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Multipotent Adult Progenitor Cells (MAPC) contribute to hepatocarcinoma neovasculature ☆

Miguel Barajas [a,b,1], Federico Franchi [a,1], Carlos Clavel [b], Xabier L. Aranguren [b], M. Gabriela Kramer [a], Gloria Abizanda [b], Juana Merino [c], Cristina Moreno [c], Leire Gárate [b], Anunciata Guitart [a], Iñigo Narvaiza [a], María Gutiérrez-Perez [b], Jose-Ignacio Riezu-Boj [a], Carmen Berasain [a], Jesús Prieto [a], Felipe Prósper [b,*]

[a] *Department of Medicine and Division of Hepatology and Gene Therapy, Clínica Universitaria/School of Medicine and Centre for Applied Medical Research (CIMA), University of Navarra, Spain*
[b] *Area of Hematology and Cell Therapy, Clínica Universitaria/School of Medicine and Centre for Applied Medical Research (CIMA), University of Navarra, Spain*
[c] *Department of Immunology, Clínica Universitaria/School of Medicine, University of Navarra, Spain*

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Abstract

The use of stem cells as a vehicle of therapeutic genes is an attractive approach for the development of new antitumoral strategies based on gene therapy. The aim of our study was to assess the potential of bone marrow-derived Multipotent Adult Progenitor Cells (rMAPCs) to differentiate in vitro and in vivo into endothelial cells and to be recruited to areas of tumor vasculogenesis. In vitro, rMAPCs obtained from Buffalo rats differentiated into cells expressing endothelial markers and demonstrated functional endothelial capacity. Intravenous injection of undifferentiated rMAPC transduced with a lentivirus expressing GFP in an orthotopic rat model of hepatocellular carcinoma, resulted in tumor recruitment of the injected cells and in vivo differentiation into endothelial cells in the tumor area with contribution to vasculogenesis. In summary, our results suggest that rMAPCs can be efficiently recruited by vascularized tumors and differentiate to endothelium and thus may represent a useful vehicle for delivery of therapeutic genes to sites of active tumor neovascularization.

Keywords: MAPC; Stem cell; Gene therapy; Cancer; Angiogenesis

Gene therapy has been proposed as a promising therapeutic strategy for the treatment of liver cancer (review in [1]). However, the direct injection of current viral and non-viral vectors into the tumor mass allows delivery of therapeutic genes only to a small number of cancerous cells [2]. This low efficiency of transduction/transfection is usually accompanied by transient transgene expression and both factors have been associated with the limited antitumor response observed in clinical trials against hepatocellular carcinoma [3]. Therefore, the ex vivo administration of genetic engineered cells that can be efficiently recruited by tumors would represent a good alternative for the delivery of therapeutic genes to a broad tumor area.

Survival of mammalian cells depends on the proximity of the vascular system to obtain both nutrients and oxygen [4]. Tumor growth and metastasis also require the continuous formation of neovasculature around which tumor cells

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* Corresponding author. Fax: +34 948 296500.
E-mail address: fprosper@unav.es (F. Prósper).
[1] These authors contributed equally to this article and should be considered equal first authors.

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will proliferate (review in [5]). Although angiogenesis—the formation of capillaries from pre-existing endothelial cells—was the first described mechanism of tumor vascularization [6], recent studies have demonstrated that vasculogenesis, the formation of new blood vessels by the recruitment, differentiation and assembling of endothelial progenitors, also plays an important role in the formation of new vessels both in physiological and pathological conditions [7].

Increasing evidence indicates that bone marrow (BM) is a major source of endothelial progenitor cells that can be mobilized into the peripheral blood in response to cytokines or tissue injury and contributes to tumor development [8,9]. However, some recent studies have questioned the hematopoietic origin of the BM derived endothelial progenitor cells [10] or even their capacity to directly contribute to tumor vasculogenesis [11]. On the other hand, a population of BM stem cells named Multipotent Adult Progenitor Cell (MAPC) has been recently described, with the potential to differentiate in vivo and in vitro into mesodermal, endodermal and ectodermal derived tissues [12–14]. In a xenogeneic mouse model, human MAPCs have demonstrated its contribution to neovasculogenesis both in subcutaneous tumor and wound healing models [13].

In this study, we demonstrate that MAPCs obtained from the BM of Buffalo rats (rMAPCs) can be differentiated in vitro and in vivo into endothelial cells and can be recruited in vivo by liver tumors. Our data suggest that

Table 1
List of primers used for MAPC differentiations

Name		Sequence (5′–3′)	Product size (bp)	Accession number
Oct3/4	F	CTGTAACCGGCGCCAGAA	237	NM_001009178
	R	TGCATGGGAGAGCCCAGA		
GAPDH		(purchased from Applied Biosystems)		
Endodermal				
α1AT	F	TCAACAGGCCAGACAGTGAG	143	M32247.1
	R	TGAGTCGGCAAAGTTGACAG		
αFP	F	CTGTCACTGCTGATTTCTCTGG	146	NM_012493.1
	R	GTCCTTTCTTCCTCCTGGAGAT		
Albumin	F	GAGAAGGTCACCAAGTGCTGTAGT	142	NM_134326.1
	R	CTGGGAGTGTGCAGATATCAGAGT		
G6P	F	ACCCTGGTAGCCCTGTCTTT	150	NM_013098.1
	R	GGGCTTTCTCTTCTGTGTCG		
HNF3β	F	GGAAACATTGGGGGAACTTT	99	NM_012743
	R	GTGTGGCCCAGCTATTTAGG		
HNF6	F	CTGTGAAACTCCCCCAGGTA	195	NM_022671
	R	TCATCCCGCATAAGTGTGAA		
Neural				
GFAP	F	CCAAGCACGAGGCTAATGACT	101	NM_017009.1
	R	GCGCATTTGCCTCTCCAA		
Nestin	F	GGAGTGTCGCTTAGAGGTGC	327	NM_012987.1
	R	TCCAGAAAGCCAAGAGAAGC		
NF200	F	GCCCCGCAGACATCAGAT	191	NM_012607.1
	R	TGGTGTGGCTGGTGTCTTCTC		
Nurr1	F	TCCTGACTGGCTCTATGGAGATC	121	U72345.1
	R	AGAACAAATAATTCTAAGAAAGC		
Otx1	F	GCTGTTCGCAAAGACTCGCTAC	425	NM_013109.1
	R	ATGGCTCTGGCACTGATACGGATG		
Tau	F	CCCAGGTCGGCCACTTT	165	X79321.1
	R	CCTCCGTTGGACTCTTCACTTC		
Th2	F	CTTTGTGTCCGAGAGCTTCAAT	102	NM_012740.2
	R	GCCAGTGTGTACGGGTCAAAC		
Endothelial				
Flk-1	F	CCAAGCTCAGCACACAAAAA	191	NM_013062.1
	R	CCAACCACTCTGGGAACTGT		
Flt-1	F	TCCGAAAACTGAAGCGGTCT	151	NM_019306.1
	R	CCTAGTTTAAGTCTCTCCCGCG		
Tie-1	F	AGCTGCCTACATCGGAGACATAC	84	XM_233462.3
	R	CAAGGTCCCTGAGCTGAACTG		
Tie-2	F	AACCAACAGTGATGTCTGGTCCTAT	85	NM_013690.1
	R	GCACGTCATGCCGCAGTA		
VE-Cadherin	F	GGCCAACGAATTGGATTCTA	196	XM_226213.3
	R	GTTTACTGGCACCACGTCCT		
vWF	F	AGCCCGTCGTGATTTTCCT	69	U50044
	R	TTCAACCATGCCATGTTCTAGAA		

genetic engineering of rMAPCs may be a useful strategy to convey the expression of therapeutic genes to the tumor mass.

Materials and methods

Isolation and characterization of rMAPCs and cell lines. rMAPCs were obtained from the BM of Buffalo rats as previously described [15]. Briefly, BM was harvested by flushing the femurs and tibias of male Buffalo rats. Mononuclear cells were plated in MAPC expansion media [15]. Two days later, nonadherent cells were removed and adherent cells cultured for 2 more weeks. Only those wells where single clones were observed were expanded. After 3 or 4 weeks, cultures were depleted of $CD45^+/Ter119^+$ cells using micromagnetic beads (Miltenyi Biotech, Sunnyvale, CA, USA). CD45-/Ter119-cells were expanded at densities between 100 and 500 cells/cm^2 on FN-coated flasks (Cellstar).

rMAPCs were characterized phenotypically by fluorescence-activated cell sorting (FACS) analysis using antibodies against rat CD45-PE, CD44-PE, RT1A-PE, RT1B-PE, CD90 (Thy-1)-PE, CD73-PE and the corresponding isotype control antibodies (all from BD, Pharmingen).

For immunofluorescent staining primary antibodies against Oct3/4 (Santa Cruz), Flk-1 (Santa Cruz), Tie-1 (Santa Cruz), vWF (Chemicon), Albumin (Dako), HNF3β (Santa Cruz), β3-tubulin (Sigma), MAP2 (Sigma), GFAP (Sigma) and Tau (Santa Cruz) were used. Secondary antibodies coupled to FITC or PE were purchased from Molecular Probes. For controls, cells were labeled with unspecific immunoglobulins followed by incubation with the secondary antibody side by side with the slides stained with the specific antibodies. Functional endothelial potential was assessed by the in vitro matrigel assay and the uptake of acetylated-LDL as previously described [15].

The hepatocellular carcinoma (HCC) McA-RH7777 cell line syngenic for Buffalo rats was obtained from ATCC (Rockville, MD). Cells were cultured in DMEM supplemented with 20% heat-inactivated horse serum (ICN) and 5% heat-inactivated FBS (Biochrom).

RNA isolation and real time quantitative RT-PCR (qRT-PCR). RNA was obtained using the RNeasy Mini extraction kit (Qiagen). The RNA extracted was quantified and 1 μg RNA/reaction was reverse transcribed in a 100 μl total volume reaction using Taqman Reverse Transcrition Reagents Kit (Applied Biosystems). The cDNA obtained underwent 40 rounds of amplification (ABI PRISM 7700). Primers used are summarized in Table 1. mRNA levels were normalized using GAPDH as housekeeping gene (Applied Biosystems) and compared with levels of a positive control: rat embryo (Ambion) (for Oct3/4 expression), rat adult liver (for hepatocyte differentiation), Total RNA (BD) (for neural differentiation) and rat spleen (for endothelial differentiation).

Tissue processing, immunofluorescence and histochemistry. Explanted livers including the tumor were embedded in paraffin and blocks were cut in 5 μm sections, deparaffinized and stained with hematoxylin and eosin or used for immunofluorescence or immunohistochemistry analysis. The detection of transplanted cells was primarily based on the presence of GFP-positive signals (1:1000, rabbit polyclonal [pAb], Molecular Probes). For immunohistochemistry staining, the Envision (DAKO) and ABC (Vector Laboratories) systems were used. Immunolabeling with Rhodamine-conjugated Lectin (*Phaseolus vulgaris* Erythroagglutinin; 2 μg/ml) and a mouse monoclonal antibody anti-pan macrophage (0.2 mg/ml; Abcam) were used for detection of endothelial cells and macrophages, respectively. All sections were examined with a Nikon Eclipse E800 fluorescence microscope.

Rat model of orthotopic HCC and rMAPC recruitment study. Five- to eight-week-old male Buffalo rats were purchased from Harlan (Barcelona, Spain). The animals were kept in pathogen-free animal facilities in accordance with the *Guide for the Care and Use of Laboratory Animals* [16]. HCC tumors were established by intrahepatic injection of 10^6 McA-RH7777. A single tumor nodule (6–10 mm in diameter) was observed 7 days after injection [17].

rMAPCs were transduced with a third-generation lentivirus vector containing GFP cDNA under a CMV promoter (LvGFP) using a MOI of 50–200 in the presence of polybrene (4 μg/ml). For the analysis of in vivo recruitment and differentiation of rMAPCs, 1×10^6 undifferentiated

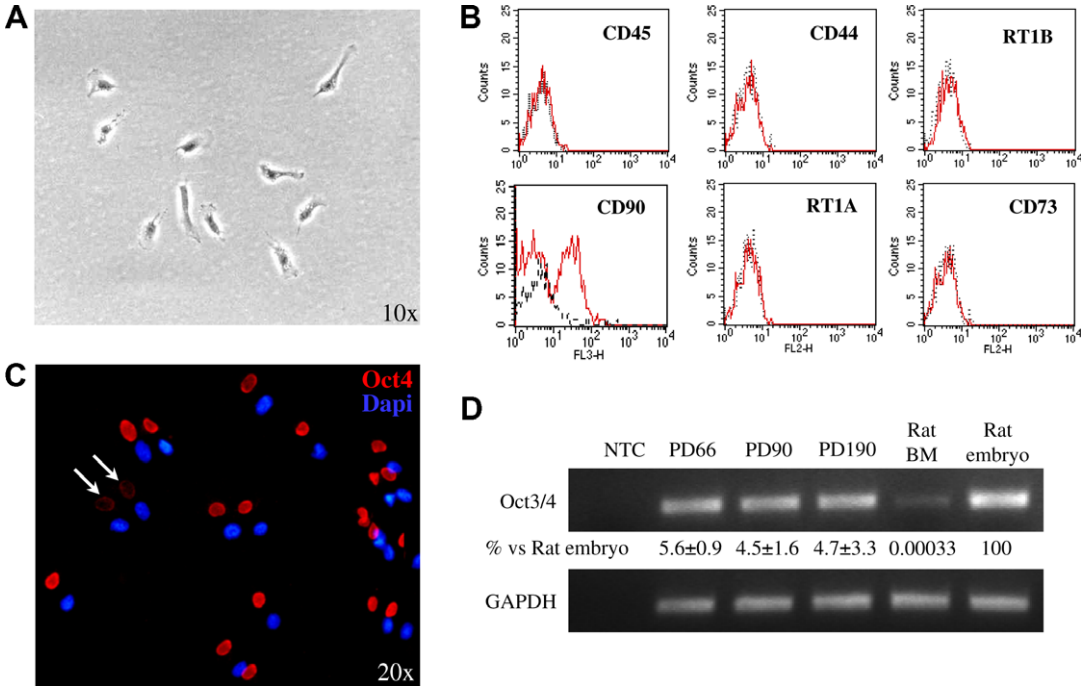


Fig. 1. Characterization of rMAPCs. (A) Morphology of rMAPCs after 50 cell doublings observed under bright field microscopy. (B) FACS analysis of rMAPC-GFP expanded for more than 80 cell doublings. Plots show isotype control IgG staining profile (black line) versus specific antibody staining profile (red line). (C) Immunofluorescence staining for Oct4 on rMAPCs at 50 PDs. Arrows indicate cells that were negative for Oct4. (D) qRT-PCR blot showing the expression of Oct3/4 mRNA in rMAPCs at different cell doublings (PD). qRT-PCR results are expressed as mean (±SEM) of experiments performed in triplicates. A representative example of more than five experiments is shown.

rMAPCs-GFP were injected intravenously in a total volume of 0.1 ml of PBS one week after intrahepatic tumor injection. One week later, animals were sacrificed and flushed the systemic circulation through the left ventricle with PBS, followed by 4% paraformaldehyde (PFA). Lectin-stained tumor periphery sections were used to evaluate the percentage of rMAPCs-GFP/Lectin$^+$ engraftment. The number of rMAPCs-GFP/Lectin$^+$ cells versus total tumor endothelial cells were quantified in three areas/sections and determined by two independent, blinded observers.

Statistical analysis. All data are expressed as mean ± standard deviation. Statistical significance was assessed using Student's unpaired *t*-test.

Results

Isolation and characterization of rMAPCs

We have established five different new cell lines of rMAPCs from Buffalo rats using methods previously described with minor modifications. Cells were grown at low density (100–500 cells/cm^2) and maintained for up to 190 population doubling (PD) with a doubling time of 36–48 hours. Cytogenetic analysis performed every 30 PDs showed a normal karyotype in each case (not shown). Based on cell size and cytoplasm complexity a population of small cells (4–6 μm) with reduced cytoplasm was observed (Fig. 1). FACS revealed rMAPCs to be negative for CD45R, CD44, RT1A, RT1B, CD73 and CD31 expression and positive for CD90 (Thy-1) expression (Fig. 1). The phenotype was consistent with the initial description of MAPCs [13].

Expression of the transcription factor Oct3/4 involved in maintenance of ES cells in undifferentiated state [18] was detected both at the mRNA level as well as the protein level (immunofluorescence) in rMAPCs (Fig. 1). rMAPCs expressed Oct3/4 at levels of 5 ± 2% of levels expressed in rat embryo and 10,000-fold higher than rat derived BMMNC (Fig. 1).

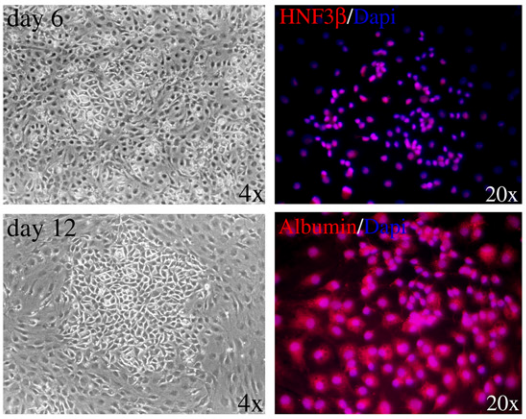
Multilineage differentiation potential of rMAPC

The multipotentiality of rMAPCs was further demonstrated by inducing differentiation along endodermal, ectodermal and mesodermal lineages. When rMAPC were cultured in the presence of FGF4 and HGF, mRNA expression for HNF3β, HNF6, αFP, α1-antitrypsin and Glucose-6-Phosphatase was upregulated albeit at different

A

	rMAPCs	Day 6	Day 10	Day 14
HNF6	< 0.01	1.7±0.1	1.6±0.1	10.9±0.3
HNF3β	0.09±0.02	0.3±0.1	3.6±0.1	1.0±0.3
αFP	< 0.01	5.5±0.7	19.0±1.7	102.1±5.2
Albumin	< 0.01	0.2±0.1	9.5±0.8	26.2±0.3
α1-AT	< 0.01	129.4±21.2	253.2±19.1	335.7±6.6
Glucose-6P	< 0.01	258.4±7.8	292.1±14.2	221.7±8.6

B



C

	rMAPCs	Day 6	Day 12	Day 16
Th2	< 0.01	6.0±0.2	14.6±0.4	31.4±1.6
GFAP	< 0.01	1.0±0.1	7.6±0.1	13.5±0.2
OTX1	< 0.01	131.3±2.0	252.5±27.5	283.7±14.3
Nestin	< 0.01	2,402.1±38.7	357.7±50.8	1,803.5±73.2
NF200	< 0.01	798.1±3.1	2,152.8±15.6	1,808.1±16.4
Tau	< 0.01	130.4±1.5	207.5±3.0	201.4±4.0
Nurr1	< 0.01	98.2±2.5	101.6±8.9	113.3±0.5

D

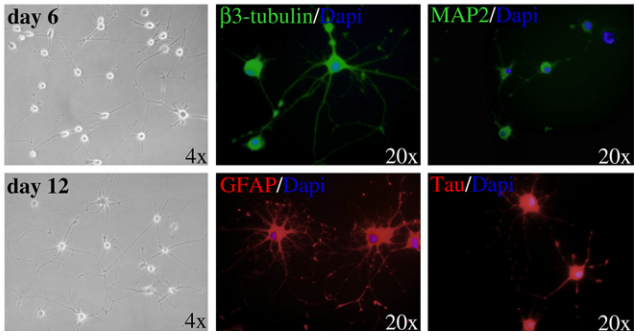


Fig. 2. Hepatocyte and neuroectoderm differentiation of rMAPCs. (A) Hepatocyte differentiation from rMAPCs: expression of hepatocyte markers was upregulated after 6–14 days in the presence of HGF and FGF-4, shown by qRT-PCR. mRNA levels are expressed in % versus a positive control (rat adult liver RNA) and were normalized using GAPDH as housekeeping gene. (B) Phase contrast imaging and immunofluorescence staining for albumin and HNF3-β (red) in rMAPCs at day +14. (C) Neuronal differentiation from rMAPCs: expression of neuronal markers was upregulated after 4–16 days in the presence of bFGF (week 1), Shh, FGF-8 (week 2) and BDNF (week 3), as shown by qRT-PCR (mRNA levels are expressed in % versus a positive control—total RNA—and were normalized using GAPDH as housekeeping gene). (D) Phase contrast imaging and immunofluorescence of neuronal markers (β3-tubulin and MAP2 in green and GFAP and Tau in red). The mean (±SEM) of three different experiments in triplicates is shown in (A) and (C).

levels (Fig. 2A). Albumin mRNA was only detected after 10 days of differentiation. Expression of albumin and HNF3β was confirmed at the protein level by IF at day +14 (Fig. 2B).

In order to induce neuroectodermal differentiation, rMAPCs were cultured in a 3 step culture as described [12]. mRNA expression for neural markers was analyzed from day +4 to +16 (Fig. 2C). Nestin, Otx1, NF200 and Tau expression was significantly upregulated (up to 1800-fold in comparison with rat total RNA) while expression of Th2, Nurr1 and GFAP was also increased but at lower level (between 1 and 100-fold in comparison with total rat RNA) (Fig. 2C). By immunofluorescence, rMAPCs expressed β3-tubulin, MAP2, GFAP and Tau at day +21 (Fig. 2D) suggesting the acquisition of a neural phenotype.

Induction of differentiation to endothelial cells was achieved by culturing rMAPCs in the presence of 100 ng/ml of $VEGF_{165}$. Under these conditions expression of Tie-1, Tie-2, VE-Cadherin, vWF, Flk-1 and Flt-1 was significantly upregulated in the cultured cells in comparison with undifferentiated rMAPCs, both at mRNA and protein level (IF) (Fig. 3A and C). Furthermore, we could confirm that rMAPC-ECs were functional as indicated by their capacity to take up acetylated-LDL (Fig. 3B) and the ability to form vascular tubes on matrigel (Fig. 3C).

rMAPCs contribute to vasculogenesis in a rat hepatocarcinoma model

To determine whether rMAPCs could participate in tumor vasculogenesis, 1×10^6 undifferentiated rMAPCs labeled with LvGPF (rMAPC-GFP) were injected intravenously into rats bearing intrahepatic tumors ($n = 6$). One week after rMAPCs-GFP injection, the animals were sacrificed and tumor samples were collected for histological analysis. Engraftment of GFP expressing cells was detected mostly at the periphery of the tumor and not inside the tumor or the healthy liver. Quantification showed a mean of 44.15 ± 4.73 cells/mm^2 GFP-positive cells in those areas with engrafted cells (Fig. 4). The tumor periphery is an area of significant vasculogenesis–angiogenesis so we examined whether rMAPCs had contributed to neovascularization. Expression of the endothelial specific lectin (*Phaseolus vulgaris* Erythroagglutinin) was detected in $69 \pm 8.35\%$ of GFP-positive cells suggesting that rMAPCs-GFP cells injected intravenously were recruited by the tumor and were induced to differentiate into cells of endothelial lineage (Fig. 4). The contribution of rMAPC-ECs to tumor vessels was however limited, 3.5% of the total endothelial cells in the periphery of the tumor were of donor origin. That rMAPC did not differentiate in vivo to hepatocytes

A

	rMAPCs	Day 3	Day 7	Day 11
Tie-1	< 0.01	20.0±1.6	26.6±0.5	15.3±0.4
Tie-2	< 0.01	3.8±0.2	4.3±0.5	8.8±0.4
VE-Cad	< 0.01	35.8±4.2	29.2±1.8	56.0±2.0
vWF	< 0.01	5.1±0.1	10.6±0.1	30.5±0.1
Flk-1	< 0.01	62.4±1.4	59.8±4.3	40.4±2.9
Flt-1	< 0.01	192.5±7.1	131.9±5.6	10.3±0.9

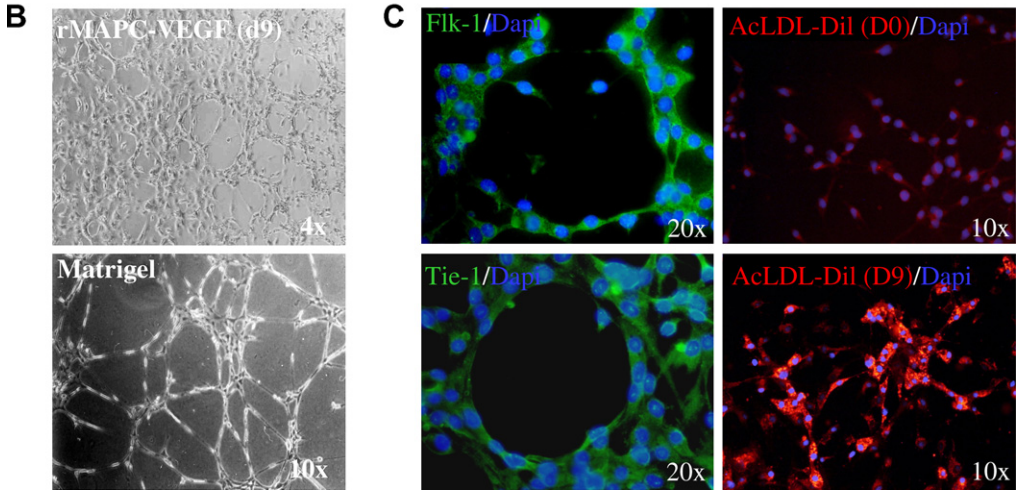


Fig. 3. Endothelial differentiation of rMAPCs. (A) Expression of endothelial markers was upregulated after 3–11 days in the presence of $VEGF_{165}$, shown by qRT-PCR. mRNA levels are expressed in % versus a positive control (rat spleen) and were normalized using GAPDH as housekeeping gene. The mean (±SEM) of three different experiments in triplicates is shown. (B, C) Phase contrast (B) and immunofluorescence (C) staining of rMAPC-ECs. After 11 days, rMAPCs were positive for endothelial markers Flk-1 and Tie-1. Functionality of rMAPC-ECs is shown by their ability to take up DiL-AcLDL (C) and to form vascular tubes in matrigel (B). A representative experiment of more than five experiments is shown.

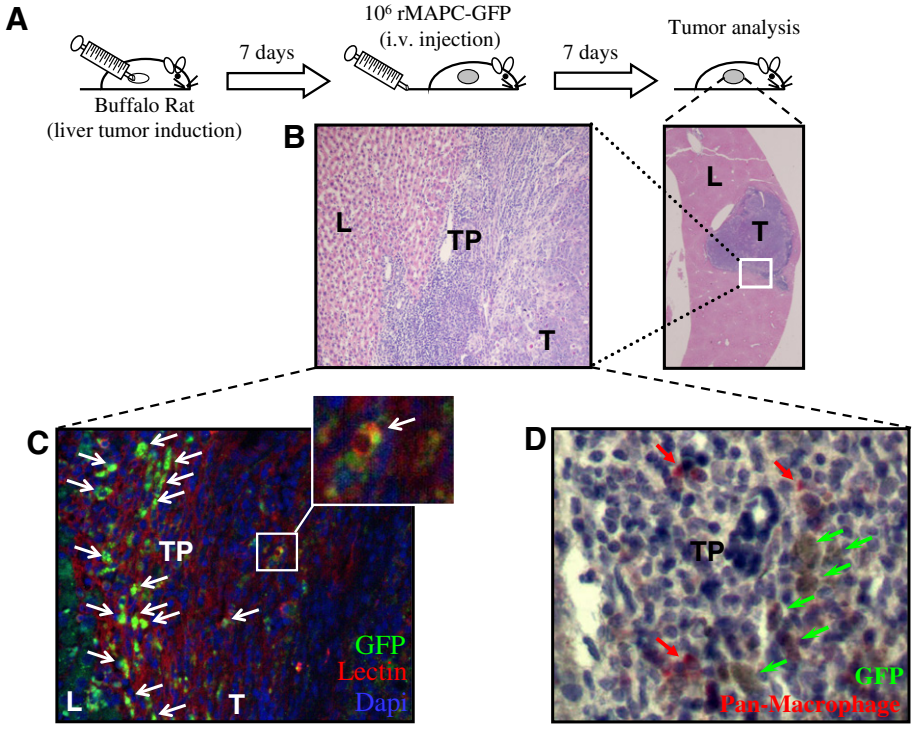


Fig. 4. In vivo recruitment of rMAPCs into liver tumors. (A) rMAPCs were intravenously injected in Buffalo rats bearing an intrahepatic hepatocarcinoma. The animals were sacrificed 7 days after rMAPCs injection and the liver tumors were processed for analysis. (B) Tumor samples were stained with hematoxilin–eosin to show the morphological differences between the tumor area (T), the healthy liver (L) and the tumor periphery (TP). (C) Immunofluorescence analysis shows the presence of significant number of GFP^+ cells (arrows) in the periphery of the tumor. Double immunofluorescence combining an anti-GFP antibody (green) and the endothelial specific *Phaseolus vulgaris* lectin (red) demonstrates the presence of endothelial-like cells derived from rMAPCs-GFP cells injected intravenously (inset). (D) Double immunohistochemistry staining of 5 μm paraffin sections through liver tumor with anti-GFP (green arrows) and anti-pan macrophage antibodies (red arrows). GFP^+ cells recruited in the periphery of the tumors (brown) were negative for pan macrophage antigen (pink).

or hematopoietic cells—mainly monocytes macrophages—was suggested by the lack of GFP-positive cells labeled with antibodies against macrophages/monocytes (Fig. 4) or hepatocytes (not shown).

Discussion

HCC is frequently diagnosed in advanced stages of the disease when there is no curative treatment available. Gene therapy has been used in these situations but despite promising preclinical data, (review in [19]) the results in patients have been rather modest [3]. The combination of gene therapy with cell therapy represents an attractive approach to enhance the antitumor effect of gene therapy-based strategies. For the treatment of tumors with high angiogenic activity such as HCC, a promising strategy could be the use of stem cells able to differentiate into endothelial cells that could be actively recruited by the tumor to form neovessels. The engineering of these cells with vectors encoding therapeutic genes could allow the expression of the antitumor molecules inside the tumor mass, thus enhancing the therapeutic effect.

The use of rMAPCs provides some advantages in comparison with other population of stem cells. The in vivo endothelial potential and the capacity of MAPC to contribute to tumor vasculogenesis has been previously described [13] and makes rMAPCs a useful population capable of incorporating into the tumor vessels. Other mesodermal derived stem cells like mesenchymal stem cells (MSC), despite their multipotentiality, have not been demonstrated to differentiate in vivo into endothelial cells [20]. Although the potential of other sources of EPC to home to areas of tumor vasculogenesis has been shown [21], rMAPCs in contrast to EPC, $CD34^+$ or $AC133^+$ cells [22] can be more easily expanded in vitro and transduced with therapeutic genes making them an attractive vehicle for delivery of genes to the tumor.

The possibility to achieve induction of gene expression specifically in MAPCs differentiated to endothelium in vivo would add a plus in terms of safety and efficacy to this therapeutic approach. Preliminary data suggest that VE-cad promoter would fulfill such conditions (data not shown). In addition, we have described long-term expression of VE-cad in human MAPC-ECs [23] indicating that this promoter would allow constitutive expression of a given therapeutic gene in the tumor. Having the tools, a number of candidate antitumor gene products, including toxic prodrugs, anti-angiogenic and immonostimulatory factors, could now be tested in our HCC rat model. The use of a syngeneic model of orthotopic HCC that better represents a clin-

ical situation further strengths the conclusion of our study that MAPCs may be a useful vehicle for gene therapy in cancer.

MAPC, being multipotent, could be expected to be found in other tissues of the body, contributing not only to vasculogenesis but also to other tissue types. However, we found no significant GFP expression in any of the organs analyzed, including the normal liver parenchyma, heart, spleen, lungs or kidney (data not shown). It has been reported that induction of tissue damage can increase the in vivo contribution of stem cells to tissue repair [24]. Thus, the lack of GFP-positive cells in other tissues could be related to the fact that stem cells may require additional signals associated with tissue damage or increased cell turnover for engraftment, which were not present in our model.

A recurrent issue in stem cell plasticity is whether the differentiated cells result from true differentiation of stem cells or from fusion between host mature cells and stem cells [25,26]. In particular, fusion events in the liver have been well characterized in models of liver regeneration using hematopoietic cells. In those studies, fusion generated predominantly hepatocyte-like cells but not endothelial cells [27]. In our study, GFP-positive cells were confined to the periphery of the tumor and the vast majority co-expressed the specific endothelial lectin *Phaseolus vulgaris*. We could not find cells with double labeling for GFP and macrophage markers in the tumor stroma, excluding that GFP-positive cells were due to fusion with macrophages. Together our data suggest that rMAPCs are able to incorporate into the tumor vessels.

In conclusion, our study indicates that bone marrow-derived MAPCs are efficiently recruited by highly vascularized tumors like HCC. These cells can serve as vehicles to convey the expression of therapeutic gene products to the inside of the tumor mass.

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