New Methods in Cardiovascular Biology

Novel Genetic Approach for In Vivo Vascular Imaging in Mice

Benjamin B. Bartelle, César A. Berríos-Otero, Joe J. Rodriguez, Anne E. Friedland, Orlando Aristizábal, Daniel H. Turnbull

Rationale: The formation and maintenance of a functional vasculature is essential for normal embryonic development, and genetic changes that affect the vasculature underlie pathogenesis in many human diseases. In vivo imaging in mouse models is required to understand the full complexity of mammalian vascular formation, which is a dynamic and 3-dimensional process. Optical microscopy of genetically expressed fluorescent reporter proteins offers high resolution but limited depth of penetration in vivo. Conversely, there are a plethora of molecular probes for alternative in vivo vascular imaging modalities, but few options for genetic control of contrast enhancement.

Objective: To develop a reporter system for multimodal imaging of genetic processes involved in mammalian vascular biology.

Methods and Results: To approach this problem, we developed an optimal tagging system based on Biotag-BirA technology. In the resulting Biotag reporter system, coexpression of 2 interacting proteins results in biotin labeling of cell membranes, thus enabling multimodal imaging with “avidinated” probes. To assess this approach for in vivo imaging, we generated transgenic mice that expressed the Biotag-BirA transgene from a minimal Tie2 promoter. A variety of imaging methods were used to show the utility of this approach for quantitative analysis in embryonic and adult models of vascular development, using intravascular injection of avidinated probes for near infrared, ultrasound, and magnetic resonance imaging.

Conclusions: The present results demonstrate the versatility of the Biotag system for studies of vascular biology in genetically engineered mice, providing a robust approach for multimodal in vivo imaging of genetic processes in the vasculature. (Circ Res. 2012;110:938-947.)

Key Words: imaging ■ Tie2 ■ endothelium ■ mouse ■ vascular biology ■ reporter genes ■ molecular imaging

Alternative in vivo imaging modalities provide 3D data with far greater penetration in mammalian tissues, albeit with lower spatial resolution than optical microscopy. For small-animal vascular imaging, available methods include near infrared (NIR), ultrasound microscopy (UBM), and magnetic resonance microimaging (micro-MRI). Although these modalities vary in their vascular imaging properties, including penetration and spatial resolution, they share a common need for probes or contrast agents that are targeted to molecules expressed on the luminal surface of vascular endothelial cells. Antibodies and peptides have been conjugated to intravascular contrast agents to target them to membrane proteins such as αvβ3-integrin and vascular endothelial growth factor (VEGF) receptor (VEGFR). Because the endothelial cell sur-
face is accessible via the circulation, contrast agents can be delivered to their target cells by intravascular injection.

There are limitations endemic to targeted contrast agents, primarily the relatively low binding affinities to their cognate receptors. As a result, in vivo binding can be inefficient and can take a substantial amount of time to bind at detectable levels. Moreover, the development of agents targeted to a specific protein is a complex and time-consuming process, and each agent can take years to properly optimize, especially for low-affinity targets. Finally, targeted imaging is necessarily limited to extracellular proteins, and many critical molecules cannot be targeted simply because they are intracellular.

To address the limitations of current reporter gene and targeted imaging technologies, we developed a synthetic “Biotag” reporter system that displays a cluster of biotins on cell membranes. Avidin-biotin affinity is several orders of magnitude higher than the strongest antibody-receptor binding, and is essentially irreversible under physiological conditions. Moreover a host of “avidinated” contrast agents are available for many imaging modalities. Compared with previous biotinylation systems, our transgene is completely self-contained and can be used with any endothelial promoter as a vascular reporter system. To test this approach, we generated transgenic Ts-Biotag mice, using a minimal promoter for Tie2 (T-short, denoted Ts). Tie2 (tyrosine-protein kinase receptor 2) was chosen for its multiple dynamic roles in vascular stability and remodeling and the availability of the regulatory sequences required to generate Ts transgenic mice. In vivo imaging of Tie2 expression was demonstrated in Ts-Biotag mice in both embryonic and adult models of vascular development, which demonstrates the potential of this general method for vascular imaging of genetic processes in mice.

Methods
An expanded Methods section is available in the online-only Data Supplement.

Cell Transfection and Biotinylation Assay
The nxBiotag-BirA constructs were transfected into HEK-293 cells, and live cell staining was performed 36 hours later by incubating cells for 1 hour with avidin-FITC (Av-FITC; A2050, Sigma, St Louis, MO) or steptavidin-Alexafluor680 (SAv-680; SAv-680; 926-32231, LI-COR, Lincoln, NE). After live cell staining, cells were fixed before cytochemical exposure. Matrigel plugs, mice were injected with SAv-680 or SAv-800 and imaged with an NIR scanner (Odyssey; LI-COR) at a nominal resolution of 180 μm.

Immunocytochemistry/Immunohistochemistry
Immunostaining was performed on cells and cryohistological sections (16 μm) acquired from mouse embryos with antibodies and protocols described in the online-only Supplement. Images were acquired with a fluorescence microscope (Leica).

Animals
Ts-Biotag mice were generated by zygote injection in the Skirball Transgenic Mouse Facility and maintained under protocols approved by the Institutional Animal Care and Use Committee at NYU School of Medicine. All results were generated from the 2 highest-expressing mouse lines (Online Figure 1). Embryos were staged according to embryonic day (E), where E0.5 denotes noon of the day after overnight mating.

Matrigel Plugs
Matrigel (BD Matrigel Matrix phenol-red free; BD Biosciences, San Jose, CA) was supplemented with VEGF (100 ng/mL; V4512, Sigma) and injected subcutaneously (100 μL) into anesthetized 5- to 8-week-old mice. All probes and contrast agents were injected via tail vein with a 27-gauge needle: 150 μg (1 mg/mL) Av-FITC or 100 μg (1 mg/mL) SAv-680 or SAv-800; 10 mg (40 mg/mL) Av-DTPA-Gd.

Fluorescence and NIR Imaging
After injection with Av-FITC, dissected mouse embryos and surgically exposed Matrigel plugs were imaged with a fluorescence dissection microscope (MZ16F; Leica). For in vivo imaging of Matrigel plugs, mice were injected with SAv-680 or SAv-800 and imaged with an NIR scanner (Odyssey; LI-COR) at a nominal resolution of 180 μm.

UBM Imaging and Guided In Utero Injections
UBM experiments were performed on a Vevo-770 UBM scanner (VisualSonics, Toronto, Canada) with an RMV-704 40-MHz transducer with nominal axial and lateral resolution of 40 and 80 μm, respectively. UBM-guided injections were performed by methods similar to those described previously. For targeted UBM imaging of the embryonic vasculature, avidin microbubbles were injected directly into the embryonic cardiac ventricle at E11.5 (1 μL) and E12.5 (2 μL).

Magnetic Resonance Imaging
All MRI images were acquired on a 7-Tesla microimaging system (Bruker, Billerica, MA). For micro-MRI, E11.5 embryos were injected with an avidinated chelated gadolinium, Av-DTPA-Gd (1 μL), with the UBM-guided injection system. One hour after in utero injection, embryos were dissected and fixed, then imaged overnight with a 3D, T1-weighted gradient echo sequence at isotropic resolution of 50 μm. Matrigel plugs were imaged in vivo with a multislice 2-dimensional, T1-weighted gradient echo sequence, with 100-μm in-plane resolution and a slice thickness of 500 μm acquired in 2 minutes.

Results
The Biotag-BirA Expression System Effectively Biotinylates Cell Membranes
Our membrane biotinylation approach is based on the coexpression of 2 interacting proteins (Figure 1). BirA, a biotin
ligase, was maintained in the endoplasmic reticulum (ER) by the inclusion of an N-terminal signal sequence derived from the ER chaperone Bip, as well as a C-terminal KDEL ER-localization sequence. The resulting ER-targeted BirA (BirAER) was coexpressed, with an internal ribosome entry site (IRES), with a chimeric protein, which consisted of the transmembrane domain of the platelet-derived growth factor receptor (PDGFR-TM) fused to multiple (n) biotags: nxBiotag-PDGFR-TM, referred to herein as Biotag. The nxBiotag DNA was further modified by localizing it to the ER by the inclusion of an N-terminal signal sequence similar to BirAER. Our goal was to express and maintain both Biotag and BirA in the secretory pathway, where they could interact and generate a biotinylated membrane protein for targeting with avidinated probes.

To establish that the Biotag reporter system resulted in correct subcellular expression of the 2 component proteins, HEK-293 cells were transfectioned with the construct CMV-nxBiotag-IRES-BirAER, and the transfected cells were subsequently analyzed with immunocytochemistry. Costaining for the BirA-specific HA (human influenza hemagglutinin) epitope tag and the ER marker calnexin showed that BirA was correctly targeted to the ER (Figure 2A). The Myc tag integrated in the Biotag protein colocalized with phalloidin staining for F-actin, which largely underlies the membrane21 (Figure 2B). Finally, costaining for both the HA and Myc tags showed partially overlapping expression patterns, which confirmed that BirA and Biotag colocalize in the ER as the Biotag protein is being trafficked to the cell membrane (Figure 2C).

To determine the functionality of the membrane biotinylation generated by the Biotag expression system, live cell staining with avidin-fluorescent probes was performed on HEK-293 cells transfected with CMV-nxBiotag-IRES-BirAER, where n ranged from 1 to 12. Compared with nontransfected cells, there was an obvious increase in Av-FITC membrane staining in transfected cells. Moreover, the membrane staining increased with the number of biotags in the expression construct (Figures 3A through 3C). The differences in membrane biotinylation were quantified with a digital NIR scanner after live cell staining in multiwell plates with the NIR probe SA-680. Nontransfected cells and cells transfected with either 1xBiotag or BirAER alone were included as negative controls, whereas HEK-293 cells transfected with a previously described biotin acceptor peptide (BAP)15 served as a positive control (Figure 3D). Limitations of BAP include its large size (387 bases), which makes it difficult to incorporate into a transgene or to amplify membrane biotin via insertion of multiple biotinylation sites, as well as its dependence on an unknown biotinylation enzyme that may not be present in all cells of interest.

Statistical analysis showed significant increases in cell staining (P<0.005) when wells of cells transfected with nxBiotag-IRES-BirAER were compared with negative controls (Figure 3D; n=8 in each group). The 1xBiotag-IRES-BirAERand BAP-transfected cells, each with 1 biotin per molecule of protein, yielded approximately a 2.5-fold signal increase over background. Increasing amounts of biotin per protein molecule with the 6x and 12x versions of the construct gave higher signal enhancement, with 12xBiotag-IRES-BirAER-transfected cells showing a further 3-fold gain over BAP and 1xBiotag-IRES-BirAER (Figure 3D; P<0.05; n=8 in each group). We conclude that cell membranes can be effectively biotinylated by expression of nxBiotag-IRES-BirAERand that the level of functional biotin is proportional to the number of biotags included in the expression vector.

Ts-Biotag Mice Express Biotin in Embryonic Vascular Endothelial Cells

To investigate the potential of the Biotag reporter system for in vivo vascular labeling, we generated transgenic mouse
lines expressing 12xBiotag-‐IRES-BirAER from the Tspromoter16 (Figure 1; Online Figure I). The resulting Ts-Biotag mice showed no noticeable defects in viability, fertility, or development compared with wild-‐type (WT) littermates (data not shown). All the results presented in this report were generated from the 2 highest-‐expressing lines of Ts-Biotag mice.

To assess any possible deleterious effects of transgene expression on vascular development, we analyzed PECAM1 (platelet and endothelial cell adhesion molecule 1) and Tie2 immunohistochemistry in brain sections from E11.5 to E13.5 WT and Ts-Biotag embryos (Figures 4A and 4B; Online Figure IIA). Neurovascular density, determined by PECAM1 staining, showed no significant differences between WT and Ts-Biotag embryos at any stage (Figure 4A). When we compared expression of endogenous Tie2 normalized against PECAM1 as a marker for vascular integrity, there was also no significant difference between WT and Ts-Biotag embryos (Figure 4B); however, there was a significant increase in Tie2 expression at E12.5 compared with both E11.5 (P<0.001) and E13.5 (P=0.02; n=3 for each stage and genotype). Similar results were also observed in histological sections of the hearts of the same WT and Ts-Biotag embryos (data not shown). We conclude that vascular development was not compromised in the Ts-Biotag mouse embryos.

Transgene expression and functional tagging of vascular endothelial cells was evaluated with immunohistochemistry for biotin. We observed an obvious increase in biotin staining in Ts-Biotag compared with WT embryos at both E11.5 and E12.5 (Figure 4C). These differences were quantified by measuring the ratio of biotin to PECAM1 signal in the region where they colocalized (Figure 4D). These results showed a significant increase in vascular biotin in Ts-Biotag versus WT embryos at both E11.5 (P=0.03) and E12.5 (P=0.01; n=3 for each stage and genotype). We also observed a significant increase in the biotin-‐PECAM1 ratio when we compared E12.5 to E11.5 Ts-Biotag embryos (P<0.001; n=3 for each stage and genotype), similar to the increase in Tie2-PECAM1 (Figure 4B). These results indicate that the Ts-Biotag transgene expresses biotin in a manner correlative to endogenous Tie2 expression in embryonic mice.

**Ts-Biotag Embryonic Vasculature Can Be Targeted With Avidinated Probes**

To explore the versatility of the Biotag system for embryonic vascular imaging, we used in vivo UBM-‐guided cardiac injection of avidinated probes into E11.5 WT and Ts-Biotag embryos. Injection of Av-‐FITC resulted in bright labeling in Ts-Biotag compared with WT embryos (Figure 5A). Light scattering prevented resolution of individual blood vessels under the dissection microscope, but subsequent sectioning and costaining with PECAM1 showed clear vascular labeling in Ts-Biotag embryos and minimal or no labeling in WT embryos (Online Figures IIB and IIC). As expected, there was also a good qualitative agreement between endogenous Tie2 expression and Av-‐FITC labeling in Ts-‐Biotag embryos (Online Figures IIA and IIC). To better resolve vasculature in 3D, embryos were injected with Av-‐DTPA-‐Gd for ex vivo micro-‐MRI (Figures 5B through 5D). Robust labeling was visible in a wide range of blood vessels in Ts-Biotag embryos, including the aorta, cardinal veins, and cerebral and inter-‐somitic vessels (Figure 5E; Online Movies I and II). Quantitative analysis was performed by an automated segmentation method (threshold=[background mean intensity+3 SDs]) to generate a 3D map of labeled voxels (Figure 5C). This analysis showed an obvious and statistically significant increase in labeled voxels in Ts-Biotag versus WT embryos (Figure 5D; P<0.003; n=5 for each genotype). These results demonstrate that the vascular endothelial cells of Ts-Biotag mouse embryos can be targeted in vivo with avidinated contrast agents.

**In Vivo Targeted Ultrasound Imaging of Vasculature in Ts-Biotag Embryos**

UBM is an established real-‐time, in utero method for cardio-‐vascular imaging in live mouse embryos.3,21 To determine the utility of the Ts-Biotag mice for transgene-‐mediated UBM
contrast enhancement, we imaged E11.5 and E12.5 embryos before and after UBM-guided injection of avidin microbubbles. There was an obvious increased persistence of vascular enhancement in Ts-Biotag embryos compared with WT littermates over the time period up to 20 minutes after injection (Online Figure III). This difference was most obvious on time-averaged images, calculated from cine (2-dimensional) UBM data acquired 20 minutes after injection of avidin microbubbles (Figure 6; Online Figure III). This method of image processing was found to minimize the signal from circulating microbubbles compared with stationary (bound) microbubbles. Most embryos gave signal around the heart, probably as a result of microbubbles being trapped in the pericardium (Figure 6A). Time-averaged images of Ts-Biotag embryos (Figure 6B) showed enhancement in a much more extensive vascular network in the brain that included increased numbers of smaller cerebral blood vessels compared with WT (Figure 6A). Similar to ex vivo micro-MRI, quantitative analysis was performed on in vivo UBM images by an automated segmentation method (threshold=background mean+5 SD). These results showed an obvious and statistically significant increase in labeled pixels in Ts-Biotag versus WT embryos (Figure 6D) at both E11.5 (P<0.001; n=7 WT and 7 Ts-Biotag) and E12.5 (P<0.01; n=7 WT and 7 Ts-Biotag). Furthermore, there was an increase in labeled pixels in E12.5 versus E11.5 Ts-Biotag embryos (Figure 6D), consistent with the increase in Tie2 and vascular biotin (Figure 4), although variability of labeling prevented this from achieving significance.

To assess the potential of this method for longitudinal imaging studies, selected Ts-Biotag embryos were injected with avidin microbubbles and imaged at E11.5, then injected again and imaged at E12.5 (Figure 6C). Quantitative analysis of the longitudinal data showed an obvious and statistically significant increase in labeled pixels at E12.5 versus E11.5 (Figure 6D; P=0.02; n=3), and no difference in labeling in comparisons of single time point and longitudinal data at E11.5 (P=0.26) or E12.5 (P=0.35). These results demonstrate the feasibility of the Biotag system for longitudinal UBM imaging of vasculature during mouse embryogenesis.

Multimodality In Vivo Imaging of VEGF-Induced Vascular Development

In vivo models have shown that Tie2 signaling by angiopoietin (Ang1) enhances VEGF-induced angiogenesis,23-24 whereas upregulation of Ang1 has also been observed in response to VEGF.25 On the basis of data that showed that Ang1 signaling was sufficient to increase Tie2 expression in cell culture,26 we asked whether the Ts-Biotag transgene was upregulated in an adult model in which neovascular development was induced by VEGF alone. For this purpose, we used a subcutaneous implantation of Matrigel supplemented with VEGF.27 Similar to many tumors, Matrigel plugs have poor vascular integrity, readily extravasating small molecular probes such as DTPA-Gd (Magnevist, Berlex; Online Figure IV). Using larger molecular-weight probes based on avidin and streptavidin, we did not detect any obvious vascular permeability in the Matrigel plugs (Online Figure IV), which allowed us to use time-dependent signal changes to assess vascular labeling.

VEGF-supplemented Matrigel plugs in both WT and Ts-Biotag mice were highly vascularized 5 days after implantation. Intravascular injection of Av-FITC and exposure of the plugs under a fluorescence dissecting microscope showed virtually no labeling in WT mice compared with highly specific vascular labeling in Ts-Biotag mice (Figure 7A; n=4 WT and n=6 Ts-Biotag). At 14 days postimplantation, Matrigel plugs were still highly vascularized in both WT and Ts-Biotag mice (n=4 for each genotype), but minimal Av-FITC labeling was observed in Ts-Biotag mice (Figure 7A), which indicates that transgene expression was only elevated in developing vasculature, similar to previous reports in other Ts transgenic mice.26 Immediately after imaging the Matrigel plugs, we also dissected the brain and spleen of Ts-Biotag mice and observed low levels of Av-FITC vascular labeling in these adult tissues, far below the levels seen in the Matrigel plugs or in E11.5 mouse embryos (data not shown).

On comparison of noninvasive NIR images of Matrigel plugs after intravascular injection of the NIR probe SAv-680 into 5-day and 14-day WT and Ts-Biotag mice (Figure 7B; n=8 WT and n=15 Ts-Biotag), there was obvious labeling in
patches only in the 5-day transgenic mice, especially around the periphery of the plugs and in blood vessels feeding into the brightly labeled patches. This labeling in vessels feeding into the plugs was consistently observed in all Ts-Biotag

Figure 5. Targeted magnetic resonance microimaging (micro-MRI) of embryonic vasculature in Ts-Biotag mice. A, Avidin (Av)-FITC injection selectively enhanced vasculature in embryonic day (E) 11.5 Ts-Biotag embryos, but light scattering prevented resolution of individual blood vessels (n=8 for each genotype). B, Using Av-DTPA-gadolinium (Gd), labeling, and ex vivo 3-dimensional (3D) micro-MRI of E11.5 WT (n=5) and Ts-Biotag (n=5) embryos, the vasculature was visualized using maximum intensity projection (MIP) images. (Online Movies I and II for full 3D rendering of wild-type (WT) and Ts-Biotag embryos, respectively). In WT embryos, magnetic resonance imaging (MRI) contrast was largely confined to the injection site in the heart (H), whereas Ts-Biotag embryos showed extensive labeling, including in the cerebral blood vessels (CBV) and intersegmental vessels (ISV). DA indicates dorsal aorta. C, Vascular labeling was quantified over multiple whole embryos (n=5 for each genotype) by segmenting the voxels (red) above a threshold (mean background signal intensity+3 SD) in both WT and Ts-Biotag embryos (red voxels superimposed on central 2-dimensional section). D, Quantitative analysis showed a significant (P<0.003) increase in the number of labeled voxels in Ts-Biotag (gray bar) vs WT (black bar) embryos (n=5 for each genotype). Scale bar=1 mm.

Figure 6. Targeted in vivo ultrasound biomicroscopy (UBM) of embryonic vasculature in Ts-Biotag mice. Time-averaged images were generated from 2-dimensional cine in vivo UBM images of embryonic day (E) 11.5 and E12.5 wild-type (WT; A) and Ts-Biotag (B) embryos (n=7 for each genotype and stage; inset shows approximate level of the coronal image plane shown for each embryo), 20 minutes after injection of avidin microbubbles (Online Figure III for time-course UBM images). Threshold analysis of cerebral vasculature (threshold=mean background signal intensity+5 SD) demonstrated increased labeling in transgenic embryos compared with WT. The in vivo capability of UBM allowed for longitudinal targeted imaging. To demonstrate the practicality of this method, selected embryos were injected and imaged first at E11.5 and then again at E12.5 (n=3; coronal image plane shown, as in inset; C). Quantitative analysis of cerebral vasculature showed significantly increased labeling in Ts-Biotag vs WT embryos at both E11.5 (P<0.001) and E12.5 (P<0.01) and between E12.5 and E11.5 (P=0.02) in the longitudinal data. D, There were no significant differences between longitudinal and single time point measurements at either E11.5 (P=0.26) or E12.5 (P=0.35). B indicates brain; 4v, fourth (cerebral) ventricle; H, heart; and P, placenta. Scale bar=1 mm.
mice, even at the relatively low spatial resolution of whole-body NIR. This more distal activation of Tie2 has not been described previously in other Tie2 transgenic mice, likely because of the lack of whole-animal imaging to assess regions away from the regions of active angiogenesis. As with Av-FITC, SAV-680 labeling of Ts-Biotag Matrigel plugs was virtually undetectable 14 days after Matrigel implantation (Figure 7B).

In longitudinal studies in 5-day Matrigel, labeling of larger feeding vessels faded away approximately 6 hours after injection of SAV-680, although some areas persisted (Figure 7C; n=3). When a second avidinated probe, SAV-800, was injected 6 hours after the initial injection of SAV-680, most of the faded vessels could be relabeled, and even areas of persistent labeling readily took up new agent (Figure 7D; n=4). In addition, some new regions of labeling appeared at the 6-hour relabeling time. On the basis of the observation that persistent areas consistently relabeled in these experiments, an area that does not relabel is most likely legitimately devoid of Tie2 expression rather than saturated with photo-bleached agent. This is an important distinction, especially at longer time points up to 24 hours, when differences in relabeling were even more prevalent (data not shown). These data indicate that Tie2 regulation in vascular development changes within hours, but the Ts-Biotag pulse-label system is still effective during this highly dynamic process.

To obtain information related to vascular labeling deep below the surface of the skin, 5-day Matrigel plugs were also imaged with MRI before and after injection of Av-DTPA-Gd in WT and Ts-Biotag mice (Figure 8). WT plugs remained unchanged after injection of Av-DTPA-Gd, whereas there was an obvious increase in postinjection signal intensity in all Ts-Biotag mice (Figure 8A; n=8 WT and n=9 Ts-Biotag). Labeling was obvious in the surface vasculature feeding the plug, consistent with NIR imaging, and in vascular tissue below the plug, which was not observable by any other method. Mice awake and behaving normally during the time between injection and MRI showed highly significant differences in contrast between WT and Ts-Biotag mice (Figure 8B; P<0.001; n=4 in each group). This increase in contrast in the Ts-Biotag mice could be blocked with an equivalent dose of unlabeled avidin (Figure 8B; n=4). The blocking study indicated that the 10-mg dosage of Av-DTPA-Gd was near saturation of all available biotin, which suggests that higher doses would not significantly improve MRI contrast. Time-course MRI data acquired in (anesthetized) WT (n=4) and Ts-Biotag (n=5) mice demonstrated a gradual increase in signal-to-noise ratio in the transgenic mice over 1 hour (Figure 8C). Finally, longitudinal MRI experiments were performed in Ts-Biotag mice after implantation of Matrigel plugs (n=4). Images were acquired before and after (1 hour) Av-DTPA-Gd administration in each mouse at 4, 7, 9, and 14 days after implantation (Figure 8D). The results showed peak contrast enhancement at 7 days, with no enhancement at 14 days, consistent with the NIR results (Figure 7). Although the MRI data acquired from Matrigel plugs had spatial resolution below the level of individual blood vessels, they provided quantitative in vivo information related to Tie2 expression and vascular labeling in this adult model of angiogenesis that is impossible to obtain by other existing methods. It is also apparent from the in vivo imaging results (both NIR and MRI) that Tie2 upregulation occurs both within and distal to regions of VEGF-induced neovascularure, which reveals a broader involvement of Tie2 in vascular development not easily appreciated from higher-resolution microscopic images. Taken together, the present results show that Ts-Biotag mice can be used for multimodal imaging and quantitative in...
efficiency with avidin probes. After incorporation into the reporter molecule, significantly improving cellular labeling of the gene was shown to amplify the amount of biotin labeling per molecule. Our novel transgene expands the Biotag reporter system to display biotin on cell membranes. The results presented here clearly demonstrate the ability of the Biotag system to efficiently label vascular endothelial cells, using intravascular injection of commercially available or easily synthesized avidinated agents. Compared with the previously reported biotin acceptor peptide (BAP) or the 1xbiotag transgene, the 12xbiotag transgene resulted in a significant increase in membrane biotin (Figure 3D). Although we found the 12xbiotag construct to provide excellent biotin labeling in vivo, our in vitro results suggest that the Biotag-BirA system may be amenable to even more biotags per protein molecule, with proportional improvements in biotin labeling, although there may be some risk of impairing vascular development with very high numbers of biotags.

In the present study, we demonstrated the first labeling and analyses of Tie2-expressing blood vessels in early-stage embryos with avidin-coated ultrasound and MRI contrast agents. Furthermore, the longitudinal in vivo UBM imaging presented here is the first such analysis of mouse embryonic gene expression with ultrasound. The UBM images did require minor postprocessing before analysis in the present study; however, persistence imaging, similar to the time-averaging approach adopted here, is already available on most commercial ultrasound systems for online image processing. With recent advances in ultrasound imaging technology and image processing, we now have the means to analyze vascular development in individual embryos at multiple stages with truly in utero and real-time imaging.

Ex vivo micro-MRI images were acquired in fixed mouse embryos after in vivo labeling with Av-DTPA-Gd. Compared with previous reports of ex vivo micro-MRI of embryonic vasculature, the Ts-Biotag mice enable clearer visualization of smaller cerebral and intersomitic vessels at E11.5, which suggests that the contrast agent is more effectively delivered to these microvessels by the normal circulation than by perfusion of fixed embryos with a Gd-loaded gelatin solution. We have recently demonstrated in utero micro-MRI of late-stage (E17.5) embryonic vasculature, providing optimism that future 3D and longitudinal MRI studies will be possible in developing mouse embryos. Ferritin-expressing transgenic mice were generated previously for MRI of vascular development in both embryos and adults. Using intravascular injection of commercially available or easily synthesized avidinated agents, we further demonstrated the potential of the Ts-Biotag mice for multimodality vascular imaging, including NIR, UBM, and micro-MRI. Our results strongly suggest that Ts-Biotag mice will also be readily amenable to an extended range of applications, including studies of neovascular formation during tumor progression and wound healing, and for other imaging modalities such as micro-computed tomography with avidin-iodinated agents, or for higher-resolution, although more superficial imaging studies with multiphoton microscopy after injection of Av-FITC or other fluorescent probes.

The approach described in the present study provides a modular self-contained system for cell membrane biotinylation that can be used generally as a genetic reporter system in vascular endothelial cells. Compared with the previously reported biotin acceptor peptide (BAP) or the 1xbiotag transgene, the 12xbiotag transgene resulted in a significant increase in membrane biotin (Figure 3D). Although we found the 12xbiotag construct to provide excellent biotin labeling in vivo, our in vitro results suggest that the Biotag-BirA system may be amenable to even more biotags per protein molecule, with proportional improvements in biotin labeling, although there may be some risk of impairing vascular development with very high numbers of biotags.

Discussion

The results presented here clearly demonstrate the ability of the Biotag reporter system to display biotin on cell membranes and the utility of the Ts-Biotag transgenic mice as a tool for research in vascular development. Our novel transgene was shown to amplify the amount of biotin labeling per reporter molecule, significantly improving cellular labeling efficiency with avidin probes. After incorporation into Ts-Biotag transgenic mice, the Biotag reporter system was shown to enable efficient labeling of endothelial cells during vascular development in both embryos and adults. Using intravascular injection of commercially available or easily synthesized avidinated agents, we further demonstrated the potential of the Ts-Biotag mice for multimodality vascular imaging, including NIR, UBM, and micro-MRI. Our results strongly suggest that Ts-Biotag mice will also be readily amenable to an extended range of applications, including studies of neovascular formation during tumor progression and wound healing, and for other imaging modalities such as micro-computed tomography with avidin-iodinated agents, or for higher-resolution, although more superficial imaging studies with multiphoton microscopy after injection of Av-FITC or other fluorescent probes.

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transgene expression. In comparison, the Ts-Biotag mice provide an opportunity to test a range of MRI contrast agents and data acquisition methods to optimize in utero detection of embryonic vasculature.

Regarding the use of the Ts promoter to visualize vasculature in embryonic and adult mice, it is important to understand the normal temporal and spatial patterns of Tie2/Ts expression. Consistent with previous studies in Tie2-eGFP mice, Ts-Biotag expression is minimally detectable in most adult tissues (data not shown). In addition, recent evidence shows that the Tie2 receptor is expressed and functions during lymphangiogenesis. Future studies using avidinated probes delivered to the lymphatic system in Ts-Biotag mice could therefore provide valuable new insights into this less studied part of the circulatory system.

In addition to Tie2, a number of alternative vascular promoters are available, including Flk1, an earlier marker of vascular development that has been shown to be effective for expressing fluorescent proteins in vascular endothelial cells of transgenic mice. Also, there are many intracellular markers of vascular development and inflammation that currently defy targeted imaging approaches, such as HIF1, COX2, or any number of downstream elements that are upregulated in vascular endothelial cells but not displayed on their luminal surface. Promoters of these genes could be used to drive the expression of the Biotag reporter to visualize their expression patterns, for example, with gene targeting to knock-in the 12xBiotag-ires-BirAER cassette into the HIF1 or COX2 locus. Visualizing the spatial and temporal dynamics of genetically defined cells critically requires in vivo imaging with high temporal resolution and deep tissue penetration. The present studies represent the vanguard of vascular reporter gene imaging in mouse models. Indeed, this new technology introduces the full power of multimodal genetic imaging to the field of vascular biology.

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Disclosures

None.

References


Novelty and Significance

**What Is Known?**

- Transgenic technologies with reporter genes are available for generating mice, but such reporters are largely limited to microscopy studies of vasculature under very restricted conditions.
- Targeted contrast agents allow for in vivo imaging, but developing an agent can be impractical or impossible, leaving many important vascular targets inaccessible.
- A reporter protein engineered for optimal targeting would allow for new in vivo studies of vascular genetics when implemented in transgenic reporter mice.

**What New Information Does This Article Contribute?**

- Engineered, multibiotinylated “Biotag” protein can be used for targeted imaging of genetic processes in the vasculature of mice.
- Expressing the Biotag transgene from a minimal Tie2 (*Ts*) promoter/enhancer element, “*Ts-Biotag*” mice were generated to label vascular Tie2 expression for targeted in vivo imaging with commercially available avidinated probes for a wide variety of imaging modalities.
- Multiday longitudinal studies of Tie2 expression were demonstrated in *Ts-Biotag* mice from embryonic to adult stages with use of near infrared (NIR), ultrasound, and magnetic resonance imaging (MRI).

Imaging studies in mice are crucial for understanding the dynamic and 3-dimensional nature of mammalian vascular biology, but currently available methods have significant technical limitations. Reporter gene imaging at the molecular level is possible but is largely limited to optical imaging under very restrictive conditions. Alternative imaging modalities such as MRI and ultrasound can be used for in vivo studies of vasculature deep within multiple organ systems, but limited options exist for molecular imaging, and very few targeted agents are available. Therefore, taking a hybrid approach, we created a reporter protein specifically designed for multimodal, targeted imaging. Enhanced Biotag technology was implemented in a transgenic mouse using the *Ts/Tie2* element. The *Ts-Biotag* mouse is compatible with commercially available avidinated probes for multiple vascular imaging modalities. With this mouse, we demonstrate novel longitudinal studies of vascular genetic imaging in adults and embryos. Most significantly, the Biotag transgenic technology enables the imaging of genetic processes in the vasculature with many clinically relevant imaging modalities, thus identifying candidates for drug or targeted agent development or relating clinically detectable biomarkers to formerly inaccessible aspects of vascular biology.


Novel Genetic Approach for In Vivo Vascular Imaging in Mice
Benjamin B. Bartelle, César A. Berríos-Otero, Joe J. Rodriguez, Anne E. Friedland, Orlando Aristizábal and Daniel H. Turnbull

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SUPPLEMENTAL MATERIAL

Detailed Methods

Expression Constructs

DNA constructs were based on the cassette \textit{nxbiotag-PDGFR-TM-IRES-BirA}^{ER} (“\textit{nxBiotag-BirA}”). ER-targeted \textit{BirA}^{ER} was generated using long oligonucleotides and synthetic PCR on a codon optimized version of \textit{BirA}^{1}. The single Biotag was constructed using synthetic oligos ligated into the pDisplay vector (Invitrogen, Carlsbad, CA). A modular multi-Biotag was designed and synthesized (BioBasic, Toronto, Canada). The synthetic construct consists of a 3x Biotag flanked by a 5’ Bgl2 and 3’ BamH1 site. This 3x tag was concatenated into 6x and 12x versions by iteratively ligating into the BamH1 site. The 12x Biotag was then ligated Bgl2/BamH1 into a pDisplay vector (Invitrogen, Carlsbad, CA), then into pCDNA3.1 (Invitrogen, Carlsbad, CA) upstream of \textit{IRES-BirA}^{ER} for cell transfection. The \textit{CMV} promoter was replaced with the \textit{Ts} (\textit{Tie2}) regulatory elements to generate transgenic mice.

Cell transfection and biotinylation assay

The \textit{nxBiotag-BirA} construct was transfected into HEK-293 cells (TransIT-LT1; Mirus Madison, WI). For optical microscopy, cells were cultured on poly-L-ornithine treated glass coverslips (26023; Fisher, Hampton, NH). Live cell staining was performed 36h after transfection by incubating cells for 1h with avidin-FITC (Av-FITC, A2050; Sigma St Louis, MO) or steptavidin-Alexa680 (SAv-680, 926-32231; LI-COR, Lincoln, NE). After live cell staining, cells were fixed in 4% paraformaldehyde (10 minutes at 4\degree C) prior to immunostaining and/or imaging with a fluorescence microscope (DM RXE; Leica; Wetzler, Germany) or an NIR scanner (Odyssey; LI-COR, Lincoln, NE).

Immunocytochemistry / Immunohistochemistry
Immunostaining was performed on HEK293T cells and cryohistological sections (16-µm) acquired from mouse embryos. Staining was accomplished using the following antibodies:
mouse anti-HA (1:200 / 18181; Abcam, Cambridge, England); goat anti-Myc (1:500 / 9106; Abcam, Cambridge, England); rabbit anti-CNX (1:100 / 22595; Abcam, Cambridge, England); mouse anti-Tie2 (1:500 / 24859; Abcam, Cambridge, England); rabbit anti-biotin (1:1000 / 53494; Abcam, Cambridge, England); rat anti-CD31/PECAM1 (1:100 / 550274; BD Biosciences, Franklin Lakes, NJ). Secondary antibody staining was performed with Cy3 (polyclonal goat anti-rat IgG; Jackson Immunoresearch, West Grove, PA); Cy2 (polyclonal donkey anti-goat IgG; Jackson Immunoresearch, West Grove, PA) FITC (polyclonal goat anti-rabbit IgG; Jackson Immunoresearch, West Grove, PA). Cell F-actin staining was performed using Phalloidin-546 (1:500 / Molecular Probes, Eugene, OR)\(^9\). Nuclear staining was performed using DAPI, dilactate (1:10,000 / Sigma, St Louis, MO). Confocal images were acquired using a Leica DMIRE, CSU10 Yokogawa confocal scanner unit (Yokogawa, Tokyo, Japan) with EM-CCD Hamamatsu digital camera (Hamamatsu Photonics, Hamamatsu City, Japan) controlled using Volocity 5.2 (Improvision, Coventry, England). ICC images were acquired using a 40X oil lens 1.25NA while IHC was acquired with a 20X air lens 0.7NA.

Measurements of vascular density were made using PECAM1 stained brain sections of embryos staged E11.5-E13.5 (n=3 embryos per genotype, 3 sections per brain). Vascular staining was defined as regions of fluorescence signal + 2 standard deviations (SD) above the mean signal, and the fraction of threshold voxels was displayed as a percentage. Tie2 and Biotin expression were analyzed by selecting an ROI within the defined vasculature and measuring signal from Tie2 or Biotin staining normalized against PECAM1 (n=3 embryos per genotype, 3 sections per brain). Measurements were presented as a ratio of Tie2 or Biotin over PECAM1. All image visualization and analysis was performed using ImageJ (v1.4; NIH freeware).
Transgenic Mice

Genotype analysis on founder lines and their offspring was performed using PCR of tissue-derived (ear, tail, embryo) DNA, using the following primers: (5′:CCARCAACAAGCACATCCAG; 3′:TCCCATCTGCTCAGGTAAGG). A limb from each embryo was separated and digested in proteolysis buffer (50mM Tris pH 8.8, 1mM EDTA, 0.5% Tween20, 20µg/mL Proteinase K) for genotype analysis by PCR. The remainder of each embryo was homogenized in tissue lysis buffer (RIPA buffer [50 mM TrisHCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS] with 10µg/mL DNAse1 and 1 Complete Mini protease inhibitor tablet/10 mL [Roche Diagnostics, Basel, Switzerland]) and incubated for 30min on ice before being combined with PAGE SDS loading buffer and incubated for 5 min at 95°C.

Samples were run on a PAGE-SDS gel and transferred to a PVDF-FL membrane for Western Blot using an antibody against the Myc epitope (1:2000 / 8226; Abcam, Cambridge, England) integrated into the Bio-tag protein and against β-Actin as a normalization control(1:5000 / 9106; Abcam, Cambridge, England). The blots were then incubated with 2 secondary antibodies conjugated to Alexaflour-680 (1:800 / LI-COR, Lincoln, NE 926-3221) and 800 (1:800 / LI-COR, Lincoln, NE 926-32210) and quantitatively imaged for fluorescence on an NIR scanner (Odyssey; LI-COR, Lincoln, NE). The 3 highest expressing lines were retained for further studies.

Ultrasound contrast agents

Targeted ultrasound contrast agents (Target-Ready, MicroMarker; Visualsonics, Toronto, Canada), consisting of avidin-coated, lipid shelled microbubbles, were reconstituted according to the manufacturer’s specifications to a stock concentration of 2 x 10⁹ microbubbles/ml with a mean diameter of 2.3 to 2.9 µm. Nontargeted microbubbles (MicroMarker;
Visualsonics, Toronto, Canada), without avidin, were used for visualizing injection of optical and MRI agents.

**Synthesis of Av-DTPA-Gd**

Avidin (Av)-DTPA-Gd was synthesized by modifying a previous protocol\(^2\). Diethylene-triamine-pentaacetic dianhydride was reacted at a 20:1 molar ratio to avidin overnight at 4°C in 0.1M carbonate buffer (Ph8). The mixture was dialyzed in 1000X volume 0.1M NaCl overnight at 4°C. Gd was then directly combined with the Av-DTPA at a 10:1 ratio, and dialyzed again in 1000X volume 0.1M NaCl overnight at 4°C. The final Av-DTPA-Gd was concentrated to 40mg/ml using a 0.5mL centrifugal concentrator (Millipore, Billerica, MA).

**Ultrasound guided micro-injections**

Timed pregnant mice were anesthetized with isoflurane. A single uterine conceptus was exposed through a midline incision of an anesthetized mouse into a phosphate buffered saline (PBS) filled Petri dish, carefully maintaining intact all maternal and embryonic vascular connections. With both the mother and PBS maintained at 37°C, the imaging transducer was lowered and the beating heart located. A 50-\(\mu\)m beveled micro-injection needle was placed inside the most proximal cardiac ventricle for injection and UBM monitoring. Intracardiac injections of optical and MRI contrast agents were visualized by coinjection of 100-nl of non-targeted microbubbles.

**UBM Data Acquisition**

For each injected embryo, 2D cine loops (300 frames acquired in 10s) were acquired preinjection, immediately postinjection, and again 20 minutes postinjection. Temperature was maintained at 37°C during the imaging session, and heart rate maintained above 160 beats per
minute to ensure proper circulation of contrast agent throughout the vasculature. For longitudinal analysis, this procedure was performed on 2 E11.5 embryos per pregnant female. Mice were sutured and allowed to recover for 24h before the procedure was repeated, imaging the same embryos at E12.5.

**Micro-MRI Data Acquisition**

For micro-MRI, embryos were injected *in utero* with an avidinated, chelated gadolinium, Av-DTPA-Gd (1-µl), using the UBM-guided injection system. Embryos were dissected 1h after *in utero* injection, and maintained in warm PBS at 37°C for 30 minutes to clear the blood. Embryos were then fixed in 4°C, 4% paraformaldehyde (PFA), and mounted inside a 30-ml syringe for multi-embryo imaging. Imaging was performed using a 3D T1-weighted gradient echo sequence (echo/repetition times, TE/TR = 6.25/50 ms; flip angle, FA = 40°; matrix = 512³; isotropic resolution = 50µm; total imaging time = 7 h 17 min).

**Embryo Image Analysis**

Individual embryo images were segmented from 2D UBM or 3D micro-MRI datasets containing images of multiple embryos using a combination of Analyze (v7.0; AnalyzeDirect, Overland Park, KS), Amira (v4.1; Visage Imaging, San Diego, CA) and ImageJ (v1.4, NIH freeware). Histogram analyses were performed with ImageJ to detect the voxels more than 3 (for micro-MRI) or 5 (for UBM) standard deviations above the mean background signal intensity, measured in a region-of-interest (ROI) placed over a non-enhanced region of the embryo. Amira was used to generate micro-MRI MIPs and visualizations of threshold-segmented vasculature for both UBM and micro-MRI. For UBM, image visualization and time-averaged images were generated using the average intensity projection (AIP) function in ImageJ. Further UBM
histogram analysis was conducted within regions-of-interest (ROI) covering the head of each embryo to focus on the neurovasculature.

**Matrigel Data Collection and Analysis**

Matrigel (BD Matrigel Matrix phenol-red free; BD Biosciences, Franklin Lakes, NJ) was defrosted to 4°C on ice and supplemented with VEGF (100ng/mL, V4512; Sigma, St Louis, MO). 5-8wk WT and Ts-Biotag mice were anesthetized with isoflurane and injected subcutaneously (100µl), waiting at least 2 minutes post implantation before removing anesthesia to allow the matrigel to warm and solidify.

All probes/contrast agents were injected via tail vein using a 30g needle in the following amounts: 150µg (1mg/ml) Av-FITC; 100µg (1mg/ml) SAv-680 or SAv-800; 10mg (40mg/ml) Av-DTPA-Gd. For florescence imaging 30min after Av-FITC injection, the dermis of anesthetized mice were opened to expose the matrigel plugs. NIR imaging was also conducted 30min post injection of agent and animals were again anesthetized using isoflourane for the duration of the imaging procedure. Mice were carefully shaved and placed on an NIR scanning bed for whole animal imaging (Odyssey; LI-COR, Lincoln, NE). Scanning took ~15min/mouse.

MRI data were acquired with a 2D multi-slice T1-weighted gradient echo sequence (TE/TR = 4.7/25ms; FA = 90°; 100µm in-plane resolution; slice thickness = 500µm; total imaging time = 2min). Signal intensities from regions-of-interest covering the matrigel plug were divided by the standard deviation of the background (air) noise to compute signal-to-noise ratio (SNR), and normalized to the SNR of the pre injection image. Contrast was computed as (Post-Pre)/Pre normalized SNR. Post injection images were acquired 45 minutes - 1h post injection owing to variability in setup time. Longitudinal data was acquired at 4,7,9 and 14 days post implantation. In these experiments, signal from the matrigel ROI was normalized against that of an external GdCl₃ standard that also served to mark the location of the plug. Contrast was computed as (Post-Pre)/Pre normalized CR.
Statistical Analyses

All data were represented as mean value ± standard deviation. The two-tailed Student's t-test was used to determine statistical significance (p < 0.05).

References


Online Figure I: Western blot analyses of transgene levels in Ts-Biotag mouse lines.

Protein samples were generated by PAGE-SDS electrophoresis on homogenized tissue from E11.5 embryos, generated from the 7 Ts-Biotag mouse lines (A-G) that produced transgenic offspring from the 8 founder mice (1 founder did not produce offspring). Protein was transferred to Immobilon-P membranes and costained with Rabbit anti-Myc (9106 Abcam, Cambridge, England) and Mouse anti-β-actin (8226 Abcam, Cambridge, England). Secondary antibody costaining was performed with Goat anti-Mouse-IRDye800 (926-32210 LI-COR, Lincoln, NE) and Goat anti-Rabbit-IRDye-680 (926-32221 LI-COR, Lincoln, NE). Blots were imaged on a NIR scanner (Odyssey, LI-COR, Lincoln, NE). Quantitative expression ratios were measured, using region-of-interest (ROI) measurements of signal intensity in each band divided by the signal intensity in the background (ROI/Background). Normalized signal intensity ratios were simply calculated as the normalized Myc intensity divided by the normalized β-actin intensity, providing a quantitative measure of expression levels. Data were represented as mean ± standard deviation (n=4-7 embryos per mouse line). The 3 highest expressing lines (A-C) were maintained by breeding. All the results in this paper were generated from the two highest expressing lines (A,B)*.
Online Figure II: E11.5 Ts-Biotag embryonic Tie2 expressing vasculature is bound by avidinated probes.

IHC of E11.5 embryos showed Tie2 expression in the brain vasculature (A). Most Tie2 colocalized with PECAM1, with some additional extravascular expression. In Av-FITC (green) injected WT and Ts-Biotag embryos co-stained with PECAM1 (red) almost no Av-FITC signal is visible in WT (B). In contrast, Ts-Biotag embryos show highly specific co-localization of Av-FITC with the vascular endothelium in the Ts-Biotag embryos, similar to the Tie2/PECAM1 staining (C). (n≥8 for each group). Scale bar = 25 µm.
Online Figure III: UBM imaging of vasculature in Ts-Biotag embryos.
In vivo longitudinal UBM imaging of E11.5 WT (A) and Ts-Biotag (B) embryos, before (Pre), immediately after (Post-0min) and 20 minutes after (Post-20min) injection of Av-microbubbles, demonstrated a higher and prolonged level of vascular enhancement in transgenic embryos, this is especially apparent in time averaged images, which emphasized signal from bound over circulating microbubbles. Threshold analysis (red pixels, threshold = mean background signal + 5 standard deviations) was used to quantify vascular labeling (n=7 for each genotype). Scale bar = 1 mm.
Online Figure IV: Matrigel plug vasculature is permeable to DTPA-Gd.
Before injection of contrast agents, matrigel plugs had no obvious contrast compared to surrounding tissue (A). Injection of DTPA-Gd (Magnevist, Berlex) led to contrast beginning around the edges of the plug, moving towards the interior and nearly becoming uniform throughout the plug by 60 minutes post injection. Conversely, Av-DTPA-Gd injections into WT animals showed no increase in contrast over the entire time course (B). Quantitative ROI analysis of WT matrigel plug signal-to-noise ratio (SNR) was performed over time for DTPA-Gd and for Av-DTPA-Gd (C). The DTPA-Gd line shows an increase over time, fitting with first order saturation kinetics curve. Non-specific labeling due to extravasation of agent was not apparent in WT mice injected with Av-DTPA-Gd, most likely due to the much higher molecular weight of the agent compared to DTPA-Gd (~100x). DTPA-Gd was administered via tail vein injection at 0.2mmol/kg, 50x the Av-DTPA-Gd dosage but only 2x the standard clinical dosage. (n=4 for each contrast agent). Scale bar = 2mm.