Chapter 4. Heterotopic vascularized hindlimb composite flap transplantation model in rats

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Rationale of model development

The origin of this model is the whole-limb transplantation model that became nowadays obsolete. In its orthotopic version, and even in its heterotopic one, there are noteworthy disadvantages (unusable, dragging leg, limping, necrosis, infection, pressure sore) that made the researchers look after new flaps that retained the advantages, yet replace the original model. Recently, various types of lesser heterotopic hindlimb models were proven to retain the types of tissues, along with the immunologic properties of the whole-limb allotransplantation, with the advantages of shorter operating time, higher success rate, less morbidity and mortality for the recipient rats. Besides the abundance in tissues, from highly immunogenic skin, to muscles, tendons, vessels, nerves, bone and fat, the flap contains vascularized bone marrow, the cornerstone in rodent model for allotransplantation, central tolerance induction, and maintenance through chimerism.
Originally included in the generous name of CTA (composite tissue allotransplantation), this type of tissue transfer received recently the precise name of VCA (vascularized composite allotransplantation/allograft), which explains also the way of the transfer. Needless to say, the major role of this kind of allotransplantation is the immunologic research that will hopefully translate the results in a bench-to-bedside manner, with benefit for the increasing number of allotransplanted patients.

With regards to the importance of the model in microsurgical training and skills assessment, compared to the original whole-limb transplantation, it is true that the complexity of the heterotopic models decreased, by missing steps like bone fixation and nerve anastomosis; however, those missing steps were skipped because they brought increased morbidity and mortality to the model. Nevertheless, heterotopic hindlimb models remain an important tool in the microsurgery training armamentarium.

The herein model is a heterotopic, non-functional model of vascularized hindlimb flap already described. Several advantages contributed to the choice of heterotopic version of the model: faster, allows normal ambulation, easy follow-up of the flap, less morbidity vs. orthotopic version. The purpose of this chapter is to describe the harvesting and transplantation techniques and share with the reader the rationale, tips and tricks of the operation, for an easy-life for those who attempt to perform this model. The use of this model must be performed in agreement with international and national accepted ethical regulations, principles and guidelines for animal research.
Experimental models in rodents

This model is our preferred model of vascularized composite tissue all transplantation and represents our personal experience in the Center for Vascularized Composite Allotransplantation, Chang Gung Memorial Hospital, Gueishan, Taiwan and Center for Simulation and Training in Surgery, “Grigore T. Popa” University of Medicine and Pharmacy of Iasi, Romania.

Steps in model development/implementation

1. Prepare operating team and materials:

Preferably have 2-3 surgeons experienced in microsurgery operating concomitantly, so both hindlimbs of the donor rat can be harvested and transplanted in timely-fashion. One operator can harvest the hindlimbs, while the other two surgeons can prepare the recipient areas and perform the transplants. This setting requests increased amount of equipment (microscopes and microsurgical kits). When 2 operators are involved in the operation, use 2 microscopes and 2 sets of instruments (both harvesting and transplantation should be performed under microscope magnification). Therefore, set microscope in advance: remember, it is a 3.5-4 hrs procedure for one transplant, and if you transplant both hindlimbs from the donor rat, operating time will increase to 5-6 hours. Without proper microscope setting, fatigue will become your enemy and fatally impact your outcome. Set your flow to minimize the number of switches from direct vision operation to operating steps under microscope. Place the macroinstruments separately, away from microinstruments (they are more delicate therefore subjected to damage). When macroinstruments are not needed until the closure of the
wounds, they must be put aside. Set microinstruments at reach without taking the eyes off the microscope. Set your experiment flow so you don't need to turn the rats from supine to lateral too many times.

However, if no help is provided from an assistant/research assistant or not enough equipment for concomitant transplantation, plan your operation flow carefully step by step, so no time or resources should be wasted. Prepare all in advance so, once the rat anesthetized, you can proceed with the operation: prepare operating instruments, solutions (heparin) and operating field yourself, before inducing the anesthesia. Plan contaminated and sterile operating areas in advance so no contamination occurs.

Harvest both hindlimbs and store one of them on ice for few hours, until first transplant is completed and you can therefore move to second transplant. If you plan concomitant harvesting and preparing recipient by 2-3 surgeons, but there is a shortage of anesthesia machines, one can use intramuscular anesthesia with ketamine and xylazine for the donor and inhalation anesthesia with isoflurane for the recipient rats. When all is planned and ready, you can proceed with the following steps.

2. **Prepare rats for surgery:**

Rats must be fasted 12 hours before operation. Induce and maintain superficial anesthesia, for preparatory stages (we do prefer inhalation anesthesia with isoflurane, but any other recommended anesthesia protocol can be used provided effective anesthesia for 3-4 hours and quick recovery is
ensured). With the inhalation mask on, sealed against rat's head, follow the next steps.

Shave completely distal from the lower ribs and prepare the shaved skin using povidone-iodine. Place a sterile drape on the heating pad and place the rat supine and do all subsequent steps taking care to maintain rat body temperature constant. Secure the animal to the operating board with sterile rubber bands or adhesive tapes and push-pins to fix the hindlimbs. The operation side may remain unrestrained, as the hindlimb will be mobilized for the drawing and turning anterior to posterior during operations; we recommend delicate dissection of the pedicle to be performed with hindlimb restrained as minor inadvertent movements of the rat can compromise the operation. Fix loosely /do not fix the upperlimbs, to allow unrestrained rat breathing. Secure the inhalation anesthesia mask over the head with adequate sealing and the corresponding tubing to the operating board. Take the anesthesia to the appropriate deeper level before starting the surgery - make sure the pain reflexes are absent.

3. Hindlimb flap harvesting in donor rat:

*Preoperative drawing:* With the rat supine and the hindlimbs unrestrained, draw the operative plan as in Figures 1 and 2.
Skin incision: The line on medial side of the hindlimb overlies the femoral pedicle and the circular lines on mid-thigh and above the ankle, respectively, delineate the skin paddle (4 x 3 cm).
For the skin paddle, start incising the line on the inner thigh or, for a more regular rectangular shape of the skin flap, excise a strip of skin and underlying fat in fan shape, tapered proximal to the knee and based at the inguinal ligament, 3 to 5 mm large. Complete the skin island with the proximal and distal circular incisions (Figures 3 and 4).
Experimental models in rodents

Fig. 3. The skin incisions on the medial aspect of the thigh

Fig. 4. The skin incision on the posterior aspect of the thigh
Tendons and muscles: While proximal to the ankle, identify the subcutaneous structures, three groups of tendons corresponding to the thigh muscle groups. Ligate the anterior and posterior tibial pedicles before sectioning, ensure good hemostasis. Cut the tendons, including Achilles tendon, using electrocautery. Tendons will retract proximally and push them by scraping along the tibia with a scalpel, until the fibular bifurcation is exposed. Complete hemostasis if needed. Do not cut the bone at this stage. If bone would be cut at this stage while femoral pedicle is not ligated, there will be unnecessary blood loss from bone stump during the remaining of the dissection. Bone cut after femoral pedicle ligation will minimize the blood loss through the bone medulla (Figure 5).
Experimental models in rodents

**Fat pad:** Skin will retract due to its elasticity and will expose the underlying subcutaneous tissue, organized proximally in form of the inguinal fat pad. With dissecting scissors, separate the skin from the fat pad. Ligate the superficial epigastric vessels and dissect the fat pad away from the thigh, but let it attached to the abdominal wall. Variation: the fat pad can be dissected away from the abdominal wall and included in the hindlimb flap, pedicled on the epigastric vessels, as in figure 5.

**Vessels dissection:** under microscope, dissect and skeletonize the femoral vessels individually (artery and vein, respectively) from the inguinal ligament to 2-3 mm proximal to epigastric pedicle branching point. If longer pedicle is intended, cut the inguinal ligament and dissect the femoral vessels more proximally. However, by doing so, one can gain only a few millimeters in vessels length, but this comes with some drawbacks: proximal to the ligament there are several side branches difficult to ligate and the ligation sutures too close to the vessel ends will get in the way when performing microanastomosis to the recipient vessels. Also, the diameter of the vessels increases as dissection proceeds more proximally, creating a considerable donor to recipient vessels size discrepancy. Skeletonize vessels using 8/0 or 9/0 suture ligation, or with bipolar cautery, set at very low intensity. Dissect the femoral nerve (situated laterally from the artery) and cut it proximally.

**Muscle:** at a smaller magnification, using an electrocautery, cut and cauterize thigh muscles starting medial to the femoral pedicle, together with nerves and connective tissues, at the distal third of the thigh. Circle around the thigh and cut
posterior and lateral muscles, taking care not to damage the femoral pedicle.

At this point, for the first cases we used to leave a strip of 4-5 mm thick rectus femoris muscle around the skeletonized femoral vessels; the cuff protected against elongation, torsion or avulsion of the pedicle during forceful operating steps such as bone cutting or muscle cutting with cautery accompanied by strong muscle contractions. We performed the bone sectioning, femoral vessels ligation and division, and remaining muscle strip sectioning in this order, for three reasons: first, is our preference to check again the bleeding and to revise hemostasis, before the complete detachment of the flap; the second reason is the longer ischemia time if we would have cut the pedicle before bone and muscle sectioning (steps that were pretty long in the beginning); the third reason is the need for the muscle strip to become a nice padding with adequate length between the bone stump and the femoral pedicle, to prevent friction injury and rupture of the vessels by the uncovered bone, therefore we preferred to tailor the muscle length according to needs, as the last step. We still recommend this sequence for the beginners.

However, with practice, we changed this approach, i.e. ligate and severe the femoral pedicle right after skeletonization, flip the vessels 180 degrees over the distal thigh, with the remaining of the muscle and bone sectioning done under ischemia, because we go faster and in the economy of the whole operation, this sequence we adopted recently appears timely-fasion. Moreover, with the vessels reflected away from the muscle incision site, we can very fast cut through the muscles with electrocautery until the bone is reached without injuring
the pedicle. No matter which sequence you choose, ligate the vessels individually as proximal as possible if extra length is intended. Cut vessels immediately distal from ligature site and write down the ischemia starting time. Thigh muscles must be cut at mid-thigh or in distal third of the thigh. Scrape thigh muscle insertion from the femoral surface and cut the femoral bone with rongeur bone cutters. Cut the tibia distal to the bone bifurcation, try to make all the bone cuts clear and even, without bone spikes or sharp edges that can damage the vessels. Once both bones are cut distally, the hindlimb flap is completely separated from the donor rat. Under microscope magnification, irrigate the femoral artery with heparin saline (10 units/mL) through a 24G angio-catheter sheath; if the catheter is too big, dilate progressively the artery with the micro forceps or specially-designed dilator.

Handling the artery only by the adventitia, insert partially the catheter in the lumen (around 1 cm), advancing parallel to the vessel walls, preventing thus intima injuries, and inject slowly, with constant pressure, 10 mL of heparinized saline. Initially blood will flow out through the femoral vein, and slowly there will be clear saline coming out. This step concludes the hind limb harvesting (Figure 6).

![Fig. 6. The hindlimb flap, separated from the rat. On the background, femoral artery is cranial and the femoral vein is caudal.](image)
If one proceeds to transplantation directly, transfer the hindlimb as it is, to the operator. If transplantation is delayed, wrap the flap in wet saline gauze and place on ice, but avoid direct contact of wet gauze and ice, by interposition of dry gauze on top of the ice. The same technique is used for the second hindlimb. After hindlimb harvesting, the donor rats will be euthanized, using recommended protocols.

4. Heterotopic hindlimb flap transplantation.

*Prepare the recipient vessels:* Prepare, drape and secure the rat supine, in the same manner as mentioned above. Incise along the inguinal ligament over 3-cm line. Immediately under the dermis lies the inguinal fat pad, easily recognized by its yellow bright color (Figure 7).

![Fig.7. In the recipient rat, 3 cm incision over the inguinal ligament, for pedicle access and preparation](image)
Ligate the epigastric vessels at their origin and cut the fat pad distally, but live it attached proximally to the abdominal wall. Retracting the fat pad medially will expose the recipient femoral pedicle. Separate the femoral artery from the vein. Skeletonize the femoral vessels, from inguinal ligament downstream, proximal to epigastric vessels stump, ligating the sizable side branches from the artery (i.e. Murphy's branch) and vein, using 9-0 Nylon; for smaller branches, use the bipolar cautery. If longer femoral vessels are intended, incise the inguinal ligament and free the vessels more proximally. Free the distal part of the femoral vessels as far as distally possible, just proximal to the branching point of the epigastric vessels; noteworthy, if pushed too much distally, dissection in this branching area becomes tedious. With the artery and vein individually skeletonized along their length, one can move now to the back of the rat. If dissection is tedious and not delicate enough or even brutal, with occurrence of stretch marks or pinching of the vessels (especially the vein), allow the vessel to recover from spasm, for the time one works on the rat's back.

Tip: If vessels mismatch is considerable between donor and recipient vessels, you may place temporarily a microvascular clamp distal to epigastric artery stumps, on the superficial femoral artery, and let the pulsatile blood flow dilate the distal part of the vessel. However, one must keep in mind, this extra time with the vessels clipped may count for increase in thrombotic complication rate, as the stasis is a critical factor for thrombosis and also the prolonged pressure on the vessel may produce microscopic ischemia lesions, contributing to thrombosis as well. Even though at this stage this may count as a theoretical factor only, it is important to remember this for your own good practice guidelines.
As an exercise, considering the recipient femoral vessels length and arc of rotation, anticipate the future anastomosis site and try to approximate the position of the flap inset and donor pedicle reach to meet the recipient vessels. The aim of the mental exercise is to foresee if the pedicle is in danger of being tensioned, kinked or avulsed during ambulation, and find ways to prevent such complications. It might be difficult in the beginning but with practice, it will come. One should not forget microsurgery is a combination of reflection and technique. One without the other will surely mean a disaster (in our case, the failure of the flap).

**Prepare the recipient site for the flap:**
Position the rat in right lateral decubitus and observe the left hip area. Flex and extend the thigh to notice the hip muscle mound movement. Considering donor and recipient vessels length and prospected anastomosis site, thigh movements, flap size and the inguinal incision, make a plan to where to place the incision for flap inset, in order to avoid tension on the flap, pedicles or anastomosis.

As a general rule, place 3-cm curvilinear incision on the lateral side of the hip, right above the hip muscle mound. Preserve 1-1.5 cm intact skin paddle between the proximal ends of the 2 incisions. Undermine the skin proximally and distally to create a pocket similar to the size of the donor hindlimb flap, roughly 4/3 cm (Figure 8).
Undermine the skin anteriorly to create a tunnel towards the groin area, over the muscles mound of the hip, enough to accommodate the proximal part of the hindlimb flap; the tunnel will serve also as passage of the donor pedicle anteriorly, to reach the recipient vessels (Figure 9). Perform hemostasis if needed.
Inset of the hindlimb flap: bring the flap in the previously created defect on top of the hip area; place the flap with the skin paddle outside and the proximal side anteriorly towards the groin area. Check (and do not just assume) donor pedicle passage through the tunnel under the intact skin area, in the vicinity of the recipient vessels. Fix the flap circumferentially to the adjacent skin with absorbable 3/o or 4/o stitches through skin and muscle fascia. If you intend more stable fixation, add a few stitches through the donor hip muscle, to fix the flap to the surrounding recipient muscle. Take care not to injure the pedicle during this step (Figure10).
It may occur in the beginning that the donor flap is misplaced: if it is too posteriorly and the anastomosis would be in tension, prolong anteriorly the hip incision, advance and re-inset the flap. If the flap is placed too anteriorly, the only danger concerns the effects on the pedicles and anastomosis, such as kinking and twisting. If not strongly angulated, the pedicle remains patent because, during ambulation, the muscle contractions favor the blood circulation through the pedicles.

For the early cases, we used to just anchor the flap in the beginning, turn the rat supine, perform the anastomosis, then turn again the rat on a side to complete the flap inset and check the anastomosis again before inguinal incision closure. Now we prefer to complete the flap inset at this stage then turn the rat supine, complete microanastomosis, close inguinal wound and wake up the rats; in this way we turn less often the rat from supine to lateral and moreover, we do the microsurgical work...
in one step, without interruption and switch from direct vision to microscope.

*Microvascular anastomosis:*
The donor site in the form of right hindlimb transferred on the left side of recipient rat has noteworthy advantages. After flap inset and bringing the donor and recipient pedicles together, the vessels have the same spatial disposition, with vein on top (cranial) and the artery in a more distal (caudal) position (Figure 11).

![Image](Fig.11. Parallel placement of the vessels, ready for the anastomosis, with the vein on top (cranial) and the artery placed caudally)

This parallel placement of the vessels allows for unrestricted handling of the vessels with no torsion or twisting of the vessel around each other. Moreover, the position of the pedicles are top right for donor to bottom left for recipient, which makes the easiest anastomosis technique, bringing the needle always form top right to left bottom, the ergonomic technique for right handed surgeons. Last, but not least, the artery placed
more caudal, during the ambulation becomes deeper in the angle between the hip muscle mound and dorsal muscles, with the vein on a more superficial position, therefore less prone to compression and thus protecting the anastomosis site. If the transplantation is performed to the right side of the recipient rat, the microsurgical technique will be incomparable more difficult, the hand will have to move from right bottom to the upper left position, highly uncomfortable for right handed surgeons.

If the flap muscle in the vicinity of the pedicle is too bulky, it will cover the pedicle and make the access and the handling of the vessels more difficult, therefore it needs trimming in order to perform the microanastomosis easier. This is the reason we advised for muscle trimming in the donor rat at the mid-thigh or even distal third of the thigh. The muscle cuff should be long enough only to allow fixation of the flap to surrounding tissues and to prevent direct contact of the sharp edges of the femoral bone with the vessels.

The anastomosis technique is described elsewhere and it is not our purpose here to get through the technique. We strongly recommend to perform this model only after achieving good basic skills in microsurgery and to strive for proficiency level. However, because it is a model more complex than simple vascular anastomosis under microscope, we will share our strategy and a few tips for this step.

We already emphasized about long operating time under microscope, how to not change too often from direct vision to microscope, but rather use the microscope vision at different magnification, throughout the operation, and how to avoid
turning too often the rats back and forth from supine to lateral. The microanastomosis after gross operating steps (like muscle sectioning, bone cutting and flap inset) can be challenging, due to fatigue and fine fingers tremor. Find your own operating steps flow that will put your hands at ease and help you to do the microsurgery in good conditions. If you feel the complete flap inset will induce you hesitating movements during microsurgery, choose to only fix the flap with 2-3 stay-stitches and complete the inset after the vessels anastomosis. As mentioned before, we prefer complete flap inset, turn the rat supine, perform the artery anastomosis and use this step as hands warming up before ending the microsurgical steps with the more challenging vein anastomosis. Minimal vessels size mismatch may be present, originating from differences in level of the pedicle stumps, over 3 cm vessels span: proximal for the donor vessels and distal for the recipient vessels. Even though, we didn’t encounter any difficulty in performing the microvascular anastomosis and it is our belief that, with good microsurgical skills on board, this matter should raise no problem at all.

In practical terms, ligate the recipient vessels proximal to the epigastric vessels branching point. Do not worry about the survival of recipient rat hindlimb in the absence of the main femoral pedicle, in more than one hundred transplants performed, no arterial insufficiency was noted, because the dorsal vascularization of the hindlimb suffices to avoid acute ischemia. Place one of the approximator clamps for recipient femoral artery before dividing it and the other clamp of the approximator on the donor artery. With the approximator clamps at maximum opening, place the 2 ends of the arteries uneven, longer for the recipient artery. At the end of the
anastomosis you will use this extra length to place a single clamp immediately proximal to the arterial anastomosis site and to remove the approximator in order to use it for the veins. This is helpful when limited resources are available (1 approximator per set), but also has other practical reasons. Of course you can keep the approximator on the artery while working on the vein, but placing a second one for the vein will make things too crowded in the already narrow working space. Moreover, remember this setting is not as in the simple vessel anastomosis model. The approximator will never flip for 180 degrees so train you to imagine ways to perform anastomosis in a less-than-standard spatial disposition of the vessels. Place the approximator keeping in mind to avoid the crowding of the clamps, while doing the vein, which is more demanding technically. Relieving the pressure of the approximator clamp and moving the pressure site of the single clamp slightly distally counts for decreased thrombotic complications that can appear due to long time pressure during the vein anastomosis.

We do follow the same steps in preparing the vessels stumps, i.e. irrigation (with heparin saline), adventicectomy and dilation before starting the suturing. Separate 6 to 9 stitches are placed to complete the anastomosis. Green or blue color background is very useful and saves time; placed on top of the artery it can separate working space for vein anastomosis and if properly tailored, helps in maintaining the water media in which the vein is more visible and easy to work on.

Once the artery anastomosis performed and setting ready for next step, ligate the recipient femoral vein and place it in approximator opposite to the donor vein and perform vein anastomosis using 10-0 micro suture.
Remove background, release approximator from the vein, first distal then proximal clamp and last one the artery single clamp. Observe for the reflow, artery pulsation, and vein fullness and perform Acland’s “milking” test to check for anastomosis patency. The remaining heparin saline solution from the flap will "bath" the anastomosis with protective antithrombotic media. Check the position of the vessels at ease and during passive hip and thigh movements and make sure before closing there will be no kinking, torsion, elongation and tension in the anastomosis site.

**Closure:**
If possible, as Acland advised, pull the fat pad over the pedicles and anastomosis site in order to provide hemostatic padding around anastomosis and nice soft tissue padding and mechanical protection against strong surrounding tissues, during ambulation. If the fat pad was not included in the hindlimb composite flap, use the recipient fat pad attached to the abdominal wall (Figure 12).

![Image](image.jpg)

*Fig. 12.* After the anastomosis, the pedicles can be covered with the inguinal fat for soft tissue padding
Close the inguinal incision using the same absorbable material (Figure 13). Clean rat skin from all traces of blood, because the smell of blood can provoke auto cannibalism. Cut the anesthesia and place the rats individually in cages, on right lateral side position. Keep detailed evidence of operating times, accidents, incidents, operation abandon or failure and the causes. Keep also record of all important facts throughout the follow-up period.

**Follow-up:**
Immediate postoperatively follow the waking up process, the resuming of the ambulation and feeding. After isoflurane anesthesia, the recovery is fast and resuming ambulation the same. Use recommended protocols for postoperative analgesia,
either injection or by gastric feeding tube, while the rat is still under anesthesia.

The flap is placed in an easily noticeable and accessible position; therefore daily clinical evaluation is straightforward to assess the evolution of the transplant. When the appearance of the flap is doubtful, pinprick or scoring the flap skin with a needle can help you judge flap circulation. The most important physical signs to be assessed include the color, temperature, and quality of capillary filling, bleeding from a cut edge of the flap and tissue turgor. A pink color with 1-to-2-second refill is consistent with good perfusion. When the flap exhibits arterial insufficiency, it looks pale, flaccid, without capillary refill or bleeding and is cooler. When vein occlusion with venous insufficiency occurs, the flap looks congested, blue, with brisk capillary refill and dark bleeding at pinprick.

When there is the case of allotransplantation (between MHC-mismatch donor and recipient rats), the flaps should be inspected daily for clinical signs of rejection such as edema, desquamation, hair loss, epidermolysis, exudation, and skin necrosis. A visual scoring system helps in evaluating the rejection, graded from 0 (pink skin, no sign of rejection) to 4 - blue purple skin, no bleeding from the flap, which indicates rejection.

Benefits and limitations

The heterotopically transplanted hindlimb osteomyocutaneous flap in rats is an excellent model for training in flap transfer and in allotransplantation research. The operation combines fine dissection skills, working under microscope using microinstruments, reflection about the inset, operating steps
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flow, saving and rational using of the personal and material resources, microsurgical anastomosis, and follow up. It is actually a highlight of combination between technique and reflection. Moreover, the advantages mentioned in the introductory chapter, recommend this flap over the hindlimb orthotopic transplantation models in rats. The flap transplantation is a lengthy procedure that requires proficiency level in microsurgical techniques and a high degree of reflection regarding intraoperative decision-making algorithm. Nevertheless, we find it a rewarding operation with high-degree of satisfaction when it is successful. Thus, we strongly emphasize the tips and trick generously shared with the reader throughout the manuscript, with sole aim to relieve frustration and approach this operation with high success rate.

To a larger scale, this operation is a preparatory step and a very good example of the bench-to-bedside principle. With the advent of refined microsurgical reconstructions, the hindlimb flap is in no way different than the custom made, free-style small-sized flap used in human for highly specialized soft-tissue defects. Moreover, the patients with complex defects amenable only to all transplantation will benefit from the results of the extensive all transplantation research that is using this experimental model in rats.

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