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Initial clonogenic potential of human endothelial progenitor cells is predictive of their further properties and establishes a functional hierarchy related to immaturity

Ségolène Ferratge [a], Guillaume Ha [a,1], Gilles Carpentier [b,1], Nassim Arouche [a], Rümeyza Bascetin [c,d,e], Laurent Muller [c,d,e], Stéphane Germain [c,d,e], Georges Uzan [a,*]

[a] INSERM U1197, Hôpital Paul Brousse, Villejuif, France
[b] ERL CNRS 9215, Laboratoire CRRET, Université Paris Est Créteil, Faculté des Sciences et Technologies, Créteil, France
[c] Center for Interdisciplinary Research in Biology, Collège de France, Paris, France
[d] Inserm U1050, Paris, France
[e] CNRS UMRS 7241, Paris, France

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ABSTRACT

Endothelial progenitor cells (EPCs) generate *in vitro* Endothelial Colony Forming Cells (ECFCs) combining features of endothelial and stem/progenitor cells. Their angiogenic properties confer them a therapeutic potential for treating ischemic lesions. They may be isolated from umbilical cord blood (CB-ECFCs) or peripheral adult blood (AB-ECFCs). It is generally accepted that CB-ECFCs are more clonogenic, proliferative and angiogenic than AB-ECFCs. Nevertheless, only a few studies have focused on the functional heterogeneity of CB-ECFCs from different individuals. Moreover, AB-ECFC loss of function is yet to be precisely described. We have focused on these two issues that are critical for clinical perspectives.

The detailed clonogenic profile of CB-ECFCs and AB-ECFCs was obtained and revealed a high inter individual heterogeneity and the absence of correlation with age. Most CB-ECFCs yielded initial colonies and had functional properties similar to those of AB-ECFCs. Conversely, a high clonogenicity was associated with an enhanced proliferative and angiogenic potential and stemness gene overexpression, confirming that immaturity, lost by AB-ECFCs, was a prerequisite to functionality. We thus demonstrated the importance of selecting CB-ECFCs according to specific criteria, and we propose using the initial clonogenicity as a relevant marker of their potential efficacy on vascular repair.

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1. Introduction

Since their discovery by Asahara's team in 1997, endothelial progenitor cells (EPCs) have aroused researcher interest for angiogenic cell therapies to treat ischemia (Asahara, 1997). The term "EPCs" refers to several heterogeneous cell populations with different phenotypes and angiogenic potential. *In vitro*, two main cell types have been identified: early EPCs or Colony Forming Unit-Endothelial Cells (CFU-ECs) with a myeloid phenotype characterized by a paracrine angiogenic effect and late EPCs or Endothelial Colony Forming Cells (ECFCs), also known as Outgrowth Endothelial Cells (OECs) (Yoder et al., 2007) which participate directly in neoangiogenesis.

Umbilical cord blood-derived Endothelial Colony Forming Cells (CB-ECFCs) display an endothelial phenotype ($CD31^+/CD144^+/VEGFR\text{-}2^+/CD45^-/CD14^-$) associated with progenitor cell features such as clonal growth, high proliferation (Bompais et al., 2004; Ingram et al., 2004), stemness gene expression and an enhanced reprogramming efficiency into induced pluripotent stem cells compared to mature endothelial cells (Guillevic et al., 2016). They also show a relative differentiation plasticity since they may acquire specialized endothelial cell features under appropriate external instructive stimuli (Boyer-Di Ponio et al., 2014). Moreover, CB-ECFCs may integrate vessel wall and participate directly in neoangiogenesis in rodent models (Melero-Martin et al., 2007; Au et al., 2008). Besides, several studies have shown their efficacy to enhance revascularization in ischemic brain (Moubarik et al., 2011), limb (Schwarz et al., 2012) and myocardium (Kang et al., 2013). However, some of these features are lost by ECFCs derived from adult peripheral blood (AB-ECFCs). Indeed, they present a decreased clonogenicity, a

Abbreviations: EPCs, endothelial progenitor cells; ECFCs, endothelial colony forming cells; CB, cord blood; AB, adult blood.

* Corresponding author at: INSERM U972, Hôpital Paul Brousse, 12-14 Avenue Paul Vaillant Couturier, 94807 Villejuif, France.
E-mail address: georges.uzan@inserm.fr (G. Uzan).

[1] Equal contribution.

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low proliferation and their angiogenic potential is impaired, even when they are obtained from healthy donors (Ingram et al., 2004; Au et al., 2008). These findings raise many questions about the validity of the efficacy of autologous cell therapy and highlight the need to better understand ECFC functional impairment in aging in order to identify targets to prevent this dysfunction. Thus, despite a potential alloantigenicity which may require MHC-matched donors, umbilical cord blood remains described as one of the most valuable sources of functional ECFCs to be adapted for cell therapy. Nevertheless, the clinical use of CB-ECFCs requires optimization and rationalization, especially by selecting the most angiogenic cells.

Since the discovery of these cells (Ingram et al., 2004), secondary colonies have been generated in several studies to assess the immaturity of ECFCs from blood or vascular wall and hierarchize them (Alphonse et al., 2015; Ingram et al., 2005; Patel et al., 2016). The aim of this work was to identify relevant information directly from the initial number of colonies obtained at the first isolation step of CB-ECFCs and AB-ECFCs.

For several years that we grow CB-ECFCs, we have observed a high heterogeneity in colony yields (from 0 to over 100 colonies). In the present study, we investigated whether their initial clonogenicity impacted CB-ECFC properties and could be used as a predictive marker of their functionality. A key issue was also to demonstrate that ECFC stemness was a functional prerequisite which is completely lost by AB-ECFCs.

2. Materials and methods

2.1. ECFC isolation and culture

Human samples were collected and handled in compliance with the declaration of Helsinki. Umbilical cord blood samples from healthy full-term newborns were obtained through a partnership with the Cord Blood Bank of St Louis Hospital (Paris, France) which is authorized by the French Regulatory Authority (authorization no. PPC51) and participates in scientific research. Human peripheral blood from healthy male adults was obtained through a partnership with the French Establishment of Blood (EFS, Ile de France, authorization 14/5/011). Legal age to give blood ranges from 18 to 70 years old. This activity was declared to and authorized by the French Ministry of Research under number AC-2008-376, and to the French Organization for standardization under number 201/51848.1.

Mononuclear cells (MNC), obtained by density gradient centrifugation, were seeded onto rat tail collagen type I (Becton Dickinson BD, Le Pont de Claix, France) -coated wells as previously described (Boyer-Di Ponio et al., 2014).

ECFC colonies appeared after 7–20 days of culture. From passage 1 (P1), cells were seeded at 5000 cells/cm^2 and grew in EGM-2 MV medium (Lonza, Köln, Germany). Population doubling (PD) was determined by the following formula: $\log^2 (n_f/n_o)$, where n_o is the cell number initially seeded and n_f the cell number at confluence obtained at each passage. Cumulative population doubling (CPD) is the sum of all previous PDs. Population Doubling Time (PDT) was calculated using the following formula: t/PD, where t is the culture time interval between each passage.

2.2. BrdU and CFDA SE proliferation assays

Proliferative potential of CB and AB-ECFCs was assessed using BrdU Cell Proliferation Assay Kit #6813 (Cell Signaling) and Vybrant® CFDA SE Cell Tracer Kit (Life technologies) according to the manufacturer's instructions (More details in supplementary material).

2.3. Senescence-associated β-galactosidase activity assay

Cells were seeded in triplicate in 12-well plates at 5000 cells/cm^2. At 80% of confluence, cells were fixed and senescence-associated β-galactosidase activity was revealed using the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich, St-Louis, USA) according to the manufacturer's instructions. Positive (blue) and negative cells were counted manually from 3 phase contrast pictures per well using ImageJ cell counter.

2.4. Western blotting

To reveal p16 protein expression, 30 μg of total protein extract, obtained using CelLytic™ M Cell Lysis reagent (Sigma-Aldrich, St-Louis, USA), were blotted onto nitrocellulose membranes (Bio-Rad, USA). The non-specific binding sites were blocked by incubation in Odyssey Blocking Buffer (Li-Cor biosciences, Lincoln, USA) for 1 h at room temperature (RT) under gentle agitation. The membranes were then cut in two parts and incubated overnight at 4 °C, one with p16 primary rabbit polyclonal antibody (0.4 μg/mL, Santa Cruz Biotechnology) and the other with β-actin primary mouse IgG1 antibody (1 μg/mL, MBL Life Science) diluted in Odyssey Blocking Buffer. After washing, they were incubated with Odyssey's secondary antibody for 45 min at RT and scanned using the Odyssey imaging system.

To reveal p21 and p53 protein expression, 20 μg of total protein extract were blotted onto PVDF membranes (GE Healthcare, Life Sciences, Germany). The non-specific binding sites were blocked by incubation in TBS-Tween 0.2% + BSA 5% for 1 h at room temperature (RT) under gentle agitation. The membranes were then cut in two parts and incubated overnight at 4 °C, one with p21 primary rabbit polyclonal antibody (1 μg/mL, Santa Cruz Biotechnology) and the other with p53 primary mouse monoclonal IgG1 antibody (1 μg/mL, Santa Cruz Biotechnology) diluted in TBS-Tween 0.2% + BSA 5%. After washing, they were incubated with HRP coupled secondary antibody and β-actin directly HRP coupled antibody (1 μg/mL, Santa Cruz Biotechnology) for 45 min at RT. HRP was detected with an Immobilon Western kit (Millipore, Molsheim, France).

2.5. Flow cytometry

Fluorescence-activated flow cytometry was performed using BD Accuri™ C6 flow cytometer (BD) and a minimum of 10,000 events were analyzed for each sample. The cells were harvested, washed in 0.5% Bovine Serum Albumin (BSA) in PBS and incubated for 45 min at 4 °C with primary antibodies to analyze the expression of endothelial cell surface marker proteins: FITC-conjugated mouse anti-human CD31 IgG1 (dilution: 1/50, BD Pharmingen, USA), PE-conjugated mouse anti-human CD144 IgG1 (dilution: 1/10, Beckman Coulter), Alexa Fluor 647-conjugated mouse anti-human CD309 (VEGFR2) IgG1 (dilution: 1/5; BD Pharmingen, USA), FITC-conjugated mouse anti-human CD45 (dilution: 1/50, Beckman Coulter) and PE-conjugated mouse anti-human CD34 IgG2a (dilution: 1/50, Miltenyi Biotec, Germany). All the antibodies were used at concentrations suggested by the suppliers according to the cell number. Antibodies and matched isotype controls were incubated for 30 min at 4 °C. Cell viability was assessed with 7-aminoactinomycin D (dilution: 1/20, BD Pharmingen, USA).

2.6. RNA extraction, reverse transcription and quantitative RT-PCR

Total RNA was extracted using RNeasy mini or micro kit (Qiagen, Courtaboeuf, France). Reverse transcriptions were performed with High Capacity cDNA RT Kit (Applied Biosystems, Fischer Scientific, Illkirch, France) according to the manufacturer's instructions.

Expression of *NOSIII* and the stemness genes *DNMT3B*, *GDF3* and *SOX2* was evaluated by Quantitative Taqman RT-PCRs in triplicate according to the manufacturer's instructions using 7000 Real-Time PCR system (Applied Biosystems). Expression of cell cycle regulator genes was screening with *TaqMan® Array Human Cyclins and Cell Cycle Regulation 96-well Plate* (Applied Biosystems) according to the manufacturer's instructions. The relative transcriptional level of each

gene was quantified using the 2-[/delta][/delta] Ct method. Endogenous *GAPDH* was used as a housekeeping gene.

2.7. Assessment of nitric oxide production

Nitric oxide (NO) was detected *via* DAF-2/DA probes purchased from Molecular Probes (Eugene, Oregon, USA). They become fluorescent when they react with NO. The protocol followed the manufacturer's instructions. ECFCs were seeded into 12-well plates at a density of 1.10^5 cells/well and incubated with 1 μM DAF-2/DA in EBM2, 0.5% FBS for 1 h at 37 °C. Light exposure was avoided as much as possible throughout the experimentation. After washing cells to remove excess probe, samples were trypsinized and centrifuged, supernatant was removed and cells resuspended in PBS, 0.5% BSA followed by immediate FACS analysis. BD Accuri™ C6 flow cytometer was used to quantify fluorescence (excitation wavelength: 488 nm and emission wavelength: 530 nm) at the single-cell level. Data are expressed as the mean fluorescence reported to the fluorescence of control cells not incubated with probes. Fibroblasts were used as non endothelial control cells.

2.8. Wound healing assay

In vitro wound healing assays were performed using culture inserts according to the manufacturer's instructions (IBIDI, Germany). Briefly, ECFCs were seeded at 6.10^4 cells/mL (70 μL per well), in order to obtain a confluent layer in 24 h, in the two wells of the insert separated by a wall. Then culture inserts were removed creating a reproducible cell-free gap of about 500 μm for each condition. The wound area filling was immediately captured by videomicroscopy (Olympus, Metamorph software) (objective ×10) for 20–24 h. Cells were incubated at 37 °C under 5% CO_2. The wound area decrease was analyzed as follows: after a shadow removal using FFT band pass filtering, a gradient analysis was performed to get a suitable segmentation of the cell layer. Batch processing of images was performed in order to obtain, for each time point, the wound outline and its surface. The wound filling *versus* time was linear in all cases. Consequently, the wound regression speed was assimilated to the slope of this linear relation, obtained by simple linear regression.

2.9. Chemotactic migration assay

ECFC migration in response to VEGF gradient was assessed using 24-well culture inserts on polyethylene terephthalate (PET) track-etched membranes with 8-μm pore diameter (Corning, USA) coated with 20 μg/mL fibronectin from bovine plasma (Sigma-Aldrich, St-Louis, USA). After overnight starvation in EBM2 basal medium supplemented with 0.2% FBS (Lonza, Köln, Germany), ECFCs were loaded in starving medium into the upper part of the microchamber at $1.5.10^4$ cells/well. The lower chambers contained the same medium with or without 50 ng/mL of human VEGF 165-aa (Miltenyi Biotec, Germany). After 5 h of incubation, membranes were fixed in 4% paraformaldehyde and stained with May-Grünwald Giemsa coloration variant (RAL 555 kit, RAL diagnostics, France). Before mounting, cells present on the upper surface of membrane filter were removed by wiping with a cotton bud. Membranes were photographed using a camera associated with an inverted microscope with a 10× objective in phase contrast mode. The number of cells per field was automatically counted using a procedure programmed with the macro language of the ImageJ software as previously described (Chevalier et al., 2014).

2.10. Matrigel™ in vitro assay

Network formation was achieved by seeding ECFCs (1×10^5 cells/well) onto phenol red-free Matrigel™ (Becton Dickinson) in a 24-well plate for 24 h at 37 °C with 5% CO_2. Five pictures per well were taken after 5 and 24 h using a camera associated with an inverted microscope with a 4× objective in phase contrast mode. Images were analyzed using a customized version of the Angiogenesis Analyzer developed for the ImageJ software (http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ, G. Carpentier, Image J News, 20 October 2012) as previously described (Chevalier et al., 2014).

2.11. 3D sprouting assay

Three-dimensional fibrin gel assays were performed as previously described (Nakatsu et al., 2003). ECFCs were seeded onto Cytodex beads (GE Healthcare) and embedded in a 2.5 mg/mL fibrin gel in presence of EBM-2 supplemented with VEGF (5 ng/mL). Fibroblasts were plated on the top of the gel. Images were acquired with a Leica DMIRBE microscope. Number of sprouts and cumulative tube length per bead was measured using MetaMorph 7.7 software.

2.12. Statistical analysis

For statistical analysis, Graphpad Prism 5.0 software was used (Graphpad Software Inc., San Diego, CA). All data are presented as the mean ± SEM. Gaussian distribution was tested using the D'Agostino & Pearson omnibus normality test. For analysis between two groups, a student's *t*-test was used. Welch's correction was used when variances were not homogeneous. Correlations were determined using the Pearson or Spearman (no Gaussian distribution) r coefficient. For analysis between more than two groups, a statistically significant difference was determined using a one-way ANOVA analysis followed by Bonferroni and Dunnett post-test. P values < 0.05 were considered significant.

3. Results

3.1. Heterogeneity of CB-ECFCs and clonogenic classification

Clonogenic data from 65 human umbilical cord blood samples and 74 peripheral healthy male adult blood samples (aged 19–68) were collected. In accordance with other studies, we found that peripheral adult blood generated less ECFC colonies (AB-ECFCs) than cord blood (CB-ECFCs). The mean number of colonies obtained was 17 for 3 million mononuclear cells (MNC) per mL of cord blood *versus* 4 for 11 million MNC per mL of adult blood, regardless of the age of the adult donor (Fig. 1A).

Umbilical cord blood appeared as a very heterogeneous source of ECFCs, yielding 1 to >100 colonies with a gap between 50 and 100 colonies. We thus classified cord blood samples into 3 clonogenicity groups: highly (>100 colonies), intermediate (10–50 colonies) and low (<10 colonies) clonogenic samples. No highly clonogenic samples were obtained from all tested adult donors. Moreover, we confirmed that cord blood colonies appeared earlier (between 7 and 12 days) than adult blood colonies (after 12 days). All highly clonogenic samples gave rise to colonies 7 days after MNC seeding (Fig. 1B). Regarding the proportion of each group, umbilical cord blood and peripheral adult blood were mostly not (24.6% and 17.6% of samples, respectively) or low clonogenic (44.6% and 71.6% of samples, respectively) while a high clonogenicity remained rare (10.8%). Nevertheless, 30.8% of cord blood samples could generate >10 colonies *versus* only 10.8% of adult blood samples (Fig. 1C).

3.2. Clonogenic and proliferative potential of AB-ECFCs is independent of the donor age

Regarding peripheral adult blood, we observed no significant difference according to donor age neither for the colony yield nor for the delayed appearance of the first colony (Fig. 2A-B). However, donors over 50 were less likely to produce no colonies than donors aged 19–30 and 31–50 (2.5 and 2 folds, respectively). Thus, it could be easier to isolate ECFCs from older individuals. Nevertheless, the intermediate age

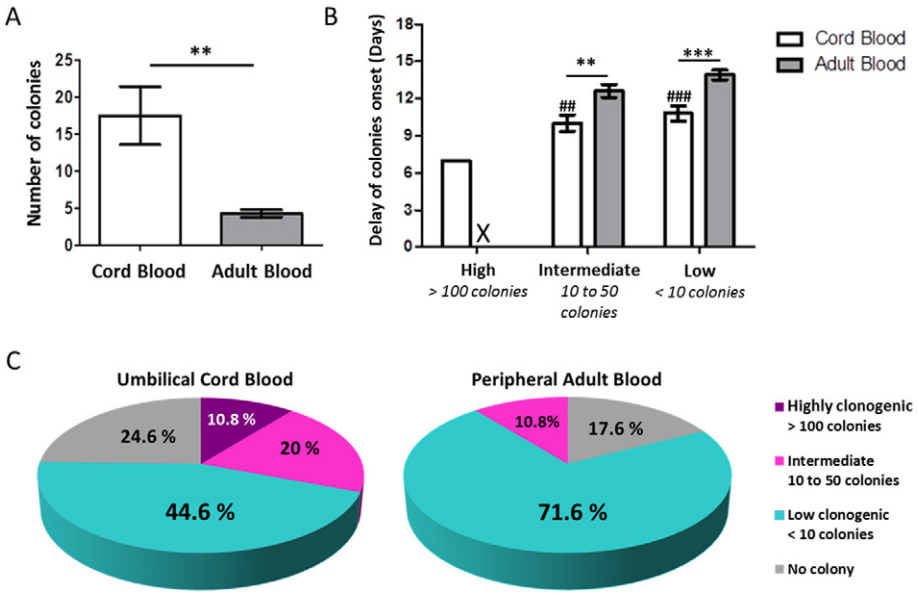

Fig. 1. Clonal features of ECFCs from adult and umbilical cord blood. A. Number of ECFC colonies generated from umbilical cord blood ($n = 65$) and peripheral adult blood ($n = 74$). B. Delayed appearance of ECFC colonies from cord blood ($n = 40$) and adult blood ($n = 48$) samples according to their clonogenicity. Data represent the mean and error bars represent SEM ($^{**}p < 0.01$, $^{***}p < 0.001$). # represents a significantly different delayed appearance of colonies between intermediate and low clonogenic CB-ECFCs compared to highly clonogenic samples ($\#\#p < 0.01$, $\#\#\#p < 0.001$). C. Clonogenicity distribution of CB-ECFCs ($n = 65$) and AB-ECFCs ($n = 74$) classified as highly (>100 colonies), intermediate (10–50 colonies), low (<10 colonies) and non-clonogenic samples.

group was the richest in intermediate clonogenic samples (Fig. 2C). Proliferation curves showed various profiles regardless of donor age. Many samples failed to expand after colony passage and only 2–4 PDs were obtained before rapid cell death. Although some AB-ECFCs could achieve >10 divisions, most samples were not able to achieve this level even those from the youngest donors. Despite a slight decrease in CPDmax in the youngest individuals, ECFC proliferative potential was not statistically related to the age in healthy adult male donors (Fig. 2D-E). Moreover, the clonogenicity and CPDmax did not correlate with the donor age (Fig. 2F).

In further experiments, AB-ECFCs from donors aged 50–65 years were mainly used as a reference for low proliferation and functionality. It should be noted that most assays performed in this study required a cell amount that could only be achieved with the most proliferative samples from adult blood. Despite this bias, these samples remained dysfunctional.

We first assessed the proliferative potential of each clonogenic class of CB-ECFCs.

3.3. ECFC proliferation is related to their initial clonogenicity

CB-ECFCs may proliferate for several months *in vitro*, up to >-6 months for the most proliferative cells, whereas most AB-ECFC sample cultures are limited to 1–3 months. Under our seeding conditions, this was equivalent to about 20 passages for CB-ECFCs and only 5 passages for AB-ECFCs. Interestingly, CPD curves showed that CB-ECFCs were divided into 2 distinct populations according to their initial clonogenicity (Fig. 3A). Indeed, the mean number of CPD was 53 in the highly and intermediate clonogenic groups and 31 in the low clonogenic group. We also confirmed that AB-ECFC proliferation was reduced as they performed on average only 10 PDs (Fig. 3B left). Regarding the PDT during the first 5 passages, the mean number of PDs was respectively 1.5 and 4 times faster in highly and intermediate clonogenic samples than in low clonogenic CB-ECFCs and AB-ECFCs. Nevertheless, only the difference with adult cells was statistically significant. Beyond P5, the gap between samples yielding >10 colonies and low clonogenic samples increased and became statistically significant. We thus observed a significant slow-down of the PDT of low clonogenic CB-ECFCs that was multiplied by 2 (4 days) between P5 and P10 and by 3 (6 days) beyond P10. In contrast, highly and intermediate CB-ECFCs still doubled in <3 days even at late passages (Fig. 3B right).

The reduced proliferative potential of AB-ECFCs at P5 and low clonogenic CB-ECFC at P10 was also confirmed by BrdU (Supplementary data, Fig. S1A) and CFSE assays (Supplementary data, Fig. S1B). As these experiments require a sufficient number of cells, we had to use AB-ECFCs enough proliferative in order to perform them. But these samples are in minority and so not representative of the mean proliferative potential of AB-ECFCs. Because of this, we also assessed BrdU incorporation of three pools of AB-ECFC low proliferative, one pool containing two adult samples from the same age class (Supplementary data, Fig. S1A, dotted line curve).

Thus, the initial clonogenicity appeared as an indicator of the proliferative potential of ECFCs. Furthermore, there was a significant correlation between the number of yielded colonies and the CPDmax in AB-ECFCs ($r = 0.51$; $^{***}p = 0.0004$) and CB-ECFCs ($r = 0.67$; $^{**}p = 0.0035$) (Supplementary data, Fig. S2).

Concurrently, low clonogenic CB-ECFCs and AB-ECFCs entered in senescence earlier than the other samples. As early as P5, 60% of AB-ECFCs were already senescent, as evidenced by the β-galactosidase assay, and we observed a strong activation of the cell cycle inhibitor p16. Although the number of senescent poorly clonogenic ECFCs remained low at P5 (<5%), it was 2 times that of highly and intermediate clonogenic CB-ECFCs. Then, the proportion of senescent cells increased progressively in low clonogenic samples to reach 60% at P15 whereas in highly and intermediate clonogenic CB-ECFCs, the mean number of senescent cells did not exceed 20% of the culture even at late passages. These results were confirmed by the profile of p16 which was strongly overexpressed in low clonogenic CB-ECFCs and in AB-ECFCs (Fig. 3C and D). No particular expression profile can be noticed for p21 and p53 (Fig. 3D).

Expression of Cyclins (*CCN*), Cyclin Dependent Kinases (*CDK*), Cyclin Dependent Kinases Inhibitors (*CDKN*), E2F factors and other key regulators of the cell cycle was also screened in RT-quantitative PCR in pre-senescent or senescent cells at passage 10 and 5 in CB-ECFCs and AB-ECFCs respectively (Supplementary data, Fig. S3). A significant accumulation of cyclin D1 associated to a weak expression of cyclins A, B and E are observed for low clonogenic CB-ECFCs and AB-ECFCs. These data indicate a blockage in G1 phase, consistent with a senescent stage (Atadja et al., 1995; Burton et al., 2007; Shackelford et al., 1999). Moreover,

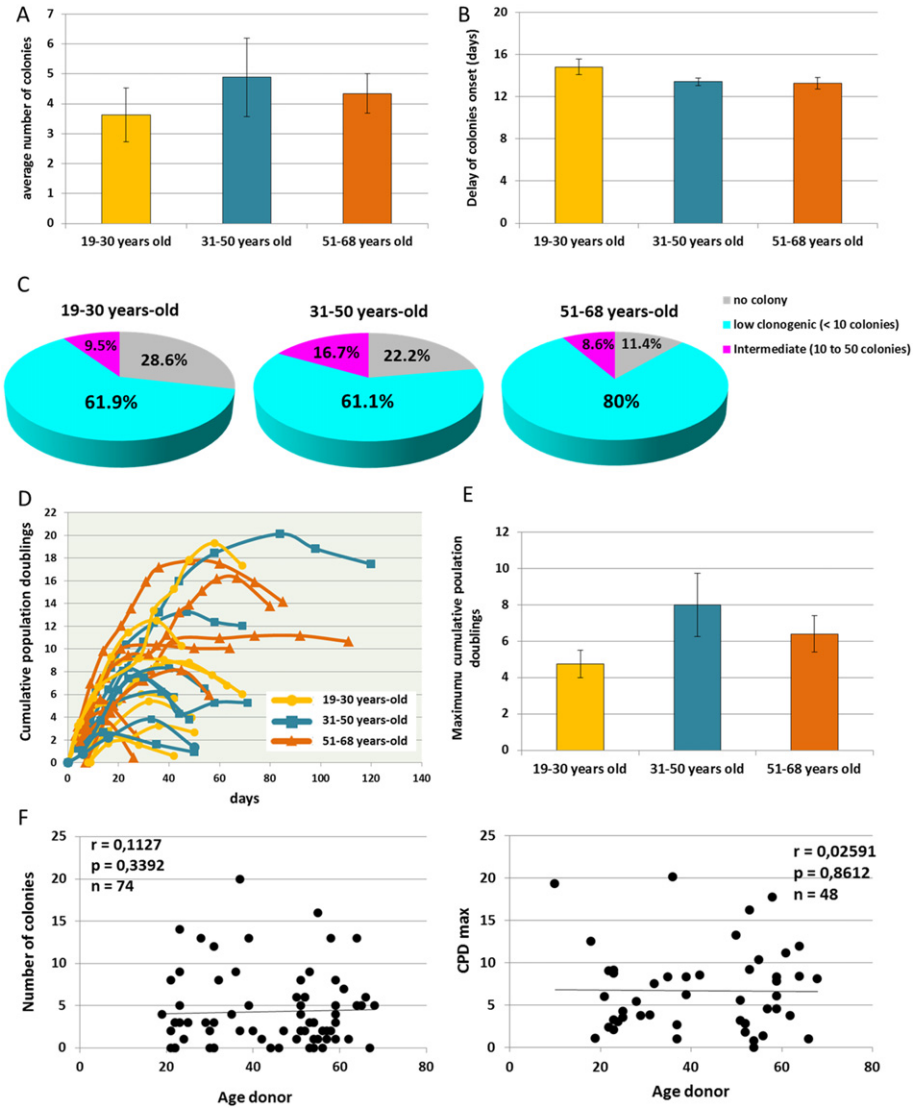

Fig. 2. Clonogenic and proliferative potential of AB-ECFCs according to donor age. A. Number of ECFC colonies obtained from peripheral adult blood of donors aged 19–30 years ($n = 21$), 31–50 years ($n = 18$) and 51–68 years ($n = 35$). B. Delayed appearance of ECFC colonies from peripheral adult blood of donors aged 19–30 years ($n = 11$), 31–50 years ($n = 12$) and 51–68 years ($n = 25$). C. Distribution of intermediate (10–50 colonies), low (<10 colonies) and non-clonogenic samples from peripheral adult blood of donors aged 19–30 years ($n = 21$), 31–50 years ($n = 18$) and 51–68 years ($n = 35$). D. Representative proliferation curves of 8 AB-ECFC samples for each age group. *E. maximum* CPD of AB-ECFCs from donors aged 19–30 years ($n = 15$), 31–50 years ($n = 10$) and 51–68 years ($n = 23$). F. Absence of correlation between the number of colonies and maximal CPD with the donor age.

cyclin D2, CDK 6 and 7 are specifically up-regulated in AB-ECFCs. It should be noted that a dramatic overexpression of cyclin D2 can be also involved in cell growth arrest (Meyyappan et al., 1998).

Concordantly with western blot profile, we observed, at a transcriptional level, a strong induction of p16 and p15 both in low clonogenic CB-ECFCs and AB-ECFCs and, to a lesser extent, an induction of p21 expression, only for adult samples. On the contrary, p18 is downregulated in these two groups. p16 and p15 are associated to premature G1 arrest notably due to oncogenic Ras/Raf/MAPK pathway (Malumbres et al., 2000). Besides, according to our results, RAF1 is particularly overexpressed in AB-ECFCs.

The cell cycle control requires the action of the transcriptional factors E2F, targeting genes involved in DNA synthesis, among others. Some of these factors are transcriptional activators (E2F1, 2 and 3a) and the others repressive ones (E2F 3b, 4, 5, 6 and 7) (Cobrinik, 2005). Low clonogenic CB-ECFCs and AB-ECFCs exhibit a significantly decreased expression of E2F1 and E2F2 whereas E2F3 (the primer contained in the TaqMan® Array used does not permit to distinguish the variant 3a and 3b) and E2F4 are specifically overexpressed in AB-ECFCs. Furthermore, E2F1, 2 and 3a are preferentially bounded and inhibited by pRB encoded by RB1 (Cobrinik, 2005). This gene seems to be up regulated in adult samples.

Interestingly, AB-ECFCs exhibit an increased expression of GSK-3β (glycogen synthase kinase-3β) which can phosphorylate Cdc25A (Cell Division cycle 25A) and so promote its proteolysis (Kang et al., 2008). The phosphatase Cdc25A activates CDK1, CDK2, CDK4 and CDK6, permitting thus G1/S transition and the progression in S phase (Jinno et al., 1994; Shen and Huang, 2012). Cdc25A gene is down regulated in low clonogenic CB-ECFCs and AB-ECFCs, and to a lesser extent, in intermediate CB-ECFCs compare to high clonogenic samples. Finally, according to our results, p53 expression profile does not correlate with ECFC senescence.

Bring together, these data confirm at a molecular scale the blockage in G1 phase and so the senescent profile of low clonogenic CB-ECFCs at P10 and AB-ECFCs at P5. Senescence in these cell populations seems to be essentially driven by p16 and p15 and not by p53/p21 pathway (except for adult samples which exhibit a notable induction of p21, at least at a transcriptional level). The entry in senescence could be initiated by oncogenic signals as we noted an increased expression of RAF1 in these two groups, mostly in adults. Besides, some genes are specifically

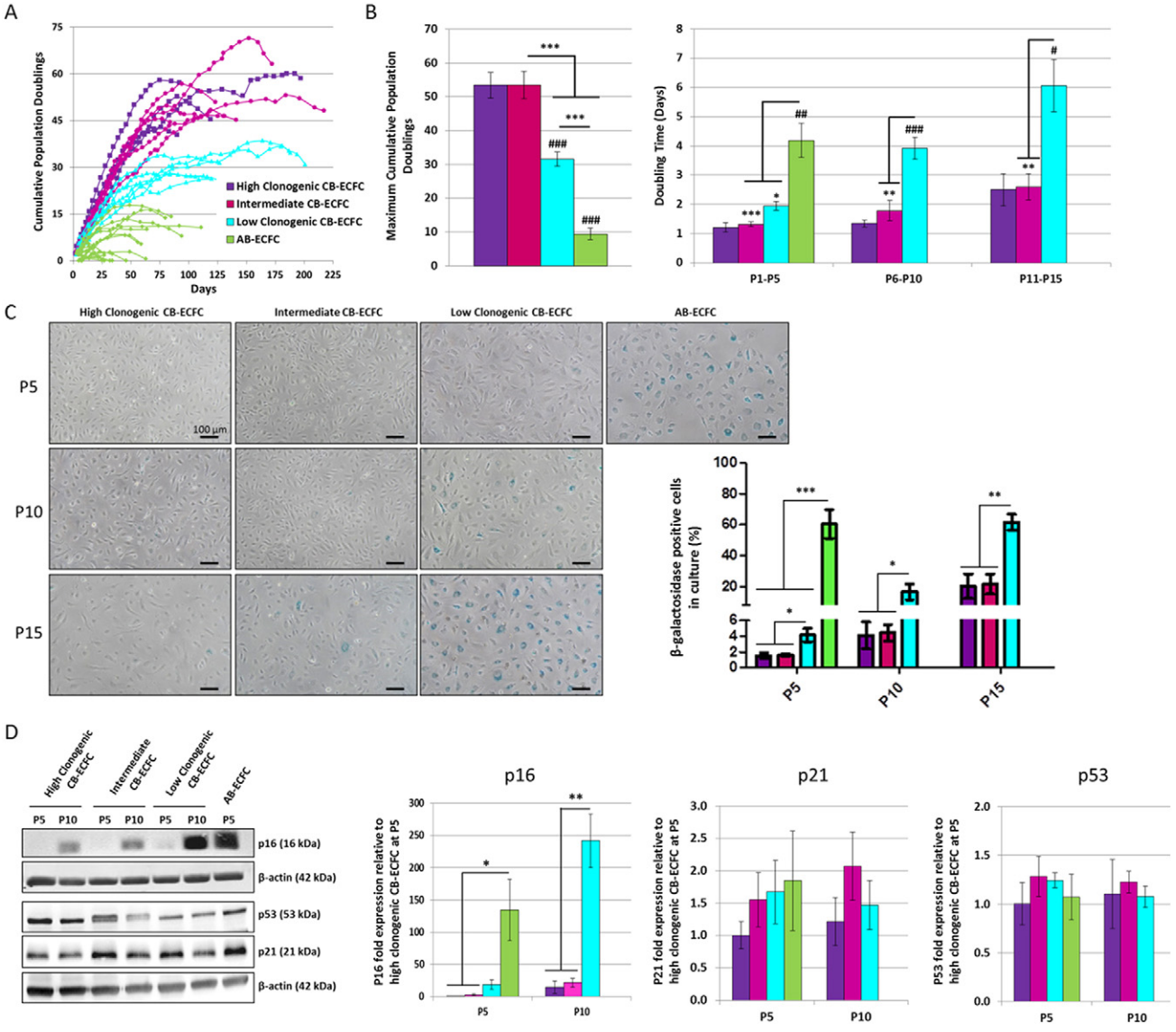

Fig. 3. Proliferative potential and replicative senescence of CB-ECFCs and AB-ECFCs. A. Proliferation curves of ECFCs from highly clonogenic ($n = 4$), intermediate ($n = 6$) and low clonogenic ($n = 6$) umbilical cord blood (CB-ECFCs) and peripheral adult blood (AB-ECFCs, $n = 10$). B. Proliferation parameters: maximum CPD (left) and PDT (right) at passage 1–5, 6–10 and 11–15. C. Representative phase contrast pictures (10× objective) of senescence-associated β-galactosidase assays (scale bars represent 100 μm) and senescent cell quantification, $n = 5$ for each group. C. p16, p21 and p53 expression shown by western blot and quantification. B-actin was used as a loading control. $N = 3$ for each group, error bars represent SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). # represents a significant difference from highly clonogenic CB-ECFCs (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$).

overexpressed by AB-ECFCs as those coding for cyclin D2, CDK6, E2F3, E2F4, GSK-3β and pRB. This distinctive profile can indicate the more advanced senescent stage of adult samples compared to low clonogenic CB-ECFCs, or it can potentially reveals the different pathways between replicative senescence provoked by prolonged culture and senescence linked to aging. Lastly, this gene pattern could be also indicative of a loss of immaturity as, for instance, E2F4 is generally associated to differentiation (Cobrinik, 2005).

To deepen the characterization of each clonogenic group, we studied their stemness/progenitor marker profile.

3.4. AB-ECFCs and low clonogenic CB-ECFCs show a similar loss of stemness markers

CB-ECFCs from the different clonogenic groups showed a similar expression of the main endothelial markers, CD31 (PECAM-1), CD144 (VE-cadherin) and VEGFR-2 (KDR) and were negative for the hematopoietic marker CD45. The same phenotype was observed in AB-ECFCs (Supplementary data, Fig. S2).

We first analyzed the expression of CD34, known to be progressively lost during the first passages of CB-ECFCs. Then, we analyzed gene expression levels of *DNMT3B*, *GDF3* and *SOX2* that we have previously identified as CB-ECFC immaturity markers in a study comparing the human embryonic stem cell line H9, CB-ECFCs and human aortic endothelial cells from adult donors (Guillevic et al., 2016).

According to the cytometry analysis at P5, CD34 expression was better preserved in highly and intermediate clonogenic CB-ECFCs (40–50% of $CD34^+$ cells) than in low clonogenic CB-ECFCs and AB-ECFCs (10–20% of $CD34^+$ cells) (Fig. 4A and B). Moreover, among the previously described stemness genes, the genes encoding the DNA-methyltransferase *DNMT3b*, the growth factor *GDF3* and the transcriptional factor *SOX2* were significantly downregulated by 50–90% in low clonogenic CB-ECFCs and AB-ECFCs compared to highly clonogenic CB-ECFCs as shown by RT-qPCR (Fig. 4C).

Thus, some CB-ECFCs and AB-ECFCs showed a loss of immaturity reflected by a low number of colonies and a limited proliferation with accumulation of senescent cells, associated with a downregulation of

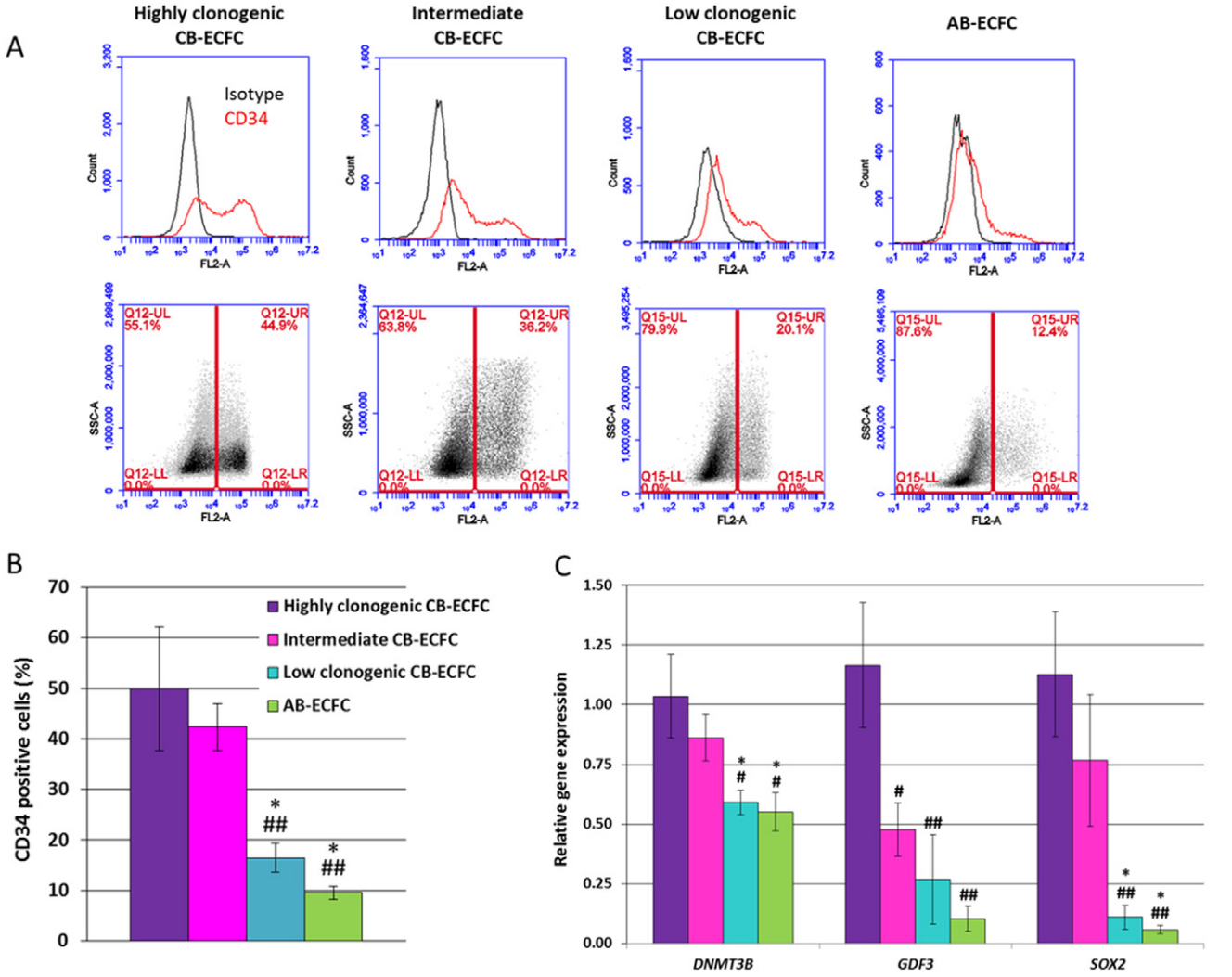

Fig. 4. CD34 and stemness gene expression in CB-ECFCs and AB-ECFCs. A. Representative expression of CD34 by fluorescence cytometry. B. Quantification of the percentage of $CD34^+$ cells in highly clonogenic ($n = 4$), intermediate ($n = 6$), low clonogenic ($n = 7$) CB-ECFCs and AB-ECFCs ($n = 4$) at P5. C. Relative transcript level of *DNMT3b*, *GDF3* and *SOX2* in the 3 clonogenic groups of CB-ECFCs and in AB-ECFCs ($n = 5$ for each group). Transcript levels were normalized to *GAPDH* and relative to the mean of highly clonogenic CB-ECFCs (calibrator). Error bars represent SEM. * represents a significant difference compared to intermediate clonogenic samples (*$p < 0.05$). # represents a significant difference compared to highly clonogenic samples ((#$p < 0.05$, ##$p < 0.01$).

progenitor markers. For establishing a link between ECFC stemness and functionality, we investigated if these impairments affected ECFC endothelial properties and angiogenic potential.

We next assessed classical functional properties of endothelial cells: NO production, wound healing, chemotactic migration in response to VEGF, network formation in Matrigel™ *in vitro* assay and 3D capillaries formation. These assays were performed at early passages, between P3 and P6.

3.5. Highly clonogenic CB-ECFCs overexpress eNOS and produce more NO

Endothelial cells play a fundamental role in regulating vascular smooth muscle cell vasomotricity *via* the release of NO, a major vasodilator. It is mainly produced by the NO synthase III (NOS III), also known as endothelial NOS (eNOS). eNOS transcripts were significantly overexpressed in highly clonogenic CB-ECFCs compared to low clonogenic CB-ECFCs and AB-ECFCs (Fig. 5A). Similarly, NO cellular content was increased in this group. No difference was observed between intermediate and low clonogenic CB-ECFCs but only samples yielding >10 colonies produced significantly more NO than adult samples (Fig. 5B). NO level was similar in AB-ECFCs and adult fibroblasts (data not shown).

3.6. Low CB-ECFCs and AB-ECFCs conserve migratory capacities

We first performed a wound healing assay to assess the migratory potential of each clonogenic group of CB-ECFCs and AB-ECFCs. Wound regression was analyzed by time-lapse videos. Despite a slight decrease in the mean wound regression speed in AB-ECFCs, no significant difference was observed (Supplementary data Videos S1–S4 and Fig. S3).

Since this assay partly depended on sample proliferative capacities, we also assessed the chemotactic response to VEGF. A similar profile was also obtained for each clonogenic group of CB-ECFCs and AB-ECFCs (Supplementary data, Fig. S4). According to our findings, the migration capacities of low clonogenic CB-ECFCs and AB-ECFCs seemed similar to those of highly and intermediate clonogenic CB-ECFCs.

3.7. Low clonogenicity and aging impact network density in Matrigel™

Functional endothelial cells may form a multi-connected network when they are seeded onto Matrigel™. We assessed the quality of this network for each clonogenic group of CB-ECFCs and for AB-ECFCs by quantifying the number of segments, junctions and meshes 5 h and 24 h after seeding.

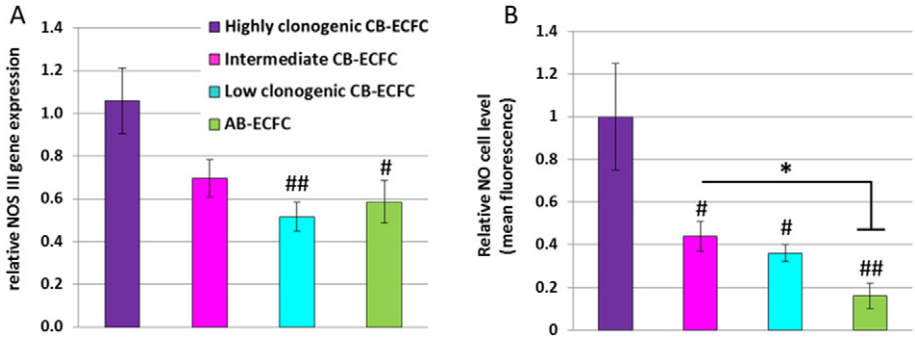

Fig. 5. Assessment of eNOS expression and NO production in AB-ECFCs and CB-ECFCs. A. Relative transcript level of eNOS (NOS III) in the 3 clonogenic groups of CB-ECFCs and in AB-ECFCs ($n = 5$ for each group). Transcript levels were normalized to *GAPDH* and relative to the mean transcript level in highly clonogenic CB-ECFCs (calibrator). B. Mean fluorescence intensity generated by reaction between DAF-2/DA probes and cellular NO, relative to the mean fluorescence intensity in highly clonogenic CB-ECFCs ($n = 4$ for each group). Error bars represent SEM. * represents a significant difference compared to intermediate clonogenic samples ($^*p < 0.05$). # represents a significant difference compared to highly clonogenic samples ($\#p < 0.05$, $\#\#p < 0.01$).

This analysis revealed that in networks formed by highly and intermediate clonogenic CB-ECFCs, there were 20–25% more segments, junctions and meshes 5 h after seeding than in networks formed by low clonogenic CB-ECFCs and AB-ECFCs. Then beyond 5 h post-seeding, the networks regressed until reaching a plateau for the three parameters studied. At 24 h, in highly clonogenic CB-ECFCs, the networks contained 30–40% more segments, junctions and meshes than in low clonogenic CB-ECFCs and AB-ECFCs. Nevertheless, there was a high variability which could explain why this difference in segment and mesh numbers between highly and low clonogenic CB-ECFCs was not significant despite the increasing gap between these 2 groups (Fig. 6).

3.8. Efficacy at generating capillaries in 3D hydrogel is predicted by high clonogenicity

Finally, an essential property of ECFCs for their use in cell therapy is their capacity to form new vessels, thus to participate in neoangiogenesis. To assess this property, we performed a tubulogenesis assay, also known as 3D sprouting assay. We obtained a very distinct profile between CB-ECFCs generated >10 colonies and poorly clonogenic or adult samples. Indeed, highly and intermediate clonogenic CB-ECFCs could form several capillaries (on average 6 sprouts per beads) with a mean total length of 3000 μm and 2000 μm, respectively whereas low clonogenic CB-ECFCs and AB-ECFCs failed to form numerous and long vessels. Their mean total length capillaries did not exceed 1000 μm (Fig. 7).

3.9. What parameters could affect upstream CB-ECFC colony yield?

Altogether, the previous results highlighted the significant impact of initial colony yield on further CB-ECFC functionality. A high clonogenicity may be considered a selective marker for the most angiogenic ECFCs. Nevertheless, in the perspective of CB-ECFC therapeutic use, such selection appears limited by the scarcity of samples yielding >10 colonies. Thus, it appeared necessary to identify factors prior isolation and culture which could influence initial clonogenicity. For this purpose, we collected all available obstetric and sample data accessible while maintaining donor anonymity.

According to our data on samples, the baby gender and weight and the pregnancy term had no impact on the colony yield. In fact, the weight and term were biased data as we received only full-term and healthy new-born cord bloods. Moreover, the delay between blood removal and MNC isolation had no effect, mainly because most of the time, we used samples within the same time interval (between 20 and 24 h). The concentration of MNC seemed to be more informative than the blood volume or MNC number. Moreover, the age of the mother also appeared particularly interesting (Supplementary data, Fig. S5A). Since ECFCs belong to the MNC population, samples with a MNC concentration lower than $2 \cdot 10^6$ cells/mL (31% of all samples) were mainly poorly or non-clonogenic. Thus, by selecting samples containing between $2 \cdot 10^6$ MNC/mL and $5 \cdot 10^6$ MNC/mL we can hypothetically enrich the proportion of blood yielding >10 colonies from 30.8% to 41.9% (Fig. S5B gate 1). Combining this MNC number range with the age of the mother enables to improve this yield up to 55.6%. Interestingly, intermediate clonogenic ECFCs were almost exclusively found in samples from mothers under 35 (Fig. S5B gate 2). Conversely, highly clonogenic ECFCs were mainly derived from cord blood of newborns whose mothers were over 37 (Fig. S5B gate 3). Nevertheless, this last gate represented <15% of samples.

4. Discussion

Based on the substantial size of the studied populations, we were able to determine the clonogenic profile of ECFCs from umbilical cord blood and peripheral adult blood (65 and 74 samples, respectively). We identified three groups based on their initial clonogenicity: highly (>100 colonies), intermediate (10–50 colonies) and low (<10 colonies) clonogenic samples.

Two proliferative populations of CB-ECFCs were distinguished based on a threshold of 10 colonies. Beyond this clonogenic limit, CB-ECFCs showed a comparable proliferative potential (a maximum of 50 CPD, PD in 1 day at early passages and in 2–3 days at late passages), a low accumulation of senescent cells even after P15 and a higher angiogenic potential *in vitro*. Thus, in addition to the higher initial number of cells conferred by a high clonogenicity, this parameter appeared as the earliest accessible predictive marker for the further functionality of CB-ECFCs. However, the parameters related to an intermediate or high clonogenicity, before MNC isolation, remain to be determined.

Despite the generally accepted higher yield of CB-ECFCs compared to AB-ECFCs also confirmed here, in this study, the CB samples mainly appeared as non- (24.6%) or poorly (44.6%) clonogenic. Nevertheless, it should be noted that the blood units for research purpose are actually samples withdrawn from the therapeutic circuit of cord blood bank, due to a too small volume or low concentration of $CD34^+$ cells. Thus, this yield could be possibly increased with a better donor selection. Moreover, these results are independent of the operator isolating ECFCs and we have used for several years the canonical protocol described in the literature based on the works of David Ingram and Mervin Yoder. Additionally, our provider, the French cord blood bank of St Louis Hospital, centralizes samples collected by different midwives from several maternities in Paris area. Due to the high sample variety, we cannot exclude a geographic bias of our results. Nevertheless, the cord blood

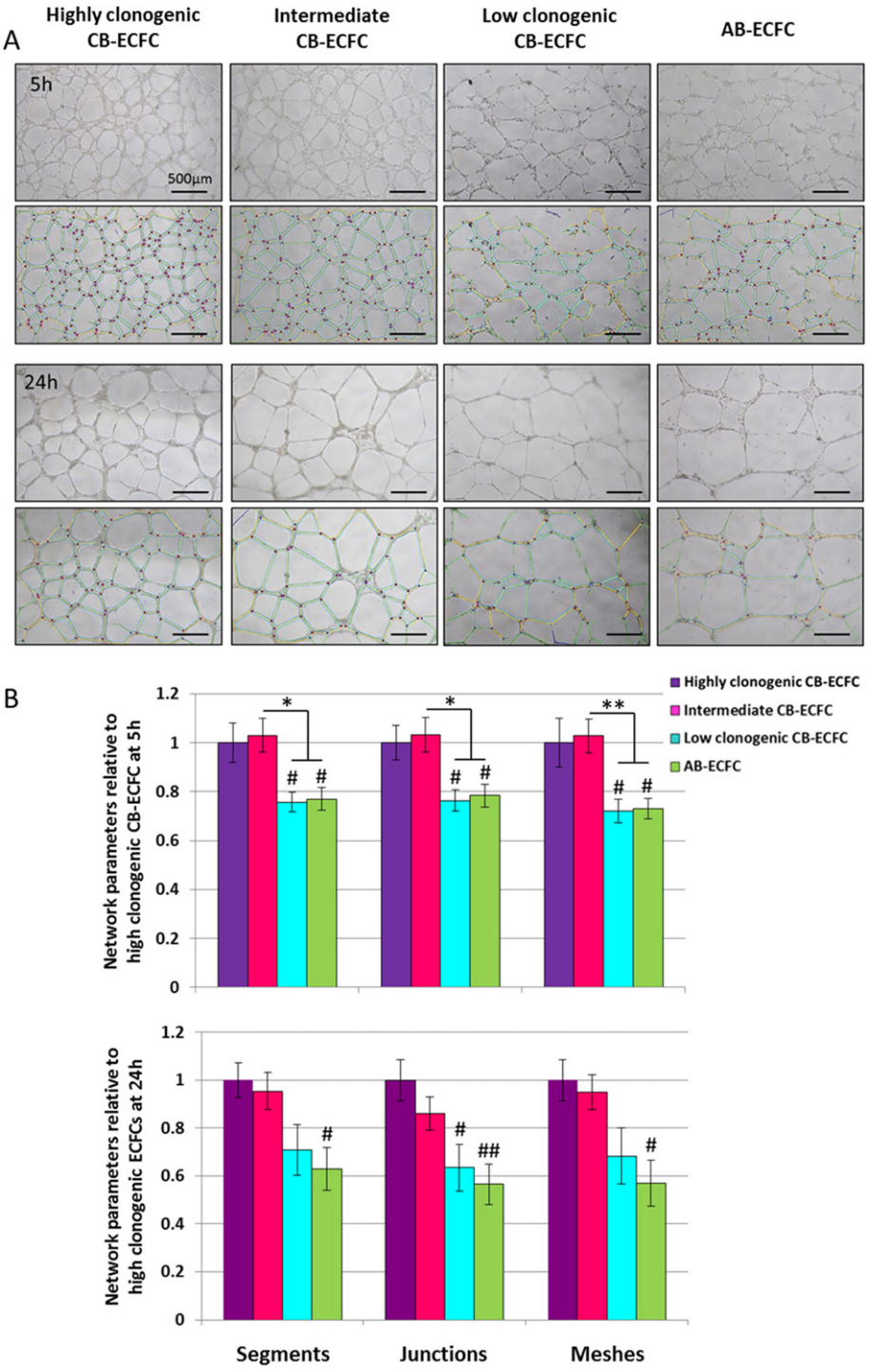

Fig. 6. Network Matrigel™ profile of CB-ECFCs and AB-ECFCs. A. Representative phase contrast pictures (4× objective) of networks onto Matrigel™ 5 h and 24 h after seeding associated with their respective extracted network skeletons identifying segments (yellow), junctions (red) and meshes (green). Scale bars represent 500 µm. (B) Quantification of network parameters 5 h (left) and 24 h (right) after seeding. Values are the mean ± SEM from triplicate in 5 independent experiments ($n = 5$, # and * represents a significant difference compared to highly and intermediate clonogenic CB-ECFCs respectively, $p < 0.05$).

clonogenicity is unlikely related to the blood collection process. Only the concentration of MNC and the age of the mother appeared as determinant parameters. However, none of the analyzed factors enabled to early completely exclude poorly and non-clonogenic samples. Interestingly, >90% of intermediate clonogenic CB-ECFCs were from mothers aged under 35 (mother mean age: 32 years) whereas highly clonogenic CB-ECFCs were from older mothers (mother mean age: 38 years). We were not informed whether the mothers were multiparous or if they received a particular hormonal treatment which could have influenced the fetal concentration of CB-ECFCs. The impact of other clinical features of the mothers has been recently described such as the pre-pregnancy body mass index which positively correlates with the number of ECFCs (Moreno-Luna et al., 2014).

Regarding peripheral adult blood, our results were opposed to those described in a previous study on aging conducted in monkeys (Shelley et al., 2012). Indeed, we found that ECFC clonogenicity and proliferation

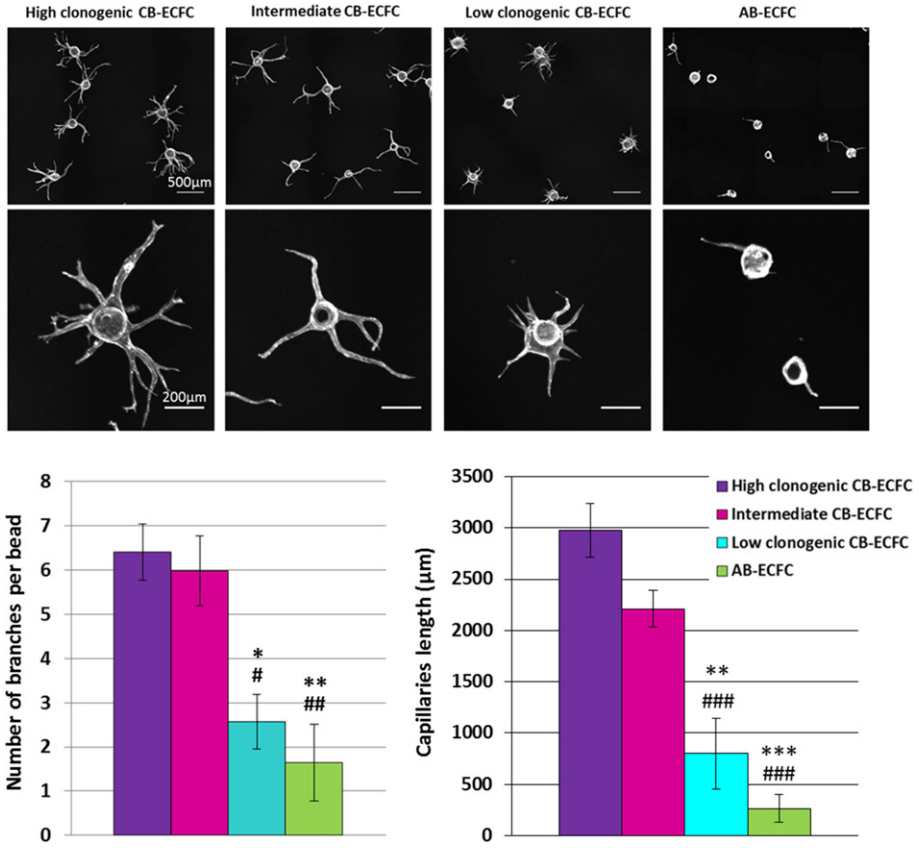

Fig. 7. Capillaries formation of ECFCs according to initial clonogenicity and aging. Representative pictures and quantification of branches and cumulative tube length per bead generated by highly ($n = 4$), intermediate ($n = 5$) and low clonogenic ($n = 5$) CB-ECFCs, and AB-ECFCs ($n = 4$). Experiments were performed in duplicate. Scale bars represent 500 µm (top) and 200 µm (bottom). Graphs represent mean values ± SEM. # (### $p < 0.05$) and * (** $p < 0.01$, ** $p < 0.05$) represent a significant difference compared to highly and intermediate clonogenic CB-ECFCs, respectively.

did not correlate with the donor age and did not decrease with aging. Nevertheless, our data only involved adults over 18 and ECFC yield in childhood should also be informative. Interestingly, the non-clonogenic fraction was reduced in adults over 50. According to some studies, the number of ECFCs increases in patients with cardiovascular diseases (Güven et al., 2006; Massa et al., 2009; Tagawa et al., 2015) and the incidence of such diseases correlate with age. Although our samples were from healthy donors, we should take into account that the exclusion from blood donation in France is essentially limited to infectious risks. Cardiovascular or cancer risk factors such as smoking or physical inactivity are not taken into account, thus the good health of donors is assumed but relative.

Most AB-ECFCs were poorly clonogenic, entered rapidly in senescence and had limited functional capacities compared to CB-ECFCs. Moreover, their transcriptional level for the stemness genes *DNMT3b*, *GDF3* and *SOX2,* that we have previously described (Guillevic et al., 2016), were similar to those found in adult mature endothelial cells. Although the efficacy of AB-ECFC injection as an autologous cell therapy remains challenging, these cells could be useful to study vascular aging *in vitro* as they may be obtained using a minimally invasive method. According to our results, senescence in AB-ECFCs is mainly mediated by p16 and p15 CDK inhibitors and seems to be associated to oncogenic signals such as an increased expression of RAF1.

Furthermore in AB-ECFCs, the transcript level of eNOS was significantly decreased and associated with a low NO production, reflecting an endothelial dysfunction which is known to characterize pathophysiological vascular aging like atherosclerosis (Minamino and Komuro, 2004). Besides, a clinical study based on a NOS overexpression strategy to enhance autologous angiogenic cell therapy in myocardial infarction is ongoing (ClinicalTrials.gov, Identifier: NCT00936819). Highly clonogenic CB-ECFCs overexpressed eNOS and constitutively released high levels of NO, so they appear particularly interesting for angiogenic cell therapy, especially as they can form numerous long capillaries as observed in 3D tubulogenesis assay. On this point, although Matrigel™ assay is often used to quickly test angiogenic potential, we can note that 3D tubulogenesis assay allows revealing a more marked difference between the tested samples.

Our study also provided new information about endothelial aging since a loss of immaturity in AB-ECFS compared to CB-ECFCs was evidenced by the downregulation of CD34 and the defined set of stemness genes. CD34 is described as a marker of quiescence. *In vivo*, both very immature stem cells and mature endothelial cells are $CD34^+$ and quiescent, thus CD34 is not a genuine stemness marker. Nevertheless, according to a recent study, the most proliferative CB-ECFCs are derived from the $CD34^+$ fraction (Patel et al., 2016). Accordingly, we observed that 40–50% of highly and intermediate clonogenic CB-ECFCs were $CD34^+$ while 4 times less low clonogenic CB-ECFCs and AB-ECFCs were $CD34^+$. It could be assumed that the initial number of quiescent cells could be higher in the most clonogenic samples, supplying the pool of proliferative cells with a progressive loss of quiescence throughout their expansion.

DNMT3b could be an interesting target since this *de novo* DNA methyltransferase plays a role in epigenetic stemness and early regulation of mesodermal differentiation (Jackson et al., 2004). Moreover, its expression in adult peripheral MNC linearly decreases during aging (Ciccarone et al., 2016). GDF3 (Growth differentiation Factor 3), a member of the TGF-β superfamily, is involved in the maintenance of embryonic stem cells at an undifferentiated stage (Levine and Brivanlou, 2006). Its role in endothelial cells or aging is not yet described. According to our results, its transcriptional expression in ECFCs appeared more related to

their clonogenicity than to their proliferation, since intermediate clonogenic samples, whose proliferative potential was similar to that of highly clonogenic ECFCs, expressed significantly less *GDF3*.

Sox2 belongs to the master transcriptional factors of the feedback loops maintaining pluripotency and self-renewal in mouse and human embryonic stem cells (Boyer et al., 2010). Interestingly, according to a study published in 2010, human endothelial/mesenchymal-like progenitor cells transduced with *SOX2* show an enhanced cardiovascular differentiation leading to better angiogenic capacities (Koyanagi et al., 2010). Moreover in a pathological context, VEGF may promote Sox2 expression in breast tumor cells leading to an enhanced self-renewal (Zhao et al., 2014). Its potential involvement in physiological progenitor cell-mediated angiogenesis remains to be elucidated.

5. Conclusion

The number of colonies at the first isolation step could only represent a more or less rich ECFC content of the donor but we demonstrated that the initial clonogenicity was also related to distinctive intrinsic stemness, proliferative and functional features. Moreover, our results enabled linking ECFC stemness and their further functionality since the most immature cells, defined by a highly clonogenic and proliferative potential associated with the expression of specific stemness genes, were also the most angiogenic and released significantly more NO. The next step of our investigations will be to determine the significance of this profile in *in vivo* models of angiogenesis. Moreover, it is important to define more precisely the molecular mechanisms involved in ECFC immaturity through the study of the role of DNMT3b, GDF3 and SOX2 in these cells.

Finally, this study demonstrated the functional heterogeneity of CB-ECFCs and thus the need to identify criteria for selecting, first, the most functional cells and then, the blood units containing the most active progenitors, as for cord blood hematopoietic stem cell therapies. With the aim to investigate CB-ECFCs in the context of clinical studies, the establishment of a functional hierarchy of these cells should fit into a global optimization strategy in terms of isolation, culture and expansion. This also involves cryopreservation and serum replacement issues. Moreover, a recent report on the low immunogenicity of CB-ECFCs, demonstrated by their tolerance and preserved angiogenic potential in immunocompetent mice (Flex et al., 2016), could help to revive interest for allogenic grafts of cord blood EPCs which is for long neglected for autologous approaches.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2017.04.009.

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