindIII + BamHI digestion products ligation.
1kb plus ladder; Pcr mCherry fragment A ligated the VEpr-GFP fragment; Pcr mCherry fragment A ligated with no VEpr-GFP fragment, Pcr mCherry fragment A non ligated (from left side to right side lanes).
1kb plus ladder; Pcr mCherry fragment B ligated the VEpr-GFP fragment; Pcr mCherry fragment B ligated with no VEpr-GFP fragment, Pcr mCherry fragment B non ligated (from left side to right side lanes).

Bacterial colonies were only obtained from the transformation of the ligation products where we did not perform gel purification of the mCherry backbone (A). Unfortunately all the colonies were negative for the presence of the VEpr-GFP fragment of interest (Figure 42). The

presence of the fragment of interest from the VEpr-GFP clone was checked by enzymatic digestion of the DNA from the different colonies with NotI and HindIII (Figure 11).

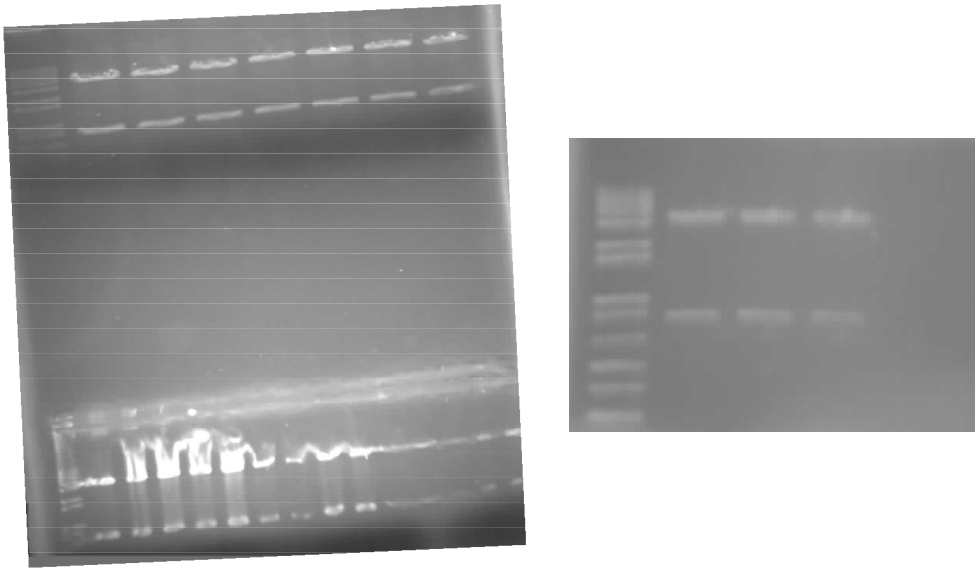


Figure 42-Restriction digestion with Not I and Hind III of the different clones from VEpr-mCherry A ligation to confirm the presence of VEpr.

In order to overcome these problems a digestion of the mCherry clone was performed with HindIII and BamHI (Table XII, Table XIII and Figure 43, Figure 44 and Figure 45) followed by dephosphorilation (with AP) and gel extraction, but this time the transformation of the ligation products was performed with ultra competent cells.

Table XII - Conditions used for HindIII digestion of VEpr-GFP and Pcr mCherry clone

Vector/clone	Water (µL)	DNA (µL)	NEB buffer 2 10x (µL)	HindIII (µL)
Pcr-mCherry	80,23	8,68	10	1

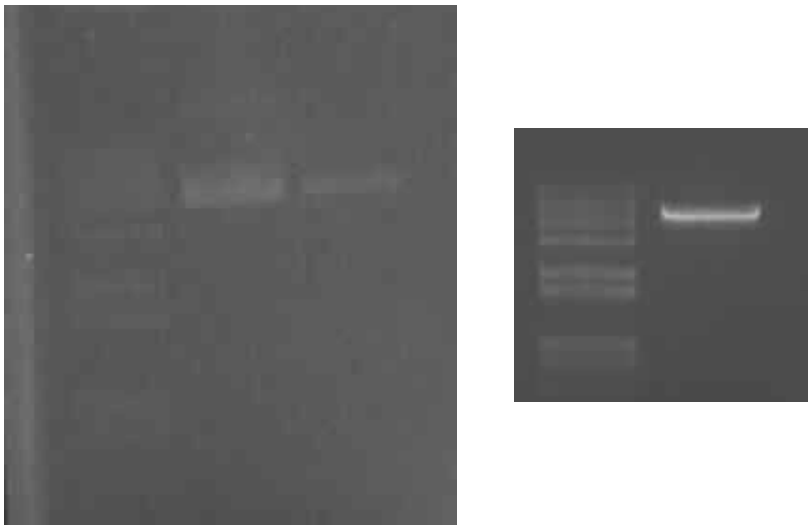


Figure 43 - HindIII digestion products.
Left side image:1kb plus ladder; Pcr mCherry uncuted; Pcr mCherry after HindIII digestion (from left side to right side lanes). Right side image: 1kb plus ladder; purified Pcr mCherry after HindIII digestion (from left side to right side lanes)

Table XIII - Conditions used for BamHI digestion of VEpr-GFP and Pcr mCherry clone

Vector/clone	Water (µL)	DNA (µL) after cutting with Hind II and desalted	Bam/Sal NEB buffer 10x (µL)	BamHI (µL)
Pcr-mCherry	49	40	10	1

After BamHI digestion, the Pcr-mCherry clone was dephosphorilated and then gel purified (Figure 44).

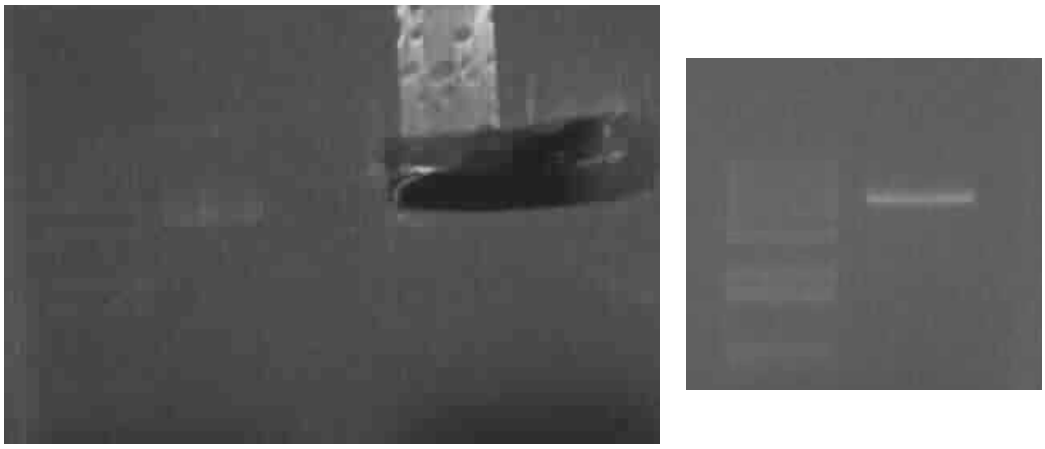


Figure 44 - HindIII + BamHI digestion products.
Left side image: 1kb plus ladder; Pcr mCherry uncuted; empty; Pcr mCherry cutted (gel extraction);(from left side to right side lanes).
Right side image: 1kb plus ladder; gel purified Pcr mCherry fragment (from left side to right side lanes).

Figure 45 - HindIII + BamHI digestion products ligation.
1kb plus ladder; Pcr mCherry fragment ligated the VEpr-GFP fragment; Pcr mCherry fragment ligated with no VEpr-GFP fragment, Pcr mCherry fragment non ligated (from left side to right side lanes).

With the colonies obtained from the transformation of the ligation products (Figure 45) bacterial minicultures were established and the purified DNA (mini spin kit QIAGEN) digested with NotI and Hind III. This digestion showed that eleven colonies were positive for the fragment of interest from the VEpr-GFP clone (Figure 46).

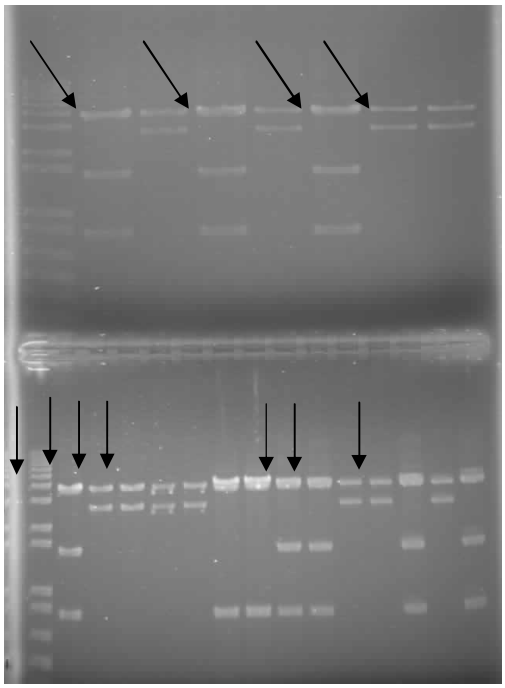


Figure 46 – Restriction digestion with Not I and Hind III of the different clones from VEpr-mCherry ligation to confirm the presence of VEpr (arrows indicate positive clones).

Some of these positive clones were selected and maxicultures were established for two positive clones (17 and 34) that showed a good linearization pattern after Hind III digestion (Figure 47).

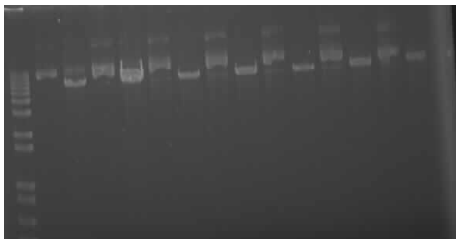


Figure 47 – Hind III digestion of VEpr-mCherry positive clones**.** 1kb plus ladder; clone 17 uncuted; clone 17 cuted; clone 34 uncuted; clone 34 cuted; clone 11 uncuted; clone 11 cuted; clone 24 uncuted; clone 24 cuted; clone 26 uncuted; clone 26 cuted; clone 31 uncuted; clone 31 cuted; clone 32 uncuted; clone 32 cuted (from left side to right side lanes).

After this step, the DNA amount within this maxicultures was analyzed and is presented on Table XIV.

Table XIV – DNA amount of maxicultures from clones 17 and 34

Clone	DNA amount (ng/µL)
17	17,8
34	52,6

The orientation of the fragment of interest was confirmed with a digestion presented in Figure 16and Figure 48.

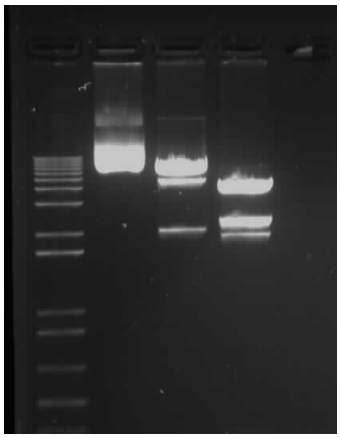


Figure 48 - Restriction digestion with HincII and PspOMI of the 34 clone from VEpr-mCherry ligation to confirm the orientation of VEpr. 1kb plus ladder; clone 34 uncuted; clone 34 cuted with PspOMI; clone 34 cuted with HincII (from left side to right side lanes).

Regarding the second step of sub cloning the initial strategy was to cut the PLenti6V5 and the VEpr-mCherry clones with ClaI plus Spume (followed by AP dephosphorilation) and HpaI plus Not I, respectively (Figure 13 and Figure 49). This strategy was based on the fact that the enzyme klenow large fragment could be used to generate a blunt end in the PLenti6v5 clone after cutting with ClaI and as Spume and Not I produce compatible ends we would have ligation of the fragments of interest.

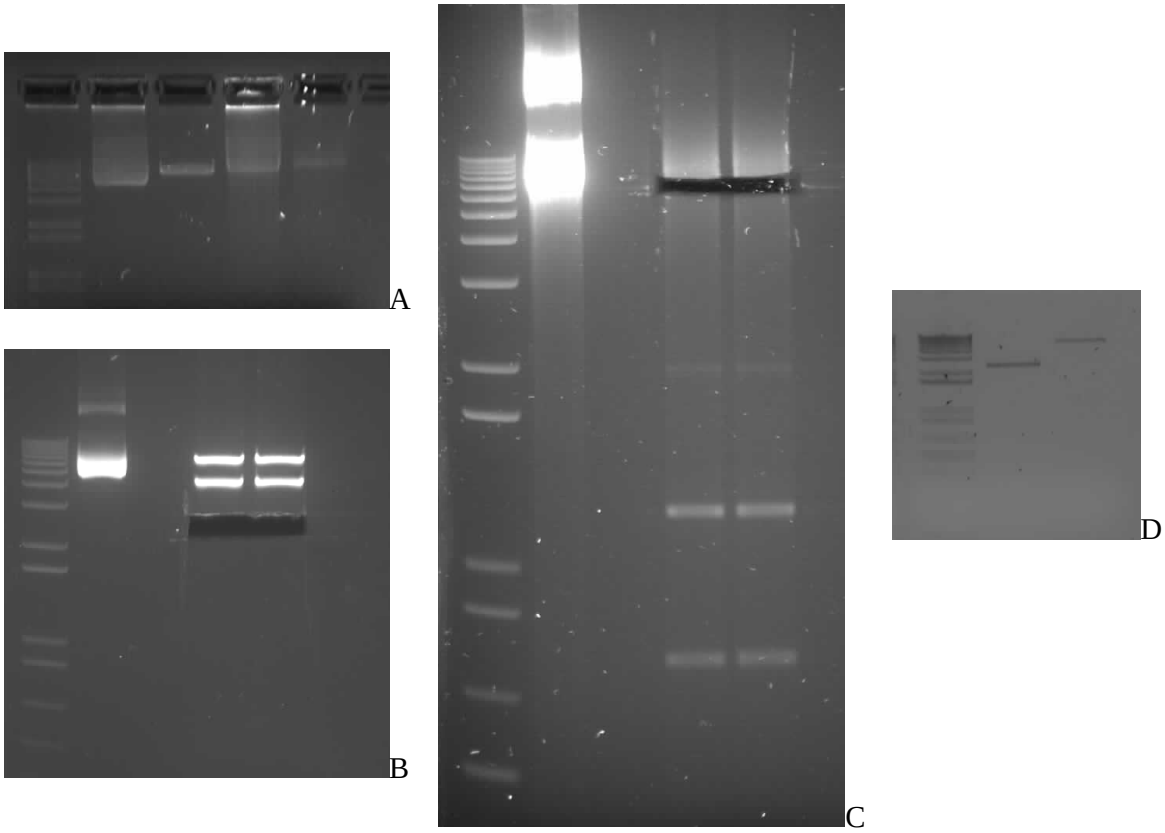


Figure 49- Second cloning step (first strategy). A - 1kb plus ladder; clone 34 uncuted;; clone 34 cuted with HpaI; PLenti6v5 uncuted; PLenti6v5 cutted with ClaI(from left side to right side lanes). B - 1kb plus ladder; clone 34 uncuted; empty; clone 34 cuted with HpaI + NotI (gel extraction) (from left side to right side lanes). B - 1kb plus ladder; PLenti6v5 uncuted; empty; PLenti6v5 cuted with ClaI + Spume (gel extraction) (from left side to right side lanes). C - 1kb plus ladder ; 34 cuted with HpaI + NotI gel purified; PLenti6v5 cuted with ClaI + Spume gel purified

Several problems arouse although the type of competent cells was changed the generation of bacterial colonies from transformation of different ligation products was not successful. This result might have been related with the blunting process with large fragment of klenow, because although this enzyme has lost 5' to 3' exonuclease activity it still has 3' to 5' exonuclease activity and might contribute to degrade the DNA of the PLenti6v5 clone. Therefore we ended up using another strategy with HpaI and PsPOMI to digest the PLenti6V5 clone (Figure 14) and maintaining the restriction strategy for the VEpr-Mcherry clone and consequently avoiding the need to use the klenow large fragment to blunt any end because the enzymes used in the different clones produce compatible ends.

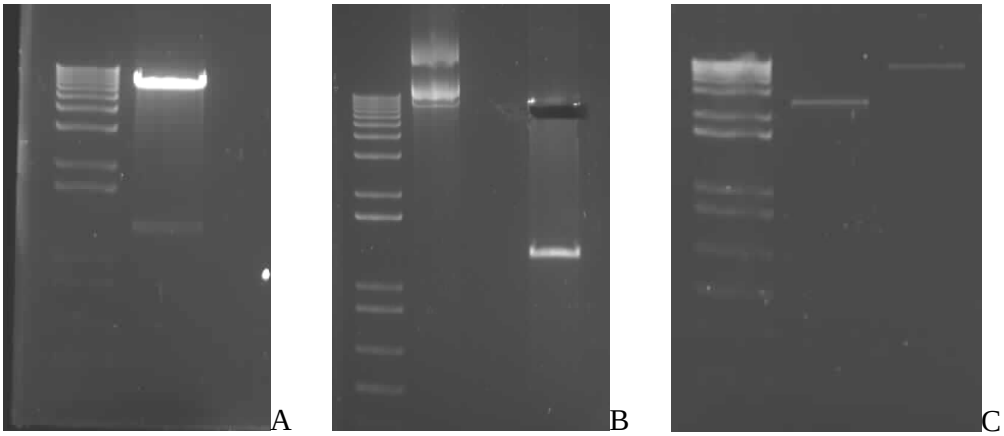


Figure 50 - Second cloning step (final strategy). A - 1kb plus ladder; PLenti6v5 cutted with HpaI+PsPOMI (from left side to right side lanes). B - - 1kb plus ladder; PLenti6v5 uncuted; empty; PLenti6v5 cutted with HpaI+PsPOMI (from left side to right side lanes) C - - 1kb plus ladder; gel purified PLenti6v5 cutted with HpaI+PsPOMI; gel purified clone 34 cutted with HpaI and NotI (from left side to right side lanes).

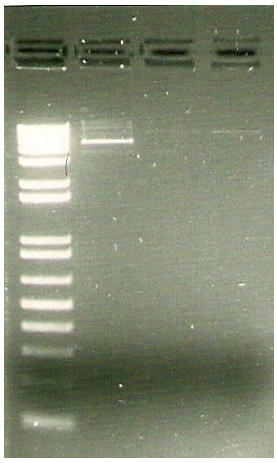


Figure 51 – VEpr-mCherry and PLenti6v5 digestion products ligation.
1kb plus ladder; VEpr- mCherry fragment ligated with the PLenti6v5 fragment; VEpr- mCherry fragment ligated with no PLenti6v5 fragment; VEpr- mCherry fragment non ligated (from left side to right side lanes).

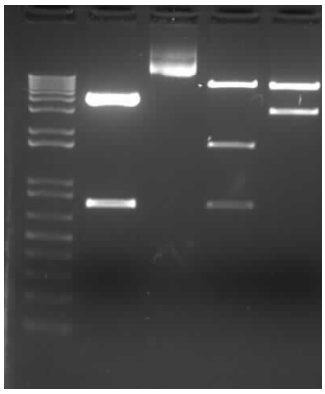


Figure 52- VEpr-mCherry digestion to confirm the presence of the fragment of interest. 1kb plus ladder; Pcr mCherry cutted with EcoRI; uncuted VEpr-mCherry clone (clone 34); VEpr-mCherry clone (clone 34) cutted with Eco RI; VEpr-mCherry clone (clone 34) cutted with Eco NI (from left side to right side lanes).

With this new approach we were able to obtain one positive colony for the fragment of interest from VEpr-mCherry that was confirmed with a restriction digestion using EcoRI and EcoNI (Figure 16 and Figure 52) and posterior establishment of a respective maxiculture with respective amount of DNA presented in Table XV.

Table XV – DNA quantification of VEpr-mCherry clone

Clone	DNA amount (ng/µL)
VLI9	101,2

3.3. Viral work

After performing the viral packaging as described in material and methods section viral titration was assessed. HUVECs were infected with different amounts of viral suspension and 5 days after infection the percentage of red fluorescent cells was analyzed.

3.3.1. Infection of HUVECs with the lentiviral vector for viral titration

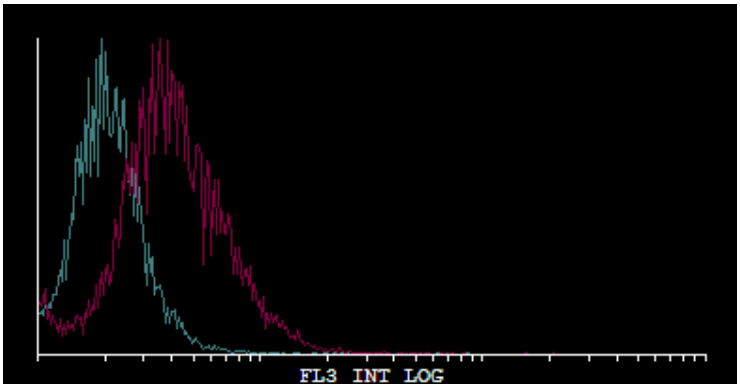


Figure 53- Fluorescence labelling of untransduced (blue) and transduced (red fluorescence) HUVECs.

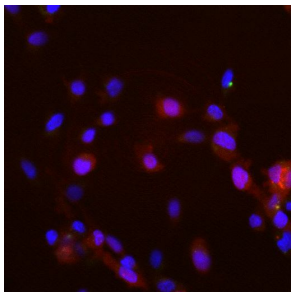


Figure 54- Transduced and blasticidin selected HUVECs

The data presented here (Table XVI, Figure 53 and Figure 54) above shows that 5 days after the viral infection the HUVECs present red fluorescence. Cells infected with increasing amounts of viral concentrate 50µL of viral suspension had a 6,22% red labelled cells, with

100µL of viral suspension 11,59% presented red fluorescence and using 300µL of viral suspension 23,59% cells show integration of the viral DNA (red fluorescence).

Table XVI – Fluorescent patterns of transduced HUVECs

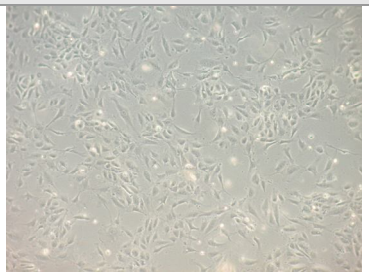
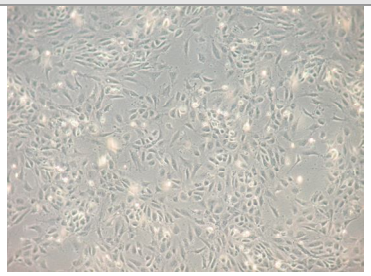
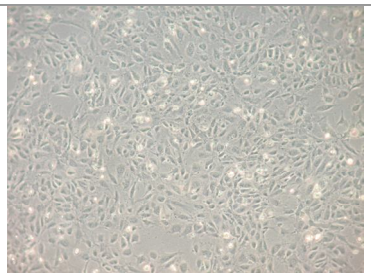
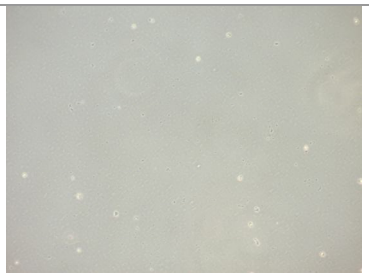
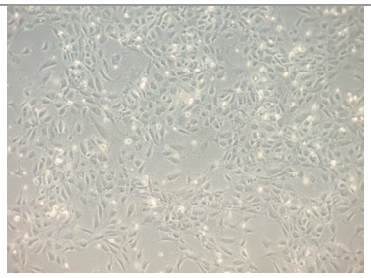
Viral suspension added (µL)	Number of cells	Percentage of cells Mcherry^{+} (5days after infection)	Mean of fluorescence intensity
50	65500	6,22	3,34
100	65500	11,59	3,82
300	65500	23,29	4,45

3.3.1.1. Blasticidin selection of HUVECs after infection with the lentiviral vector

The selection with blasticidin as reported in several studies (refs), eliminates non resistant cells by inhibition of translational. This was observed in untransduced HUVECs where all the cells died after 5 days with blasticidin (5µg, 10µg and 25µg per ml of medium - Figure 55) within the regular endothelial medium. Maintaining PLenti-VEpr-mCherry infected HUVECs (transduced) in culture during 5 days we observed by FACS (Table XVI) that the percentage of labelled cells tends to increase with increasing concentrations of blasticidin (Table XVII) but not significantly. This might be explained by the fact that the number of viral integrants within the HUVEC transduced population is highly heterogeneous and therefore the expression of mCherry will also be heterogeneous (Figure 54). Additionally, it is possible that some cells that are blasticidin resistant present really low levels of fluorescence due to the fact that mCherry is monomeric and the fluorescence intensity (50-75% of EGFP (ref)) it produces is sub-optimal for the settings of the cytometer available at Biocant. Another reason for the presence of non-fluorescent cells after blasticidin selection is the short half-life of this protein (15 minutes) (Shaner et al., 2004). This means that in a proliferating population of cells we will

always have cells that are low or non-fluorescent because there is no transcription during mitosis so during early G1 the cells won't have much red fluorescence.

Nevertheless, one of the main advantages of using mCherry is due to the fact that this monomeric protein has less toxic effects within the cells than other oligomeric fluorescent proteins (e.g. GFP) (Shaner et al., 2004).

Blasticidin concentration (µg/mL)	Untransduced HUVECs	Transduced HUVECs
0		
5		
10		

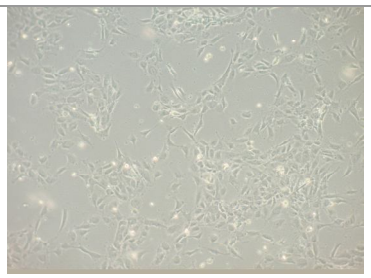
25		

Figure 55- Cell behaviour during blasticidin selection of untransduced and transduced HUVECs

Table XVII – Fluorescent labelling of untransduced and transduced HUVECs

Condition	Gated % of red (FL3) labelled cells	Total % of red (FL3) labelled cells
HUVECs untransduced	2,29	1,55
HUVECs transduced without blasticidin selection	95,57	58,08
HUVECs transduced with blasticidin selection (5µg/mL)	94,35	57,21
HUVECs transduced with blasticidin selection (10µg/mL)	95,20	60,82
HUVECs transduced with blasticidin selection (25µg/mL)	93,76	62,16

The infected HUVECs selected with blasticidin present red fluorescence (resulting from the viral transduction) and show immunolabeling of CD31 as presented in Figure 56.

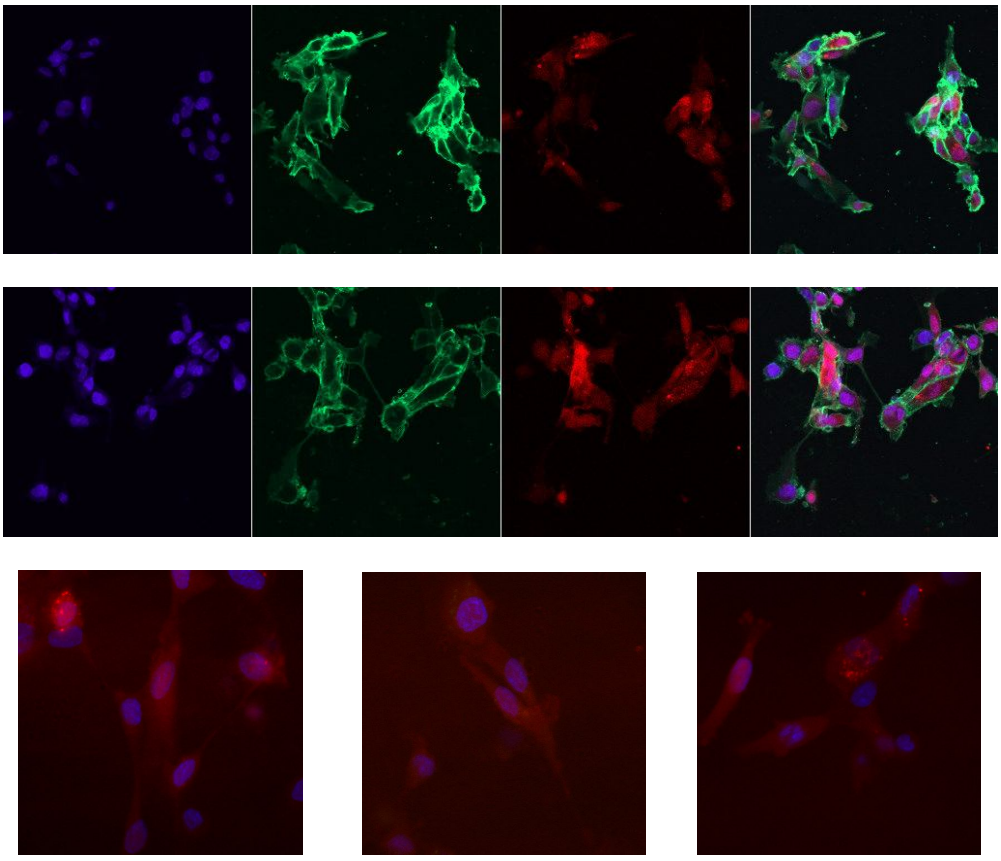


Figure 56 – Fluorescent labelling of transduced HUVECs, mCherry protein (red fluorescence); CD31 immunolabeling (green fluorescence).

3.3.2. Infection of iPS cells with the lentiviral vector

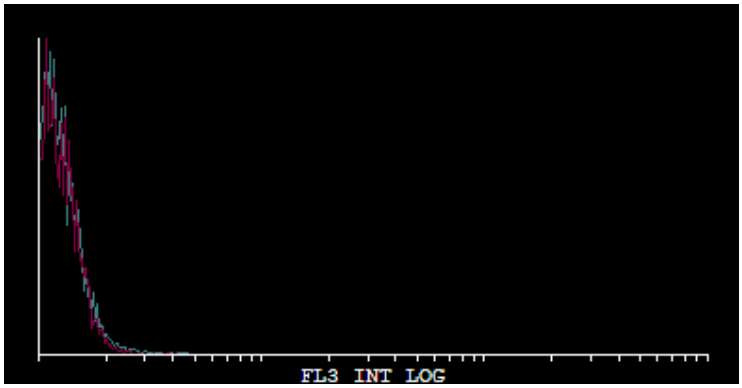
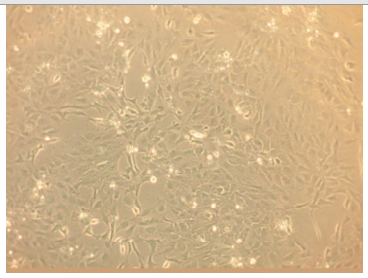
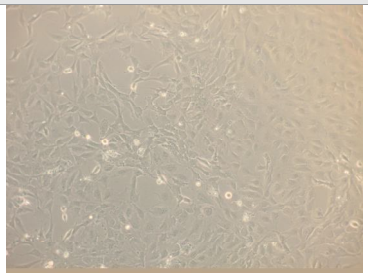
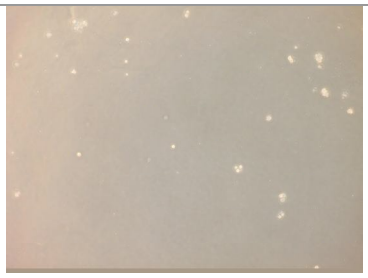
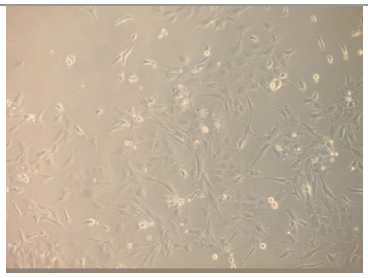


Figure 57- Fluorescence labelling of untransduced (blue) and transduced (red fluorescence) iPSCs.

The data presented above (Figure 57) demonstrate that 12 days after the viral infection of iPSCs there is no change in the red fluorescence pattern of infected iPSCs (transduced) versus non transduced iPSCs.

3.3.2.1. Blasticidin selection of iPS after infection with the lentiviral vector

Regarding the iPS selection, in the control situations the cell death was proportional to the concentration of blasticidin and within 72 hours after culturing untransduced iPSCs with blasticidin there were no more live cells. Within the experimental situations it was noticed some cell death but cell proliferation was occurring normally and therefore denoting the presence of blasticidin resistant iPSCs (Figure 58). Nevertheless, the FACS analysis shows no alteration between the control situation and the infected iPSCs (Figure 59). Therefore the selection with blasticidin did not promote any alteration in terms of vascular endothelial-cadherin expression.

Blasticidin concentration (µg/mL)	Untransduced iPSCs	Transduced iPSCs
0		
5		

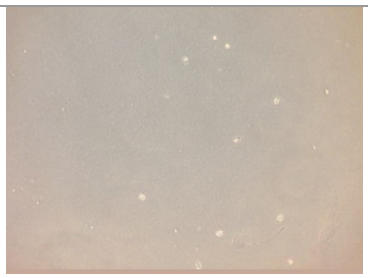
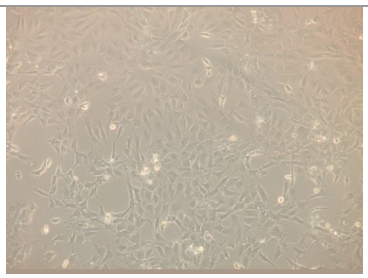
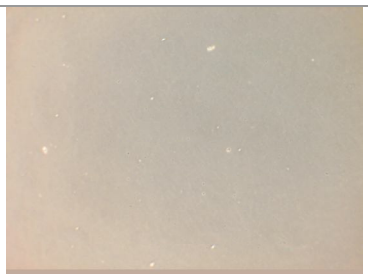
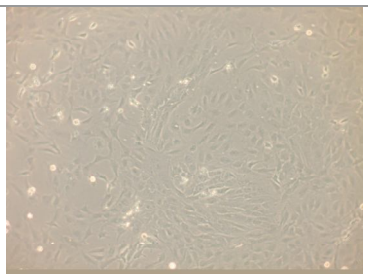
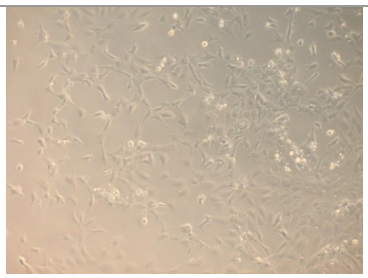
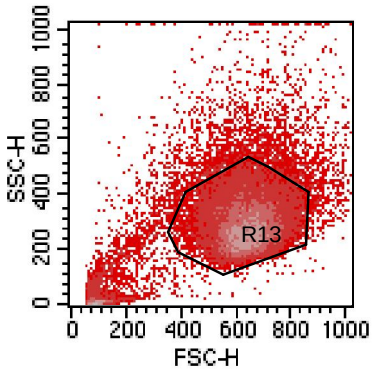
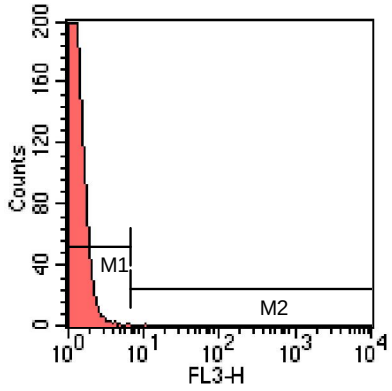
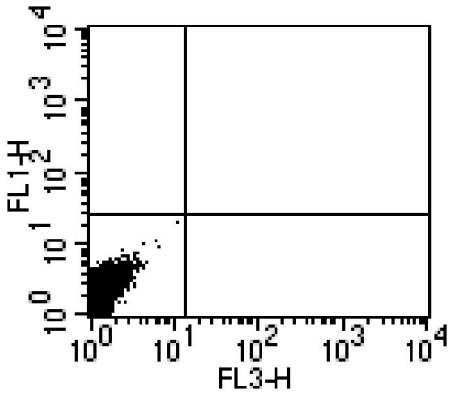
8		
10		
20		

Figure 58- Cell behaviour during blasticidin selection of untransduced and transduced iPSCs.

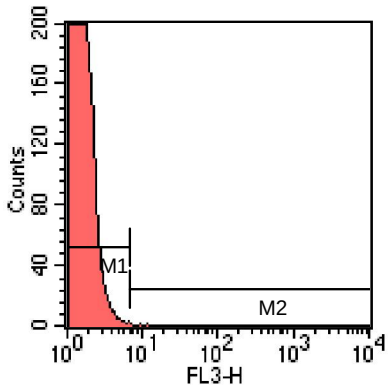


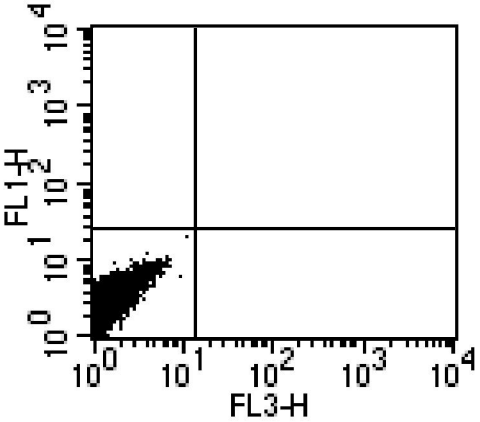
Untransduced iPSCs



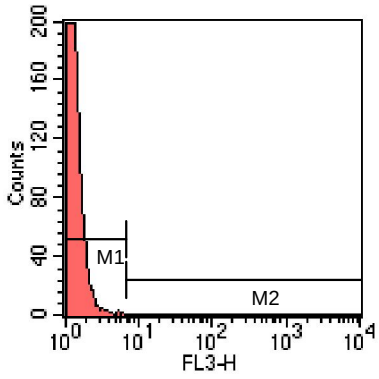


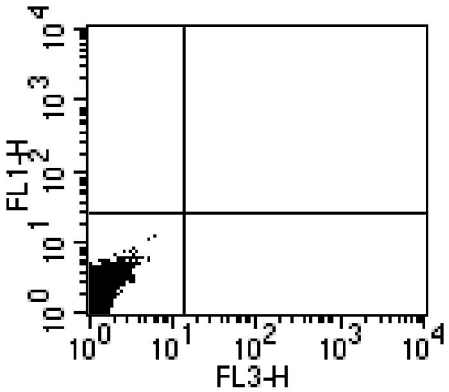
Untransduced iPSCs



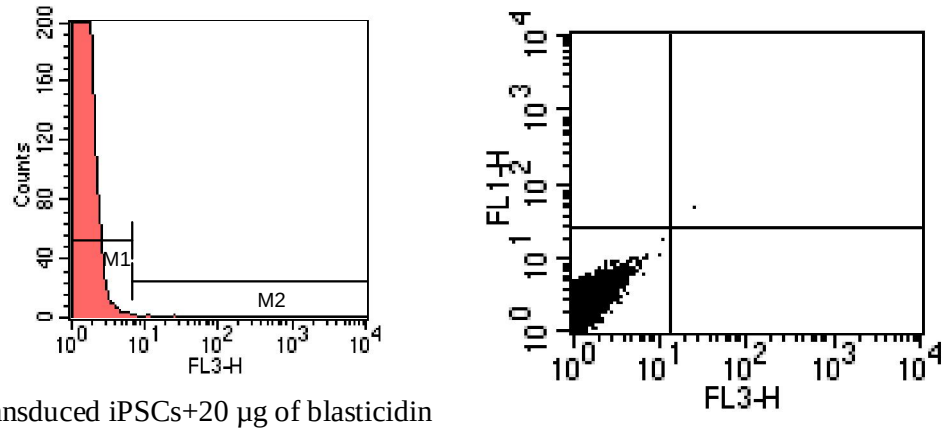


Transduced iPSCs + 5 µg of blasticidin





Transduced iPSCs+10 µg of blasticidin



Transduced iPSCs+20 µg of blasticidin

Figure 59- Cell fluorescent behaviour during blasticidin selection of untransduced and transduced iPSCs.

3.3.3. Genomic integration of viral work by HUVECs and iPSCs

After infecting the cells (HUVECs and iPSCs) the viral integration was accessed by polymerase chain reaction (PCR) with specific primers (Table V) to the mCherry fragment. After adjusting the PCR conditions the amplification of the fragment of interest was observed (Figure 60).

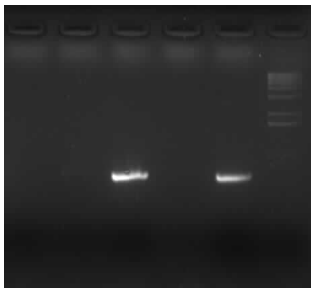


Figure 60 – PCR amplification products (1,2%agarose gel). Water; HUVECs untransduced; HUVECs transduced; iPSCs untransduced; iPSCs transduced; 1kbplus ladder (from left side to right side lanes)

3.4. On going work

3.4.1. Differentiation of VE-cadherin promoter cherry iPS cells with and without blasticidin selection

The blasticidin selected iPSCs and also unselected iPSCs were treated according to different endothelial differentiation protocols, already described (Levenberg et al., 2002; Lagarkova et al., 2008). Unfortunately the iPSCs in culture are not yet differentiated into endothelial cells, they present negligible immunolabeling of CD31 and VE-cadherin.

This demonstrate that the reporter system that is described here may have a really important role in the optimization of the differentiation protocols, because in addition to the low efficiencies obtained with the different protocols the cell type used to generate endothelial cells might also have a great impact in the differentiation output. Therefore, using the screening tool described here will permit also the monitorization of differentiation of different cell sources since the packaging of this virus enables the infection of every mammalian cell type (hESCs, iPSCs, EPCs and others).

Chapter 4

Conclusion

4. Conclusion

The work described here, lead to the successful generation of a molecular biology tool that permits the screening of endothelial differentiation. In order to achieve this purpose there is some on going work to generate a stable cell line of transduced (Plenti6V5-VEpr-mCherry) iPSCs. These cells after blasticidin selection will be sorted for pluripotency markers (CD326 with MACS system) to ensure their pluripotency. Furthermore a clonal selection, expansion and differentiation will prove the integration of the viral DNA and the establishment of the desired iPSC cell line. Additionally this tool can also be applied to other cells and also to study processes like transdifferentiation (Haase et al., 2009; Graf & Enver, 2009). Therefore, this reporter system can be applied to a variety of cells and study their endothelial potential in different environmental conditions.

The monitorization of endothelial differentiation with this tool can be adjusted to high throughput screening methods already described by Ng et al 2005, 2008 that have developed a 96-well spin EB method. Additionally, Koike et al. 2007 have constructed a 96-well murine ESCs differentiation system and studied the effect of EB seeding density in the output of cardiomyocyte differentiation. Other studies have developed 384 well plate (Outten et al., 2011) screening platforms. Generation of biomaterial high throughput screening platforms is associated with micropatterned surfaces. In these platforms the effect of different polymers and matrices on cell differentiation and the use of different chemical components like cytokines, growth factors, miRNAs, siRNA can be concomitantly tested. These kinds of biomaterial platforms circumvent the need of either using large number of cells (miniaturized platforms) or interrupting the differentiation process in different time points in order to access the differentiation state of the cells. Therefore the screening process can be retransformed into a less time and resource consuming process.

Furthermore the ability of internalization of the nanoparticle and adhesion of iPSCs to different substrates (that may present potential endothelial induction characteristics), concerted with the reported system developed, might be a potent way to try to improve the differentiation process. In one hand the nanoparticle delivery system might be used to improve the delivery of differentiation factors and explore the importance of the specific time point delivery of differentiation factors that have major effects in the endothelial differentiation. On the other hand, the use of different substrates might improve the differentiation process by giving endothelial specific cues to the cells.

The use of the endothelial reporter system here described will permit the screening of different endothelial differentiation strategies, namely the use of different nanoparticle cargo combination and different matrices. During this screening process when the cells present red fluorescence is a sign of their commitment to the endothelial differentiation pathway, since the promoter associated with the fluorescent protein codes for a late specific endothelial marker (Kita-Matsuo et al., 2009).

We hope that this work may contribute in the future for a better understanding of the vasculogenesis and the angiogenic process and their implication in prevalent diseases as cancer and ischemic conditions.

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Annexes

Annexe I – DNA sequences of the clones used

pCR -mcherry clone: 4071bp

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCT
GGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTG
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PLenti6 ires egfp: 8274bp

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VEpr-GFP construct: 8712bp

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