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A tyrosine-rich amelogenin peptide promotes neovasculogenesis in vitro and ex vivo

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ABSTRACT

The formation of new blood vessels has been shown to be fundamental in the repair of many damaged tissues, and we have recently shown that the adult human periodontal ligament contains multipotent stem/progenitor cells that are capable of undergoing vasculogenic and angiogenic differentiation in vitro and ex vivo. Enamel matrix protein (EMP) is a heterogeneous mixture of mainly amelogenin-derived proteins produced during tooth development and has been reported to be sometimes effective in stimulating these processes, including in clinical regeneration of the periodontal ligament. However, the identity of the specific bioactive component of EMP remains unclear. In the present study we show that, while the high-molecular-weight Fraction A of enamel matrix derivative (a heat-treated form of EMP) is unable to stimulate the vasculogenic differentiation of human periodontal ligament cells (HPC) in vitro, the low-molecular-weight Fraction C significantly up-regulates the expression of the endothelial markers VEGFR2, Tie-1, Tie-2, VE-cadherin and vWF and markedly increases the internalization of low-density lipoprotein. Furthermore, we also demonstrate, for the first time, that the synthetic homolog of the 45-amino acid tyrosine-rich amelogenin peptide (TRAP) present in Fraction C is likely to be responsible for its vasculogenesis-inducing activity. Moreover, the chemically synthesized TRAP peptide is also shown here to be capable of up-regulating the angiogenic differentiation of the HPC, based on its marked stimulation of in vitro cell migration and tubule formation and of blood vessel formation assay in a chick embryo chorioallantoic membrane model ex vivo. This novel peptide, and modified derivatives, might thereby represent a new class of regenerative drug that has the ability to elicit new blood vessel formation and promote wound healing in vivo.

1. Introduction

The formation of new vascular networks from progenitor cells is considered to be essential for the repair and regeneration of many types of tissue in vivo, including the periodontal ligament (PDL) that supports the teeth [1,2]. This tissue is composed primarily of fibroblasts, osteoblasts and osteoclasts, and has recently been reported to also contain a precursor/stem cell-like population that can undergo multi-lineage differentiation [3–9]. Thus, in the presence of the appropriate biological mediators, human periodontal ligament cells (HPC) have been reported to be capable of forming new vascular networks that ensure the supply of sufficient blood for the repair/healing of damaged PDL and the regeneration of healthy new tissue in vivo [1,2].

The complex process of neovasculogenesis comprises both vasculogenic and angiogenic differentiation during adult wound healing as well as in developing microenvironments [1,10]. Both vasculogenesis, the differentiation of progenitor/stem cells into endothelial cells [1,10,11], and angiogenesis, the development of an organized network of tubular structures originating from endothelial cells (EC) [1,12,10], are regulated in vitro and in vivo by a number of biological mediators, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), insulin-like growth factor, transforming growth factor-beta (TGF-β), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) [1,13–17], and also by enamel matrix proteins (EMP) [18–22].

EMP is a heterogeneous mixture of components consisting primarily of peptide isoforms derived from the full-length amelogenin gene transcript and is secreted during tooth development [23]. Crude preparations of EMP and a heat-treated preparation de-

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rived from it, enamel matrix derivative (EMD; Institut Strauman), have been widely used clinically for regenerating periodontal tissues, including PDL, alveolar bone and cementum, a mineralized tissue found uniquely in the periodontium [13,24–26,54–56,64]. However, a number of clinical studies carried out to evaluate the effects of these materials on periodontal soft tissue wound healing have resulted in discrepant conclusions. For example, periodontal wounds treated topically with EMD exhibited rapid and complete healing and PDL regeneration, compared with the control sites that were not treated with EMD which exhibited only partial healing and PDL regeneration [28]. In contrast, in a different study it was shown that a test patient group treated with EMD exhibited only partial PDL regeneration, comparable to the control group that did not receive EMD [27]. Similarly, discrepancies were also observed in a number of randomized controlled clinical studies and histological evaluations, as previously noted and discussed in the reviews by Sculean et al. [65], Giannobile and Somerman [66] and Venezia et al. [67].

The above studies highlight the variability of responses to EMP/EMD therapy of periodontal wound sites. The reasons for these conflicting results are not yet understood, possibly because the range of parameters involved in determining the clinical outcome is not yet clear. However, there is much evidence that new blood vessel (BV) formation is likely to be a central and indispensable feature of periodontal healing and regeneration [27,28,18–22], and it is thus possible that the effects of EMP/EMD on the establishment of a sufficient blood supply may be fundamental to the clinical efficacy of this material.

A number of studies have therefore examined the effects of EMD on angiogenic differentiation using model systems in tissue culture. However, these results have been inconclusive, possibly because some experimental protocols have used freshly isolated and non-heat-treated EMP while others have used commercially prepared and heat-treated EMD [19,29,22,18,30,4]. Thus, although EMP was reported to strongly stimulate migration/chemotaxis and the formation of a tubular network of human microvascular endothelial cells (HMVEC) in vitro [19,29], both Johnson et al. [19] and Thoma et al. [30] showed that EMD did not induce HMVEC migration/chemotaxis in a cell wound healing assay. Since the composition of EMP and EMD preparations is likely to vary qualitatively as well as quantitatively, a number of attempts have also been made to delineate the specific protein fraction(s) in these preparations that have angiogenic activity [19,30]. Johnson et al. [19] examined the effects of EMP fractions on HMVEC angiogenesis in vitro and observed that both a low- and a high-molecular-weight fraction of this preparation were able to stimulate chemotaxis and tube formation, although the latter fraction also contained some low-molecular-weight peptides. Similarly, Thoma et al. [30] examined the effects of EMD on a murine angiogenesis model in vivo and also reported that both a low- (<6 kDa) and a high-molecular-weight (<15 kDa) fraction of heat-treated EMD exhibited angiogenic activity. Although specific components were not identified, it is notable that proteins of relatively low molecular weight were again found to be present in all of the fractions prepared from EMD [30]. Such studies highlight the possibility that the discrepant effects of EMP and EMD on angiogenesis in vitro and in vivo might be related to the use of heterogeneous protein preparations containing multiple components that have different effects on angiogenesis.

Further studies aimed at identifying the specific bioactive components of EMD have separated two subfractions, by industrial-scale protein fractionation methodologies [31]: Fraction C, comprising <6 kDa peptides (mainly a 5.3 kDa tyrosine-rich amelogenin peptide (TRAP)), and Fraction A, containing a mixture of >6 kDa peptides, including a leucine-rich amelogenin peptide, sheathlin proteins and the full-length amelogenin protein [31]. In the present study we therefore examined the effect of EMD and these Fractions A and C, as well as a chemically synthesized 5.3 kDa TRAP peptide that is the main component of the low-molecular-weight Fraction C, on HPC, which are capable of undergoing vasculogenic differentiation when cultured in endothelial medium in vitro [3]. In addition, since we have also previously shown [3,4] that such cells are able to undergo angiogenic differentiation, forming tubular-like structures with minimal sprouting, the present study examined the effects of EMD components on HPC cell angiogenesis in vitro, including chemotaxis and the ability to form BV-like structures. The chick embryo chorioallantoic membrane (CAM), a highly vascularized extra-embryonic membrane that serves as a transient gas exchange surface similar to the lungs [32–34], was also utilized as an ex vivo model for examining the angiogenic effects of these EMD components.

2. Materials and methods

2.1. Isolation and culture of primary adult HPC and EC

HPC were obtained from the PDL tissues of three individual donors (three males, aged between 18 and 25 years) and were used as a source of progenitor/stem cells to investigate the effects of EMD component(s) on vasculogenesis, as previously described [3,4]. Patients undergoing routine third molar tooth extractions signed informed consent, in accordance with the protocol approved by the Joint Research and Ethics Committee of the Eastman Dental Institute and Hospital. Briefly, ligament tissue from the middle portion of the root of the tooth was digested with 3 mg ml^{-1} collagenase type I and 4 mg ml^{-1} dispase (both from Sigma, Gillingham, UK) for 1 h at 37 °C, as previously described [3,4]. Single-cell suspensions of HPC were obtained by passing the cells through a stainless steel filter (70 μm gauge; Falcon, Becton Dickinson, Cowley, UK) and cultured in growth medium (GM) comprising α-modified Eagle's medium (Gibco Life Technologies Ltd, Paisley, UK) and 10% fetal calf serum (PAA Laboratories, Yeovil, UK) supplemented with 200 U ml^{-1} penicillin, 200 μg ml^{-1} streptomycin and 2 mM L-glutamine (all from Gibco) at 37 °C in a humidified atmosphere of 5% CO_2 in air. As noted above, three separate HPC populations, each obtained from a different donor, were pooled together and used between passages 3 and 6.

Human umbilical vein endothelial cells (HUVEC; Lonza, Slough, UK) were used as the positive control/source of EC, as they have previously been shown to be capable of angiogenic differentiation [35,19,22]. The HUVEC were used between passages 3 and 6.

2.2. Effects of EMD and the EMD fractions on HPC cell vasculogenic differentiation

2.2.1. Immunocytochemical evidence of vasculogenesis

HPC were seeded into 24-well plates at a density of 2.5×10^4 cells $well^{-1}$, cultured in GM for 2–3 days and the medium replaced with endothelial basal medium-2 containing increasing concentrations of EMD components (1–100 μg ml^{-1}). EBM-2 supplemented with the endothelial growth factors (GF) VEGF (150 ng ml^{-1}) and EGF (50 ng ml^{-1}) (EBM-2 + GF) (Peprotech, London, UK), a medium which has previously been shown to induce murine and human mesenchymal stem cell vasculogenesis in vitro [36,37], was used to facilitate vasculogenic differentiation, as previously described [36,37]. The EBM-2, EBM-2 + EMD components and EBM-2 + GF were changed every 3–4 days. After 5 weeks, immunostaining of replicate cultures was carried out, as previously described [3,4], for two markers that are expressed by terminally differentiated/mature endothelial cells: VE-cadherin, an EC adhesion protein [3,4,36,37], and von Willebrand factor (vWF), a blood glycoprotein localized in EC-specific vesicles [3,4,36,37]. Briefly, cells were fixed

in 4% paraformaldehyde (Merck, Poole, UK) for 15 min at room temperature (RT) and permeabilized using 0.1% Tritox X-100 (Sigma) for 15 min at RT. They were then treated with a blocking solution containing 10% normal goat serum (NGS) in phosphate-buffered saline (PBS) for 1 h and incubated for 1 h at RT with primary mouse monoclonal anti-VE-cadherin (Insight Biotechnology, London, UK) and anti-vWF (Abcam, Cambridge, UK) antibodies diluted 1:100 in PBS containing 1% NGS. Incubation was then carried out with goat anti-mouse Alexa Fluor secondary antibody (Invitrogen, Paisley, UK) diluted 1:200 in PBS containing 1% NGS for 1 h at RT. VE-cadherin- and vWF-positive cells were visualized by their green fluorescent staining. Nuclei were stained blue using Hoechst dye. The proportions of VE-cadherin- and vWF-positive cells were determined by manual counting of five separate fields of each culture.

Total RNA was extracted from replicate cultures for conventional reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of the VEGF receptor-1 (VEGFR1) and VEGFR2 (measured at week 1) genes, involved at an early stage of VEGF-mediated vasculogenic differentiation. The Tie-1 and Tie-2 genes, involved in an angiopoietin-mediated late stage of vasculogenesis, were measured after 2 weeks, and the VE-cadherin gene, involved in producing EC adhesion protein at a late stage of vasculogenesis, was measured at week 3, as described in previously [3,4]. Since mRNA transcripts are expressed much earlier than the corresponding proteins, the VE-cadherin gene was measured at week 3 and immunostaining of VE-cadherin protein was carried out at week 5. Primer sequences are noted in Supplementary Table 1. To obtain a semi-quantitative estimate of the relative level of mRNA transcript expression, the intensity of the cDNA band corresponding to each PCR product was measured by densitometry using ImageJ (NIH software) and normalized to that of GAPDH used as an internal control.

2.2.2. Functional assay of vasculogenic differentiation by flow cytometry (FCM)

Receptor-mediated endocytosis of acetylated low-density lipoprotein (LDL), involved in the transport of triglycerides and cholesterol into the blood, is a key functional indicator of terminal vasculogenic differentiation [38,57]. To determine whether HPC exhibited this activity when cultured in differentiation medium and the effects of EMD component(s), the cells were cultured in 24-well plates in GM for 2–3 days and then the medium was replaced with EBM-2 alone, EBM-2 + EMD components (test sample) and EBM-2 + GF (positive control sample). After 5 weeks, cultures were washed with PBS and incubated for 2 h with EBM-2 containing 100 ng ml^{-1} Alexa Fluor-labeled acetylated LDL (Invitrogen, Paisley, UK), fixed with 4% paraformaldehyde (PFA) for 15 min at RT and the nuclei stained using Hoechst dye. Concurrently, FCM was carried out on duplicate cultures to measure the intracellular levels of Alexa Fluor-labeled acetylated LDL, according to the procedure described by [8], as follows. The cells were detached from the culture dish by incubating with trypsin–ethylenediaminetetraacetic acid (EDTA) (0.25% trypsin, 1 mM EDTA) (Gibco) for 5 min at 37 °C, centrifuged, fixed in 4% PFA and permeabilized using 0.1% Triton X (Sigma) for 15 min at RT. The cells were then centrifuged and resuspended in 200 μl of PBS and the fluorescence intensity of 10,000 individual cells measured using a flow cytometer (FACScan; Becton–Dickinson, Cowley, UK). Data analysis was carried out using the WinMDI 2.8 software program.

2.3. Effects of EMD components on angiogenesis

2.3.1. Chemotaxis assay

Chemotaxis/migration of endothelial precursors to the wound site followed by formation of angiogenic structures by the accumulated endothelial precursors is one of the initial events of angiogenic differentiation in vivo [39,29]. To examine the effect of EMD components on HPC cell chemotaxis, a two-dimensional cell "wound healing" migration assay was performed, as previously described [18,40]. Briefly, 2.5×10^4 EC $well^{-1}$ were cultured in GM alone in a 24-well plate for 3–4 days or until confluent, after which a "wound" was created in vitro by scraping off the cells using a pipette tip (200 μl) in a straight horizontal line (1 mm wound in width). The cultures were then washed with PBS and recultured with EBM-2 alone, EBM-2 + EMD components (test samples) and EBM-2 + GF (positive control samples) for 12 h. For analysis of cell migration (i.e. "wound healing activity"), microscopic images of the wound were taken immediately after scraping the cells (0 h) and after 6 and 12 h in the absence and presence of EMD components and GF. Cell migration was quantified by measuring the area of the wound (in pixel counts) using ImageJ software, using the following equation: area of wound (no cells) at 0 h minus area of wound at 6 or 12 h in the absence and presence of EMD component(s) and GF. Full healing of a monolayer wound is the full closure of wounded area as a result of cell migration, while no healing is equivalent to zero (the cell-free wound area is the same at the 0 h test time period). Replicate experiments were also performed using HUVEC as a positive control.

2.3.2. Angiogenic structure formation in vitro

The formation of angiogenic structures by endothelial precursors in vitro was performed using an angiogenesis assay kit (Millipore, Billerica, MA, USA), as described in previous reports [3,34,35,41]. The assay comprises a gel of basement membrane proteins (laminin, collagen type IV, heparan sulfate proteoglycan, entactin and nidogen) on which the endothelial precursors are cultured and align to form polygonal tube-like structures in a multi-step process involving cell adhesion, migration, growth and differentiation [34,39,41]. In this assay, 10^4 HPC were plated on gel-coated 96-well plates and cultured in the presence of EBM-2 alone, EBM-2 + EMD components (test samples) and EBM-2 + GF (positive control sample). After 5 and 15 h, digital images were obtained using bright-field microscopy. For quantitative analysis, branch points of polygonal tube-like structures in each culture condition were scored in five random fields, as described by the manufacturer of the kit and other reports [35,22]. Similar experiments were also carried out using HUVEC as a positive control.

2.4. CAM assay of angiogenesis ex vivo

The assay of the chick embryo CAM, a specialized highly vascular extra-embryonic tissue formed during avian embryogenesis, has been widely used to examine angiogenic compounds including bFGF [42,43], VEGF [44], EGF [45], retinoids [46] and somatostatin [47], mainly because of its accessibility for monitoring BV formation over the course of the experiment and relatively rapid outcome [34,32,33]. It was therefore used in the present study to determine the effects of EMD components on angiogenesis ex vivo. Fertilized White Leghorn chick eggs (Joice and Hill Poultry Ltd, Peterborough, UK) were incubated at 37 °C in 70–80% relative humidity in air for 3.5 days. The eggs were then carefully cracked open and the developing embryos transferred into 10 cm^2 Petri dishes and recultured. On day 7, UV-sterilized filter disks (3 mm^2) were placed on the CAM where BV were visible and 15 μl of EMD components containing 1, 25, 50 and 100 μg ml^{-1} (test samples) were added dropwise onto the disks. Replicate eggs were treated with 15 μl of 100 μg ml^{-1} bovine serum albumin in cell culture grade water (Thermo Scientific) as control samples. The eggs were then reincubated for a further period of 3 days, and the capillaries that had sprouted from the arteries of the CAM around the disks were photographed and counted in each

micrograph, then analyzed using ImageJ software as previously described [48,35].

2.5. Statistical analysis

The RT-PCR data are shown as the mean fold-change ± standard error (SE) of three separate experiments compared with that of control cells cultured in EBM-2 alone (defined as 1.0). One-way analysis of variance was used to assess statistically significant differences ($p < 0.05$), followed by Bonferroni corrections ($p < 0.05$) for multiple comparisons between the means (SPSS 11.0 software, Chicago, IL).

3. Results

3.1. Vasculogenic differentiation

3.1.1. Effects of increasing concentrations of EMD and the EMD fractions on vasculogenic differentiation of HPC

Vasculogenic differentiation of progenitor/stem cells into EC is considered pivotal in new BV formation in embryological development and also in adult wound healing [1,10], and this process has also been reported previously in adult HPC cultured in endothelial basal medium (EBM-2) for 5 weeks [3]. To examine whether EMD and the EMD fractions modulate vasculogenesis, the HPC were incubated in EBM-2 alone and EBM-2 + EMD. The representative micrographs in Fig. 1 show that only few VE-cadherin immunostained cells were present in cells cultured in EMD alone, whereas in the presence of Fraction C (30 μg ml^{-1}) the VE-cadherin-positive cells were more evident and the staining was found to be localized at the lateral borders of the cells (junctions), as previously reported using endothelial precursors derived from human umbilical cord [49,50] (Fig. 1). In contrast, in the presence of Fraction A no VE-cadherin-positive cells were detected. The results in Table 1 show that when cultured in EBM-2 alone for 5 weeks, 8.5 ± 1.3% of the HPC were positive for VE-cadherin, very similar to the proportion of VE-cadherin-positive cells observed when they were cultured in EBM-2 in the presence of 1, 3 and 10 μg ml^{-1} EMD (9.2, 9.7 and 10.2% positive cells, respectively). However, in the presence of higher concentrations of EMD (30 and 100 μg ml^{-1}), 13.0 and 15.4% of the HPC were VE-cadherin-positive, significantly greater than the proportion of positive cells when cultured in EBM-2 alone (8.5%; Table 1). Increasing concentrations of Fraction C also markedly increased the number of VE-cadherin-expressing cells, with 30 μg ml^{-1} resulting in 29.2% positive cells, although the presence of 100 μg ml^{-1} Fraction C did not further increase the relative proportion of positive cells. It is notable that the proportion of VE-cadherin-positive cells in the presence of 30 and 100 μg ml^{-1} Fraction C was also significantly higher than observed in the presence of the same concentrations of EMD, as shown in Table 1. Further experiments were therefore carried out to establish this apparent ability of Fraction C to actively promote vasculogenic differentiation by using a chemically synthesized form of the TRAP peptide, the main component of Fraction C, while Fraction A was not examined further since the results in Table 1 demonstrated that this fraction significantly suppressed HPC vasculogenesis.

Table 1
Effect of increasing concentrations (l-100 μg/ml) of EMD and the EMD Fractions on VE-cadherin staining of PDL cells cultured for 5 weeks in EBM-2. The numbers are the % of VE-cadherin-positive cells, as described in the Materials and methods. The values are the means ±SE of three measurements of three separate experiments.

Conc. (μg/ml)	VE-cadherin-positive cells (% of total cells)		
	EMD	Fraction C	Fraction A
1	9.2 ± 1.6	9.3 ± 1.9	3.1 ± 1.4*
3	9.7 ± 1.7	15.1 ± 2.2*,§	2.6 ± 1.7*
10	10.2 ± 1.8	20.7 ± 3.0*,§	l.l ± 0.8*
30	13.0 ± 2.2*	29.2 ± 3.4*,§	0.0
100	15.4 ± 1.2*	27.4 ± 4.0*,§	0.0

The control cultures (EBM-2 alone) had 8.5 ± 1.3% VE-cadherin positive cells after 5 weeks.
* Indicates significant difference compared with EBM-2 alone ($p < 0.05$).
§ Indicates significantly higher than EMD ($p < 0.05$).

3.1.2. Effects of TRAP on vasculogenic differentiation

As noted above, TRAP has previously been reported to be a main component of Fraction C [31], and in the present study this peptide was obtained by chemical synthesis and its pro-vasculogenic activity, examined as shown in Fig. 2 and Table 2. In control HPC cultured in EBM-2 alone, VE-cadherin staining was found to be localized at the lateral borders (junctions) of the HPC. vWF staining was observed in diffuse intracellular vesicular structures within the HPC (possibly comparable to Weibel–Palade-like bodies, characteristically associated with endothelial cells, [49,50]). Similar cellular localization was observed in the HPC cultured in the presence of TRAP (Fig. 2), although quantitative assessment of the effect of the peptide showed that TRAP markedly enhanced vasculogenic differentiation of the HPC. Thus, as with Fraction C, the presence of 30 μg ml^{-1} TRAP resulted in 26.2% of the cells exhibiting VE-cadherin staining after 5 weeks of culture, comparable to the proportion of VE-cadherin-positive cells observed in HPC cultures containing GF (21.7%) ($p < 0.05$) used as a positive control and significantly higher than the 8.5% positive cells when cultured in EBM-2 alone ($p < 0.05$) (Table 2). Similarly, in the presence of TRAP, 23.1% of the HPC were found to also express vWF (a late endothelial cell protein previously localized in Weibel–Palade vesicles [51]), again comparable to the proportion of cells in the positive control cultures containing GF (17.4%) ($p < 0.05$) and sig-

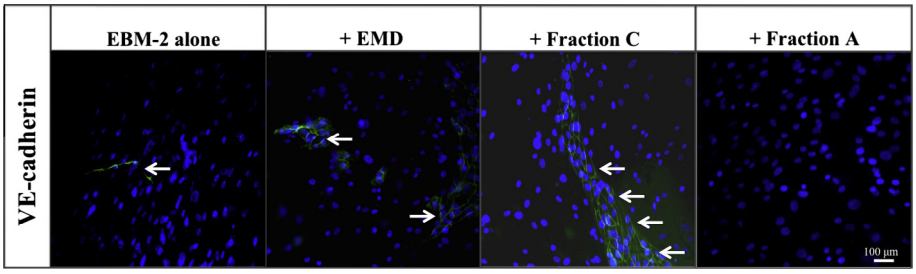

Fig. 1. Effects of increasing concentrations of EMD and the EMD fractions on VE-cadherin immunostaining of HPC. Representative micrographs of VE-cadherin of HPC cultured for 5 weeks in EBM-2 alone and EBM-2 containing EMD (100 μg ml^{-1}), Fraction C (30 μg ml^{-1}) and Fraction A (100 μg ml^{-1}), then immunostained for VE-cadherin. The nuclei are stained blue with Hoechst dye. Note the apparently greater numbers of VE-cadherin-positive cells (green fluorescence) following incubation with Fraction C, as indicated by the white arrows.

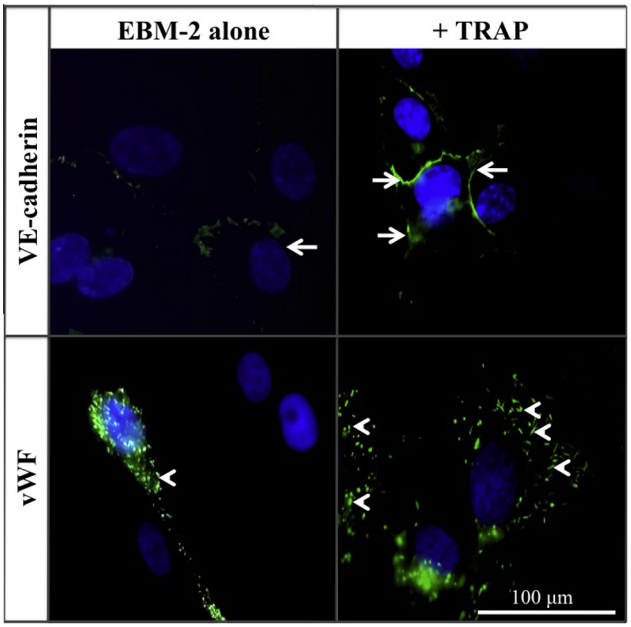

Fig. 2. Effects of TRAP on vasculogenic differentiation of HPC. Representative micrographs of HPC immunostained for VE-cadherin and vWF after culture in EBM-2 alone, EBM-2 + TRAP (30 μg ml^{-1}) and EBM-2 + GF for 5 weeks. The nuclei are stained blue with Hoechst dye and the white arrows show the green fluorescence of VE-cadherin staining at the lateral borders (junctions) of the cells. The white arrowheads show the punctate green fluorescence staining of vWF. Note apparently greater numbers of VE-cadherin and vWF-positive cells when cultured in the presence of TRAP.

Table 2
Effects of TRAP (30 μg/ml) on VE-cadherin and vWF staining of PDL cells cultured for 5 weeks in EBM-2. Cells cultured in EBM-2 + GF was used as a control. The numbers are the % of VE-cadherin- and vWF-positive cells. The values are the means ± SE of three measurements of three separate experiments.

Markers	VE-cadherin and vWF-positive cells (% of total cells)		
	EBM-2 alone	+TRAP	+GF
VE-cadherin	8.5 ± 1.3	26.2 ± 3.7*	21.7 ± 3.1*
vWF	7.1 ± 2.8	23.1 ± 5.6*	17.4 ± 4.7*

* Indicates significant difference compared with EBM-2 alone ($p < 0.05$).

nificantly higher than the 7.1% positive cells when cultured in EBM-2 alone ($p < 0.05$). These findings indicate that the TRAP peptide, like Fraction C of EMD, appears to stimulate HPC vasculogenic differentiation in vitro, comparable to that elicited by the presence of vasculogenic growth factors.

3.1.3. Effects of TRAP on vasculogenic gene expression

It was shown previously that the expression of a number of endothelial genes was elevated when HPC were cultured in EBM-2 alone [3]. Notably, the addition of TRAP was found to further up-regulate endothelial gene expression by the HPC, as shown in the representative RT-PCR gels in Fig. 3. Thus the presence of 30 μg ml^{-1} TRAP significantly increased the relative level of the early endothelial gene VEGFR2 mRNA transcript (by 3.9-fold), of the late Tie-1 and Tie-2 tyrosine kinases essential for angiopoietin-mediated vasculogenesis [11] (by 6.2 and 6.8-fold, respectively) and of the VE-cadherin mRNA (by 2.4-fold). Similar but less pronounced relative gene expression profiles were observed in the positive control HPC cultures containing GF (Fig. 3). In contrast, the relative level of the VEGFR1 gene transcript was unaffected when the cells were cultured in the presence of TRAP and in the presence of GF, as shown in Fig. 3.

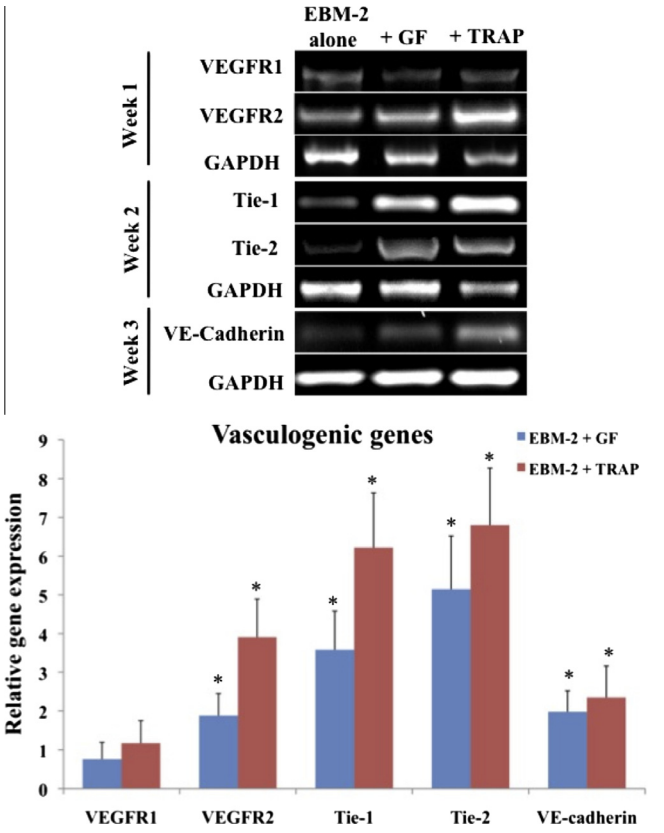

Fig. 3. Effect of TRAP on vasculogenic genes of HPC. (i) A representative RT-PCR gel showing the expression of the early vasculogenic genes VEGFR1 and VEGFR2 and the late genes Tie-1, Tie-2 and VE-cadherin by HPC cultured for 3 weeks in EBM-2 alone, EBM-2 + GF and EBM-2 + TRAP. (ii) The values are the changes in PCR product band intensity relative to GAPDH of cells cultured in the presence of GF and TRAP compared with EBM-2 alone, defined as 1.0. The values are the means ± SE of triplicate measurements. *Significant difference compared with EBM-2 alone ($p < 0.05$). Note the apparent up-regulation of HPC mRNA transcripts of VEGFR2, Tie 1, Tie 2 and VE-cadherin in the presence of TRAP.

3.1.4. Effects of TRAP on vascular cell functional activity

HPC were also examined for the effects of TRAP on internalization of acetylated LDL, a key functional indicator of terminal vasculogenic differentiation [38], by incubating the cells for 5 weeks in the absence and presence of 30 μg ml^{-1} TRAP (and GF as a positive control). The results in Fig. 4(i) show that, after 2 h of incubation with green fluorescent-tagged acetylated LDL, cultures incubated with TRAP and with GF appeared to contain a similarly high proportion of fluorescent-labeled cells (i.e. those that had internalized acetylated LDL), compared with many fewer positive cells when cultured in GM or EBM-2 alone. Measurement of the relative levels of green fluorescence using FCM showed that approximately 72% of the cells internalized acetylated LDL when cultured with TRAP and nearly 67% when cultured with GF (positive control), whereas only 26.2% of the cells were positive after culture in EMB-2 alone ($p < 0.05$) (Fig. 4(ii)). Thus the presence of TRAP appears to promote a significant increase in acetylated LDL uptake, similar to that found in the presence of GF, indicating a marked enhancement of vascular cell activity.

3.2. Angiogenic differentiation in vitro

3.2.1. Effects of TRAP on chemotactic migration to wound sites in vitro

Following vasculogenic differentiation of progenitor/stem cells, the newly formed endothelial precursors migrate to the wound site of damaged tissue and develop into an organized network of tubu-

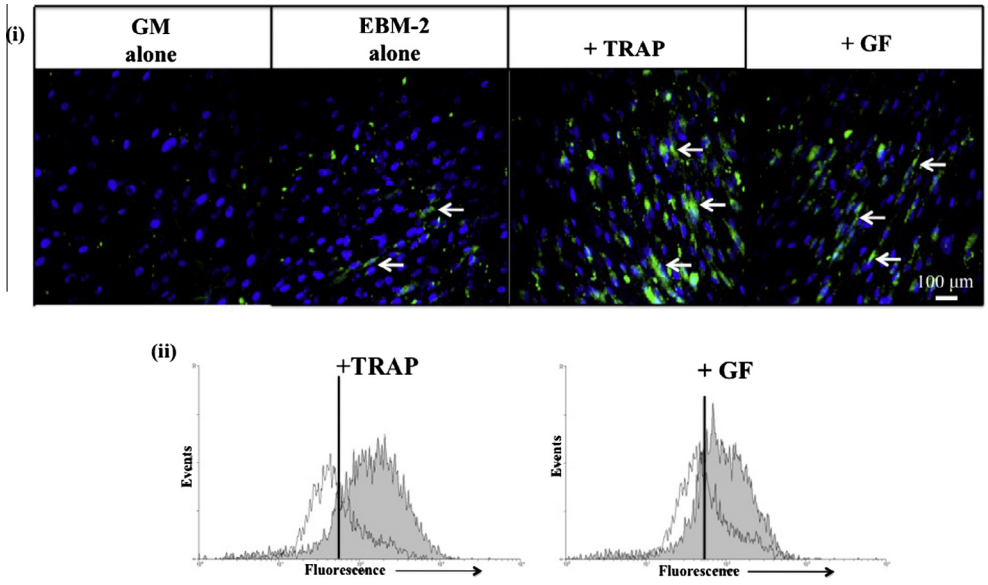

Fig. 4. Assessment of LDL-uptake by HPC treated with TRAP. Uptake of fluorescent-labeled LDL by HPC cultured in EBM-2 alone, EBM-2 + TRAP and EBM-2 + GF for 5 weeks. (i) White arrows show the internalization of LDL as visualized by fluorescence microscopy. Cells showing green fluorescent staining show internalized LDL and nuclei of the cells are stained blue. (ii) Representative FCM histogram of the levels of intracellular fluorescent-labeled LDL. In (ii), the shaded profiles show the fluorescence distribution of the TRAP and GF-treated cells, while the open profile shows the control cells cultured in EBM-2 alone. The vertical lines have been arbitrarily selected at a value of 75 fluorescence units. The events on the left side of the vertical lines are considered as non-fluorescent cells (negative cells) and events on the right side of the vertical line are considered as fluorescent cells (positive cells). Note the increased proportion of green fluorescent cells in the presence of TRAP and GF, compared with control conditions.

lar structures leading to the formation of BV in vivo, a defining feature of angiogenic differentiation [39,1,10,16,29]. In vitro it has been shown, using a two-dimensional wound healing assay, that such putative precursors cells can apparently be induced to migrate to "wound" sites by angiogenic growth factors, including VEGF and EGF [40,52]. In view of the above results indicating that TRAP was capable of inducing vasculogenic differentiation, further studies were carried out to determine whether this peptide also affected angiogenesis, as measured by its effect on HPC chemotaxis in a two-dimensional in vitro wound healing assay as described in Materials and methods. The results in Fig. 5 show that HPC migrated to the wound site during 6 h of exposure to the TRAP peptide, resulting in full closure of the wound site by 12 h, in contrast to cultures maintained in differentiation medium alone, which showed only partial closure at 12 h. Moreover, the results in Table 3 show that HPC in control cultures (incubated with EBM-2 alone) migrated over an area of approximately 22,500 pixels after 6 h and 32,986 pixels after 12 h, whereas there was a significant increase in the migration of the HPC when they were cultured with EBM-2 and TRAP for 6 h (58,360 pixels) and for

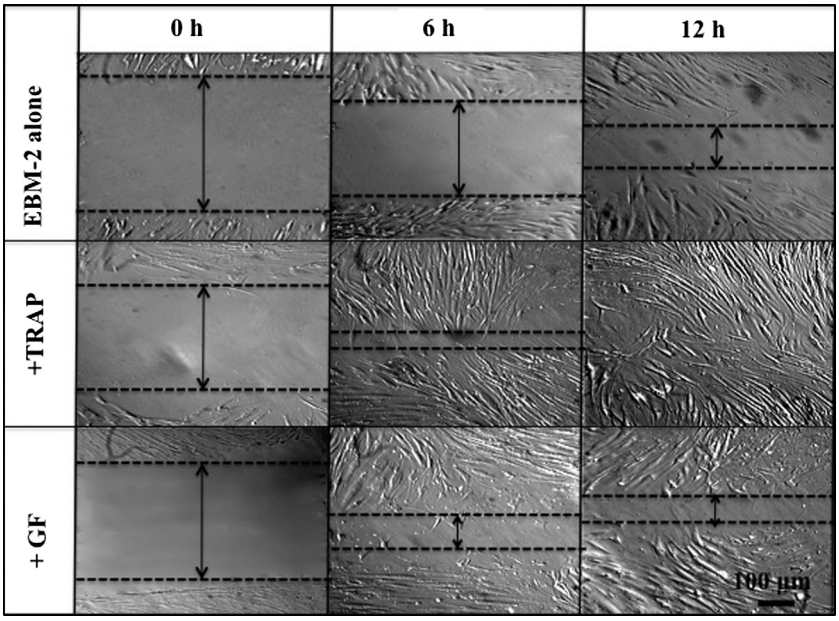

Fig. 5. Determination of the effects of TRAP on HPC chemotaxic activity in a cell wound healing assay. Representative microscopic images of HPC cultured in EBM-2 alone, EBM-2 + TRAP (30 µg ml^{-1}) and EBM-2 + GF for 6 and 12 h following the creation of an in vitro cell wound, as described in Materials and methods. The area between the black dashed lines shows the size of the wound (space without cells), which was measured in pixels. Magnification ×10. Note the apparent full closure of the cell wound after 12 h of incubation with TRAP, compared with only partial closure in the presence of EBM-2 alone and GF.

Table 3
Effects of TRAP on migration of PDL cells when cultured in EBM-2 alone. EBM-2 + TRAP (30 μg/ml) and EBM-2 + GF for 6 and 12 h after creating the cell wound in vitro. The numbers are the average (of 3 separate experiments) of the measurement of area (in pixel counts × 10^3) of PDL migration (area of wound (no cells) at 0 h minus area of wound at 6 or 12 h in the absence and presence of TRAP and GF).

Conditions	Cell migration (in pixels × 10^3)	
	6 h	12 h
EBM-2 alone	22.5 ± 0.37	33.0 ± 2.16
+TRAP	58.3 ± 1.19*	68.0 ± 1.47*
+GF	49.0 ± 2.10*	53.1 ± 2.24*

* Indicates significant difference compared with EBM-2 alone ($p < 0.05$).

12 h (67,996 pixels) ($p < 0.05$). As in cultures containing TRAP, there was also a marked increase in HPC migration in the positive control of EBM-2 + GF (49,053 and 53,136 pixels after 6 and 12 h, respectively, $p < 0.05$; Table 3). It is notable that similar effects of TRAP (and GF) on migration to wound sites were observed using HUVEC as positive control cells (Supplementary Material 1). The above findings thus indicate that TRAP induces the initial chemotactic phase of angiogenic differentiation and BV formation, comparable to that induced by angiogenic GF.

3.2.2. Effects of TRAP on formation of branched angiogenic-like tubular structures in vitro

The accumulation of platelets (thrombocytes) at a fibrin clot in vivo provides a growth-factor-rich environment that attracts endothelial precursors to migrate to the wound site and induces their organization into tubular-like structures that ultimately form BV [53]. In vitro tubule-like structure formation by endothelial precursors can be demonstrated morphologically when endothelial precursors are cultured on a gel of basement membrane proteins in the presence of angiogenic factors [34,35,41]. The present study therefore examined whether TRAP is also capable of inducing the formation of branched, tubular angiogenic-like structures, as described in Materials and methods. The results in Fig. 6(i) show that a complex polygonal tubular network, morphologically characteristic of angiogenic differentiation, was formed when HPC were cultured in EBM-2 in the presence of TRAP for 5 h, whereas relatively fewer of these structures were present in control cultures of HPC (in EBM-2 alone), as determined by manual counting of branching points (described in Materials and methods) of polygonal tubular-like structures. Thus, in the presence of TRAP there were nearly 110 branch points of such structures, similar to the 82 branch points observed in the presence of EBM-2 + GF and significantly more than the 22 branch points when the cells were cultured in EBM-2 alone ($p < 0.05$; Fig. 6(i)). In addition, HPC cultured for an extended period of 15 h in the presence of TRAP demonstrated the formation of distinct elongated BV-like structures (Fig. 6(ii)), in contrast to HPC cultured with EBM-2 alone in which these structures were not detected (data not shown). Similar results were obtained when HUVEC (positive control cells) were cultured on a basement membrane protein gel in the presence of EBM-2, EBM-2 + TRAP and EBM-2 + GF (data not shown). These results thus indicate that TRAP induces the organization of HPC into tubular-like structures in an in vitro angiogenesis assay, comparable to the inductive effects of angiogenic GF.

3.3. Effects of TRAP on CAM angiogenesis ex vivo

Since TRAP appears to be capable of stimulating angiogenic differentiation of HPC and mature EC in vitro, as indicated by the results presented above, the present study used the CAM model to examine whether TRAP also exhibited angiogenic activity ex vivo, as described in Materials and methods. The results in Fig. 7 show that when 7-day-old chick embryos were treated with 25 and 50 μg ml^{-1} TRAP for 3 days, the disks that had been placed over the CAM appeared to be surrounded by capillaries that had sprouted from arteries (Fig. 7). Although 1 μg ml^{-1} TRAP had no significant effect on the appearance of such "allantoic capillaries" (capillaries sprouted from the arteries of the developing CAM of chick embryo responsible for gas exchange and removal of the waste products [34,42,43]) compared with the control CAM, the presence of 25 and 50 μg ml^{-1} TRAP significantly increased the number of capillaries in the CAM (2.6- and 2.8-fold, respectively; $p < 0.05$). However, increasing the TRAP concentration to

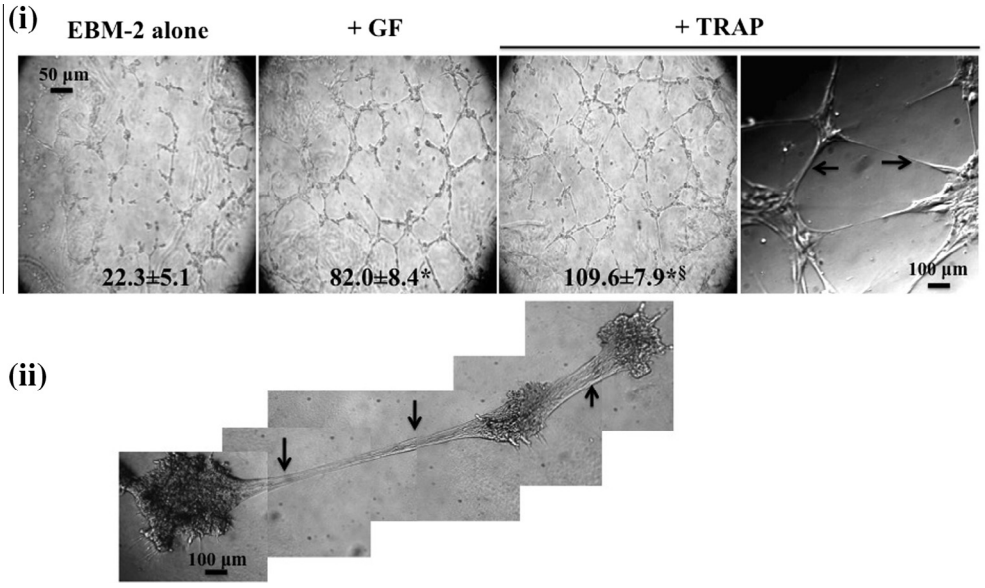

Fig. 6. Angiogenic structure formation by HPC in vitro. (i) HPC cultured for 5 h in EBM-2 alone, EBM-2 + TRAP (30 μg ml^{-1}) and EBM-2 + GF. The numbers are the angiogenic structure branching points. The black arrows show the polygonal tubule-like structures formed by HPC. The values are the mean ± SE of five measurements of branching points in randomly selected image areas in three separate experiments. *Significant difference compared with EBM-2 alone ($p < 0.05$). §Significant difference compared with EBM-2 + GF ($p < 0.05$). Magnification ×4 and ×20. (ii) An elongated BV-like structure formed by HPC cultured for 15 h in EBM-2 + TRAP. Magnification ×20. Note the apparent complex polygonal structure formed by HPC cultured in the presence of TRAP.

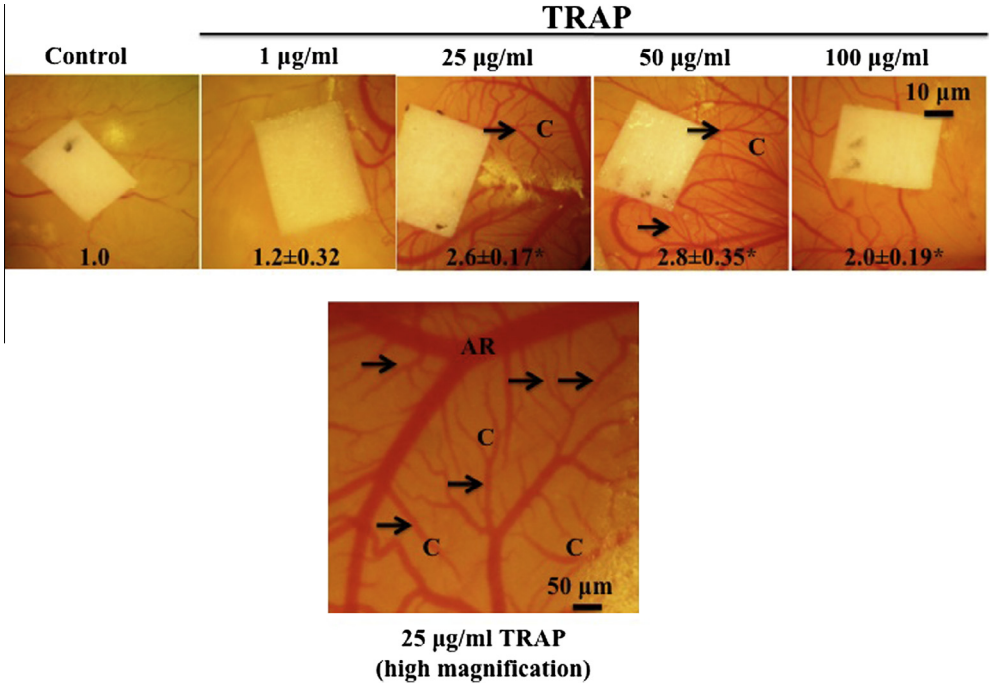

Fig. 7. Effects of increasing concentrations of TRAP on angiogenesis ex vivo. BV development after 3 days of treatment of chick embryo CAM ex vivo with TRAP (1–100 μg ml^{-1}). Embryos treated with bovine serum albumin were used as a control. The black arrows show vascular bundle-like "allantoic capillaries" (C). Magnification ×1.5. The numbers show the relative capillary density (number of total capillaries in a micrograph) in the presence of TRAP compared with the control CAM. The values are the mean ± SE of measurements of six separate experiments. *Significant difference compared with the control (bovine serum albumin), defined as 1.0 ($p < 0.05$). The higher magnification (magnification ×2.5) image of the CAM treated with 25 μg ml^{-1} shows an artery (AR) from which allantoic capillaries have sprouted. Note the apparently increased number of capillaries when the CAM was treated with 50 μg ml^{-1}TRAP.

100 μg ml^{-1} did not further increase the overall number of capillaries (2.0-fold increase compared with the control CAM; $p < 0.05$), and the tissue near the filter disk did not exhibit the extensive sprouted capillaries observed in the presence of 25 and 50 μg ml^{-1} TRAP, as noted above. These results thus indicate that 25–50 μg ml^{-1} TRAP is the optimal concentration for the strong stimulation of BV formation in this ex vivo model.

4. Discussion

The process of neovascularization, fundamental in both development and the repair/regeneration of adult tissue [1,10,16], involves vasculogenic differentiation of stem cells into endothelial precursors [1,10,16] and angiogenic formation of an organized network of BV-like tubular structures by endothelial precursors [1,12,10]. It has previously been shown that HPC cells cultured in medium that facilitates vasculogenesis are able to form endothelial-like cells [3,4], and the results of the present study suggest that EMD and a low-molecular-weight fraction of EMD (Fraction C) strongly stimulated the HPC vasculogenic differentiation of the HPC cells, in contrast to the high-molecular-weight EMD Fraction A, which suppressed this process. Moreover, the present study also showed for the first time that the main component of Fraction C, a TRAP derived by N-terminal proteolytic clipping of the full-length amelogenin appeared to enhance HPC cell vasculogenesis, based on up-regulation of expression of the endothelial markers VEGFR2, Tie-1, Tie-2, VE-cadherin and vWF by the HPC cells. The apparent marked stimulation of vasculogenic differentiation of HPC cells by the TRAP peptide was found to be comparable to the effects of known GF (VEGF and EGF) on vasculogenic differentiation of human umbilical cord blood-derived mesenchymal stem cells and the murine embryonic mesenchymal cell line C3H/10T/1/2, in which the expression of VE-cadherin, vWF, Tie-1 and Tie-2 is also up-regulated [36,37].

The present study has also shown that HPC cells cultured in vasculogenic conditions in the presence of TRAP were able to internalize markedly higher levels of acetylated LDL, indicating that TRAP induced the expression of "scavenger" receptors, a key functional characteristic of EC. Notably, the proportions of HPC cells that were able to internalize acetylated LDL after incubation with EBM-2 alone and with EBM-2 + TRAP were found to be significantly higher than the HPC cell subpopulation that expressed VE-cadherin and vWF after incubation in these same two media. The reason for this is not yet known, thus further investigations are required to get clarification on this discrepancy.

Under selective vasculogenesis-inducing conditions, the present study showed that TRAP stimulated HPC cell expression of the early endothelial marker gene VEGFR2 (a tyrosine kinase receptor for the VEGF ligand), the late genes Tie-1 and Tie-2 (tyrosine kinase receptors for angiopoietin that is expressed exclusively by EC [58]) and the late gene VE-cadherin (an endothelial cell adhesion molecule). Notably, the early endothelial gene VEGFR1 was found to be unaffected by the TRAP peptide, similar to that previously reported in HPC cells when cultured under non-selective growth conditions in the presence of EMD [59]. Although the reason for the lack of VEGFR1 transcript up-regulation by TRAP is not yet understood, this receptor has previously been identified as a "src" oncogene involved in abnormal cell proliferation and development of a neoplastic phenotype [60,61], knockdown of this gene resulting in loss of unlimited population doubling and ability to form tumors [62]. Thus, while these data indicate that the VEGFR1 receptor may be of functional importance in neoplastic cells [62], it is unlikely to be involved in the normal cellular physiology of the non-transformed HPC used here.

Previous studies have suggested that both non-fractionated (non-heat-treated) EMP and heat-treated EMD stimulated the chemotactic migration of HUVEC and HMVEC in vitro [18,19,22,29], a process which, together with the formation of tubular-like structures, is a fundamental feature of angiogenic differentiation

[34,63]. In contrast, other studies have reported the lack of effect of EMD on HUVEC and HMVEC chemotaxis [18,22], the discrepancies arising possibly because of the use of different preparations of EMP and EMD containing different proportions of the constituent proteins, including the TRAP peptide, and also the presence of GF (e.g., TGF β and PDGF) in the non-heat-treated preparation [19,29]. In the present study, chemically synthesized TRAP was shown to markedly stimulate the chemotactic migration of both HPC and HUVEC, suggesting that the presence of this peptide in EMD is likely to have been at least partly responsible for the pro-chemotactic activity of the cells. Moreover, the present results also provided strong evidence that the TRAP peptide promoted the formation of branched structures characteristic of angiogenic differentiation. Although a recent study using a non-heat-treated preparation of EMP reported that neither a high- (>15 kDa) nor low-molecular-weight (<6 kDa) fraction of EMP was able to increase the formation of angiogenic-associated tubular structures by HMVEC [19], both of these fractions were found to be cross-contaminated with both high- and low-molecular-weight components [19]. In contrast, the data presented here using the chemically prepared TRAP show the ability of this peptide to induce HPC to form significantly greater numbers of complex polygonal tubule-like structures than found in control cultures (in the absence of TRAP), indicating that this synthetic peptide is able to stimulate angiogenesis in vitro.

The pro-angiogenic activity of TRAP was further established using an ex vivo chick embryo CAM model, which demonstrated that the presence of this peptide increased the number of allantoic vessels compared with control eggs treated with bovine serum albumin. A previous study using a murine angiogenesis model also showed that a low-molecular-weight fraction of heat-treated EMD (<6 kDa), presumably containing the TRAP peptide, exhibited strong angiogenic activity in vivo [30]. However, in this study the high-molecular-weight fraction (<15 kDa) was found to have similar activity in vivo, although the specific active component(s) of these fractions were not identified and both the fractions were found to contain components of differing molecular sizes (as noted above) that may have had differential effects on angiogenesis, unlike the synthetic TRAP peptide used here.

The identification of a small peptide with potent neovasculogenic activity, as described here, may be an important additional tool in the repertoire of therapies for periodontal wound healing. Thus this peptide, and possibly smaller synthetic sequences derived therefrom, might constitute a consistent, reproducible and far less costly means to treat a common and widespread disorder with a far more predictable clinical outcome. Further, our findings highlight the future potential of a possibly new class of therapeutic drug for promoting early stages in the repair and regeneration of many types of tissue that are dependent on the formation of new BV, possibly even including TRAP-mediated neovasculogenesis in patients suffering from atherosclerosis.

5. Conclusions

The results of this study demonstrate that the low-molecular-weight Fraction C obtained from EMD promotes vasculogenic differentiation in vitro. Further, the data presented here have also shown, for the first time, that the amelogenin-derived TRAP peptide component of Fraction C is capable of stimulating vasculogenic and angiogenic differentiation in vitro and ex vivo. This type of peptide, and chemically modified compounds derived therefrom, might thereby represent a novel class of drug able to elicit new blood vessel formation and promote wound healing in vivo.

6. Declaration of interest

(a) This work was supported by Institut Straumann (Basel, Switzerland) and Engineering & Physical Science Research Council, UK (EPSRC).
(b) The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.
(c) Institut Straumann is the manufacturer and sole proprietor of the commercial product EMD used here.
(d) The authors alone are responsible for the content and writing of the paper.
(e) No additional external funding was received for this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2013.11.027.

Appendix B. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 1–4 and 7, are difficult to interpret in black and white. The full color images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2013.11.027.

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