Matricellular proteins: Recombinant expression of Smoc2 and Periostin in vascular cells

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Abstract: Extracellular matrix (ECM) proteins are a large family of macromolecular proteins which play crucial role in a wide range of biological activities such as cell adhesion, migration, proliferation, survival and differentiation. Matricellular proteins are a novel group of ECM proteins which are postulated to have regulatory activities and modify the features of ECM and reactivity of cells. This project aims to investigate the role of two new members of matricellular proteins namely: Smoc2 and Periostin. Both proteins should be expressed and recombinantely purified and initial attempts need to define their biological functions. For this purpose, different cell types were used such as two types of pericyte-like cells (PVC cells): PVC/MI (Anxa5 $^{LacZ/LacZ}$; knock out) and PVC/MII (Anxa5 $^{+/LacZ}$; heterozygote) and HEK-293(Human embryonic kidney). The results indicate that Smoc2 and Periostin can be expressed and purified from HEK-293 cells. In contrast, transfection and expression in pericyte-like cells was very inefficient. Therefore, Smoc2 and Periostin proteins can be expressed and purified after improvements of transfection protocols and optimisation of experiments. Apart from expression and purification of proteins, initial experiments showed more effects on endothelial differentiation and cell binding capacities, but as both proteins can be expressed future investigation are require.
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1. Introduction

Proteins are essential component of the body which consist of polypeptides.Each protein is composed of unique chain of amino acids and playing biological role accordingly. Extracellular matrix proteins (ECM) are a well-known member of protein family which consists of approximately 300 different proteins with broad spectrum of activities (Hynes and Naba, 2012).Extracellular matrix proteins (ECM)confer not only structural support to tissue, but also are involved in key biological functions such as cell proliferation, adhesion, survival, migration and differentiation (Daley et al., 2008).

Matricellular proteins are a novel group of ECM proteins and defined as a family of protein involved in the modification of ECM reactivity (Bornstein and Sage, 2002).They bind to cell-surface receptors, growth factors and cytokines, resulting in modulating cell-matrix interactions and modification of features of the ECM proteins, as well as modification of the reactivity of cells (Bornstein and Sage, 2002). Matricellular proteins consist oftenascins, SPARC family (secreted protein acidic and rich in cysteine/osteonectin), osteopontin and thrombospondins (Rocnik et al., 2006). It has been stated that matricellular proteins are over expressed in tissue remodelling, wound healing and embryogenesis (Rocnik et al., 2006).Matricellular proteins through their regulatory effects on growth factors, MMP and ECM activity, stimulate adhesion of cells which leads to signalling by growth factors receptors(GF-Rs) and integrins. matricellular proteins have been brought to attention in terms of their role in interaction with cells and ECM as a regulator.

SPARC (secreted protein acidic and rich in cysteine/BM40/ osteonectin) is the best analysed member of matricellular proteins which mediate interactions between cell and matrix(Lane and Sage, 1994). Other members of the protein family with related function arethrombospondins (TSP) 1 and 2, osteopontin (OPN), tenascins (TN)-C and X, and the SPARC-related proteins, SC1 and QR1 which are structurally distinct, but also modify the function of ECM components (Brekken and Sage, 2000).SPARC binds structural matrix proteins (e.g. vitronectin, collagen) and modulate interaction between ECM and the cells by modifying the features of ECM and reactivity of the cells(Brekken and Sage, 2000).

Smoc2 (SPARC-related modular calcium-binding protein 2) is a member of SPARC-related proteins (Rocnik et al., 2006) with not fully understood functions in biological activities(Liu et al., 2008). An important property of SPARC-related proteins is the presence of a C-terminal extracellular calcium-binding domain with two EF-hand calcium-binding motifs and a follistatin-like domain(Rocnik et al., 2006). Smoc1 and Smoc2 are almost structurally the same, whereas other related proteins have less or additional domains (Figure 1). Smoc2 protein is believed to play a critical role in cell migration, angiogenesis, proliferation and

growth factor signalling(Pazin and Albrecht, 2009). Due to interactions between Smoc2 and integrins (αvβ1 and αvβ6), it has been suggested that interactions between ECM and integrinsare mediated by Smoc2(Pazin and Albrecht, 2009).More importantly, Smoc2 can have angiogenic and mitogenic influences owing to interact with growth factors (VEGF, PDGF and FGF)(Liu et al., 2008; Rocnik et al., 2006).

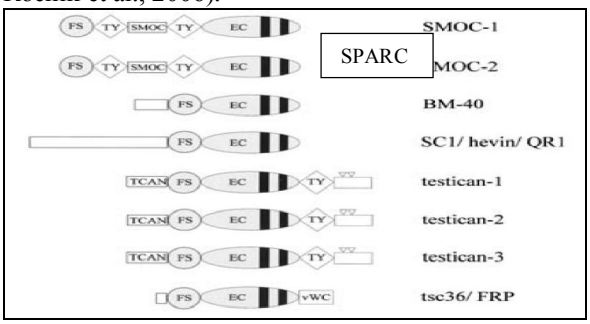


Figure 1: Domain organization of SMOC-2 and related proteins. Differences in structure of Smoc2 and Smoc1 proteins with SPARC family of proteins, as well as other matricellular proteins (Vannahme et al., 2003).

Periostin is a 90kDa secreted cell adhesion protein which is expressed in connective tissues such as skin and bone (Hamilton, 2008).Periostin consists of an amino-terminal EMI domain, a tandem repeat of 4 Fas I domains, and a carboxyl-terminal domain including a heparin-binding site at its C-terminal end (Kudo, 2011). Due to effects of Periostin on collagen fibrillogenesis, it is assumed that Periostin is associated with characteristics of connective tissues in health and diseases(Hamilton, 2008). It is indicated that Periostin binds to αvβ3 and αvβ5, inducing cell motility (Gillan et al., 2002). As a result, Periostin is considered as a putative protein to promote angiogenesis (Morra and Moch, 2011).

Angiogenesis is the development of new vessels originating from pre-existing vascular system (Longo et al., 2002). It plays crucial roles during embryogenesis, wound healing and reproductive function in adult (Folkman, 2007). Misregulation of such process is involved in wide range of diseases such as psoriasis, cancer (Bisht et al., 2010), diabetes (Kolluru et al., 2012), and age-related macular degeneration(Bressler, 2009). There are various molecules and pathways involving in angiogenesis. Different growth factors (Ruegg and Mutter, 2007), and cell signalling (Kuhnert et al., 2011; Siekmann et al., 2008; Thurston and Kitajewski, 2008), cell-cell interaction (Chantrain et al., 2006; Tan et al., 2009)and cell-ECM proteins interaction (Fiedler and Eble, 2009; Saharinen and Alitalo, 2011; Taneja et al., 2010), are well known factors and pathways which are involved in angiogenesis (Fig. 6).In angiogenesis point of view, the ECM is identified as storage of pro-angiogenic factors (Schultz and Wysocki, 2009), so that for instance, deficiency of basement membrane proteins (eg, collagen IV) leads to vascular defects (Poschl et al., 2004).Interactions between endothelial cell and mural cells are critical in angiogenesis. It has been indicated that specialised cell-cell contacts are formed as the result of contact between endothelial cells and vascular pericytes in vivo (Diazflores et al., 1992). Furthermore, close contact of endothelial cells and pericytes results in stability of the endothelial tube, increase survival of endothelial cell and regulate hemodynamic processes (Zhou, Z. *et al*., unpublished). Requirement of pericytes to vascular endothelium triggers chemical and physical signalling with endothelium (Hall, 2006).

As mentioned before, matricellular proteins modulate and modify feature of ECM proteins and cells. Therefore, it is speculated that matricellular proteins, like Smoc2 and Periostin may have interaction with vascular cells or may be considered as regulators of angiogenesis. With this regard, this paper aims to try to: detect expression of Smoc2 and Periostin proteins in tissues and cells, recombinant expression of Smoc2 and Periostin proteins, purify recombinant proteins, evaluate functional studies of Smoc2 and Periostin proteins by using expressed and purified recombinant proteins.

2. Materials and Methods

2.1. Immunofluorescence staining

2.1.1. Immunofluorescence staining of tissue

To find distribution of Smoc2 and Periostin in tissue, a slide with four 10µ sections was chosen (Taken from a mouse with Pdgfrb-Cre: Rosa26-LacZ genotype; mouse was provided by Ulrike Mayer and sectioned by Ernst Poschl). Sections were fixed with 1% PFA/PBS for 10 min, washed with PBS and fixed again in methanol in -20ºC. They were blocked in5% NDS/PBS for an hour. First antibody was anti-Smoc2-pAb IgG/Goat (1:25) and NG2 PG-pAb/Rabbit (1:200) for first section, anti-Smoc2-pAb/Goat (1:25) and Nidogen-1 pAb/ Rabbit (1:500) for second section, anti-Postn-pAb/Goat (1:25) and NG2 PG-pAb/Rabbit (1:200) for third section and forth section was used as a control of staining (no first antibody was used). After overnight exposing to first antibody, sections were washed 3 times with PBS and treated with secondary Abs: Donkey-anti goat-cy2 and Donkey-anti rabbit-cy3 and DAPI (all 1:200) for four sections for an hour. Secondary antibodies were centrifuged and kept in dark before apply. Tissue was observed after applying mounting solution.

2.2. Immunofluorescence staining of the 3D monoculture

First, collagen gels were washed with PBS, and then fixed with 80% methanol/20% DMSO for 2 hours. Gels then were rehydrated to PBS: 50% methanol/PBS,20% methanol/PBS and 0.1% Tween-20/PBS(each 1 hour). They were blocked 2 times each 1 hour in 10% Normal donkey serum in PBS, followed by exposing them to the first antibody in blocking reagent (Mouse-anti human CD31, 1:500) (BD Pharmingen) overnight at 4ºC. Then they were washed 7 times each 1 hour with TBS/0.1% Tween-20 and again treated with secondary antibody in blocking reagent for (Goat-anti –rat-Cy2) 48 hours. The initial washing step included DAPI (HOECHST 1:500). Finally they were prepared to be observed with dropping mounting solution on the gels.

2.3.Plasmid Transformation

Plasmids were already constructed in order to be transformed to the bacteria (DH5α) and to have sufficient amount of plasmid for transfection of cells and gaining recombinant expression of Smoc2 and Periostin.

Briefly, 1ul of each pcDNA/His-mCherry-Smoc2-4.3 and pcDNA/His-mCherry-Postn1 was mixed with 50ul of DH5α in two separate tubes and were incubated for 30min on the ice, for 40 sec at 40ºC in a water bath and incubated again on the ice for 2 min and added to LB for 60 min in shaking incubator at 37ºC. 15ul of cells/LB were cultured on LB/Amp plates and kept at 37ºC for an overnight. One colony of each plate was picked and added to 5ml LB medium for 4 hours incubation and shaking at 37ºC. Next the culture was added to 250ml LB with 250ul 1000×Amp which followed by 24 hours incubation and shaking at 37ºC.

2.4. DNA isolation

To isolate DNA, procedure was done according to QIAGEN Plasmid Purification Protocol. Grown bacteria in LB/Amp were centrifuged at 6000rpm for 15 min at 4ºC, then supernatants were discarded and 8ml of Buffer P1 was added to the pellets. Next, 8 ml of Buffer 2 was added which followed with inverting and incubation at room temperature for 5 min. 8 ml of Buffer 3 was added and followed with inverting and incubation for 15 min was done. They were centrifuged twice for 30 min and 15 min (20000 rpm) at 4ºC. The columns were equilibrated with 4 ml Buffer QBT and then supernatants were applied to the columns and washed 2 times with Buffer QC and eluted with 5ml Buffer QF. For precipitating DNAs 3.5 ml isopropanol was mixed with eluted DNAs and were centrifuged for 30min (15000) at 4ºC. After discarding the supernatants, 2ml 70% ethanol was added to the pellets and again centrifuged with the same condition. The supernatants were discarded and after pellets were air-dried, pelletswere dissolved in 500ul of TE buffer. Microspectrophotometery showed the concentration of 856ng/ul and 590ng/ul for pcDNA/His-mCherry-Smoc2-4.3 and pcDNA/His-mCherry-Postn1.

2.5. Restriction map

Reactions were prepared according to supplier's recommendation (Roche) with 0.5-ug of DNA plus an appropriate buffer (0.5- 1ul) in a total volume of 20ul.

2.6. DNA- Agarose Gel

1 % agarose gel was prepared for DNA analysing according to supplier's recommendation (Roche).

2.7. Cell culture

During conducting the project, three different types of cells were cultured for investigations:

HEK-293 cells, PVC/MI and PVC/MII cells. All types were usually plated in 10cm plate and in 10 ml of 10%FCS/DMEM. Once the cells became confluent, they were washed with PBS and for detaching cells; 1ml of trypsin was applied to do so. To stop trypsin functions, 5 ml of 10%FCS/DMEM was added to the trypsin. Cells then were centrifuged (1000 rpm) and dissolved in adequate amount of 10% FCS/DMEM and replated 1:5 in 10 cm plate with 10 ml of 10%FCS/DMEM.

2.8. Transfection

To see whether cells (HEK-293 cells,PVC/MI and PVC/MII cells) can express the recombinant proteins and also for collecting the conditional medium for purification, all three type of cells were transfected with pcDNA/His-mCherry-Smoc2 andpcDNA/His-mCherry-Postn. For this purpose different methods and approaches were used.

2.8.1. jetPRIME® method

Procedure was followed according to supplier's recommendation (Roche) (Table 1).

In a 6-well plate 150,000 PVC cells were plated in each well before transfecting the cells. Procedure briefly is provided below:

Table 1:BrifelyjetPRIME® method which was applied for PVC cells, according to Roche.

wells		Jet Buffer (µl)	DNA(µg)	Vortexing 1 sec	Jet Prime (µl)	Vortexing 1 sec, followed by centrifuging for 1 sec and keeping 10 min at RT
control	1	200	_		_	
	2	200	_		2	
Smoc2	3	200	1		2	
	4	200	2		4	
Postn1	5	200	1		2	
	6	200	2		4	

Cells were incubated an overnight at 37ºC and 5% CO2.

2.8.2. Other methods which were employed to transfcet the cell

Apart from jetPRIME® method, other methods for transfectiong cell FuGENE® 6 Transfection Reagent, Lipofectamine ™ Reagent and calcium-phosphate were applied to transfect the plasmids to the cells. However, they were not as effective as jetPRIME®.

2.8.3. Selection of transfected cells

In order to select just transfected HEK-293 cells, usually 250ug/ml to 500ug/ml of G418 (50mg/ml) were regularly added to their media. For selection of transfected PVC/MI, we also treated them with 400ug/ml of G418 from the same concentration.

2.9. Collection of serum-free and 10% FCS containing media from HEK-293/His-mCherry-smoc2

Conditioned media (serum-free and serum-contain media) were regularly collected in 50 ml tubes and kept at -20ºC.

2.10. Protein purification

To purify the proteins from either serum free or serum-containing media and detect the interested recombinant proteins, purification of medium was performed according to Clontech (TALON Affinity chromatography).

Collected media adjusted to 300 mMNaCl and 50 mMNaphosphate. On the other hand, 3ml of TALON Resin was poured in a column and equilibrated with 1× equilibration buffer (50mM Na phosphate pH: 8 and 300mM NaCl). Then, adjusted medium was added to the Resin. Resin was washed with washing buffer (equilibration buffer plus 15mM imidazole). Following step was adding elution buffer (equilibration buffer+ 150mM imidazole). Finally, eluted proteins were collected in 15 fractions each 1ml and they were kept in fridge.

2.11. Protein analysis

In order to detect the recombinant proteins either in eluted protein fractions or directly from collected serum-free and serum-containing media, they had to be precipitated and prepared to be run on polyacrylamide gel for immunoblotting and chemiluminescence assay.

2.11.1. Protein precipitation

1 ml of aliquots were mixed with 139ul 1%Triton X-100 and 259ul 55%TCA which followed by 15 min incubation on the ice, then 15 min centrifugation in 4ºC (13000 rpm), washing with cold acetone and again 15 min centrifugation in 4ºC (13000 rpm). After removing the organic waste, the pellets were left at room temperature to be air-dried and were dissolved in 100ul 1×SDS-loading buffer). However, for subjecting to the electrophorese gel 25ul of each was used to do so.

2.11.2. Polyacrylamide gel

To do western blot and analysis of proteins, precipitated proteins were run on either 8% or 10% polyacrylamide gels. Gels were made in according to supplier's recommendations (Roche) (Table2):

Table 2: making 8% and 10% polyacrylamide gels according to Roche.

Material	%8 polyacrylamide gel (15ml)	%10 polyacrylamide gel (15ml)	Stacking gel (4ml)
H2O	6.9 ml	5.9 ml	2.7
%30 acryl-bisacrylamide mix	4.0 ml	5.0 ml	0.67
1.5M Tris(pH 8.8)	3.8 ml	3.8 ml	0.5 (pH 6.8)
%10 SDS	0.15 ml	0.15 ml	0.04
%10 amuniumpersulfate	0.15 ml	0.15 ml	0.04
TEMED	0.009 ml	0.006 ml	0.006

All gels were run with the voltage of 110 (V), then prepared for coomassie blue staining.Gels then were transferred on PVDF (MILLIOPORE) membrane for immunoblotting.

2.11.3. Immunoblotting

After overnight transferring, the membrane were stained with PonceauRed solution for 10 min. after washing with water, all appeared bands were marked and membranes were washed with PBST (%0.1 Tween) three times. The membranes were blocked with %5 NDS/PBS then exposed to the first antibody (anti-Smoc2-pAb IgG/Goat (1:1000) into %2 NDS/PBS and kept in 4ºC overnight. Washed again with PBST 3×10min and exposed to the secondary antibody in %2 NDS/PBS: Peroxidase-conjugated Mouse anti-goat IgG (1:2000) for an hour and then washed with PBST 3×10 min (last time just with PBS).

2.11.4. Chemiluminescence assay

To apply for chemiluminescence, all membrane were exposed to chemiluminescence substrate (Super Signal® West Pico) (Thermo SCIENTIFIC) for 15 min in a dark condition and analysed by an appropriate machine (FUJIFIM LAS-3000).

2.11.5. Stripping blots for reprobing

To strip and reprobe the membrane in order to detect His-tag on the protein bands, the membranes were washed three times with PBST. Then they were treated with 10ml of stripping buffer and incubated in 55ºC, then washed 3×10 min with PBST and blocked with %5 NDS/PBS for overnight.

For probing, we used His-tag probe (HisProbe™-HRP) (Thermo SCIENTIFIC) (1:5000) for overnight exposing to the membranes. Then, membranes were washed three times with PBST and again preparing for chemiluminescence analysing.

2.12. FACS analysis

To understand precise number of cells which were expressing the recombinant proteins, FACS analysis was performed for PVC/MI/His-mCherry-Smoc2 and PVC/MI/His-mCherry-Postn. The cells were detected in channel 2 (488nm laser with a wave length of 585) (Accuri C6).

After PVC/MI cells were transfected with pcDNA/His-mCherry-Smoc2 and Postn, cells were prepared for FACS analysis. Two different Transfected cells in 6-well plate were trypsinased and each dissolved in 5ml DMEM. Then 200ul of each was washed in 5% BSA/PBS and centrifuged 5 min in 1000rpm. Then the pellet of each two types was dissolved in 500ul of FACS buffer (5% BSA/PBS).

As FACS analysis was conducted twice, for second time PVC/MI/pcDNA/His-mCherry-Smoc2 and PVC/MI/pcDNA/His-mCherry-Postn were analysed, but in this case 80% of cells were used for FACS analysis.

3. Results

3.1. Detection of distribution of Smoc2 and Periostin in tissue

Immunofluorescence staining of mouse muscle tissue (Pdgfrb-Cre: Rosa26-LacZ) was conducted in order to detect Periostin and Smoc2 proteins in the tissue. Expression of such proteins was recognised by using first polyclonal antibodies against interested proteins and fluorescent antibodies against the first antibodies. To detect the distribution of Smoc2 and Periostin, antibodies were used to detect Smoc2 and Periostin proteins and alsoNG2 as a marker for pericytes (Fig. 2). To identify the ECM proteins in the basement membrane, an antibody against Nidogen-1 was used. Although the detected Periostin proteins was rather weak (C), Smoc2 proteins were clearly detected in small capillaries (B) and big capillaries (A). To define more clearly, tissue was stained with NG2 antibody, indicating blood vessels and also Nidogen-1 antibody to recognise basement membrane around muscle fibres, as well as blood vessels. The yellow colours imply that the expression of Smoc2 and Periostin proteins by pericytes while they are located on basement membrane.

3.2. Plasmid transformation and restriction mapping

3.2.1. Expression plasmids

Plasmids were constructed by using the expression vector (pcDNA3). Both plasmids contain an expression cassette under the control of CMV promoter. The N-terminal sequence contains signal peptide of BM-40, used previously recombinant expression of ECM proteins (Nischt et al., 1991). The signal peptide sequence is fused to an RGS-His 6 tag to facilitate purification. After a short linker (13 aa) mCherry coding sequence (708 aa) is included. A flexible linker domain (26 aa). The coding sequence of the secreted part of mSmoc2 was fused to the domain. As a result, protein which is expressed would be His-mCherry-Smoc2. For Periostin, the construction is the same. Both plasmids the signal peptide region of Smoc2 and Periostin are not included and secretion is facilitated by the signal peptide of BM-40 (51 aa).

3.2.2. DNA preparation and restriction map

Constructed plasmids were transformed to DH5α bacteria and DNAs were isolated from bacteria (QIAGEN). Final concentration of DNA after isolation was about 856ng/ul and 590ng/ul for pcDNA/His-mCherry-Postnand pcDNA/His-mCherry-Smoc2, respectively.

Restriction mapping was performed in order to verify whether acquired DNAs were constructed properly. Figures 3and 4 show the fragments of bands after digestion with restriction enzymes on 1% agarose gel. In addition, tables 3 and 4 present the expected and detected DNA fragments. Taken together, all fragments were detected as they were expectedwith an exception which is about digestion of plasmid with *Nhe I* in plasmid containing Periostin gene (due to contamination of restriction enzyme).

3.3. Recombinant expression of Smoc2 and Periostin proteins

To study whether eukaryotic cells can express the recombinant proteins and also to purify the recombinant proteins, different cell lines were used such as PVC/MI (pericyte-like cell Anxa5 $^{lacZ/LacZ}$; knockout), PVC/MII (pericyte-like cells Anax5 $^{+/LacZ}$; heterozygote) to investigate about angiogenesis and HEK-293 (Human embryonic kidney cells) for recombinant expression of proteins.

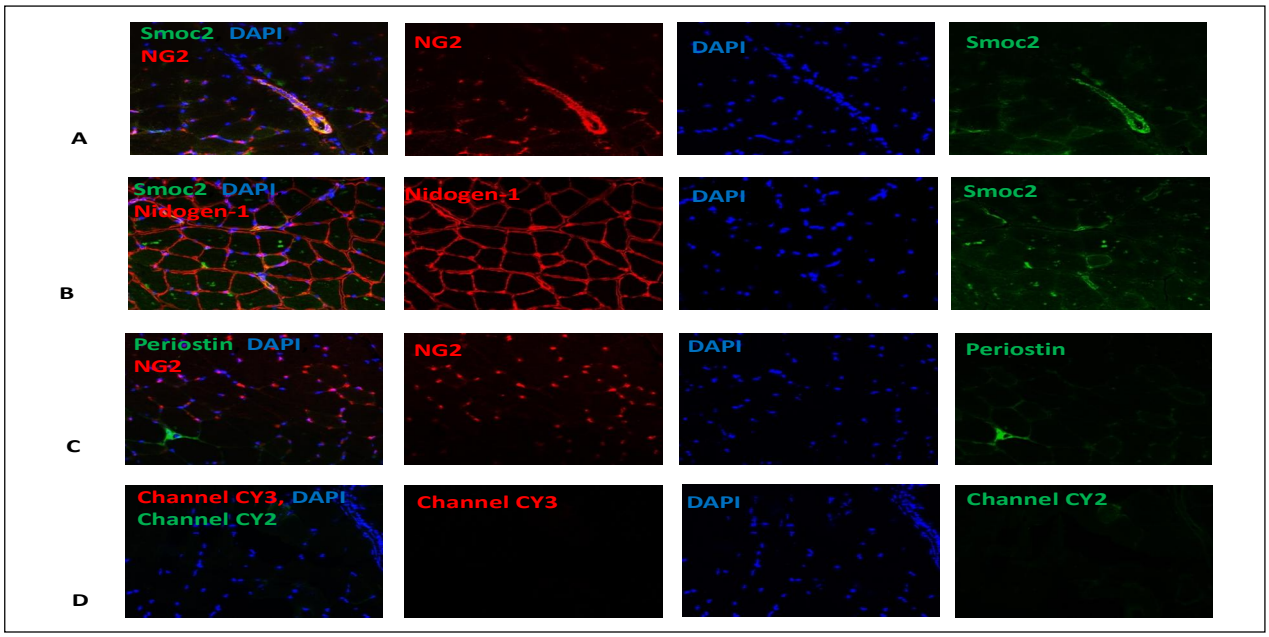


Figure 2. Immunofluorescence staining of muscle tissue (Pdgfrb-Cre: Rosa26-LacZ). A shows detected proteins Smoc2 (Green)and NG2 (Red), as well as nuclei of cells (Blue). Yellow colours particularly on the capillary of the tissue indicate that the expression of Smoc2 and NG2 proteins are originated from the same cells (Perycites). That pattern is exactly similar with **C** which displays the expression of Periostin proteins (Green), nuclei (Blue) and NG2 (Red). **B**, expression of Smoc2 proteins (Green) and Nidogen-1 (a marker of basement membrane) (Red) are indicated which illustrates the basement membrane around muscle fibre and blood vessels. **D** shows the control of the experiment, so that the cells just were treated with secondary antibodies. **Magnification is 20X.** Microscope:ApoTome ZEISS.

Table 3. Numbers and molecular weight of pcDNA/His-mCherry-mPostn-1fragments afterelecterophoresis. As there is just one site for some restriction enzymes (*Xba I, Nhe I* and *EcoRV*) a linear DNA is appeared on the gel, while for other enzymes depending of the number of sites to react, different fragments are appeared on the gel.

Restriction Enzyme(s)	Expected Fragment(s) length (bp)	Detected Fragment(s) length (kDa)
Uncut	Sc/oc	_
Hind III	6794, 1652	≃ 6.7, 1.6
Nco I	3345, 2297, 874, 863, 735, 332	≃3.3, 2.2,0.8, 0.7, 0.3
Pst I	4867, 2064, 1515, 358	≃4.8,2.0, 1.4, 0.3
Sma I	Linear	≃ 8.0
EcoRV	Linear	≃8.0
Sma I + Pst I	4749, 3697, 3339, 1824, 240	≃4.7, 3.6, 3.2, 1.7,0.2
Sma I + Nco I	4482, 3619, 2745, 2413, 116	≃ 4.4, 3.5, 2.7, 2.4,0.1
Sma I + EcoRV	5115, 3331	≃ 5.0, 3.3
Nhe I	Linear	_
Xba I	Linear	≃ 8.0, 5.0

3.3.1. Transfection of plasmids to HEK-293 cells

Cells were transfected with jetPRIME® approach. As figure 5 displays, most of the cells express the pcDNA/His-mCherry-Smoc2 proteins. In order to select transfected cells, they were treated with 250 and 400ug/ml of G418.

3.3.2. Transfection of plasmids to pericyte-like cells (PVC/MI and PVC/MII)

3.3.2.1. Transfection of plasmids to PVC/MIIcells

Cells were transfected with jetPRIME® approach. Similar to HEK-293 cells, PVC/MII did not express any pcDNA/His-mCherry-Postn proteins. Also, just one cell was infected with the pcDNA/His-mCherry-Smoc2 and expresses the recombinant protein. Vesicles inside the cells which are prepared to be secreted outside are obviously

shown (Fig. 6).Other methods for transfection of PVC/MII were employed such as Lipofectamine ™ Reagent, Fugene® 6 Transfection Reagent. However, the cells were not infected with mentioned methods (Table 5).

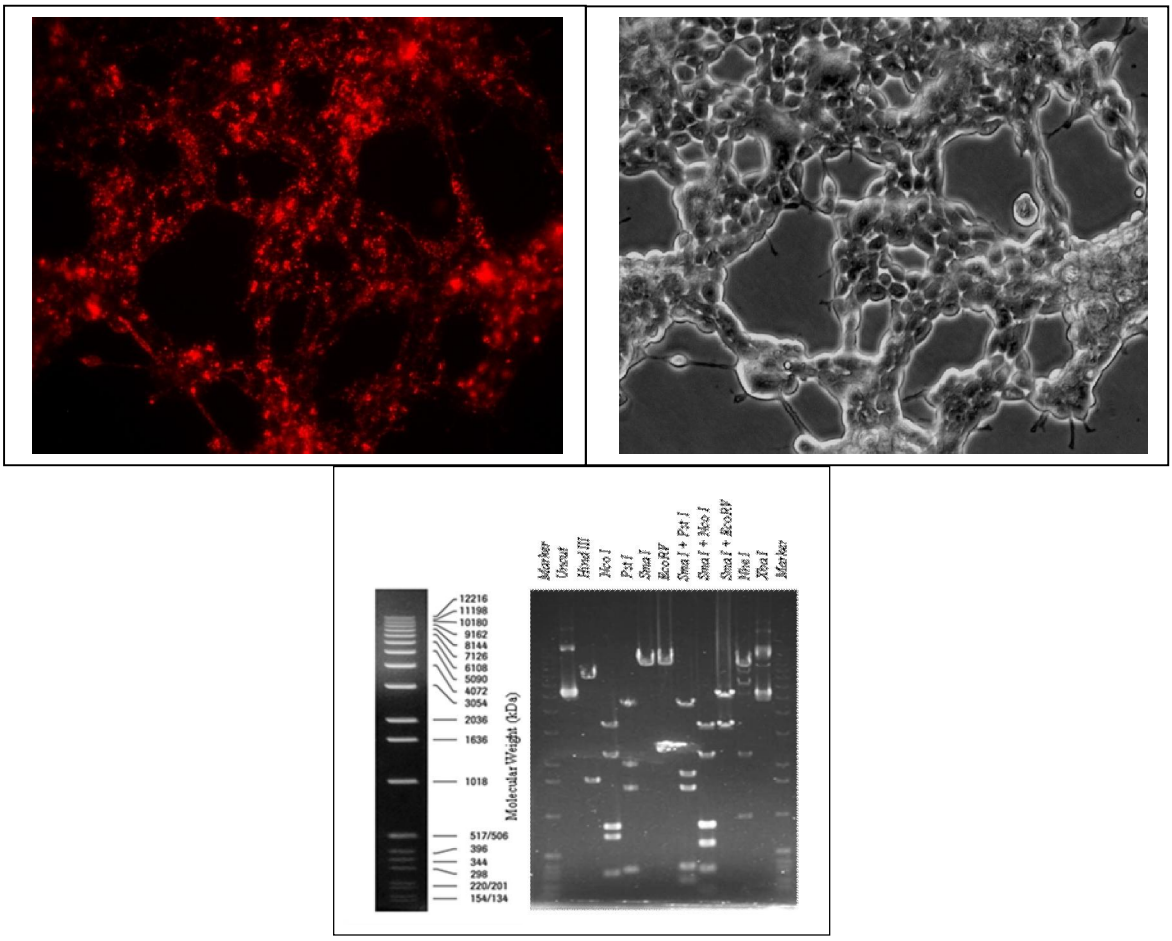


Figure 3. DNA electrophoresis of pcDNA/His-mCherry-mPostn-1. Different fragments of DNA are appeared on the gel due to function of restriction enzymes. All fragments display the molecular weights as it was expected. As the result of contamination *of NheI* enzyme with other enzymes, more than on fragment is appeared on the gel. The marker (1 kb Marker X Roche) determines the molecular weight of each fragment.

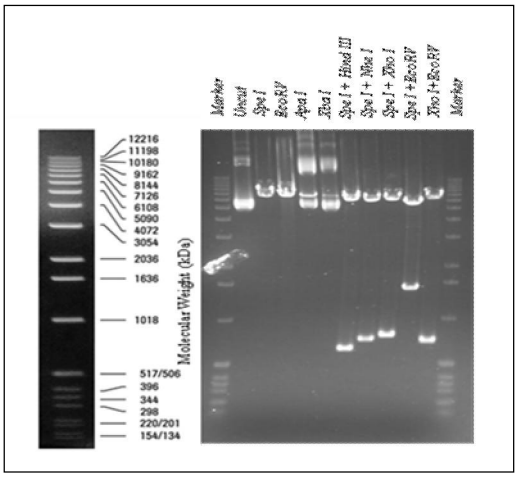


Figure 4. DNA electrophoresis ofpcDNA/His-mCherry-mSmoc2. Different fragments of DNA are appeared on the gel due to function of restriction enzymes. All fragments show the molecular weights as it was expected. The marker (1 kb Marker X (11498037001) Roche) on the left side determines the molecular weight of each fragment.

Table 4. Numbers and molecular weight of pcDNA/His-mCherry-mSmoc2 fragments after electrophoresis. As there is just one site for some restriction enzymes (*Xba I, Nhe I* and *EcoRV*) a linear DNA is appeared on the gel,

Restriction Enzyme(s)	Expected Fragment(s) length (bp)	Detected Fragment(s) length (kDa)
Uncut	Sc/oc	-
Spe I	Linear	≃ 8.1
EcoRV	Linear	≃ 8.1
Apa I	Linear	≃ 7.0
Xba I	Linear	≃7.0
Spe I+ Hind III	6882, 640	≃ 8.1, 0.6
Spe I + Nhe I	6777, 745	≃ 8.0, 0.7
Spe I + Xho I	6738, 748	≃ 8.0, 0.7
Spe I + EcoRV	6010, 1512	≃6.0, 1.5
Xho I + EcoRv	6794, 728	≃ 8.1, 0.7

while for other enzymes depending of the number of sites to react, different fragments are appeared on the gel.

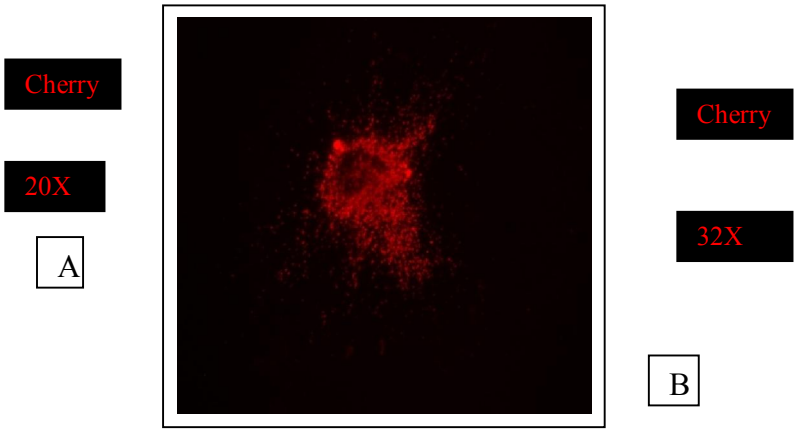


Figure 5.Expression of recombinant proteins in HEK-293 cells.A: illustrates fluorescence light of channel 2 fluorescent implying expression of cherry proteins.**B**: shows the phase contrast picture of transfected HEK-293 cell. (Microscope: Axiovert 40 CFL ZEISS).

3.3.2.2. Transfection of plasmids to PVC/MI cells

To transfect the plasmids to PVC/MI, two methods were performed: transfecting by calcium phosphate approach and jetPRIME® approach. In transfection of PVC /MI with calcium-phosphate method no transfected cells were found. Whereas, in transfection of cells by jetPRIME® approach just 1% of cells expressed the pcDNA/His-mCherry-Postn proteins and 10 % expressed the pcDNA/His-mCherry-Smoc2 proteins (Fig. 7). Cells were treated with 400ug/ml of G418 to select just transfected cells. In the transfected experiments only few cells were expressing the recombinant proteins. Therefore, we used Flow cytometry for detecting expressing cellsin large pool of cells. Recombinant proteins were detected by their Cherry domains which can be detected in channel 2 of a cytometer (Accuri C6). Part of signals can be detected in FL2 channel. Additionally, FSC and SSC were detected, characterising the size and granularity of cells, respectively. Surprisingly, after two weeks transfection, no specific expressing cell was detected, although they were G418 resistant (Fig. 8 A and B).

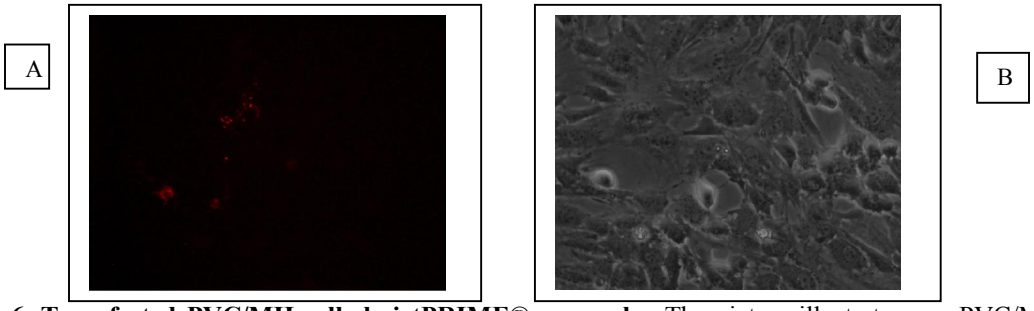


Figure 6. Transfected PVC/MII cells byjetPRIME®approache. The picture illustrates one PVC/MII cellwhich expresses the recombinantproteins. Microscope: Axiovert 40 CFL ZEISS.

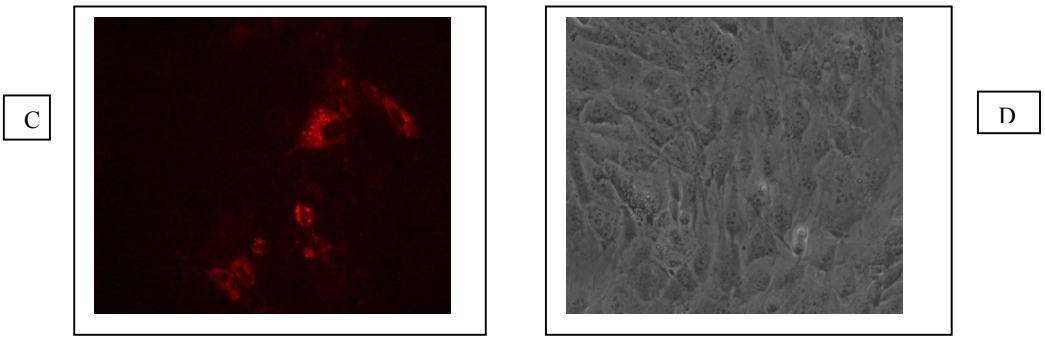


Figure 7. Transfecting PVC/MI cellsbyjetPRIME® method. A: represents PVC/MI cells expressing the recombinant proteins. **B:** illustrates the phase contrast photo of the cells presented in previous picture. **C:** displays expression of recombinant proteins by PVC/MI cells. Vesicles inside the cells show how proteins are prepared to be secreted to the extracellular environment. **D:** illustrates the phase contrast photo of the cells presented in picture C.

Table 5.effectivity of different transfection methods for three types of cells. (++) indicates the most effective approach for on specific cell line, (- +) represents partially effective, (0) indicates no effective at all. (NT) represents not tested.

Transfection methods	HEK-293 cells	PVC/MII cells	PVC/MI cells
Lipofectamine ™ Reagent	NT	0	NT
Fugene®6 Transfection Reagent	0	- +	NT
Calcium-phosphate	NT	NT	0
jetPRIME® ®	++	- -	- +

3.4. Purification of recombinant proteins

In order to detect recombinant proteins by immunoblotting and also understand the function of proteins, purification of recombinant proteins was carried out according toClontech (TALON Affinity chromatography). For this purpose, HEK-293 cells were regularly kept in serum-free DMEM (plus 400ug/ml G418) and also 10% FCS/DMEM, in order to collect the conditioned media.

The principal of purification is that, recombinant proteins bind to the resin which consist of a tetradentatechelator charged with cobalt which confer a high affinity for His-tagged (recombinant protein) proteins (Chaga et al., 1994). Recombinant proteins then are eluted with a buffer which is composed of Na phosphate, NaCl and imidazol to break the bonds. In our case, all eluted proteins were usually collected in 15 fractions (1 ml each).

3.5. Detection of proteins by immunoblotting

After protein purification of collected media, eluted proteins were precipitated and run on the either 8% or 10% polyacrylamide gel. To detect recombinant proteins on the gels, immunoblotting was carried out after transferring gels on the PVDF membranes.

3.5.4. Immunoblottingof serum-free and serum-containing media (purified and supernatant media).

In order to make sure whether all recombinant proteins are still in collected media and also in eluted protein fraction, immunoblotting of all was carried out. The procedure was that to precipitate 0.5 ml of any the most possible eluted protein fractions from serum-containing and serum-free media of HEK293/His-Cherry-Smoc2, as well as the conditioned media of HEK293/His-Cherry-Smoc2 (collected on 09/May/2012, 10/May/2012 and 11/May/2012). All precipitated proteins were electrophoresed on 8% polyacrylamide gel in two different conditions: reduced and non-reduced conditions. They then transferred on the PVDF membranes and exposed to the Smoc2 antibody and were prepared for chemiluminescence immunoassay. Photos illustrated the bands with various molecular weights (Fig. 9 A and B). However, we could identify the bands representing His-Cherry-Smoc2 and also degraded His-Cherry-Smoc2 proteins. The membranes again were prepared to be exposed to His-tag probes and doing chemiluminescence immunoassay. Overlapped bands may confirm more the presence of the interested protein (Fig.9 A and B).

3.6. 3D monoculture of HUVECs treated with Serum-free medium HEK-293/His-mCherry-Smoc2

In order to investigate the functions of Smoc2 proteins in angiogenesis, a monoculture of HUVECs in 3D culture was prepared in 6 wells of a 24-well plate in order to treat with the eluted proteins from fractions with containing the most amounts of recombinant proteins, which were already identified by doing polyacrylamide gel electrophoresis, and also collected serum-free 293/His-Cherry-Smoc2. After changing the medium of the culture every day replacing

with collected media and serum-free/ 5X growth factors in ratio of 1:4 for a period of one week and after formation of tubes by HUVECs, they were immunostained and visualised by fluorescence microscope. Nucleus and membranes of HUVECs were immunostained nicely by DAPI and CD31 antibodies, respectively (Fig. 10). Overlapping of green areas (CD31) and blue areas (nucleus) confirm the status of HUVECs in the culture. However, as we expected to observe the presence and involvement of Smoc2proteins (tagged with Cherry proteins) we could not detect any fluorescent light representing the existence of such proteins (Fig. 10).

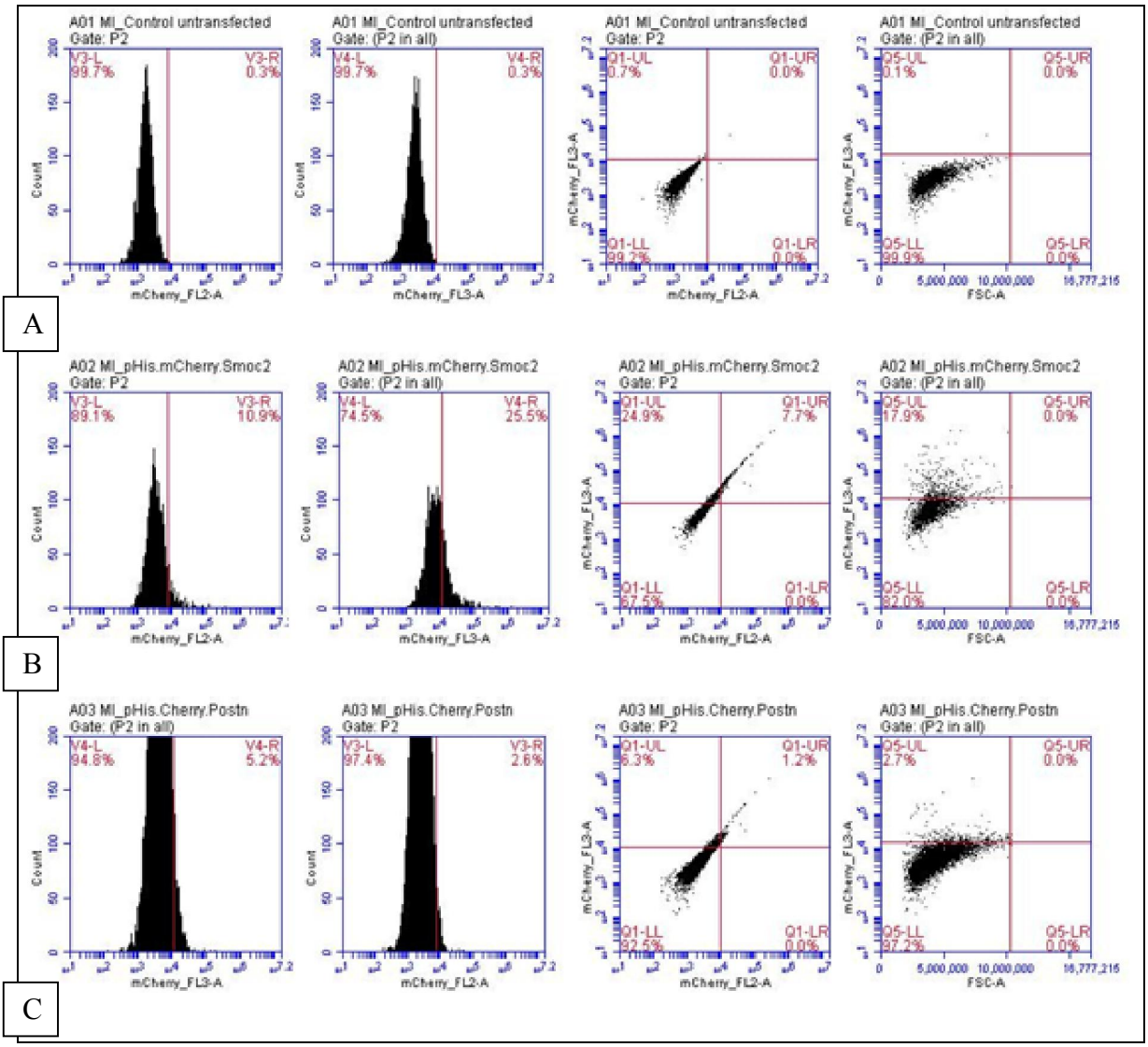


Figure 8 A. FACS analysis of PVC/MI/His-mCherry-Smoc2and PVC/MI/His-mCherry-Postn after two days transfection. A: indicates the control of experiments which untransfected PVC/MI cells were analysed. **B**: shows the expression of recombinant proteins (His-mCherry-Smoc2) by PVC/MI cells. As it is shown, about 7.7% of cells (shown in Q1-UR) were expressing the recombinant proteins. C: illustrate the percentage of cells expressing recombinant proteins (His-mCherry-Smoc2) which is about 1.2% (shown in Q1-UR).

4. Discussion

Due to the fact that matricellular proteins modify the features of ECM and also reactivity of cells, it was speculated that such proteins may also play important roles during angiogenesis. Smoc2 and Periostin as two members of matricellular proteins may interact with vascular cells and influence angiogenesis due to their interactions with ECM proteins such as integrins(Kuhn et al., 2007; Liu et al., 2008; Morra and Moch, 2011; Rocnik et al., 2006). With this regard, detection, expression, purification and initial tests for biological functions were conducted.

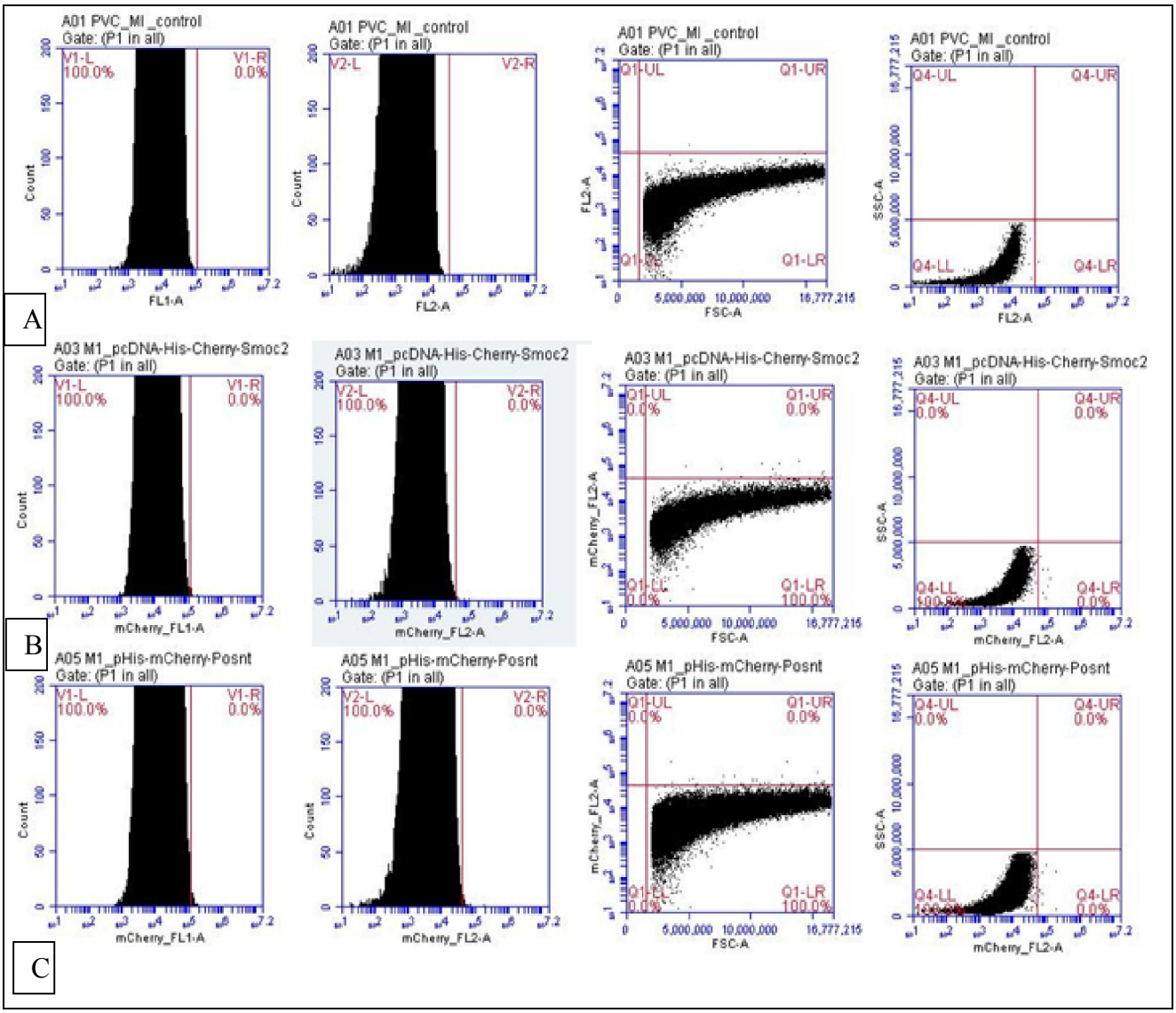


Figure 8 B. FACS analysis of PVC/MI/His-mCherry-Smoc2and PVC/MI/His-mCherry-Postn. A: presents the control of experiment which untransfected PVC/MI cell were analysed. **B** and **C** show no expression of recombinant proteins neither Smoc2 nor Periostin was detected, respectively.

4.1. Expression of matricellular proteins in HEK-293 cells

Expression of recombinant proteins in HEK-293 cells after being transfected with pcDNA/His-mCherry-Smoc indicates that the constructed plasmids are able to be transfected and express recombinant Smoc2in cells. As result of HEK-293 cell showed, most of the cells expressed the recombinant proteins, and the expression of proteins was detected through the observation of the fluorescent Cherry proteins tag by fluorescence microscopy. However, in transfection of HEK-293 with pcDNA/His-mCherry-Postn, we did not see any expression of such proteins. Yet, there is not any clear reason for this finding. As transfection is very effective in HEK-293 and the structure and sequence of the construct was verified, we would like to speculate that either the Postn protein is not stable in these cells or causes death in expressing cells. This has to be tested in future experiments.

4.2. Expression of matricellular proteins in pericyte-like cells

Different methods of transfection were applied for pericyte-like cells. PVC/MI and PVC/MII cells, the results of transfection indicate that these cells are rather resistant to transfection. PVC/MI cells were transfected in a very low number as data of FACS analysis showed. Although expressing PVC/MI/His-mCherry-Smoc2 and PVC/MI/His-mCherry-Postn transfected could be found, the expression of recombinant proteins was gradually decreased, so that after two weeks no cells secreting recombinant proteins could be detected by FACS analysis (as

detected by mCherry fusion tag). As the transfected cells were selected by adding G418 to the medium, it shows that transfection was done successfully. One potential reason for that could be that, cells died after they overexpressed the recombinant proteins and washed away as cells were regularly kept in fresh medium. Another possibility could be due to partially integration of plasmids to the eukaryotic chromosome. In other words, part of the plasmids was integrated into the eukaryotic genome which contains just the gene which expresses the resistance to G418 and the rest did not integrated to DNA. It also may be speculated that the transfected cells lost their adhesive ability to the cell culture plates and they were washed away during changing the medium of cells, such an idea would imply that the overexpression of matricellular proteins may affect survival (due to overexpression) or adhesion properties (due to modification of ECM). This has to be analysed in more details by additional experiments and may give important clues to function of this group of protein.

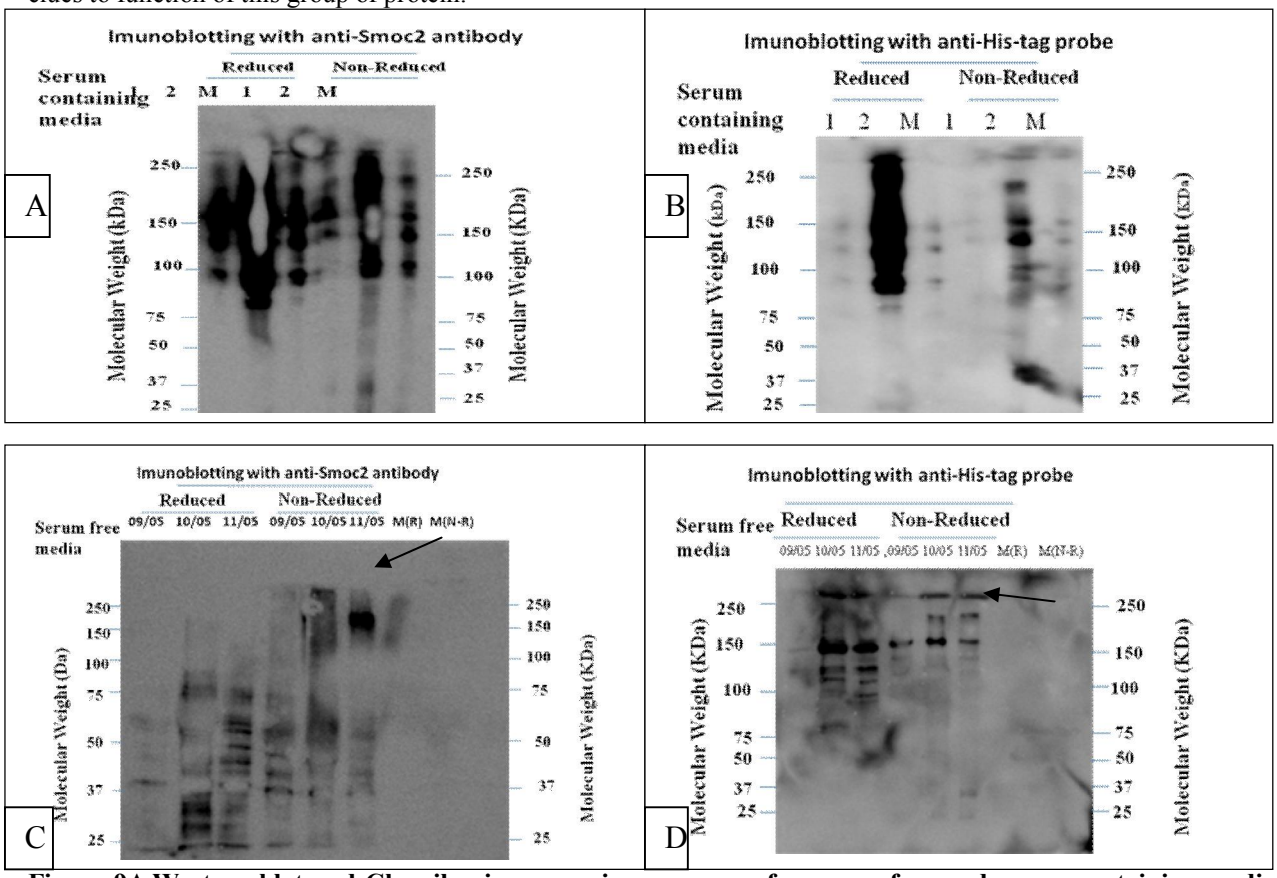
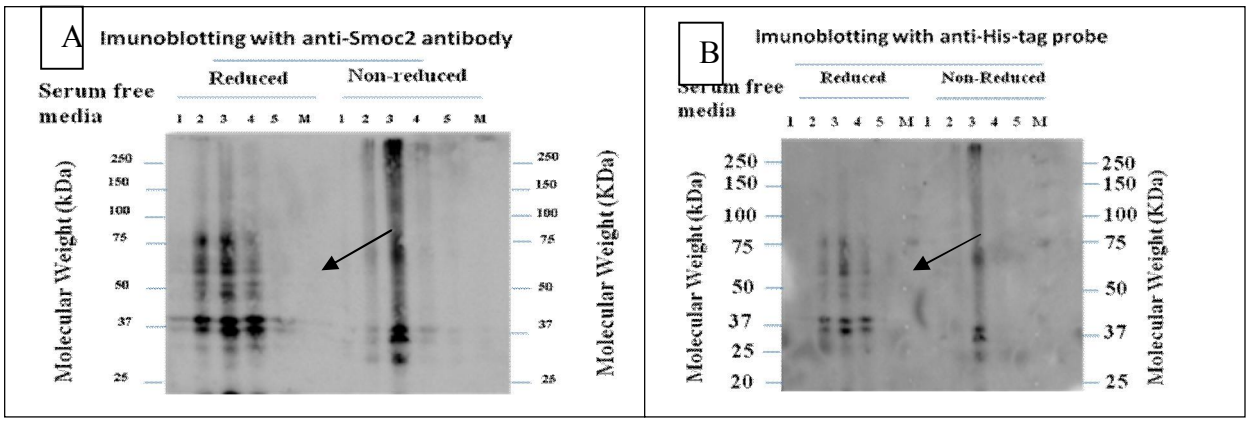


Figure 9A.Western blot and Chemiluminescence immunoassay for serum-free and serum-containing media with anti-Smoc2 antibody and anti-His-tag probe. A and B display the immunoblotting of serum containing media which were exposed to anti-Smoc2 and anti-His –tag probe respectively**. C and D** represent the same experiments but for serum-free media. Some overlapped bands are arrowed**.** M: Marker.



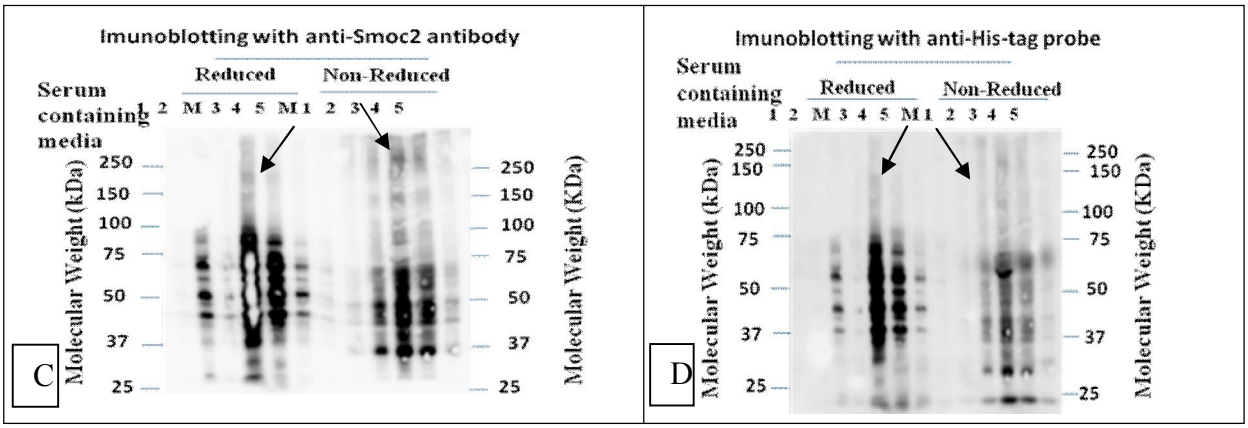


Figure 9 B.Western blot and Chemiluminescence immunoassay for serum-free and serum-containing media with anti-Smoc2 antibody and anti-His-tag probe. A and B display the immunoblotting of serum-free media which were exposed to anti-Smoc2 and anti-His-tag probe respectively**. C and D** represent the same experiments but for serum-containing media. Some overlapped bands are arrowed**.** M: Marker.

4.3. Morphological change in transfected pericyte-like cells (PVC)

Although the rate of transfection in both types of cells was very low, both types of PVC cells showed morphological changes after transfection. A few transfected PVC/MII with pcDNA His-mCherry-Smoc2 by FuGENE® 6 Transfection Reagent showedsome changes in their shape and size, while it was not seen in control cells of experiment. As the transfected cells had irregular shape and showed sign of formation of vesicles and fragmentation. It may be thought that such cells were undergoing to apoptosis. However, experiments to detect cell death like staining with Annexin V probes and Propidiumiiodid for dying and dead cell, respectively, should be performed to support that idea.

In PVC/MI after transfection to pcDNA/His-mCherry-Postn also morphological changes was observed. In this case expressing cells seem to differentiate to adipocyte cells (due to the presence of lipid vesicles inside the cytoplasmic). However, no experiment was carried out to confirm the speculation. Staining for lipid deposition or the detecting the expression of adipocyte-specific marker genes (PPAR gamma mRNA by RT-PCR) would be useful.

4.4. Purification of secreted Smoc2 proteins

Purification of proteins from serum-free and serum-containing media HEK-293/His-mCherry-Smoc2 was carried out. Purified proteins were analysed by polyacrylamide gels and revealed that in both serum-containing and serum-free media recombinant proteins can be detected through staining with coomassie blue and by Smoc2-specific immunoblotting. The results indicate that proteins with an expected molecular weight (≃ 76 kDa) can be detected. Therefore, the secreted monomeric protein shows the expected molecular weight and indicates that Smoc2 protein can be successfully expressed. In addition, doing immunoblot under reduced and non-reduced condition suggests that the reducing agents (eg.DTT) break numerous S-S bridges of proteins(Geiger and Arnon, 1976). The Smoc2 protein has 24 cysteine amino acids and may therefore form up to 12 Cys-Cys bridges. Breaking the Cys-Cys bridges may have noticeable differences in movement of protein through the gel, resulting in appearing in different molecular weights. The appearance of higher molecular weight bands in the range of 200 to 250 kDa indicates the formation of higher molecular weight multimers. The size may indicate the formation of trimers, but this has to be defined by additional studies. Other protein bands with lower molecular weights may represent degraded recombinant proteins, respectively.

4.6. Effects of matricellular proteins on angiogenic differentiation

It was stated previously that ECM proteins modify and change the features of ECM. Otherwise, previous studies indicated that Smoc2 and Periostin are both significantly up-regulated during angiogenic differentiation in vitro. Therefore, the effects were tested on tube formation of endothelial cells in 3D culture. No significant effect on tube formation and survival of endothelial cells was seen. This could be due to the same effects as discussed before. Therefore, more studies are needed to define any effects of these proteins.

4.7. Summary, outlook and future investigations

The results show that Smoc2 and Periostin proteins can be expressed and purified from HEK-293 cells. Otherwise, pericyte-like cells PVC/MI and PVC/MII cells could be transfected only with very low efficiency and no stably expressing clones could be selected. Initial functional assays with purified proteins were performed, but did

not reveal yet significant result. Therefore, future studies should focus on the following strategies: improvement of transfection protocols, optimisation of the protein purification protocols, optimisation of selection of stably expressing cells, reduction of degradation of proteins, use of improved functional assays.The project was started as yet there is only little information available.For further investigations, transfection of pericyte-like cells with optimised methods would be an approach to transfect the cells to express the proteins. As immunoblottingof serum-free and serum-containing media showed, protein bands with higher molecular weight as expected could be studied to indicate whether they are multimeric recombinant proteins. Alternatively, they are recombinant proteins may are bound to other proteins.Due to time limitation, only primarily experiments could be performed in order to address morphological changes of transfected cell, as well as investigation on differentiation of transfected PVC/MI. Therefore, further studies should include: expression and purification of more recombinant proteins for in vitro studies, improvement of transfection methods for PVC cells (option would be viral transfection systems), and functional studies involving adhesion, migration and antigenic differentiation in 2D or 3D cultures.

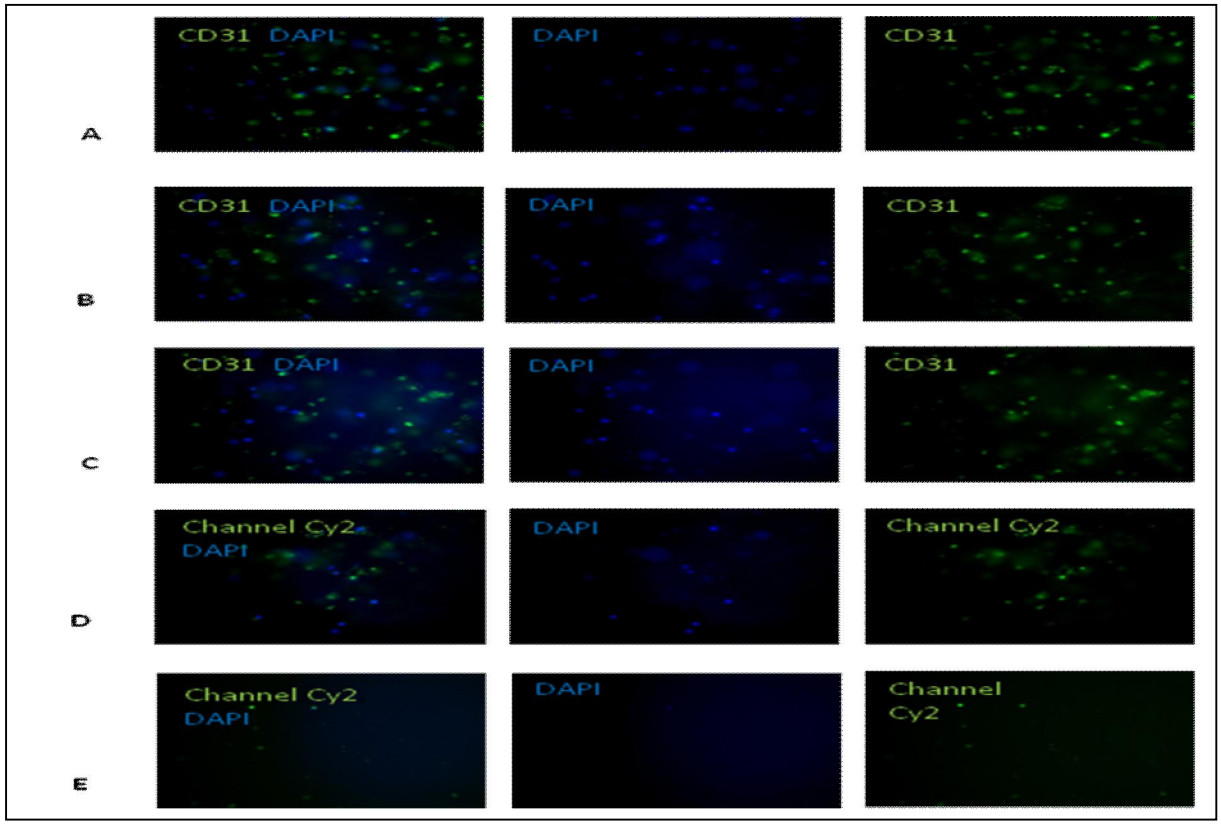


Figure 10. Immunofluorescence staining of the3D culture.A and B show the staining of HUVECs which were treated with collected serum-free HEK-293/His-Cherry-Smoc2 (collected on 09/May/2012) and 5X growth factors in ratio of 1:4. Although the nuclei and CD31 (marker of endothelial cells) were observed, but Cherry proteins were not detected. Such pattern is also similar for **C and D** which cells treated with the same amount of media but the date of collection of medium was different, 10/May and 11/May, respectively. Finally **E** indicates the control of experiment, so that HUVECs were treated just with serum-free medium and 5X growth factors in ratio of 1:4.
Magnification is 10X. Microscope: Axiovert 40 CFL ZEISS.

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List of abrreviations

aa: Amino acid
Amp: Ampicillin
Ab: Antibody
BSA: Bovine serum albumin
CMV: Cytomegalovirus
DMEM: Dulbecco's Modified Eagle Medium
DMSO: Dimethyl sulfoxide

DTT: Dithiothreitol
ECM: Extracellular matrix
FACS: Fluorescence activated cell sorting
FCS: Fetal calf serum
FGF:Fibroblast growth factors
HEK-293 cells: Human embryonic kidney-293 cells
HUVEC: Human umbilical vein endothelial cell
LB: Lysogeny broth
mAb: Monoclonal antibody
pAb: Polyclonal antibody
MMP: Matrix metalloproteinase
NDS:Normal donkey serum
PBS: Phosphate buffered saline
PBST: Phosphate Buffered Saline Tween -20
PDGF: Platelet-derived growth factor
Pdgfrb: Platelet-derived growth factor receptor
PFA:Paraformaldehyde
PVC/MI: Pericyte-like cells (anxa5$^{lacZ/lacZ}$mouseknockout)
PVC/MII: Pericyte-like cells (anxa5 $^{+/lacZ}$mouseheterozygote)
PVDF: Polyvinylidene fluoride
Postn: Periostin
rpm: Rounds per minute
SDS-PAGE: Sodium dodecyl sulfatepolyacrylamidegel electrophoresis
Smoc2: SPARC-related modular calcium-binding protein 2
SPARC: Secreted protein acidic and rich in cysteine
TBS: Tris-buffered saline
TCA: Trichloroacetic Acid
TGF: Transforming growth factor
VEGF: Vascular endothelial growth factor

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