Chapter 8

In Vivo Assays for Assessing the Role of the Wilms' Tumor Suppressor 1 (Wt1) in Angiogenesis

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Abstract

The Wilms' tumor suppressor gene (*WT1*) is widely expressed during neovascularization, but it is almost entirely absent in quiescent adult vasculature. However, in vessels undergoing angiogenesis, *WT1* is dramatically upregulated. Studies have shown *Wt1* has a role in both tumor and ischemic angiogenesis, but the mechanism of *Wt1* action in angiogenic tissue remains to be elucidated. Here, we describe two methods for induction of in vivo angiogenesis (subcutaneous sponge implantation, femoral artery ligation) that can be used to assess the influence of *Wt1* on new blood vessel formation. Subcutaneously implanted sponges stimulate an inflammatory and fibrotic response including cell infiltration and angiogenesis. Femoral artery ligation creates ischemia in the distal hindlimb and produces an angiogenic response to reperfuse the limb which can be quantified in vivo by laser Doppler flowmetry. In both of these models, the role of *Wt1* in the angiogenic process can be assessed using histological/immunohistochemical staining, molecular analysis (qPCR) and flow cytometry. Furthermore, combined with suitable genetic modifications, these models can be used to explore the causal relationship between *Wt1* expression and angiogenesis and to trace the lineage of cells expressing *Wt1*. This approach will help to clarify the importance of *Wt1* in regulating neovascularization in the adult, and its potential as a therapeutic target.

Key words Angiogenesis, Ischemia, Cardiovascular disease, Wilms' tumor, *Wt1*

1 Introduction

The Wilms' tumor suppressor gene (*Wt1*) is a transcription factor which has recently been implicated in angiogenesis; neovascularization from pre-existing blood vessels. The angiogenic response, whilst essential to tumor growth in cancer, is also triggered by arterial occlusion and hypoxia, and hence, can contribute to reperfusion of ischemic tissues in cardiovascular disease, reducing ischemic injury [1, 2]. Therefore, increasing the speed or magnitude of the angiogenic response is of considerable therapeutic interest to improving outcomes in a number of cardiovascular conditions including, myocardial infarction, stroke and peripheral limb ischemia.

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Despite being integral to embryogenesis, *Wt1* expression is notably sparse in the healthy adult organism, with expression only in a few cell types. *Wt1* is largely absent from non-regenerating adult vasculature (except for reports of low expression in a few smaller arteries [3]) but is up-regulated in endothelial cells, fibroblasts and pericytes in vasculature undergoing angiogenesis [4–6]. Furthermore, following induction of myocardial infarction (MI) in rats, *Wt1* is expressed in the coronary vasculature of the ischemic tissue, yet not in the adjacent healthy tissue [6]. In order to assess the role of *Wt1* in new vessel formation, angiogenesis can be induced in a number of in vivo models.

A simple, reproducible method of inducing angiogenesis is the well-described model of subcutaneous sponge implantation [7, 8]. In brief, polyurethane sponges are implanted subcutaneously in vivo and become infiltrated by a number of cell types (including fibroblasts, immune cells and endothelial cells), with angiogenesis occurring to provide a blood supply. Sponges are routinely harvested 20 days after implantation (when considerable angiogenesis has occurred) but can be retrieved at any time deemed suitable. Angiogenesis in these sponges can be measured in vivo (e.g. Using Fluorescence Molecular Tomography) and ex vivo (e.g. by measuring hemoglobin or by histological analysis). Mechanisms underlying angiogenesis can be assessed using immunohistochemistry, quantitative PCR and FACs to isolate cell populations. Angiogenesis in this model can be manipulated by pharmacological treatment (systemic or directly administration into the sponge [7]) or by impregnation of the sponge with an appropriate cell population in Matrigel prior to implantation [9].

Ischemic angiogenesis can be assessed using a model of femoral artery ligation [10]. Reperfusion in this model comprises a combination of proximal collateral formation (arteriogenesis) and distal angiogenesis [11]. Experiments are routinely maintained for between 4 and 28 days following induction of ischemia and laser Doppler analysis during the experiment allows a quantitative measure of the time-dependent restoration of blood flow. Tissues can be harvested at appropriate time-points for analysis of vascular density (using histology/immunohistochemistry), cell populations (FACS) and measurement of transcript expression (real time PCR). As with the sponge implantation model, the angiogenic response to hindlimb ischemia can be modulated by pharmacological manipulation [12] or cell administration [13].

Both these in vivo models of angiogenesis (sponge implantation, hindlimb ischemia) can be applied to rodent models of disease [14, 15] and to genetically modified animals [7]. When carried out using mice with tissue-specific *Wt1* KO, data from these models provides information on the functional effect of *Wt1* on angiogenesis and reperfusion whilst subsequent analyses (qPCR, histology, cell sorting) of sponge and muscle tissue allows insights into the mechanisms underlying the influence of *Wt1* on angiogenesis.

All methods described herein can be applied to genetically modified mice, to provide further insight into the actions of *Wt1* in vivo. *Wt1*+ cells can be traced using *Wt1* reporter mice (such as the *Wt1*-GFP or *Wt1*. Cre-ERT2; mTmG mouse lines) [16, 17] revealing the translocation of these cells and the site of *Wt1* influence. Insight into the effect of *Wt1* on recovery from ischemia can be deduced using mice with inducible knockout of *Wt1* from specific cells (e.g. the vascular endothelium) [18] and quantification of the angiogenic response as outlined above.

2 Materials

Prepare and store all reagents at room temperature (RT) using dH_2O for preparation if necessary, unless otherwise stated.

2.1 Subcutaneous Sponge Implantation

A comprehensive list of all equipment required to undertake the procedure is outlined below. All surgical tools should be autoclaved prior to use.

1. Blunt tip serrated forceps (0.8 × 0.7 mm).
2. Hemostats (12.5 cm).
3. Surgical scissors (22 mm straight tip).
4. Wound closure clips (1.75 × 7.5 mm).
5. Electric shaving clippers.
6. Polyurethane sponge (0.5 × 0.5 × 1 cm).
7. Sterile surgical drapes.
8. Heated mat.
9. Autoclave tape.
10. Anesthetic rig with medical-grade O_2 supply.
11. CO_2 chamber with CO_2 supply.

2.2 Femoral Artery Ligation

A comprehensive list of all equipment required to undertake the procedure is outlined below. All surgical tools should be autoclaved prior to use.

1. Standard tip fine forceps (0.1 × 0.06 mm).
2. 2× Angled tip fine forceps (0.1 × 0.06 mm).
3. Blunt tip serrated forceps (0.8 × 0.7 mm).
4. Haemostats (12.5 cm).
5. Surgical scissors (22 mm straight tip).
6. Fine surgical scissors (3 mm straight tip).
7. Scalpel.
8. Straight bulldog clips (28 mm).

9. Wire wound retractor.
10. 5-0 silk suture thread.
11. 5-0 nonabsorbable monofilament sutures, 19 mm 3/8 circle reverse cutting needle.
12. Electric shaving clippers.
13. Sterile surgical drapes.
14. Sterile H_2O for injections.
15. Heated mat.
16. Autoclave tape.
17. Surgical microscope.
18. Anesthetic rig with medical-grade O_2 supply.
19. CO_2 chamber with CO_2 supply.
20. MoorLDI2-HIR laser Doppler.
21. MoorLDI V6 software.

2.3 Solutions and Reagents for Surgical Procedures

1. Sterile H_2O for injections.
2. PBS solution.
3. Isoflurane.
4. Vetergesic (buprenorphine).
5. Povidone Iodine solution.
6. EMLA cream.
7. 1% lidocaine solution.
8. Paraformaldehyde (4% in PBS).
9. Ethanol (70% in dH_2O).

2.4 Materials for Isolating GFP+ Cells from Sponges Using FACS

1. Leibovitz's L-15 Medium (Invitrogen, UK).
2. Primary tissue culture hood (Ultimat Biological Safety Cabinet, Medical Air Technology Ltd., UK).
3. 60-mm Petri dish.
4. Scalpel blades.
5. PBS.
6. BSA (4 mg/mL, Sigma-Aldrich, UK).
7. Collagenase B (Roche, UK).
8. Stuart SI_3OH hybridization Oven (Bibby Scientific Ltd., UK).
9. 10% Fetal calf serum.
10. 100, 70 and 40 μm sterile cell strainers (Scientific Laboratory Supplies Limited, England).
11. Red Blood Cell lysis buffer (Biolegend, UK).
12. 50 mL centrifuge tubes.

2.5 Components for RNA Extraction and cDNA Synthesis

1. Ultra Turrax homogenizer (Fisher Scientific, UK).
2. Centrifuge.
3. Techne™ TC-512 thermal cycler (Fisher Scientific, UK).
4. Nanodrop spectrophotometer.
5. 2 mL Eppendorf tubes.
6. RNase-free water.
7. Chloroform.
8. GelRed™ (Biotium, USA).
9. 1× TAE buffer.
10. Agarose.
11. QIAzol® Lysis Reagent (Qiagen, UK).
12. RNeasy® Mini Kit (Qiagen, UK).
13. Quantitect® Reverse Transcription Kit (Qiagen, UK).

3 Methods

3.1 Subcutaneous Sponge Implantation

1. Anesthetize mice with inhaled isoflurane (5 %) in O_2 and maintain anesthesia thereafter at 2–3 % isoflurane in O_2.
2. Remove a roughly 3 cm^2 area of fur from the dorsal neck region using clippers, with the midpoint centered between the scapulae.
3. Sterilize exposed skin using Povidone Iodine solution.
4. Inject (subcutaneous) the analgesic Vetergesic (buprenorphine) diluted 1:10 in sterile H_2O at a dose of 0.05 mg/kg body weight.
5. Cover the animal with sterile drapes and make a 1.5 cm horizontal incision between the scapulae using a scalpel.
6. Using blunt dissection with hemostat forceps, subcutaneously extend the incision with the point of the blunt forceps facing away from the body cavity by inserting and opening the forceps to create two tunnels extending caudally over the left and right hindquarters.
7. Insert one $0.5 \times 0.5 \times 1$ cm sterile polyurethane sponge into each subcutaneous pocket, with the longest axis of the sponge lying parallel to the body.
8. Close the wound using wound clips and apply EMLA cream topically.
9. Allow mice to recover from anesthetic on a heated mat and monitor over the course of the experiment.
10. Cull mice by CO_2 asphyxiation at desired time points.
11. Expose sponges by cutting from the site of incision in an anterior–posterior direction and then across as necessary (Fig. 1).

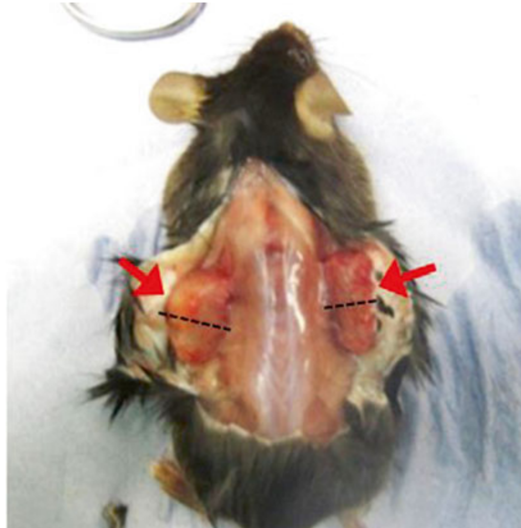

Fig. 1 Exposed subcutaneous sponges in situ 21 days after implantation. Sponges are indicated by *red arrows* and the *dotted line* represents the axis along which sponges were cut for processing

12. Excise sponges by cutting through the membrane between the sponge and the muscle or skin, keeping the points of the scissors facing away from the sponge to avoid damage while peeling away the skin.
13. Sponges can then be cut in half across the shortest axis.
14. Sponges can then be; preserved in 4 % paraformaldehyde in PBS at 4 °C overnight, then placed in 70 % ethanol; or snap frozen on dry ice and stored at −80 °C prior to further analysis.

3.2 Extraction of RNA from Subcutaneous Sponge and cDNA Synthesis

1. Remove sponge samples from −80 °C and homogenize in 700 μL QIAzol® Lysis Reagent in a suitable Eppendorf tube for 2 min or until of a uniform consistency.
2. Divide the sample equally between two further Eppendorf tubes and add a further 350 μL of QIAzol® Lysis Reagent to each.
3. Using an Ultra Turrax homogenizer (Fisher Scientific) homogenize each sample for 1 min and leave for 5 min.
4. Add 200 μL of chloroform to each sample and leave to stand for 2 min at RT.
5. Centrifuge samples at 12,000 × *g* and 4 °C for 15 min.
6. Combine aqueous fractions from the same sample and carry out RNA extraction using an RNeasy Mini Kit as per manufacturer's instructions. Store RNA at −80 °C.
7. Determine RNA concentration and integrity using a Nanodrop spectrophotometer and GelRed™ agarose gel electrophoresis.

8. Using the quantities determined by the Nanodrop spectrophotometer, dilute each sample to a final concentration of 0.5 ng/μL in 12 μL using RNase-free water.
9. Synthesize cDNA from RNA using a Quantitect® Reverse Transcription Kit as per manufacturer's instructions, a Techne TC-512 thermal cycler and a reaction mix of 20 μL.
10. Store cDNA at −20 °C prior to qPCR analysis.

3.3 Isolating GFP+ Cells from Subcutaneous Sponges Using FACS

1. Cull mice by asphyxiation in CO_2, and remove the sponges and kidneys immediately, placing them in separate falcon tubes containing Leibovitz's L-15 Medium. The kidneys of the experimental mice in these experiments act as positive tissue controls as the glomeruli are known to express *Wt1* in the GFP+ mice.
2. Under a primary tissue culture hood transfer the sponges into a 60-mm Petri dish, and disrupt as finely as possible using forceps and scalpel blades.
3. Resuspend tissue homogenate in 10 mL prewarmed PBS plus BSA (4 mg/mL) with 1 mg/mL Collagenase B per sample.
4. Next, incubate the samples for 45 min at 37 °C in a hybridization oven.
5. Stop the digestion process by adding 15 mL Leibovitz's L-15 Medium containing 10% FCS.
6. Pass the digested sponge through a 100 μm sterile cell strainer into a 50 mL centrifuge tube; permitting the removal of any undigested fibrous tissue. Pass the resulting suspension through a 70 μm sterile cell strainer to remove contaminating tubular fragments.
7. Wash the sieved cells by centrifugation (10,000 × *g*, 5 min).
8. Discard the supernatant and re-suspended the pellet in 1 mL Red Blood Cell lysis buffer and leave for 3 min at room temperature.
9. Wash the pellet by adding 15 mL PBS and centrifuging for a further 5 min at 10,000 × *g*.
10. Discard the supernatant and re-suspend the pellet in 1 mL PBS containing 2% FCS.
11. Finally, filter the sample through a 40 μm sterile cell strainer prior to FACS.
12. In our lab FACS was performed using the BD FACSAri™ II System (BD Biosciences, UK) equipped with five lasers and fluorescent detectors. Once **steps 1–11** have been performed to isolate the cells from GFP^+ and GFP^- mice sponge and kidney, prepare them as single cell suspensions in PBS/5% FCS. Incubate the cells in the dark at 4 °C for 15 min with the appropriate antibody combinations, with 5 min PBS/5% FCS

washes between stainings. Create sorting gates using sponge and kidney cells from GFP^- mice. Isotype control antibodies and OneComp eBeads (Catalogue ♯01-1111, eBioscience, UK) act as negative controls. Perform analysis using FlowJo Software Version 7.6.5 (TreeStar Inc., USA).

3.4 Induction of Hindlimb Ischemia

1. Anesthetize mice with inhaled isoflurane (5 %) in O_2 and maintain anesthesia thereafter at 2–3 % isoflurane in O_2.
2. Remove fur from the ventral surfaces of both hindlimbs extending up to the lower abdomen.
3. Immobilize mice (supine) on a non-reflective surface and secure hindlimbs parallel to the tail.
4. Using a laser Doppler scanner and Moor LDI software (5.3) carry out a preoperative laser Doppler scan, with the scan region comprising the whole of both hindlimbs and the corresponding length of tail.
5. Following completion of the laser Doppler scan, sterilize exposed skin of the left hindlimb by rubbing with Povidone Iodine solution.
6. Inject (subcutaneously) the analgesic Vetergesic (buprenorphine) diluted 1:10 in sterile H_2O at a dose of 0.05 mg/kg body weight.
7. In the supine position, on a heated mat, cover the animal with sterile drapes and secure the limbs extended with tape. Perform the rest of the surgical procedure using a surgical microscope at appropriate magnification.
8. Using fine forceps and surgical scissors make a 1 cm incision down the medial thigh, starting at the mid-point of the inguinal ligament.
9. Using blunt dissection with fine forceps, separate the subcutaneous fat to reveal the femoral vessels.
10. Secure the surgical field with a small retractor and topically apply 1 % lidocaine liquid to lubricate the field and cause arterial dilatation.
11. Displace the femoral nerve inferiorly to ensure it is not damaged.
12. Superior to the popliteal artery apply a temporary ligation with 5-0 suture silk to the femoral artery and vein, controlling tension by securing with a bulldog clip.
13. Isolate the femoral artery from the vein distal to this ligation using blunt dissection to reveal a section of femoral artery ~20 mm in length.
14. Apply two permanent double-knotted permanent ligatures to the femoral artery using 5-0 suture silk, first proximally, then distally.
15. Remove the temporary ligation and transect the femoral artery between the knots, 5 mm inferior to the proximal and superior

to the distal knots, respectively, using fine surgical scissors. Remove the ~10 mm length of femoral artery.

16. Close the skin using 5/0 nonabsorbable monofilament sutures and apply EMLA cream topically.
17. Allow mice to recover from anesthetic on a heated mat and monitor over the course of the experiment.
18. Perform a postoperative laser Doppler scan to confirm ablation of blood flow in the left hindlimb (Fig. 2) and then further laser Doppler scans to monitor hindlimb reperfusion at appropriate intervals (usually, 3, 7, 14, 21, 28 days) until termination of the experiment.
19. Carry out postexperimental color quantification of laser Doppler images using MoorLDI software (5.3).

3.5 Tissue Extraction Post-Hindlimb Ischemia

1. After 28 days and completion of all laser Doppler scans, cull mice by asphyxiation in CO_2.
2. Using forceps and surgical scissors, remove the skin from the left and right hindlimbs. All following stages are carried out on both the left and right hindlimbs.
3. Using blunt dissection on the medial thigh expose the adductor muscle. Isolate from other muscles and transect as distally and proximally as possible using fine scissors.
4. Isolate the gastrocnemius muscle from the lower limb and transect as proximally and distally as possible using fine scissors.

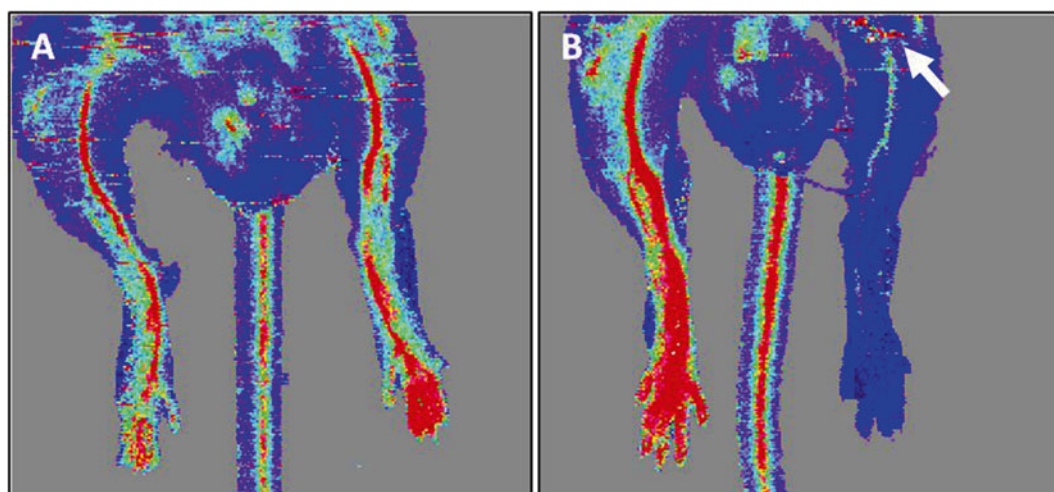

Fig. 2 Representative laser Doppler scans of mouse hindlimbs. Laser Doppler imaging (**a**) preoperatively and (**b**) postoperatively reveal abolition of blood flow in the left hindlimb following femoral artery ligation and reactive hyperemia in the right hindlimb and tail. Color from *red* to *dark blue* represents speed of blood flow and *arrow* indicates distal site of femoral artery ligation

5. Remove, using blunt dissection, the small section of dark brown muscle clearly visible on the inner gastrocnemius.
6. Cut both muscles in half across the shortest axis. Fix half of each muscle in 4 % paraformaldehyde in PBS at 4 °C overnight, then place in 70 % ethanol. Freeze the other half at −80°c prior to further analysis.

3.6 RNA Extraction from Muscle Tissue and cDNA Synthesis

1. Remove muscle samples from −80 °C and homogenize in 800 μL QIAzol® Lysis Reagent in a suitable Eppendorf tube for 2 min or until of uniform consistency.
2. Add 200 μL of chloroform to each sample and leave to stand for 2 min at R.T.
3. Centrifuge samples at 12,000 × *g* and 4 °C for 15 min.
4. Carry out RNA extraction using an RNeasy Mini Kit as per manufacturer's instructions. Store RNA at −80 °C.
5. Determine RNA concentration and integrity using a Nanodrop spectrophotometer and GelRed™ agarose gel electrophoresis.
6. Using the quantities determined by the Nanodrop spectrophotometer dilute each sample to a final concentration of 0.5 ng/μL in 12 μL using RNase-free water.
7. Synthesize cDNA from RNA using the Quantitect® Reverse Transcription Kit as per manufacturer's instructions, a Techne TC-512 thermal cycler and a reaction mix of 20 μL.
8. Store cDNA at −20 °C prior to qPCR analysis.

4 Notes

1. It is possible to investigate the effect of different drugs or factors on angiogenesis in the subcutaneous sponge implantation model, by impregnating sponges with these factors prior to insertion [7, 9].
2. Sponges can be compacted with the hemostat forceps for ease of implantation, as they will expand once securely in situ.
3. On removal of sponges, differential impregnation of sponges by blood vessels, at different time points, or due to different treatments/genotypes is often evident by visual inspection. Furthermore, when fixing sponges in 4 % paraformaldehyde, sponges pre-implantation or those with relatively little organic tissue will float, while sponges with a high density of organic tissue sink.
4. Due to its thin wall, the femoral vein is relatively fragile and easily punctured, especially when attempting to separate it from the femoral artery. The risk of rupturing the vein can be reduced by keeping the point of the forceps pointing away

from the vein and towards the artery, which is, by comparison, less prone to rupture.

5. In the event of rupture of the femoral artery or vein, hemostasis can usually be achieved by application of gentle pressure to the site of bleeding with a sterile cotton bud.
6. In the event of persistent or recurrent bleeding from the femoral vein, it may be necessary to ligate the femoral artery without complete separation of the artery from the vein. In this instance, cut each end of the artery proximal and distal to the ligations and remove as much of the artery as possible. Transect remaining section of artery along its length with fine scissors to prevent any recovery of blood flow.
7. The femoral artery branches at a number of points close to the site of ligation. Variation in the local anatomy between mice can influence the possible placement of the distal ligature.
8. The extent of hindlimb perfusion, at baseline and during experiments, inevitably varies between mice, especially due to size and weight of the mouse. To eliminate this variation and obtain a clearer representation of the relative change in blood flow, it is advisable to express hindlimb perfusion as a ratio relative to the tail perfusion [19]. It is also possible to use the right hindlimb to normalize values, but reactive postoperative hyperemia may act as a confounding factor.
9. When extracting RNA from tissues and following initial centrifugation to separate solutions, if only a small amount of aqueous phase is present, with a large amount of white, colloidal phase, add another 200 μL of Qiazol reagent and repeat centrifugation.
10. Suggested histochemical and immunohistochemical stains for assessment of sponges and hindlimb muscle tissue include; Hematoxylin and Eosin (H&E) staining for general tissue morphology; triple immunostaining for CD31 (endothelial cells), α-smooth muscle actin (αSMA) (smooth muscle cells, fibroblasts, pericytes and myofibroblast) and the nuclear stain DAPI; CD31 immunoperoxidase (DAB) staining for visualization and quantification of vessels; picrosirius red collagen staining; F4.80 DAB staining for macrophages; *Wt1*/CD31/αSMA/DAPI staining; *Wt1* DAB staining.

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