Allogenic adipose-derived stem cell therapy overcomes ischemia-induced microvessel rarefaction in the myocardium: systems biology study

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Background

- Increased rate of survival in patients suffering from acute myocardial infarction
- Development of heart failure at a later time point due to reduced perfusion, microvessel rarefaction, irreversible cardiomyocyte loss and consequent adverse cardiac remodeling
- Implantation of adult stem cells into the ischemic damaged myocardium to repair/regenerate the injured cells within the infarct zone → A solution?
- Administration of autologous stem cells exerts limited benefits in ischemic heart disease patients (Cardiovascular factors and metabolic disorders influence the effects of adult/progenitor stem cells negatively).

- Allogenic adipose-derived stem cells from obese patients display an impaired angiogenic potential and clustering of risk factors reduce ASC and bone-marrow-derived cell pluripotency and self-renewal (Healthy donors?)
- Mesenchymal stem cells (MSCs)
  - immunomodulatory and immunosuppressive properties
  - immunoprivileged due to lack of expression of class II major histocompatibility complex and costimulatory molecules on the cell surface. → No donor-specific alloimmune reactions after allogeneic administration!
- Among all MSCs those derived from the adipose tissue (ASCs) hold great potential for allogenic use because
  - of their inherent and proven low immune reactivity,
  - they are highly abundant,
  - they are easily accessible and expandable in culture.

- Promising results across a wide range of therapeutic applications including bone-cartilage defect, inflammatory-based disease and IHD.
- IHD → cardiac beneficial effects likely through paracrine/autocrine-related mechanisms.
- Most of the benefits associated with the administration of ASCs are largely mediated by the actions of cytokines and growth factors secreted by the ASCs rather than by ASC transdifferentiation and engraftment.
- <u>In this study:</u>

  The authors sought to determine in a pig model of MI using clinical standard perioperative procedures (antiplatelet treatment) and operating blindly whether coadministration of ASCs and conditioned media (CM) could overcome myocardial rarefaction and whether the effects were due to ASCs or their secretome.

Methods

Ethical approval

- Institutional Animal Care and Use Committees
- Animal Experimental Committee of the local government in accordance with Spanish law

Experimantal design

- Pigs (n = 20) subjected to MI induction
- One week post MI 4 groups:
  - ASCs (1 × 10^7 cells);
  - CM (30 ml);
  - ASCs and their CM;
  - Phosphat-buffered saline (PBS) (control; 30 ml).
- ASC coronary delivery was performed with an over-the-wire catheter and total balloon occlusion for 2 min.
- CM was administered intravenously.
- Cell preparations were administered blindly by the surgical operators.

ASC isolation, characterization and preparation for infusion

- Subcutaneous adipose tissue was harvested from the neck of healthy pigs and processed for ASC isolation and expansion under hypoxic conditions.
- The day before infusion, the expanded ASCs were washed and serum-free medium was added.
- After 24 h, the secretome of ASCs released to the medium (CM) was collected, centrifuged and filtered. Then 30 ml of sample was kept at 4 ˚C until infusion.
- ASCs (1 × 10^7 cells) were resuspended in 2 ml PBS and kept at 4 ˚C until intracoronary administration.
- Previously, ASCs had been characterized by cell surface marker expression by flow cytometry and differentiation potential to mesenchymal cell lineages.
- Aliquots of ASCs and CM were kept for proteomics.

In-vitro ASC function: proof-of-principle characterization

a) 3D cultures: ASC endothelial cell differentiation was evaluated by capillary network formation with HMEC-1 (microvascular endothelial cells) on coculture in 3D matrigel plugs.

b) Chorioallantoic membrane assay (CAM) immunofluorescence:

- Fertilized chicken eggs were incubated for 3 days.
- A small opening was then made in the shell, exposing the CAM. The window was covered with cellophane tape and the eggs were returned to the incubator.
- Six days later, growth factor-reduced matrigel droplets (containing 10^5 ASCs, 20 µl concentrated CM (×10) or 10^5 ASCs + 20 µl concentrated CM) were applied onto the CAM.
- Matrigel droplets containing 20 µl PBS served as negative controls.
- Following 2 days of incubation the eggs were opened and photographed, and the CAM was carefully dissected and processed for histological analysis against von Willebrand factor (vWF).

c) Microvesicle release: ASC-GFP+ microvesicles release into the CM were analyzed by flow cytometry

Experimental model of MI

- MI was induced by 90-min total balloon occlusion of the LAD.
- Transthoracic echocardiography before inducing ischemia and upon reperfusion to monitor the impact of MI induction on LVEF.

GFP-lentiviral transduction of ASCs: assessment of ASC cardiac homing

Substudy (n = 4 pigs) to confirm ASC retention and homing within the infarcted region post infusion:

ASCs were transduced with GFP-expressing lentiviral vectors and 1 × 10^7 ASC-GFP+ cells were intracoronary infused into MI-induced pigs. Animals were sacrificed 24 h later and samples from multiple cardiac regions (left and right ventricle, ischemic and remote myocardium, atrium) and vascular beds (coronary arteries and different aortic regions) were rapidly obtained for GFP detection.

Cardiac magnetic resonance imaging

- 1 week post MI just after ASC, CM, ASC + CM or PBS infusion (baseline),
- 1 week post infusion (2 weeks post MI),
- 3 weeks post infusion (4 weeks post MI).

CMR sequences: “cine” (b-SSFP) imaging sequence to assess wall motion and cardiac function; and late gadolinium enhancement (LGE) to assess the amount and extent of myocardial necrosis.

CMR images were analyzed using dedicated software by a CMR-trained cardiologist blinded to the study medication.

Assessment of myocardial vascular density and angiogenesis

- Lectin staining
- Analysis of angiogenic markers including gene levels of CD105, vWF, endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor receptor type 2 (VEGFR2), VEGF1, CD31, CD62 and tissue factor (TF) as well as protein activation and/or expression of eNOS phosphorylated in Thr495, eNOS, CD105 and vWF.

Myocardial fibrosis

- analysis of transforming growth factor beta receptor (TGFβR), TGFβ, and collagen type I and type III
- histological Masson's trichromic staining

Proteomic analysis

- performed on ASCs and their secretome (CM).
- protein extracts were separated by bidimensional gel electrophoresis (2-DE) and protein spots of interest were identified by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF/TOF).

In-silico bioinformatics analysis

The statistically significant functional networks in which the identified proteins were involved were generated through the use of ingenuity pathway analysis.The functional analysis of a network identified the biological function and/or disease that were most significant to the molecules in the network. The network molecules associated with biological functions and/or diseases in the Ingenuity Knowledge Base were considered for the analysis.

Statistical analysis

- **Shapiro–Wilk test** to verify the normal distribution of the data and statistical analysis.
- Within the porcine studies, data were analyzed by a nonparametric statistical analysis and results are reported as medians and interquartile range (IQR).
- For independent factors (comparisons between groups) **Mann–Whitney analysis.**
- For repeated measurements, **Wilcoxon and Friedman analyses.**
- For the chicken egg analysis a one-way **ANOVA followed by Bonferroni's multiple comparison test.**
- For in-silico bioinformatics analysis, a right-tailed **Fisher's exact test** to calculate a p value determining the probability that each biological function and/or disease assigned to that network is due to chance alone.
- All statistical tests conducted were two-sided and $p < 0.05$ was considered significant. Statistical analyses were performed with Statview.

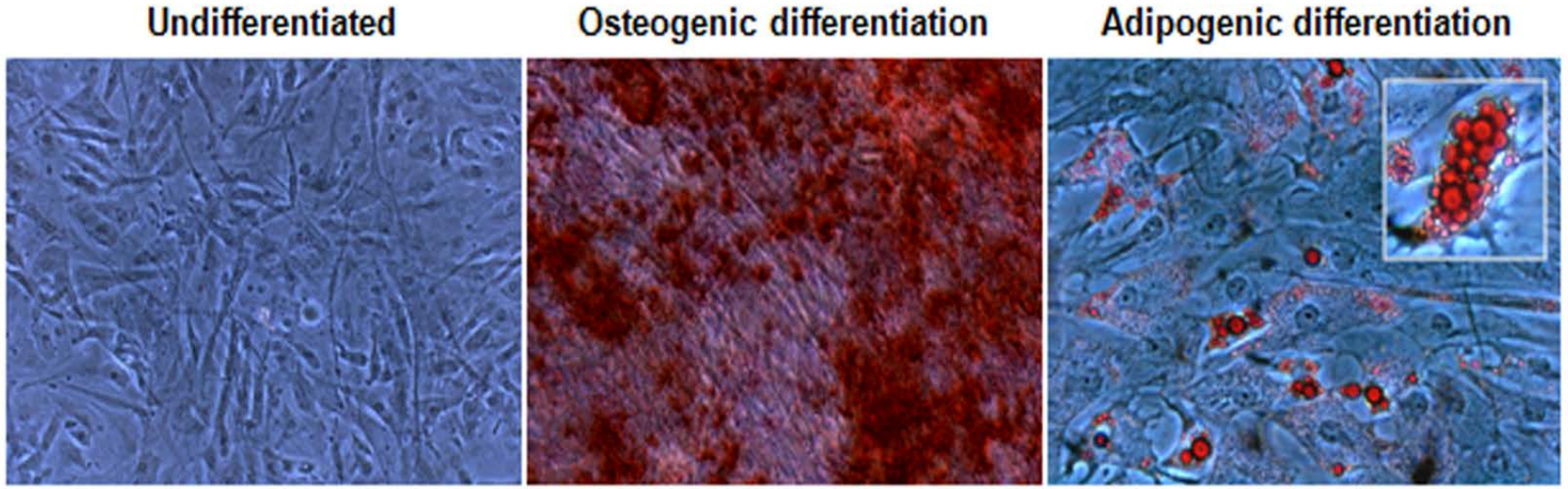
Results

ASC characterization

Flow cytometry: Cultured ASCs were positive for surface markers characteristic of ASCs including CD105, CD29 and CD90 and negative for CD45, thereby expressing the mesenchymal cell specific markers reported.

ASC differentiation potential to mesenchymal cell lineages was assessed. After 21 days of induction with specific differentiation medium, staining for lipids and calcium deposition confirmed the differentiation of ASCs towards adipogenic and osteogenic lineages, respectively.

Additional File 2. Adipogenic and osteogenic differentiation of ASCs. ASC-related differentiation towards mesodermal lineages was determined by specific staining; Alizarin red staining showed extracellular calcium deposition after osteogenic differentiation wherease adipogenic differentiation was evidenced by lipid droplets stained by Oil red.



A 3D coculture system of ASCs with endothelial cells was performed to further demonstrate the capacity of ASCs to migrate, contact and localize around the endothelial cells, providing support and stability to the developed capillary-like structures (Fig. 1a, b).

Analysis of CM evidenced the presence of ASC-released microvesicles, of which 30% were positive for both Annexin V and GFP (Fig. 1c).

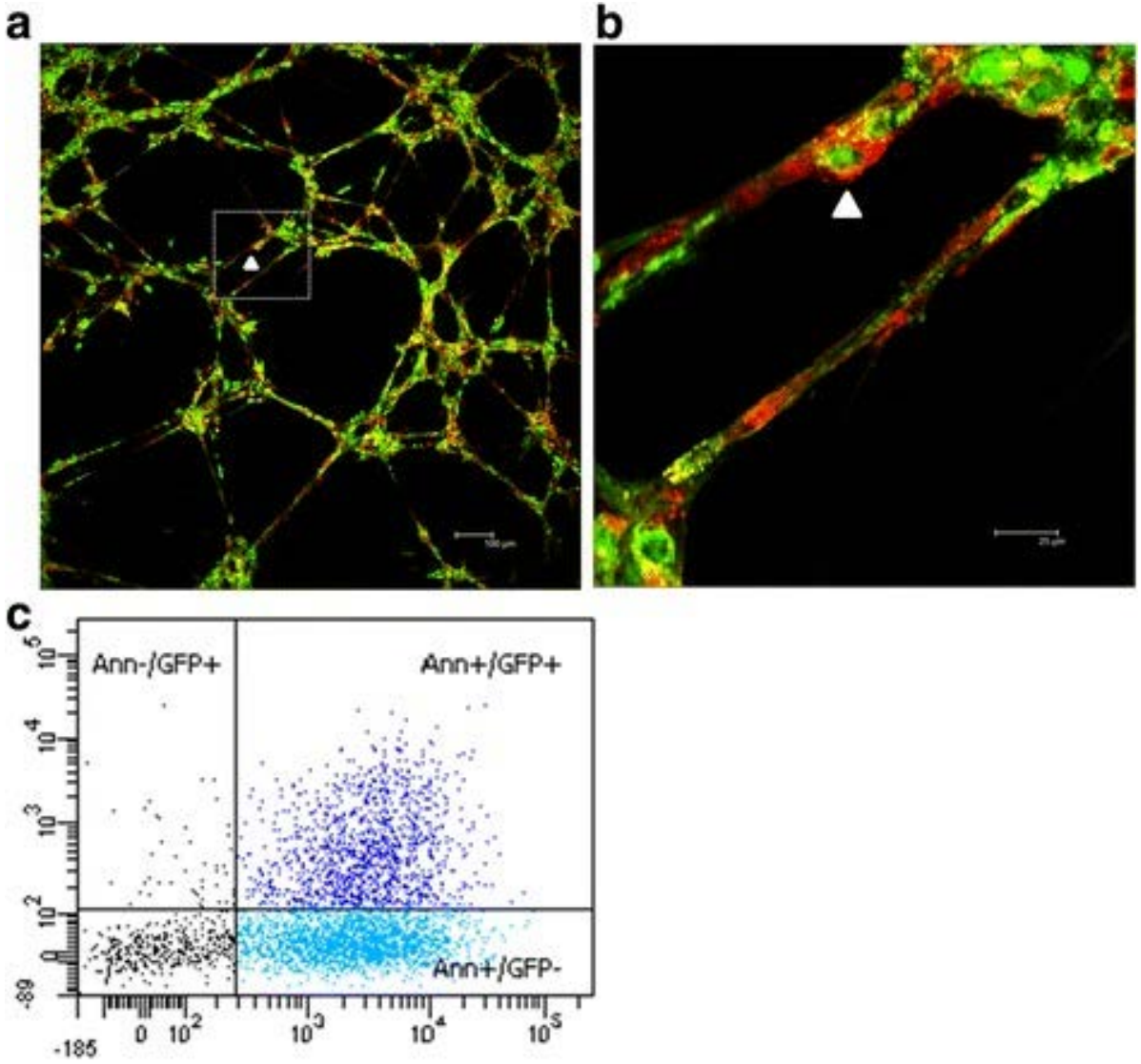


***Fig. 1 a**. ASCs (green) and endothelial cells (red) in a 3D basement membrane coculture system for 24 h. **b.** Magnification to visualize ASC incorporation (white arrowhead) within the capillary network. **c.** Flow cytometry analysis of microvesicles contained in the CM. Ann Annexin V, GFP green fluorescent protein*

Chick CAM model of angiogenesis

ASC + CM seeding in the scaffolds induced a 2-fold and 4-fold increase in angiogenesis in the border area between the CAM and the scaffolds as compared with the ASCs or CM alone as detected by vWF staining.

ASCs alone also induced an angiogenic response that reached significance versus control ($p < 0.05$) but was not as great as combined treatment.

CM seeding did not induce CAM angiogenesis.

ASC homing

GFP was detected by RT-PCR in the LAD and in the layers of the ischemic damaged myocardium (from endocardium to epicardium) at 24 h post delivery of ASCs.

In contrast, it was undetectable in the remote myocardium, circumflex and right coronary artery, atria, right ventricle and the aorta. Confocal microscopy analysis revealed the presence of GFP+ cells in the LAD and in the ischemic cardiac tissue. GFP+ cells were not detected in the nonischemic myocardium (image not shown).

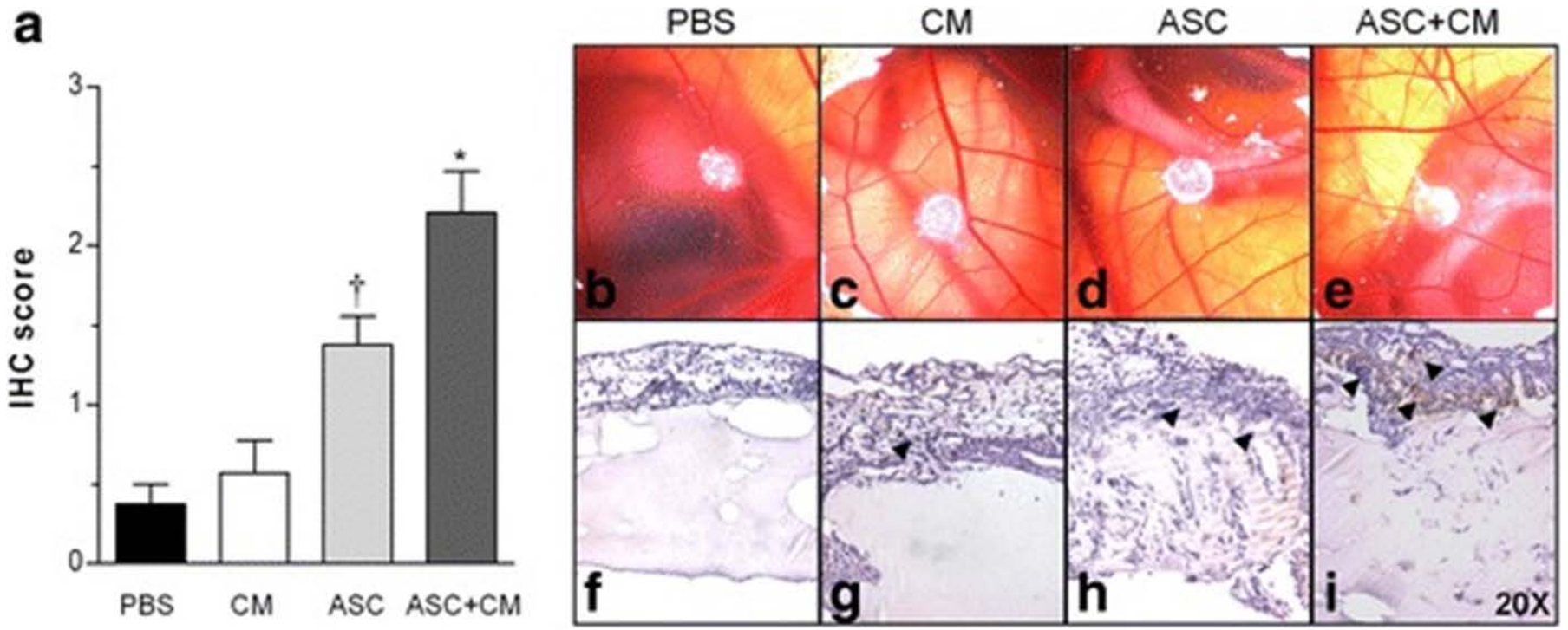


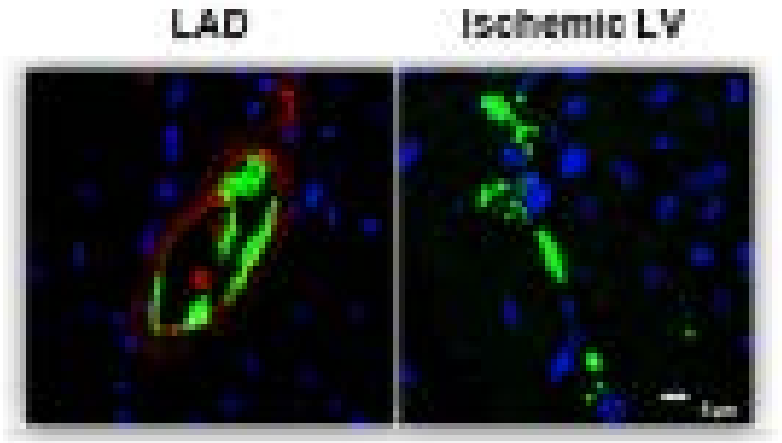
Fig. 2 a *Immunohistochemical (IHC) score for vWF staining. Each sample was analyzed double blind and independently by two investigators. Staining (brown) was semiquantitatively scored as 0, 1, 2 or 3 for absence, weak, moderate and strong staining, respectively.* ***b–e*** *Representative macroscopic pictures of 48-h incubated matrigel scaffolds in the in-vivo CAM assay.* ***f–i*** *Representative immunochemical staining of vWF in sections of matrigel–CAM junction. *p < 0.05 vs PBS, CM and ASCs; †p < 0.05 vs PBS. ASC adipose-derived mesenchymal stem cell, CM conditioned media, PBS phosphate-buffered saline.*

Additional File 3. Analysis of ASC homing. (A) GFP+ detection in different regions of the heart and aorta by real time-PCR analysis. We set cycle threshold (CT) of 28 as the cutt of value. **(B)** ASC-GFP+ cells by confocal analysis in the left anterior descending coronary artery and ischemic left ventricle (red: smooth muscle actin; green :GFP; blue: nuclei). Identification was carried out 24hours after intracoronary infusion of ASC-GFP+ cells to the infarcted myocardium. LAD: left anterior decending coronary artery; LV: left ventricle.

A.

		CTs <28 (detectable)	CTs >28 (undetectable)
Left anterior descending coronary artery (LAD)	Occluded portion	+	
	Non-occluded portion	+	
Ischemic Left Ventricle (LV)	Endocardium	+	
	Epicardium	+	
	Septum	+	
Non-ischemic LV			+
Right ventricle			+
Circumflex coronary artery			+
Right coronary artery			+
Atriums	Left atrium		+
	Righ atrium		+
Aorta	Aortic arch		+
	Abdominal aorta		+
	Thoracic aorta		+

B.



Cardiac function and damage

Echocardiography revealed that all animals showed a comparable impairment in the left ventricle ejection fraction (LVEF; average 29 (27–30)% decrease) after 90 min of complete coronary ischemia ($p < 0.05$ vs prior MI). Similarly, no variations were detected in hemodynamic parameters (heart rate and mean blood pressure) during MI induction among all groups (average: 76 (74–76) bpm and 56 (54–56) mmHg, respectively).

CMR analysis at 1 week post MI (baseline) showed that all groups displayed a comparable deterioration in LVEF and size of infarction. At this time point allogenic cell preparations were administered by coronary infusion of placebo solution or ASCs and intravenous injection of placebo or CM (Table 1).

Table 1

MRI analysis of cardiac performance and scar size

		Baseline (1 week post MI)	1 week post infusion (2 weeks post MI)	3 weeks post infusion (4 weeks post MI)
LVEF (%)	PBS	48.4 (48.3–50.1)	48.9 (46.6–50.7)	46.5 (46.3–48.1)*
	ASCs	47.7 (47.6–54.2)	48.3 (47.5–54.1)	48.7 (47.1–49.7)
	CM	47.2 (46.5–47.5)	48.2 (48.0–49.4)	49.5 (48.2–52.1)
	ASCs + CM	50.0 (46.4–53.0)	49.5 (48.7–51.0)	47.6 (47.3–49.6)
LVEDV (ml)	PBS	91.9 (85.3–94.1)	107.5 (101.0–109.1)*	118.7 (115.0–125.7)*†
	ASCs	113.0 (105.0–116.0)	123.2 (101.3–124.3)	121.0 (117.3–129.3)
	CM	87.7(81.9–122.8)	93.4 (89.0–94.1)*	119.2 (106.1–122.3)
	ASCs + CM	91.4 (89.0–98.4)	93.7 (84.0–124.4)	136.32 (82.1–153.0)
LVESV (ml)	PBS	44.0 (40.0–46.2)	55.2 (46.1–58.3)	63.4 (45.0–65.2)†
	ASCs	55.0 (49.9–60.7)	64.4 (51.4–64.9)	62.3 (60.6–66.0)
	CM	42.9 (40.9–64.8)	47.2 (46.0–49.4)	60.2 (47.5–61.5)
	ASCs + CM	46.1 (40.4–50.3)	48.0 (42.0–49.5)	64.8 (43.3–77.0)
Scar size (g)	PBS	11.6 (10.5–12.4)	10.8 (10.1–13.2)	10.4 (9.8–11.7)
	ASCs	9.9 (9.0–16.1)	9.5 (9.4–13.9)	10.8 (9.6–14.4)
	CM	11.3 (9.7–13.2)	10.1 (9.2–11.0)	9.5 (8.1–9.9)
	ASCs + CM	13.0 (10.0–15.0)	12.3 (10.2–14.0)	10.8 (7.2–11.5)

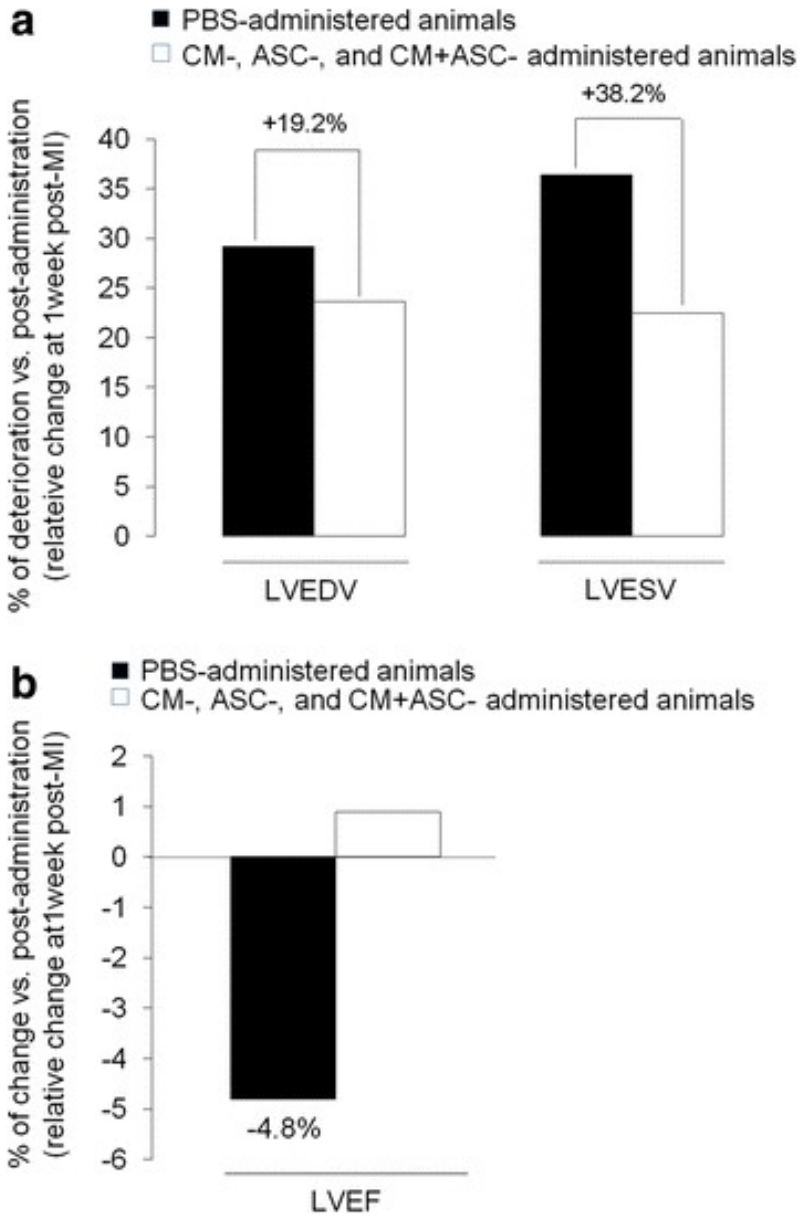
ASC adipose-derived stem cell, *CM* ASC conditioned media. *LVEF* left ventricular ejection fraction, *LVEDV* left ventricular end-diastolic volume, *LVESV* left ventricular end-systolic volume, *MI* myocardial infarction, *MRI* magnetic resonance imaging, *PBS* phosphate-buffered saline

*$p < 0.05$ vs baseline

†$p < 0.05$ vs time

Follow-up CMR analysis showed a deterioration in cardiac contractility in control animals 3 weeks post infusion as compared with baseline, with a marked impairment in left ventricular end-diastolic volume (LVEDV; 38.2% worse), left ventricular end-systolic volume (LVESV; 19.2% worse) and LVEF (4.8% worse) vs the other groups ($p < 0.05$; Fig. 3a, b).

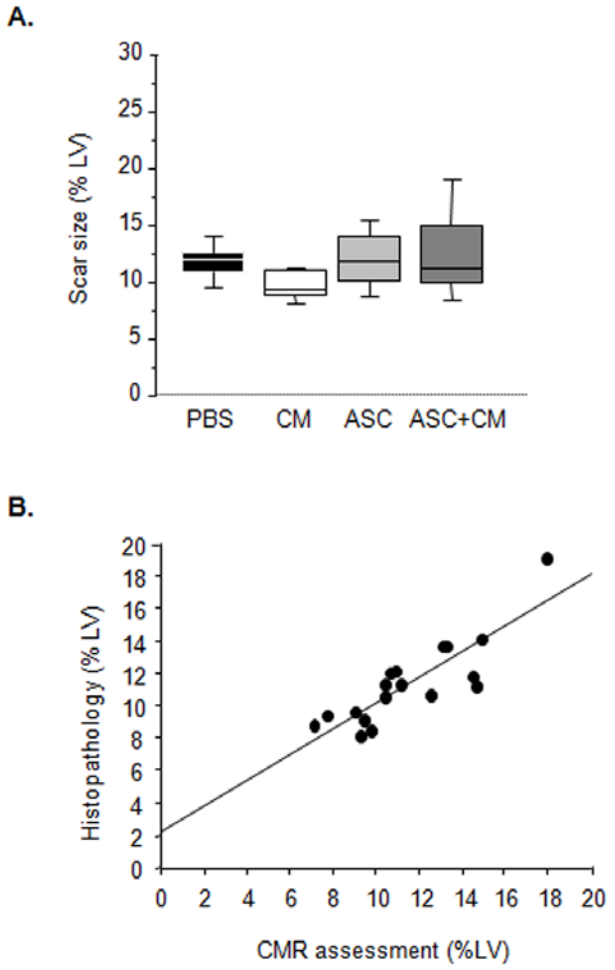
Such cardiac deterioration was not detected in those animals administered CM, ASCs or ASCs + CM in which cardiac performance remained unchanged as compared with baseline (Table 1).



No differences were detected in the size of the scar at 3 weeks post infusion among the different animal groups (Additional file 4A).

Histopathological analysis of the scar by triphenyl tetrazolium chloride (TTC) staining showed reparative fibrosis by increase collagen in the scar area which highly correlated with the CMR data ($r = 0.85$; $p < 0.01$; Additional file 4B).

Additional File 4. Correlation between infarct size assessment by histopathology and by CMR in all animals of the study. CM: adipose-derived stem cells (ASC) conditioned media.



Vessel density

Total vessel density measured by histology operators was found enhanced by 40% in the ischemic myocardium of animals that received the combination of ASCs and CM as compared with ASCs or CM alone or placebo-control ($p < 0.05$; Fig. 4). The amount of vessels detected in the infarcted area of ASC + CM animals was comparable with that observed on the remote non-ischemic myocardium.

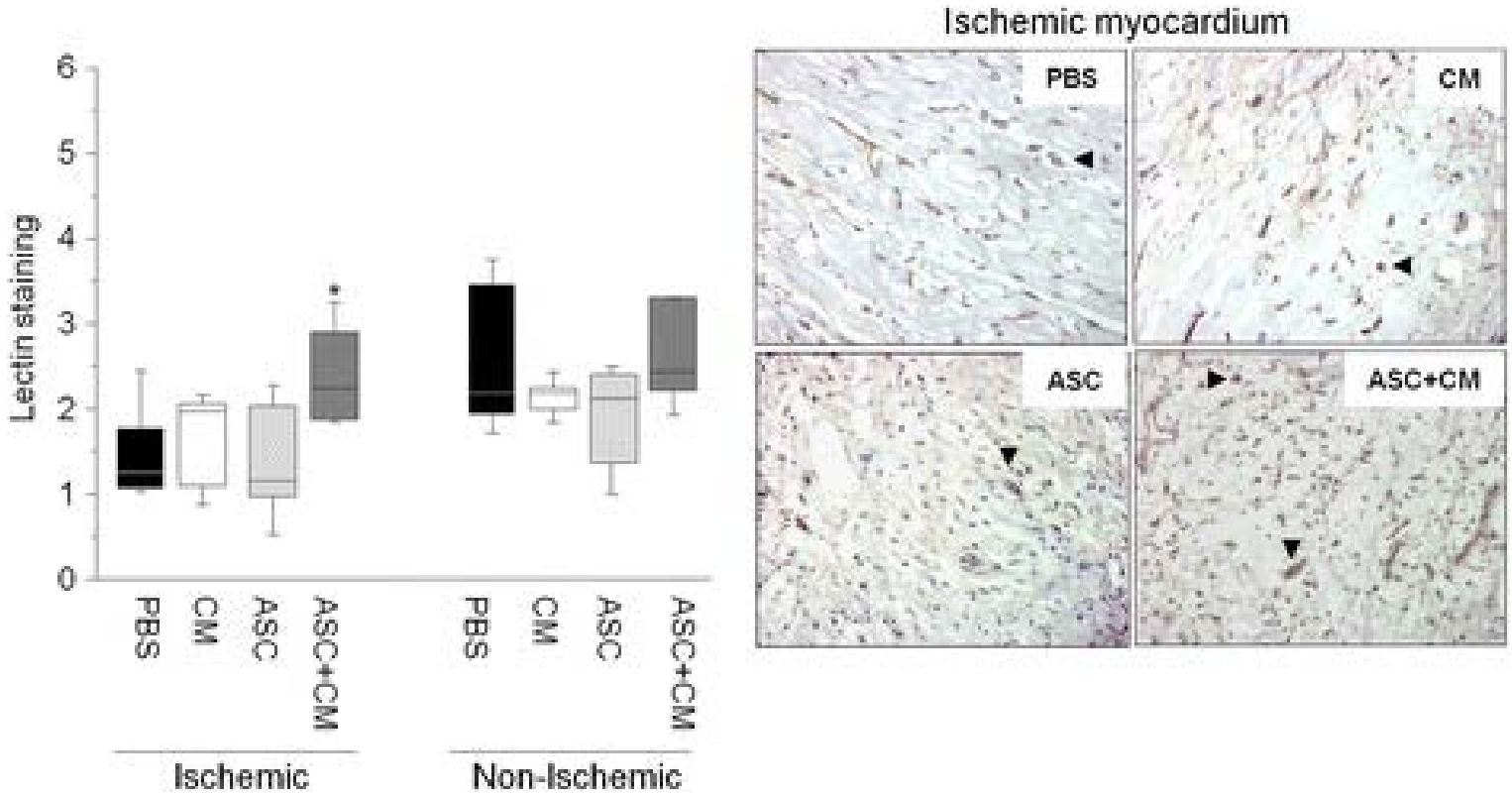


Fig. 4 *Vessel density analysis of the ischemic and non-ischemic myocardium assessed by lectin staining and representative image s of the ischemic cardiac tissue. *p < 0.05 vs ASCs, ASC releasate and PBS within the ischemic cardiac tissue. ASC adipose-derived stem cell, CM ASC conditioned media, PBS phosphate-buffered saline*

Markers of neovessel formation

- Myocardial gene and protein expression of markers involved in new vessel formation
- CD105 (Fig. 5a) and vWF (Fig. 5b) gene expression and protein levels were significantly higher in the ischemic myocardium of those animals administered ASCs + CM as compared with ASCs or CM alone and PBS control.
- Not only eNOS transcription levels but also eNOS activity was markedly enhanced within the ischemic myocardium of animals administered ASCs + CM (Fig. 5c).

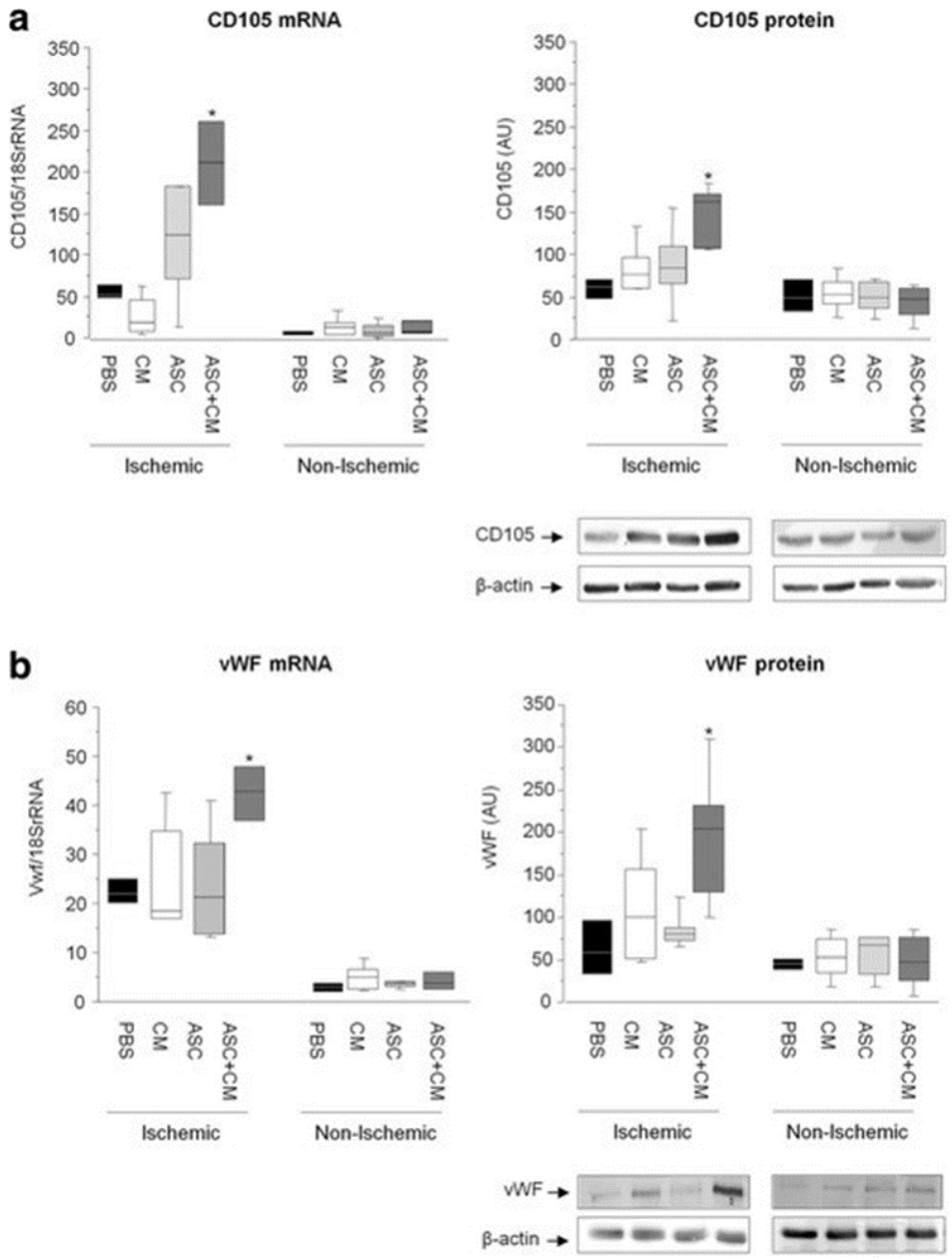
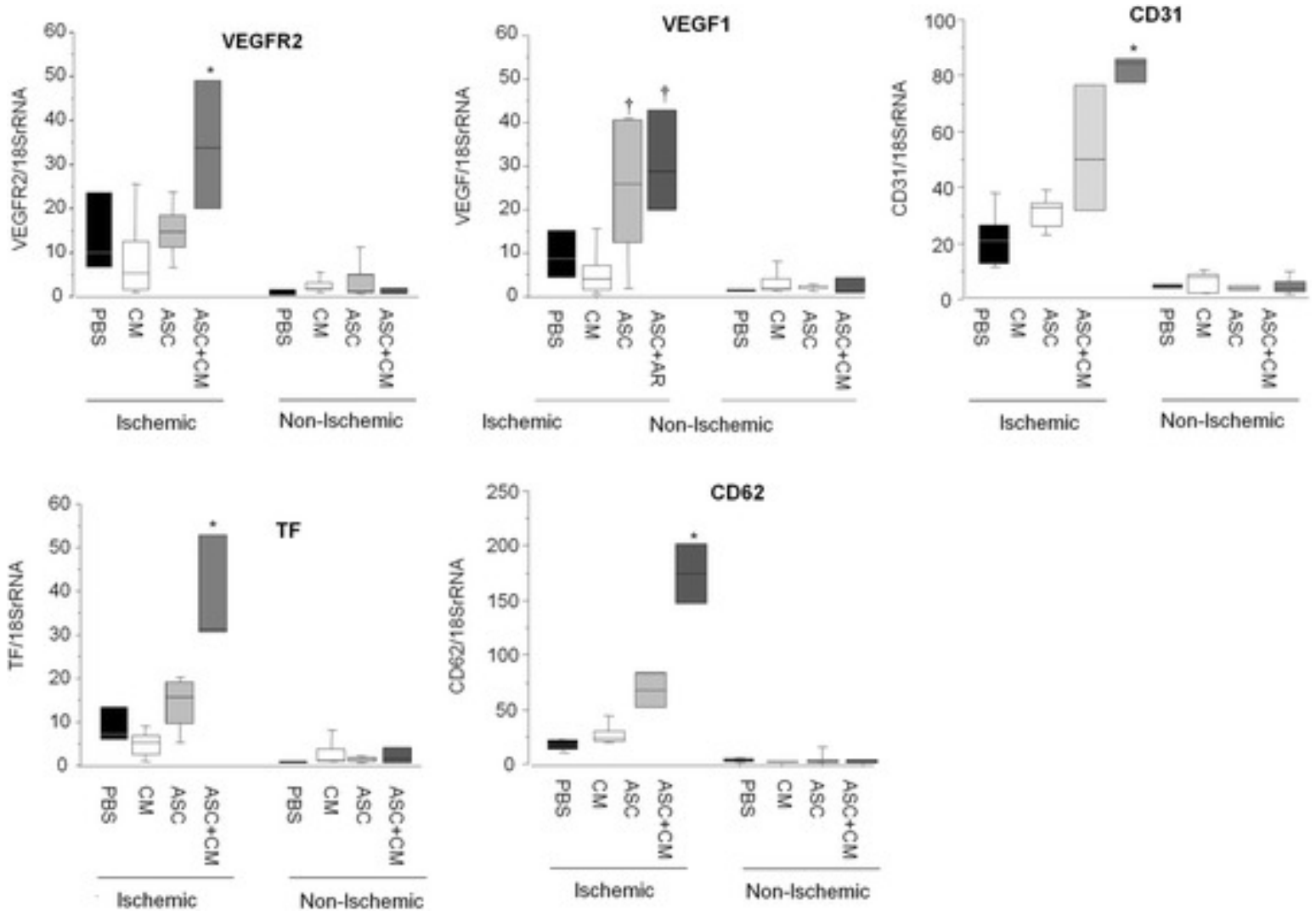


Fig. 5 *Molecular analysis (gene and protein expression) of markers involved in neovessel formation within the cardiac tissue:* ***a*** *CD105,* ***b*** *von Willebrand factor (vWF) and c endothelial nitric oxide synthase (eNOS). *p < 0.05 vs ASC, CM and PBS within the ischemic cardiac tissue; †p < 0.05 vs PBS within the ischemic myocardium. ASC adipose-derived stem cell, CM ASC conditioned media, PBS phosphate-buffered saline*

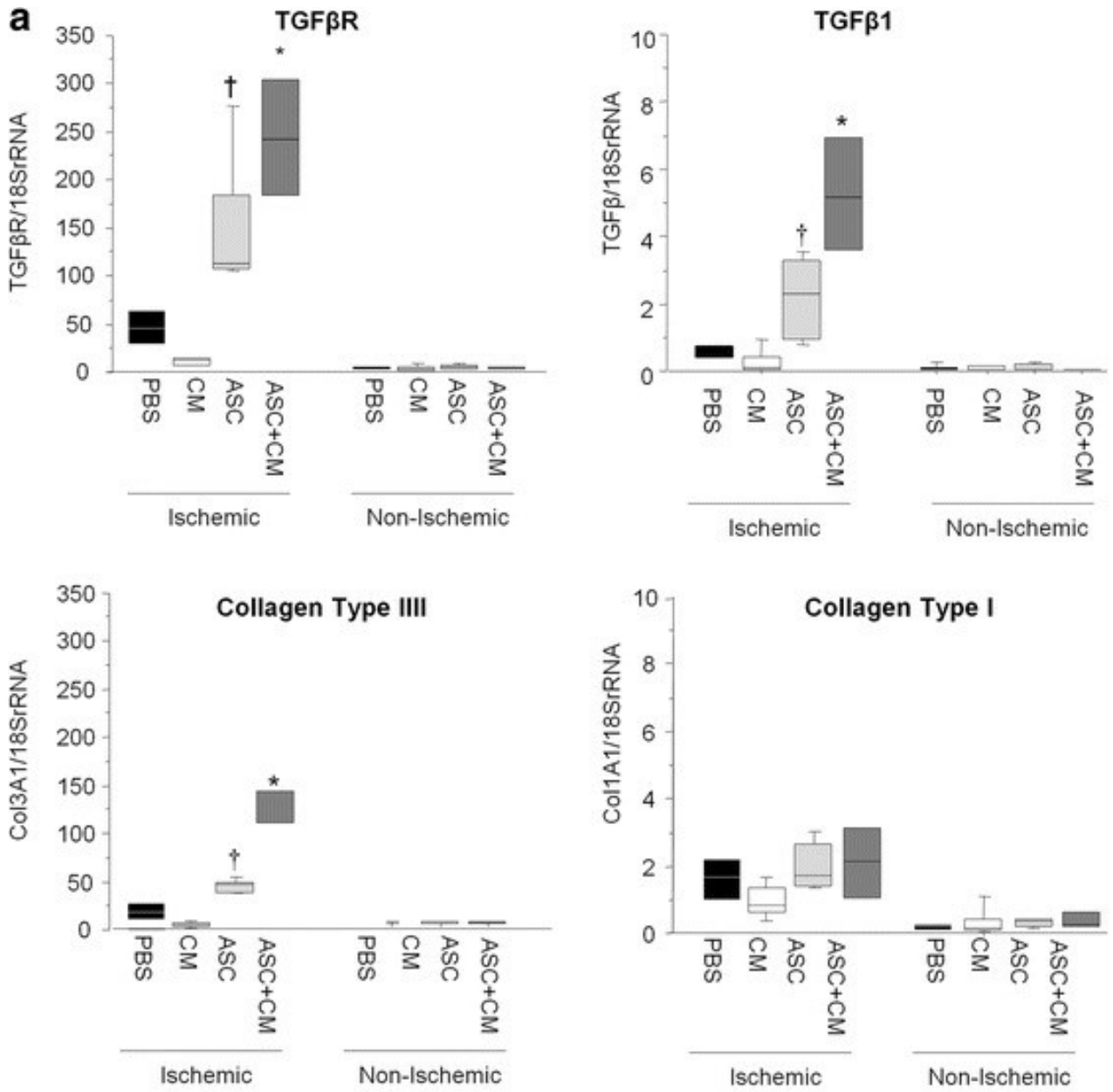
Transcription of VEGFR2, TF, CD62 and CD31 was also found to be significantly increased in those animals receiving ASCs + CM (Fig. 6), further supporting, at a molecular level, a proangiogenic synergistic effect of ASC and ASC releasate (CM) administration. VEGF1 was found to be upregulated by ASCs + CM and ASCs (Fig. 6).

Fig. 6 *Myocardial gene expression of several markers involved in new vessel formation. VEGFR2 vascular endothelial growth factor receptor 2, TF tissue factor, CD62 P-selectin, CD31 platelet. *p < 0.05 vs ASCs, CM and PBS within the ischemic cardiac tissue; †p < 0.05 vs CM and PBS within the ischemic cardiac tissue. ASC adipose-derived stem cell, CM ASC conditioned media, PBS phosphate-buffered saline*



Reparative fibrosis

Gene expression of TGFβR/TGFβ/ collagen type III (Fig. 7a) and collagen deposition (Fig. 7b) was found to be upregulated in the evolving scar of animals administered ASCs as compared with animals administered PBS and CM ($p < 0.05$). This effect was further enhanced in animals coadministered ASCs + CM ($p < 0.05$ vs ASCs alone). Fibrosis was barely detectable in the nonischemic myocardium (Fig. 7a).



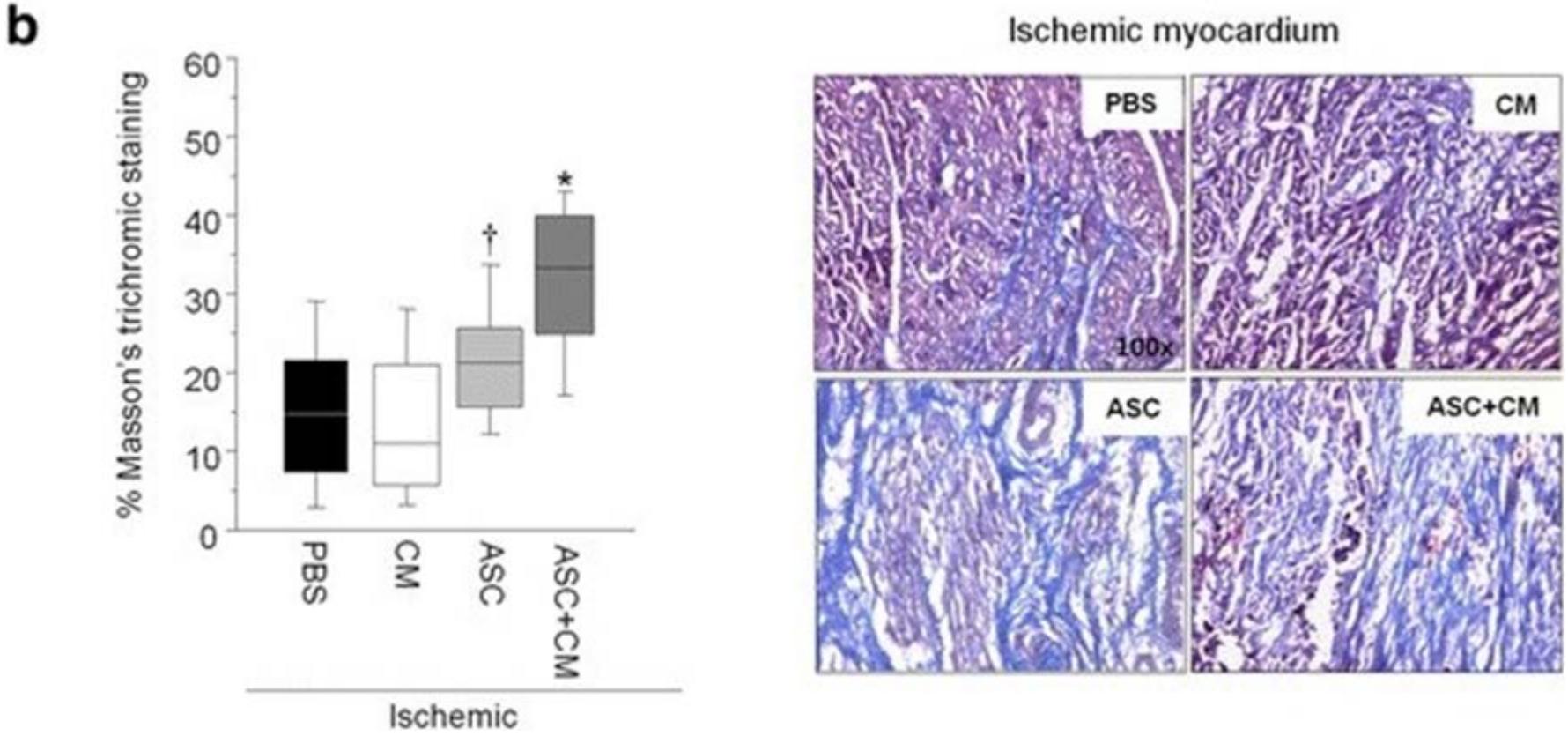


Fig. 7 *Transcript levels of fibrotic markers* ***(a)*** *and collagen deposition in the ischemic myocardium assessed by Masson's trichromic staining* ***(b)****. *p < 0.05 vs all; †p < 0.05 vs CM and PBS within the ischemic cardiac tissue. ASC adipose-derived stem cell, CM ASC conditioned media, PBS phosphate-buffered saline*

ASC-related proteome

- ASC secretome (CM)
- ASC proteome (ASC cytosol and membrane fractions)

a) ASC secretome analysis:

A comparative proteomic approach between CM and cell-free culture medium (negative control).

Fetuin alpha-1 antitrypsin, ApoA-I, serum albumin and serotransferrin were identified in both CM and cell-free culture medium and therefore their presence was attributed to the culture medium (Fig. 8a). As expected, GFP was solely identified in the secretome of ASCs and its detection supported its extracellular release.

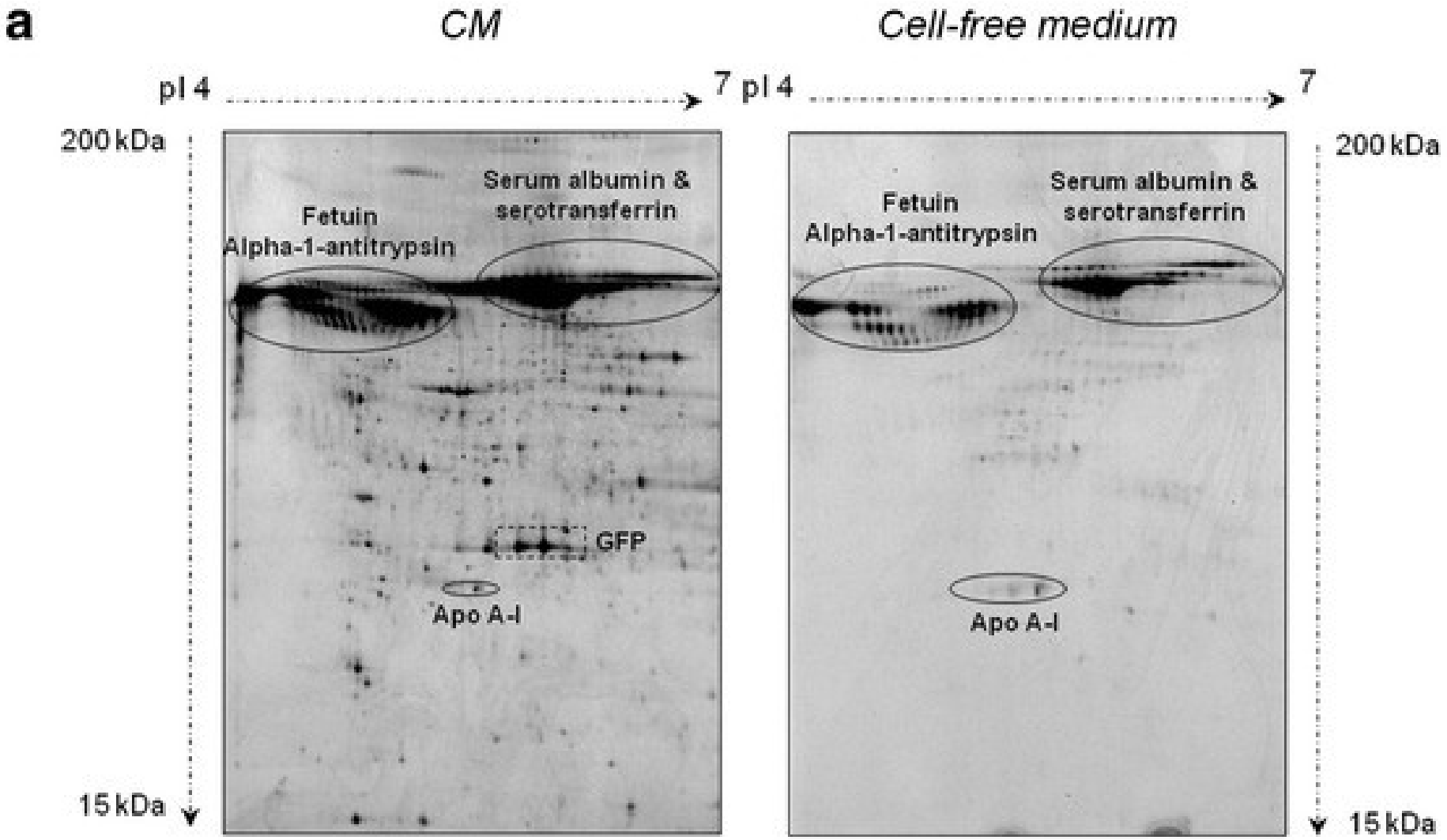
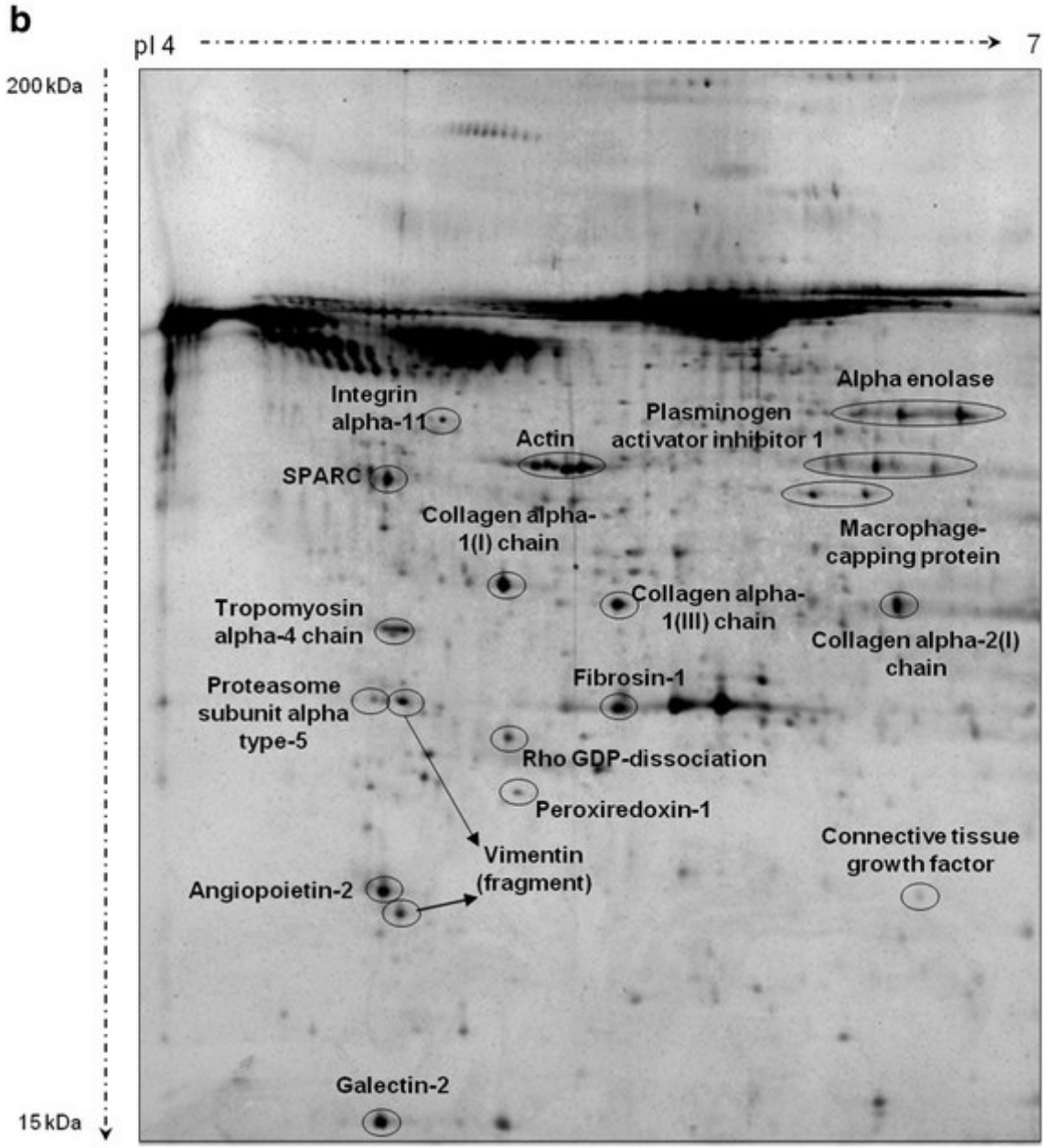
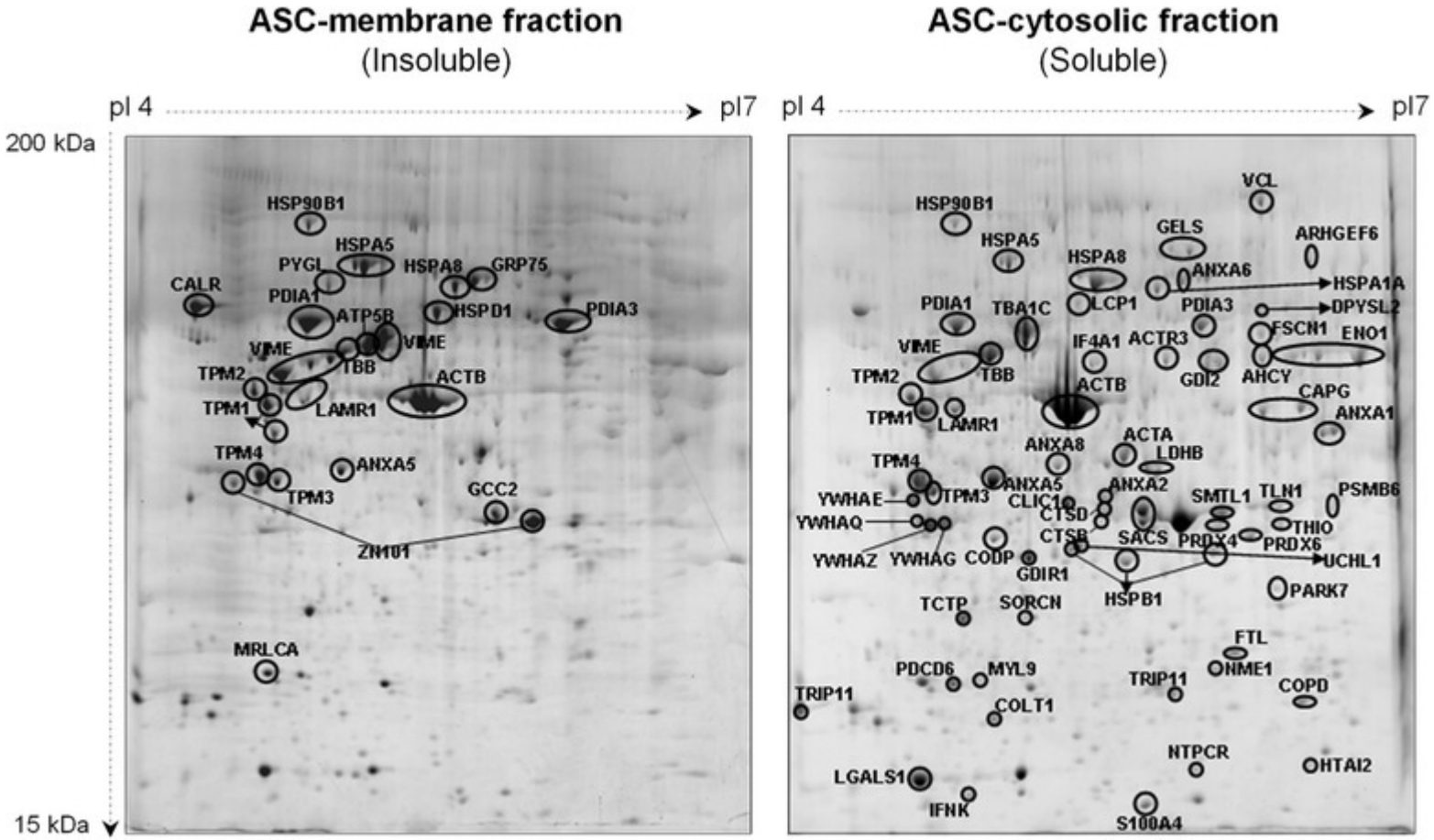

a
CM
Cell-free medium
pI 4
7
pI 4
7
200 kDa
15 kDa
Fetuin
Alpha-1-antitrypsin
Serum albumin &
serotransferrin
GFP
Apo A-I
Fetuin
Alpha-1-antitrypsin
Serum albumin &
serotransferrin
Apo A-I
200 kDa
15 kDa

Figure 8b depicts the proteins released by ASCs into the media during cell culture (ASC secretome). Proteins were identified related to five functional categories with potential paracrine properties within the CM. These functional groups were related to angiogenesis, cell proliferation/ differentiation/ apoptosis regulation, protein processing/ chaperone activity and structural proteins.



b) ASC proteome analysis:

ASC cytosolic and membrane fractions (Fig. 9). Proteins identified in the ASC proteome (Additional file 5) were related to 11 different functional categories: angiogenesis, antioxidant/redox homeostasis, cell proliferation/ differentiation/apoptosis regulation, coagulation/hemostasis, defense response, metabolism, protein processing/chaperone activity, proteolysis, signaling/gene transcription, structural and transport/trafficking proteins.



c) ASC interactome:

Analysis of the functional groups of proteins identified in both fractions (CM and ASCs; Additional file 5) in order to determine the potential interactions (i.e., interactome) behind the synergistic effect observed upon ASC + CM administration.

Table 2 presents the functional groups of the proteins identified in the ASC-related proteome.

Structural proteins are found in both the ASC secretome and the ASC proteome, although with a differential protein contribution of each fraction.

Additionally, the ASC secretome mainly contained proteins related to angiogenesis and cell proliferation/differentiation/ apoptosis regulation whereas the ASC proteome was mostly represented by proteins involved in protein processing regulation and chaperone activity.

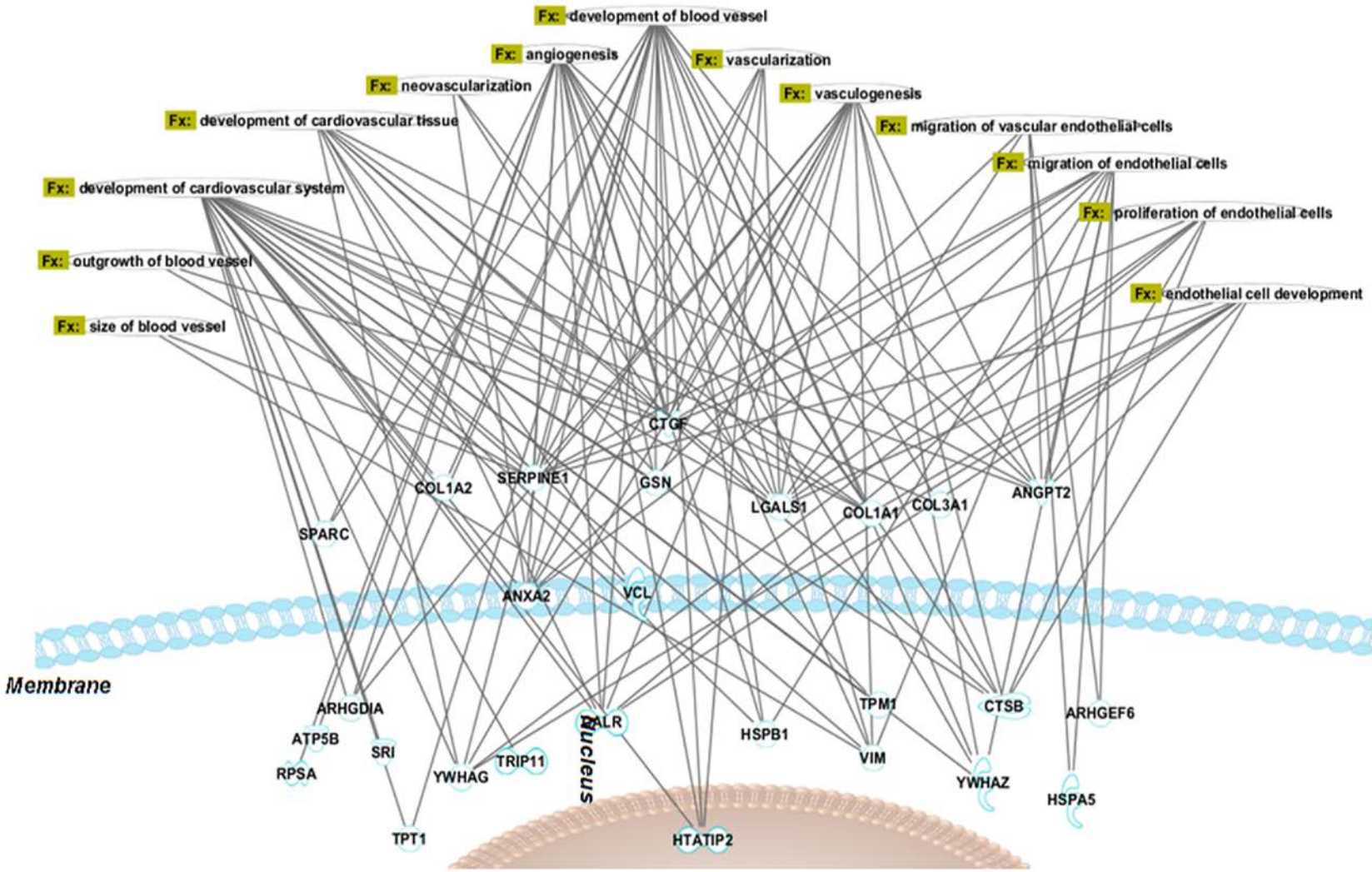
Table 2 Functional groups in the ASC interactome and the percentage of identified proteins

Functional group in donor cell	CM (%)	ASC (%)
Angiogenesis	17	5
Antioxidant/redox homeostasis	0	4
Cell proliferation/differentiation/apoptosis regulation	28	12
Coagulation/hemostasis	0	1
Defense response	0	1
Metabolism	0	3
Protein processing/chaperone	6	22
Proteolysis	0	3
Signaling/gene transcription	0	14
Structural	50	26
Transport/trafficking	0	8

ASC adipose-derived stem cell, *CM* conditioned media

Analysis of the functional networks in which the proteins identified in the ASC interactome were involved by performing an in-silico bioinformatic analysis using the IPA software. This analysis revealed that the most representative network in the ASC interactome was related to blood vessel development and neovascularization.

Additional File 6. ASC Interactome-Angiogenesis Network



Discussion 1

- Intravenous administration of CM with an intracoronary infusion of ASCs enhances neovessel formation in the ischemic myocardium as compared with the delivery of CM or ASCs alone.
- Evidence of a positive synergistic effect between ASCs and CM over the evolving reparative scar
- Limited clinical benefits in the setting of MI
- Recent data from the APOLLO and the PRECISE trials (phase I/IIb trials) suggest that administration of ASCs preserves cardiac function.
- A single intravenous delivery of CM obtained from ASCs (1 × 10^7 cells) exerts similar protective effects to that of ASCs in cardiac performance (CMR detected parameters) but not in the recovery of the rarefaction (loss of microvessels) where the effect is improved by ASCs plus secretome administration.
- Administration of stem cells capable of directly inducing vessel formation or secreting proangiogenic factors holds great promise to repair the ischemic damaged tissue.

Discussion 2

- Evidence indicates that ASCs have higher angiogenic capacities than bone marrow MSCs.
- A combination of CM plus ASCs significantly enhances neoangiogenesis as compared with CM or ASCs alone, suggesting a synergistic interaction among ASCs and CM.
- Further studies are required to determine the effects of ASCs + CM on LVEF and cardiac remodeling upon a more severe ischemic insult and after longer survival periods.
- The effect of administering ASCs + CM early after revascularization deserves to be investigated.
- ASC interactome clearly shows a key role for ASC-related proteins in angiogenesis.
- Administration of ASCs favors TGFβ/ TβRII-related collagen synthesis in the evolving scar, helping to maintain myocardial shape and structure.

Conclusion

Peripheral vein administration of ASC paracrine mediators in combination with local coronary delivery of ASCs synergistically contributes to enhance the neovascularization of the infarcted myocardium through multiple effectors that interact through a complementary and coordinated protein network.

Further studies should approach the evaluation of effects at periods longer than 3 weeks.

In addition, further studies are needed to ascertain which of the released molecules are endowed with beneficial effects in order to improve delivery of purified paracrine regulators of angiogenesis and neovessel formation.