



HAL
open science

Evaluation of endothelial cell culture as a model system of vascular ageing

Louise Boisen, Kim Ryun Drasbek, Anna Sofie Pedersen, Peter Kristensen

► **To cite this version:**

Louise Boisen, Kim Ryun Drasbek, Anna Sofie Pedersen, Peter Kristensen. Evaluation of endothelial cell culture as a model system of vascular ageing. *Experimental Gerontology*, 2010, 45 (10), pp.779. 10.1016/j.exger.2010.06.003 . hal-00625938

HAL Id: hal-00625938

<https://hal.science/hal-00625938>

Submitted on 23 Sep 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

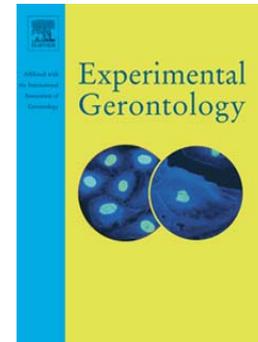
Evaluation of endothelial cell culture as a model system of vascular ageing

Louise Boisen, Kim Ryun Drasbek, Anna Sofie Pedersen, Peter Kristensen

PII: S0531-5565(10)00196-8
DOI: doi: [10.1016/j.exger.2010.06.003](https://doi.org/10.1016/j.exger.2010.06.003)
Reference: EXG 8753

To appear in: *Experimental Gerontology*

Received date: 3 January 2010
Revised date: 11 May 2010
Accepted date: 21 June 2010



Please cite this article as: Boisen, Louise, Drasbek, Kim Ryun, Pedersen, Anna Sofie, Kristensen, Peter, Evaluation of endothelial cell culture as a model system of vascular ageing, *Experimental Gerontology* (2010), doi: [10.1016/j.exger.2010.06.003](https://doi.org/10.1016/j.exger.2010.06.003)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

a

Evaluation of endothelial cell culture as a model system of vascular ageing

Louise Boisen, Kim Ryun Drasbek, Anna Sofie Pedersen, Peter Kristensen*

Department of Molecular Biology, Aarhus University, Aarhus, Denmark

*Corresponding author:

Peter Kristensen

Department of Molecular Biology

Aarhus University

Gustav Wieds Vej 10 C

DK-8000 Aarhus C

Denmark

Email: pk@mb.au.dk

Phone: +45 89 42 52 75

Fax: +45 86 12 31 78

Running title:

Endothelial ageing and tube formation

Keywords:

Ageing, Endothelial cells, senescence, tube formation, SA beta-galactosidase activity, lysosome staining, gamma-H2A.X staining

Abstract

The purpose of this study was to evaluate the relevance of long-term endothelial cell culture as a model system of vascular ageing. Micro- and macrovascular endothelial cells were serially passaged until replicative senescence and their ability to form tube-like structures when cultured on Matrigel was assessed throughout their lifespan. For both cell types low passage cultures adopted a homogeneous cobblestone morphology, while senescent cultures were extremely heterogeneous. Furthermore, both cell types showed a reduction in tube formation ability with in vitro ageing, which is in accordance with the reduction in angiogenic potential observed with ageing in vivo. Examination of senescence associated β -galactosidase activity revealed an increased activity in cells forming tubes as compared to cells cultured on plastic, which could be attributed to an increased lysosomal content of cells undergoing tube formation. As this increased senescence associated β -galactosidase activity was unrelated to the replicative age of the cells, senescence associated β -galactosidase activity may not be a relevant senescence marker for differentiating endothelial cells. The age-related reduction in tube formation ability suggested that long-term culture of endothelial cells may be a valid model system of vascular ageing, which makes it an ideal platform for high throughput screening of compounds influencing angiogenesis.

Introduction

Common to all vessels of the vasculature is the endothelium, which is a single layered sheet of squamous, polarised cells that are primarily responsible for all signalling and transportation from blood to tissue and vice versa (Cines et al, 1998). As the body ages, so does the vasculature and ageing of the vasculature is accompanied by a number of changes such as reduced vasoregulation (Taddei et al, 2001), delayed and/or altered angiogenesis (Agah et al, 2004, Sadoun & Reed, 2003), reduced repair capacity and altered blood vessel composition (Izzo Jr. & Mitchell, 2007).

Different types of endothelial cells have been isolated and cultured to understand the biology and pathobiology of the vasculature and the angiogenic response. The most widely used type isolated from human umbilical vein (named HUVEC) show a characteristic cobblestone shape when cultured, although cell borders are indistinct and hard to visualise (Jaffe et al, 1973). Senescent HUVEC cultures have been described as very heterogeneous with greatly enlarged cells containing multiple cytoplasmic protrusions coupled with positive staining for senescence associated β -galactosidase (SA β -gal) activity (Kalashnik et al, 2000, Unterluggauer et al, 2003).

In vivo, angiogenesis is mediated by the microvasculature and the angiogenic response of human microvascular endothelial cells (HMVEC) has been suggested to be stronger than that of HUVEC because of differing expression of matrix metalloproteases (Jackson & Nguyen, 1997). However, HMVEC are generally more fastidious in their culture requirements than HUVEC making long-term culture more challenging.

Functional studies of endothelial cells have been carried out in culture, as endothelial cells cultured in the presence of an extracellular matrix (ECM) organise themselves into tube-like structures. Depending on the conditions, the tube structures will form in two or three dimensions (Staton et al, 2009). This response is seen as a mimic of natural endothelial behaviour during angiogenesis, and as such has been used as a simple in vitro angiogenesis assay. As expected for endothelial cells, low

passage HUVEC form tube-like structures when cultured on top of gels formed by ECM proteins such as collagen-1 or Matrigel (Unger et al, 2002). However, such studies have not been reported for microvascular endothelial cells, although these cells are directly involved in angiogenesis. While endothelial cells in vivo normally are quiescent, cultured endothelial cells are kept in an artificially activated state characterised by extensive proliferation. Therefore, cultured endothelial cells are not ideally suitable for the study of the quiescent state. In contrast, these cell cultures are in principle well suited for the study of activated endothelium e.g. the study of angiogenesis. Furthermore, as primary cells in culture eventually reach replicative senescence, functional studies of cultured endothelial cells could turn out to be an excellent model system of angiogenesis during ageing.

In this study, we have cultured both micro- and macrovascular endothelial cells until the onset of replicative senescence. Minor differences in morphology between the two cell types at corresponding ages were found, while the senescent cells were distinguishable from young cells on the basis of morphology and SA β -gal activity. Finally, studying the function of the activated endothelial cells, we found that in vitro tube formation of the cells diminished as the culture aged and, thus, the number of senescent cells increased. This observation suggests that long-term culture of endothelial cells may serve as a suitable model of vascular ageing. Intriguingly, a pronounced and increasing SA β -gal activity was observed in cells regardless of age when cultured on Matrigel. However, this increase was not accompanied by a corresponding increase in γ -H2A.X positive cells after cultivation on Matrigel. This suggests that the increased SA β -gal activity did not reflect a similar increase in senescent cells and questions the validity of this particular marker in the evaluation of senescence in differentiated endothelial cells.

Materials and Methods

Cell culture

HMVEC isolated from adult dermis as well as HUVEC were obtained from Clonetics Cell Systems (Lonza, Basel, Switzerland). HUVEC NS2 were kindly provided by Professor Pidder Jansen-Dürr of the Austrian Academy of Sciences in Innsbruck (Austria).

HMVEC were cultured in Endothelial Cell Basal Medium-2 (Lonza) supplemented with EGM-2 singlequots (Lonza) containing h-EGF, VEGF, h-FGF-B, R3-IGF-1, hydrocortisone, and FBS (2% final concentration). HUVEC were cultured in Endothelial Cell Basal Medium (Lonza) supplemented with EGM Singlequots (Lonza) containing h-EGF, Hydrocortisone, GA-1000, bovine brain extract (BBE), FBS (2% final concentration). HUVEC NS2 were cultured in EGM in cell culture flasks coated with 0.2% gelatine in EGM.

The cells were grown in an atmosphere of 5% CO₂ at 37°C and subcultured by trypsinisation with trypsin-EDTA (Lonza). Cells were passaged such that the monolayers never exceeded 75-90% confluency. The cells were propagated until senescence and cell numbers were determined when subcultured by passing a 1/50 dilution of cells through a Coulter Counter. Population doublings (PD) were estimated using the following equation:

$$PD = \frac{\log_{10}(\# \text{cells harvested at end of passage}) - \log_{10}(\# \text{cells seeded at beginning of passage})}{\log_{10} 2}$$

For each passaging the resulting PD estimation was added to the sum of PD from the previous passages to achieve the cumulative population doubling level (CPDL), which was plotted against time to obtain a growth curve.

Tube formation assay

Wells of 96-well (Corning, Lowell, MA) or 4-well (Nunc, Roskilde, Denmark) cell culture plates were coated with BD Matrix Growth Factor Reduced Matrigel™ (BD Biosciences, San Jose, CA) at 4°C. The gel was allowed to polymerise in the 37°C CO₂-incubator for at least 30 minutes before

seeding of 20 000 (96-well) or 80 000 (4-well) cells on top of the gel. The cells were allowed to attach and differentiate overnight. Tubes were visually examined with phase contrast using either 2.5x or 10x objective and pictures were recorded digitally with a Zeiss Axiocam MRc camera (Carl Zeiss Micro Imaging, Göttingen, Germany) attached to the microscope. Due to Matrigel coating and light refraction through the gel and media, dark spots in the centres and sometimes edges of wells were visible in the pictures.

Senescence evaluation – SA β -gal activity assay

Cells were examined for senescence associated β -galactosidase activity, which is a widely accepted marker for cellular senescence (Dimri et al, 1995). Briefly, cells were washed twice with phosphate buffered saline (PBS) and fixed with 2% formaldehyde, 0.2% glutaraldehyde solution in PBS for 3-5 min at room temperature (RT). The fixed cells were washed twice with PBS before addition of 1 mg/ml X-gal in NN-dimethylformamide, 2 mM $MgCl_2$, 150 mM NaCl, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 40 mM citric acid/Na phosphate buffer, pH 6.0. Cells were incubated with the staining solution overnight at 37°C and washed twice with H_2O and allowed to dry before visual examination in an inverted light microscope. Cells forming tubes on Matrigel were stained using the same procedure, although the stained tubes were not dried prior to examination.

Lysosome detection

The lysosomal content of the cells was evaluated using a fluorescent LysoTracker probe (Invitrogen, Carlsbad, CA). HUVEC NS2 cells were seeded on plastic and Matrigel in 24-well plates (Corning) at a density of 40 000 cells per well and allowed to attach/form tubes overnight. The following day the growth medium was replaced with fresh, warm growth medium containing 100 nM LysoTracker Red DND-99, and the cells were incubated for 2 hours in the CO_2 -incubator. The LysoTracker solution was replaced with normal growth medium and the cells examined for red

fluorescence under an inverted fluorescence microscope (Leica DMI 3000B (Wetzlar, Germany) equipped with an Olympus DP72 CCD camera (Olympus, Ballerup, Denmark).

Kinetic study of LysoTracker and SA β -gal staining

To study the staining of lysosomes with LysoTracker Red DND-99 and SA β -gal activity, low and high passage HUVEC NS2 cells (40 000 cells/well) were plated in 24-well plates with or without prior coating with Matrigel and assayed 4, 8, 12, and 16 hours after plating. At each time point, live cells were stained with LysoTracker for 30 min before fluorescent pictures were obtained with the same exposure conditions (200 ms exposure) at all time points using an inverted fluorescence microscope. Simultaneously, the wells for SA β -gal activity visualization were fixed at each time point and incubated with X-gal solution for 16 hours before acquisition of pictures using differential interference contrast (DIC).

Immunocytochemical staining

Low and high passage HUVEC NS2 cells were seeded in 24-well plates with or without prior coating with Matrigel and allowed to grow for 16 hours before fixation in 2% paraformaldehyde diluted in PBS for 15 min. The cells were permeabilized with 0.1% Triton-X-100 for 5 min prior to blocking in 5% FCS for 1 hour. After washing, the wells were incubated with primary antibody, rabbit-anti- γ -H2A.X (Abcam, Cambridge, UK) for 1 hour followed by incubation for 45 min with goat-anti-rabbit-Alexa546 (Invitrogen). Stained cells were overlaid with VectaShield antifade with DAPI (Vector Laboratories, Burlingame, CA) before visualization using an inverted fluorescence microscope equipped with filter sets for DAPI and red fluorescence.

Cellular expression of endothelial cell markers

Cells were analysed for expression of endothelial cell markers von Willebrand Factor (vWF) and platelet endothelial cell adhesion molecule/CD31. Cells were washed trice in cold PBS before fixation for 2 min at -20°C with a methanol/acetone mixture. After three additional washes, the

culture flasks were blocked for 1 hr RT with 1% BSA. Cells were incubated with primary antibodies, rabbit anti-vWF (Abcam, Cambridge, UK) and mouse anti-CD31 (Abcam) shaking overnight at 4°C. The cells were incubated for 2½-3 hrs with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (DAKO, Glostrup, Denmark) and Rhodopsin-conjugated anti-mouse antibody (Invitrogen) shaking in the dark before counterstaining with DAPI (Invitrogen) and examination for green, red and blue fluorescence under an inverted fluorescence microscope.

Results

Cell cultures

The human microvascular cell line, HMVEC, was continuously passaged until senescence, which ensued after approximately 40 PD (Figure 1A). The growth curve showed the expected pattern of rapid growth for the first 35 PD, or about 40 days, where after growth slowed down over a period of about 20 days and eventually subsided, creating a plateau. As the growth curve also shows, the nearly non-growing culture was rather stable with no significant loss of cells over time. Low passage HMVEC cultures were very homogeneous with small, spindle-shaped cells growing in monolayers. When cells approached confluence they adopted the typical cobblestone morphology of endothelial cells (not shown). HMVEC appeared reasonably homogeneous with no significant SA β -gal activity for more than half of their lifespan (Figure 1E). The cellular rate of proliferation started slowing down around 30 CPDL (Figure 1A), which coincided with the appearance of enlarged and oddly shaped cells. The proportion of senescent cells increased in the last quarter of the culture's lifespan (Figure 1F) until the very end, where the culture was dominated by a heterogeneous SA β -gal positive population of greatly enlarged cells with multiple cytoplasmic protrusions (Figure 1D and G). The cells stained positively for endothelial markers, vWF and CD31, throughout their lifespan (not shown).

The long-term culture of HUVEC largely followed the pattern seen for HMVEC culture with the cells undergoing replicative senescence at about 36-38 CPDL after approximately 80 days in culture (not shown). Low passage HUVEC exhibited the characteristic cobblestone morphology described for endothelial cells, with the majority of cells being rather small and slightly spindle shaped. As the cultures aged, they gradually became more heterogeneous with oddly shaped cells starting to appear. Generally, cells of abnormal shape and size stained positive for SA β -gal activity, which resulted in a gradual increase in SA β -gal positive cells during ageing (not shown) and as seen in HMVEC cultures, the HUVEC culture eventually became dominated by greatly enlarged SA β -gal positive cells with multiple cytoplasmic protrusions when growth subsided (not shown). In contrast to the HMVEC cultures, there was a substantial cell loss in the last few passages, where the cells detached from the flask and died.

Tube formation assay

The tube formation assay was used to test the function of the activated endothelial cells throughout their lifespan. At several time points HMVEC were assessed for their tube formation ability as shown in Figure 2. Low passage cells formed many short tubes and the overall picture resembles that of a honeycomb (Figure 2A). As the cells aged and entered the second half of their lifespan, the tubes became longer and the branch points fewer (Figure 2B-E). Finally, as the growth rate slowed down and the culture reached senescence, the cells eventually stopped forming tubes altogether (Figure 2E and F) while the cells appeared to form clusters, which may represent the node points of the tubes.

As for HMVEC, the tube formation ability of HUVEC was affected by cellular ageing. While low passage (not shown) and mid-life (Figure 3A) cells did form tubes when cultured on Matrigel, this ability appeared to diminish as the culture approached senescence, with senescent cells hardly forming tubes at all (Figure 3C). As seen in Figure 3, the ageing cells appeared to concentrate in

branch points rather than spread out and form tubes. This behaviour could reflect a reduced ability to migrate and/or differentiate with advancing age.

Correlation between tube formation ability and appearance of senescent cells in culture

To see if the appearance of senescent cells in culture could be definitely linked with reduced tube formation, the SA β -gal assay was attempted for HUVEC NS2 cells cultured on Matrigel. Cells cultured on Matrigel did stain positively for β -gal activity in an age-related manner, but, interestingly, stained cells appeared earlier and were much more prominent when cultured on Matrigel than when cultured on plastic (Figure 4). Because SA β -gal activity has been shown to be lysosomal β -gal activity, it seemed relevant to evaluate whether the increased SA β -gal activity observed in the tubes could be caused by an increased lysosomal content. To achieve this goal, cells seeded on Matrigel and cells seeded on plastic were incubated with a LysoTracker probe that accumulates and fluoresces in the acidic organelles of cells. As seen in Figure 5, the tubes were strongly fluorescent (Figure 5C) while fluorescence in the cells seeded on plastic generally was much weaker with only the occasional cell showing strong fluorescence (Figure 5D). The picture for cells assayed for SA β -gal activity was similar, with tubes being strongly positive (Figure 5E) and cells seeded on plastic less positive (Figure 5F).

To investigate whether the increased LysoTracker/SA β -gal activity in tube forming cells was a result of differentiating endothelial cells, the LysoTracker fluorescence and SA β -gal activity were evaluated at different time points after seeding in both low passage and high passage HUVEC. As Figure 6 shows, there was a clear increase in LysoTracker fluorescence with advancing time in the cells cultured on Matrigel (Figure 6A-H), while the fluorescence of cells cultured on plastic remained constant at all time points (staining after 4 hours shown in Figure 6I-L). Furthermore, it was evident that the LysoTracker fluorescence was stronger in high passage cells than in low passage cells throughout the experiment (Figure 6A-H), albeit the difference was most pronounced

at the early time points (Figure 6A-B, E-F). Supporting this, the age-associated difference in LysoTracker intensity was also evident in the cells seeded on plastic (Figure 6, I-L). In parallel, cells were assayed for SA β -gal activity where a similar but less pronounced increase in activity with time was observed in cells seeded on Matrigel (not shown).

Evaluation of endothelial senescence with DNA damage marker during differentiation

As the increased SA β -gal activity in endothelial cells cultured on Matrigel indicated that SA β -gal activity might not be a good marker for endothelial cell senescence, low and high passage HUVEC were analysed for the presence of a DNA damage response signified by the expression of γ -H2AX. As seen in Figure 7, high passage cells showed punctuate nuclear staining with a γ -H2AX antibody at a similar level whether cultured on plastic or Matrigel (Figure 7D and H), while nuclear staining of low passage cells was extremely limited (Figure 7B and F).

Discussion

Endothelial cell culture has been used extensively to describe many of the changes happening with ageing of the vasculature. However, the application of a continuously dividing cell culture system, which eventually undergoes senescence, to the study of a cell type that in vivo is mostly quiescent has some obvious disadvantages. This study was undertaken to investigate the validity of the Hayflick model on a particular aspect of vascular ageing, namely the well-documented reduction in blood vessel formation happening with ageing.

To cover different parts of the vascular endothelium both macro- (HUVEC) and microvascular (HMVEC) primary cell cultures were used. As described, HUVEC is the most extensively studied endothelial cell type, primarily because they are relatively easy to isolate and culture. Therefore, this cell type was chosen rather than more recently available and not thoroughly characterised somatic macrovascular endothelial cell types, although they might have been more interesting from

a physiological point of view. The dermal HMVEC isolated from adults were chosen because they represent a cell type that is often activated and involved in angiogenesis.

Morphologically, HUVEC and HMVEC cultures were quite similar in young and middle age with slight variations in shape from the traditional cobblestone appearance to more of a spindle shape, although the degree of confluence in the cultures did influence the cell shape. As the cells aged, they started showing typical signs of senescence with changes in shapes and increased size as well as SA β -gal activity. In fact, the most prominent difference between senescent HUVEC and HMVEC was the observed quite substantial cell loss during the last few passages of HUVEC cultures. This might be due to apoptosis as senescent HUVEC has been reported to undergo extensive apoptosis (Hampel et al, 2004). In contrast, apoptosis did not seem to be prominent in the senescent HMVEC cultures, which could be maintained for several weeks with no obvious changes in cell density. This is in accordance with earlier reports of stable senescent HMVEC cultures (Vasile et al, 2001, Watanabe et al, 1997). These observations indicate that not all types of endothelial cells behave alike in culture and that some cell-type specific characteristics may be retained in culture making the use of endothelial cells isolated from different parts of the vasculature appropriate.

During culturing, the functionality of the endothelial cells was tested using the tube formation assay. Interestingly, cellular senescence appeared to have a profound effect on cellular behaviour as tube morphology was influenced by the age of the assayed cells. For both HMVEC and HUVEC, cellular ageing was accompanied by a gradual increase in the length of the tubes coupled with a decrease in the number of branch points. As the cells entered the senescence plateau, tube formation was nearly completely abolished with the cells apparently forming clusters. Thus, these results suggest that *in vitro* ageing of endothelial cells causes a loss of differentiation potential as evidenced by the reduction in tube formation from senescent cultures. Interestingly, most reports on

long-term cultures of endothelial cells do not assess tube formation in any form, but the few reports available have concluded that senescence of HUVEC does indeed lead to loss of tube formation ability (Ha et al, 2007, Yang et al, 1999). Reports on long-term culture of HMVEC are scarce and apparently none of them have addressed tube formation ability, although migration of HMVEC was reportedly reduced with ageing (Reed et al, 2000).

In an attempt to further investigate the correlation between endothelial cell senescence and tube formation ability, HUVEC NS2 cells were assessed for their ability to form tubes throughout their lifespan. To visualize the proportion of senescent cells, the tubes were stained for SA β -gal activity, as were control cells seeded on plastic. The staining procedure revealed that endothelial cells seeded on Matrigel did give rise to age-related SA β -gal staining although the apparent proportion of stained cells on Matrigel was higher than the proportion of cells grown on plastic. To further investigate the consistent difference between cells grown on plastic and Matrigel, another staining molecule was used as the SA β -gal assay does not measure cellular age per se but rather the abundance of lysosomes in the cytoplasm of the cells (Kurz et al, 2000). Thus, the early appearance of SA β -gal staining could be due to an upregulation of the number of lysosomes during cell differentiation when cultured on Matrigel. By tracking the acidic organelles including the lysosomes with fluorescent LysoTracker probes, it was found that tube forming cells exhibited a much stronger level of fluorescence than individual cells seeded on plastic, which correlated well with the difference in SA β -gal staining intensity. Moreover, it was found that the fluorescence intensity as well as the SA β -gal activity increased with time after seeding on Matrigel but not on plastic, which supported the idea that endothelial cells upregulate the number of lysosomes during differentiation. The observed evolution in fluorescence over time was seen in both high passage and low passage cells (Figure 6A-H) supporting this theory of increasing numbers of lysosomes. However, no articles have been published supporting the notion that endothelial cells cultured on

Matrigel should contain more lysosomes than endothelial cells in standard culture. Alternatively, the difference in staining may be explained by the actual differentiation process endothelial cells undergo during tube formation: endothelial cells undergoing tubulogenesis in vivo and in 3D culture systems form a lumen through the production and fusion of multiple acidic cytoplasmic vesicles (Lubarsky & Krasnow, 2003, Ulmasov et al, 2009). Whether endothelial tube formation in two dimensions is accompanied by lumen formation is questionable, but Matrigel does lead endothelial cells down the differentiation pathway (Staton et al, 2009), and as such, it is not inconceivable that the endothelial cells will form intracellular acidic vacuoles when cultured on Matrigel. The vacuoles that contribute to lumen formation are not lysosomes (Ulmasov et al, 2009) and presumably do not contain β -galactosidase. However, the fact that they are acidic might explain why endothelial cells cultured on Matrigel exhibited a higher degree of SA β -gal staining than expected for their age. If SA β -gal is in fact normal lysosomal β -gal, the enzymatic activity is maximal around pH 4. The staining procedure is performed at pH 6.0. However, if the endothelial cells contain a considerable amount of acidic vesicles, that might be enough to shift the pH of the buffer sufficiently low as to allow a greater enzymatic activity and consequently a more intense staining. In accordance with this, the LysoTracker probe does not fluoresce in lysosomes specifically, but rather in acidic vacuoles in general.

The observation that SA β -gal activity increased in cells cultured on Matrigel regardless of age naturally questions the validity of this presumed marker of cellular senescence in assessing the cellular age of differentiating endothelial cells. In order to confirm that the observed increase was unrelated to the replicative age of the cells, cells cultured on plastic and on Matrigel were analysed for the expression of γ -H2A.X, which is a component of the DNA damage response and has been shown to be expressed in the nucleus of senescent cells (Wang et al, 2009). As expected, γ -H2A.X staining was found in the nuclei of high passage cells with a similar frequency regardless of the

culture conditions (Figure 7). In contrast, in low passage cells the staining was negligible both on plastic and Matrigel as would be expected based on the replicative age of the seeded cells. Consequently, a DNA damage response does not appear to be initiated during differentiation on Matrigel. Previously, various studies have questioned the relevance of SA β -gal activity as a relevant marker of cellular senescence. Yang and Hu (2005) found that SA β -gal activity in human foreskin fibroblasts increased not only with cellular age, but also in confluent quiescent cells, in serum-starved cells and in cells treated with H_2O_2 . Additionally, a number of studies have failed to find a correlation between the intensity of SA β -gal staining and donor age in vivo (Cristofalo, 2005). In conjunction with these observations and based on the presented data, SA β -gal activity as a measure of cellular senescence is questionable when assaying differentiating endothelial cells.

In conclusion, from the presented results it appears possible that senescence of the endothelium contributes to the age-related reduction in angiogenesis, and that long-term endothelial cell culture can serve as an appropriate, albeit simple, model of the reduction of angiogenic potential occurring during vascular ageing. This model will enable the high throughput screening of compounds and antibodies having an effect on angiogenesis during ageing. Furthermore, these results indicate that endothelial differentiation when cultured on Matrigel is accompanied by an increase in the number of lysosomes as illustrated by the LysoTracker probe and SA β -gal activity. Additionally, this observation highlights that SA β -gal activity may not be particularly relevant as a marker of senescence during endothelial differentiation and should in any case not be used as the sole marker when evaluating endothelial senescence but should be confirmed by independent markers.

Acknowledgements

We thank Professor Pidder Jansen-Dürr (Austrian Academy of Sciences, Innsbruck, Austria) for the HUVEC NS2 cells. We appreciate the technical support of Helle Jacobsen. Financial support was obtained through the FP6 EU project, PROTEOMAGE (LSHM-CT-2005-518230).

References

Agah A, Kyriakides TR, Letrondo N, Björkblom B, & Bornstein P (2004) Thrombospondin 2 levels are increased in aged mice: Consequences for cutaneous wound healing and angiogenesis. *Matrix Biology* **22**: 539-547

Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt A-, & Stern DM (1998) Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* **91**: 3527-3561

Cristofalo VJ (2005) SA β Gal staining: Biomarker or delusion. *Exp Gerontol* **40**: 836-838

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M, & Campisi J (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A* **92**: 9363-9367

Ha J-, Kim M-, Oh H-, Lee BH, Ahn H-, Shin J-, Baek SH, & Joe YA (2007) Outgrowing endothelial progenitor-derived cells display high sensitivity to angiogenesis modulators and delayed senescence. *FEBS Lett* **581**: 2663-2669

Hampel B, Malisan F, Niederegger H, Testi R, & Jansen-Dürr P (2004) Differential regulation of apoptotic cell death in senescent human cells. *Exp Gerontol* **39**: 1713-1721

Izzo Jr. JL & Mitchell GF (2007) Aging and arterial structure-function relations. *Advances in Cardiology* **44**: 19-34

- Jackson CJ & Nguyen M (1997) Human microvascular endothelial cells differ from macrovascular endothelial cells in their expression of matrix metalloproteinases. *International Journal of Biochemistry and Cell Biology* **29**: 1167-1177
- Jaffe EA, Nachman RL, Becker CG, & Minick CR (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* **52**: 2745-2756
- Kalashnik L, Bridgeman CJ, King AR, Francis SE, Mikhalovsky S, Wallis C, Denyer SP, Crossman D, & Faragher RGA (2000) A cell kinetic analysis of human umbilical vein endothelial cells. *Mech Ageing Dev* **120**: 23-32
- Kurz DJ, Decary S, Hong Y, & Erusalimsky JD (2000) Senescence-associated β -galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J Cell Sci* **113**: 3613-3622
- Lubarsky B & Krasnow MA (2003) Tube morphogenesis: Making and shaping biological tubes. *Cell* **112**: 19-28
- Reed MJ, Corsa AC, Kudravi SA, McCormick RS, & Arthur WT (2000) A deficit in collagenase activity contributes to impaired migration of aged microvascular endothelial cells. *J Cell Biochem* **77**: 116-126
- Sadoun E & Reed MJ (2003) Impaired angiogenesis in aging is associated with alterations in vessel density, matrix composition, inflammatory response, and growth factor expression. *Journal of Histochemistry and Cytochemistry* **51**: 1119-1130

Staton CA, Reed MWR, & Brown NJ (2009) A critical analysis of current in vitro and in vivo angiogenesis assays. *Int J Exp Pathol* **90**: 195-221

Taddei S, Viridis A, Ghiadoni L, Salvetti G, Bernini G, Magagna A, & Salvetti A (2001) Age-related reduction of NO availability and oxidative stress in humans. *Hypertension* **38**: 274-279

Ulmasov B, Bruno J, Gordon N, Hartnett ME, & Edwards JC (2009) Chloride intracellular channel protein-4 functions in angiogenesis by supporting acidification of vacuoles along the intracellular tubulogenic pathway. *Am J Pathol* **174**: 1084-1096

Unger RE, Krump-Konvalinkova V, Peters K, & James Kirkpatrick C (2002) In vitro expression of the endothelial phenotype: Comparative study of primary isolated cells and cell lines, including the novel cell line HPMEC-ST1.6R. *Microvasc Res* **64**: 384-397

Unterluggauer H, Hampel B, Zwerschke W, & Jansen-Dürr P (2003) Senescence-associated cell death of human endothelial cells: The role of oxidative stress. *Exp Gerontol* **38**: 1149-1160

Vasile E, Tomita Y, Brown LF, Kocher O, & Dvorak HF (2001) Differential expression of thymosin β -10 by early passage and senescent vascular endothelium is modulated by VPF/VEGF: Evidence for senescent endothelial cells in vivo at sites of atherosclerosis. *FASEB Journal* **15**: 458-466

Wang C, Jurk D, Maddick M, Nelson G, Martin-Ruiz C & von Zglinicki T (2009) DNA damage response and cellular senescence in tissues of aging mice. *Aging Cell* **8**: 311-323

Watanabe Y, Lee SW, Detmar M, Ajioka I, & Dvorak HF (1997) Vascular permeability factor/vascular endothelial growth factor (VPF/ VEGF) delays and induces escape from senescence in human dermal microvascular endothelial cells. *Oncogene* **14**: 2025-2032

Yang J, Chang E, Cherry AM, Bangs CD, Oei Y, Bodnar A, Bronstein A, Chiu C-, & Herron GS (1999) Human endothelial cell life extension by telomerase expression. *J Biol Chem* **274**: 26141-26148

Yang NC, Hu ML (2005) The limitations and validities of senescence associated- β -galactosidase activity as an aging marker for human foreskin fibroblast Hs68 cells. *Exp Gerontol* **40**: 813-819

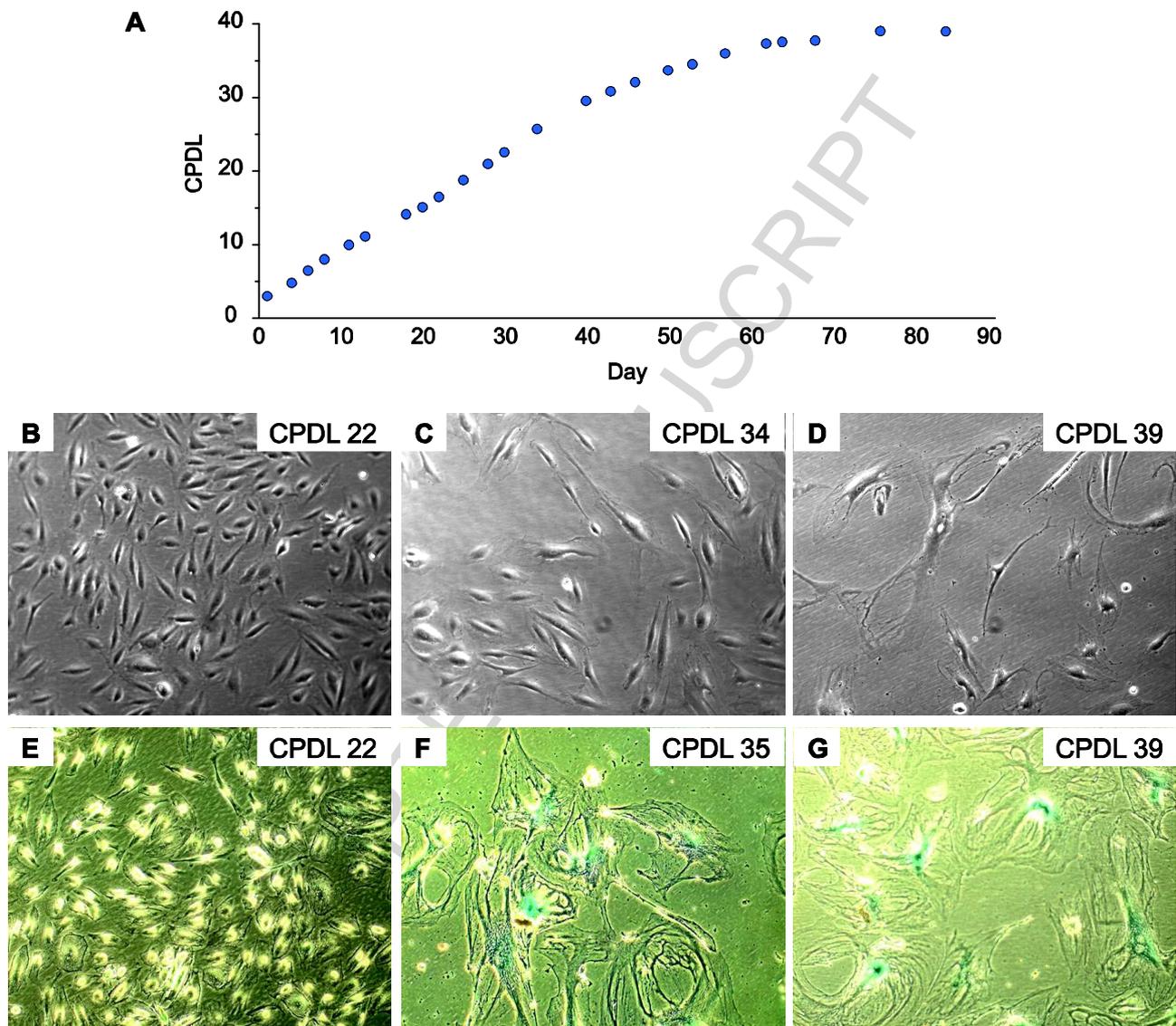


Figure 1. Cultivation of primary human microvascular endothelial cells (HMVEC). A: growth curve showing the cumulative population doubling level (CPDL) during culturing from young to senescent. B-D: cellular morphology changes with advancing cellular age from small cobblestone shaped young cells to large cells with multiple protrusions at old age. E-G: the activity of SA β -gal increases with advancing cellular age. B: CPDL 22, C: CPDL 34, D: CPDL 39, E: CPDL 22, F: CPDL 35, G: CPDL 39. Scale bar 100 μ m.

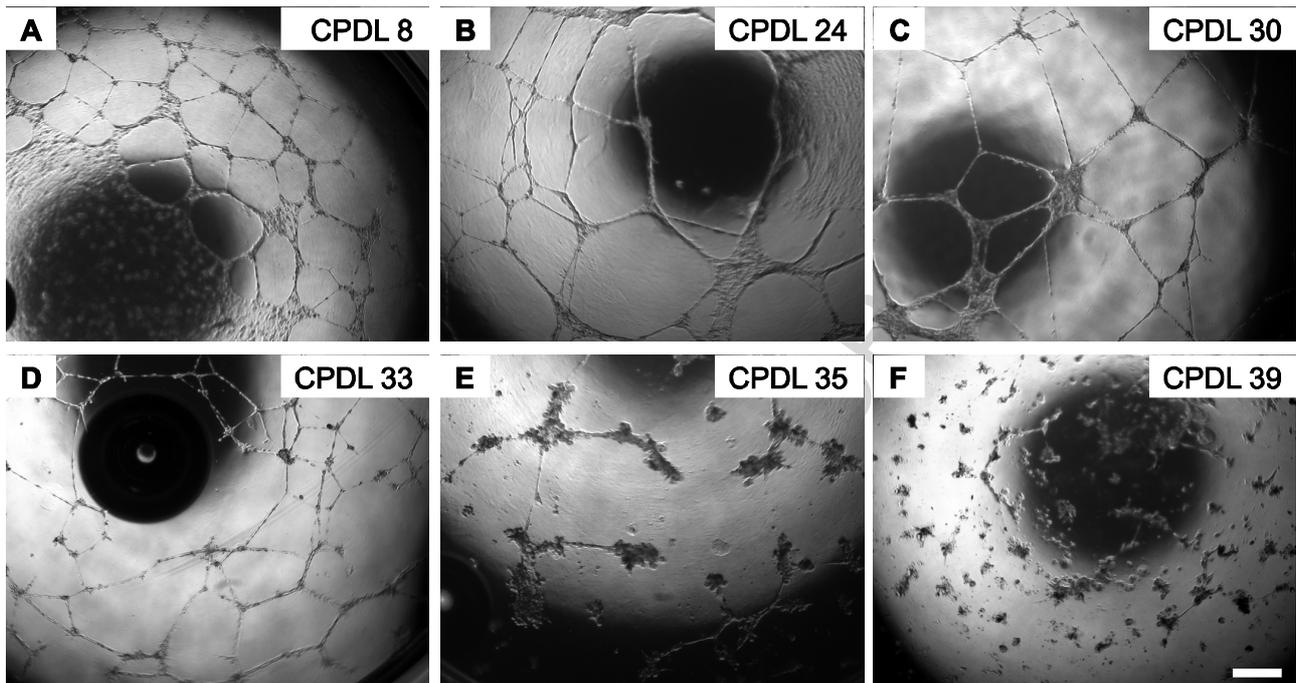


Figure 2. Tube formation ability of HMVEC decreases with advancing cellular age. A network of short and branched tubes were observed at CPDL 8 (A), while older cultures at CPDL 24 (B), 30 (C) and 33 (D) showed longer and fewer branch points. In the oldest cultures, very few tubes were seen at CPDL 35 (E) and virtually none at CPDL 39 (F) although the cells formed clusters resembling branch points. Scale bar 400 μm .

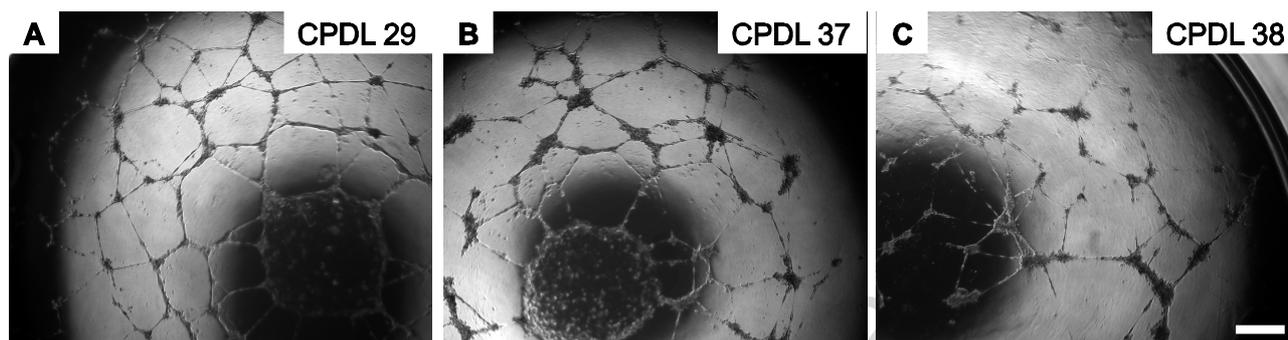


Figure 3. Tube formation ability of HUVEC decreases with advancing cellular age. A gradual move from a network of short and branched tubes at CPDL 29 (A) over longer tubes with fewer branch points at CPDL 37 (B) to very few tubes and clusters of cells at CPDL 38 (C) was observed. Scale bar 400 μm .

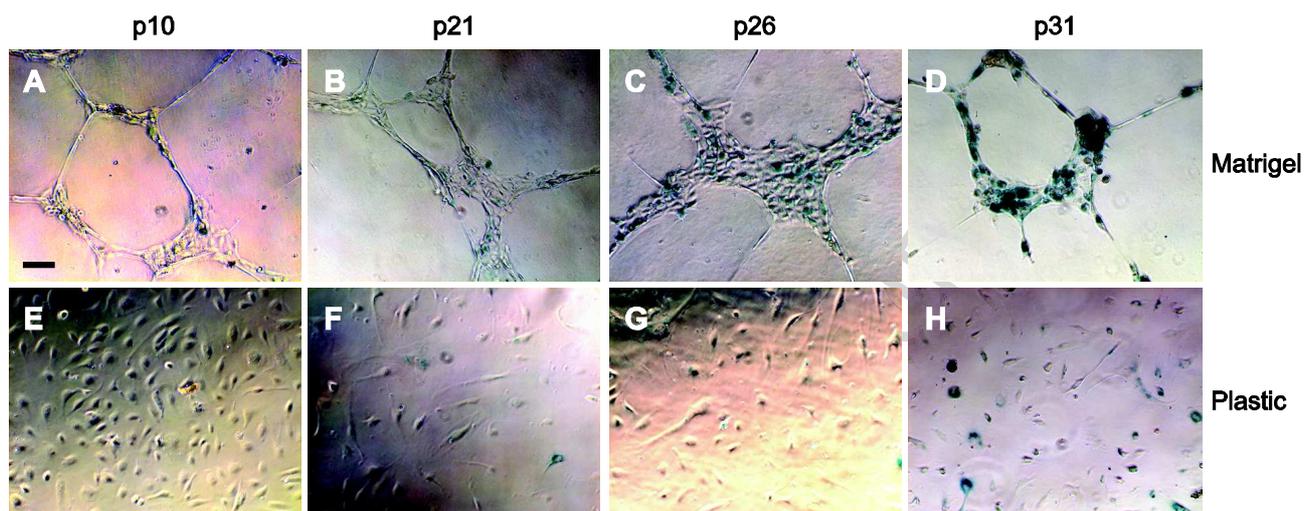


Figure 4. HUVEC NS2 tubes stain positively for SA β -gal activity in an age-related manner. HUVEC NS2 cells were either seeded on Matrigel (A-D) or on plastic (E-H) at different population doublings. Slight SA β -gal staining was seen in young p10 (A) Matrigel cultures with increasing staining in the older cultures p21 (B) and p26 (C) without staining cells grown on plastic (E-G). Pronounced SA β -gal staining was seen in old cells p31 (D and H) regardless of culture conditions. Scale bar 100 μ m.

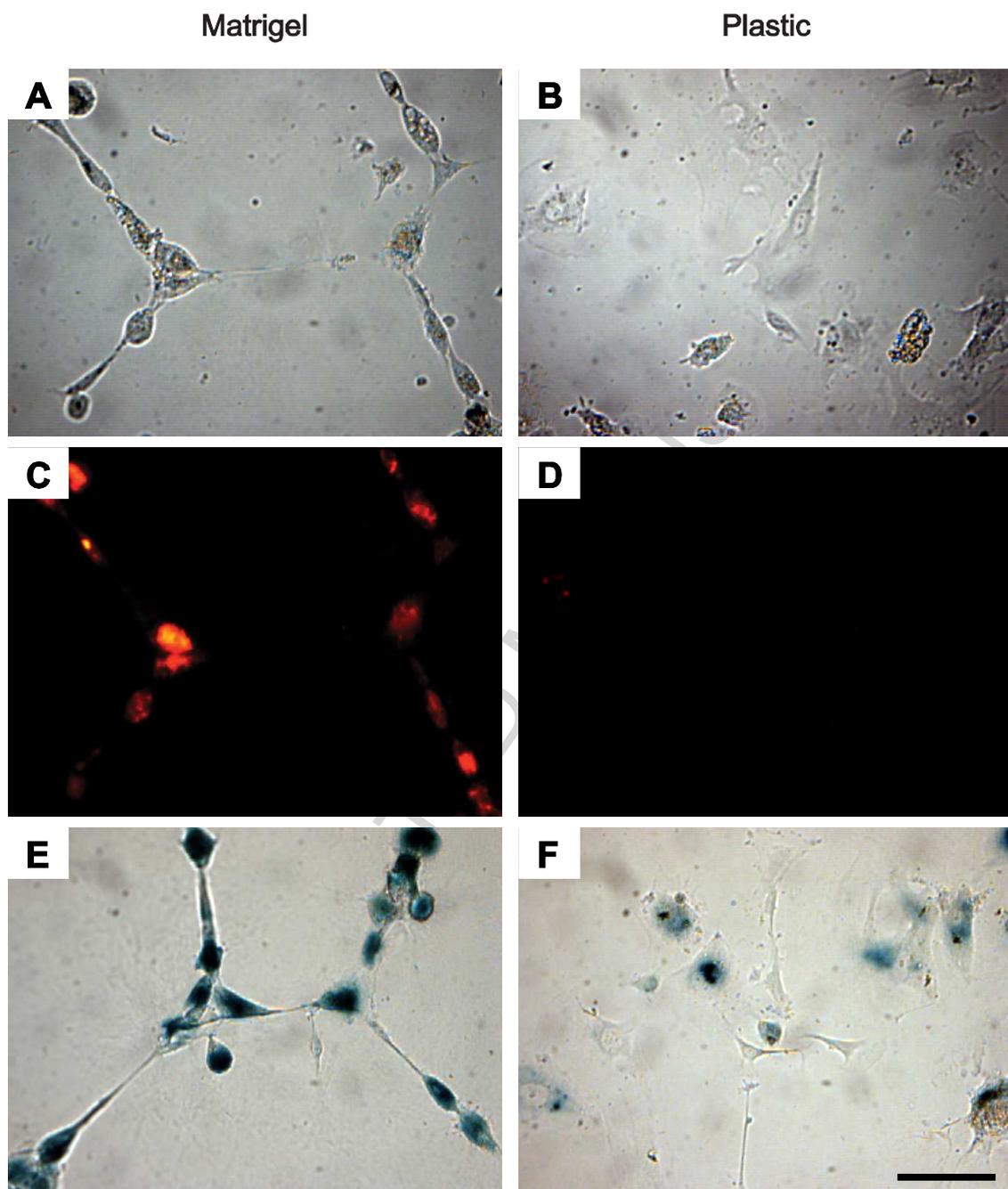


Figure 5. Correlation of LysoTracker and SA β -gal staining on HUVEC NS2 p28 tubes. HUVEC NS2 cells were cultured on Matrigel (A, C and E) or plastic (B, D and F). Light microscopy was used to visualize SA β -gal stained cells (E and F) as well as LysoTracker stained cells (A and B), which exhibit a red fluorescence upon localization to acidic vacuoles (C and D). Both LysoTracker and SA β -gal staining were more pronounced in tube forming cells (C and E). Scale bar 100 μ m.

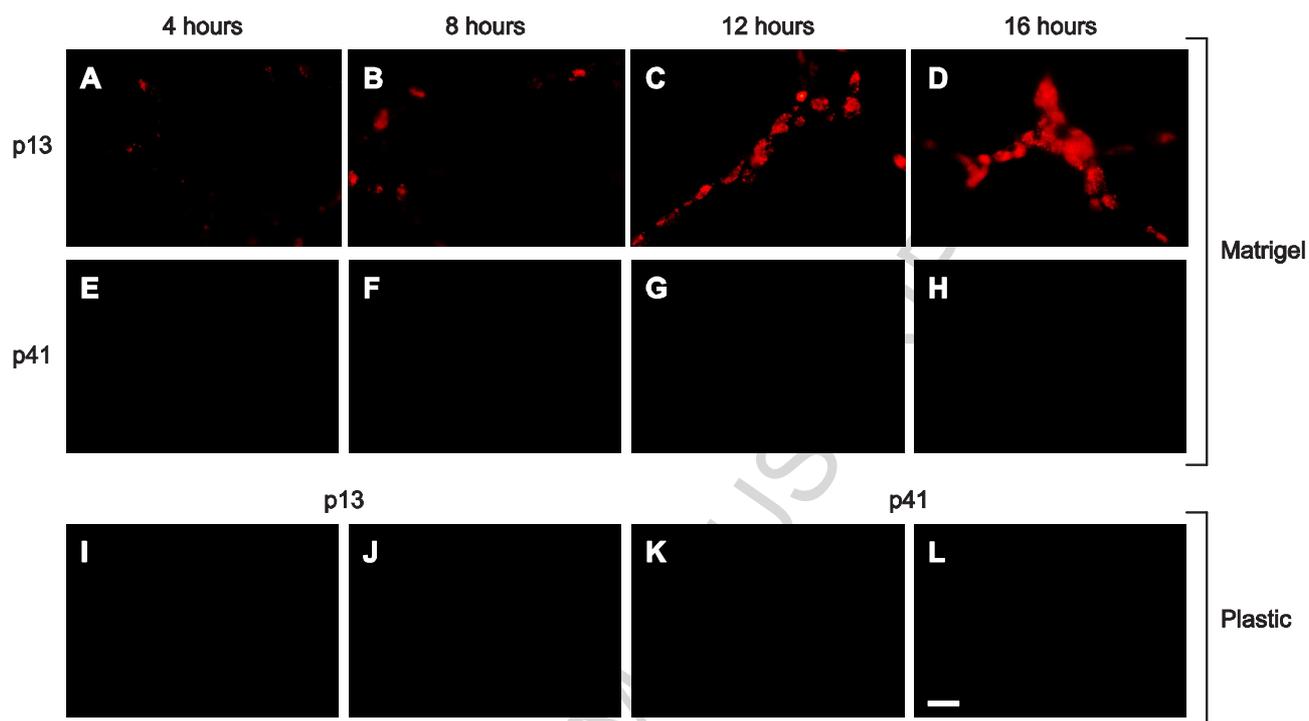


Figure 6. Kinetic study of LysoTracker staining. HUVEC NS2 p13 and p41 cells were cultured on Matrigel (A-H) or plastic (I-L) and incubated with the LysoTracker probe 4, 8, 12 and 16 hours after seeding. A clear increase of LysoTracker staining with time for both low passage (A-D) and high passage (E-H) cells was evident, although the staining was more pronounced for high passage cells throughout the experiment as would be expected. No evolution of LysoTracker staining was observed for cells seeded on plastic during culturing (I-L, 4 hour time point shown) but as anticipated a higher proportion of high passage cells was stained (L) compared to low passage cells (J). Scale bar 25 μm .

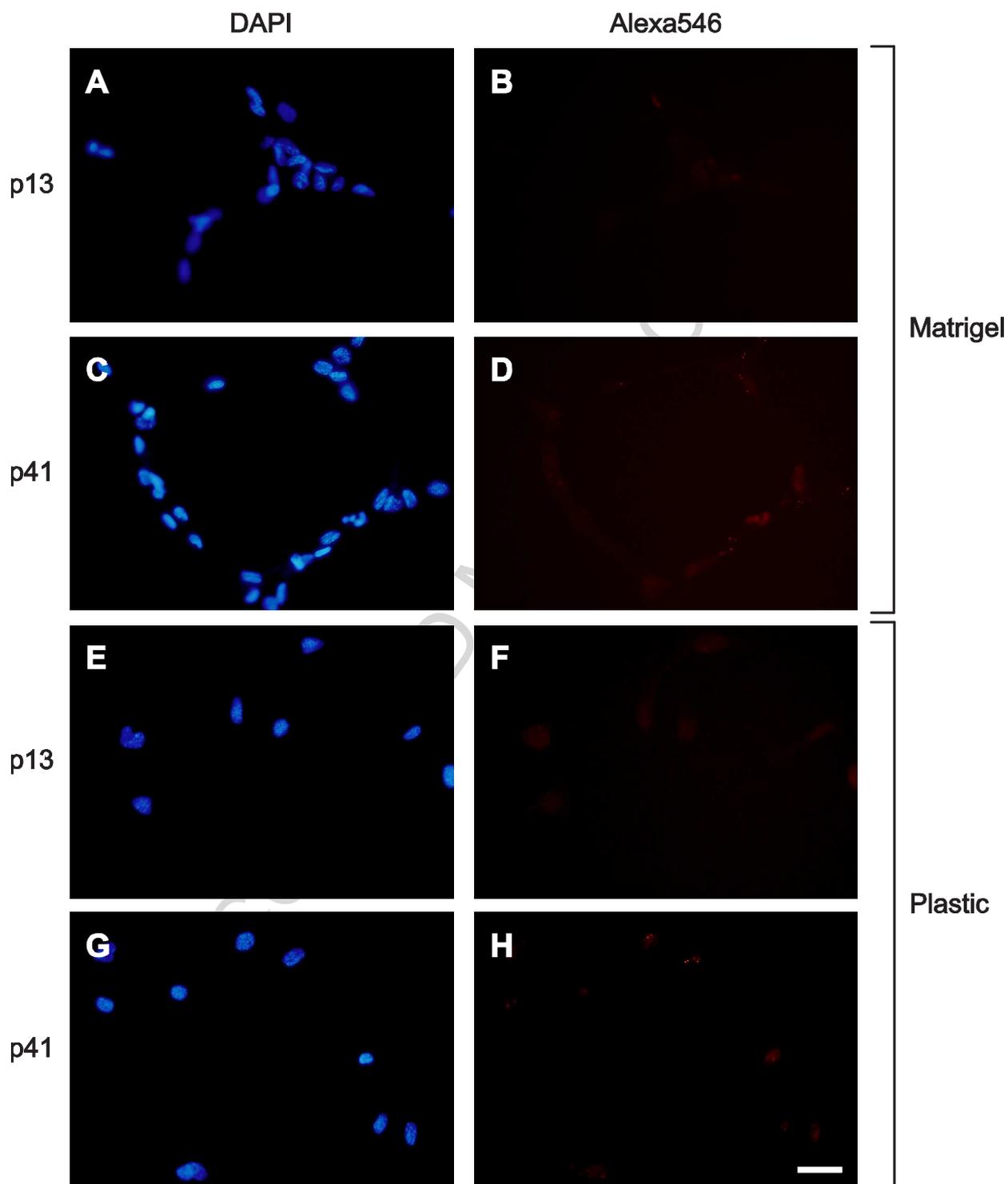


Figure 7. Immunocytochemical staining to evaluate the amount of senescent HUVEC NS2 cells following differentiation. HUVEC NS2 p13 and p41 cells were cultured on Matrigel (A-D) or plastic (E-H) and immunostained with the DNA damage response marker, γ -H2A.X antibody (B, D, F and H) using DAPI to visualize the cell nucleus (A, C, E and G). As expected, the proportion of nuclear γ -H2A.X punctates was higher among high passage HUVEC (D and H) than in low passage

HUVEC (B and F), while no difference between cells cultured on Matrigel (B and D) and cells cultured on plastic (F and H) was observed. Scale bar 25 μm .

ACCEPTED MANUSCRIPT