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Copyright Year	2014	
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Corresponding Author	Family Name	**Salatino**
	Particle	
	Given Name	**Mariana**
	Suffix	
	Division	Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental (IBYME)
	Organization	Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)
	Address	Vuelta de Obligado 2490, C1428, Buenos Aires, Argentina
	Email	mariansalatino@gmail.com
Author	Family Name	**Croci**
	Particle	
	Given Name	**Diego O.**
	Suffix	
	Division	Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental (IBYME)
	Organization	Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)
	Address	Vuelta de Obligado 2490, C1428, Buenos Aires, Argentina
Author	Family Name	**Laderach**
	Particle	
	Given Name	**Diego J.**
	Suffix	
	Division	Laboratorio de Glicómica Funcional, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales
	Organization	Universidad de Buenos Aires
	Address	Buenos Aires, Argentina
Author	Family Name	**Compagno**
	Particle	

	Given Name	**Daniel**
	Suffix	
	Division	Laboratorio de Glicómica Funcional, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales
	Organization	Universidad de Buenos Aires
	Address	Buenos Aires, Argentina
Author	Family Name	**Gentilini**
	Particle	
	Given Name	**Lucas**
	Suffix	
	Division	Laboratorio de Glicómica Funcional, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales
	Organization	Universidad de Buenos Aires
	Address	Buenos Aires, Argentina
Author	Family Name	**Dalotto-Moreno**
	Particle	
	Given Name	**Tomas**
	Suffix	
	Division	Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental (IBYME)
	Organization	Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)
	Address	Vuelta de Obligado 2490, C1428, Buenos Aires, Argentina
Author	Family Name	**Dergan-Dylon**
	Particle	
	Given Name	**L. Sebastián**
	Suffix	
	Division	Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental (IBYME)
	Organization	Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)
	Address	Vuelta de Obligado 2490, C1428, Buenos Aires, Argentina
Author	Family Name	**Méndez-Huergo**
	Particle	

	Given Name	**Santiago P.**
	Suffix	
	Division	Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental (IBYME)
	Organization	Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)
	Address	Vuelta de Obligado 2490, C1428, Buenos Aires, Argentina
Author	Family Name	**Toscano**
	Particle	
	Given Name	**Marta A.**
	Suffix	
	Division	Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental (IBYME)
	Organization	Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)
	Address	Vuelta de Obligado 2490, C1428, Buenos Aires, Argentina
Author	Family Name	**Cerliani**
	Particle	
	Given Name	**Juan P.**
	Suffix	
	Division	Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental (IBYME)
	Organization	Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)
	Address	Vuelta de Obligado 2490, C1428, Buenos Aires, Argentina
Author	Family Name	**Rabinovich**
	Particle	
	Given Name	**Gabriel A.**
	Suffix	
	Division	Laboratorio de Glicómica Funcional, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales
	Organization	Universidad de Buenos Aires
	Address	Buenos Aires, Argentina

Abstract	Formation of an aberrant and heterogeneous vascular network is a key pathological event in the multistep process of tumor growth and metastasis. Pro-angiogenic factors are synthesized and released from tumor, stromal, endothelial, and myeloid cells in response to hypoxic and immunosuppressive microenvironments which are commonly found during cancer progression. Emerging data indicate key roles for galectins, particularly galectin-1, -3, -8, and -9 in the regulation of angiogenesis in different pathophysiologic settings. Each galectin interacts with a preferred set of glycosylated receptors, triggers different signaling pathway, and promotes sprouting angiogenesis through different mechanisms. Understanding the role of galectins in tumor neovascularization will contribute to the design of novel anti-angiogenic therapies aimed at complementing current clinical approaches. Here we describe selected strategies and methods used to study the galectin-1 regulation by hypoxia and its role in blood vessel formation.
Keywords (separated by “-”)	Galectin - Angiogenesis - Tumor neovascularization - Hypoxia

Chapter 19

Regulation of Galectins by Hypoxia and Their Relevance in Angiogenesis: Strategies and Methods

[AU1]

Mariana Salatino, Diego O. Croci, Diego J. Laderach, Daniel Compagno, Lucas Gentilini, Tomas Dalotto-Moreno, L. Sebastián Dergan-Dylon, Santiago P. Méndez-Huergo, Marta A. Toscano, Juan P. Cerliani, and Gabriel A. Rabinovich

Abstract

Formation of an aberrant and heterogeneous vascular network is a key pathological event in the multistep process of tumor growth and metastasis. Pro-angiogenic factors are synthesized and released from tumor, stromal, endothelial, and myeloid cells in response to hypoxic and immunosuppressive microenvironments which are commonly found during cancer progression. Emerging data indicate key roles for galectins, particularly galectin-1, -3, -8, and -9 in the regulation of angiogenesis in different pathophysiologic settings. Each galectin interacts with a preferred set of glycosylated receptors, triggers different signaling pathway, and promotes sprouting angiogenesis through different mechanisms. Understanding the role of galectins in tumor neovascularization will contribute to the design of novel anti-angiogenic therapies aimed at complementing current clinical approaches. Here we describe selected strategies and methods used to study the galectin-1 regulation by hypoxia and its role in blood vessel formation.

Key words Galectin, Angiogenesis, Tumor neovascularization, Hypoxia

1 Introduction

Angiogenesis is the physiologic mechanism that leads to formation of new blood vessels from preexisting ones and involves the coordinated action of different soluble factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-1 and -2, angiopoietins, and cell adhesion molecules such as integrins [1]. This process can be examined in vitro by studying three critical steps: endothelial cell proliferation, migration, and tube formation in response to different extracellular or intracellular stimuli [1]. Angiogenesis is a hallmark of cancer and various ischemic diseases like retinopathies [2]. The identification of new players of angiogenic

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programs and the elucidation of the precise molecular pathways linking tumor hypoxia to angiogenesis are essential for the design of rational anti-angiogenic therapies.

In addition to modulation of tumor immunity (reviewed by Salatino et al. in this issue), emerging evidence indicates a key role for galectin-1 in the modulation of vascular signaling programs. This glycan-binding protein is up-regulated in hypoxic microenvironments [3, 4] through hypoxia-inducible factor (HIF)-dependent [5] or HIF-independent pathways involving activation of nuclear factor (NF)-κB and production of reactive oxygen species (ROS) [3]. Thijssen and colleagues demonstrated that galectin-1 is expressed in tumor-associated endothelial cells, an effect which is associated with the promotion of an angiogenic phenotype [6]. In addition, endothelial cells can also take up galectin-1 which activates H-Ras signaling and Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase (Erk) kinase (Mek)/Erk cascade, thus stimulating endothelial cell proliferation and migration [7]. This pathway has been proposed to be activated through binding to neuropilin-1 on the surface of endothelial cells [8]. Interestingly, galectin-1 promotes tumor angiogenesis in different tumor models including Kaposi's sarcoma, melanoma, and prostate cancer [3, 6, 7, 9, 10]. Disruption of galectin-1-*N*-glycan interactions, using a galectin-1-specific monoclonal antibody or through inhibition of complex *N*-glycan branching, abrogates hypoxia-driven angiogenesis and tumorigenesis in a model of Kaposi's sarcoma [3], suggesting that blockade of galectin-1 may contribute not only to potentiate tumor immunity, but also to ameliorate hypoxia and block neovascularization in different tumor types. Furthermore, other galectins including galectin-3 and galectin-8 also contribute to tumor angiogenesis [11–14]. The $\alpha_v\beta_3$ integrin has been proposed to be a major galectin-3-binding protein [11] and CD166 (activated leukocyte cell adhesion molecule; ALCAM) has been identified as a candidate receptor for galectin-8 in normal vascular ECs [13]. Here we describe a selection of methods used to study the role of galectins, particularly galectin-1, in the modulation of tumor angiogenesis and their regulated expression by hypoxic microenvironments.

2 Materials (*See* Note 1)

2.1 Hypoxia Induction in Modular Incubation Chamber

1. Modular Incubator Chamber (MIC-10, Billups-Rothenberg).
2. Petri dishes.
3. Cells to be evaluated.
4. Appropriate cell culture medium (follow guidelines for individual cell culture).
5. O_2 gas cylinder.

6. N_2 gas cylinder.
7. CO_2 gas cylinder.
8. Closing clamps.
9. Oxygen sensor.
10. Conventional incubator.

2.2 Evaluation of Hypoxia. Hif-1α Detection by Western Blot

1. Cells to be evaluated (e.g., tumor cells; endothelial cells).
2. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4.
3. Protein Extraction Buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 10 mM EDTA; 1 % v/v NP-40) with protease and phosphatase inhibitors cocktails (Sigma).
4. 18 mm cell scraper (Corning).
5. 2× Laemmli sample buffer (BioRad).
6. Amersham Hybond-ECL (GE Healthcare).
7. 1.6 ml tubes (Axygen).
8. Tris-buffered saline (TBS): 150 mM NaCl, 50 mM Tris, pH: 7.4.
9. tTBS (TBS with 0.1 % Tween 20).
10. Blocking buffer: tTBS with 5 % nonfat milk or bovine serum albumin (BSA).
11. HIF-1α primary antibody (MA1-516, Pierce).
12. HRP-conjugated secondary antibody (Vector Labs).
13. Immobilon chemiluminescent HRP substrate (WBKLS01-00, Millipore).
14. PVDF membrane (Millipore).
15. 7.5 % SDS-polyacrylamide electrophoresis gel.
16. GBOX incubator (Syngene).
17. Bradford assay kit (Pierce).

2.3 Detection of Soluble VEGF

1. Cells to be evaluated.
2. Cell culture medium.
3. 15 ml tubes (BD).
4. P60 petri dish (GBO).
5. Human VEGF DuoSet ELISA Kit (R&D System).

2.4 Assessment of Angiogenesis In Vitro

2.4.1 Endothelial Cell Tubulogenesis

1. Conditioned media (*see* **Note 2**).
2. Primary Human Umbilical Vein Endothelial Cells (HUVEC) or Bovine Aortic Endothelial Cells (BAEC) (*see* **Note 3**).
3. Matrigel Reduced Growth Factor Basement Membrane Matrix (BD Biosciences).

4. DMEM medium (D1) supplemented with 1 % heat-inactivated FBS (PAA, the Cell Culture Company), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin (Life Technologies).
5. 24-well plates (GBO).
6. Crystal Violet aqueous solution 0.1 % (Sigma-Aldrich).
7. Recombinant human Gal-1 (rGal-1).
8. Anti-Gal-1 monoclonal antibody (F8.G7).
9. Lactose (Sigma).
10. Incubator set at 37 °C, 5 % CO_2.
11. Inverted phase microscope.
12. Digital camera (Nikon).

2.4.2 Endothelial Cell Migration

1. Conditioned media (*see* **Note 2**).
2. Primary Human Umbilical Vein Endothelial Cells (HUVEC) or Bovine Aortic Endothelial Cells (BAEC) (*see* **Note 3**).
3. DMEM medium (D1) supplemented with 1 % heat-inactivated FBS (PAA, the Cell Culture Company), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin (Life Technologies).
4. Endothelial cell migration 24-multiwell transwells, 8 μm (BD Biosciences).
5. 24-well plates (GBO).
6. rGal-1.
7. Lactose (Sigma).
8. Incubator set at 37 °C, 5 % CO_2.
9. 0.1 % crystal violet solution (Sigma-Aldrich).
10. Distilled water.
11. Q-tips.
12. Inverted microscope.
13. Chemoattractant (*see* **Note 4**).
14. rVEGF (R&D).

2.5 Assessment of Angiogenesis In Vivo

2.5.1 Matrigel Plug Assay

1. Matrigel Reduced Growth Factor Basement Membrane Matrix (BD Biosciences).
2. 1 ml syringe (Neojet).
3. 23 G needle (BD).
4. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4.
5. rVEGF-A (R&D system).
6. FGF-2 (R&D system).

7. TNF-α (R&D system).
8. Heparin (Fluka, Sigma).
9. Serum-Free Conditioned Media from Kaposi's sarcoma.
10. Gal-1 shRNA.
11. rGal-1.
12. Anti-Gal-1 monoclonal Ab (F8.G7).
13. 1.6 ml tubes (Axygen).

2.5.2 Inoculation of Matrigel Plugs to Evaluate Angiogenesis In Vivo

1. 23 G needle.
2. Matrigel mix (*see* Subheading 3.5.1).
3. Athymic nude mice.
4. C57BL/6 *Lgals1*$^{-/-}$ (Gal-1 KO).
5. C57BL/6 WT (Jackson).

2.5.3 Determination of Angiogenesis In Vivo in Matrigel Plugs

1. Scale.
2. H_2O.
3. P60 Petri dish (GBO).
4. Drapkin's reagent (Sigma).
5. Spectrophotometer.
6. Mouse hemoglobin (Sigma).
7. RPMI (Gibco).
8. 50 ml conical tubes (BD/Falcon).
9. Collagenase II solution (0.03 % in PBS/Sigma).
10. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4.
11. 100 μm cell strainer (BD bioscience).
12. 1 % fetal bovine serum (FBS) (Gibco).
13. Paraformaldehyde (1 % and 4 % w/v buffer).
14. PE-conjugated anti-CD34 antibody (clone RAM34 BD biosciences).
15. Phosphate buffered saline with 1 % FBS and 0.05 % NaN_3.
16. Ketamine (Holliday scott).
17. Xylazine (Richmond).
18. Optimum cutting temperature (OCT) medium (Biopack).
19. Cryostat.
20. PBS 10 % normal rat serum (Sigma).
21. Acetone (Cicarelli).
22. Anti-PECAM-1/CD31 antibody (clone MEC13.3 Novus Biologicals).

23. PBS 1 % BSA.
24. Slides.
25. AlexaFluor 596-conjugated goat anti-rat antibody (Cell Signaling).
26. Fluoromount slide mounting media (Southern Biotech).
27. DAPI (Life technologies).

2.6 Special Equipment

1. Flow cytometer.
2. Fluorescence microscope.

3 Methods

3.1 Induction of Hypoxia in Modular Incubator Chamber

1. Prepare cell cultures at 60–70 % of confluence.
2. Open the chamber incubator, place a Petri dish containing water and place the cells inside the incubator. Close the incubator ensuring that it is hermetically closed.
3. Maintain a separate cell culture in normoxia as control.
4. To generate an hypoxic atmosphere, flush a mixture of 1 % O_2, 5 % CO_2, and 94 % N_2 at 2 psi during 10 min. Turn off the gas flow and isolate the chamber by closing clamps (*see* **Notes 5** and **6**).
5. Place the chamber in a conventional incubator for 18–24 h (*see* **Notes 5** and **6**).

3.2 Evaluation of Hypoxia. HIF-1α Detection by Western Blot

1. Open the chamber and immediately place cell cultures on ice.
2. Remove culture medium, and wash with PBS twice.
3. Add protein extraction buffer (30 μl for 60 mm dishes) and use a scraper on hypoxia-treated and control cells.
4. Collect the total volume and centrifuge at 16,000 × *g* for 20 min in a 4 °C precooled centrifuge.
5. Transfer the supernatant to a fresh 1.6 ml tube on ice and discard the pellet.
6. Remove a small volume (5 μl) of lysate to perform Bradford assay according the manufacturer's recommended protocol.
7. Determine the protein concentration for each cell lysate. Prepare 20–40 μg of total cell lysate in 2× Laemmli sample buffer and boil it for 3 min.
8. Run samples on a 7.5 % SDS-PAGE gel and transfer to a PDVF membrane.
9. Block the membrane with TBS 0.1 % Tween-20 (tTBS) with 5 % nonfat milk at room temperature for 1 h on constant stirring.

10. Incubate for 18 h with HIF-1α primary antibody diluted 1:500 in tTBS 1 % nonfat milk at 4 °C on constant stirring.
11. Wash three times with tTBS at room temperature for 10 min and incubate with HRP-conjugated secondary anti-mouse antibody diluted 1:3,000 in tTBS for 1 h at room temperature.
12. Wash three times, incubate with Immobilon chemiluminescent HRP substrate, and capture the luminescent image in a GBOX incubator.

3.3 Detection of Soluble VEGF by ELISA

1. Collect culture medium supernatants from 24 h cell cultures in P60 Petri dishes. Subject samples to quick centrifugation (spin) in 15 ml tubes to eliminate cellular debris. Store supernatants at −80 °C until use.
2. Determine VEGF concentration in the supernatants with a VEGF ELISA kit following the manufacturer's instructions.

3.4 Assessment of Angiogenesis In Vitro

3.4.1 Endothelial Cell Tubulogenesis (See **Note 7**)

1. Seed 150 μl of Matrigel per well in prechilled 24 well plate. Incubate 2 h at 37 °C.
2. Add the conditioned media (CM) to be tested. Avoid freeze/thaw cycles of the CM. Different dilutions of the CM should be assayed.
3. Adjust to a final volume of 400 μl.
4. Add 25,000 endothelial cells in 100 μl D1 per well.
5. Add 1 μM recombinant galectin-1 (rGal-1) or other relevant galectin to evaluate tubulogenesis. Use 30 mM lactose or anti-Gal-1 monoclonal antibody to selectively block Gal-1 function (*see* **Note 8**).
6. Incubate at 37 °C and 5 % CO_2. Visualize slides at phase contrast microscope every hour for 24 h.
7. When tubular structures are apparent, take photomicrographs of several fields.
8. Quantify tubular structures (*see* **Note 9**).

3.4.2 Endothelial Cell Migration

[AU3]

1. Seed 40,000 endothelial cells per well in 250 μl D1 in the upper chamber of the endothelial cell migration 24-multiwell transwells (*see* **Note 10**).
2. The bottom well is filled with 750 μl CM containing the chemotactic factor to be tested, or other modulators of endothelial cell migration.
3. Add 1 μM rGal-1 or 1 μM rGal-1 plus 30 mM lactose to evaluate the effects of Gal-1 on endothelial cell migration.
4. Incubate for 18–24 h at 37 °C, 5 %CO_2.

5. Stain transwells with 0.1 % crystal violet solution for 10 min.
6. Wash transwells with distilled water.
7. Remove excess Matrigel with a Q-tip.
8. Examine at inverted microscope and count the number of cells. Data are expressed as cells per cm^2. A "fold-migration" value may be calculated as the number of cells migrating in response to rGal1 or VEGF (positive control), relative to the number of cells in the absence of mediator.

3.5 Assessment of Angiogenesis In Vivo

3.5.1 Matrigel Plug Assay

Matrigel preparation:

1. It is important to keep Matrigel HC as cold as possible (lower than 10 °C). It is recommended to maintain all materials and reagent (syringes, needles, solutions, pipettes, etc.) on ice prior to use.
2. Mix 300 μl of Matrigel with 200 μl of experimental solution in 1.6 ml tubes.
   (a) Experimental solutions:
   - Positive control: PBS + VEGF (10 ng/ml) + FGF-2 (20 ng/ml) + TNF-α (5 ng/ml), + heparin 10,000 IU (positive control mix) [16].
   - Serum-Free Conditioned Media (SFCM) from Kaposi's sarcoma (KS) cells exposed or not to a hypoxic atmosphere (1 % O_2, 5 % CO_2, and 94 % N_2 during 18 h) and transduced or not with Gal-1 shRNA encoded retrovirus [3].
   - PBS + rGal-1 (1.5 μM).
   - PBS + rGal-1 (1.5 μM) + anti-Gal-1 monoclonal antibody F8.G7 (1 μM).
3. Vortex tubes.
4. Inject the solution subcutaneously using a 23 G precooled needle. Injections should be done quickly to prevent the gel from solidifying.

3.5.2 Inoculation of Matrigel Plugs to Evaluate Angiogenesis In Vivo

1. Using 23-G precooled needle inject 0.5 ml of Matrigel mix subcutaneously into anesthetized female athymic nude mice (for SFCM studies) or female C57BL/6 *Lgals1*$^{-/-}$ (KO) or WT (for rGal-1 studies).
2. After 7 days, euthanize mice and remove the Matrigel plugs.
3. Angiogenesis can be evaluated by studying three independent parameters (*see* Subheading 3.5.3):
   (a) Hemoglobin content in pellets.
   (b) Number of endothelial cells.
   (c) Microvascular density.

3.5.3 Determination of Angiogenesis In Vivo in Matrigel Plugs

Hemoglobin content

1. Remove Matrigel plugs, weight and mechanically disaggregate them in 1.5 ml of H_2O in a Petri dish.
2. Incubate for 20 min at RT.
3. Centrifuge for 5 min at 10,000 × *g*.
4. Discard cell pellet.
5. Incubate supernatant with Drabkin's reagent according to the manufacturer's instructions.
6. Read absorbance of tubes at 540 nm. A standard curve of mouse hemoglobin should be simultaneously performed. Plot absorbance vs. cyanmethemoglobin concentration (mg/ml) and interpolate. The final concentration of hemoglobin in Matrigel pellets is calculated as mg/ml per 100 mg of pellets.

Number of endothelial cells

1. Remove Matrigel pellets, and mechanically disaggregate them in 5 ml of RPMI medium in a Petri dish.
2. Transfer to a 50 ml conical tube, add 5 ml of collagenase II solution, and incubate for 10 min at 37 °C water bath. After incubation, add 25 ml of PBS and filter the suspension through a 100 μm cell strainer.
3. Wash the filtered solution by adding 5 ml PBS and centrifuge for 5 min at 800 × *g*.
4. Remove the supernatant and wash the pellet with PBS 1 % FBS.
5. Stain cells with 1 μg of PE-conjugated anti-CD34 antibody in 100 μl of PBS 1 % FBS 0.05 % NaN_3 for 45 min in ice.
6. Wash cells with PBS and centrifuge for 5 min at 800 × *g*.
7. Fix cells with 1 % paraformaldehyde.
8. Analyze the percentage of PE^+ $CD34^+$ cells by flow cytometry.

Analysis of microvascular density (MVD)

1. Anesthetize animals (ketamine/xylazine, 140/14 mg/kg) and perfuse with PBS and 4 % paraformaldehyde.
2. Remove Matrigel pellets and embed them in frozen Optimum Cutting Temperature (OCT) medium and freeze at –70 °C.
3. Cut frozen Matrigel into 40–100 μm sections with a cryostat.
4. Air-dry sections at RT and fix in acetone for 10 min at –20 °C.
5. Air-dry for 5 min. Wash three times with PBS.
6. Block nonspecific binding through incubation for 1 h at RT with PBS 10 % normal rat serum.
7. Incubate sections with 1.5 μg of anti-PECAM-1/CD31 antibody in 200 μl of PBS 1 % BSA ON at 4 °C.
8. Wash with PBS three times.

9. Incubate for 1 h at RT with 0.2 μg of AlexaFluor 596-conjugated goat anti-rat antibody in 100 μl PBS.
10. Wash slides and mount in Fluoromount slide mounting medium containing DAPI as counterstaining fluorophore.
11. Determine microvessel density (MVD) by counting the number of microvessels per mm^2 in ten randomly selected fields (200×).

4 Notes

1. Galectins, particularly galectin-1, -3, and -8, have recently emerged as novel pro-angiogenic molecules responsible of the generation of tumor vascular networks [3, 6–14]. In this chapter we enumerate and discuss some of the strategies used to study the regulation of galectin-1 by hypoxic microenvironments and the stimulatory function of galectins in angiogenesis in vitro and in vivo. These lectins may act as "cytokine-like molecules" and contribute to angioproliferative and immunosuppressive nature of different pathologic conditions [18]. Understanding the molecular and cellular mechanisms underlying the pro-angiogenic function of galectins will contribute to delineate novel therapeutic strategies. We hope that the strategies and methods described here will facilitate and encourage scientists to further evaluate the role of galectins in neovascularization processes in different pathophysiologic settings.
2. To obtain serum-free conditioned media (CM), cells are cultured in normal culture media (RPMI, 10 % FCS) until reaching ~80 % confluency. Then, medium is discarded, cells are washed three times with sterile PBS, and serum-free (SF) RPMI media is added. After ~24 h, CM is collected, filtered with 0.22 μm syringe, filter and distributed in 1 ml aliquots.
3. For Primary Human Umbilical Vein Endothelial Cells (HUVEC) or Bovine Aortic Endothelial Cells, passages 8 or lower are recommended.
4. We recommend the use of oxygen sensors for more precise measurement of the intra-chamber O_2 levels during the experiment.
5. In order to eliminate the O_2 diluted in the medium it is recommended to re-gas the chamber once after 2 h or bubbling the gas into the cell culture medium before starting the experiment.
6. Hypoxia regulates a large number of genes through the binding of HIF-1α to Hypoxia Response Element (HRE) sequences. Many genes are under the regulation of HRE such as VEGF-A and erythropoietin. Therefore, measurement of up-regulation of pro-angiogenic mediators is an effective and reliable method to evaluate induction of HIF-dependent hypoxia.

7. Suggested Experimental Controls: Negative control: CM is replaced by D1, Positive control: 10 ng/ml rVEGF diluted in D1
8. Quantification can be done through multiple ways. One is based on counting the number of tubules/cm^2. On the other hand, morphometric analysis can be performed and tubules length can be measured using the ImageJ software.
9. The filter pores are small enough ~8 μm to allow passage of actively migrating cells; otherwise they rest upon the filter.

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Author Queries

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