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PhD Thesis

FREQUENCY, ANGIOGENIC POTENTIAL, PHENOTYPE AND MOLECULAR SIGNATURE OF ENDOTHELIAL COLONY-FORMING CELLS ISOLATED FROM PATIENTS WITH CLASSIC KAPOSI'S SARCOMA

MED/04

PhD Student: Francesca Calcaterra

Tutor: Prof.ssa Silvia Della Bella

Doctoral School Coordinator: Prof. Massimo Locati

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Abstract

Background

Kaposi's Sarcoma (KS) is a lymphangioproliferative disease whose causative agent is herpesvirus HHV-8. KS is characterized by hyperproliferation of HHV-8 infected spindle cells - cells of endothelial origin that represent the typical component of KS lesions. In previous studies we reported that circulating endothelial progenitor cells (EPCs), identified as $CD45^{dim}/CD34^{+}/KDR^{+}$, are increased in patients with classic KS (cKS) and we also demonstrated that in cKS patients EPCs - isolated and cultured as Endothelial colony-forming cells (ECFCs) - are HHV-8 infected and can act as viral reservoir. Therefore, in this study we investigated whether ECFCs isolated from cKS patients are endowed with features typical of spindle cells in order to evaluate whether they may represent the precursors of spindle cells.
Moreover, in preliminary studies we surprisingly observed that not only ECFCs isolated from cKS patients but also ECFCs isolated from healthy donors expressed LYVE-1 and podoplanin – lymphatic markers expressed by spindle cells in KS lesions. Since several studies supported the hypothesis that adult lymphangiogenesis could be promoted by the presence of bone marrow derived lymphatic endothelial progenitor cells (LEPCs) we also investigated the lymphangiogenic potential of ECFCs to evaluate whether they can act also as LEPCs. In particular, we evaluated the expression of lymphatic markers in basal condition and we investigated whether the lymphatic differentiation of ECFCs could be fostered by fibronectin a component of the tumor microenvironment whose presence correlates with tumor lymphangiogenesis and metastasis. In addition, we also evaluated whether migration stimulating factor (MSF), an oncofetal isoform of fibronectin released by cancer stromal cells and tumor associated macrophages, could promote lymphatic differentiation of ECFCs.

Methods

83 cKS patients and 86 healthy HHV-8 seronegative donors were enrolled in the study. ECFCs were isolated using a protocol previously optimized in our lab. PBMCs were seeded in EGM-2 medium in culture plates coated with fibronectin and ECFC colonies were identified by microscopic visual inspection as colonies of cells with cobblestone-like morphology. Once isolated, ECFC colonies were expanded in culture plates coated with collagen. During the isolation phase, the time of appearance and the frequency of ECFC colonies were analyzed. During the following expansion phase, ECFC phenotype and the presence of HHV8-infection - assessed as expression of the viral latent nuclear antigen (LANA) - were analyzed by immunofluorescence. ECFC were functionally characterized by evaluating their cell viability, proliferative potential, vasculogenesis ability by Matrigel assay and cytokine production by ELISA. The molecular signature of ECFCs was also analyzed by gene array analysis.
To investigate the lymphatic differentiative potential of ECFCs the expression of typical lymphatic markers (PROX-1, podoplanin, LYVE-1 and VEGFR-3) was analyzed by Real Time PCR and confocal microscopy. To evaluate the possible role of fibronectin in promoting lymphatic differentiation, ECFCs were cultured on either fibronectin or collagen and the effects of stimulation with MSF were also analyzed.

Results

In our study ECFC colonies appeared earlier ($p<0.001$) and with higher frequency ($p<0.001$) when isolated from cKS patients than healthy donors. Moreover, the frequency of ECFC colonies was higher in cKS patients with rapidly evolving disease than in cKS patients with slowly evolving

disease ($p<0.05$). All screened ECFC colonies isolated from cKS patients contained HHV8-infected cells. During the expansion phase, ECFCs isolated from cKS patients were endowed with a higher proliferative potential ($p<0.05$), a higher vasculogenic ability in vitro ($p<0.05$) and a higher production of IL-6 ($p<0.05$) than ECFCs isolated from healthy donors. In addition, preliminary analysis of the gene expression profile revealed that patients and healthy controls segregated by clustering, thus confirming that gene expression profile differs between ECFCs isolated from cKS patients and healthy donors.
A further relevant result of this study was the observation that ECFCs are endowed with the ability to express the typical lymphatic markers PROX-1, podoplanin, LYVE-1 and VEGFR-3. In particular, lymphatic markers were expressed by donor-derived ECFCs cultured on both fibronectin and collagen and they were upregulated by ECFC treatment with MSF ($p<0.05$).

Conclusion

In this study we demonstrated that ECFCs obtained from cKS patients are endowed with features that may be particularly relevant to KS pathogenesis, suggesting that ECFCs may act as putative precursors of the spindle cells and contribute to the development of KS lesions.
Moreover, we demonstrated that ECFCs isolated from peripheral blood of adult healthy donors expressed markers typical of lymphatic endothelium, suggesting that ECFCs may act also as LEPCs thus participating in lymphangiogenic and lymphovasculogenic processes.

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1.**Introduction**

1.1 Endothelial Progenitor Cells (EPCs)

1.1.1 EPCs in the development of blood vascular system and postnatal neovascularization

In vertebrates, the vascular system is composed by a complex network of vessels essential for the transport of gases, fluids, macromolecules and cells [Adams RH et al, 2007]. Its development represents one of the earliest events that take place during embryonic organogenesis [Coultas L et al, 2005]. In particular, in mouse embryo, vascular system development starts at day 6.5-7 of gestation when mesodermal precursors, known as hemangioblasts, aggregate forming structures called "Blood Islands" [Dzierzak E et al, 1998].

Hemangioblasts are multipotent precursors, able to differentiate in both hematopoietic and endothelial precursors [Carmeliet P et al, 2003]. In embryo, the development of the vascular system is based on two mechanisms: vasculogenesis and angiogenesis. In particular, vasculogenesis - which consists in de novo formation of blood vessels by aggregation of endothelial precursors - allows the formation of the primitive vascular plexus starting from blood island mesodermal precursors whereas angiogenesis – which consists in blood vessel formation starting from pre-existing vessels – mediates the remodelling process of the primitive vascular plexus that lead to the formation of a functional vascular system [Coultas L et al, 2005].

In postnatal life, new blood vessel formation occurs in several physiological and pathological conditions. In particular, it is required for the proper postnatal development and contributes to physiological processes which necessitate of endothelial cell proliferation such as tissue repair and regeneration or angiogenesis of female reproductive system during ovarian cycle and pregnancy [Marçola M et al, 2015]. Moreover, neovascularization in adult life is also a key process of some important pathological conditions such as tumor growth (so called tumor-angiogenesis) [Marçola M et al, 2015].

Until 15 years ago, it was thought that in postnatal life only angiogenesis contributes to new blood vessel formation whereas vasculogenesis took place only during embryonic development. In 1997 and 1998 respectively, two groups described for the first time circulating endothelial progenitor cells (EPCs) in adults [Asahara T et al, 1997; Shi Q et al, 1998]. In particular, they reported that human $CD34^+$ cells isolated from bone marrow, umbilical cord blood and peripheral blood could differentiate into endothelial cells in vitro and in vivo and could contribute to neo-endothelialization and neovascularization in adult organisms in in vivo murine models [Asahara T et al, 1997; Shi Q et al, 1998]. Therefore, it was proposed that also in postnatal life both

angiogenesis and vasculogenesis could contribute to neovascularization and that both mature endothelial cells and EPCs could be involved in these processes. In particular, it was hypothesized that EPCs could play a crucial role in postnatal vascularization since mature endothelial cells are terminally differentiated and endowed with a low proliferative potential [Peichev M et al, 2000; Ishikawa M et al, 2004].

In the following years, further studies were performed to better define EPC phenotype and functions. On the basis of "precursor cell" definition, the authors proposed that cells identified as EPCs must possess specific features. In particular, EPCs must express stemness markers (i.e. CD34, CD133 or CD117) [Peichev M et al, 2000] and endothelial markers such as Kinase insert Domain Receptor - KDR (also called vascular endothelial growth factor receptor 2 – VEGFR-2 – or flk-1). In support of this hypothesis, Pelosi et al reported that $CD34^+/KDR^+$ bone marrow-derived cells were endowed with high proliferative potential and could generate both hematopoietic and endothelial precursors in vitro [Pelosi E et al, 2002]. Moreover, EPCs must be characterized by the ability to differentiate into mature endothelial cells, acquiring - during in vitro culture - the expression of typical endothelial markers (i.e. basal expression of CD31, CD144, Von Willebrand factor (vWF) and eNOS and e-selectin expression after stimulation with proinflammatory cytokines) [Asahara T et al, 1997; Kaushal S et al, 2001]. Finally, Urbich et al proposed that EPCs must be endowed with a high proliferative potential and the ability to incorporate in pre-exsisting vessels in order to support endothelial repair and vascularization [Urbich C et al, 2004]. Since in literature several protocols for EPCs in vitro culture and characterization are reported, EPC phenotype and functions were deeply debated; therefore this topic will be discussed in a dedicated chapter.

EPCs are usually located in the bone marrow inside the "stem cell niche" and only a low number of EPCs circulate in peripheral blood. EPCs can be mobilized from the bone marrow by physiological and pathological stimuli coming from the peripheral tissues. In particular, EPC mobilization from the bone marrow is promoted by angiogenic factors (VEGF-A), cytokines (GM-CSF, SDF-1), hormones (erythropoietin, estrogens) and drugs (statins) [Dimmeler S et al, 2001; Vasa M et al, 2001 a; Heeschen C et al, 2003; Strehlow K et al, 2003; Walter DH et al, 2002]. In fact, high levels of angiogenic factors and cytokines – in particular VEGF and SDF-1 - can be detected in hypoxic peripheral tissues where they induce EPC mobilization [Marçola M et al, 2015; Xiao Q et al, 2008; Yamaguchi J et al, 2003; Shintani S et al, 2001; Kalka C et al, 2000]. The immune cells located in hypoxic tissues also promote EPC mobilization participating in the production of soluble factors. In particular, macrophages and T-lymphocytes produce VEGF that induces the production of

monocyte chemoattractant protein (MCP)-1 by endothelial cells [Jaipersad AS et al, 2014]. MCP-1 increases the permeability of endothelial layer and attracts EPCs [Fujiyama S et al, 2003; Hong KH et al, 2005].

EPC mobilization relies on the activation of complex mechanisms [Heissig B et al, 2002]. Several studies demonstrated that EPC mobilization, beyond the action of soluble factors (mainly VEGF and SDF-1), required proteinases and metalloproteases [Takahashi T et al, 1999]. In particular, soluble factors such as SDF-1 create a chemotactic gradient that promote EPCs mobilization whereas proteases and metalloproteases degrade extracellular matrix, cleaving the interaction between EPCs and the bone marrow stroma allowing EPC mobilization. Moreover, the metalloprotease MMP-9 cleaves the ligand c-Kit which is present on the membrane of bone marrow cells (m-Kit) thus promoting the release of c-Kit in its soluble form (s-Kit, also called stem cell factor- SCF) that promotes EPC mobilization [Heissig B et al, 2002]. Also nitric oxid (NO) produced by stromal cells and growth factors produced by hematopoietic stem cells foster EPC mobilization [Aicher A et al, 2003].

Once EPCs are released from bone marrow into the bloodstream, they migrate toward the angiogenic sites where they home and differentiate acquiring all the phenotypic features typical of mature endothelial cells. During the homing process, EPCs adhere and then transmigrate through the cytokine-activated endothelial cells to reach the ischemic tissues. EPC adhesion to endothelial cells is mediated by integrins. In particular, β2-integrin and α4β1-integrin mediate adhesion and are also involved in EPC transmigration [Chavakis E et al, 2005]. Moreover, EPC adhesion to denuded vessels is mediated by vitronectin receptors –αVβ3 and αVβ5 integrins – and β1-integrins that bind extracellular matrix [Urbich C et al, 2004].

In the site of angiogenesis, EPCs promote revascularization directly by proliferating, integrating in the pre-existing vessels and differentiating into mature endothelial cells; moreover, they support neovascularization also in a paracrine manner, by releasing soluble factors that in turn sustain angiogenesis.

In the last years, several studies described the presence of EPCs inside niches within the vascular walls [Psaltis PJ et al, 2015]. These cells, called vascular wall EPCs (VW-EPCs) were described by Ingram et al in 2005 [Ingram DA et al, 2005]. In this study, the authors analyzed by clonogenic assays mature endothelial cells derived from vessel walls. In particular, they analyzed two different population of mature endothelial cells - HUVEC (Human Umbilical Cord Endothelial Cells) and HCAEC (Human Coronary Artery Endothelial Cells) - and they observed that both populations

contained a hierarchy of low and high proliferative EPCs endowed with self-renewal capacity [Ingram DA et al, 2005]. Fang at al also identified a population of endothelial colony-forming cells within endothelial cells isolated from pulmonary vessels of adult mice [Fang S et al, 2012]. These observations support the hypothesis that adult neovascularization could be supported also by EPCs resident within the vessel walls [Psaltis PJ et al, 2015].

1.1.2 Role of EPCs in physiological and pathological conditions

In healthy subjects, the endothelial cell turnover is low in basal condition and can be reactivated in some physiological conditions. In fact, neo-endothelialization and neovascularization processes are required for the manteinance of endothelial homeostasis and also occur in female reproductive system during ovarian cycle and pregnancy [Marçola M et al, 2015].

Since mature endothelial cells are endowed with a low proliferative potential, circulating EPCs seem to play a crucial role in neovascularization process in adult. In particular, in vivo studies in animal models demonstrated that labeled donor-derived progenitor cells could be detected in ischemic tissues of recipient animals after bone marrow transplantation [Crosby JR et al, 2000; Jackson KA et al, 2001]. These evidences were then confirmed also in clinical studies by observation that EPCs can be mobilized after tissue injury (i.e. burns, coronary artery bypass or acute myocardial infarction) thus contributing to neovascularization processes [Urbich C et al, 2004].

Moreover, clinical studies showed that EPCs alteration in number and/or functions can be associated to phatological conditions and among them cardiovascular diseases are the most studied and best characterized. In fact, EPC alterations represent an independent risk factor for cardiovascular diseases [Schmidt-Lucke C et al, 2005]. Several studies reported EPC dysfunction or reduction in patients affected by cardiovascular diseases such as CAD (coronary artery disease) [Lee PS et al, 2014], PAD (peripheral artery disease) [Fadini GP et al, 2006 a; Fadini GP et al, 2006 b] or congestive heart failure [Nonaka-Sarukawa M et al, 2007; Michowitz Y et al, 2007]. In addition, EPC alterations are also associated to cardiovascular risk factors such as smoking, hypertension, diabetes and hypercholesterolemia. In particular, one of the mechanisms through which smoking promotes the development of cardiovascular diseases consists in the reduction of EPC number due to the increased oxidative stress and the reduced nitric oxide bioavailability [Werner N et al, 2005]. Therefore, EPCs were proposed as a biological and prognostic marker to

assess the severity of cardiovascular diseases and to predict events in patients with CAD [Lee PS et al, 2014].

Besides cardiovascular diseases, EPC alterations have also been reported in other pathological conditions. In particular, dysfunctional and reduced EPCs have been described in diseases associated with impaired endothelial repair capacity and reduced vasculogenic ability [Calcaterra F et al, 2014] whereas increased EPC number has been reported in pathological conditions - such as tumor -which are characterized by increased vasculogenesis [Marçola M et al, 2015]. In fact, the number of circulating EPCs has been reported to be increased in patients with many different types of cancer and, in some cases, it correlates with the volume of the tumoral mass thus supporting the hypothesis that EPCs play a role in neovascularization that fosters tumor development by promoting tumor growth and metastasis formation [Bertolini F et al, 2006; Marçola M et al, 2015]. Therefore it has been proposed that EPC number could be used as a prognostic and/or diagnostic biomarker and that EPCs could represent a possible target for new strategies for tumor treatment [Bertolini F et al, 2006; Fang D et al, 2010]. Moreover, the role of EPCs in tumor neovascularization was investigated by several research groups using mouse models and reporting that EPCs are able to incorporate into the vasculature of solid tumor xenografts; nevertheless, the extent of the reported contribution is highly variable ranging from 0% to about 100% [Lyden D et al, 2001; De Palma M et al, 2003; Purhonen S et al, 2008]. The different results reported may rely on the different strategies used for EPC identification into neovascularization sites and on the different tumor model used (type of tumor and tumor stage) [Gao D et al, 2009]. In 2005, Peters et al analyzed tumor samples derived from individuals who developed cancer after bone-marrow transplantation with donor of the opposite sex. In particular, they used multicolor FISH technique with X or Y chromosome specific probes and observed that about 5% of endothelial cells in tumor were bone-marrow derived supporting the hypothesis that EPCs participate in tumor neovascularization [Peters BA et al, 2005].

In conclusion, EPCs could be a possible new target for therapies that aim to promote angiogenesis in pathological conditions characterized by defective angiogenesis and could represent a novel source of autologous cells to promote vascularization of ischemic tissues [Sukmawati D et al, 2015].

1.1.3 Strategies to study EPCs in adult peripheral blood

In 1997, Asahara's group described for the first time EPCs showing that isolated $CD34^+$ hematopoietic progenitor cells obtained from adult peripheral blood resembled the embryonic angioblasts, were able to differentiate in endothelial cells and were involved in revascularization processes [Asahara T et al, 1997]. After this first description, several approaches have been used to study and characterize EPCs. In particular, the approaches reported in literature can be divided in two main strategies: identification of circulating EPCs based on cell surface markers expression or in vitro culture of EPCs isolated from peripheral blood [Fadini GP et al, 2012; Hirschi KK et al, 2008].

1.1.3.1 EPC identification by flow cytometry

The first strategy that can be used for EPC characterization is based on the use of antigen-specific antibodies conjugated to a fluorochrome that allow the identification, by flow cytometry, of specific cell populations in blood samples. The advantages of this technique, which is deemed as the gold standard for EPC quantification in peripheral blood, are: the rapidity of execution, requirement of small volume of blood and no need for ex vivo manipulation of the sample. The inconvenience of this approach is that, even after years of research, there is not yet a consensus on the marker combination to be used for EPC identification [Timmermans F et al, 2009; Basile DP et al, 2014]. According to the definition of "endothelial progenitor", at least one marker of immaturity/stemness plus at least one marker of the endothelial lineage are required to define the antigenic phenotype of EPCs (Figure 1).

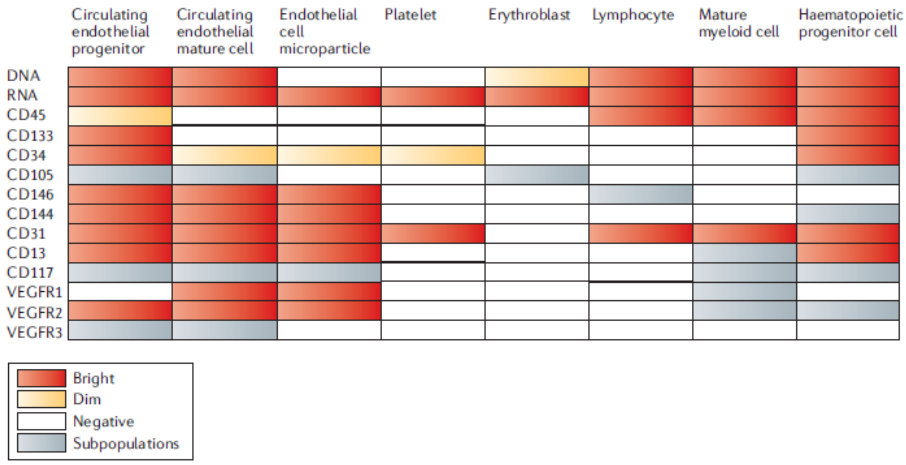


Figure 1: Antigen expression in circulating endothelial progenitor cells (EPCs), circulating endothelial mature cells, microparticles, platelets and hematopoietic cells (Bertolini F et al, Nat Rev Cancer, 2006).

In particular, CD34 and/or CD133 are used as stemness markers; CD34 is the prototypical stem cell antigen and CD133 is expressed on the surface of more immature hematopoietic cells than CD34. On the other hand, KDR is the most common endothelial marker used for EPC identification.

Unfortunately, the specificity of this combination is impaired by the fact that these markers are expressed also by circulating hematopoietic progenitors. In addition, circulating endothelial cells (CECs) are KDR^+ and may also express CD34. Therefore, the analysis of CD45 expression was introduced in the protocol for EPC identification in order to exclude hematopoietic cells which express CD45. Notably, Case et al demonstrated that the vast majority of $CD34^+/CD133^+/KDR^+$ cells in the blood are actually hematopoietic progenitor cells (HPC) expressing CD45; moreover, they showed by endothelial cell and hematopoietic cell clonogenic assays that only the rare $CD45^-$ subpopulation is not endowed with hematopoietic activity and should therefore represent the true EPCs [Case J et al, 2007].

Nevertheless, despite only $CD45^-$ subpopulation seems to contain the true EPCs and CD133 marker allows the distinction between EPCs and mature endothelial cells, the cell population identified with marker combination that includes also CD45 and CD133 is really rare and difficult to be detected with sufficient accuracy to be used in clinical studies as biomarker [Fadini GP et al, 2012]. Therefore, the most used combination for defining EPCs in clinical studies is $CD45^{dim}/CD34^+/KDR^+$ as it allows the identification of a population that has widely been demonstrated to correlate with the severity of cardiovascular disesases. In this respect, several studies have demonstrated that $CD45^{dim}/CD34^+/KDR^+$ cells can be used as valuable prognostic marker as well as a marker for treatment monitoring [Schmidt-Lucke C et al, 2010].

1.1.3.2 Isolation and in vitro culture of EPCs

The second strategy to study EPCs consists in EPCs in vitro culture. This approach allows to obtain a number of cells sufficient for their detailed phenotypic and functional characterization. EPCs obtained in vitro can also be use for in vivo experiments in animals and for cell-based therapies [Fadini GP et al, 2012].

However, after the first description by Asahara, several protocols were proposed for isolation and in vitro culture of EPCs from peripheral blood mononuclear cells (PBMCs). Despite the use of different protocols allows the isolation of different types of cells, all these cell populations were initially called EPCs on the basis of their endothelial phenotype [Prater DN et al, 2007]. This confused situation was clarified for the first time in 2007 by Ingram, Yoder and colleagues who

described and compared the three cell types commonly called EPCs and their respective cell culture protocols [Yoder MC et al, 2007]. The three cell types are named: colony forming unit (CFU)-Hill cells, circulating angiogenic cells (CAC) and Endothelial colony-forming cells (ECFCs) and they are characterized by different phenotype, angiogenic potential and functions [Madonna R, 2015]. Among these cell populations, only ECFCs are considered the true EPCs [Fadini GP et al, 2012] (Figure 2).

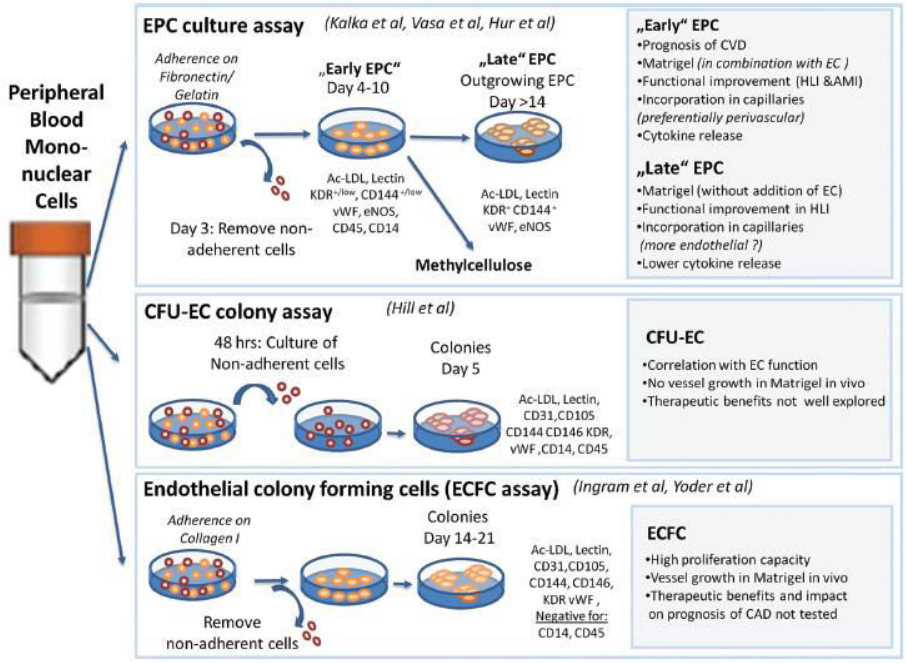


Figure 2: Cell types commonly called EPCs in the past and their respective cell culture protocols (*Fadini GP et al, Circ Res 2012).*

1.1.3.2.1 Endothelial colony-forming cells (ECFCs)

In 2004, Ingram et al described a culture method that allowed to obtain a population of EPCs called ECFCs [Ingram DA et al, 2004]. Briefly, PBMCs are seeded in culture plates coated with collagen-I and are cultured in presence of conditions that promote endothelial differentiation; ECFC colonies appeared 2-3 weeks after PBMCs seeding and are composed by cells with endothelial characteristics. Due to their phenotypic and functional features, ECFCs are considered to be the true EPCs [Ingram DA et al, 2004; Ligi I et al, 2011; Rosti V et al, 2010; Leicht SF et al, 2011; Medina RJ et al, 2010; Jodon de Villeroche V et al, 2010]. In fact, ECFCs are characterized by cobblestone-like morphology, express endothelial markers (i.e. CD31, CD34, CD105, CD144, CD146, KDR and vWF), up-take Dil-acLDL (acetylated LDL labeled with 1,1' - dioctadecyl – 3,3,3',3' - tetramethyl - indocarbocyanineperchlorate), bind Ulex europaeus lectin 1 (UEA-1) and do not express CD45, CD14 and CD115. ECFCs also display a relatively high level of telomerase and a high proliferative potential [Ingram DA et al, 2004]. In particular, basing on a clonogenic single cell

assay, Ingram et al described in ECFC population the existence of a hierarchy of EPCs. In fact, using this strategy, ECFCs can be divided according to their proliferative and clonogenic potential thus identifying a population endowed with a high proliferative potential (HPP-ECFCs) [Ingram DA et al, 2004]. In addition, ECFCs promote in vivo angiogenesis by contributing to vessel formation in models of ischemia and when implanted subcutaneously in matrices, such as collagen, or in human skin substitutes [Reviewed in Critser PJ et al, 2010].

In literature, a second protocol that allows to obtain cells endowed with characteristics similar to those of ECFCs was described [Gulati R et al, 2003; Fuchs S et al, 2006; Smadja DM et al, 2011; Imanishi T et al, 2005; Meneveau N et al,2011]. The cells obtained with this protocol are called late-EPCs or outgrowth EPCs. Briefly, mononuclear cells are seeded in culture plates coated with fibronectin and are cultured in presence of conditions that promote endothelial differentiation. Similar to ECFCs, late-EPC colonies appear after 2-3 weeks of culture. Late-EPCs also share with ECFCs other characteristics. In fact, late-EPCs have a cobblestone-like morphology, express endothelial markers (KDR, CD144, eNOS, vWF) and show the ability to form capillary structures both in vitro and in vivo matrigel models [Gulati R et al, 2003]. Moreover, also late-EPCs do not express CD45 and CD14. In particular, Gulati et al demonstrated that late-EPCs developed only from $CD14^-$ fraction of PBMCs [Gulati R et al, 2003].

Therefore, since different research groups isolate and culture cells endowed with the typical characteristics of ECFCs both on collagen and fibronectin, in a previous study published in 2013 we investigated whether the substrate used for cell isolation and culture could affect ECFC culture [Colombo E et al, 2013]. In particular, we confirmed that ECFCs can be efficiently isolated and expanded from adult peripheral blood on both culture plates coated with collagen or fibronectin. Moreover, we optimized a protocol for ECFCs isolation and expansion; in fact, we observed that ECFC isolation on fibronectin followed by ECFC expansion on collagen represented, in our hand, the most efficient strategy to culture ECFCs [Colombo E et al, 2013].

1.1.3.2.2 Other cultured EPCs

Different cell populations have been called EPCs in the past. The use of the same name for cell population endowed with different features created a confuse situation. In particular, also the cell populations described in this chapter - CFU-Hill and CACs - have been called EPCs in the past despite only ECFCs are now considered the true EPCs.

CFU-Hill colony counting method was the first method developed for EPC culture. It was originally proposed by Asahara et al [Asahara T et al, 1999] and it was later optimized to avoid the presence

of contaminant such as mature endothelial cells [Hill JM et al, 2003; Vasa M et al, 2001 a]. CFU-Hill colony counting method can be performed also with a commercially available kit (Endocult, StemCell Technologies). In particular, PBMCs are seeded at low density in culture plates coated with fibronectin and cultured for two days; nonadherent cells are then collected and replated. After 5 to 9 days of culture colonies of adherent cells arise. These colonies, called colony –forming CFU-Hill, are composed by a cluster of round cells surrounded by spindle-shaped cells. CFU-Hill cells possess characteristics typical of endothelial cells since the express CD31, CD34 CD105, CD144, CD146, vWF, KDR and uptake Dil-acLDL. Despite that, CFU-Hill cells are endowed with some features typical of monocytes/macrophages. In particular, they are characterized by a low proliferative potential, express pan-leukocyte marker CD45 and monocyte/macrophage markers CD14 and CD115, are able to phagocyte bacteria and possess a nonspecific esterase activity [Yoder MC et al, 2007]. Moreover, Rehman et al. demonstrated that CFU-Hill cells do not proliferate, but release proangiogenic factors such as VEGF, granulocyte colony-stimulating factor (G-CSF) granulocyte-macrophage colony-stimulating factor (GM-CSF) and hepatocyte growth factor (HGF) [Rehman J et al, 2003; Kalka C et al, 2000].

Another cell population erroneously defined EPC is represented by CACs whose culture protocol was described by Vasa in 2001 [Vasa M et al, 2001 b]. Briefly, PBMCs are seeded in culture plates coated with fibronectin and are cultured under “endothelial” differentiation conditions using a specific culture medium supplemented with serum and endothelial growth factors. The fourth day non adherent cells are removed and CACs with a spindle-shaped morphology remain adherent to the plate. CACs are also called early-EPCs since they appear few days after PBMCs seeding. Like CFU-Hill cells, CACs produce high levels of proangiogenic factors, are endowed with a low proliferative potential and express both endothelial cells markers and the markers of monocytic/macrophagic lineage CD45 and CD14 [Yoder MC et al, 2007]. In addition, recent studies highlighted the fact that the angiogenic properties of CACs isolated in vitro with this method could due to the platelet-derived proteins [Critser PJ et al, 2010]. In fact, Prokopi et al observed that contaminated PBMCs fraction used for CACs isolation was also enriched in platelets; during culture, platelets and platelet microparticles were taken up by monocytes. Through this mechanism many platelet proteins, which are also used as endothelial markers, were transferred on the membranes of monocytes that apparently acquired an endothelial phenotype. In particular, Prokopi et al described this mechanism using a proteomic analysis demonstrating that CACs expressed surface endothelial-platelet antigens such CD31, several integrins and Ulex Europaeus

lectin binding but they did not express mRNA for the same surface proteins [Prokopi M et al, 2009].

In conclusion, both CFU-Hill cells and CACs can not be considered the true EPCs since they are monocytes/macrophages which express also endothelial markers. In fact, they originate from $CD14^+$ fraction of PBMCs, as confirm by several studies that used several different mieloproliferative disorders to proved that early outgrowth EPCs derived from hematopoietic cells [Piaggio G et al, 2009; Gunsilius E et al, 2000]. Other typical features of CFU-Hill and CACs are: secretion of high amount of pro-angiogenic factors and low proliferative potential [Rehman J et al, 2003]. Therefore it has been proposed these cell populations should be better defined as angiogenic macrophages that, by secreting angiogenic factors and chemokines, promote angiogenesis in a paracrine way [Pearson JD, 2010]. In fact, in vivo studies highlighted the role of monocytes in promoting angiogenesis by secretion of pro-angiogenic factors and releasing of enzymes that degrade matrix [Anghelina M et al, 2006] and, in 2009, Krenning et al summarized in a review the evidences that demonstrate that EPCs can act similarly [Krenning G et al, 2009].

1.2 The lymphatic vascular system

1.2.1 Structure and functions of the lymphatic vascular system

Lymphatic vessels are located in most of the internal organs and in the skin but not in the bone marrow, central nervous system and avascular tissues such as cornea, epidermis, cartilage, hair and nails [Alitalo K et al, 2005]. The lymphatic vasculature begins with blind-ended lymphatic capillaries that drain lymph - the protein-rich interstitial fluid- from the interstital spaces within organs. The lymphatic capillaries are thin-walled vessels with a diameter of approximately 30–80 µm; they lack fenestrations and are not surrounded by pericytes – that usually surround blood capillaries - or smooth muscle cells. Lymphatic capillaries are characterized by the presence of a discontinuous basement membrane and are composed of a single layer of overlapping oak-leaf-shaped lymphatic endothelial cells (LECs) that form loose intercellular junctions. In fact, unlike blood vascular endothelial cells (BECs) that are characterized by zipper-like junctions, LECs posses button-like junctions thus making lymphatic capillaries highly permeable to fluids and allowing also the entrance of large macromolecules, migrating cells and pathogens. LECs that compose lymphatic capillaries are anchored through filaments to collagen fibers of extracellular matrix. When interstitial pressure is high the lymphatic capillaries are kept open by forces applied through the filaments that regulate the valve-like opening into the lumen of lymphatic vessels [Alitalo K et al, 2011; Alitalo K et al, 2012] (Figure 3).

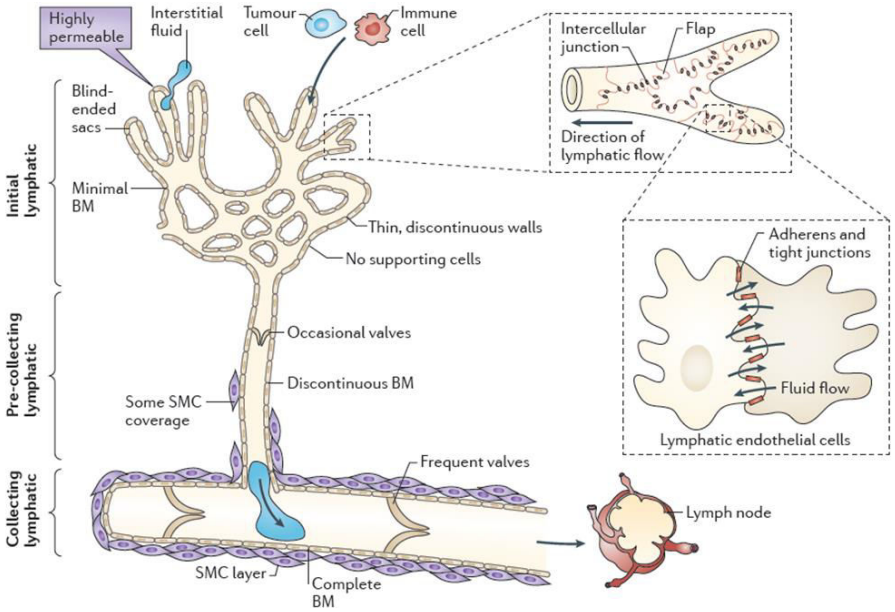


Figure 3: Lymphatic vasculature structure and features (Stacker et al, Blood, 2014).

The capillaries drain lymph into larger collecting ducts, sparsely covered by smooth muscle cells, called pre-collecting vessels and thereafter to collecting lymphatic vessels that are characterized by the presence of a layer of smooth muscle cells, basement membrane and valves. Collecting lymphatic vessels share some features with small veins; in fact, lymph propulsion is supported by contraction of smooth muscle cells and surrounding skeletal muscles, as well as arterial pulsations and the presence of valves prevents backflow [Alitalo K et al, 2011]. The collecting lymphatic vessels drain lymph into sentinel lymph nodes acting as afferent lymphatic vessels. Sentinel lymph nodes are the first organs that receive cells and fuids drained from peripheral tissues. Cells and fluids are then transferred by the efferent vessels of the sentinel lymph nodes to distal lymph nodes [Alitalo K et al, 2012]. Finally, lymph returns to the blood circulation through the thoracic duct and the lymphatic duct draining into the left and right subclavian veins, respectively [Alitalo K et al, 2005; Oliver G et al, 2004; Alitalo K et al, 2011].

Lymphatic vessels were described for the first time by Aselli in 1622 but despite its important role in physiological and pathological conditions, the lymphatic system drew scientific community attention only in the last twenty years. The lymphatic system is typical of higher vertebrates that are characterized by large body size and a complex cardiovascular system. For these reasons they need a secondary vascular system in order to maintain the fluid balance in the body [Alitalo K. et al, 2005]. Therefore, the main function of lymphatic system is to drain lymph from the interstitial spaces back to blood circulation. However, the maintenance of colloid osmotic volume is not the only function of lymphatic system. Besides lymphatic vessels, the lymphatic system is composed also by lymphoid organs and is involved in the immune response [Oliver G et al, 2004]. Lymphoid organ are classified as primary and secondary. In particular, in primary lymphoid organs – thymus and bone marrow – lymphocytes are formed and mature to become immunocompetent whereas in secondary lymphoid organs - lymph nodes, tonsils, spleen, Peyer's patches and mucosa associated lymphoid tissue (MALT) – mature lymphocytes are responsible for mounting the specific defense of the. In fact, drained lymph contains macromolecules and cells - mainly antigen presenting cells (APCs) - that, once reached the lymph nodes, initiate specific immune responses. Moreover, in pathological conditions, lymphatic system plays a crucial role also in systemic dissemination of infectious agents and tumor cells thus contribuiting to tumor metastatization [Alitalo K et al, 2011]. In addition, lymphatic system plays also a role in lipid absorbtion from intestinal tract. In particular, lacteal lymphatic vessels, located inside the intestinal villi, are

deputated to the absorbtion of lipid particles – called chylomicrons – released by enterocytes [Oliver G et al, 2004; Alitalo K et al, 2011].

1.2.2 Development of the lymphatic vascular system in mammals

In the past years, two different models have been proposed to explain the lymphatic vascular system development. On the one hand, in 1902, Sabin proposed that LECs derive from endothelial cells localized in the cardinal vein. Accorting to Sabin's model, a subset of endothelial cells located in the cardinal vein differentiates toward lymphatic phenotype and forms the primary lymphatic sacs from which the lymphatic vascular system develops through remodelling and sprouting mechanisms ("the centrifugal system") [Sabin F, 1902]. On the other hand, in 1910, Huntington and McClure proposed a model of "centripetal sprouting" in which primary lymph sacs could arise in the mesenchime by concrescence of discontinuous and independent lymph vesicles [Huntington GS et al, 1910]. Despite the presence of lymphatic vessels deriving from mesenchymal lymphangioblasts has been reported in Xenopus [Ny A et al, 2005] and chick embryos [Wilting J et al, 2003], the model proposed by Sabin is the most accepted for the development of the lymphatic system in mammals, since it was confirmed by both imaging in zebrafish [Yaniv K et al, 2006] and by in vivo models of lineage tracing in mice [Srinivasan RS et al. 2007]. According to Sabin's hypothesis, mice models confirmed that the lymphatic vascular system develops in parallel, but secondary to the development of the blood vascular system. In particular, at embryonic day (E) 9 endothelial cells into the cardinal vein start to express the lymphatic vessel endothelial Hyaluronan (HA) receptor (LYVE-1) which is the first lymphatic marker to be expressed and therefore LYVE-1 expression can be considered the first evidence that venous endothelial cells are able to respond to a lymphatic-inducing signal. At day E9.5, the expression of the transcription factor SOX18 fosters the expression of the homeobox transcription factor (PROX-1) in a subset of LYVE-1^+ endothelial cells located in the anterior cardinal vein. PROX-1, acting as a master gene regulator in lymphatic differentiation, induces endothelial cells to acquire a lymphatic phenotype (i.e. expression of lymphatic molecules including vascular endothelial growth factor receptor 3 - VEGFR-3) through the interaction with the venous orphan nuclear receptor COUP-TFII [Alitalo K et al, 2011]. Therefore, LYVE-1^+/PROX-1^+/VEGFR-3^+ LECs originated from the cardinal vein migrate and sprout in response to the stimulus of VEGF-C produced by surrounding mesenchyme thus giving rise to primary lymphoid sacs [Kukk E et al,1996]. In later stages LECs acquire a mature phenotype consisting in the expression of other typical lymphatic markers such as podoplanin and neuropilin-2 (NRP-2). In particular, NRP-2 is involved VEGF-VEGFR signaling. In fact, by acting as co-

receptor for VEGF-C, NRP-2 renders LECs more responsive to VEGF-C arising from the lateral mesenchyme [Xu Y et al, 2010; Tammela T et al, 2010].

From primary lymphoid sacs, LECs migrate and sprout forming the primary lymphatic plexus that further develops into the mature lymphatic vascular system [Alitalo K et al, 2005]. In line with the process of lymphatic vascular development described in mice models and according to the observation that blood and lymphatic systems are similar in structure and share the expression of some molecular markers, Oliver et al suggested that BECs can be considered as the default endothelial cells and that PROX-1 is necessary and sufficient to induce their trans-differentiation toward lymphatic phenotype. In particular, Oliver et al proposed a model for lymphatic vascular development in mammals that is composed by 4 sequential stages characterized by the acquisition of LEC competence, LEC bias, LEC specification and LEC differentiation and maturation of lymphatic vessels (Figure 4).

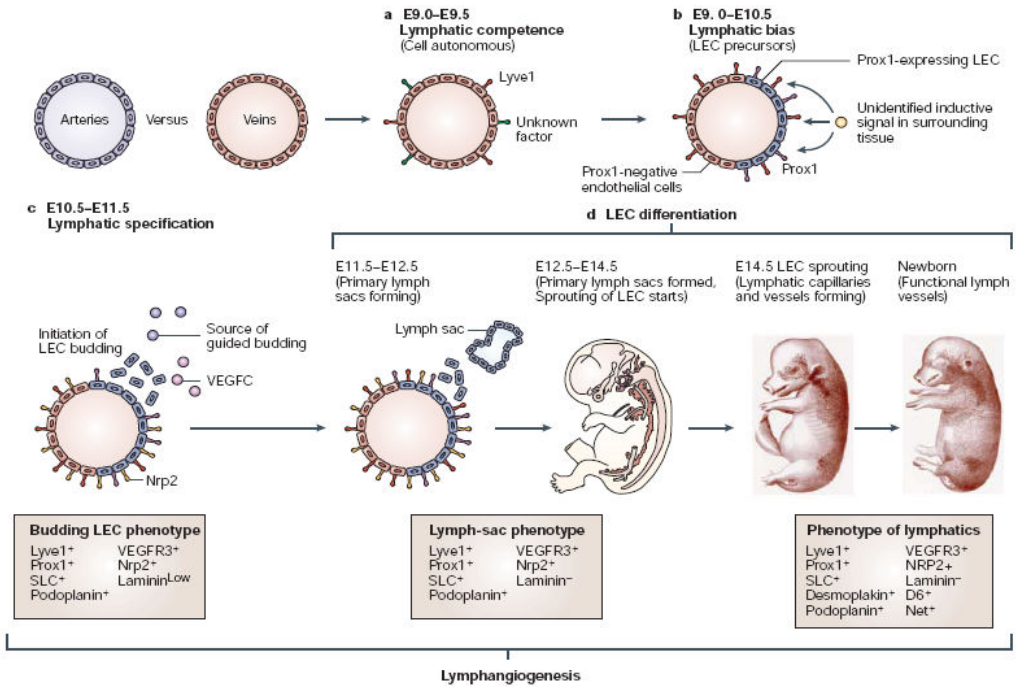


Figure 4: Model for differentiation of lymphatic vascular system proposed by Oliver G et al (Oliver G et al, Nat Rev Immunol, 2004).

In fact, they stated that, as a first step in lymphatic vascular development, the subset of vascular endothelial cells from which the lymphatic vessels originated must became competent to respond to initial signals that induce lymphatic differentiation. In particular, competent endothelial cells, according to this model, seem to be represented by the subset of endothelial cells that start to express PROX-1 in response to still unidentified signals. Once competent, PROX-1^+ cells became committed (biased) and can differentiate only into LECs. Therefore PROX-1^+ endothelial cells differentiate by upregulating the expression of typical lymphatic molecules and dowregulating the

expression of BEC markers thus acquiring the typical phenotype of LECs. Finally, LECs that originate in cardinal vein sprout forming lymphatic sacs from which mature lymphatic vessels develop [Oliver G et al, 2004].

The two models proposed - respectively by Sabin and by Huntington and McClure - to describe the lymphatic vascular system development were in contrast. Sabin proposed a centrifugal model in which LECs develop from cardinal vein and sprout forming lymphatic vessels whereas Huntington and McClure proposed a centripetal model in which LECs originate from mesodermal precursors. In addition, the evidences reported in literature in the last years supported exclusively on model or the other. Recently, Nicenboim J. et al tried to reconcile the two models originally proposed by Sabin and Huntington and McClure. In particular, they proposed that LECs originate from veins but they do so starting from specialized mesoderm derived angioblasts located in a venous niche [Nicenboim J et al, 2015]. In fact, using a zebrafish model, the authors described for the first time a niche of specialized angioblasts within the cardinal vein from which cells destined to differentiate in LECs can arise. Such angioblasts can also generate arterial and venous fates. Moreover, they identify Wnt5b as a novel lymphatic inductive signal. In particular, they showed that it can also induce 'angioblast-to-lymphatic' transition in human embryonic stem cells [Nicenboim J et al, 2015].

1.2.3 Immunophenotype of lymphatic endothelial cells (LECs)

Until few years ago, lymphatic and blood vessels within tissues could be distinguished only at the histological level using morphological criteria based on the fact that lymphatic vessels are thinner and do not have the external layer of pericytes. In fact, several markers that are expressed by both blood and lymphatic vessels were known (i.e. CD31) but a specific marker for lymphatic vessels identification lacked [Oliver G et al, 2004]. Recently, researchers identified markers specifically expressed by lymphatic cells that can be used for identification of lymphatic vessels in tissues and for studying the lymphatic system and the mechanisms that regulate lymphatic vessel growth in order to identify possible therapeutic targets. In particular, among the lymphatic markers the most extensively used for LEC in vitro characterization and lymphatic vessel identification in tissue sections are: PROX-1, VEGFR-3, LYVE-1 and podoplanin [Tammela T et al, 2010] (Figure 5).

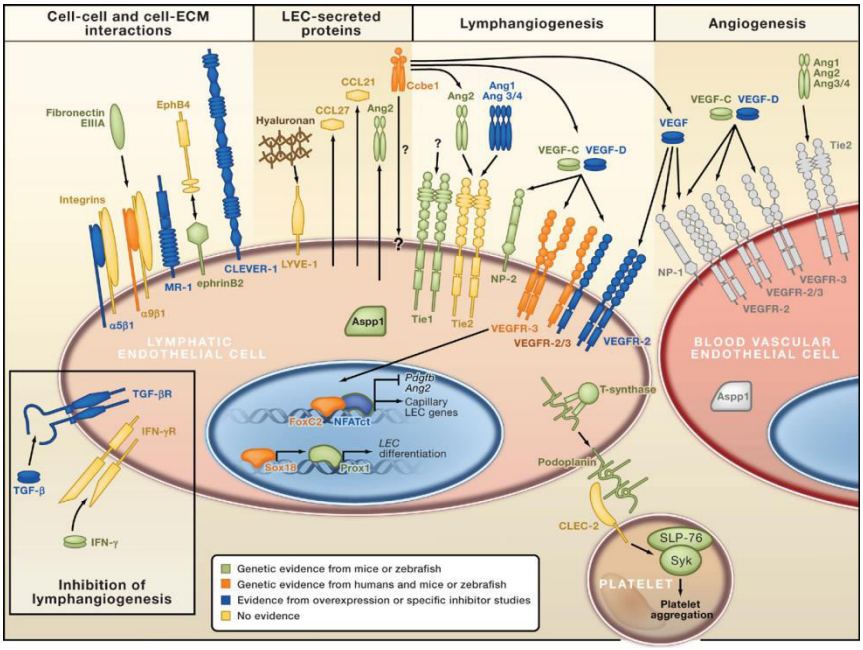


Figure 5: Key Molecular markers of lymphatic endothelial cells (Tammela T et al, Cell, 2010).

1.2.3.1 PROX-1

PROX-1 is a transcription factor and is considered the master control gene that regulates the expression of other lymphatic markers on LECs [Tammela T et al, 2010]. PROX-1 is located in the nucleus and can be use as constitutive marker of LECs both in normal and pathologic human tissues. In fact, it is expressed by LECs and it is not expressed by BECs as confirmed by the observation that its expression is mutually exclusive with that of PAL-E, a molecule used as marker for blood vessel identification in tissue sections [Wilting J et al, 2002]. PROX-1 expression starts in the early stage of lymphatic system development. In particular, in mice embryogenesis, starting from day E9.5 a subpopulation of endothelial cells PROX-1^{+} can be detected on one side of the anterior cardinal vein and several studies described how lymphatic system develops starting from this population [Oliver G et al, 2004]. The importance of PROX-1 as the regulator of lymphatic system development was confirmed by animal models knockout for PROX-1. PROX-$1^{-/-}$ mice embryos died perinatally in most genetic backgrounds - with the exception of NMRI mice that developed chylous ascites and adult-onset obesity - since they didn't develop lymph sack and lymphatic vessels [Alitalo K et al, 2005]. In fact, in PROX-$1^{-/-}$ mice the endothelial cells in the cardinal vein were not able to migrate, maintained the phenotype typical of BECs and did not express lymphatic endothelial markers [Oliver G et al, 2004]. Further studies also demonstrated that the overexpression of PROX-1 in mature BECs on the one hand promotes the expression of LEC specific genes and on the other one downregulates the expression of genes that are highly expressed by BECs [Wigle JT et al, 2002; Petrova TV et al, 2002]. The signals that induce the

expression of PROX-1 in cells destinated to lymphatic system development are still object of study [Tammela T et al, 2010]. In 2009, Francois et al proposed that homeobox transcription factor SOX18 could be required to induce the expression of PROX-1 and therefore may be involved in initiating the differentiation of endothelial cells of the cardinal vein towards lymphatic phenotype. The authors reported that, in endothelial cells located in the cardinal vein, SOX18 expression preceded the expression of PROX-1 and that PROX-1 promoter contained SOX18-binding sites [François M et al, 2008]. Moreover, they demonstrated that SOX18 silencing - by gene targeting of SOX18 locus or by homozygous expression of a dominant-negative mutant – abolished the expression of PROX-1 causing edema and embryonic death [François M et al, 2008].

1.2.3.2 VEGFR-3

The first lymphatic marker to be described was VEGFR-3, a tyrosine kinase receptor that belongs to the family of vascular endothelial growth factor receptors. It is activated by its ligands VEGF-C and VEGF-D. VEGFR-3 expression starts in early stages of lymphatic system development, at day E12.5 in mice embryos and – by mediating signaling of VEGF-C - it plays a crucial role in lymphangiogenesis by fostering the sprouting of PROX-1^{+} cells in the cardinal vein and by promoting survival and proliferation of LECs [Adams RH et al, 2007].

In embryonic development, VEGFR-3 is expressed also by BECs and it is also involved in early blood vascular functions. This was shown in experiments in which VEGFR-3 deletion caused embryonic death and alteration in blood-vessel remodeling. However, after the early stages of development, VEGFR-3 expression is downregulated in BECs and becomes restricted to LECs in the late stage of development and in adulthood [Dumont DJ et al, 1998]. The importance of VEGFR-3 mediated signaling in lymphangiogensis was demonstrated in murine models where the homozygous deletion of VEGF-C caused severe lymphatic hypoplasia and embryos did not develop any lymphatic vasculature [Karkkainen MJ et al, 2004]. In fact, in mice knockout for VEGF-C, PROX-1^{+} cells located in the cardinal veins initially differentiated in LEC but then were unable to migrate and form primary lymph sacs. Notably, despite VEGF-D is known to also supports lymphangiogenesis processes, VEGF-D deletion did not impair the development of lymphatic vasculature [Karkkainen MJ et al, 2004]. Moreover, Karkkainen et al described the presence of a missense mutation in VEGFR-3 gene - that causes the inactivation of tyrosin-kinase domain – in subjects affected by Milroy disease, a rare autosomal dominant disease characterized by lymphoedema [Karkkainen MJ et al, 2000]. The same research group described Chy mice as a possible model for studying hereditary lymphoedema and its possible therapy since they have a

similar mutation and develop lymphoedema [Karkkainen MJ et al, 2001]. Therefore, VEGF-C-VEGFR-3 pathway seems to play a crucial role in regulating lymphangiogenesis**.**

1.2.3.3 LYVE-1

LYVE-1 is considered one of the most specific markers for the identification of lymphatic endothelium since it allow to distinguish lymphatic from blood vessels in many different peripheral tissues [Jackson DG et al, 2004]. Despite LYVE-1 is expressed by sinusoids in liver and spleen and by a subset of macrophages that infiltrate tumors and inflamed tissues, LYVE-1 expression is absent in primary and cultured fibroblasts, epithelial cells and hematopoietic cells. In histological analysis of tissue sections of intestine, skin and secondary lymphoid tissues, LYVE-1 stains specifically only lymphatic vessels which are also negative for the markers typically used for blood vessel identification (i.e. CD34, vWF, CD44). Moreover, LYVE-1^{+} vessels are also positive for the expression of other lymphatic markers such as PROX-1, podoplanin and VEGFR-3 [Jackson DG et al, 2004].

LYVE-1 is an integral membrane glycoprotein that mediates binding and internalization of HA. HA binding is allowed by the presence of the HA-binding domain called “LINK” module located at the N-terminous of LYVE-1 extracellular domain. Moreover, LYVE-1 is characterized by high structural similarities with CD44 – its closest homologue expressed on lymphocytes - that is a HA receptor involved in the homing of inflammatory leukocytes [Banerji S et al, 1999].

During embryonic lymphangiogenesis, LYVE-1 is the first expressed marker of lymphatic endothelial commitment. In adults, LYVE-1 remains highly expressed in lymphatic capillaries but its expression decreases in the collecting lymphatic vessels [Alitalo K et al, 2005; Tammela T et al, 2010]. The role of LYVE-1 in regulation of lymphatic vascular function has not yet been clarified since in vivo studies demonstrated that mice that lack the expression of LYVE-1 develop functional lymphatic vessels [Gale NW et al, 2007]. Two possible functions have been proposed for LYVE-1 expressed on LECs. The first hypothesis is that LYVE-1 can act as an HA transporter thus participating to the process of HA catabolism. In particular, up-take HA can either be degraded inside LECs or released in the lumen of lymphatic vessels, via transcytosis. By this pathway, HA can be transported to other sites deputed to its catabolism: lymph nodes and liver. This hypothesis is supported by the fact that LECs express LYVE-1 on both luminal and baso-lateral side, thus allowing HA transcytosis [Prevo R et al, 2001]. The second possibility is that LYVE-1 by binding HA may play a role in the regulation of adhesion, entry and migration of cells in lymphatic vessels [Jackson DG et al, 2004].

1.2.3.4 Podoplanin

Podoplanin - also called T1α, E11 antigen, gp38, and PA2.26 – is a small O- and N-glycosylated transmembrane protein. In mouse embryos, podoplanin expression starts in PROX-1^{+} LECs sprouted from the cardinal vein at day E11.5. In adults, it is co-expressed with VEGFR-3 in the lymphatic endothelium [Breiteneder-Geleff S et al, 1999] whereas it is not expressed in larger lymphatic vessels and in all the blood vessels. Moreover podoplanin is also expressed in some non-endothelial cells, such as kidney podocytes, osteoblastic cells and lung alveolar type I cells [Breiteneder-Geleff S et al, 1997]. Podoplanin seems to regulate several LEC functions as described in in vitro studies in which podoplanin promotes LEC adhesion, migration, and tubulogenesis formation. In vivo, it was reported that podoplanin deficient mice are characterized by dysfunctional lymphatic vessels [Schacht V et al, 2003]. Despite the role of podolanin in adults is still under investigation, several studies showed that podoplanin plays a crucial role in the process of separation of lymphatic vessels from the blood vessels in embryonic development [Tammela T et al, 2010]. In fact, podoplanin binds CLEC-2 (C-type lectin receptor 2) on platelets inducing their activation and aggregation [Suzuki-Inoue K et al, 2007]. Platelet aggregation in turn prevents blood from flowing into newly formed lymphatic vessels that bud from the cardinal vein. Uhrin et al demonstrated the role of podoplanin in inducing platelet aggregation showing that podoplanin knockout or pharmacological inhibition of platelet aggregation inhibited the formation of platelet aggregates in correspondence of the separation zone of cardinal veins and podoplanin^{+} lymph sacs [Uhrin P et al, 2010].

1.2.4 Lymphatic endothelial progenitor cells (LEPCs)

Similar to the development of blood vascular system, also the process of lymphatic vessel formation (lymphatic neovascularization) depends on two mechanisms: de novo formation of lymphatic vessels starting from stem or progenitor cells (lymphovasculogenesis) and formation of new lymphatic vessels starting from pre-existing vessels (lymphangiogenesis).

Until about 10 years ago, lymphatic vessel formation in adulthood was thought to occur only by lymphangiogenesis [Karpanen T et al, 2001; He Y et al, 2004]. Nevertheless, starting from 2003 several studies suggest that lymphvasculogenesis may also occur in adulthood thus contributing to lymphatic neovasculaziation processes [Kerjaschki D et al, 2006; Maruyama K et al, 2005]. Since hematopoietic stem cells and EPCs play a well established role in adult neovascularization processes [Asahara T et al, 2011] it was hypothesized that a similar mechanism could occur also in

lymphatic neovasculaziation process. In particular, it was suggested that lymphatic endothelial progenitor cells (LEPCs) derived from bone marrow could participate in lymphatic vessel formation. In these years, many efforts have been made to validate this hypothesis. In 2003, Salven et al reported first evidence about LEPCs. In particular, they described in human fetal liver the presence of a $CD34^+/VEGFR\text{-}3^+/CD133^+$ sub-population that in vitro was characterized by a high proliferative potential and the expression of both lymphatic and blood vascular markers. These observations suggested that these cells could act both as EPCs and LEPCs [Salven P et al, 2003]. Moreover, two different studies reported that -in inflammatory conditions- after bone marrow transplantation, donor-derived cells labeled with GFP were localized and/or incorporated in newly formed lymphatic vessels [Maruyama K et al, 2005; Religa P et al, 2005]. In 2006, Kerjaschki et al confirmed these observations also in human. In fact, they reported that male-recipient derived LECs were clearly detectable in lymphatic vessels of kidney-transplanted from female donor [Kerjaschki D et al, 2006].

In the last years, different cell populations have been described as potential LEPCs. In 2010, Lee at all identified a population of $podoplanin^+/CD11b^+$ macrophages derived from the bone marrow that contribute to lymphatic neovascularization both by transdifferentiating in lymphatic endothelial cells and by producing VEGF-C and other lymphangiogenic factors that promote proliferation of local pre-existent LECs [Lee JY et al, 2010; Kerjaschki D et al, 2006]. In other studies, LEPCs have been identified in the peripheral blood as cells that express CD34 in combination with the lymphatic markers VEGFR-3. Tan et al reported that $CD34^+/VEGFR\text{-}3^+$ cells, isolated by fluorescence-activated cell sorting from human cord blood mononuclear cells, acquired - after two weeks of culture in presence of VEGF-C - a lymphatic phenotype since they expressed typical lymphatic markers such as PROX-1, LYVE-1 and are characterized by high activity of 5'-nucleotidase, an enzyme preferentially expressed in LECs than in BECs [Tan YZ et al, 2014].

All these observations supported the hypothesis the lymphatic precursors cells derived from bone marrow or organs can actually participate to post-natal lymphatic vessels formation [Park C et al, 2011]. Nevertheless, the identity and the characteristics of LEPCs have not yet been clearly defined and therefore, more extensive studies will be required to characterize the putative precursors of LECs involved in neo-lymphangiogenesis also in adulthood.

1.2.5 Tumor-associated lymphangiogenesis

In adulthood, lymphangiogenesis is mainly inactive in physiological conditions and occurs only during luteal phase of menstrual cycle in corpus luteum formation and during pregnancy in endometrium [Alitalo K et al, 2005]. On the contrary lymphangiogenesis is observed in pathological conditions such as wound healing, allograft rejection, tissue and organ regeneration, autioimmunity, acute and chronic inflammation, and tumor [Alitalo K et al, 2005; Karpanen T et al, 2008]. In particular, cancer represents a major cause of lymphangiogenesis in adult [Alitalo A et al, 2012].

In fact, in the past, it was thought that lymphatic vascular system passively contribute to tumor cell dissemination in lymph nodes and distant organs providing channel through which tumor cells can transit. In support to this hypothesis several clinical studies showed that lymphangiogenesis in the proximity of solid tumors correlates with mestastasis in lymph node and distant organs in patients with melanoma or breast cancer [Achen MG et al, 2005]. This observation confirms that lymphatic vessels could represent a route that cancer cells can use to disseminate in lymph nodes and, in a second time, in other organs [Alitalo A et al, 2011]. Nevertheless, recent evidence suggests that lymphatic vasculature plays an active role in tumor spread and the tumor-associated lymphangiogenesis directly facilitate tumor dissemination [Li T et al, 2012]. In particular, recent studies documented that tumor associated lymphangiogenesis occurs also within draining lymph nodes, even before tumor cells colonize them [Qian CN et al, 2006]. Qian et al suggested the “seed and soil” theory according to which cancer (the “seed”) is able to remotely modify and prepare the lymph node (the “soil”) for its arrival. In particular, lymphangiogenesis in lymph nodes is mainly mediated by soluble factors produced within the primary tumor site (mainly VEGF-C and VEGF-A). Since high levels of prolymphangiogenic growth factors have been detected in blood of patients with cancer, it is possible that such soluble factors may act not only in sentinel lymph nodes but also in distant organs [Witte MH et al, 2011].

Several studies are therefore investigating the role of the tumor microenvironment, in particular soluble factors and components of extracellular matrix (ECM), in promoting tumor lymphangiogenesis in order to identify possible targets and develop new therapeutic strategies.

1.2.5.1 Role of soluble factors

The tumor microenvironment is rich in signaling molecules that regulate lymphangiogenesis in addition to blood vessels angiogenesis. In particular, tumor-induced lymphangiogenesis is

promoted by VEGF-C and VEGF-D. Such molecules are lymphatic growth factors and several studies reported that they are overexpressed in different type of cancers [Alitalo K et al, 2012]. In particular, VEGF-C and VEGF-D can be released in the tumor microenvironment by several cellular types: tumor cells themselves, stromal cells (i.e. cancer associated fibroblast – CAF), tumor-infiltrating macrophages (TAMs) and platelets [Neuchrist C et al, 2003; Schoppmann SF et al, 2002; Alitalo K et al, 2011; Kerjaschki D, 2005].

Between these growth factors, VEGF-C is considered the most important in supporting tumor associate lymphangiogenesis [Achen M et al, 2005]. In fact, several clinical studies reported that high levels of VEGF-C correlate with lymph node metastatization [Alitalo A et al, 2011]. Moreover, in vivo studies - performed in murine models of different types of tumor - confirmed that VEGF-C overexpression promotes tumor lymphangiogenesis increasing lymphatic vessel diameter and density and increasing metastatization in lymph nodes and organ [Skobe M et al, 2001; Karpanen T et al, 2001]. In addition, VEGF-C overexpression promotes lymphangiogenesis even in mouse models of tumors characterized by low frequencies of lymph node metastatization such as the murine fibrosarcoma T241 cell line and the human prostate cancer cell line LAPC9 [Burton JB et al, 2008; Hoshida T. et al, 2006].

Moreover, despite VEGF-D promotes lymphangiogenesis and metastatization in mouse models, VEGF-D levels in patients correlate with lymph node metastasis only in a small proportion of the reported clinical studies. Therefore VEGF-D seems to play a minor role in tumor lymphangiogenesis compared to VEGF-C [Alitalo A et al, 2012].

The pro lymphangiogenic function of both VEGF-C and –D is mediated by their ligation to their receptor VEGFR-3. Therefore, the inhibition of VEGFR-3 function using blocking antibodies or soluble receptors that sequester the ligands could represent possible therapeutic strategies to reduce tumor lymphangiogenesis. Despite further studies are needed to clarify the role of lymphangiogenesis and VEGF-C/VEGFR-3 pathway in tumor growth, it has been demonstrated that VEGFR-3 blockade reduces tumor growth rate in solid tumor such as breast cancer, melanoma and squamous cell carcinoma [Alam A et al, 2012; Lund A et al, 2012; Alitalo AK et al, 2013].

Moreover, also overexpression of VEGF-A was reported to promote lymphangiogenesis [Björndahl MA et al, 2005]. In particular, Björndahl et al reported that, in mice models of T241 fibrosarcoma, VEGF-A overexpression promoted peritumoral lymphatic vessel growth thus promoting formation of lymph node metastasis. In addition, in a corneal tumor model, the authors showed that

lymphangiogensis was fostered by VEGF-A through a VEGF-C/VEGF-D/VEGFR-3-independent pathway [Björndahl MA et al, 2005].

1.2.5.2 Role of the extracellular matrix (ECM)

The extracellular matrix (ECM) is a highly organized meshwork of collagens, proteoglycans, glycoproteins, and growth factors. The major function of ECM is to provide support and anchorage to cells [Chen J et al, 2012]. In the last years, several studies revealed that the ECM not only has structural functions but, through integrin-mediated interaction, it can also regulate cell survival, differentiation, growth and migration. However, very little is known about the role of ECM in the regulation of tumor lymphangiogenesis. Evidences suggest that fibronectin may be involved in ECM-dependent lymphangiogenesis [Bae YK et al, 2013; Ou J et al, 2013; Kramer RH et al, 1985].

In particular, in vitro, fibronectin interacts with integrins α5β1 and α4β1 expressed on LECs, fostering the survival and growth of these cells. In vivo, the levels of fibronectin expression in tumor stroma correlates with lymphovascular invasion, lymph node metastasis and poor clinical outcome in breast cancer and other human tumors [Bae YK et al, 2013; Ou J et al, 2013]. Fibronectin is also highly expressed in Kaposi's Sarcoma, [Kramer RH et al, 1985] a tumor characterized by the proliferation of the typical spindle cells, cells of endothelial origin that express typical lymphatic markers such as VEGFR-3, LYVE-1 and podoplanin [Dupin N et al, 2006].

Futher evidences for a role of fibronectin in promoting lymphangiogenesis in human cancer derive from observation that EDA-fibronectin - an alternatively spliced oncofetal isoform of fibronectin whose most specific receptor is integrin α9 - promotes lymphangiogenesis. Moreover, its expression correlates with distant metastasis in colorectal carcinoma where integrin α9 is expressed on both LECs and tumor cells [Xiang L et al, 2012]. Another oncofetal truncated isoform of fibronectin – namely migration stimulating factor (MSF) - was described in tumor microenvironment [Schor SL et al, 2003] but its role in lymphangiogenic process has never been investigated.

Altough fibronectin and its oncofetal isoforms are the ECM proteins that have been mainly involved in human cancer associated lymphangiogenesis, other ECM proteins may participate to this process. In particular, in literature was reported that also other ECM components, such as tenascin-C and osteopontin, seem to be involved in promoting tumor-lymphangiogenesis; in fact, also their stromal expression is associated with increased lymph node metastasis in various tumor types [Ishihara A et al, 2002; Rudland PS et al, 2002].

1.3 Kaposi's Sarcoma (KS)

1.3.1 Epidemiology and clinical variants

In 1872, the Hungarian dermatologist Moritz Kaposi first described *idiopatisches multiples Pigmentsarkom der Haut* which became known as Kaposi's Sarcoma (KS) [Braun M, 1982].

KS is a multifocal lymphangioproliferative disease, characterized by proliferation of endothelial cells and immune system deregulation. KS presents typically with cutaneous lesions with macular, papular, nodular, or plaque-like appearance but rarely it also involve visceral organs [Braun M, 1982].

In 1994, Chang et al. discovered a new herpesvirus - called Human Herpes Virus 8 (HHV-8) or Kaposi's Sarcoma-associated herpesvirus (KSHV) - that was present in KS lesions of an AIDS patient [Chang Y et al, 1994]. This was the first evidence that a virus - HHV-8 - was the causative agent of KS as confirmed in further studies [Whitby D et al, 1995; Kedes DH et al, 1996; Martin JN et al, 1998; O'Brien TR et al, 1999].

However, HHV-8 is necessary but not sufficient for KS development. Most of primary HHV-8 infections are asymptomatic and in healthy people, the immune system is able to control the infection that persists in a latent form. HHV-8 reactivates and supports the development of KS lesions both in case of immunedeficiency – due to pathological (i.e. AIDS) and iatrogenic (i.e. transplants) conditions – and in the elderly - that are characterized by a low-grade of chronic state of inflammation (defined as inflammaging) [Antman K et al, 2000; Ascoli V et al, 2009].

Due to the close association between KS and HHV-8 infection, KS epidemiology reflects, at least in part, HHV-8 seroprevalence. The modes of HHV-8 transmission are still unclear, and both vertical and horizontal (sexual and non-sexual) transmission modes have been described. In particular, saliva-mediated HHV-8 transmission is also likely to occur since saliva of HHV-8 infected individuals was reported to contain high titer of virus [Rohner E et al, 2016]. Among the general population, the prevalence of HHV-8 is higher in sub-Saharan Africa (percentage of HHV-8 seropositive subjects within the total population: ≥50%) than in Europe, United States, Asia and Australia (<4%). In Western World, a higher HHV-8 prevalence is observed in men who have sex with men (MSM) and in the Mediterranean region (4-35%) [Rohner E et al, 2016].

Based on epidemiological and clinical features, four clinical variants of KS are known: classic KS (cKS), endemic, AIDS-associated and iatrogenic KS. Despite they are all characterized by HHV-8 infection and similar hystological features, these clinical variants develop in specific populations

and differ one from each other in rates of progression and anatomical sites os KS lesions [Antman K et al, 2000].

Classic Kaposis's Sarcoma (cKS)

cKS is a rare disease mainly found in the Mediterranean region, where HHV-8 seroprevalence is higher. In fact, cKS incidence is 10-fold higher in Italy, Greece, Israel and Turkey than in other European and North American Regions. In particular, the Italian islands Sardinia and Sicily are characterized by the highest incidence rates of cKS in Europe [Schwartz RA et al, 2008; Iscovich J et al, 2000]. cKS occurs mainly in middle-aged and elderly people of Mediterranean or Jewish descent [Iscovich J et al, 2000]. Despite HHV-8 can be transmitted both sexually and through saliva, only few familial KS cases were described thus suggesting that infectious (viral), genetic, and environmental factors, either independently or in combination may contribute to KS pathogenesis [Armyra K et al, 2014; Mancuso R et al, 2011].

cKS usually presents as cutaneous lesions that initially appear on the lower extremities. The onset of KS lesions on lower extremities is supported by low oxygenation of the tissues and therefore venous stasis and lymphedema are considered co-factors in KS development. In fact, several studies demonstrated that chronic exposure to hypoxia promotes viral reactivation in HHV8-infected cells [Davis DA et al, 2001; Haque M et al 2003]. During disease progression KS can also involve the upper body and rarely the visceral organs.

Among the four clinical variants, cKS is characterized by slow progression and good prognosis. Accordingly, treatment is individualized on the basis of the stage of disease and range from nonintervention to local therapy (i.e. intralesional chemotherapy, surgical excision) or systemic therapy (i.e. chemotherapy, radiotherapy) [Schwartz RA et al, 2008; Vano-Calvan S et al, 2011]. However, after treatment HHV-8 DNA is still detectable in PBMCs and in post-lesional skin, contributing to the relapses that frequently occur in cKS events [Dourmishev LA et al, 2003].

Moreover, cKS can represent a useful model of virus-associated tumor taking advantage from the absence of confounding factors such as iatrogenic immunosuppression or co-infection with other viruses (i.e. HIV) that are present in the other variant of KS.

Endemic KS

Endemic KS, also called African KS, is frequent in sub-Saharan Africa (Zaire, Uganda and Tanzania), where HHV-8 infection is endemic and the virus is mostly transmitted during childhood, vertically – from mother to child – and horizontally – for example between siblings [Plancoulaine S et al,

2000; Andreoni M et al, 2002]. Endemic KS affects both children (median age of 3 years and a male-to-female ratio of 1,7:1) and adults (median age of 35 years and a male-to-female ratio of 15:1) [Schwartz RA et al, 2008]. It presents in four distinct clinical patterns: one is similar to cKS but affects predominantly young adults; the other three forms are aggressive and similar to AIDS-associated KS. In particular, among the aggressive forms, one variant is a cutaneous disease which invades locally bones and soft tissues; another variant is characterized by florid muco-cutaneous and visceral manifestations; a third variant occurs in young children and is represented by a fulminant disease rapidly disseminating to visceral organs and lymph nodes [Hengge UR et al, 2002].

AIDS-associated KS

Co-infection by HIV-1 represents the major risk factor unique to this KS variant. In fact, AIDS-associated KS affects HIV-positive patients affected by AIDS and is the most common malignancy in bisexual and homosexual men with AIDS [Dourmishev LA et al, 2003]. AIDS-associated KS is a very aggressive form and is characterized by frequent mucosal and visceral progression [Schwartz RA et al, 1996]. In previous studies it was hypothesized that the frequency and the aggressiveness of the AIDS-associated KS variant could be caused not only by the immunodeficiency related to HIV but also by HIV infection itself [Vogel J et al, 1988].

The introduction of HAART therapy (Highly Active Anti-Retroviral Therapy) for treatment of HIV infection produced a reduction of the incidence of KS in AIDS affected patients. In particular, in USA and Europe, a reduction between 33% and 95% has been observed in the KS incidence in HAART-treated patients. On the other hand, in Africa - where HIV infection is epidemic and the access to HAART treatment is limited – the incidence of AIDS-Associated KS is increasing [Semeere AS et al, 2012].

Iatrogenic KS

Iatrogenic KS develops in subjects undergoing immunosuppressive therapy, in particular in organ-transplant recipients. Several studies reported an increase in KS incidence in transplant recipients (100 fold or more) compared to general population in particular in renal and lung transplant recipient [Zavos G et al, 2014; Piselli P et al, 2009]. In fact, immunosuppressive therapy can favour HHV-8 reactivation in HHV-8 seropositive recipients. Nevertheless, cases of seroconversion after transplant were described, supporting the hypothesis that HHV-8 infection can also derive from transplanted organ [Hosseini-Moghaddam SM et al, 2012; Barozzi P et al, 2003; Luppi M et al,

2003]. The manifestations of Iatrogenic KS range from chronic to aggressive visceral organ involving form.

Withdrawal or reduction of the immunosuppressive treatment usually is accompanied by KS remission. In particular, it was observed that the treatment with calcineurin inhibitor (i.e. Cyclosporine) promoted KS insurgence. In fact, it was documented that when the treatment was switched from Cyclosporine to mTOR (mammalian Targets Of Rapamycin) inhibitor, such as Sirolimus, KS lesions regressed [Stallone G et al, 2005].

1.3.2 Histopathology of KS

1.3.2.1 Spindle cells in KS lesions

The German pathologists Kobner (in 1883) and Philippson (in 1902) were the first to use the term spindle cells, talking about KS. Spindle cells, whose name derives from their spindle-shaped morphology, are the typical cells of KS lesions. In particular, they are the main component of KS lesions in the late stages of disease. Spindle cells are all HHV-8 infected and only a small percentage expresses markers of HHV-8 lytic replication while the majority of the cells are latently infected [Gessain A et al, 2005].

Even though spindle cells are considered the tumoral component of KS, their features differs from those of common tumor cells. In fact, spindle cells in KS lesions often are oligo- or polyclonal [Gill PS et al, 1998; Duprez R et al, 2007] and lack the genetic instability typical of the majority of tumor cells. Even in late stage lesions, spindle cells are typically diploid and do not have characteristic chromosomal rearrangement. Spindle cells differ from typical tumor cells also in their vitro behaviour; despite they can grow in culture [Ensoli B et al, 1998; Corbeil J et al, 1991; Lebbe C et al, 1997], spindle cells do not form foci, do not grow in soft agar and need exogenous growth factors for their survival and proliferation [Ensoli B et al, 1998]. Moreover, in in vivo models, spindle cells do not form persistent tumor when injected in nude mice [Herndier BG et al, 1994]. In fact, upon inoculation in nude mice, spindle cells, obtained from primary KS lesion, give rise to transient tumor, promote the formation of new vessels of murine origin in surrounding tissues but they survive for a short period of time and, after plug reabsorption, vessels of murine origin also disappear [Mutlu AD et al, 2007]. All these observations suggest that growth factors in the tumor microenvironment are necessary for spindle cells survival and proliferation [Ensoli B et al, 2001]. Therefore, it has been proposed a model in which interactions among different factors are necessary for KS development: spindle cells produced pro-inflammatory and pro-angiogenic

factors that recall inflammatory cells that produce cytokines which in turn promote spindle cells survival and growth. In particular, the presence of a state of inflammation seems to play an important role in KS development especially in cKS since human aging is characterized by the presence of a condition of chronic and low-grade inflammation.

1.3.2.1.1 Origin of the spindle cells

The origin of the spindle cells has been investigated for long time and several types of cells have been taken into account [Gessain A et al, 2005]. Electron-microscopy analysis was not sufficient to define the origin of spindle cells since these cells have some features typical of endothelial cells but some particular endothelial marker such as Weible-Palade bodies (storage granules of von Willebrand factor) are present only in a small fraction of spindle cells. Moreover, spindle cells are characterized by some ultra-structural features typical of other cell types such as fibroblasts, myo-fibroblats, smooth muscle cells and pericytes. Further immunohystochemical studies did not clarify the origin of the spindle cells because they express endothelial markers (including CD31, CD34, FVIII and VE-cadherin), markers of monocytes-macrophages (including CD68 and PAM-1) and markers of others cell types such as fibroblasts and smooth muscle cells [Browning PJ et al, 1994]. However, the scientific community reached the consensus on the fact that spindle cells derived from the endothelial lineage. In fact, spindle cells express factor VIII which is produced exclusively by endothelial cells [Burgdorf WH et al, 1981]. Moreover, spindle cells derived from primary KS lesions express several endothelial markers such as CD31, CD34, CD36, CD54, ELAM-1, ULEX-1 and do not express markers of other cell types such as CD2 and CD19 [Russell JR et al, 1995; Browning PJ et al, 1994].

Once researches defined that the spindle cells derive from endothelial cells, they investigated whether KS spindle cells derived from BECs or LECs. Several studies proposed that they derived from lymphatic endothelium because they lack markers preferentially expressed by BEC (vWF, PAL-E and Enos) while they express typical lymphatic markers such as podoplanin, LYVE-1 and VEGFR-3 [Weninger W et al, 1999; Dupin N et al, 1999; Pyakurel P et al, 2006; Cheung L et al, 2005].

More recent studies supported the hypothesis that BECs latently infected by HHV-8 acquire an intermediate phenotype between BECs and LECs by up-regulating the expression of lymphatic markers and down-regulating the expression of markers preferentially expressed at high levels in BECs as a direct consequence of HHV-8 infection. Wang et al observed by gene expression micro

array analysis that cells contained in KS lesions (>80% spindle cells in nodular KS biopsy) were more similar to LECs than to BECs [Wang HW et al, 2004]. Hong et al. observed that HHV-8-infected BECs underwent a lymphatic reprogramming. In particular PROX-1, a transcription factor with a key role in lymphatic differentiation, was up-regulated and genes highly expressed on BECs were down-regulated [Hong YK et al, 2004]. Carroll et al reported that HHV-8 can infect in vitro both BECs and LECs minimizing the difference of gene expressions between the two cell populations. They observed that HHV-8-infected immortalized human dermal microvascular cells (HDMECs) up-regulated the expression of typical lymphatic endothelium markers such as PROX-1, podoplanin, LYVE-1 and VEGFR-3 [Carroll PA et al, 2004; Yoo J et al, 2010]. Moreover, it was reported that HHV-8 virion envelope-associated glycoprotein B can activate VEGFR-3 on HDMECs [Zhang X et al, 2005].

Based on these observations it may be hypothesized that spindle cells may derive from mature endothelial cells infected in the site of KS lesions or they may derive from infected EPCs that once infected by HHV-8, can acquire an intermediate phenotype between BECs and LECs [Wang HW et al, 2004; Hong YK et al, 2004]. However, in this possible scenario, it should still be clarified the sites and modality in which EPCs are infected by HHV-8. In fact, EPCs may be infected in the bone marrow or in the sites of KS lesions by monocytes or B cells, cell types that are typically infected by HHV-8 otherwise they themselves could be latently infected and therefore could enhance KS lesions development. The hypothesis that KS lesions develop from dissemination of previously infected endothelial precursor cells is supported by several observations; in particular, that pro-inflammatory micro-environment could promote spindle cells development and proliferation from infected endothelial precursor cells. In fact, it has been observed that KS lesions often develop in patients with systemic inflammation at sites of previous local inflammation, such as a surgical wound (Koebner's phenomenon) [Webster-Cyriaque J et al, 2002].

Moreover, Barozzi et al reported that, after kidney transplant, HHV-8 negative recipients developed KS and lesions contained neoplastic cells derived from HHV-8-infected donors. Despite mature endothelial cells derived from kidney could be the precursors of spindle cells, they are terminally differentiated and characterized by a low proliferative potential, therefore, the more probable hypothesis is that infected endothelial precursors cells localized in the transplanted kidney could disseminate in the immunosuppressed recipients and act as precursors of spindle cells [Barozzi P et al, 2003].

1.3.2.1.2 In vitro culture of KS spindle cells

Several models of spindle cells culture were reported in literature. Spindle cells can be isolated from epidermal skin biopsies of KS lesions [Kaaya EE et al, 1995]. Moreover, spindle cells can also be cultured from peripheral blood mononuclear cells. In this case spindle cells are cultured in medium supplemented with pro-inflammatory cytokines and/or in conditioned medium (CM) derived from activated T-cells. In fact, CM contains pro-inflammatory cytokines -such as: IL-2, IL-6, TNFα, TNFβ, GM-CSF, PDGF, TGFβ – that in vivo induce KS progression and Barillari et al showed that CM is necessary for culturing spindle cells derived from AIDS-associated KS lesions [Barillari G, 1992]. CM can also induce mature endothelial cells to acquire spindle cell phenotype, to produce angiogenic factors and to promote in vitro angiogenesis [Browning PJ et al, 1994].

Another important feature of in vitro cultured spindle cells is that, during culture, they lose HHV-8 episomes [Aluigi MG et al, 1996; Lebbe C et al, 1997]. The reason why, after several passages, spindle cells lose HHV-8 episomes in vitro is unknown and this condition does not reflect what happens in vivo. In fact, in vivo, spindle cells are always infected by HHV-8 even in late stage KS lesions [Sgadari C et al, 2003]. These observations support the hypothesis that lytic replication and production of new virions are required for counterbalancing the episomal loss occurring during spindle cells proliferation [Grundhoff A et al, 2004].

By contrast, HHV-8 infection persists in several B cell lines derived from Primary Effusion Lymphoma (PEL), another disease associated with HHV-8 infection. Therefore, at least for B cells, a stable latency can be maintained both in vivo and in vitro under standard culture conditions [Pica F et al, 2000].

1.3.2.2 Histopathological and clinical staging of KS

In all epidemiological variants, the histopathology of KS is similar. Macroscopically, KS lesions can be classified as patch (or macular), plaque and nodule according to their histological features. During disease progression KS lesions with different histological features can also co-exist [Gessain A et al, 2005].

Macular lesions are frequent at the onset of KS and are characterized by spreading through the dermis of capillaries and small vessels-like structures - composed by small and irregular endothelial cells - by hemosiderin deposition and the presence of little inflammatory infiltrate enriched in plasma-cells and lymphocytes [Ackerman AB et al, 1979].

Plaque KS lesions are confluent and palpable since the blood vessels infiltration of dermis increases leading to the formation of angiomatoid areas and glomerulous-like vascular structures surrounded by *spindle cells* organized in short fascicles. This kind of lesions can invade all the reticular dermis and can also reach subcutaneous fat; moreover they are characterized by the presence of perivascular inflammatory infiltrate [Ackerman AB et al, 1979].

In nodular KS lesions, spindle cells become the predominant component of KS lesions and organize in sheets forming large fascicles. The vessels inside the lesion are characterized by fragmented basal lamina and irregular endothelial layer. Erythrophagocytosis and endothelial cell necrosis were also observed [Regezi JA et al, 1993; Modlin RL et al, 1983].

The definition of criteria for clinical staging of patients affected with KS has been a challenge. During the years the criteria and the consequent classification, have been changed since new hystopathological and clinical aspects were gradually taken into account.

In particular, in 2003 Brambilla et al proposed one of the most used and accepted KS classification [Brambilla L et al, 2003; Cappelletti M et al, 2012]. In this classification the authors introduced new clinical parameters such as evolution and the possible presence of complications in order to help clinicians in patient management and treatment (Table 1).

Stage	Skin Lesion	Localization	Behaviour	Evolution	Complications
I Maculo-nodular (±v)	Nodules and/or macules	Lower Limbs	Non aggressive	Slow (A) Fast (B)	•Lhymphedema •Lhymphorrea •Hemorrhage •Pain •Functional impairment •Ulceration
II Infiltrative (±v)	Plaques	Lower limbs	Locally aggressive	Slow (A) Fast (B)	
III Florid (±v)	Angiomatous nodules and plaques	Limbs, lower prevalent	Locally aggressive	Slow (A) Fast (B)	
IV Disseminated (±v)	Angiomatous nodules and plaques	Limbs, Trunk, Head	Disseminated aggressive	Slow (A) Fast (B)	

Table 1: KS staging according to classification proposed by Brambilla et al.

In particular, KS classification proposed by Brambilla et al takes into account several aspects: the histological features of the lesions (nodular, infiltrative, florid, disseminated, with or without visceral involvement), the localization of the lesions (lower limb, limbs ,trunk and head) the clinical behaviour (classified as not aggressive, locally aggressive and disseminated aggressive) the rate of disease evolution (classified as slow or fast) and the possible presence of complications (lymphedema, lymphorragia, hemorrhage, pain, etc.).

The rate of disease evolution is defined as fast (B) when one or more new lesions appear or the total area of the lesions increases within three months from the last observation. Visceral lesions (gastroenteric tract, oral cavity, lymph nodes and lung) are rare and more frequent in late stages (III and IV stage) with fast evolution rate [Brambilla L et al, 2003].

Therapy able to eradicate HHV-8 is not yet available; anti-herpes drugs, like Cidofovir or Ganciclovir, inhibits viral DNA polymerase but are effective only on virus in active replication phase but not when virus is in latent phase. KS treatment is personalized and is usually defined on the basis of clinical variant, tumor extension and general clinical condition of the patients. Possible treatment for KS, whose goal is reduction and control of lesions progression, ranges from elastocompression to intralesional chemotherapy and systemic chemotherapy. Small and superficial lesions can be surgically excised; sporadic and isolate lesions are usually treated with topic and intralesional chemotherapy like vincristine [Brambilla L et al, 2010; Regnier-Rosencher E et al, 2013]. Extended superficial lesions are treated with low doses of radiation-therapy (800-1000 cGy as single dose or 1500-2000 cGy for a week) directed on the lesions and their hedges [Fatahzadeh M et al, 2012]. High-doses of radiotherapy or systemic chemotherapy protocols are used when KS lesions are deep or in case of visceral involvement [Fatahzadeh M et al, 2012; Vaz P et al, 2011]. Anti-angiogenic therapy represents another possible treatment for KS [Coras B et al, 2004]. cKS and endemic KS are efficiently treated with the methods previously described; iatrogenic KS regresses after reduction or suspension of immune-suppressor therapy [Nagy S et al, 2000] in AIDS-associated KS, treatment is mainly based on HAART therapy that induces KS lesions regression by slowing down progression of AIDS [Jones JL et al, 1999].

1.3.3 Etiopathogenesis of KS

HHV-8 is considered the etiological agent of all clinical variants of KS [Gao SJ et al, 1996]. Nevertheless, HHV-8 is necessary but not sufficient for KS development. In fact, other factors such as immunosuppression and the presence of a pro-inflammatory microenvironment are required.

1.3.3.1 Human Herpes Virus 8 (HHV-8)

The herpesvirus HHV-8 was identified for the first time in tissues of a AIDS-associated KS patient by Chang et al in 1994 [Chang Y et al, 1994]. Later, HHV-8 infection was also observed in all the other KS clinical variants (cKS, endemic KS and iatrogenic KS) [Huang LM et al, 2000].

HHV-8, like Epstein-Barr virus (HHV-4) and herpesvirus Saimiri, belongs to the gammaherpesvirinae subfamily. Seven different HHV-8 subtypes (A, B, C, D, E, F and Z) have been

identified by sequencing ORF K1, a high variable region located at the left extremity of HHV-8 genome. The subtypes are differently distributed in the world. A and C subtypes are the most common in USA, Europe, Middle East and Northern Asia; subtype B is present in Africa and subtype D predominated in the Pacific islands; in addition subtypes E, Z and F have been recently described respectively in Brazilian Amerindians, Zambian children and Ugandan Bantu tribe. It is still unknown whether the different HHV-8 subtypes are associated with different rate of KS development and progression [Ouyang X et al, 2014; Cordiali-Fei P et al, 2015].

However, evidences reported by Cordilali-Fei et al supported the hypothesis that subjects infected with different HHV-8 subtypes may be characterized by different susceptibility to develop KS. In fact, in a small group of 27 HHV-8 infected patients all originated from central and south Italy, they observed that in both A and C subtypes two main clusters were present and they turned out to be distinctively associated to cKS or non-cKS subjects [Cordiali-Fei P et al, 2015].

From the inside to the outside, HHV-8 structure is characterized by: an icosadeltahedral caspid composed by 162 capsomers that contains viral DNA, the tegument and the envelope composed by a double layer of lipids and viral glicoproteins [Edelman DC et al, 2005]. HHV-8 genome is composed by a double strand DNA molecule of 165-170 kb. It is linear in the capsid and circularizes when is released inside the host cell where it localizes inside the nucleus [Renne R et al, 1996]. Viral genome conformation shift from episomal to linear when HHV-8 infection shifts from latent to lytic phase. In fact, HHV-8, like the other herpesviruses, is able to establish both latent and lytic infection. Some factors such as UV or immunosuppresion can promote the switch from latent towards lytic infection. During the latent phase, HHV-8 replicates as closed circular episome using host replication machinery. In the lytic phase, HHV-8 replication proceeds on the basis of rolling circle model: viral genome is replicated as a linear molecule off from an episome [Wen KW et al, 2010].

HHV-8 genome encodes for molecules that inhibit apoptosis, favour progression through cell cycle and allow viral immune evasion.

During latency, no-infectious or no-function virions are produced and only few viral molecules are expressed. In particular, viral transcripts expressed during the latent phase promote spindle cells proliferation and hyperplasia and favour HHV-8 immune evasion.

The Latency-Associated Nuclear Antigen (LANA) is encoded by ORF73 and plays a crucial role in episome persistence; N-terminal LANA binds histones H2A/H2B in mitotic chromosomes and C-terminal LANA binds HHV-8 terminal repeat (TR) DNA. Thus, LANA tethers the viral genome to host

mitotic chromosomes mediating distribution of HHV-8 episomes to daughter nuclei [Ballestas ME et al, 1999; Sun Q et al, 2014]. Sun et al, also demonstrated that LANA promotes HHV-8 DNA replication recruiting host cell machinery [Sun Q et al, 2014]. Moreover, LANA is able to affect gene expression of the host cell recruiting transcription factors [Renne R et al, 2001]. In particular, LANA can inhibit RB–E2F tumor suppressor pathways and p53-mediated transcription activity and apoptosis both by inhibiting p53 itself and its transcription [Wen KW et al, 2010].

viral Cyclin (vCyc) is encoded by ORF72 and is homolog D2 cellular Cyclin. vCyc activates cellular cyclin-dependent kinase 6 (CDK6) by promoting cell cycle transition from G1 to S phase. Moreover, it is reported to have oncogenic potential [Li M, et al, 1997; Zhi H et al, 2015]. In fact, vCyc acts as a pleiotropic cyclin mediating phosphorilation of a higher number of proteins than ones phosphorilated by cellular homolog [Chang Y et al, 1996].

vBcl-2 is homolog of cellular Bcl-2 and inhibits apoptosis [Sarid R et al, 1997]. It was reported that several factors expressed in KS lesions activates vBcl-2. For example, IFN-γ, by promoting the expression of CD40, induces the expression of vBcl-2 [Ensoli B et al, 2001].

viral FLICE inhibitory protein (vFLIP) is encoded by ORF71 and activates NFkB signaling which is involved both in viral latency and oncogenesis. In fact, NFkB signaling activation is important for inhibition of lytic replication and for transforming potential of vFLIP. Furthermore, vFLIP inhibits apoptosis thus supporting survival of infected cells [Schulz TF et al, 2006; Wen KW et al, 2010].

During the lytic phase, other genes are expressed that encode for molecules endowed with tumorigenic activities that promote the development of an angiogenic and inflammatory microenviroment in KS lesions [Mesri EA et al, 2010]. In particular, viral IL-6 (vIL-6) is encoded by ORF K2 and is homolog of human IL-6. vIL-6 acts as an angiogenic factor, also to increase the expression and signaling of VEGF and to promote cell proliferation [Suthaus J et al, 2011].

In addition, Viral G protein-coupled receptor (VGPCR), encoded by ORF 74, is homolog of human IL-8 receptor and binds CXC and CC chemokines. Its activation is constitutive and it induces VEGF expression thus promoting angiogenesis. vGPCR is also characterized by a potent oncogenic activity [Yang TY et al, 2000].

HHV8 infects several different types of cells. In KS patients, HHV-8 was detected by PCR in 95% of analysed KS lesions, in all KS clinical variants [Huang LM et al, 2000] both in lathent and lytic phase [Polstra AM et al, 2003 a; Polstra AM et al, 2003 b]. HHV-8 infects mainly spindle cells, most of them are latently infected whereas only a small percentage are lytically infected [Stürzl M et al, 1999 a; Stürzl M et al, 1999 b].

Besides KS, two lymphoproliferative diseases – Multicentric Castelman'sDisease (MCD) and Primary Effusion Lymphoma (PEL) – are associated to HHV-8 infection and derived from infected B-cells characterized by alteration in their differentiation process [Chadburn A et al, 2008; Gantt S et al, 2011]. In fact, T- and B- cells can be infected by HHV-8 [Ensoli B et al, 2001; Nsubuga MM et al, 2008; Hassman LM et al, 2011; Della Bella S et al, 2010]. Moreover, HHV-8 also infects monocytes and macrophages mainly in lytic form [Stürzl M et al, 1999 a; Dupon M et al, 1997; Blasig C et al, 1997].

HHV-8 DNA was also detected in circulating $CD34^+$ cells isolated from PBMCs of KS patients [Henry M et al, 1999]. Moreover, it has been shown that HHV-8 can infect in vitro $CD34^+$ cells both latently and lytically, and infection can persist during in vitro differentiation and also in vivo, after injection in NOD/SCID mice [Wu W et al, 2006]. These observations suggest that $CD34^+$ cells could be a viral reservoir strengthening the hypothesis that HHV-8 could infect bone-marrow precursors from which infection could disseminate to hematopoietic and endothelial progeny. According to this hypothesis, Pellet et al. observed that circulating endothelial cells were HHV-8 infected in 50 % of KS patients [Pellet C et al, 2006]. Moreover Della Bella et al demonstrated that circulating endothelial progenitor cells, isolated and cultured in vitro as ECFCs from peripheral blood of cKS patients, are latently infected by HHV-8 and are also able to support lytic viral replication of HHV-8 [Della Bella S et al, 2008].

1.3.3.2 Role of the immune system in KS pathogenesis

HHV-8, like other herpesviruses, has developed mechanisms to protect itself from the immune system control. Latency is a strategy of passive immune evasion; in fact, in the latent phase, viruses express few genes thus reducing the expression by infected cells of viral antigens that may trigger an immune response [Rezaee SA et al, 2006]. However, lytic replication is necessary for the production and release of the virions and the consequent viral dissemination in the host. Therefore, HHV-8 has developed also strategies of active immune evasion, as during lytic replication several viral proteins are expressed and can be identified by the immune system [Coscoy L et al, 2007; Hu Z et al, 2014].

In particular, in order to escape mechanisms of the innate immune system, HHV-8 protein KCP (KSHV complement-control protein) inhibits the activation of the complement cascade that is designated to direct lysis of viral particles and infected cells and to promote their phagocytosis and elimination by macrophages [Spiller OB et al, 2003]; the same protein is also able to inhibit type I

IFN expression and its signaling cascade thus blocking IFN-mediated anti-viral immunity [Lee HR et al, 2015].

Apoptosis of infected cells is a commom mechanism involved in blocking viral replication and dissemination in the host, as apoptotic cells are phagocyted by macrophages. HHV-8 developed several strategies aimed to inhibit apoptosis [Rezaee SA et al, 2006]. In particular, HHV-8 viral proteins vFLIP, viral inhibitor of apoptosis protein (vIAP, encoded by ORF K7) and vBcl-2 (encoded by ORF 16) promote survival of infected cells by blocking apoptosis. In fact, besides previously described vFLIP, vIAP acts by inhibiting capsases, whereas vBcl-2 negatively regulates the release and activity of pro-apoptotic molecules [Dourmishev LA et al, 2003].

Moreover, HHV-8 developed mechanisms to evade mechanisms of the adaptive immune response, too. Viral antigens in association with major histocompatibility complex (MHC) class I molecules (HLA-A, B and C) exposed on infected cells can be recognized by cytotoxic $CD8^+$ T-cells and trigger specific antiviral immune responses. In order to avoid this mechanism, HHV-8 encodes for modulator of immune recognition 1 (MIR1) and MIR2. These viral proteins inhibit the presentation of viral antigens to cytotoxic T-cells by promoting, in infected cells, the internalization of HLA molecules and their subsequent degradation in lysosomes [Coscoy L et al, 2000]. Moreover MIR1 e MIR2 also inhibits the expression of other molecules that act as co-stimulatory molecules for T-cells such as CD86 and the adhesion molecule ICAM-1. Down regulation of these molecules on HHV-8 infected antigen presenting cells (APCs) - like dendritic cells, macrophages and B-cells – impair the proper activation of adaptive immune responses [Coscoy L et al, 2001].

HHV-8 could also evade from immune system control inducing alterations in dendritic cells (DCs) [Campbell DM et al, 2014]. Della Bella et al reported that frequencies of myeloid and plasmacytoid DC in patients affected with cKS were lower than in healthy controls. It is possible that alteration of DC compartment may represent an immune evasion strategy used by the viruses to establish and maintain viral persistence. DC of cKS patients were also characterized by lower production of IL-12 – which promotes cell-mediated immune responses - and increased production of IL-6 [Della Bella S et al, 2006].

Viral chemokines support the development of a microenviroment that promote survival of infected cells and consequent viral propagation. In particular, vMIP-I (ORF K6), vMIP-II (ORF K4) and vMIP-III (ORF K4.1) selectively chemoattracts $CD4^+$ Th2 cells switching the immune response towards humoral response thus avoiding the cytotoxic cell mediated response which is deputed to

control and elimination of viral infections [Stine JT et al, 2000; Holst PJ et al, 2003]. vMIP-II can also impairs Th1 immune responses and inflammatory responses by acting as antagonist of several endogenous chemokines, such as RANTES or RANTES o MIP-1α [Jensen KK et al, 2004].

1.3.3.3 Involvement of the endothelial compartment in KS pathogenesis

KS development is a multi-step process in which, in addition to HHV-8 infection, alterations of the immune system, pro-inflammatory cytokines and angiogenic factors play a crucial role in sustaining development and maintenance of KS lesions [Ensoli B et al, 2001]. KS is characterized by three parallel processes: a proliferative component (involving chiefly spindle cells), an inflammatory component, and an angiogenic component. Typical histological features of KS lesions included spindle cell growth, proliferation of endothelial cells that may form dysfunctional vessels, extravasated erythrocytes, edema, lymphocytic infiltrate and - in later stages of disease - neoangiogenesis.

Although endothelial proliferation in KS lesions may rely on the acquisition of hallmarks of cancer by the spindle cells, several intermediary cytokines and angiogenic factors have been demonstrated to play a crucial role in sustaining KS development. In particular, KS lesions are characterized by high level of endothelial growth factors such as VEGF, basic fibroblast growth factor (bFGF) and fibroblast growth factor 2 (FGF-2) that are necessary for lesion development and maintenance [Samaniego F et al, 1998]. In KS lesions, besides angiogenic factors, pro-inflammatory cytokines (i.e. IFNγ, TNFα, IL-1β, IL-2, IL-6 and IL-8) are also produced by both spindle cells and inflammatory cells like lymphocytes and monocytes-macrophages [Sirianni MC et al, 1998; Monini P et al, 1999; Ensoli B et al, 1992]. Such factors promoted, in vitro, endothelial cell migration and matrix invasion [Thompson EW et al, 1991] and induced, in vivo, angiogenesis and formation of lesions similar to KS [Albini A et al, 1994]. Moreover inflammatory cytokines induce cell proliferation in an autocrine and paracrine manner, activate vascular endothelium, recruit other inflammatory cells in KS lesions by increasing inflammation and promote the expression of adhesion molecules (i.e. ICAM-1, ELAM-1 and V-CAM-1) on inflammatory cells [Fiorelli V et al, 1998; Sodhi A et al, 2000].

VEGF is a prototypical angiogenic factor and it has been detected both in KS lesions and in in vitro culture of spindle cells [Cornali E et al, 1996]. Like bFGF, VEGF expression in spindle cells is promoted by inflammatory cytokines such as PDGF-β. VEGF acts synergistically with bFGF stimulating endothelial cell growth, angiogenesis and edema formation.

bFGF is involved in the formation of KS lesions. In fact, Ensoli et al observed that bFGF in synergy with HIV-1 Tat protein can induce lesions similar to KS in mice [Ensoli B et al, 1994]. They also demonstrated that bFGF is produced by spindle cells and support spindle cells and endothelial cells proliferation in autocrine and paracrine manner [Ensoli B et al, 1994].

PDGF-β is a potent mytogen produced by spindle cells. It acts on spindle cells themselves in paracrine and autocrine manner [Stürzl M et al, 1995].

IFN-γ synergistically acts with IL-1 and TNFα and induces CD40 expression on spindle cells and vascular endothelial cells in the areas proximal to the tumor. CD40 signaling inhibits apoptosis probably by inducing the expression of bcl-2 [Pammer J et al, 1996]. Sirianni et al also observed that PBMCs in KS patients produced higher levels of IFN-γ than in healthy control. [Sirianni MC et al, 1998].

IL-1 is produced and released by spindle cells in KS lesions and promotes spindle cells growth in autocrine manner. IL-1 acts by interacting with bFGF. Moreover, in association with TNF and IFN-γ, IL-1 induces endothelial cell activation, acquisition of the phenotype typical of spindle cells and promotes leucocyte recruitment [Ensoli B et al, 1992].

IL-8 is a chemokine expressed in KS lesion by spindle cells and activated endothelial cells. IL-8 acts as a chemotactic factor for neutrophils [Masood R et al, 2001; Li X et al, 2011].

IL-6 is produced in KS lesions by inflammatory cells, spindle cells and activated endothelial cells. IL-6 promotes angiogenesis and tumor cell growth. Because the vast majority of infected cells express IL-6, it was supposed that a viral protein expressed during the latent phase of HHV-8 infection could induce IL-6 expression. In support to this theory, An et al demonstrated that LANA can act as transcription repressor and/or activator. In particular, they observed that LANA by interacting with AP1 RE (response elements) present in IL-6 promoters can up-regulate IL-6 expression. Since AP1 RE are present in several promoters, LANA could induce the expression of other factors such as VEGF or other cytokines and molecules that regulate cell growth [An J et al, 2004]. In addition, HHV-8 genome contains a viral homolog of IL-6 called vIL-6. vIL-6 acts on target cells by binding only one subunit of the human IL-6 receptor (gp130) without the need of specific IL-6 receptor α (CD126) chain for mediating its effects [Polizzotto MN et al, 2013; Yang J et al, 1994; Osborne J et al, 1999].

These observations support the hypothesis that KS development is based on an inflammatory process triggered by HHV-8 infection and reactivated by a proinflammatory microenvironment [Ensoli B et al, 1998].

1.3.4 EPCs in KS

Previous studies carried out in our laboratory supported the hypothesis that EPCs could play a role in KS pathogenesis. In particular, we analyzed by flow cytometry circulating EPCs in cKS patients and reported that circulating EPCs, identified as $CD45^{low}/CD34^{+}/KDR^{+}$ or $CD45^{low}/CD34^{+}/KDR^{+}/CD133^{+}$ cells, were increased in cKS patients compared with healthy controls. As we did not observed any correlation between the number of EPCs and the plasmatic levels of factors such as VEGF, TNF-α and GM-CSF, we hypothesized that circulating EPCs in cKS patients may be affected by HHV-8 infection through direct and indirect mechanisms [Taddeo A et al, 2008]. Moreover, we demonstrated that ECFCs obtained from cKS patients were HHV-8 infected [Della Bella S et al, 2008]. By documenting the presence oh HHV-8 DNA in both cells and supernatant of ECFCs isolated from cKS patients, we demonstrated that HHV-8 can sustain both latent and lytic infection in these cells. The viral infection persisted in long term cultures after multiple passages, supporting that ECFCs may represent a HHV-8- reservoir in c KS patients [Della Bella S et al, 2008]. Based on this observation, we hypothesized that ECFCs may represent putative precursors of spindle cells in KS patients.

2. Aim of the study

Kaposi's Sarcoma (KS) is a lymphangioproliferative disease whose causative agent is the herpesvirus HHV-8 [Chang Y et al, 1994]. KS presents typically with cutaneous lesions but can also involve visceral organs and it is characterized by hyperproliferation of HHV-8-infected spindle cells - the typical cells of KS lesions. Spindle cells are cells of endothelial origin but it is still unclear if they derive from mature endothelial cells infected by HHV-8 in peripheral tissues or whether they originate from previously infected circulating cells such as bone-marrow derived endothelial progenitor cells (EPCs).

EPCs play a crucial role in endothelial homeostasis and in vasculogenic processes both in physiological and pathological conditions [Urbich C et al, 2004]. Although different types of cells - isolated from peripheral blood - have been called EPCs in the past, only endothelial colony-forming cells (ECFCs) are considered the true EPCs [Fadini GP et al, 2012].

In previous studies, we observed that the number of circulating EPCs, identified by flow cytometry as $CD45^{dim}/CD34^{+}/KDR^{+}$, is increased in patients with classic KS (cKS) when compared with healthy controls [Taddeo A et al, 2008]. Moreover, we also observed that ECFCs isolated from cKS patients are HHV-8 infected and can act as viral reservoir [Della Bella S et al, 2008].

Therefore, in this study we investigated whether ECFCs isolated from cKS patients are endowed with features typical of spindle cells in order to evaluate whether they may represent the precursors of spindle cells.

In particular, we compared ECFCs isolated from cKS patients and healthy controls and we evaluated the following parameters:

- during ECFC isolation, we analyzed:
    - the frequency of subjects who gave rise to at least one ECFC colony within 30 days from PBMCs seeding
    - the time of ECFC colony appearance
    - the frequency of isolated ECFC colonies
- during ECFC expansion, we analyzed:
    - the morphology and phenotype of ECFCs
    - ECFC cell senescence and proliferative potential
    - the vasculogenic potential
    - the cytokine production
    - the molecular signature

Moreover, several studies reported that spindle cells in KS lesions express LYVE-1, podoplanin and VEGFR-3 [Pyakurel P et al, 2006] and that in vitro HHV-8-infected blood endothelial cells (BECs) express lymphatic markers including PROX-1 [Hong YK et al, 2004]. Nevertheless, in preliminary experiments we observed that LYVE-1 and podoplanin were expressed by ECFCs isolated not only from cKS patients but also from healthy controls.

In the last years, several studies supported the hypothesis that a population of lymphatic endothelial progenitor cells (LEPCs) could be involved in lymphangiogenic processes [Park C et al, 2011]. However, the identity of LEPCs has not yet been fully elucidated.

Therefore, in this study we investigated the lymphatic differentiative potential of ECFCs and whether it could be influenced by the substrate used for ECFC culture. In particular, we evaluated the impact of fibronectin compared with collagen, as the presence of fibronectin and its oncofetal isoforms (i.e. EDA-FN and EDB-FN) in the tumor microenvironment positively correlates with lymphangiogenesis and metastasis [Bae YK et al, 2013; Ou J et al, 2013; Xiang L et al, 2012]. Moreover, we evaluated whether migration stimulating factor (MSF), an oncofetal truncated isoform of fibronectin typically present in tumor microenvironment [Solinas G et al, 2010], could promote the lymphatic differentiation of ECFCs.

To this aim, we isolated ECFCs from healthy donors and evaluated their lymphatic differentiative potential in basal conditions and after MSF stimulation. ECFCs were cultured on either fibronectin or collagen and the expression of typical lymphatic markers (PROX-1, podoplanin, LYVE-1 and VEGFR-3) was evaluated by:

- detection of specific transcripts
- detection of protein expression

3. Material and methods

3.1 Reagents

The following reagents were used for PBMCs isolation from peripheral:

- Heparin sodium, For Hospital s.r.l., Potenza, Italy
- Falcon 50 ml conical tubes, BD, New Jersey,USA
- Ficoll-Paque PREMIUM, GE Healthcare, Invitrogen, California, USA
- Hanks' Balanced Salt Solution (HBSS) w/o Ca^{2+} and Mg^{2+}, Lonza, Basel, Switzerland
- Trypan Blue, Sigma-Aldrich, Missouri, USA

The following reagents were used for ECFC isolation and expansion:

- mw6 and mw24 culture plates, BD, New Jersey USA
- T25, T75 and T175 flasks, BD, New Jersey, USA
- Tripsin-EDTA, Lonza, Basel, Switzerland
- Sterile water, Baxter, Rome, Italy
- Phosphate Buffered Saline (PBS) w/o Ca^{2+} and Mg^{2+}, Lonza, Basel, Switzerland
- Acetic acid, Merck Millipore, Billerica, MA, USA
- Fibronectin from human plasma, Sigma-Aldrich, Missouri, USA
- Collagen Solution, Type I from rat tail, BD, New Jersey,USA
- EGM-2 BulletKit (EBM-2 Basal Medium (500 ml) + EGM-2 SingleQuot Kit Suppl. & Growth Factors), Lonza, Basel, Switzerland
- Fetal Bovin Serum (FBS), Lonza, Basel, Switzerland
- Trypan Blue, Sigma-Aldrich, Missouri, USA

The following reagents were used for cytofluorimetric characterization of ECFCs:

- ACCUTASE™ SOLUTION, Merck Millipore, Billerica, MA, USA
- LIVE ⁄ DEAD® Fixable Aqua Dead Cell Stain Kit, Invitrogen, California, USA
- FACS Tubes, BD, New Jersey, USA
- Mouse monoclonal antibody anti- human CD14 PerCp Cy5.5-conjugated, BD, New Jersey, USA
- Mouse monoclonal antibody anti- human CD31 FITC-conjugated, BD, New Jersey, USA
- Mouse monoclonal antibody anti- human CD45 PerCp Cy5.5-conjugated, eBionscience, California, USA
- Mouse monoclonal antibody anti- human CD54 PE-conjugated, R&D, Minnesota, USA
- Mouse monoclonal antibody anti- human CD144 FITC conjugated, Serotec, Kidlington, UK

- Mouse monoclonal antibody anti- human CD146 PE-conjugated, Biocytex, Marseille, France
- Mouse monoclonal antibody anti- human VEGFR-2 PE-conjugated, R&D, Minnesota, USA
- Mouse monoclonal antibody anti- human CXCR4 PE Cy7-coniugato, eBioscience, California, USA
- Sterile water, Baxter, Rome, Itay
- PBS w/o Ca^{2+} and Mg^{2+}, Lonza, Basel, Switzerland
- HBSS w/o Ca^{2+} and Mg^{2+}, Lonza, Basel, Switzerland
- FBS, Lonza, Basel, Switzerland
- Stabilizing Fixative, BD, New Jersey, USA
- Wash buffer solution: HBSS w/o Ca^{2+} and Mg^{2+} + FBS 2% p/v

The following reagents were used for ECFC characterization by immunofluorescence:

- EGM-2 BulletKit (EBM-2 Basal Medium (500 ml) + EGM-2 SingleQuot Kit Suppl. & Growth Factors), Lonza, Basel, Switzerland
- mw24 culture plates, BD, New Jersey USA
- Coverslips, diameter 14 mm, Thermo Scientific, Massachusetts, USA
- Microscope slides, Thermo Scientific, Massachusetts, USA
- Sterile water, Baxter, Rome, Italy
- PFA, Sigma-Aldrich, Missouri, USA
- PBS w/o Ca^{2+} and Mg^{2+} , Lonza, Basel, Switzerland
- PBS with Ca^{2+} and Mg^{2+}, Lonza, Basel, Switzerland
- Dil–labeled acetylated Low-density lipoprotein(Dil-ac-LDL), Molecular Probes, Oregon, USA
- Lectin from Ulex europaeus (UEA-1) FITC-conjugated, Sigma, Missouri USA
- DAPI, Invitrogen, California, USA
- Fluor Preserve Reagent, Calbiochem, California, USA
- Fixing solution: PBS with Ca^{2+} and Mg^{2} + 2% PFA p/v

The following reagents were used for LANA detection by immunofluorescence:

- mw24 culture plates, BD, New Jersey USA
- Coverslips, diameter 14 mm, Thermo Scientific, Massachusetts, USA
- Microscope slides, Thermo Scientific, Massachusetts, USA
- Rat monoclonal antibody anti- anti HHV-8 ORF73 (LANA) (Rat IgG2c) Advanced Biotechnologies Inc.

- Goat anti-rat IgG (H+L) secondary antibody, Alexa488-conjugated, Invitrogen, California, USA
- DAPI: Nucleic Acid Stain, Invitrogen, California, USA
- PFA, Sigma-Aldrich, Missouri, USA
- PBS w/o Ca^{2+} and Mg^{2+}, EuroClone, UK
- PBS with Ca^{2+} and Mg^{2+}, EuroClone, UK
- Sterile water, Baxter, Rome, Italy
- Bovine Serum Albumin, fraction V (BSA), Sigma-Aldrich, Missouri, Usa
- Normal Goat Serum (NGS), Dako, Carpinteria, CA
- TRITON X100, Sigma-Aldrich, Missouri, Usa
- Tween20, International PBI, Milan, Italy
- Wash buffer:Tween20 0.05% in PBS with Ca^{2+} and Mg^{2+}
- Permeabilization solution: Triton X-100 0.3% in PBS with Ca^{2+} and Mg^{2+}
- Blocking solution: BSA 2% + NGS 5% in PBS with Ca^{2+} and Mg^{2+}
- Fixing solution: PFA 4% in PBS w/o Ca^{2+} and Mg^{2+}
- Fluor Preserve Reagent, Calbiochem, California, USA

The following reagents were used for in senescence-associated β-galactosidase assay:

- Cellular Senescence Assay Kits, Merck Millipore, Billerica, MA, USA
- PBS w/o Ca^{2+} and Mg^{2+}, EuroClone, UK
- Glycerol, Sigma-Aldrich, Missouri, Usa

The following reagents were used for in vitro vasculogenic assay:

- Flat bottom mw96 culture plates, BD, New Jersey, USA
- Matrigel, BD, New Jersey, USA
- EGM-2 BulletKit (EBM-2 Basal Medium (500 ml) + EGM-2 SingleQuot Kit Suppl. Growth Factors) Lonza, Basel, Switzerland

The following reagents were used for cytokine detection in ECFC culture supernatant:

- DuoSet® ELISA Development Systems - R&D Systems, Minnesota, USA

The following reagents were used for total RNA extraction retrotranscription:

- RNeasy® Plus Micro kit, Qiagen, California, USA
- High Capacity cDNA Reverse Transcription kit, Applied Biosystem, California, USA

The following reagents were used for gene array analysis:

- Illumina TotalPrep RNA Amplification kit. Ambion/Applied Biosystems, Texas, USA
- Illumina HumanHT-12 v4 Expression BeadChip arrays, Illumina Inc., California, USA

The following reagents were used for evaluation by Q-PCR of the expression of genes encoding for lymphatic markers:

- Sterile water, Baxter, Rome, Italy
- TaqMan® Universal PCR Master Mix, Applied Biosystem, California, USA
- TaqMan probe GAPDH FAM, Applied Biosystem, California, USA
- TaqMan probe PROX-1 FAM, Applied Biosystem, California, USA
- TaqMan probe podoplanin FAM, Applied Biosystem, California, USA
- TaqMan probe LYVE-1 FAM Applied Biosystem, California, USA
- TaqMan probe VEGFR-3 FAM, Applied Biosystem, California, USA

The following reagents were used for detection of lymphatic markers by immunofluorescence:

- mw24 culture plates, BD, New Jersey USA
- Coverslips, diameter 14 mm, Thermo Scientific, Massachusetts, USA
- Microscope slides, Thermo Scientific, Massachusetts, USA
- Mouse monoclonal antibody anti- human Podoplanin (IgG1), Serotec, Kidlington, UK
- Rabbit polyclonal antibody anti- human LYVE-1 Abcam USA
- Goat polyclonal antibody anti human PROX1, R&D, Minnesota, USA
- Mouse polyclonal antibody anti human VEGFR-3, R&D, Minnesota, USA
- Donkey anti-Mouse IgG1 secondary antibody, AlexaFluor488-conjugated, Invitrogen, California, USA
- Donkey anti-Rabbit IgG (H+L) secondary antibody, AlexaFluor568-conjugated, Invitrogen, California, USA
- Donkey anti-Goat IgG (H+L) secondary antibody, AlexaFluor647-conjugated, Invitrogen, California, USA
- DAPI: Nucleic Acid Stain, Invitrogen, California, USA
- PFA, Sigma-Aldrich, Missouri, USA
- PBS w/o Ca^{2+} and Mg^{2+}, EuroClone, UK
- PBS with Ca^{2+} and Mg^{2+}, EuroClone, UK
- Sterile water, Baxter, Rome, Italy

- BSA, Sigma-Aldrich, Missouri, Usa
- TRITON X100, Sigma-Aldrich, Missouri, Usa
- Human serum (HS) AB, Lonza, Basel, Switzerland
- Tween20, International PBI, Milan, Italy
- Wash buffer Tween20 0.05% in PBS with Ca^{2+} and Mg^{2+}
- Block-Perm solution: Triton X-100 0.1% + BSA 1% + HS 5% + FBS 5% in PBS with Ca^{2+} and Mg^{2+}
- Blocking solution: BSA 1% + HS 5% + 5% FBS in PBS with Ca^{2+} and Mg^{2+}
- Fixing solution: PFA 4% in PBS w/o Ca^{2+} and Mg^{2+}
- Fluor Preserve Reagent, Calbiochem, California, USA

The following reagent was used for the stimulation of ECFC culture:

- Migration stimulating factor (MSF), kind gift from Dr. Barbara Bottazzi (Immunopharmacology Lab, Istituto Clinico Humanitas)

The following reagents were used for cell line cultures:

- Dulbecco's Modified Eagle Medium (DMEM), Sigma-Aldrich, Missouri, USA
- Endothelial Cell Growth Medium MV2, Promo Cell,Heidelberg, Germany
- Endothelial Cells Medium, Promo Cell, Heidelberg, Germany
- RPMI, Lonza. Basel, Switzerland

3.2 Cell lines

Besides ECFCs in this study we used other cell types – summarized in Table 2 - as positive and negative controls for the optimization of immunofluorescence staining protocols.

Cell Type	Function	Technique	Marker
BCBL-1	Positive control	IFA	LANA
HDLEC	Positive control	IFA	PROX-1 Podoplanin LYVE-1 VEGFR-3
FIBROBLAST	Negative control	IFA	PROX-1 LYVE-1
HUVEC	Negative control	IFA	Podoplanin
SW480	Negative control	IFA	VEGFR-3

Table 2: Summary of cell types used as positive and negative controls for the optimization of the immunofluorescence staining protocols.

The body-cavity-based lymphoma cell line BCBL-1 is a commercial cell line established from the peritoneal effusion of a 40-year-old man (HIV+) with primary effusion lymphoma (PEL). BCBL-1 cells were shown to be latently infected by HHV-8, but not by Epstein-Barr virus (EBV).
BCBL-1 cells grow singly in suspension in RPMI 1640 + FBS (10%) + L-glutammine (3%) + Penicillin-Streptomycin (1%).

HDLECs are mature lymphatic endothelial cells isolated from human derma or skin. They grow in adhesion and were cultured in Endothelial Cell Growth Medium MV2 (EGM MV2). HDLECs are characterized by endothelial morphology and by the expression of the typical lymphatic markers such as Podoplanin, PROX-1, LYVE-1 and VEGFR-3.

Human fibroblasts were a kind gift from Dr. Danese (Istutito Clinico Humanitas). They were isolated from colon of healthy subjects. Fibroblasts were seeded at a density of 20.000 cell/cm^2 and were cultured in Dulbecco's Modified Eagle Medium (DMEM) + 10% FBS + 1% L-Glutamine + 1% penicillin-streptomycin.

HUVECs are mature endothelial cells isolated from Human Umbilical Veins and are one of the most used model for the study of endothelium. They grow in adhesion in Endothelial Cell Basal Medium additioned with specific endothelial growth factors. HUVEC can be expanded by splitting them every 3-4 days and seeding at a density of 8000 cells/cm^2.

SW480 cell line is a commercial cell line established from a primary adenocarcinoma of the colon. SW480 are characterized by epithelial morphology and grow in adhesion. They were seeded at a density of 30.000 cells/cm^2 and were cultured in DMEM + 10% FBS, 1% L-Glutamine + 1% penicillin-streptomycin.

3.3 Subjects enrolled

86 healthy controls and 83 patients with cKS were enrolled in this study. All cKS patients were followed in the Institute of Dermatological Sciences of the Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena. All cKS patients enrolled had histologically confirmed diagnosis of KS, were positive for anti-HHV-8 antibody and were negative for HIV. Patients in systemic chemotherapy and patients in treatment with drugs that have been reported to affect EPC number and functions (Statins, ACE- Inhibitors, calcium antagonists and metformin) were excluded.

cKS patients were classified according to the stage of disease. Staging was performed in accordance with the classification proposed by Brambilla et al [Brambilla L et al, 2003] that takes into account the prevalent type of lesions, localization, clinical behavior, evolutive pattern and presence of complications (Table 3).

		Evolutive Pattern								
		A			B			Bc		
		n.	Age (range)	Sex (ratio m:f)	n.	Age (range)	Sex (ratio m:f)	n.	Age (range)	Sex (ratio m:f)
Stage	I	12	60-81	10:2	19	50-96	16:3			
	II	3	67-76	3:0	21	58-89	18:3			
	III	2	60	2:0	12	51-93	7:5	2	79-85	1:1
	IV	1	72	1:0	5	60:79	5:0	4	65-86	4:0

Table 3: cKS patients enrolled in the study classified on the basis of the stage and the evolutive pattern of disease according to the classification proposed by Brambilla et al.

We collected a sample of heparinized fresh venous blood from all the subjects enrolled in the study. A signed informed consent was obtained from all participants.

3.4 Isolation and expansion of Endothelial Colony Forming Cells (ECFCs)

ECFCs were isolated and cultured by using a protocol recently optimized in our lab [Colombo et al, 2013]. In particular, ECFCs were isolated from PBMCs isolated from fresh samples of heparinized blood collected from both cKS patients and healthy controls.

3.4.1 Isolation of Peripheral Blood Mononuclear cells (PBMCs)

Heparinized blood samples were centrifuged at 1400 rpm for 10 minutes to separate plasma from blood cellular components. After plasma removal, blood cellular components were diluted with HBSS (ratio of 1:3) and Ficoll-Paque was dispensed. The falcon tubes containing 2 phases -the blood cellular components diluted in HBSS on the top and Ficoll-Paque on the bottom of the falcon tube- were centrifuged at 400 rcf for 30 minutes. PBMCs were obtained by Ficoll density gradient centrifugation. In fact, after centrifugation, starting from the bottom of the tube 4 phases containing different cell types can be identified: the bottom layer made up of erytrocytes and granulocytes, the layer of Ficoll-Paque, a thin ring composed by PBMCs and - on the top - the residual plasma and HHBS used for the dilution of blood cellular components. PBMCs were carefully collected and then were washed twice with HBSS.

PBMCs were then resuspended in EGM-2 + 5% FBS culture medium and were counted in a Burker counting chamber. Cell viability was tested with trypan blue.

Culture medium Endothelial Growth Medium (EGM-2) was prepared by adding to the Endothelial Basal Medium (EBM2) fetal bovine serum (FBS), antibiotics (gentamicin, amphotericin B –GA1000) and growth factors that promote endothelial cell differentiation and growth (hydrocortisone, hFGF, VEGF, R3-IGF-1, hEGF, heparin and ascorbic acid). The complete cell culture medium EGM-2 + 5% FBS was used for ECFC isolation and culture.

3.4.2 ECFC culture

3.4.2.1 ECFC isolation

ECFC colonies were isolated from PBMCs obtained from fresh peripheral blood. Once isolated, PBMCs were resuspended in EGM2 + 5% FBS and seeded in mw24 culture plates precoated with fibronectin. Fibronectin was diluted in PBS w/o Ca^{2+} and Mg^{2+} to the final working concentration of 1 μg/cm^2 and fibronectin coating was incubated for 45 minutes at room temperature to allow fibronectin polymerization before cell seeding. PBMCs, seeded at a final density of 2,5 x 10^6/cm^2, were cultured at 37°C with 5% CO_2. After one day of culture, non-adherent cells and debris were removed, adherent cells were washed with EGM-2 + 5% FBS medium and fresh medium was added to each well. Medium was changed every 2 days until the first passage. PBMC cultures were monitored by daily visual inspection using an inverted microscope in order to detect the initial appearance of visible ECFC colonies. ECFC colonies generally appeared 2-3 weeks after PBMC seeding and can be identified as well circumscribed monolayers of cobblestone-like cells.

In order to evaluate the effect of the substrate on the efficiency of ECFC isolation in cKS patients, a portion of PBMCs isolated from 4 cKS patients was seeded in mw24 culture plates coated with collagen. In particular culture plates were coated with collagen diluted in acid acetic 0,02 N at the final working concentration of 50 μg/ml. Collagen coating was incubated for 90 minutes at room temperature to allow collagen polymerization. Before cell seeding, the excess fluid was removed from the coated surface and the culture plates were washed twice with PBS w/o Ca^{2+} and Mg^{2+}.

To evaluate the efficiency of ECFC isolation, the number of PBMC seeded, the number of colonies isolated and the time of colonies appearance – calculated as the number of days starting from the day of PBMCs seeding – were recorded for each subjects enrolled in the study.

3.4.2.2 ECFC expansion

Once identified, ECFC colonies can be detached with Trypsin/EDTA and plated onto 6-well tissue culture plates precoated with collagen. Subconfluent cells were further expanded until cell senescence. In particular, ECFC were expanded in culture plates coated with collagen, in EGM-2 + 5% FBS and they were seeded at a density of 5000 cells/cm^2.

In order to evaluate whether the substrate used for ECFC culture can affect ECFC lymphatic differentiative potential, 15 ECFC colonies isolated from healthy controls were expanded in culture plates coated with fibronectin.

3.4.2.3 ECFC in vitro stimulation with MSF

In order to evaluate whether MSF could promote the expression of lymphatic markers on ECFCs, ECFCs cultured on both fibronectin and collagen were stimulated with MSF.

In preliminary experiments the optimal MSF concentration and the optimal time of stimulation were defined by treating ECFCs with different MSF concentration (10 pg/ml, 100 pg/ml, 10 ng/ml and 100 ng/ml) and for different times (6h ,12h, 24h, 48h and 72h).

Once the optimal MSF concentration and time of incubation have been defined (10 ng/ml for 24 hours), ECFCs cultured on both fibronectin and collagen were stimulated with MSF to evaluate the effect of MSF on the lymphatic differentiative potential of ECFCs. Untreated ECFCs derived from the same ECFC colonies were used as basal.

After stimulation, ECFCs were detached with Trypsin and they were counted in a Burker counting chamber. Cell viability was tested with trypan blue. Cell suspensions were transferred in 1.5 ml eppendorfs and were then centrifuged (maximum speed for 5 minutes). After centrifuge, supernatants were discarded and cell pellets were stored at -80°C for total RNA extraction.

3.5 ECFC characterization

3.5.1 ECFC immunophenotype

3.5.1.1 Evaluation of hematopoietic and endothelial markers expression by flow cytometry

In order to evaluate the immunophenotype of ECFCs isolated from both cKS patients and healthy controls, ECFC expression of hematopoietic markers (CD45 and CD14) and endothelial markers (CD31, CD54, CD144, CD146 and VEGFR-2) and CXCR4 (SDF-1 receptor) were evaluated by flow cytometry.

In particular, ECFCs detached with Accutase were counted and stained with Live/Dead – a viability dye that allows the identification of dead cells. ECFCs were then washed, resuspended in 100 µl of wash buffer (HBBS + 2% FBS) at the final concentration of $1,5x10^6$ cells/ml and were stained with the following monoclonal antibodies: anti-CD31 FITC-conjugated, anti-CD54 FITC-conjugated anti-VEGFR-2 PE-conjugated, anti-CD144 FITC-conjugated, anti-CD146 PE-conjugated, anti-CXCR4 PE Cy7-conjugated anti-CD45 PerCp Cy5.5-conjugated, anti-CD14 PerCp Cy5.5- conjugated. Data were acquired on a FACSCanto II flow cytometer (Becton-Dickinson) and analysis was performed using FlowJo 9.8.5 software (Tristar, Ashland, OR). Cells were electronically gated according to light scatter properties to exclude cell debris.

3.5.1.2 Ulex Europaeus Agglutinin I (UEA I) and Dil-ac-LDL immunofluorescence staining

To further confirm that ECFCs isolated from both cKS patients and healthy controls were endowed with the typical features of ECFCs, the ability to up-take Dil-ac-LDL and bind UEA-1 were evaluated by immunofluorescence.

In particular, ECFCs were incubated for 1 hour at 37°C with Dil-ac-LDL (10 µg/m in EGM). After incubation, cells were washed twice with PBS with Ca^{2+} and Mg^{2+}, fixed for 15 minutes (PFA 2% in PBS with Ca^{2+} and Mg^{2+}) and incubated for 1 hour at 37°C with UEA-1–FITC conjugated (10 µg/ml). cell nuclei were then stained with DAPI. The coverslips with stained cells were mounted onto microscope slides. Stained cells were analyzed on a confocal microscope.

3.5.2 Evaluation of the expression of lymphatic markers

3.5.2.1 Evaluation of the expression of genes typically expressed by LECs

The expression of genes encoding for PROX-1, podoplanin, LYVE-1 and VEGFR-3 was evaluated in ECFCs by Q-PCR.

3.5.2.1.1 RNA extraction, quantification and retro-transcription

Total RNA was extracted from ECFC pellets using a commercial kit (RNeasy® Plus Micro – Qiagen). RNA extraction was performed according to the manufacturers' instructions. RNA purity and concentration were measured using NanoDrop 1000 UV-VIS Spectrophotometer.

1 µg of total RNA extracted from ECFCs was retro-transcribed to cDNA using a commercial kit (High Capacity cDNA Reverse Transcription kit - Applied Biosystem)**.** In particular, the reverse transcription reaction mix has been prepare by combining the components indicated in Table 4.

Reagent	Volume or Quantity
RT Buffer 10x	5 µl
RT Random Primers 10x	5 µl
dDNTPs 100 mM	2 µl
MULTISCRIBE TM REVERSE TRANSCRIPTASE	2.5 µl
RNase Inhibitor	0.05 µl
RNA	1 ug
Water	To 50 µl of final volume

Table 4: Reagents used for the preparation of reverse transcription reaction mix.

Thermal cycling parameters used for RT-PCR are indicated in Table 5

Temperature	25°C	37°C	85°C
Time	10 min	2 h	5 min

Table 5: Thermal cycling parameters used for RT-PCR.

Resulting cDNA was stored at -80°C.

3.5.2.1.2 Q-PCR

The expression of genes typically expressed by LECs - PROX-1, podoplanin, LYVE-1 and VEGFR-3 - was evaluated on ECFCs by Q-PCR.

cDNA was used as the template for Q-PCR and in particular, Q-PCR assay employing the TaqMan probe was performed. GAPDH was used as housekeeping gene.

For each analyzed gene, every sample was analyzed in triplicate and the same amount of starting cDNA was used in each reaction. In particular, 20 ng of cDNA were used per 10 µl amplification reaction.

The reagents used to prepare the PCR reaction mix are shown in Table 6.

Reagent	Volume/reaction
Master mix	5 µl
TAQMAN Probe	0.5 µl
cDNA (20 ng/µl)	1 µl
Water	3.5 µl

Table 6: Reagents used to prepare the PCR reaction mix.

Samples were run on ABI Prism 7900HT system (Applied Biosystem); thermal cycling parameters used for Q-PCR are indicated in Table 7.

	Incubation	Activation Amplitaq Gold	Real-time PCR	
			n. cycles= 45	
			Denaturation	Annealing/Extension
Temperature	50° C	95° C	92°C	60°C
Time	2 min	10 min	15 min	1 min

Table 7: Thermal cycling parameters used for RT-PCR.

The expression level of each gene was normalized to the GAPDH housekeeping gene and was calculated as $2^{(-\Delta CT)}(X10^6)$.

3.5.2.2 Evaluation of lymphatic markers expression by confocal microscopy

3.5.2.2.1 Evaluation of PROX-1, Podoplanin and LYVE-1 expression by multicolor confocal microscopy

For the evaluation of PROX-1, podoplanin and LYVE-1 expression, a protocol for by multicolor immunofluorescence staining was optimized. Multicolor immunofluorescence staining allowed the evaluation of lymphatic markers coexpression.

Three days before the analysis, 20.000 ECFCs were seeded on coverslips coated with fibronectin or collagen. Once confluent, cells were fixed with the fixing solution (4% PFA in PBS w/o Ca^{2+} and Mg^{2+}) for 20 minutes at room temperature. Therefore, fixed cells were washed twice with PBS with Ca^{2+} and Mg^{2+}, blocked for 1 hour (blocking solution: BSA 1%, HS 5%, FBS 5% in PBS with Ca^{2+} and Mg^{2+}) and then incubated 2 hours at room temperature with the primary antibodies (Mouse anti-human Podoplanin and Rabbit anti-human LYVE-1, both diluted 1:50 in blocking solution).

After primary antibody incubation, cells were washed three times with the wash buffer (Tween20 0.05% in PBS with Ca^{2+} and Mg^{2+}) and incubated 1 hour at room temperature with the secondary antibodies (Donkey-anti-Mouse secondary antibody AlexaFluor488-conjugated and Donkey anti-Rabbit secondary antibody, AlexaFluor568-conjugate; both secondary antibodies were diluted 1:1000 in blocking solution). After secondary antibody incubation, cells were washed three times with the wash buffer and they were fixed with PFA 1% in PBS w/o Ca^{2+} and Mg^{2+} for 5 minutes.

After fixation, cells were washed with the wash buffer and were stained for PROX-1. Briefly, cells were blocked (blocking solution: BSA 1% in PBS with Ca^{2+} and Mg^{2+}, incubated for 20 minutes at room temperature), permeabilized (block-perm solution: BSA 1%, FBS 5%, HS 5%, TRITON X100

0,01 % in PBS with Ca^{2+} and Mg^{2+}, incubated for 30 minutes at room temperature) and incubated with the primary antibody (Goat anti human PROX-1, diluted 1:100 in block-perm solution, incubated overnight at +4°C).

After primary antibody incubation, cells were washed three times with the wash buffer and were incubated with the secondary antibody (Donkey anti-Goat secondary antibody, AlexaFluor647-conjugated, diluted 1:1000 in block-perm solution, incubated 1 hour at room temperature).

After secondary antibody incubation, cells were washed with the wash buffer, stained with DAPI (diluted 1:50000 in water, Incubated 10 minutes at room temperature) and washed three times with the wash buffer. The coverslips with stained cells were mounted onto microscope slides.

Stained cells were analyzed on a confocal microscope. For every sample, 10 casual microscopic fields (60x magnification) were acquired and analyzed.

Cells stained only with the secondary antibody were used to evaluate the background due to the aspecific binding of the secondary antibodies and to the cell autofluorescence. HDLECs were used as positive control. Fibroblasts were used as negative control for PROX-1 and LIVE-1 staining; HUVECs were used as negative control for podoplanin staining.

3.5.2.2.2 Evaluation of VEGFR-3 expression by confocal microscopy

For the evaluation of VEGFR-3 expression, 3 days before the analysis, 20.000 ECFCs were seeded on coverslips coated with fibronectin or collagen.

Once confluent, cells were fixed with the fixing solution (4% PFA in PBS w/o Ca 2^{+} and $Mg2^{+}$) for 20 minutes at room temperature. Therefore, fixed cells were washed twice with PBS with Ca^{2+} and Mg^{2+}, blocked for 1 hour (blocking solution: BSA 1% in PBS with Ca^{2+} and Mg^{2+}), permeabilized for 30 minutes (BSA 1%, FBS 5%, HS 5%, TRITON X100 0,01% in PBS with Ca^{2+} and Mg^{2+}) and incubated 2 hours at room temperature with the primary antibody (Mouse anti-VEGFR-3, diluted 1:50 in blocking solution).

After primary antibody incubation, cells were washed three times with the wash buffer (Tween20 0.05% in PBS with Ca^{2+} and Mg^{2+}) and incubated 1 hour at room temperature with the secondary antibody (Donkey-anti-Mouse AlexaFluor488-conjugated, diluted 1:1000 in blocking solution). After secondary antibody incubation, cells were washed three times with the wash buffer, stained with DAPI (Incubated 10 minutes at room temperature, diluted 1:50000 in water) and washed three times with the wash buffer. The coverslips with the stained cells were mounted onto microscope slides.

Stained cells were analyzed on a confocal microscope. For every sample, 10 casual microscopic fields (60x magnification) were acquired and analyzed

Cells stained only with the secondary antibody were used to evaluate the background due to aspecific binding of the secondary antibody and to cell autofluorescence. HDLECs were used as positive control. SW480 cell line – that does not express VEGFR-3 - was used as negative control.

3.5.3 Evaluation of HHV-8 infection

In order to evaluate the presence of HHV-8 infection in ECFC colonies, Latency-Associated Nuclear Antigen (LANA) was detected by confocal microscopy.

Cells were fixed with the fixing solution (4% PFA in PBS w/o Ca^{2+} and Mg^{2+}) for 15 minutes at room temperature. Therefore, fixed cells were washed twice with PBS with Ca^{2+} and Mg^{2+}, permeabilized (permeabilization solution: TRITON X100 0,3% in PBS with Ca^{2+} and Mg^{2+}, incubated for 20 minutes at room temperature) blocked (blocking solution: BSA 2%, NGS 5% in PBS with Ca^{2+} and Mg^{2+}, incubated 20 minutes at room temperature) and then incubated with the primary antibody (Rat anti-LANA –ORF73, diluted 1:1000 in blocking solution, incubated overnight at +4°C).
After primary antibody incubation, cells were washed three times with the wash buffer (Tween20 0.05% in PBS with Ca^{2+} and Mg^{2+}) and incubated with the secondary antibody (Goat-anti-Rat secondary antibody AlexaFluor488-conjugated diluted 1:1000 in blocking solution, incubated 1 hour at room temperature).
After secondary antibody incubation, cells were washed with the wash buffer, stained with DAPI (diluted 1:50000 in water, incubated 10 minutes at room temperature) and washed three times with the wash buffer. The coverslips with stained cells were mounted onto microscope slides. Stained cells were analyzed on a confocal microscope. For every sample, 15 casual microscopic fields (60x magnification) were acquired and analyzed
Cells stained only with the secondary antibody were used to evaluate the background due to the aspecific binding of the secondary antibodies and to the cell autofluorescence. BCBL1 cell line was used as positive control.

3.5.4 Cell senescence

Senescent cells were characterized by low viability – assessed by Trypan Blue exclusion test – and altered morphology – assessed by microscopic inspection. Senescence was further confirmed by positive staining for senescence-associated ß-galactosidase. Senescence-associated ß-

galactosidase assay was performed with a commercial kits (Cellular Senescence Assay Kits, Merck Millipore) used according to the manufacturers' instructions. Senescent cells were identified by observation under an inverted microscope since senescence-associated ß-galactosidase catalyzes the hydrolysis of Xgal, which results in the accumulation of a distinctive blue color in senescent cells.

3.5.5 Cell proliferation

In order to evaluate the proliferative ability of ECFC isolated from healthy controls and cKS patients, at each culture passage detached ECFCs were counted and the cell number and the day of culture were recorded.

3.5.6 *In vitro* vasculogenesis

Matrigel capillary tube formation assay was performed to evaluate the tubule formation ability of ECFCs isolated from cKS patients and healthy controls.

Matrigel is a commercially available basement membrane preparation - isolated from Engelbreth-Holm-Swarm (EHS) mouse sarcoma - that allows to mimic in vitro the cell-ECM interaction.

In Matrigel capillary tube formation assay, 80 µl of Matrigel were added into wells of flat bottom 96 well culture plates. Plates with Matrigel were incubated at 37°C for 45 minutes to allow matrix polymerization into insoluble gels. ECFCs were seeded onto the surface of Matrigel-coated wells at a density of 15×10^3cells/well. Each sample was analyzed in triplicate. The plates were incubated at 37 °C in the CO_2 incubator. After 24 hours, the plates were examined using an inverted phase-contrast microscope. 5 photographs of different microscopic fields were taken for each well (4x magnification). In each photograph, the number of capillary-like structures formed by seeded cells was evaluated.

3.5.7 Cytokine production

IL-6, VEGF, FGFb, TNFα, IFNγ and IL-3 were quantified in ECFC supernatants by specific enzyme-linked immunosorbent assay (ELISA). Cytokine concentration was measured with commercial kits (R&D Systems, Minneapolis, MN) used according to the manufacturers' instructions.

At each passage, ECFC supernatants were collected and centrifuged to eliminate debris and death cells. Cell-free culture supernatants were filtered and stored at – 20°C.

3.5.8 Gene array analysis

ECFCs isolated from cKS patients and healthy controls were compared for gene expression profiling.

Total RNA was extracted from ECFC pellets using a commercial kit (RNeasy® Plus Micro – Qiagen). RNA extraction was performed according to the manufacturers' instructions. RNA purity and concentration were measured using NanoDrop 1000 UV-VIS Spectrophotometer, RNA integrity was evaluated by using an 2100 BioAnalyzer (Agilent Technologies) and samples with a RNA Integrity Number (RIN) lower than 8 were discarded.

RNA extracted from ECFCs was reverse transcribed using the commercial kit Illumina TotalPrep RNA Amplification kit according to the Illumina protocol (Ambion) and cRNA was generated after a 14 hours of in vitro transcription. Gene expression of ECFCs was analyzed by Illumina direct hybridization assays. Washing, staining and hybridization were performed according to the standard Illumina protocol. cRNA obtained from each sample was hybridized onto Illumina HumanHT-12 v4 Expression BeadChip arrays. Hybridization and scanning were performed according to the manufacturer's indications on an Illumina iScan System and data were processed with BeadStudio v.3.

3.6 Statistical analysis

The frequency of subjects who give rise to at least one ECFC colony was shown as percentage and *P* value was calculated as $\chi_c 2$.

The time of ECFC colonies appearance and the frequency of ECFC colonies isolated - evaluated during ECFC isolation phase - were shown as median (interquartile range) and T bars represent 5 and 95 percentile. *P* value calculated by the Mann-Whitney U-test or Wilcoxon signed-rank test, as indicated.

In ECFC expansion phase analysis, data were shown as as median (interquartile range) and T bars represent 5 and 95 percentile. *P* value calculated by the Mann-Whitney U-test or Wilcoxon signed-rank test, as indicated.

In the evaluation of ECFC lymphatic differentiative potential, data of gene expression were shown as median and interquartile and *P* value was calculated by the Mann-Whitney U-test or Wilcoxon signed-rank test, as indicated whereas the data of lymphatic markers expression were shown as percentage or as median (interquartile range) and T bars represent 5 and 95 percentile and *P* value was calculated by $\chi_c 2$ or by the Mann-Whitney U-test or Wilcoxon signed-rank test, as indicated.

4. Results

4.1 Comparison between ECFCs isolated from cKS patients and healthy controls

EPCs play a crucial role in endothelial homeostasis and in vasculogenic processes both in physiological and pathological conditions [Urbich C et al, 2004]. Although different types of cells - isolated from peripheral blood - have been called EPCs in the past, only ECFCs are considered the true EPCs. In fact, ECFCs are endowed with high proliferative potential and are able to give raise to new vessels in vivo and form capillary-like structures in vitro [Fadini GP et al, 2012].

Since in previous studies we demonstrated that circulating EPCs, identified as $CD45^{dim}/CD34^{+}/KDR^{+}$, are increased in cKS patients [Taddeo A et al, 2008] and that in cKS patients ECFCs are HHV-8 infected and can act as viral reservoir [Della Bella S et al, 2008], in this project we evaluated whether EPCs isolated and cultured as ECFCs from cKS patients are characterized by features that resemble the spindle cells, the typical cells of KS lesions. In particular, ECFCs were isolated from cKS patients and healthy controls and were analyzed for the parameters described in the following chapters.

4.1.1 Efficiency of ECFC colonies isolation

In order to assess whether the frequency of ECFCs may be altered in patients with cKS, we compared the appearance of ECFC colonies obtained after seeding PBMCs isolated from cKS patients and healthy donors, by applying a method recently optimized in our lab [Colombo et al, 2013]. Briefly, PBMCs isolated from fresh peripheral blood samples were seeded in culture plates coated with fibronectin, in EGM-2 medium + 5% FBS, and ECFC colonies were identified as cluster of adherent cells with cobblestone-like morphology. Once identified, ECFC colonies were detached and further expanded for several passages in culture plates coated with collagen.

From each subject, PBMCs were isolated from a sample of fresh peripheral blood. PBMC cultures were monitored for 30 days starting from PBMCs seeding by visual inspection using an inverted microscope, in order to identify the ECFC colonies on the basis of their morphological appearance. For each subject, the number of seeded PBMCs, the time of ECFC colonies appearance and the number of ECFC colonies obtained were recorded.

As shown in Figure 6A, the proportion of donors giving rise to at least one ECFC colony was similar among cKS patients and healthy controls (cKS vs controls: 80.72% vs 73.26% subjects who give rise to at least one ECFC colony, n=83, n=86, *P*=n.s.).

The subjects who gave rise to at least one ECFC colony were further characterized and in particular, we evaluated the frequency of ECFC colonies isolated and the time of ECFC colonies appearance. As shown in Figure 6B, the frequency of ECFCs was strikingly higher in cKS patients than healthy controls (median, cKS vs controls: 15.0 vs 2.6 ECFC colonies/10^8 seeded PBMCs, n=67, n=63, *P*<0.0001). Moreover, ECFC colonies isolated from cKS patients appeared some days earlier than those from healthy controls (median cKS vs controls: 13.6 vs 19.0 days, n=67, n=63, *P*<0.0001) (Figure 6C).

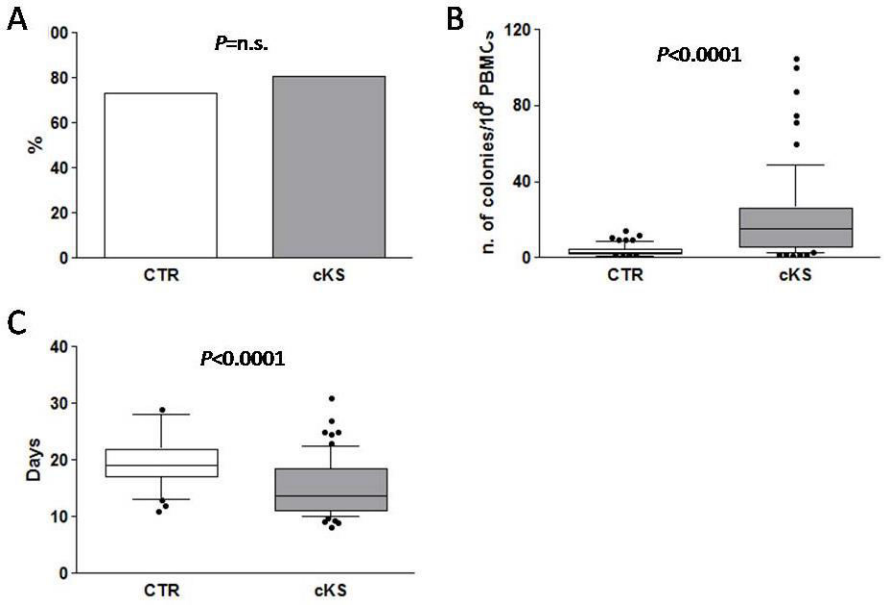


Figure 6: ECFC isolation from healthy controls and cKS patients. (A) Frequency of subjects who gave rise to at least one ECFC colony. Percentage of subjects who gave rise to at least one ECFC colony within 30 days after PBMC seeding in healthy controls (CTR, white bar) and cKS patients (cKS, gray bar). Data shown as percentage. P value calculated by the $\chi_c 2$ test. (B) Frequency of ECFC colonies. Frequency of ECFC colonies isolated from healthy controls (CTR, white box) and cKS patients (cKS, gray box). The frequency of ECFC colonies isolated was expressed as number of ECFC colonies isolated/10^8 seeded PBMCs. Data shown as median (interquartile range) and T *bars represent 5 and 95 percentile. P value calculated by Mann-Whitney test. (C) Time of appearance of ECFC colonies. Time of appearance of ECFC colonies isolated from healthy controls (CTR, white box) and cKS patients (cKS, gray box). Data shown as median (interquartile range) and* T *bars represent 5 and 95 percentile. P value calculated by Mann-Whitney test.*

These findings were relatively stable over time. In fact, 5 cKS patients with a stable stage of disease over a period of six months and 5 healthy controls were analyzed for circulating ECFCs at a second time point and showed the persistence of similar ECFC frequencies (median in cKS: 14.6 and 16.8 ECFC colonies/10^8 PBMCs, n=5, *P*=n.s.; median in controls: 3.1 and 2.9, n=5, *P*=n.s.) and similar time of appearance (median in cKS: 15.3 and 13.9 days, n=5, *P*=n.s.; median in controls: 18.3 and 18.5, n=5, *P*=n.s.) (Data not shown).

Moreover, in the group of cKS patients, we evaluated whether the stage and evolutive pattern of disease could influence the efficiency of ECFC isolation.

As shown in Figure 7, the increased frequency of ECFC colonies was more pronounced in patients with rapidly evolving (all stages B) than slowly evolving (all stages A) disease (median in stage B vs stage A: 16.3 vs 7.7 ECFC colonies/10^8 seeded PBMCs, n=46, n=15, P<0.05) (Figure 7A), whereas no correlation between the frequency of ECFC colonies and clinical stage (I, II, III, IV) was observed (median in stage I-II vs stage III-IV: 15.62 vs 15 ECFC colonies/10^8 seeded PBMCs, n=46, n=21, P=n.s.) (Figure 7B). No correlation between the frequency of subjects who gave rise to at least one ECFC colony and clinical stage or evolutive pattern was observed (percentage in stage I-II vs stage III-IV: 83.64% vs 75% subjects who give rise to at least one ECFC colony, n=55, n=28, P=n.s.; percentage in stage B vs stage A: 80.7% vs 83.3%, n=57, n=18, P=n.s.) (Data not shown). No correlation between the time of appearance of ECFC colonies and clinical stage or evolutive pattern was observed (median in stage I-II vs stage III-IV: 13.6 vs 13.65 days, n=46, n=21, P=n.s.; median in stage B vs stage A: 13.6 vs 13.67, n=46, n=15, P=n.s.) (Data not shown).

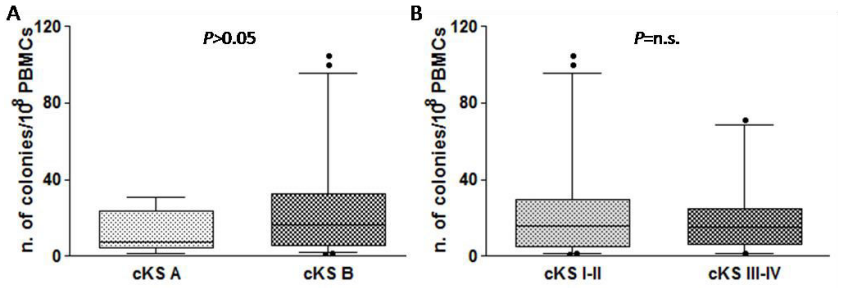


Figure 7: Frequency of ECFC colonies isolated from cKS patients stratified on the basis of the evolutive pattern and the stage of disease. (A) Frequency of ECFC colonies isolated from cKS patients stratified on the basis of the evolutive pattern of disease. Frequency of ECFC colonies isolated from cKS patients with slowly evolving disease, stage A (cKS A) and cKS patients with rapidly evolving disease, stage B (cKS B). The frequency of ECFC colonies isolated was expressed as number of ECFC colonies isolated/10^8 seeded PBMCs. Data shown as median (interquartile range) and T bars represent 5 and 95 percentile. p value calculated by Mann-Whitney test. (B) Frequency of ECFC colonies isolated from cKS patients stratified on the basis of the stage of disease. Frequency of ECFC colonies isolated from cKS patients with early stages of disease, stage I and II (cKS I-II) and cKS patients with advanced stages of disease, stage III and IV (cKS III-IV). The frequency of ECFC colonies isolated was expressed as number of ECFC colonies isolated/10^8 seeded PBMCs. Data shown as median (interquartile range) and T bars represent 5 and 95 percentile. P value calculated by Mann-Whitney test.

In order to assess whether the use of collagen rather than fibronectin used for coating plastic plates may differently affect the efficiency of ECFC colony isolation from cKS patients, in few experiments we compared the appearance of ECFC colonies obtained after seeding PBMCs from cKS on either substrate. To this aim, the PBMCs obtained from 4 cKS patients were divided into

portions, with half of them seeded on fibronectin-coated plates and the rest seeded on collagen-coated plates.

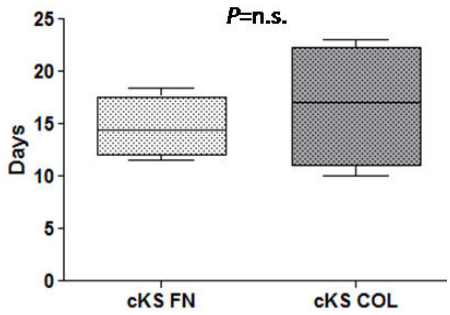


Figure 8: Effects of fibronectin and collagen on isolation of ECFC colonies in cKS patients. Time of appearance of ECFC colonies obtained from cKS patients whose PBMCs were seeded on fibronectin (cKS FN) or collagen–coated culture plates (cKS COL). Data shown as median (interquartile range) and T bars represent 5 and 95 percentile. P value calculated by Wilcoxon signed-rank test.

Similarly to our previous observation in healthy donors [Colombo et al, 2013], also ECFCs from cKS patients were isolated more efficiently when fibronectin was used, as ECFC colonies appeared some days earlier when PBMCs were seeded on fibronectin than collagen (median fibronectin vs collagen: 14.4 vs 17.1 days, n=4, n=4, *P*=n.s.) (Figure 8).

4.1.2 In vitro expansion of ECFCs

Once isolated, ECFC colonies can be expanded in vitro for several passages allowing their phenotypic and functional characterization.

In particular, we compared ECFCs isolated from cKS patients and healthy controls and we evaluated: the immunophenotype and morphology, the life span, the proliferative and in vitro vasculogenic potential of ECFCs. Moreover, we evaluated also ECFC cytokine production and ECFC molecular signature.

4.1.2.1 Immunophenotype

In order to assess whether ECFCs obtained from cKS patients may have a different immunophenotype compared with healthy controls, in few cultures we analyzed the expression of endothelial and non-endothelial markers.

As shown in Figure 9, ECFCs isolated from both cKS patients and healthy controls were endowed with characteristics typical of ECFCs. In facts, immunophenotyping by flow cytometry revealed that ECFCs isolated from both cKS patients and healthy controls did not express the pan-leukocyte marker CD45 and the monocyte/macrophage marker CD14 (Figure 9A) whereas they expressed typical endothelial cell-surface antigens such as CD31, CD54, CD144, CD146 and VEGFR-2 and CXCR4 (SDF-1 receptor) (Figure 9B). In addition, as confirmed by confocal microscopy analysis

ECFCs isolated from both cKS patients and healthy controls incorporated Dil-ac-LDL and bound lectin UEA- 1 (Figure 9C).

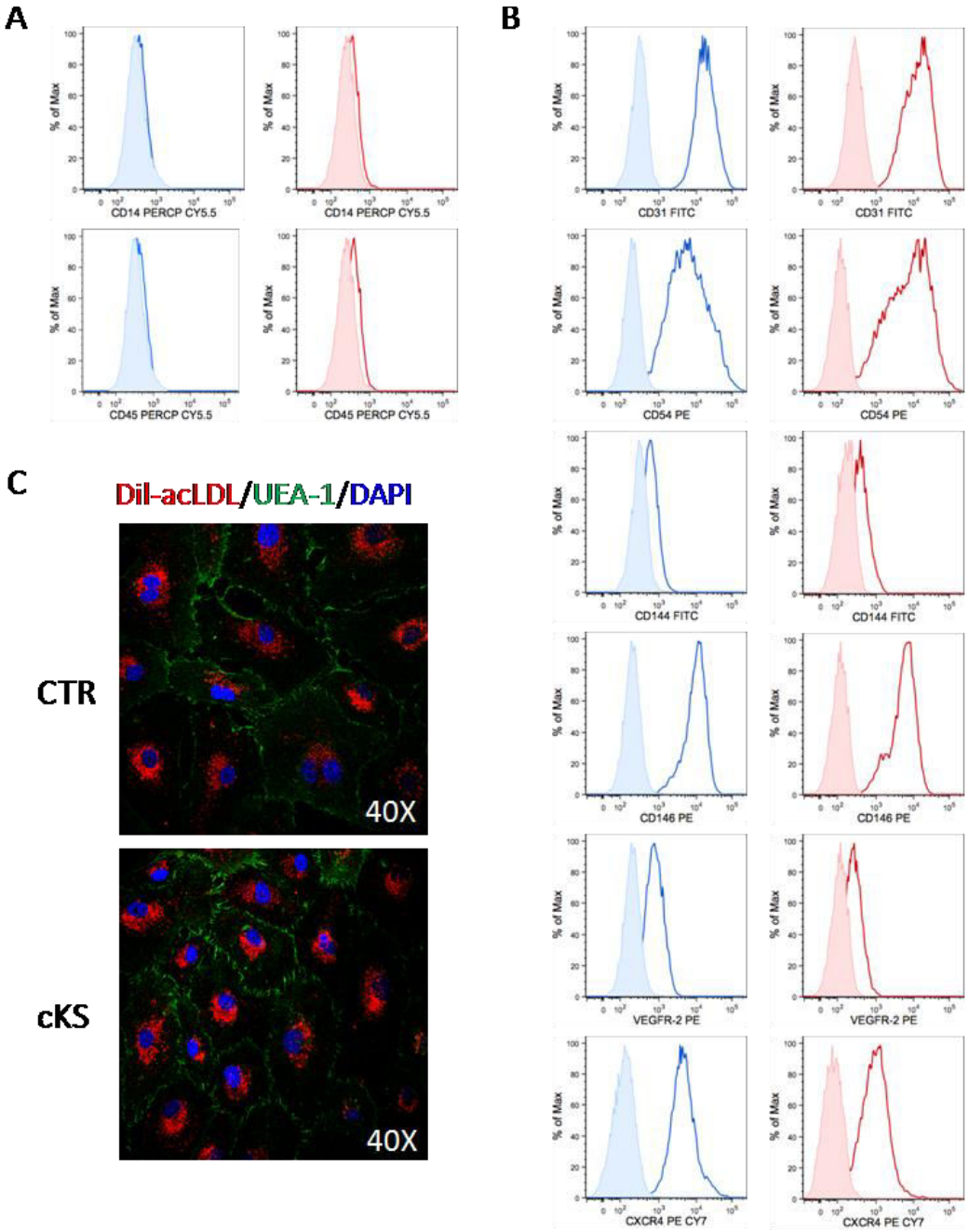


Figure 9: Immunophenotype of ECFCs isolated from healthy controls and cKS patients. (A) CD14 and CD45 expression and (B) endothelial markes (CD31, CD54, CD144, CD146 and VEGFR-2) and CXCR4 (SDF-1 receptor) expression in ECFCs isolated from healthy controls (blue histograms) and cKS patients (red histograms). Data shown as flow cytometric histograms. For each marker, representative flow cytometric histograms of one ECFC colony isolated from a healthy control and one ECFC colony isolated from a cKS patient are shown. In each flow cytometric histogram the overlay of stained (empty curve) and unstained cells (filled curve) is shown. (C) Representative immunofluorescence photographs showing the uptake of Dil-ac-LDL (in red) and the binding of lectin UEA-1 (in green) in ECFCs isolated from healthy controls and cKS patients. Photographs were obtained using an Olympus Fluoview FV1000 confocal microscope, 40x magnification.

4.1.2.2 Detection of HHV-8 infection

In a previous study we reported that ECFCs isolated from cKS were HHV-8-infected as we detected HHV-8 DNA in both cells and culture supernatants [Della Bella S et al, 2008].

In order to confirm the presence of HHV-8 infection also with a different technique, we investigated the presence of LANA - a nuclear antigen typically expressed during the latent phase of HHV-8 infection - by immunofluorescence assay. In particular, LANA detection by confocal microscopy allowed to evaluate the percentage of HHV-8-infected cells in ECFC cultures and to evaluate the antigen localization within the cells.

We first set up the protocol for LANA detection in ECFC cultures. In particular, we defined the optimal concentration and incubation conditions for the primary antibody (diluition 1:1000; final working concentration of 1 µg/ml, incubated overnight at +4°C).

The body-cavity-based lymphoma (BCBL)-1 cell line was used as positive control for LANA staining. In fact, BCBL-1 cells are latently infected with HHV-8 and LANA is expressed costitutively in nearly all cells.

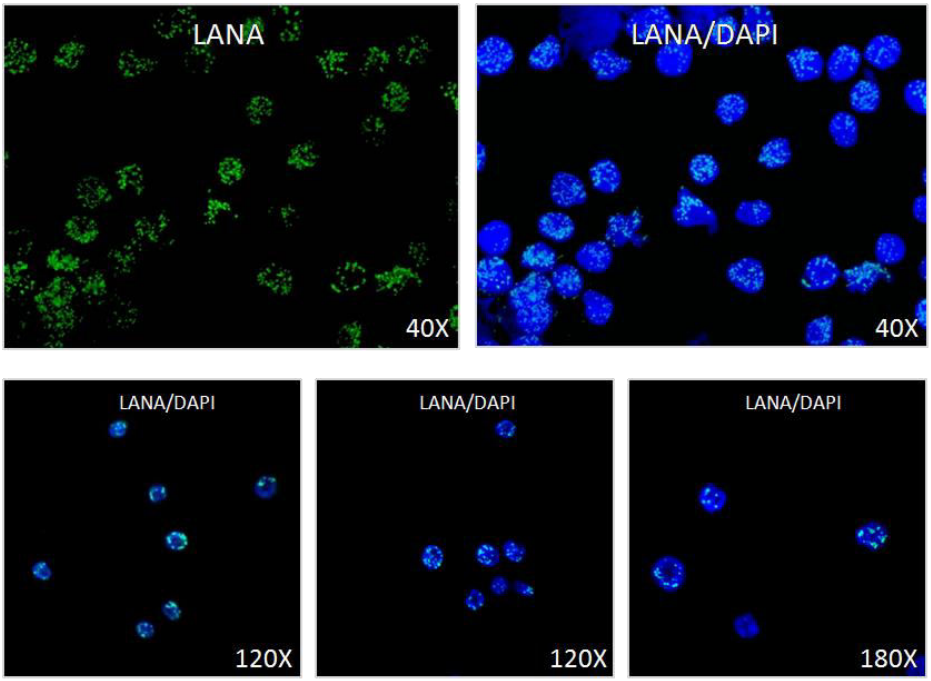


Figure 10: LANA detection in BCBL-1 cells. Representative immunofluorescence photographs of BCBL-1 cells stained for LANA (green). Cells were stained for LANA and cell nuclei were stained with DAPI (blue) Photographs were obtained using an Olympus Fluoview FV1000 confocal microscope; photographs at 40x, 120x and 180x magnification are shown.

As shown in figure 10, the optimized protocol allowed the detection of LANA that is expressed with the typical intranuclear punctate pattern in nearly all BCBL-1 cells.

The optimized protocol was used to evaluate LANA expression - and therefore the presence of HHV-8 infection - in ECFC colonies isolated from cKS patients and healthy controls.

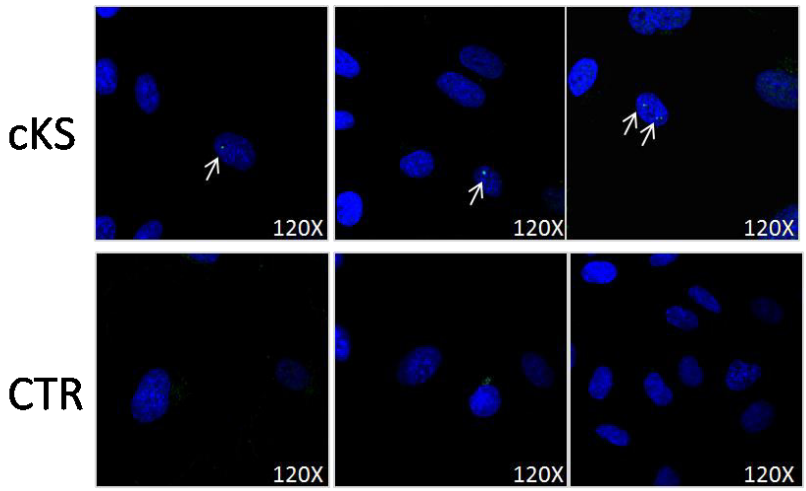


Figure 11: LANA detection in ECFC cultures. Upper row: representative immunofluorescence photographs showing ECFCS isolated from cKS patients stained for LANA; lower row: representative immunofluorescence photographs showing ECFCS isolated from HHV-8 seronegative healthy controls stained for LANA. Cells were stained for LANA (intranuclear green dots, indicated by white arrows) and cell nuclei were stained with DAPI (blue). Photographs were obtained using an Olympus Fluoview FV1000 confocal microscope; photographs at 120x magnification.

As shown in figure 11, confocal analysis for LANA expression revealed that all the screened ECFC colonies isolated from cKS patients expressed LANA antigen thus confirming that they were HHV-8 infected. Notably, the expression level of LANA was lower in KS-ECFCs than in BCBL-1 and only a portion of cells of each screened ECFC colony expressed LANA. The percentage of $LANA^+$ cells was variable among the analyzed colonies, ranging from 5% to 20% of total cells (ECFC colonies analyzed n=6). As expected, all the screened ECFC colonies isolated from HHV-8 seronegative healthy controls were negative for LANA expression (ECFC colonies analyzed n=5).

4.1.2.3 Senescence

In order to evaluate the senescence of ECFC colonies, we identified as senescent cells those with low viability (viable cells <60%) and altered morphology. Cell senescence was further confirmed by the β-galactosidase assay, based on the expression of high levels of the senescence-associated enzyme β-galactosidase by senescent cells (Figure 12).

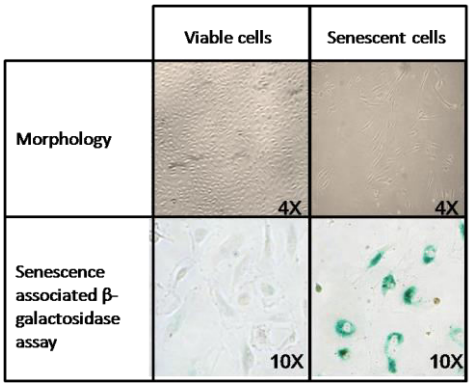


Figure 12: Senescence evaluation in ECFC cultures. Representative photographs of viable cells characterized with typical cobblestone-like morphology and absence of senescence-associated β-galactosidase activity (left column); representative photographs of senescent cells characterized by altered morphology and expression of high levels of senescence-associated β-galactosidase, as indicated by the presence of blue/green precipitate (right column). Photographs were obtained using an Olympus inverted microscope IX51, 4x and 10x magnification.

Similarly to ECFCs isolated from healthy controls, ECFCs obtained from cKS patients maintained stable endothelial morphology and exhibited features of primary cells by ultimately becoming senescent after long term culture as confirmed by senescence associated β-galactosidase assay (median cKS vs controls: 7 vs 6 passage, n=45, n=45, *P*=n.s.) (data not shown).

4.1.2.4 Proliferative potential

In order to assess whether ECFCs obtained from cKS patients may have an in vitro behaviour that may evoke the in vivo behaviour of KS spindle cells, we analyzed the proliferation curves of ECFC colonies obtained from cKS and healthy donors. To this aim, we analyzed and compared 45 consecutive ECFC colonies obtained from each group.

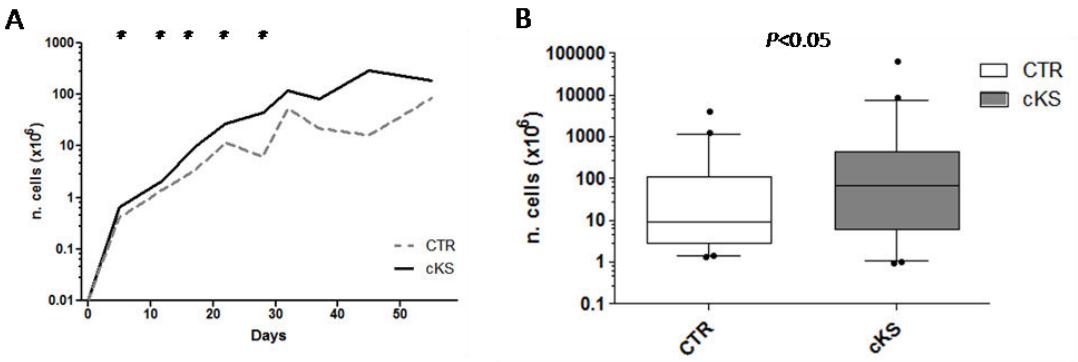


*Figure 13: Proliferative potential of ECFCs isolated from healthy controls and cKS patients. (A) Ex vivo expansion of ECFCs isolated from healthy controls (CTR) and cKS patients (cKS). Data shown as median curves. P values calculated by Mann-Whitney test (*P<0.05, #P<0.01, §P<0.001. (B) Maximum cell yield obtained after ex vivo expansion of ECFCs isolated from healthy controls (CTR) and cKS patients (cKS). Data shown as median (interquartile range) and T bars represent 5 and 95 percentile. P value calculated by Mann-Whitney test.*

ECFCs isolated from cKS patients were endowed with a higher proliferative potential than ECFCs isolated from healthy controls. Median curves which described the cell yield at each passage of ECFC cultures are reported in Figure 13A. Despite the cell number increased with increasing days of culture in ECFCs isolated from both groups, the cell yield was higher in cultures of ECFCs isolated from cKS patients at each time point (<10 days of culture: n=60 vs 54, *P*<0.05; 10-14 days: n=67 vs 57, *P*<0.05; 15-19 days: n= 40 vs 34, *P*<0.05; 20-24 days: n=35 vs 28, *P*<0.05; 25-29 days: n=25 vs 23, *P*<0.05; 30-34 days: n=13 vs 9, *P*=n.s.; 35-39 days: n=8 vs 6, *P*=n.s.; 40-49 days: n=6 vs 12, *P*=n.s.; 50-59 days: n= 2 vs 3, *P*=n.s.). Accordingly, also the maximum cell yield obtained after ECFC ex vivo expansion was higher in cultures of ECFCs isolated from cKS patients than healthy controls (median cKS vs controls: 69.55 vs 5.58 number of cells $x10^6$, n=45 vs 45, *P*<0.05) (Figure 13B).

To further confirm the higher proliferative ability observed in ECFC colonies isolated from cKS patients, we evaluated the proliferation rate of ECFCs isolated from both cKS patients and healthy controls.

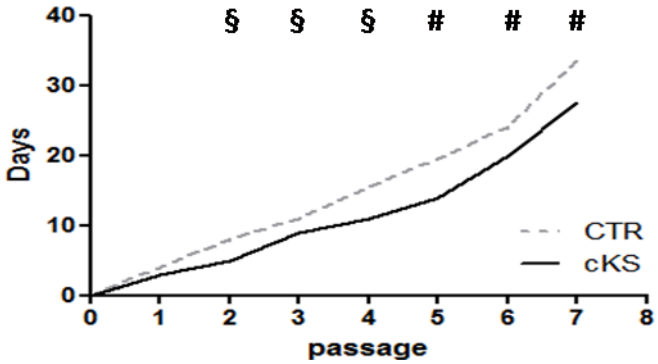


*Figure 14: Proliferation rate of ECFCs isolated from healthy controls and cKS patients. Proliferation rate of ex vivo expanded ECFCs isolated from healthy controls (CTR) and cKS patients (cKS). Data shown as median curves of day of culture at each culture passage. P values calculated by Mann-Whitney test (*P<0.05, #P<0.01, §P<0.001).*

As shown in Figure 14, ECFCs isolated from cKS patients proliferated faster than ECFCs isolated from healthy controls. In fact, ECFCs isolated from cKS patients reached late passages earlier than ECFCs isolated from healthy donors (cKS vs controls: n=28 vs 28, passage 1: *P*=n.s.; passage 2: *P*<0.001; passage 3: *P*=<0.001; passage 4: *P*=<0.001; passage 5: *P*=<0.01; passage 6: *P*=<0.01; passage 7: *P*=<0.01).

Moreover, in the group of cKS patients, we evaluated whether the stage and the evolutive pattern of disease could influence the proliferative potential of ECFCs isolated from cKS patients. No

correlation between the proliferative potential and clinical stage (I, II, III, IV) or evolutive pattern (A,B) of disease was observed (data not shown).

4.1.2.5 In vitro vasculogenic potential

In order to evaluate whether ECFCs isolated from cKS patients were characterized by a higher vasculogenic potential than ECFCs isolated from healthy controls, we performed Matrigel capillary tube formation assay. In particular, we evaluated the ability of ECFCs to form capillary-like structures when seeded in Matrigel matrix (Figure 15).

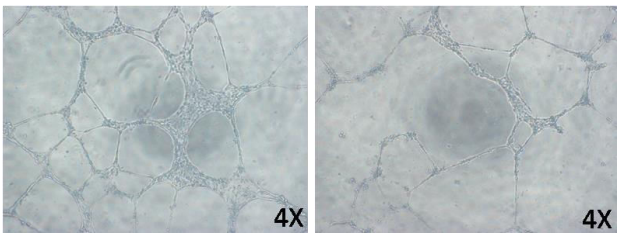


Figure 15: Matrigel capillary tube formation assay. Representative phase-contrast photographs showing the capillary-like structures formed by ECFCs seeded in Matrigel matrix. Photographs were obtained using an Olympus inverted microscope IX51, 4X magnification.

Although ECFCs isolated from both cKS patients and healthy controls were able to form capillary-like structures when seeded in Matrigel matrix, ECFCs isolated from cKS patients were endowed with a higher vasculogenic potential than ECFCs isolated from healthy controls.

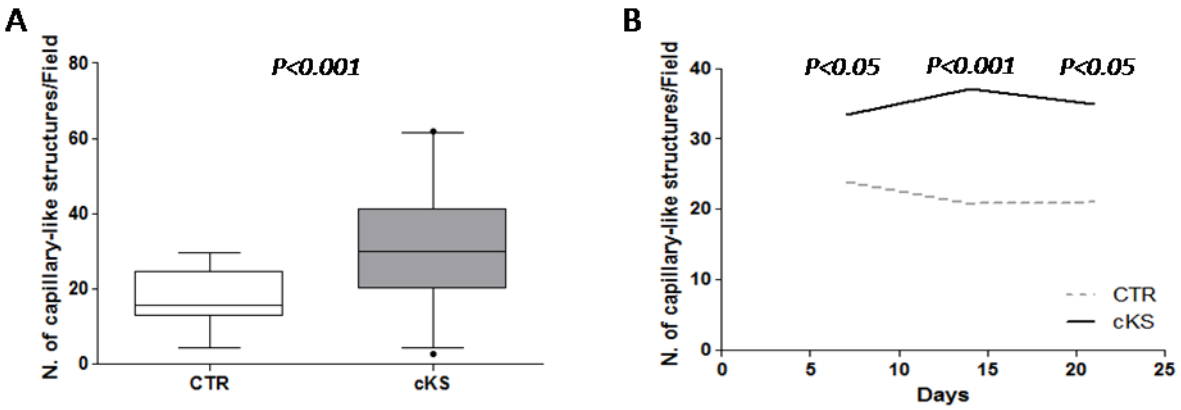


Figure 16: Vasculogenic potential of ECFCs isolated from healthy controls and cKS patients. The capillary-like structure formation was evaluated 24h after ECFC seeding in Matrigel matrix by observation with inverted microscopy. (A) Number of capillary-like structures formed by ECFCs isolated from healthy controls (CTR, white box) and cKS patients (cKS, gray box). Data shown as median (interquartile range) and T bars represent 5 and 95 percentile. p value calculated by Mann-Whitney test. (B) Vasculogenic potential evaluated at different time points in ECFCs isolated from healthy controls (CTR) and cKS patients (cKS). Data shown as median curves. p values calculated by Mann-Whitney test.

As shown in Figure 16A, ECFCs isolated from cKS patients formed a significantly higher number of capillary–like structures than ECFCs isolated from healthy controls (median cKS vs controls: 30 vs

15.8 capillary-like structures/field, n=19, n=15, *P*<0.001). To evaluate whether the vasculogenic potential of ECFCs may vary during ex vivo expansion, the angiogenic potential of 4 ECFC colonies isolated from healthy controls and 4 ECFC colonies isolated from cKS patients was assessed at different time point during the ex vivo expansion .As shown in Figure 16B, ECFCs isolated from cKS patients - after 24 hours of culture in Matrigel - displayed a higher vasculogenic potential at each analyzed time point (median number of capillary-like structures/field, cKS vs controls, at day 7: 33.43 vs 23.89, *P*<0.05.; at day 14: 37.17 vs 20.28, *P*<0.01; at day 21: 35 vs 21, *P*<0.05).

We further evaluated whether the stage and the evolutive pattern of disease could influence the angiogenic potential of ECFCs isolated from cKS patients. No correlation between the angiogenic ability of ECFCs and the stage or the evolutive pattern of disease was observed (data not shown).

4.1.2.6 Cytokine production

To investigate the mechanisms possibly involved in sustaining the higher proliferative and vasculognic potential observed in ECFCs isolated from cKS patients than healthy controls, we evaluated the cytokine production of ECFCs. In particular, we analyzed in our cultures the main proangiogenic cytokines secreted by endothelial progenitor cells [Krenning G et al, 2009] and the cytokines typically present in KS lesions [Sirianni MC et al, 1998]. To this aim, we measured by ELISA assay the levels of IL-6, IL-3, IFNγ, TNFα, FGFb and VEGF in the supernatants recovered from each ECFC culture just before cell passaging. VEGF, IFNγ, TNFα and IL-3 were undetectable at any passage in ECFCs isolated from both healthy controls and cKS patients, in accordance with a previous study [Hur J et al, 2004].

FGFb was detectable in ECFC cultures isolated from both healthy controls and cKS patients but no differences in FGFb concentration were observed between the two groups (data not shown).

As shown in Figure 17, IL-6 was detectable in ECFCs isolated from both healthy controls and cKS patients. Moreover, IL-6 concentration - expressed as pg/10^5 cells – increased during ECFC ex vivo expansion (Figure 17A) and was higher in culture supernatants of ECFCs isolated from cKS patients than healthy controls at each time point analyzed (cKS vs controls, <10 days of culture: n=34 vs 33, *P*=n.s.; 10-14 days: n=40 vs 37, *P*<0.05; 15-19 days: n=19 vs 35, *P*<0.05; 20-24 days: n=14 vs 30, *P*<0.05; 25-29 days: n=8 vs 16, *P*<0.05; 30-34 days: n=5 vs 7, *P*<0.05; 35-39 days: n=4 vs 7, *P*<0.05; 40-49 days: n= 3 vs 9, *P*<0.05; 50-59 days: n=3 vs 3, *P*=n.s.). In addition, the maximum levels of IL-6 detected in the supernatants of ECFC cultures were significantly higher in ECFCs isolated from cKS

patients than healthy controls (median cKS vs controls: 2467.48 vs 847.37 IL-6 pg/10^5 cells, n=29, n=40, *P*<0.05) (Figure 17B).

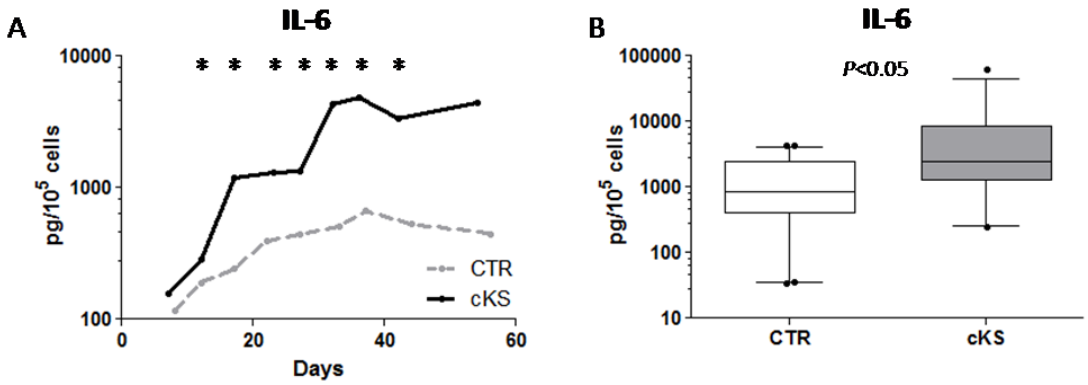


*Figure 17: IL-6 production by ECFCs. (A) IL-6 levels, expressed as pg/10^5 cells, were measured in culture supernatants of ECFCs isolated from healthy controls (CTR, gray dashed line) and cKS patients (cKS, black line). Data shown as median curves. P values calculated by Mann-Whitney test (*P<0.05, $^{\#}$P<0.01, §P<0.001). (B) Maximum levels of IL-6, expressed as pg/10^5 cells, detected in culture supernatants of ECFCs isolated from healthy controls (CTR, white box) and cKS patients (cKS, gray box). Data shown as median (interquartile range) and T bars represent 5 and 95 percentile. P value calculated by Mann-Whitney test.*

We also evaluated whether stage and evolutive pattern of disease could influence IL-6 production of ECFCs isolated from cKS patients. No correlation between the levels of IL-6 produced by ECFCs and the stage or evolutive pattern of disease was observed (data not shown).

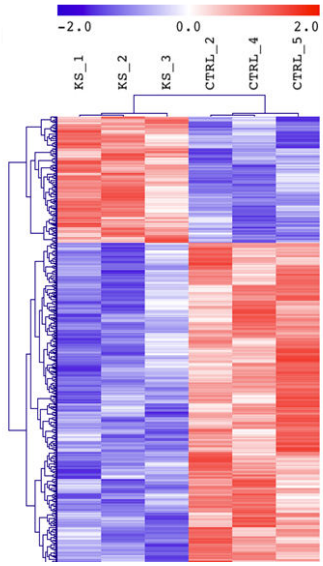
4.1.2.7 Gene array analysis

In order to investigate the molecular pathways possibly involved in the different behaviour of ECFCs isolated from cKS patients, we performed a gene-array analysis comparing the molecular signature of ECFC isolated from cKS patients and healthy controls.

ECFCs isolated from 3 healthy controls and 3 cKS patients were analyzed by gene array. Total RNA isolated from ECFCs was reverse-transcribed and cRNA was hybridized onto Illumina Human HT-12 v4 Expression BeadChip arrays.

Preliminary analysis revealed that, as shown in the heat map in Figure 18A, patients and healthy controls segregated by clustering, thus confirming that gene expression profile differs between ECFCs isolated from cKS patients and healthy donors.

A



B

Gene upregulated in cKS ECFC
FAM60A
CDV3
PTPN12
C9orf30
TRIT1
LOC647150
ISOC1
TARS
PSMD14
SCYL2
LOC728809
DNAJC10
POLR2J2
C14orf104
HDAC2
SLC38A1

Figure 18: Gene expression profile of ECFCs isolated from healthy controls and cKS patients. (A) Hierarchical clustering of samples and transcripts. The heat map shows the relative expression levels of each transcript (rows) in each sample (column). Rows and columns are hierarchically clustered. (B) List of upregulated genes in ECFCs isolated from cKS patients compared with healthy controls (fold change >1.5 and P<0.05.)

In particular, as shown in Figure 18B, 16 genes were found to be upregulated (fold change >1.5 and P<0.05) in ECFCs isolated from cKS patients compared to ECFCs isolated from healthy controls. More detailed analysis are still in progress in order to evaluate the possible role of the genes that have been found to be differentially expressed in determining the typical behavior observed in ECFCs isolated from cKS. In particular, deubiquitinase PSMD14 - whose expression was significantly up-regulated in ECFCs isolated from cKS patients - was reported to be involved in regulation of cell proliferation. In fact, Byrne et al documented that knockdown of human PSMD14 induced cell cycle arrest and senescence in carcinoma cell lines [Byrne A et al, 2010].

Moreover, gene set enrichment analysis (GSEA) will be performed to evaluate whether specific gene-sets are differentially expressed by ECFCs isolated from cKS patients. In particular, genes specifically involved in angiogenesis, cell proliferation, IL-6 pathway and VEGFR-3 pathway and genes that are differentially expressed by BECs and LECs will be evaluated.

4.2 Evaluation of lymphatic differentiative potential of ECFCs

Several studies reported that spindle cells in KS lesions express the lymphatic markers LYVE-1, podoplanin and VEGFR-3 [Pyakurel P, 2006] and in vitro HHV-8-infected BECs upregulate the expression of lymphatic markers including PROX-1 [Hong YK et al, 2004]. Therefore, we initially planned to evaluate the expression of LYVE-1 and podoplanin in ECFCs isolated from cKS patients and healthy controls. Unexpectedly, in preliminary experiments performed in few ECFC cultures analyzed by immunofluorescence microscopy, we observed that podoplanin and LYVE-1 were expressed not only by ECFCs isolated from cKS patients but also by ECFCs isolated from healthy controls (Figure 19).

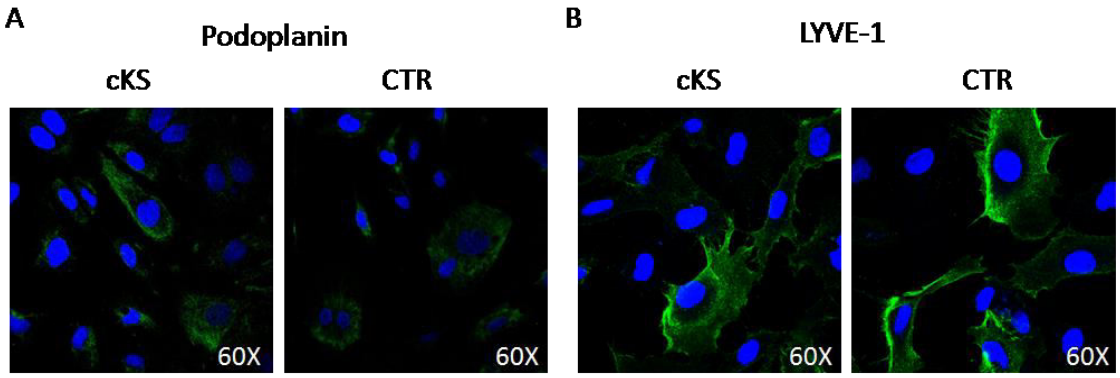


Figure 19: ECFCs isolated from cKS patients and healthy controls stained for podoplanin and LYVE-1. (A) representative photographs showing immunofluorescence staining for podoplanin (green) in ECFCs isolated from cKS patients (cKS) and healthy controls (CTR); (B) representative photographs showing immunofluorescence staining for LYVE-1 (green) in ECFCs isolated from cKS patients (cKS) and healthy controls (CTR). Cell nuclei were stained with DAPI (blue). Photographs were obtained using an Olympus Fluoview FV1000 confocal microscope, 60X magnification.

These results were totally unexpected, as the expression of lymphatic markers by ECFCs had never been reported. Moreover, in recent years several studies supported the hypothesis that a population of lymphatic endothelial progenitor cells (LEPCs) could be involved in lymphangiogenic processes [Park C. et al, 2011]. Nevertheless, the identity of LEPCs has not yet been fully elucidated.

Therefore, we studied the expression of lymphatic markers on ECFCs isolated from healthy controls.

4.2.1 Evaluation of lymphatic differentiative potential of ECFCs in basal conditions

In order to investigate the lymphatic differentiative potential of ECFCs, the expression of mRNA encoding for PROX-1, Podoplanin, LYVE-1 and VEGFR-3 in ECFCs was investigated by Q-PCR. We further confirmed the expression of the lymphatic markers by confocal microscopy.

Moreover, we evaluated whether fibronectin – an ECM protein commonly used as substrate for ECFC culture – may foster ECFC differentiation toward the lymphatic phenotype. In fact, several clinical studies reported that the presence of fibronectin in tumor stroma correlates with lymphangiogenesis and lymph node metastases [Bae YK et al, 2013; Ou J et al, 2013]. Therefore, we compared the expression of PROX-1, Podoplanin, LYVE-1 and VEGFR-3 between ECFCs cultured on fibronectin and ECFCs cultured on collagen – another ECM protein commonly used as a substrate for ECFC culture.

4.2.1.1 Detection of mRNA encoding for lymphatic markers by Q-PCR

The expression of mRNA encoding for PROX-1, Podoplanin, LYVE-1 and VEGFR-3 was investigated in ECFCs by Q-PCR. GAPDH was used as the housekeeping and the expression level of each marker was shown as $2^{-\Delta CT}$ ($X10^6$).

ECFCs cultured both on fibronectin and collagen expressed the transcripts encoding PROX-1, LYVE-1 and VEGFR-3. Also podoplanin was expressed by ECFCs but this lymphatic marker was expressed only by some of the ECFC colonies screened and the percentage of ECFC colonies that expressed podoplanin was similar between ECFC colonies cultured on fibronectin and on collagen (fibronectin vs collagen: 72.73% vs 54.5% of ECFC colonies that expressed podoplanin, *P*=n.s.) (Data not shown).

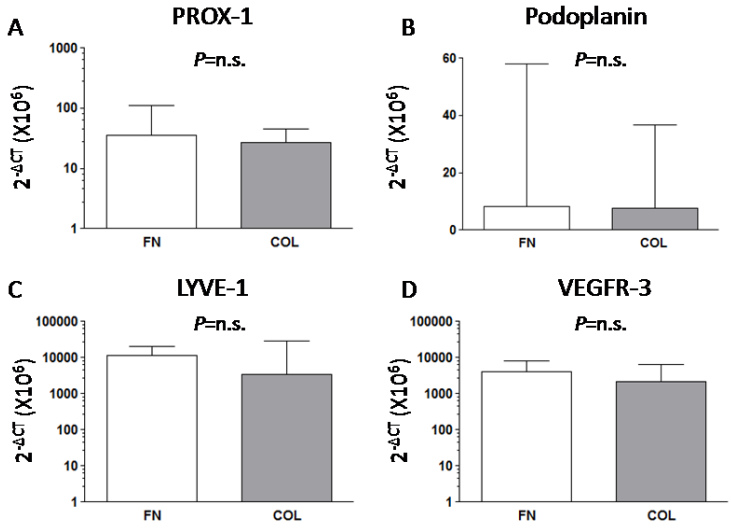


Figure 20: Expression of mRNA encoding for PROX-1, podoplanin, LYVE-1 and VEGFR-3 evaluated by Q-PCR. The expression of (A) PROX-1, (B) podoplanin, (C) LYVE-1 and (D) VEGFR-3 was assessed in ECFCs cultured on fibronectin (FN, white bar) or collagen (COL, gray bar). GAPDH was used as the housekeeping and the expression level of each marker was shown as $2^{-\Delta CT}$ ($X10^6$). Data shown as median±interquartile range. P value calculated by Mann-Whitney test.

Moreover, the expression levels of the analyzed markers were variable between colonies. As shown in figure 20, ECFCs cultured on fibronectin and collagen expressed PROX-1, podoplanin, LYVE-1 and VEGFR-3 at similar levels (median $2^{-\Delta CT}(X10^6)$ on fibronectin vs collagen, PROX-1: 35.84 vs 27.31, n=11 vs 11, *P*=n.s.; podoplanin: 8.31 vs 7.57, n=8 vs 6, *P*=n.s.; LYVE-1: 11406 vs 3431, n=11 vs 11, *P*=n.s.; VEGFR-3: 4010 vs 2219, n=11 vs 11, *P*=n.s.).

In order to evaluate whether ECFCs isolated from cKS patients express the lymphatic markers – a typical feature of KS spindle cells [Pyakurel P, 2006] and in vitro HHV-8-infected BECs [Hong YK et al, 2004] - and in order to confirm our previous experiments performed by immunofluorescence, we evaluated by Q-PCR the expression of genes encoding for PROX-1, podoplanin, LYVE-1 and VEGFR-3 also in ECFCS isolated from cKS patients.

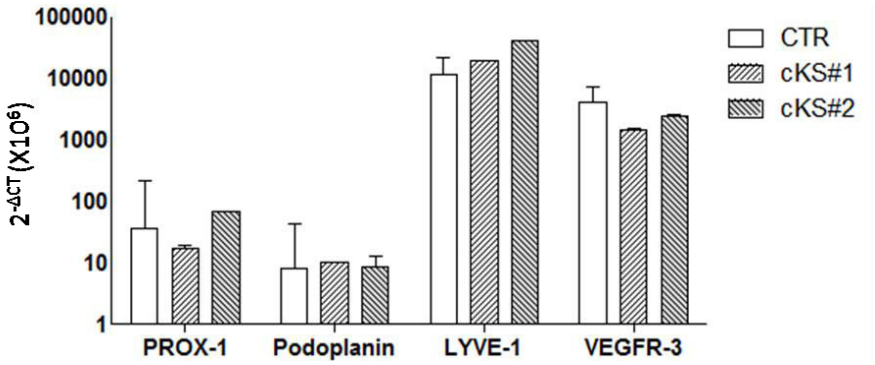


Figure 21: Expression of mRNA encoding for lymphatic markers evaluated by Q-PCR in ECFC isolated from cKS patients compared with healthy controls.The expression of PROX-1, podoplanin, LYVE-1 and VEGFR-3 - expressed as $2^{-\Delta CT}(X10^6)$ - was assessed in 2 ECFCs colonies isolated from cKS patients (respectively cKS#1 cKS#2). Data shown as median±SD.

As shown in Figure 19, in preliminary experiments performed in 2 ECFC colonies isolated from cKS patients, we observed that ECFCs isolated from cKS patients expressed the lymphatic markers PROX-1, podoplanin, LYVE-1 and VEGFR-3. Moreover, ECFC colonies isolated from cKS patients expressed the lymphatic markers at similar levels of ECFC colonies isolated from healthy controls.

Further experiments are still in progress in order to evaluate whether the lymphatic marker expression is similar or it is increased in ECFCs isolated from cKS patients compared to ECFCs isolated from healthy controls.

4.2.1.2 Detection of lymphatic markers by confocal microscopy

To confirm the results observed at the transcriptional level by Q-PCR, we further analyzed the expression of PROX-1, Podoplanin, LYVE-1 and VEGFR-3 by confocal microscopy. In particular, we analyzed ECFCs colonies cultured on both fibronectin and collagen.

4.2.1.2.1 Optimization of protocols for lymphatic markers detection by confocal microscopy

In order to evaluate by confocal microscopy the lymphatic marker expression on ECFCs, we first optimized the staining protocols. HDLECs were used as positive controls, as they express all the lymphatic markers investigated at high levels [Johnson LA et al, 2013]. Moreover, different negative controls were used to verify staining specificity. In particular, cell types that do not express lymphatic markers were used as negative controls [Leclers D et al, 2006, Norgall S et al, 2007, Lorusso B et al, 2015]

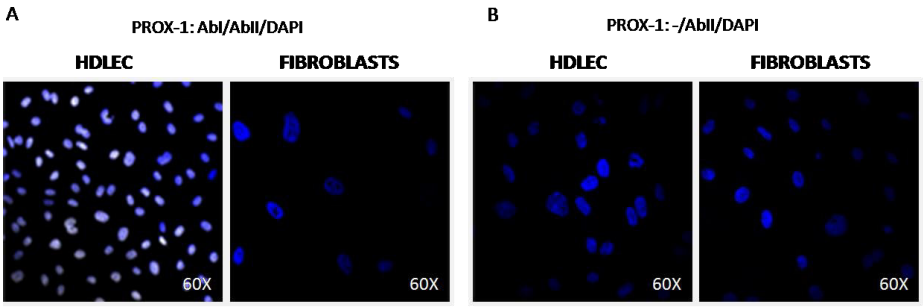


Figure 22: HDLECs and fibroblasts, used as positive and negative controls, respectively, stained for PROX-1. (A) representative photographs showing immunofluorescence staining for PROX-1 in HDLECs and fibroblasts; (B) representative immunofluorescence photographs of HDLECs and fibroblasts stained only with the secondary antibody. Cells were stained for PROX-1 (white) and with DAPI for nuclear staining (blue). Photographs were obtained using an Olympus Fluoview FV1000 confocal microscope, 60X magnification.

As shown in Figure 22, the optimized protocol allowed the specific staining of PROX-1. In fact, PROX-1 signal localized in the nuclei of HDLECs, whereas PROX-1 signal was absent in fibroblasts and in HDLECs stained only with the secondary antibody.

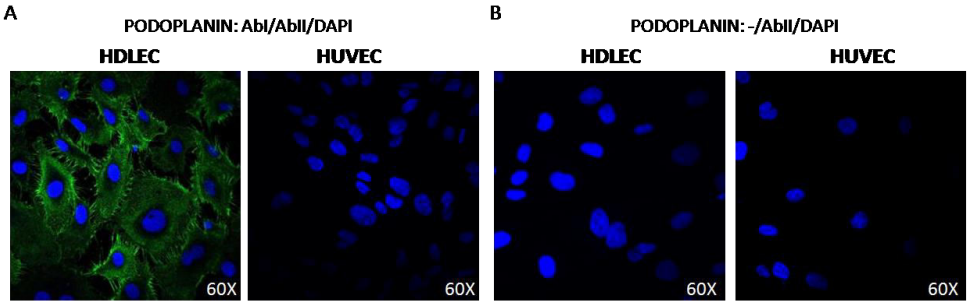


Figure 23: HDLECs and HUVECs, used as positive and negative controls, respectively,stained for podoplanin. (A) representative photographs showing immunofluorescence staining for podoplanin in HDLECs and HUVECs; (B) representative immunofluorescence photographs of HDLECs and HUVECs stained only with the secondary antibody. Cells were stained for podoplanin (green) and with DAPI for nuclear staining (blue). Photographs were obtained using an Olympus Fluoview FV1000 confocal microscope, 60X magnification.

As shown in Figure 23, the optimized protocol allowed the specific staining of podoplanin. In fact, podoplanin signal localized on the membrane of HDLECs whereas podoplanin signal was absent in HUVECs and in HDLECs stained only with the secondary antibody.

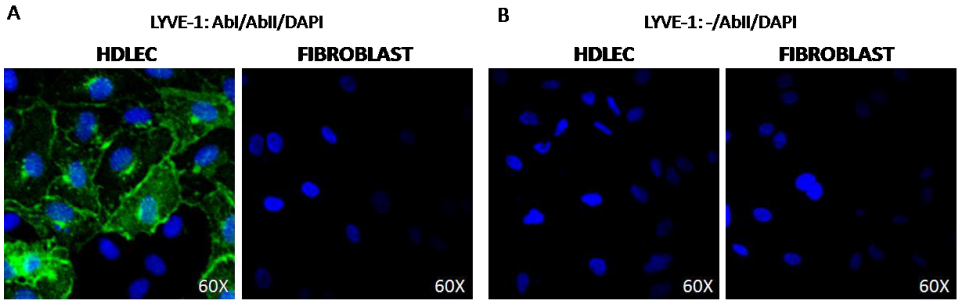


Figure 24: HDLECs and fibroblasts, used as positive and negative controls, respectively, stained for LYVE-1. (A) representative photographs showing immunofluorescence staining for LYVE.1 in HDLECs and fibroblasts; (B) representative immunofluorescence photographs of HDLECs and fibroblasts stained only with the secondary antibody. Cells were stained for LYVE-1 (green) and with DAPI for nuclear staining (blue). Photographs were obtained using an Olympus Fluoview FV1000 confocal microscope, 60X magnification.

As shown in Figure 24, the optimized protocol allowed the specific staining of LYVE-1. In fact, LYVE-1 signal localized on the membrane of HDLECs whereas it was absent in fibroblasts and in HDLECs stained only with the secondary antibody.

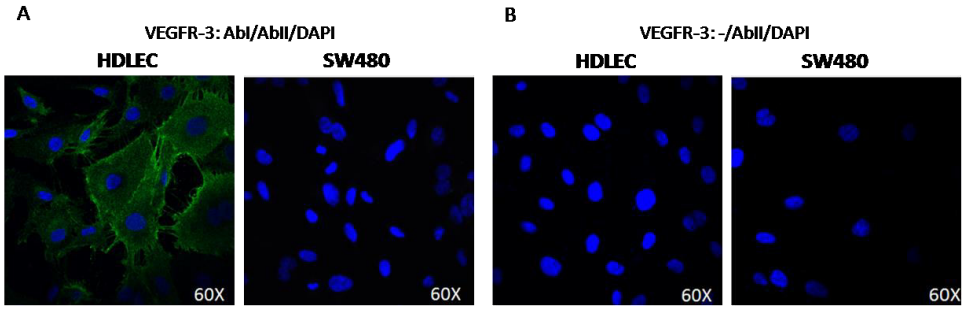


Figure 25: HDLECs and SW480 cell line, used as positive and negative controls, respectively, stained for VEGR-3. (A) representative photographs showing immunofluorescence staining for VEGFR-3 in HDLECs and SW480; (B) representative immunofluorescence photographs of HDLECs and SW480 stained only with the secondary antibody. Cells were stained for VEGFR-3 (green) and with DAPI for nuclear staining (blue). Photographs were obtained using an Olympus Fluoview FV1000 confocal microscope, 60X magnification.

As shown in Figure 25, the optimized protocol allowed the specific staining of VEGFR-3. In fact, VEGFR-3 signal localized on the membrane of HDLECs whereas it was absent in fibroblasts and in HDLECs stained only with the secondary antibody.

4.2.1.2.2 Lymphatic markers detection in ECFCs by confocal microscopy

In order to confirm the expression of lymphatic markers by ECFCs at the protein level, we evaluated the expression of PROX-1, podoplanin, LYVE-1 and VEGFR-3 on ECFCs by using the optimized protocol and analyzing cells by confocal microscopy.

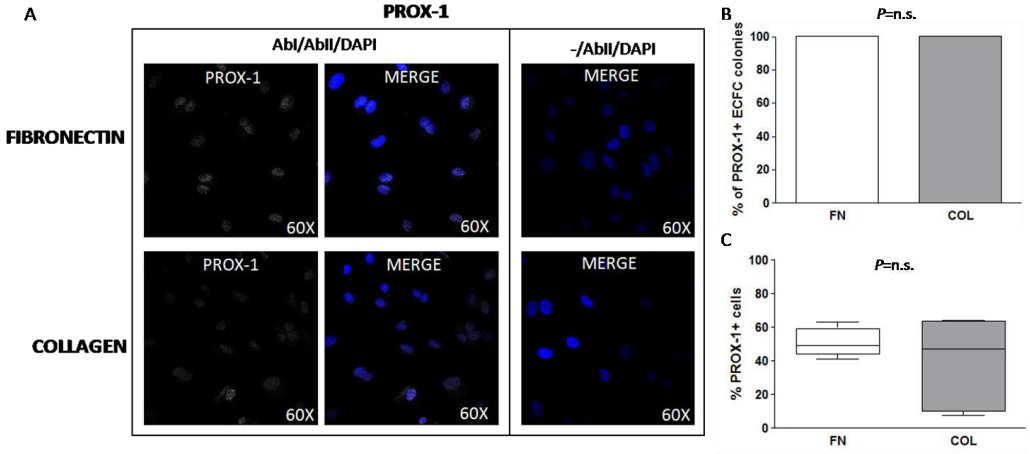


Figure 26: PROX-1 expression in ECFCs cultured on fibronectin and collagen: (A) ECFCs cultured on fibronectin and collagen stained for PROX-1. Representative photographs showing immunofluorescence staining for PROX-1 in ECFCs cultured on fibronectin (upper row) and collagen (lower row). ECFCs were stained for PROX-1 (white) and with DAPI (blue). ECFCs stained only with the secondary antibody were used as negative control (right column). Photographs were obtained using an Olympus Fluoview FV1000 confocal microscope, 60X magnification (B) Percentage of PROX-1$^+$ ECFC colonies in ECFC colonies cultured on either fibronectin (FN, white bar) or collagen (COLL, gray bar). Data shown as percentage. P value calculated by $\chi_c 2$ test. (C) Percentage of PROX-1$^+$ cells within ECFC colonies cultured on fibronectin (FN, white box) or collagen (COLL, gray box). Data shown as median (interquartile range) and T bars represent 5 and 95 percentile. P values calculated by Mann-Whitney test.

As shown in Figure 26A, ECFC colonies cultured on both fibronectin and collagen expressed the transcription factor PROX-1. We also observed that the intensity of PROX-1 expression was highly variable among PROX-1$^+$ cells of the same colony and among different ECFC colonies. We evaluated the percentage of ECFC colonies positive for PROX-1 expression and the percentage of PROX-1$^+$ cells within ECFC colonies. As shown in Figure 26B, all the screened ECFC colonies were positive for PROX-1 expression both on fibronectin and collagen (fibronectin vs collagen: 100% vs 100%, n=5, n=5, *P*=n.s.). Moreover, we observed that PROX-1 was expressed only by a proportion of cells and that the percentage of PROX-1$^+$ cells was similar in ECFC colonies cultured on either fibronectin or collagen (median fibronectin vs collagen: 51.19 vs 38.92 percentage of PROX-1$^+$ cells, n=5, n=5, *P*=n.s.) (Figure 26C).

Similar results were observed also when podoplanin (fibronectin vs collagen: 80% vs 80% percentage of podoplanin$^+$ colonies n=5 vs 5, *P*=n.s.; median 19.5 vs 24.48 percentage of podoplanin$^+$ cells, n=4 vs 4, *P*=n.s.) and LYVE-1 (fibronectin vs collagen: 100% vs 100% percentage of LYVE-1$^+$ colonies, n=5 vs 5, *P*=n.s.; median 22.9% vs 26.3% percentage of LYVE-1$^+$ cells, n=5 vs 5, *P*=n.s.) expression was analyzed (Figure 27 and 28).

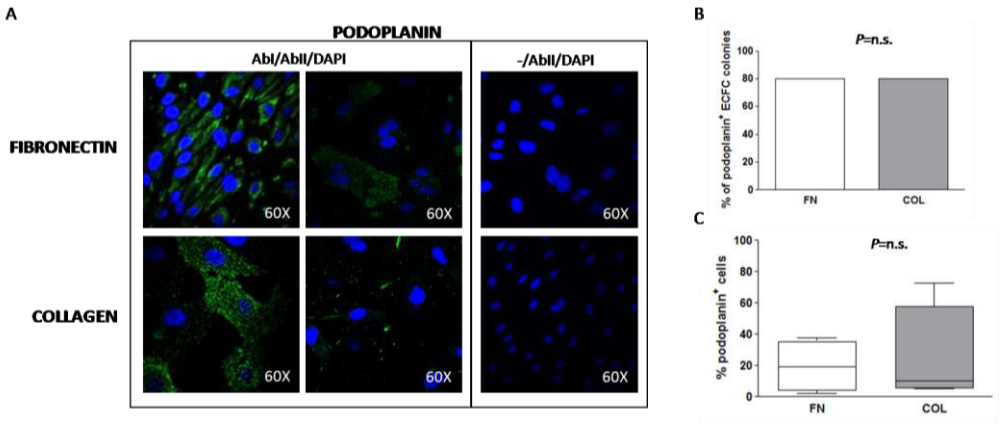


Figure 27: Podoplanin expression in ECFCs cultured on fibronectin and collagen: (A) ECFCs cultured on fibronectin and collagen stained for podoplanin. Representative photographs showing immunofluorescence staining for podoplanin in ECFCs cultured on fibronectin (upper row) and collagen (lower row). ECFCs were stained for podoplanin (green) and with DAPI (blue). ECFCs stained only with the secondary antibody were used as negative control (right column). Photographs were obtained using an Olympus Fluoview FV1000 confocal microscope, 60X magnification (B) Percentage of podoplanin$^+$ ECFC colonies in ECFC colonies cultured on either fibronectin (FN, white bar) or collagen (COLL, gray bar). Data shown as percentage. p value calculated by $\chi_c 2$ test. (C) Percentage of podoplanin$^+$ cells within ECFC colonies cultured on fibronectin (FN, white box) or collagen (COLL, gray box). Data shown as median (interquartile range) and T bars represent 5 and 95 percentile. P values calculated by Mann-Whitney test.

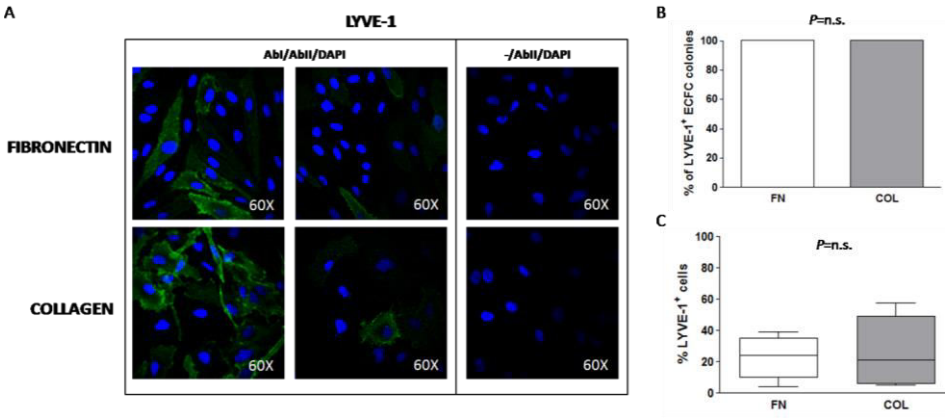


Figure 28: LYVE-1 expression in ECFCs cultured on fibronectin and collagen: (A) ECFCs cultured on fibronectin and collagen stained for LYVE-1. Representative photographs showing immunofluorescence staining for LYVE-1 in ECFCs cultured on fibronectin (upper row) and collagen (lower row). ECFCs were stained for LYVE-1 (green) and with DAPI (blue). ECFCs stained only with the secondary antibody were used as negative control (right column). Photographs were obtained using an Olympus Fluoview FV1000 confocal microscope, 60X magnification (B) Percentage of LYVE-1$^+$ ECFC colonies in ECFC colonies cultured on either fibronectin (FN, white bar) or collagen (COLL, gray bar). Data shown as percentage. p value calculated by $\chi_c 2$ test. (C) Percentage of LYVE-1$^+$ cells within ECFC colonies cultured on fibronectin (FN, white box) or collagen (COLL, gray box). Data shown as median (interquartile range) and T bars represent 5 and 95 percentile. P values calculated by Mann-Whitney test.

In addition, as shown in Figure 29, all the screened ECFC colonies cultured both on fibronectin and collagen expressed VEGFR-3 (fibronectin vs collagen: 100% vs 100%, n=5 vs 5, *P*=n.s.) and VEGFR-3 was expressed by all the cells of the screened ECFC colonies cultured on either fibronectin or collagen (fibronectin vs collagen: 100% vs 100% percentage of VEGFR-3$^+$ cells, n=5, n=5, *P*=n.s.) (Data not shown).

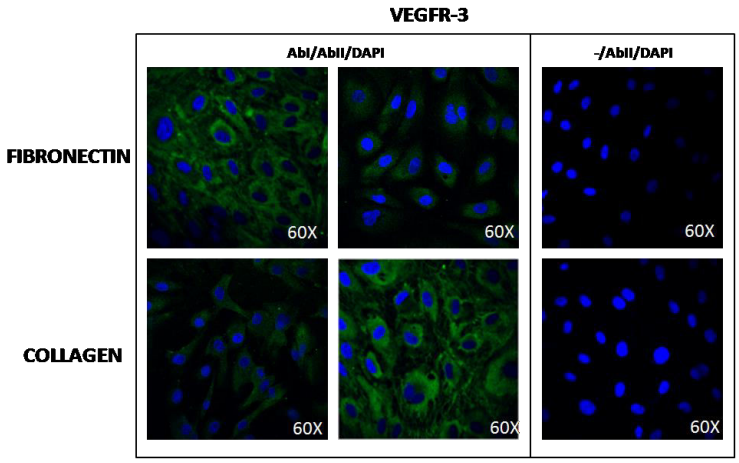


Figure 29: VEGFR-3 expression in ECFCs cultured on fibronectin and collagen: ECFCs cultured on fibronectin and collagen stained for VEGFR-3. Representative photographs showing immunofluorescence staining for VEGFR-3 in ECFCs cultured on fibronectin (upper row) and collagen (lower row). ECFCs were stained for VEGFR-3 (green) and with DAPI (blue). ECFCs stained only with the secondary antibody were used as negative control (right column). Photographs were obtained using an Olympus Fluoview FV1000 confocal microscope, 60X magnification.

4.2.1.2.3 Evaluation of lymphatic markers coexpression in ECFCs

As the lymphatic markers PROX-1, podoplanin and LYVE-1 were expressed only by a proportion of the cells that composed the ECFC colonies, we evaluated their coexpression on ECFCs by multicolor immunofluorescence staining.

The staining protocol was optimized on HDLECs which were used as positive control; afterwards the optimized protocol was used to evaluate the coexpression of PROX-1, podoplanin and LYVE-1on ECFCs cultured on both fibronectin and collagen (Figure 30).

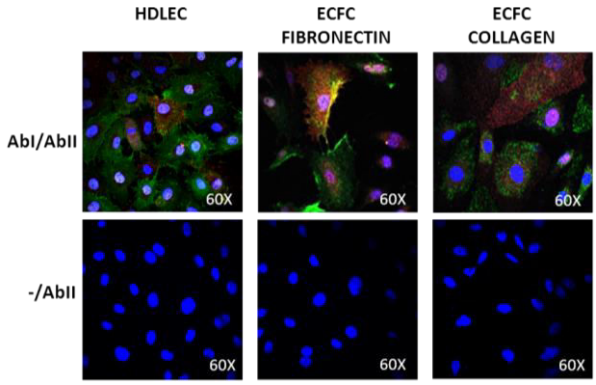


Figure 30: Analysis of lymphatic markers coexpression. Upper row row representative photographs showing HDLECs and ECFCs cultured on fibronectin and collagen stained for PROX-1, podoplanin and LYVE-1; lower row: representative immunofluorescence photographs of HDLECs and ECFCs cultured on fibronectin and collagen stained only with the secondary antibodies. Cells were stained for PROX-1 (white), podoplanin (green) and LYVE-1 (red). Cell nuclei were stained with DAPI (blue). Photographs were obtained using an Olympus Fluoview FV1000 confocal microscope, 60X magnification.

As shown in Table 8, ECFCs cultured on both fibronectin and collagen expressed PROX-1, podoplanin and LYVE-1 in different combinations. In fact, the three markers were not always coexpressed and within the same ECFC colony it was possible to observe cells which expressed the three markers in different combinations.

		Frequency of positive cells (%)		
		FIBRONECTIN	COLLAGEN	Statistical analysis
	Number of colonies	n= 3	n= 4	
	PROX-1$^+$	49.28±4.75	37.74±31.23	*P*=n.s.
	Podoplanin$^+$	29.55±10.13	10.03±32.37	*P*=n.s.
	LYVE-1$^+$	24.17±14.18	31.19±22.32	*P*=n.s.
	VEGFR-3	100	100	*P*=n.s.
PROX-1$^+$	-	24.17±5.98	9.81±11.96	*P*=n.s.
	Podoplanin$^+$	15.83±5.06	0.66±8.89	*P*=n.s.
	LYVE-1$^+$	4.35±4.10	5.11±13.04	*P*=n.s.
	Podoplanin$^+$/LYVE-1$^+$	8.33±6.36	4.12±6.61	*P*=n.s.
PROX-1$^-$	Podoplanin$^+$	5.68±3.91	8.69±5.95	*P*=n.s.
	LYVE-1$^+$	4.55±3.06	6.88±3.35	*P*=n.s.
	Podoplanin$^+$/LYVE-1$^+$	3.41±2.93	4.12±6.61	*P*=n.s.
	Podoplanin$^-$/LYVE-1$^-$	38.64±11.21	48.37±36.56	*P*=n.s.

Table 8: Lymphatic marker coexpression in ECFCs. Frequency of cells positive for lymphatic marker expression analyzed in different combinations in ECFCs cultured on both fibronectin and collagen. Data shown as median±SD. P value calculated by Mann-Whitney test.

4.2.2 Evaluation of lymphatic differentiation of ECFCs upon stimulation with MSF

In clinical studies, it has been reported that also the levels of some oncofetal isoforms of fibronectin – namely EDA-FN and EDB-FN - promote lymphangiogensis [Xiang L et al, 2012]. Therefore, we evaluated whether the lymphatic differentiative potential of ECFCs could be fostered by stimulating the cultures with an oncofetal isoform of fibronectin. In particular we stimulated ECFCs - isolated from healthy controls and cultured on both fibronectin and collagen – with MSF, a soluble oncofetal truncated isoform of fibronectin typically present in tumor microenvironment [Solinas G et al, 2010].

4.2.2.1 Optimization of ECFC stimulation with MSF

As the possible role of MSF in promoting lymphatic differentiation of endothelial cells has never been investigated so far, we first performed preliminary experiments in order to define the optimal MSF concentration and time of stimulation.

To define the optimal MSF concentration, ECFCs colonies – cultured on both fibronectin and collagen - were stimulated for 24 hours with different concentrations of MSF. After MSF stimulation, the expression of mRNA encoding for PROX-1, podoplanin, LYVE-1 and VEGFR-3 in ECFCs was investigated by Q-PCR.

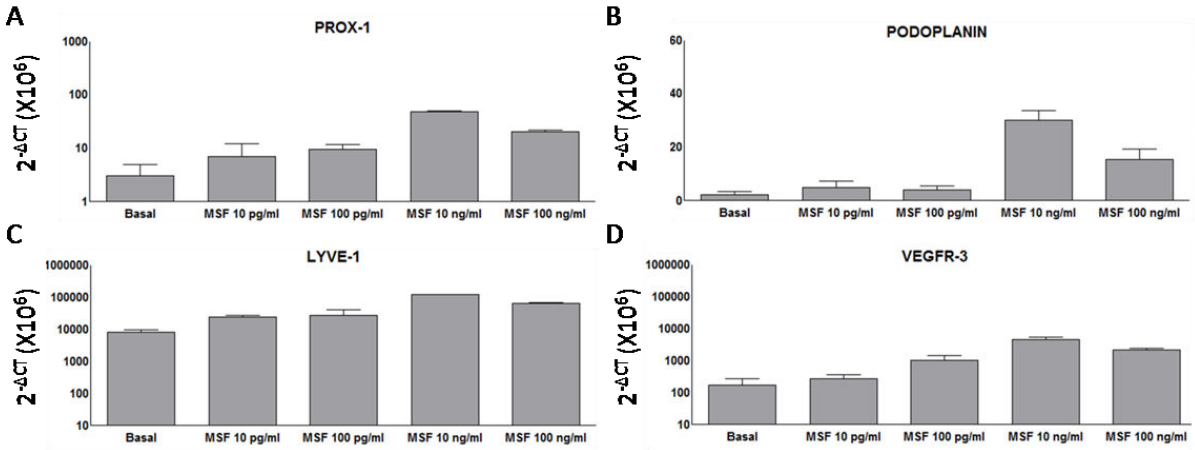


Figure 31 Expression of mRNA encoding for lymphatic markers in ECFCs treated with different concentrations of MSF. The expression levels of (A) PROX-1, (B) podoplanin, (C) LYVE-1 and (D) VEGFR-3 were assessed by Q-PCR. The expression levels of a representative ECFC colony treated with increasing concentrations of MSF was shown. The expression level of each gene was normalized to the GAPDH internal control and was shown as $2^{-\Delta CT}$ ($X10^6$).

As shown in Figure 31, ECFCs upregulated the expression of the genes encoding for all the lymphatic markers after treatment with MSF. The effect of MSF was similar in ECFCs cultured on fibronectin and collagen. In particular, the maximum stimulation of lymphatic marker expression was observed when ECFCs were treated with MSF at the concentration of 10 ng/ml.

Once defined the optimal MSF concentration for ECFC stimulation, we defined the optimal time of stimulation. To do that, ECFCs colonies cultured on both fibronectin and collagen were treated with MSF (10 ng/ml) for different times.

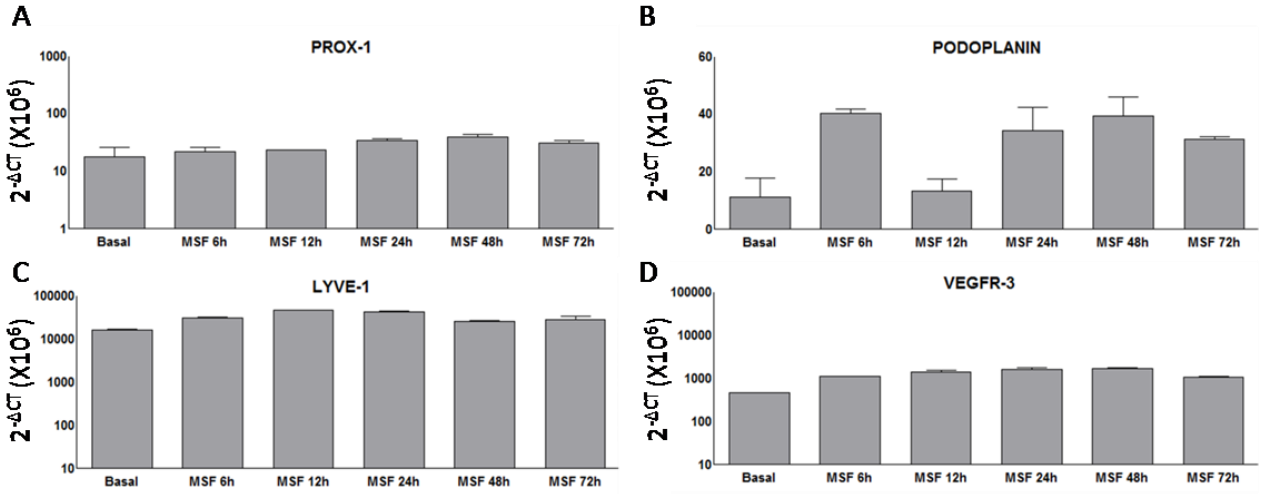


Figure 32: Expression of mRNA encoding for lymphatic markers in ECFCs treated with MSF for different times. The expression levels of (A) PROX-1, (B) podoplanin, (C) LYVE-1 and (D) VEGFR-3 were assessed by Q-PCR. The expression levels of a representative ECFC colony treated with MSF (10 ng/ml) for different times was shown. The expression level of each gene was normalized to the GAPDH internal control and was shown as $2^{-\Delta CT}$ ($X10^6$).

As shown in Figure 32, ECFCs upregulated the expression of genes encoding for lymphatic markers after treatment with MSF for different times (6, 12, 24, 48 and 72 hours) and the effect of MSF was similar in ECFCs cultured on fibronectin and collagen. In addition, since the four analyzed marker were characterized by different kinetics of upregulation upon MSF stimulation, we decided to stimulate ECFCs for 24 hours in the following experiments. In fact, the upregulation of all four analyzed lymphatic markers can be appreciated in ECFCs stimulated with MSF (10 ng/ml) for 24 hours.

4.2.2.2 Detection of mRNA encoding for lymphatic markers in ECFC colonies stimulated with MSF

The expression of mRNA encoding for PROX-1, Podoplanin, LYVE-1 and VEGFR-3 in ECFCs was investigated by Q-PCR in ECFCs cultured on both fibronectin and collagen both in basal conditions and after MSF stimulation. In particular, ECFC colonies were treated for 24 hours with MSF at the final concentration of 10 ng/ml in order to evaluate the effect of MSF in fostering the lymphatic differentiation of ECFCs..

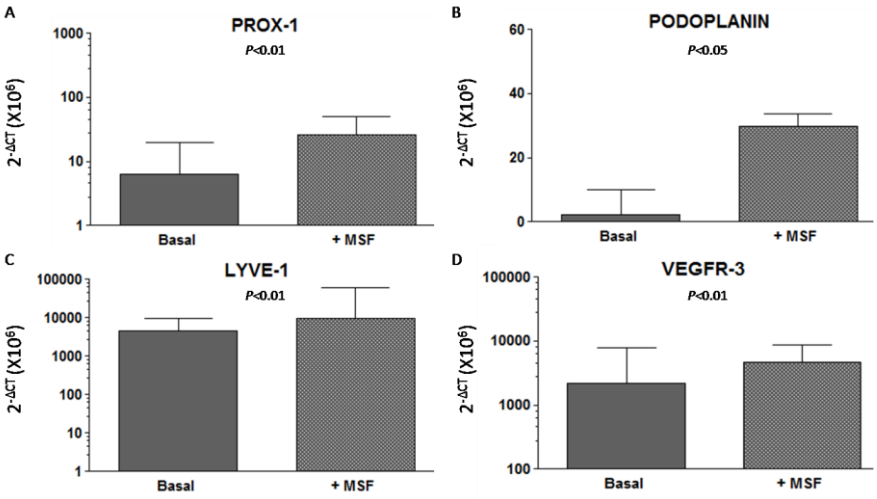


Figure 33: Expression of mRNA encoding for lymphatic markers in ECFCs treated with MSF. The expression levels of genes encoding for (A) PROX-1, (B) podoplanin (C) LYVE-1 and (D) VEGFR-3 were assessed by Q-PCR in ECFCs colonies (fibronectin + collagen) in basal condition (Basal) and after stimulation with MSF (+MSF). The expression level of each gene was normalized to the GAPDH internal control and was shown as $2^{-\Delta CT}$ ($X10^6$). Data shown as median±interquartile range. P value was calculate by Wilcoxon signed-rank test.

MSF treatment upregulated the expression of lymphatic markers in ECFCs cultured either on fibronectin and collagen (PROX-1: median fibronectin vs collagen: 38.42 vs 20.39 $2^{-\Delta CT}$($X10^6$), n=4, n=5, *P*=n.s.; LYVE-1: median fibronectin vs collagen: 27493 vs 34666 $2^{-\Delta CT}$ ($X10^6$), n=4, n=5, *P*=n.s.; VEGFR-3: median fibronectin vs collagen: 6481 vs 4320 $2^{-\Delta CT}$ ($X10^6$), n=4, n=5, *P*=n.s.) (Data not shown). As shown in Figure 33, the expression of lymphatic markers was significantly higher in ECFCs treated with MSF compared to untreated cultures. (PROX-1: median basal vs MSF stimulated: 13.26 vs 28.68 $2^{-\Delta CT}$ ($X10^6$), n=9, n=9, *P*<0.01.; LYVE-1: median basal vs MSF stimulated: 6067 vs 31478 $2^{-\Delta CT}$ ($X10^6$), n=9, n=9, *P*<0.01.; VEGFR-3: median basal vs MSF stimulated: 3972 vs 5280 $2^{-\Delta CT}$ T($X10^6$), n=9, n=9, *P*<0.01). Moreover, podoplanin was expressed only by a portion of ECFC colonies screened (fibronectin vs collagen: 25% vs 40% percentage of podoplanin$^+$ colonies, n=4, n=5 p=n.s.) (Data not shown) and MSF stimulation did not induce de novo expression of podoplanin but induced the upregulation of podoplanin expression in ECFC colonies that already expressed podoplanin in basal conditions (podoplanin: median basal vs MSF stimulated: 4.61 vs 22.44 $2^{-\Delta CT}$ ($X10^6$), n=3, n=3, *P*=n.s.) (Figure 33B).

4.2.2.3 Lymphatic markers detection by confocal microscopy in ECFCs stimulated with MSF

To further confirm the ability of MSF to induce the lymphatic differentiation of ECFCs at the protein level, the expression of PROX-1, podoplanin and LYVE-1 was also evaluated - in ECFCs in basal conditions and stimulated with MSF -by confocal microscopy.

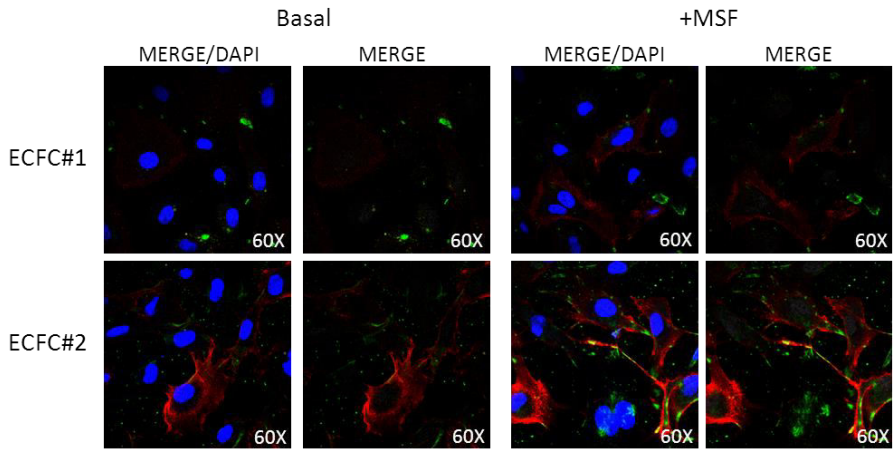


Figure 34: Analysis of lymphatic marker expression in ECFCs stimulated with MSF. (A) Left columns: representative photographs showing ECFCs cultured in basal conditions stained for PROX-1, podoplanin and LYVE-1; right columns: representative immunofluorescence photographs of ECFCs stimulated with MSF stained for PROX-1, podoplanin and LYVE-1. Cells were stained for PROX-1 (white), podoplanin (green) and LYVE-1 (red). Cell nuclei were stained with DAPI (blue). Photographs were obtained using an Olympus Fluoview FV1000 confocal microscope, 60X magnification.

Immunofuorescence analysis confirmed the results observed at the transcriptional level by Q-PCR. In fact, as shown in Figure 34, immunofuorescence analysis showed that the expression of PROX-1, podoplanin and LYVE-1 was increased in ECFCs treated with MSF.

5. Discussion

EPCs were described for the first time in 1997 by Asahara [Asahara T et al, 1997]. Since that time, everal studies demonstrated that EPCs play a crucial role in endothelial homeostasis and in vasculogenic processes both in physiological and pathological conditions [Urbich C et al, 2004].
In the past, several cell types – isolated from the peripheral blood with different methods and endowed with endothelial features - have been called EPCs. Among these cell populations, only endothelial colony-forming cells (ECFCs) are considered the true EPCs. [Fadini GP et al, 2012]. In fact, only ECFCs are endowed with all the typical features of EPCs. In particular, ECFCs express endothelial markers while lacking hematopoietic markers, are characterized by a high proliferative potential [Mead LE et al, 2008] and have the unique ability to display in vivo vasculogenic activity when implanted into immunodeficient mice [Foubert P et al, 2007; Yoder MC et al, 2007].

Kaposi's Sarcoma (KS) is a lymphangioproliferative disease which presents typically with cutaneous lesions but can also involve visceral organs. KS is characterized by the hyperproliferation of the spindle cells, the typical cells of KS lesions. Spindle cells are cells of endothelial origin which are infected by HHV-8 – the herpersvirus which is the causative agent of KS [Chang Y et al, 1994] –but it is still unclear if they derive from mature endothelial cells infected by HHV-8 in peripheral tissues or whether they originate from previously infected circulating cells such as bone-marrow derived endothelial progenitor cells (EPCs).
In previous studies, we observeed that the number of circulating EPCs, identified by flow cytometry as $CD45^{dim}/CD34^{+}/KDR^{+}$, is increased in cKS patients [Taddeo A et al, 2008]. Moreover, we reported that ECFCs isolated from cKS patients are HHV-8 –infected and support viral productive replication and may therefore act as potential virus reservoirs [Della Bella S et al, 2008].
Therefore, in this study we characterized the ECFCs isolated from cKS patients in order to evaluate whether these cells have features similar to the spindle cells in KS lesions. ECFCs were isolated and cultured by using a protocol recently optimized in our lab [Colombo E et al, 2013]. Moreover, we enrolled in the study only patients with the Mediterranean variant of KS – also called classic KS (cKS). This clinical variant of KS represents a useful model of virus-associated tumor since it is characterized by the absence of confounding factors such as iatrogenic immunosuppression or co-infection with other viruses (i.e. HIV).

During ECFC isolation we evaluated the frequency of subjects who gave rise to at least one ECFC colony, the time of ECFC colonies appearance and the frequency of ECFC colonies isolated.

The frequency of subjects who gave rise to at least one ECFC colony was similar in cKS patients and healthy controls whereas the time of ECFC colonies appearance was reduced in cKS patients. Since ECFC colonies were identified by daily culture observation of PBMC cultures using an inverted microscope, the reduced time of appearance reported in cKS patients could be due to a higher cell proliferation rate that may support a faster growth of ECFC colonies that therefore reach earlier a size that allows their identification.

The frequency of ECFC colonies isolated was strikingly higher in cKS patients than in healthy controls. In fact, the frequency of ECFC colonies isolated was about 5-fold higher in cKS patients. This observation may suggest the presence, in the group of cKS patients, of higher levels of circulating cells able to give rise to ECFCs in vitro. Moreover, the higher number of ECFC colonies observed in cKS patients is in accordance with our previous study in which we reported that circulating EPCs, identified as $CD45^{dim}/CD34^{+}/KDR^{+}$ were increased in cKS patients [Taddeo A et al, 2008]. Nevetheless, the cell population identified as $CD45^{dim}/CD34^{+}/KDR^{+}$ contained not only the cells from which ECFCs originate in vitro but also hematopoietic cells that promote angiogenesis with paracrine mechanisms. In fact, Case et al demonstrated that only the rare $CD45^{-}$ subpopulation of $CD34^{+}/CD133^{+}/KDR^{+}$ cells in the blood is not endowed with hematopoietic activity and should therefore represent the true EPCs [Case J. et al, 2007]. Although in this study we did not investigate the mechanisms underlying the increased frequency of ECFCs in the blood of KS patients, it could be argued that HHV-8 infection could foster cell proliferation and therefore could sustain the reduced time of appearance of ECFC colonies and the increased frequency of ECFCs isolated from cKS patients.

Moreover, in order to evaluate whether ECFC isolated from cKS patients were characterized by the expression of different integrins and adhesion molecules, we assessed whether fibronectin and collagen – the substrate typically used for ECFC culture - may differently affect the efficiency of ECFC isolation from cKS patients. ECFCs were isolated both on fibronectin- and collagen-coated culture plates but, similarly to our previous observation in healthy donors (Colombo et al, 2013), ECFCs from cKS patients were isolated more efficiently when fibronectin was used.

cKS patients can be classified according to the stage and the evolutive pattern of disease. In our study, cKS patients were classified according to the classification proposed by Brambilla et al [Brambilla L et al, 2003] and we therefore evaluated whether the stage or the evolutive pattern of disease could influence the efficiency of ECFC isolation. In particular, the frequency of ECFCs isolated was higher in cKS patients with rapidly evolving disease (stage B) than cKS patients with

slowly evolving disease (stage A). This observation may support the hypothesis that circulating HHV-8 – infected EPCs could be involved in the pathogenesis of KS. The higher number of cells giving origin to ECFCs in vitro that has been detected in cKS patients with stage B disease could explain, at least in part, the rapid evolution of disease observed in this group of patients that developed new KS lesions within three months from the last observation. These data are in contrast with our previous study in which we reported that circulating EPCs identified as $CD45^{dim}/CD34^{+}/KDR^{+}$ are significantly reduced in cKS patients with rapidly evolving disease than patients with slowly evolving disease [Taddeo A et al, 2008]. This could be due to the fact that, as discussed above, $CD45^{dim}/CD34^{+}/KDR^{+}$ cells and ECFCs are different populations of EPCs.

Once isolated, ECFCs were expanded for further characterization. ECFCs isolated from both cKS patients and healthy controls possessed all the typical characteristics of ECFCs. In fact they were characterized by a cobblestone-like morphology, did not express the hematopoietic markers CD45 and CD14 and expressed endothelial markers typically used for ECFC characterization. In particular ECFCs isolated from both cKS patients and healthy controls expressed CD31, CD54, CD144, CD146, and VEGRF2 and SDF-1 receptor CXCR4, internalized Dil-ac-LDL and bound UEA-1 [Ingram DA et al, 2004].

The possible presence of HHV-8 infection was evaluated by immunofluorescent detection of the latent phase antigen LANA by confocal microscopy. All the screened ECFC colonies isolated from cKS patients were positive for LANA staining thus confirming that ECFCs isolated from cKS patients were HHV-8 infected. These data are in accordance with our previous study in which HHV-8 DNA was detected by PCR analysis in both ECFCs isolated from cKS patients and their culture supernatants [Della Bella S et al, 2008]. Moreover, immunofluorescence analysis of LANA expression allowed to observe that - within the analyzed colonies - only a proportion of cells expressed LANA and iwas therefore HHV-8-infected (5%-20% of total cells). The low percentage of HHV-8 infected cells detected is in accordance with our previous study in which we reported that ECFCs harbor moderate amounts of viral genome [Della Bella S et al, 2008] and it may be partly related to the fact that the patients enrolled in the study were affected by cKS. In fact, cKS is the less aggressive variant of KS and it is characterized by a poorly aggressive course and a relatively low viral load [Pellet C et al, 2006; Antman K et al, 2000].

ECFCs expanded ex vivo were characterized in order to evaluate whether ECFCs obtained from cKS patients may have an in vitro behaviour that may evoke the in vivo behaviour of KS spindle cells.

ECFCs isolated from both cKS patients and healthy controls maintained stable endothelial morphology and exhibited features of primary cells. In fact they could be expanded for a limited number of passages before they reached a senescent state as confirmed by senescence-associated β-galactosidase assay These results are in line with those of our previous study in which we reported that ECFC colonies isolated from healthy controls can be expanded for several passages by ultimately becoming senescent after long term culture [Colombo E et al, 2013]. Moreover, the observation that ECFCs isolated from cKS patients exhibited features of primary cells is in agreement with the fact that also HHV-8-infected spindle cells derived from KS lesions are not immortalized. In fact, as shown in in vivo studies, spindle cells injected in nude mice survived for a short period of time and formed only transient tumors [Mutlu AD et al, 2007].

Moreover, ECFCs isolated from cKS were endowed with a higher proliferative potential than ECFCs isolated form healthy controls. In fact, ECFCs isolated from cKS patients proliferated faster than ECFCs isolated from healthy controls and higher cell yields were obtained at the end of the cultures when ECFCs were isolated from cKS patients than healthy controls.

The increased proliferative ability of ECFCs isolated from cKS patients could be explained by the presence of HHV-8 infection. In fact, previous studies have documented that HHV-8 infection promotes cell proliferation of infected cells. In particular, Flore et al demonstrated that HUVECs infected in vitro by HHV-8 were characterized by increased proliferation. In this model, only a proportion of the cultured cells were HHV-8-infected (about 5%) and the authors demonstrated that HHV-8 infection fostered also the proliferation of uninfected cells through paracrine mechanisms [Flore O et al, 1998]. In fact, many studies reported that several viral molecules expressed in the latent phase of HHV-8 infection (i.e. LANA, vCyc, vBcl-2 and vFLIP) act on pathways involved in cell proliferation and survival both directly (i.e. regulating cell cycle) and through paracrine mechanisms by inducing the production of soluble factors that can promote cell survival and proliferation also in adjacent uninfected cells [Ganem D et al, 2010]. In KS lesions, the microenvironment is enriched in cytokines and growth factors produced by HHV-8-infected spindle cells themselves and by the inflammatory cells recruited within the lesions. Such soluble factors play a crucial role in promoting spindle cell survival and growth. In particular, one of the mechanisms used by HHV-8 to promote cell proliferation consists in promoting IL-6 production by infected cells. In fact, HHV-8 in vitro infection induced an increase in IL-6 production in human microvascular endothelial cells (HMECs) [Speciale L et al, 2006]. In addition, An et al demonstrated that, during the latent phase of HHV-8 infection, LANA can up-regulate IL-6 expression by

interacting with AP1 RE (response elements) present in IL-6 promoters [An J et al, 2004]. In turn, IL-6 produced by infected cells promotes cell proliferation also in uninfected cells. Moreover, also vIL6 –a lytic phase HHV-8 viral molecule, homolog of human IL-6- was documented to induce IL-6 production in several cell types [Mori Y et al, 2000]. These evidence support the hypothesis that IL-6 pathway can play an important role in KS pathogenesis fostering cell proliferation and survival of spindle cells in KS lesions.

To evaluate whether IL-6 may play a role in the increased proliferation observed in ECFCs cultures obtained from cKS patients, we measured the levels of IL-6 in ECFC culture supernatants. ECFCs isolated from cKS patients produced higher levels of IL-6 than ECFCs isolated from healthy controls, thus supporting the hypothesis that, in ECFCs isolated from cKS patients, HHV-8 infection could promote IL-6 production which in turn may foster cell proliferation. Our results are also in accordance with our previous study demonstrating that IL-6 can sustain ECFC proliferation [Colombo et al, 2013].

In addition, we measured in ECFC supernatants the levels of other cytokines typically present in KS lesions. [Sirianni MC et al, 1998]. FGFb was detectable in supernatant of ECFs isolated from both healthy controls and cKS patients but no differences in FGFb concentration were observed between the two groups, whereas VEGF, IFNγ, TNFα and IL-3 were undetectable at any passage in ECFCs isolated from both healthy controls and cKS patients. Our results are in accordance with a previous study in which other authors reported that unstimulated ECFCs produce low amount of these cytokines [Hur J et al, 2004]. In addition, it is possible that the increased cytokine production and proliferative potential observed in ECFCs isolated from cKS patients could be further enhanced upon ECFC stimulation with proinflammatory cytokine or culturing ECFCs in hypoxic conditions since proinflamatory cytokines and hypoxia are typical features of KS lesions microenvironment.

Our results further demonstrated that ECFCs isolated from cKS patients were endowed with higher vasculogenic ability, as shown by in vitro matrigel tubulogenesis assay. It is possible that the higher proliferative potential displayed by ECFCs isolated from cKS patients and sustained by increased IL-6 production, may explain at least in part their enhanced ability to form capillary-like structures when seeded in Matrigel.

In order to investigate the molecular pathways possibly involved in the different behavior of ECFCs isolated from cKS patients and healthy controls, we performed a gene-array analysis to compare the molecular signature of ECFCs isolated from the two groups. Preliminary analysis revealed that patients and healthy donors segregated by clustering, thus confirming that gene expression profile

differs between ECFC isolated from cKS patients and healthy donors. Moreover, 16 genes were upregulated in ECFC isolated from cKS patients compared to healthy controls. In particular, the deubiquitinase PSMD14 - whose expression was significantly upregulated in ECFCs isolated from cKS patients - was reported to be involved in regulation of cell proliferation. In fact, Byrne et al documented that knockdown of human PSMD14 induced cell cycle arrest and senescence in carcinoma cell lines [Byrne A et al, 2010]. Therefore, it is possible that the increased expression of PSMD14 observed in ECFC colonies isolated from cKS patients may have a role in supporting ECFC proliferation. More detailed analysis will be performed in order to investigate the possible role of the diffentially expressed genes in determining the characteristic behavior observed in ECFCs isolated from cKS patients. In addition, gene set enrichment analysis (GSEA) will be performed in order to evaluate whether genes specifically involved in angiogenesis, cell proliferation, IL-6 pathway and VEGFR-3 pathway are differentially expressed by ECFCs isolated from cKS patients

Spindle cells are characterized by the expression of both BEC and LEC markers. In particular, spindle cells in KS lesions express the lymphatic markers VEGFR-3, LYVE-1 and podoplanin [Dupin N et al, 2006]. To further evaluate whether ECFCs obtained from cKS patients may have features that resembled KS spindle cells, in preliminary experiments we evaluated whether ECFCs isolated from cKS patients expressed the lymphatic markers LYVE-1 and podoplanin.

Surprisingly, in those preliminary experiments we observed that LYVE-1 and podoplanin were expressed not only by ECFCs isolated from cKS patients but also by ECFCs isolated from healthy controls. These results were unexpected, as ECFCs have been always considered as the true EPCs involved in vasculogenesis but no one had investigated their potential involvement also in lymphangiogenesis as putative LEPCs. Therefore, we investigated the lymphatic differentiative potential of ECFCs evaluating the expression of four typical lymphatic markers – namely PROX-1, podoplanin, LYVE-1 and VEGFR-3 - both at the transcriptional and protein levels. We observed, indeed, that ECFCs isolated from the peripheral blood of adult healthy controls expressed the lymphatic markers PROX-1, podoplanin, LYVE-1 and VEGFR-3 whose expression was confirmed by Q-PCR and confocal microscopy.

In 2 ECFC colonies isolated from cKS patients we further confirmed our preliminary results by reporting that also ECFCs isolated from cKS patients expressed the lymphatic markers PROX-1, podoplanin, LYVE-1 and VEGFR-3. Our results are in accordance with previous studies that showed that KS spindle cells and in vitro HHV-8 infected cells BECs are characterized by the expression of lymphatic markes such as podoplanin, LYVE-1 and VEGFR-3 [Dupin N et al, 2006; Hong YK et al,

2004]. Further experiments will be performed in order to evaluate whether the expression levels of lymphatic markers in ECFCs are increased in cKS patients.

VEGFR-3 was expressed by the totality of the cells in all the screened colonies isolated from healthy controls. Our results were in line with a previous study in which Huang et al documented the expression of VEGFR-3 in ECFCs isolated from cord blood [Huang L et al, 2011]. The high levels of VEGFR-3 expression on ECFCs may depend on the fact that ECFCs are endothelial progenitors. In fact, in early stages of development, VEGFR-3 is expressed on both BECs and LECs and only in the late stage of development and in adulthood its expression is downregulated in BEC and becomes restricted to LECs [Dumont D.J. et al, 1998].

Moreover, PROX-1, podoplanin and LYVE-1 were expressed with variable intensity only by a proportion of cells in the screened ECFC colonies. By multicolor immunofluorescence analysis we observed that PROX-1, podoplanin and LYVE-1 were expressed by ECFCs in different combinations. In fact, the three markers were not always coexpressed and cells which expressed the three markers in different combinations could be identified within the same ECFC colony. In particular we identified cells that were PROX-1^- /podoplanin$^+$ and this is in contrast with the most accepted model for lymphatic vascular system development, according to which it derives from committed PROX-1^+ cells and podoplanin expression is acquired only in later stages [Oliver G et al, 2004]. The presence of PROX-1^-/podoplanin$^+$ cells may suggest that the lymphatic differentiation process in adults may differ from the model proposed for the development of the lymphatic vascular system during embryogenesis. In addition, also PROX-1^-/LYVE$^+$ cells have been identified in our ECFC cultures. This is in accordance with the most accepted model for lymphatic vascular system development in embryogenesis since several studies reported that LYVE-1 is expressed in the early stages of lymphatic differentiation in endothelial cells of the embryonic cardinal veins *prior to* the induction of *Prox-1* [Tammela T et al, 2010].

Starting from 2003, several studies have proposed that lymphvasculogenesis may also occur in adulthood thus contributing to lymphatic neovasculaziation processes. [Kerjaschki D. et al, 2006; Maruyama K. et al, 2005] In addition, it was hypothesized that bone-marrow derived precursors could participate in lymphatic vessel formation by acting as lymphatic endothelial progenitor cells (LEPCs). Nevertheless, the identity and the characteristics of LEPCs have not yet been clearly defined and several cell populations have been described as putative LEPCs. In 2010, Lee at al identified a population of podoplanin$^+$/CD11b$^+$ macrophages derived from the bone marrow that contribute to lymphatic neovascularization both by transdifferentiating in lymphatic endothelial

cells and by producing VEGF-C and other lymphangiogenic factors that promote proliferation of local pre-existent LECs. [Lee JY et al, 2010] In other studies, LEPCs have been identified in the peripheral blood as cells that express CD34 in combination with the lymphatic markers VEGFR-3. In particular, in 2003 Salven et al first described in human fetal liver the presence of a $CD34^+/VEGFR\text{-}3^+/CD133^+$ sub-population that in vitro was characterized by a high proliferative potential and the expression of both lymphatic and blood vascular markers [Salven P. et al, 2003]. Tan et al reported that $CD34^+/VEGFR\text{-}3^+$ cells isolated by fluorescence-activated cell sorting from human cord blood mononuclear cells, acquired - after two weeks of culture in presence of VEGF-C - a lymphatic phenotype [Tan YZ et al, 2014]. The identification of lymphatic markers on ECFCs supported the hypothesis that the cell population that in vitro gives origin to ECFCs could differentiate towards both BECs and LECs thus acting not only as EPCs but also as LEPCs. Our results are in accordance with a recent study - that has been published online during the late writing phases of this thesis - in which the Authors reported that ECFCs express also markers specific of lymphatic endothelium: PROX-1, podoplanin, LYVE-1 and VEGFR-3 [DiMaio TA et al, 2015].

In adulthood, cancer represents a major cause of lymphangiogenesis. [Alitalo A et al, 2012]. Therefore we evaluated whether the lymphatic differentiative potential of ECFCs could be fostered by component of tumor microenvironment which are known to promote tumor associated lymphangiogenesis and metastasis. Since ECFCs were cultured in culture plates coated with ECM protein – typically fibronectin and collagen – we first evaluated whether the substrate used for ECFCs culture could affect their lymphatic differentiative potential. In fact, as reported in several clinical studies, the presence of fibronectin in the tumor stroma correlates with lymphovascular invasion, lymph node metastasis in breast cancer and other human tumors [Bae YK et al, 2013; Ou J et al, 2013]. We observed that ECFCs cultured on both fibronectin and collagen expressed PROX-1, podoplanin, LYVE-1 and VEGFR-3. Moreover, the analyzed lymphatic markers were similarly expressed in ECFCs cultured on the two substrates. We hypothesized that the reason why we did not observed difference between ECFCs cultured on fibronectin and on collagen may depend on the fact that the fibronectin used for plate coating was a soluble form derived from human plasma - that therefore differs from fibronectin present in tumor ECM since plasma fibronectin and cellular fibronectin possess distinct structures and rates of assembly into three-dimensional matrices [To WS et al, 2011].

Several clinical studies reported that also some alternatively spliced oncofetal isoforms of fibronectin (i.e. EDA-fibronectin and EDB-fibronectin) promote lymphangiogensis. In particular, EDA-fibronectin expression correlates with distant metastasis in colorectal carcinoma [Xiang L et al, 2012]. Therefore, we evaluated whether the stimulation with migration stimulator factor (MSF) – an oncofetal truncated isoform of fibronectin - could foster ECFC differentiation towards a lymphatic phenotype. MSF has been cloned in 2003 by Schor and colleagues [Schor SL et al, 2003]. It is expressed during wound healing and it is strongly overexpressed in a wide range of common human cancers [Schor SL et al, 2003]. In particular, MSF was known to be produced by fetal skin keratinocytes, neoplastic cells, tumor-associated vascular endothelial cells and tumor associated macrophages (TAM) [Solinas G et al, 2010]. MSF was first identified by its motogenic activity. In fact, it is characterized by a strong chemotactic activity for monocytes and tumor cells [Solinas G et al, 2010] Moreover, it was reported to be a potent stimulator of angiogenesis [Schor SL et al, 2003] but its potential role in inducing lymphatic differentiation has never been investigated.

Our study revealed that MSF induced the expression of lymphatic markers in ECFCs. In particular, MSF stimulation induced an upregulation of PROX-1, podoplanin, LYVE-1 and VEGFR-3 in ECFCs cultured on both fibronectin and collagen. The upregulation was confimed by Q-PCR and by confocal microscopy.

Our results seem to suggest for the first time that also MSF, like other oncofetal isoforms of fibronectin (EDA-fibronectin and EDB-fibronectin), may play a role in promoting lymphatic differentiation and lymphangiogenesis.

In conclusion, in this study we characterized ECFCs isolated from cKS patients and in particular, we observed that ECFCs isolated from cKS patients were endowed with features typical of KS spindle cells. Our results support the hypothesis that ECFCs could be involved in the phathogenesis of KS as precursors of spindle cells. Our hypothesis is also supported by the observation that ECFCs isolated from cKS patients expressed typical lymphatic markers such as PROX-1, podoplanin, LVYE-1 and VEGFR-3. In fact, spindle cells in KS lesions are characterized by a typical intermediate phenotype between BECs and LECs [Dupin N et al, 2006].

Moreover, in our study we described that also ECFCs isolated from healthy controls expressed the typical lymphatic markers PROX-1, podoplanin, LYVE-1 and VEGFR-3. In addition we reported that the expression of lymphatic markers in ECFCs can be promoted by stimulation with MSF, an oncofetal truncated isoform of fibronectin. Our observation seems to suggest that ECFCs can differentiate towards both BECs and LECs thus supporting the hypothesis that ECFCs can act not

only as EPCs but also as LEPCs. The identification of lymphatic markers on ECFCs has an important biological relevance since ECFCs are considered the true EPCs, are used as non-invasive tool for the study of the endothelial compartment in both physiological and pathological conditions and they are considered a promising candidate cells for endothelial regenerative medicine. Therefore, a more detailed study of the lymphatic differentiative potential of ECFCs, with particular attention to the identification of the stimuli that can induce the differentiation of ECFCs to LECs and the evaluation of the possible involvement of ECFCs in lymphangiogenic processes, will be required.

In addition, since MSF ability to induce lymphatic differentiation has never been described also the role of MSF in promoting lymphatic differentiation and lymphangiogenesis deserve to be investigated in more depth.

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