Chapter 14

The Rip1Tag2 Transgenic Mouse Model

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Abstract

The Rip1Tag2 transgenic mouse model of β-cell carcinogenesis has been instrumental in studying various aspects of tumor angiogenesis and in investigating the response to anti-angiogenic therapeutics. Thereby, the in-depth assessment of blood and lymphatic vessel phenotypes and functionality represents key experimental analyses. In this chapter, we describe basic protocols to assess tumor blood vessel morphology (pericyte coverage), functionality (perfusion, leakiness, and hypoxia), lymphatic tumor coverage, and tumor cell proliferation and apoptosis based on immunofluorescence microscopy analysis.

Key words Rip1Tag2, PNET, Insulinoma, Tumor angiogenesis, Anti-angiogenic therapy, Endothelial cells, Immunofluorescence

1 Introduction

Generated in 1985, the Rip1Tag2 transgenic mouse model of pancreatic neuroendocrine tumors (PNETs) has ever since served as a versatile tool to study various aspects of tumor angiogenesis. In this model, the simian virus 40 large T-antigen (*Tag*) oncogene is expressed under the control of the rat insulin gene promoter (*Rip*) leading to multifocal development of insulin-producing β-cell carcinoma (insulinoma) of the islets of Langerhans in the pancreas [1]. Whereas all β-cells present in the approximately 400 islets of Langerhans contain the property to express the *Tag* oncogene at birth, stochastically occurring additional genetic and epigenetic events are required for successful stepwise carcinogenesis. Such an event is for example the acquired capability to express insulin-like growth factor 2 (IGF2). Only about 1–2 % of all islets eventually progress into highly vascularized solid tumors [2]. Finally, Rip1Tag2 mice start to die between weeks 12 and 14 of age due to the tumors' excessive production of insulin resulting in fatal hypoglycemia (*see* **Notes 1** and **2**, [3]).

Primary tumor growth in the Rip1Tag2 mouse model is highly dependent on active tumor angiogenesis. The acquisition of new

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blood vessels (i.e., the angiogenic switch) has been shown to be a critical event in order to progress from hyperplastic lesions to adenoma, which is a prerequisite for further development into invasive tumors (carcinoma) [4]. Furthermore, tumor growth is prevented in Rip1Tag2 mice with a β-cell-specific deletion of the major pro-angiogenic molecule VEGF-A [5]. In the following years, the Rip1Tag2 model has revealed important insights into the functions of key angiogenesis mediators by their β-cell-specific deletion or transgenic expression [6–12]. In addition, this PNET mouse model has proven highly instrumental for preclinical validation of eligible biological or pharmacological anti-angiogenic compounds, some of which have been subsequently successfully implemented into clinical practice [13].

Tumor angiogenesis is generally considered to be overshooting, producing abnormal vessels with poor pericyte coverage and a highly fenestrated endothelial layer, thus promoting leakiness and increased interstitial fluid pressure. Intriguingly, despite an abundance of pro-angiogenic factors, regional differences in blood vessel perfusion can result in hypoxic areas with low pH [14]. The delivery of chemotherapeutic agents to tumors is hampered because of hypo-perfused areas and increased interstitial fluid pressure caused by the abnormal vasculature. Vessel-normalizing interventions have been shown to increase chemotherapy availability in tumors [15, 16]. Key to the identification of quantitative and qualitative tumor blood vessel characteristics, i.e., vessel functionality, are methods based on immunofluorescence (IF) analysis.

In this chapter, we provide a simple workflow for routine assessment of the most important parameters of tumor blood vessel characteristics in Rip1Tag2 mice. Analysis of blood vessel microvessel density (MVD; i.e., the number of CD31-positive blood vessels per tumor area) provides quantitative insights into the extent of angiogenesis. This can be further complemented by injecting fluorescently labeled lectin (typically fluorescein/FITC or Texas Red) into the mice prior to euthanization to label the vessel lumen and to assess the percentage of actually patent (i.e., perfused) tumor blood vessels. Similarly, leaky vessels are identified by extravasation of FITC-labeled dextran. Pericytes are perivascular cells localized on the abluminal site of endothelial cells, sharing a common basement membrane and stabilizing the vessel tube. To date, a marker exclusively expressed by pericytes has not been identified [17]. However, the marker neuron-glia antigen 2 (NG2) known to be expressed by pericytes is commonly used to assess pericyte coverage of tumor blood vessels in the Rip1Tag2 model when combined with a staining for CD31 [3]. In the Rip1Tag2 model, staining for α-smooth muscle actin, a marker often used to visualize perivascular cells, only results in a strong staining around large blood vessels, but not around capillaries. To estimate the consequences of an experimentally altered MVD on tumor

oxygenation, the aforementioned characterization of the tumor vasculature can be complemented with the assessment of tumor hypoxia. Intraperitoneally (i.p.) injected pimonidazole hydrochloride (HCl) is chemically reduced in hypoxic areas, and the resulting pimonidazole adducts can be visualized by IF staining.

Lymphatic vessels represent an alternative route for metastatic spread in addition to tumor blood vessels. Indeed, the transgenic expression of VEGF-C or D in β-tumor cells of Rip1Tag2 mice, VEGF family members known to induce lymphangiogenesis, massively increased peritumoral lymphatic coverage and promoted lymph node metastasis [10, 18]. Assessing the peritumoral lymphatic coverage of tumors of Rip1Tag2 mice therefore provides insights into lymphangiogenesis-promoting or -inhibiting mechanisms (*see* **Note 3**).

The ultimate readout of most experimental manipulations in the Rip1Tag2 mouse model is the impact on tumor volumes (*see* **Note 4**). To assess the basis of changes in tumor growth, analysis of tumor cell proliferation by staining for phospho-histone H3 (pH3) and of tumor cell apoptosis by staining for cleaved caspase 3 (cCasp3) can give first insights into the mechanisms underlying a potentially altered tumor burden. In addition, performing a combination staining for CD31 with pH3 or cCasp3 reveals the amount of proliferating and dying endothelial cells, respectively.

The methods presented in this chapter are meant to allow a routine workflow as a basis for further morphological, biochemical, and molecular biology experiments. While the protocols presented here have been specifically described for the use with the Rip1Tag2 transgenic mouse model, they have proven to be functional in a variety of other mouse models of cancer, including xenograft and syngeneic tumor transplantation models and other transgenic mouse models of cancer.

2 Materials

Rip1Tag2 mice

Depending on the source and genetic background, Rip1Tag2 mice start to die approximately at the age of 12–14 weeks. Due to this short survival, the mouse colony has to be constantly bred to prevent loss of the line. In addition, due to the complications caused by the tumor insulin production during pregnancy and nursing, Rip1Tag2 females are not being used for breeding. Rip1Tag2 mice should be strictly kept in a C57BL/6 background, since the genetic background of the mice significantly affects tumorigenesis [19]. Hence, heterozygous transgenic males are bred with wild-type C57BL/6 females. Both heterozygous females and males are used for experiments. Although no apparent gender-dependent phenotypic differences in terms of tumor development have been

observed, it is recommended to stratify experimental groups based on sex. Genotyping of the mice is performed by employing the primers Tag1 5′GGACAAACCACAACTAGAATGCAG and Tag2 5′CAGAGCAGAATTGTGGAGTGG. The resulting PCR product has a size of 449 kb.

2.1 Lectin or Dextran Injection and Mouse Perfusion

1. Fluorescein Lycopersicon Esculentum (Tomato) Lectin (FITC-Lectin; Vector Laboratories/Reactolab), diluted in sterile PBS to 1 mg/ml.
2. Dextran Fluorescein (FITC-Dextran; Life Technologies), anionic, lysine fixable, 70,000 MW, dilute in sterile PBS to 1.25 mg/ml.
3. Pimonidazole Hypoxyprobe (TM-1 Omni Kit Hydroxyprobe Inc.), pimonidazole HCl plus rabbit antisera, dilute in sterile PBS to 6 mg/ml.
4. Ethanol 70 % spray.
5. Surgical scissors.
6. Forceps.
7. Insulin syringe, BD Micro-Fine, 29G.
8. Anesthetic, according to the locally licensed compounds for terminal anesthesia (e.g., Pentobarbital).
9. “Butterfly,” BD Valu-Set, 25G.
10. Syringe, 10 ml.
11. PBS (sterile for diluting lectin, dextran, and pimonidazole; non-sterile for mouse perfusion and tissue processing).
12. PBS/4 % PFA.

2.2 Tissue Fixation and Cryopreservation

1. PBS/4 % PFA.
2. PBS/20 % sucrose.
3. Optimal cutting temperature (OCT) compound (Tissue-Tek).
4. Embedding mold for frozen tissues.
5. Dry ice pellets.
6. Ethanol 100 %.

2.3 Cryosectioning and Immunofluorescence Staining

1. Cryotome.
2. Microscope slides.
3. Cover slips.
4. Liquid blocker, Super Pap Pen (Daido Sangyo Co., Ltd. Tokyo, Japan).
5. PBS (non-sterile).
6. PBS/0.2 % Triton X-100.
7. PBS/5 % or 20 % normal goat serum (ngs).

8. DAPI 1:10,000.
9. Fluorescent Mounting Medium (Dako) (*see* **Note 5**).
10. Primary antibodies, dilutions:

    CD31, rat (BD Pharmingen, 550274), 1:50.

    Cleaved Caspase-3 (cCasp3; Cell Signaling, 9664), 1:50.

    Insulin, guinea pig (Dako, A0564), 1:200.

    LYVE-1, rabbit (RELIATech, 103-PA50S/0412P02-2), 1:200.

    NG2, rabbit (Chemicon, AB5320), 1:100.

    Phospho-Histone H3 (pH3), rabbit (Millipore, 06-570), 1:200.

    Pimonidazole, rabbit (Hypoxyprobe Inc.), antibody included in "Pimonidazole Hypoxyprobe TM-1 Omni Kit", 1:25.

    SV40 Large T antigen, rabbit (Santa Cruz, sc-20800), 1:50.
11. Secondary antibodies, Alexa labeled (Molecular Probes/Life Technologies).

3 Methods

3.1 Injections, Systemic Perfusion, and Excision of Pancreas (See Also Note 6*)*

1. Inject all animals with pimonidazole 60 mg/kg (inject 100 µl per 10 g mouse body weight of a 6 mg/ml solution) intraperitoneally 1–2 h prior to euthanization (*see* **Note 7**).
2. If the anesthetic licensed for terminal anesthesia provides fast narcosis, the intravenous (i.v.) injection of FITC-lectin or FITC-dextran can be performed in non-anesthetized animals, followed by anesthesia (methods adapted from refs. 16, 20, 21).
3. For the injection of FITC-lectin, inject 100 µl of FITC-lectin i.v. into the tail vein (0.1 mg/mouse, perfuse after 10 min).
4. For the injection of FITC-dextran, inject 200 µl of FITC-dextran i.v. into the tail vein (0.25 mg/mouse, perfuse after 5 min).
5. For the perfusion, anesthetize the mouse with an ultra-deep (terminal) and fast-acting anesthesia as licensed by the local veterinary office (*see* **Note 8**).
6. Pin the extremities of the animal on a dissection pad, spray it with ETOH 70%, and incise the skin and the underlying peritoneum with a horizontal cut immediately *caudal* of the costal arch using scissors. Now, the *caudal* surface of the diaphragm should be visible.
7. Grab the *processus xiphoideus* with forceps and induce a pneumothorax by incision of the diaphragm. The resulting collapse of the lungs prevents them from damage potentially caused during the dissection procedure. Introduce the scissors carefully along the interior surface of the ribs in direction of the axilla

and cut the ribs. Repeat this on the other side. Free the resulting flap containing the cut ribs and the *sternum* from remaining *caudal* attachments to the diaphragm, fold it, and pin it over one of the shoulders on the surface. Now, a free look on the collapsed lungs and the heart should be possible.

8. Introduce the needle of the butterfly into the left ventricle of the heart (due to the oxygenated arterial blood it shows a lighter red color than the right ventricle) and cut into the right *atrium* to open the circulation and provide an opening for the perfused solution.
9. For the perfusion of FITC-lectin-injected animals, perfuse the FITC-lectin-injected animal slowly with 10 ml ice-cold PBS/4% PFA for immediate fixation, immediately followed by 10 ml ice-cold PBS (*see* **Note 9**).
10. For the perfusion of FITC-dextran, perfuse the FITC-dextran-injected animals slowly first with 10 ml ice-cold PBS to wash out the intravascular dextran, immediately followed by the perfusion with 10 ml ice-cold PBS/4% PFA for fixation (*see* **Note 10**).
11. In order to dissect the pancreas (and other abdominal organs such as the liver), widen the existing abdominal incision to have free access to the abdominal organs. Move the intestine to the right side of the animal and identify the spleen. Pull the spleen carefully. This helps to identify the pancreas, which is the yellowish tissue containing red, vascularized tumors and is connected via the *ligamentum pancreato-lienale* to the spleen.
12. Dissect the pancreas, rinse it briefly in PBS to get rid of blood, measure the diameter of the macroscopic tumors using a ruler, and fix the tissue as soon as possible in cold PBS/4% PFA. Incubate the tissue in PBS/4% PFA for 2 h while rotating at 4 °C, followed by overnight incubation at 4 °C in PBS/20% sucrose.
13. On the next day, prepare a bath with dry ice pellets covering the floor of a Styrofoam container and add ethanol 100% (*see* **Notes 11** and **12**). Snap-freeze the tissues in optimal cutting temperature (OCT) freezing solution in the ethanol/dry ice bath. Store samples at −80 °C.

3.2 Cryosectioning and IF Staining

1. Put frozen OCT blocks at −20 °C (into cryotome or freezer) >30 min before starting sectioning (*see* **Notes 13–15**).
2. Cut 7–10 μm thick sections (*see* **Note 16**).
3. Let it dry for at least 30 min.
4. Encircle the sections with a liquid blocker and let it dry for some additional minutes.
5. Rehydrate in PBS 3 × 5 min (*see* **Note 17**).

6. Permeabilize in PBS/0.2% Triton X-100 for 20 min.
7. Wash in PBS 3×5 min.
8. Block with blocking buffer (PBS/5% ngs) for 1 h in a humid chamber. For staining with the anti-cCasp3 antibody perform the blocking with PBS/20% ngs.
9. Replace the blocking buffer with the desired antibodies diluted in blocking buffer. Incubate in a humid chamber overnight at 4 °C (*see* **Note 18**).
10. On the next morning, wash the specimen 3×5 min in PBS at room temperature.
11. Incubate for 1 h at room temperature with secondary antibodies directed against the species of the corresponding primary antibodies and labeled with Alexa fluorochromes suitable for the filters of the fluorescence microscope available.
12. Wash in PBS 3×5 min.
13. Incubate with DAPI diluted 1:10,000 in PBS at room temperature for 10 min in a humid chamber.
14. Wash in PBS 3×5 min.
15. Mount slides with cover slips using DAKO mounting medium (*see* **Note 19**). Avoid air bubbles.
16. Let slides dry (in the dark) for some hours at room temperature. Then transfer them to 4 °C and analyze the slides within few days.
17. We recommend acquiring the images using a 20× magnification for the assessment of MVD, pericyte coverage, vessel perfusion, and leakiness. For the analysis of the hypoxic area fraction, a 10× magnification is recommended.
18. For image analysis we routinely employ ImageJ image processing and analysis software.

4 Notes

1. Although the Rip1Tag2 mice used in different laboratories around the globe originate from the same founder line, breeding in isolation over years has led to interesting phenotypic differences even when kept in a pure C57BL/6 background. Notable differences in survival, lymph node, and liver metastasis at baseline and upon anti-angiogenic therapy, and the extent of intra- and peritumoral lymphangiogenesis, can be detected when screening the literature [3, 11, 21–26].
2. Rip1Tag2 mouse survival can be extended by the administration of food pellets consisting of 60% glucose starting from the age of around 9 weeks.

3. Insulinoma in the Rip1Tag2 model only rarely displays intratumoral lymphatic vessels. Instead, they can be found at peritumoral location. Whereas peritumoral lymphatic vessels in Rip1Tag2 single transgenic mice only cover less than 10% of the tumor circumference, almost complete lymphatic coverage can be achieved by intercrossing Rip1Tag2 mice with Rip1-VEGF-C or D transgenic mice [3, 10].
4. Tumor volumes of macroscopic tumors (≥1 mm diameter) are assessed by measuring the diameter of each tumor, calculating the volume assuming a spherical shape (volume = (4/3)*π* (diameter/2)^3), and summing up the resulting volumes per mouse.
5. DAKO mounting medium can be replaced with Mowiol.
6. Blood vessel perfusion and leakiness can in principle be analyzed in the same mouse by co-injecting FITC-dextran and Texas-Red-labeled lectin. However, this reduces the possibilities of future IF co-staining, since the "green" and the "red" channels are occupied in this setting. Additionally, please note that DAPI is always accompanying the IF stainings. Therefore, we usually split the mouse cohorts into three groups. All the animals receive an injection of pimonidazole, one-third of the animals FITC-lectin or FITC-dextran, respectively, and one-third pimonidazole only. Furthermore, tissues from the latter group can be conveniently used for RNA or protein isolation, since the pimonidazole injection alone does only require a (immediate) post-dissection fixation in PBS/4% PFA, but not a systemic PBS/4% PFA perfusion prior to organ dissection, as it is the case for FITC-lectin- and FITC-dextran-injected animals.
7. Pimonidazole HCl displays a half-life of about 0.25 h in mice. To avoid artifacts introduced by hypoxia/anoxia during the euthanasia and dissection procedure, pimonidazole levels in the systemic circulation should therefore be sufficiently low at the time of euthanasia. Therefore, the manufacturer recommends to dissect the mice 1–2 h (the chosen time point should be strictly kept for all the animals in the same experiment) after injection, using a fast euthanasia technique (cervical dislocation rather than CO_2 suffocation) [27].
8. In principle, mice could also be perfused shortly after death by CO_2 suffocation. If mice are co-injected with pimonidazole, however, CO_2 suffocation should be avoided (*see* **Note** 7). In this case, any terminal anesthesia method licensed by the relevant veterinary office can be employed to obtain a humane peri-mortal perfusion.
9. Changing from the syringe containing 10 ml PBS to the 10 ml syringe containing PBS/4% PFA should ideally be performed by a second assisting person. Like this, the needle can remain

in the exact same position in the left ventricle preventing the creation of holes in the myocardium of the left ventricle that could reduce the perfusion quality.

10. If the perfusion with PBS is incomplete, remaining intravascular FITC-dextran can be distinguished from extravascular (i.e., leaking) FITC-dextran by visualizing the blood vessels with a staining for the endothelial cell marker CD31.
11. Styrofoam containers, which are often used to ship laboratory materials, can easily be recycled for the purpose of freezing tissues. Please make sure that you take a container that does not leak.
12. To prevent vanishing of markings made on the embedding molds, use an alcohol-resistant lab marker and fill the ethanol only to a level below the markings.
13. Prevent prolonged handling of OCT blocks at room temperature because the OCT solution should not thaw. If rapid transfer from the –80 °C freezer to the cryotome or –20 °C freezer is not possible, transport the OCT blocks cooled.
14. Short-term storage of OCT blocks at –20 °C for a few days is possible, although not recommended. In addition, avoid leaving the OCT blocks in the cryotome overnight, since some machines display temperature fluctuations.
15. Protect sections from light throughout the whole experiment, especially if FITC-lectin or FITC-dextran is present in the respective tissue.
16. Since cutting sections of larger experiments containing numerous OCT blocks often take some hours, the time the different sections are drying may vary. To account for potential "batch effects," the order of the OCT blocks processed should not be according to the experimental groups, but random instead.
17. All washing and the permeabilization step (if not otherwise indicated) can be performed by putting the sections as a batch in one container gently shaking on a tumbling table at room temperature.
18. Alternatively to overnight incubation with the primary antibodies, the incubation can also be shortened to 1 h at room temperature in a humid chamber (do not forget to protect the specimen from light).
19. Insulinoma can be easily distinguished from the surrounding exocrine pancreas based on the more dense distribution pattern of the DAPI-stained nuclei. Pancreatic lymph nodes display a densely packed nuclei distribution pattern as well, but nuclei are more densely distributed and smaller than observed in insulinoma. If required, tumor cells can be specifically visualized by staining for insulin or SV40 large T antigen.

Importantly, it has been described that a subpopulation of insulinoma in the Rip1Tag2 mouse model loses insulin expression but remains positive for SV40 large T antigen expression [28]. Therefore, SV40 large T antigen appears to be a more sensitive marker to detect tumors (and metastases) in this model. On the other hand, the quality of the IF staining on cryosections for insulin is significantly better than that for SV40 large T antigen.

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