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2 3 4	Development of an endothelial cell restricted transgenic reporter rat: A resource for physiological studies of vascular biology
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48 49	Running Title: Endothelial-restricted reporter rat model

50 New and noteworthy: Transgenic mice have been instrumental in advancing molecular insight of 51 physiological processes, yet these models oftentimes do not faithfully recapitulate human physiology and 52 pathophysiology. Rat models better replicate some human conditions, like Group 1 pulmonary arterial 53 hypertension. Here, we report the development of an endothelial cell restricted transgenic reporter rat that 54 has broad application to vascular biology. This first-in-kind model offers exceptional endothelial 55 restricted tdTomato expression, in both conduit vessels and the microcirculations of organs.

56

57 Keywords:

- 58 (1) CDH5 (VE-cadherin)
- 59 (2) Cre recombinase (iCre)
- 60 (3) tdTomato
- 61 (4) Animal model
- 62 (5) Pulmonary hypertension

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Abstract

Here, we report the generation of a Cre-recombinase transgenic rat, where iCre is driven using a CDH5 66 67 promoter. The CDH5 promoter was cloned from rat pulmonary microvascular endothelial cells and 68 demonstrated approximately 60% similarity to the murine counterpart. The cloned rat promoter was 2,508 69 bp, it extended 79 bp beyond the transcription start site, and it was 22,923 bp upstream of the translation 70 start site. The novel promoter was cloned upstream of codon-optimized iCre and subcloned into a 71 Sleeping Beauty transposon vector for transpositional transgenesis in Sprague-Dawley rats. Transgenic 72 founders were generated and selected for iCre expression. Crossing the CDH5-iCre rat with a tdTomato 73 reporter rat resulted in progeny displaying endothelial-restricted fluorescence. tdTomato fluorescence was 74 prominent in major arteries and veins, and it was similar in males and females. Quantitative analysis of 75 the carotid artery and the jugular vein revealed that on average more than 50% of the vascular surface 76 area exhibited strong fluorescence. tdTomato fluorescence was observed in the circulations of every tissue 77 tested. The microcirculation in all tissues tested displayed homogenous fluorescence. Fluorescence was 78 examined across young (6-7.5 months), middle (14-16.5 months), and old age (17-19.5 months) groups. 79 Although tdTomato fluorescence was seen in middle- and old-age animals, the intensity of the fluorescence was significantly reduced compared to that seen in the young rats. Thus, this endothelial 80 81 restricted transgenic rat offers a novel platform to test endothelial microheterogeneity within all vascular 82 segments and it provides exceptional resolution of endothelium within organ microcirculations for 83 application to translational disease models.

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Introduction

Endothelium forms a highly dynamic barrier that coordinates the passage of water, solutes, 86 87 macromolecules and cells between the blood and the underlying tissue. Endothelial cells are highly 88 specialized, or differentiated, to achieve the functions of any given vascular location(2, 14). Such 89 differentiation is classified on an anatomical basis, being either continuous, discontinuous or 90 fenestrated(2). Conduit vessels, like the carotid artery and jugular vein, and capillaries in organs like the 91 skin, lung, central nervous system, and muscle, possess a continuous type of endothelium, where cells 92 reside on an underlying basement membrane. Organs like the liver, spleen, and bone marrow possess a 93 discontinuous, or sinusoidal-type, capillary endothelium, where the cells do not reside on a basement 94 membrane. The kidney glomerulus, endocrine glands and intestinal endothelium each exhibits fenestrae, 95 which are contiguous transcellular openings. These anatomical classifications relate to the variable ability 96 of endothelium to regulate permeability in accordance with site-specific environmental demands. 97 Permeability of the continuous endothelium largely occurs through intercellular junctions, and to a lesser 98 degree, through vesicular transcytosis, both of which are highly regulated processes(23, 30, 31). 99 Physiological stimuli dynamically adjust the strength of cell-to-cell junctions and the rate of transcytosis 100 in order to coordinate blood-to-tissue communication. In contrast to this process, discontinuous 101 endothelium offers less resistance to permeability and fenestrated endothelium serves to sieve molecules 102 based on molecular size and charge, although in both cases, junctional integrity remains an important 103 mechanism governing transcellular permeability. Despite these highly specialized phenotypes, 104 continuous, discontinuous and fenestrated cells retain an "endothelial" specification.

Adherens junction proteins contribute to the restrictive endothelial cell barrier in each of these cell phenotypes(10), and hence, are important determinants of endothelial specification. Vascular endothelial cell cadherin, VE-cadherin (CDH5), is the principal transmembrane protein comprising an adherens junction(16). VE-cadherin displays a highly restricted expression pattern. It is expressed selectively in endothelium and not in the underlying smooth muscle cells. However, VE-cadherin may not be equally expressed in all endothelial cell phenotypes. For example, VE-cadherin was prominently expressed in 111 pulmonary arteries, arterioles, and capillaries, but not in venules and veins, in post-mortem human 112 lung(19). In contrast, VE-cadherin reporter mouse models typically reveal a more homogenous expression 113 pattern. A 2.2kb CDH5 promoter sequence was cloned upstream of Cre recombinase and these animals 114 were crossed with various Cre-responsive reporters(18, 24) to engineer constitutive or inducible 115 expression of reporter genes. Approximately half of the endothelial cells isolated from these transgenic 116 mice expressed the reporter gene early in development, and this percentage increased throughout 117 development in blood and lymphatic vessels(18). Near uniform Cre recombinase reporter gene expression 118 was seen in adult endothelium, across multiple organs. The promoter sequence possesses regulatory 119 elements sufficient to drive expression in endothelium while reducing expression in other cell types(18). 120 A similar promoter that also contains a 200.3kb upstream region has been used to drive inducible 121 expression of genes of interest(27, 29, 35, 37, 38) for physiological studies. These studies also confirm 122 the VE-cadherin promoter sequence retains endothelial specificity.

123 While use of VE-cadherin driven reporter genes has been instrumental in advancing our 124 understanding of endothelial cell fate and properties of angiogenesis and barrier function, mouse models 125 are limited in some areas of vascular biology, especially for the study of chronic lung diseases like 126 pulmonary arterial hypertension(7, 17). In this case, severe pulmonary arterial hypertension is 127 characterized by evolving occlusive neointimal lesions thought to arise from endothelial apoptosis and 128 exuberant hyperproliferation of apoptosis-resistant cells into the lumen. Mouse models of pulmonary 129 arterial hypertension do not typically develop this form of the vasculopathy, with the exception of 130 endothelial and hematopoietic prolyl-4-hydroxylase 2 null mice(7, 17). In contrast, rat models better 131 recapitulate the pulmonary arterial hypertension phenotype characterized by neointimal occlusive 132 lesions(1, 4, 36). Therefore, we sought to develop a transgenic rat model that would enable endothelial 133 cell fate mapping during the evolution of chronic vascular disease states, like occlusive vasculopathies. 134 To develop this model, we engineered the rat VE-cadherin promoter upstream of Cre recombinase and 135 crossed this animal with a Cre-sensitive tdTomato reporter rat. Here, we report the resulting progeny 136 possesses prominent reporter activity across multiple organs, in both males and females.

Materials and Methods

138 Cloning of the CDH5 promoter from rat endothelium. Generation of CDH5-cre recombinase rats 139 was performed at the Genome Rat Resource Center at the Medical College of Wisconsin under 140 protocols approved by the institutional animal care and use committee. An approximate 2.5kB 141 fragment of the CDH5 promoter was cloned upstream of the codon-optimized HA-tagged Cre 142 (iCre) and this expression cassette was subcloned into a Sleeping Beauty (SB) transposon 143 vector(15). The SB method of transpositional transgenesis (TnT) was used to produce transgenic 144 Sprague Dawley (Crl:SD, Charles River Laboratories) rats by pronuclear microinjection as we 145 have previously described(21, 22). Three transgenic founders were produced, one of which 146 demonstrated robust endothelial-specific Cre expression when crossed to the tdTomato Reporter 147 Knock-in Rat (Horizon). This founder was then backcrossed to the parental Crl:SD strain to 148 establish a colony for further development and characterized (herein referred to as CDH5-iCre).

149 Animal Care. CDH5-iCre x tdTomato transgenic Sprague-Dawley rats were shipped from the Medical 150 College of Wisconsin to the University of South Alabama for this study. Once received, they underwent a 151 30-day quarantine, after which time, pathogen testing revealed they were all pathogen-free. Until the time 152 of the study, animals were group-housed in micro-isolation caging with enrichment, according to the 153 established guidelines for care and use of laboratory animals. Rooms were on a 12-hour light-dark cycle 154 and temperature was controlled. Sixteen animals were studied, including 10 females and 6 males. 155 Procedures were reviewed and approved by the University of South Alabama institutional animal care and 156 use committee.

Animal surgery and tissue preparation. Tissues and vessels of interest were harvested from transgenic rats to examine the expression and distribution of tdTomato fluorescent protein. For lung tissue, we utilized the agarose/gelatin-infused lung slice technique to evaluate tdTomato fluorescence, autofluorescence, and Hoechst nuclear dye (NucBlue, Invitrogen) fluorescence within a 300 µm thick lung tissue. Rats were sedated with injection of sodium pentobarbital intraperitoneally and an anesthetic

162 plane was verified by lack of toe pinch response. Rats were then injected with 0.3 mL heparin 163 intraperitoneally and following thoracotomy the pulmonary artery was cannulated through the right 164 ventricle. The left ventricle was nicked, and ~30 mL of Hanks Buffered Saline Solution (HBSS, Gibco) 165 was slowly perfused through the pulmonary circulation. Next, NucBlue (3 mL) was perfused through the 166 pulmonary artery cannula and a stopcock was used to infuse ~15 ml gelatin (6%; ThermoFisher 167 Scientific) solution into the pulmonary circulation. The right lung was then sutured at the hilum, and a 168 tracheal tube was inserted, followed by infusion of ~20 ml agarose (2.7%; ThermoFisher Scientific) 169 solution to inflate the left lung. Ice was used *in situ* to solidify the gel *en bloc*. The left lung, heart and 170 trachea were excised en bloc and placed in cold HBSS on ice at 4 °C for 1 hr. The left lung was cut using 171 a razor into ~ 1 mm thick slice at the hilum and then sliced into 300 µm thick serial sections using a 172 vibratome. Slices were added to a six-well, glass-bottom plate (cellvis) and placed in an incubator for 1 173 hr. An Andor Revolution RS3 (Andor) microscope using IQ (Andor, v3.6.1) software was used to collect 174 z-stacks (100 planes at ~ 0.4 um/plane) of selected structures within the slices using the RSL red 175 (emission: 625 nm), RSL green (emission: 525 nm), and Dapi (emission: 440 nm) filter sets.

176 Other tissues, including liver, spleen, mesentery and brain, were harvested and placed in buffered 177 saline solution. Tissues were then cut into thin (~ 0.5 mm) slices using a surgical knife. A representative 178 section of each tissue was incubated with NucBlue (ThermoFisher Scientific) for 60 minutes to visualize 179 nuclei. Blood vessels, including the aorta, carotid artery, jugular vein, inferior vena cava (IVC), and 180 basilar artery, were harvested, cut longitudinally, opened, and pinned onto SYLGARD 182 blocks (182 181 silicone elastomer, DOW Chemical) as previously described (13). Blocks were then immersed in buffered 182 solution containing NucBlue for 10 minutes. Blocks were washed and placed in a 35 mm round glass 183 culture dish (separated 100 µm from glass by two parallel supporting pins) containing PBS. All tissues 184 and vessels harvested from rats in all three age groups (6-7.5 months, 14-16.5 months, and 17-19.5 185 months) were prepared similarly. Prepared specimens were imaged using a Nikon A1R confocal 186 microscope (Nikon Instruments).

187 Hyperspectral imaging. Hyperspectral imaging analysis approaches were utilized to examine the 188 distribution and expression of tdTomato fluorescent protein across a range of tissue types, while 189 accounting for tissue type-specific autofluorescence(12). Hyperspectral z-stack images were acquired 190 using a Nikon A1R confocal microscope and several objectives, including 4X (Plan Apo λ 4X), 20X 191 (Plan Fluor 20X multi immersion DIC N2), and 60X (Plan Apo VC 60X WI DIC N2). Samples (both 192 from transgenic rats and control rats) were excited using 405 nm for NucBlue, 488 nm for 193 autofluorescence, and 561 nm for tdTomato and autofluorescence simultaneously and the emission 194 spectrum was collected using wavelengths ranging from 424 nm - 724 nm in 10nm increments. All 3 195 laser lines were set to 20% illumination intensity. The z-step (axial) interval for z-stack acquisition was 196 set at 30 µm, 2 µm, and 1 µm, for 4X, 20X, and 60X objectives, respectively. Specimens were imaged 197 first using the 4X objective, and subsequently with 20X and 60X objectives. A confocal pinhole size of 198 38.3 µm and detector voltage gain of 130 were used. Similar imaging parameters and settings were 199 utilized across all image data acquired. Imaging parameters were selected so as to maximize signal-to-200 noise ratio while minimizing photobleaching of tdTomato.

201 A spectral library containing pure spectra of each endmember (tdTomato, NucBlue, and tissue-202 specific autofluorescence) was generated to analyze spectral image data. To construct the spectral library, 203 spectral images were acquired using HEK 293 cells either transfected with tdTomato or labeled with 204 NucBlue to obtain pure tdTomato and NucBlue spectral signatures, respectively. Tissues and vessel 205 specimens from control Sprague Dawley rats were imaged to obtain tissue-specific spectral signatures. 206 Custom developed MATLAB programs were utilized to linearly unmix raw spectral data into individual 207 endmembers including tdTomato, NucBlue, and tissue autofluorescence. Unmixed images of tdTomato 208 were used to visualize and quantify the expression and distribution of tdTomato fluorescence signal in 209 different conduit vessels and tissues.

Quantitative Analysis. The age-dependent distributions of tdTomato fluorescence signal in the carotid artery and the jugular vein were quantified using ImageJ analysis software(32). Images acquired using a 4X objective were utilized for quantitative measurements. Maximum intensity projection images of 213 tdTomato and NucBlue signals were generated from unmixed tdTomato and nuclei image z-stacks, 214 respectively. A region of interest was selected to cover the maximum possible area occupied by the vessel 215 while eliminating all areas outside of the vessel, as based on the NucBlue projected image. This region of 216 interest was applied to the tdTomato maximum intensity projected image, and the tdTomato image was 217 subsequently cropped to eliminate any effects from areas outside of the tissue, thus removing any 218 potential bias from blank regions of the image. A lower-bound intensity threshold of 0.2% and 0.3% of 219 maximum signal intensity (grey scale values of 126 and 200 out of 65535) was selected for carotid and 220 jugular images, respectively, to define pixels that were tdTomato positive. The tdTomato positive 221 fractional area of the thresholded image was then calculated. The same lower-bound intensity threshold 222 was applied to all images ($n \ge 4$ rats for each age group) in each group. The age-dependent tdTomato 223 positive fractional area in carotid artery and jugular vein was plotted for each age group. Statistical 224 significance of variations in tdTomato positive fractional area per age group were calculated using one-225 way ANOVA, with multiple comparisons and Tukey post-hoc test. Significance was established at p < p226 0.05.

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Results

230 The rat CDH5 promoter shares limited homology to the equivalent mouse promoter. The rat CDH5 231 start site was identified, and the promoter region resolved by alignment against the mouse sequence. The 232 cloned mouse and rat promoters were 2,509 and 2,508 bp, respectively (Figure 1A, B). Whereas the 233 previously reported mouse promoter fragment overlaid the first exon by 86 bp, the cloned rat promoter 234 extended 79 bp past the transcription start site. These promoters shared sequence homology, equal to 60% 235 using EMBOSS, 64.4% using Lalign, 64.9% using AlignX (Vector NTI) (Figure 1C). In both genes, 236 translation initiation sites are located in the second exon separated by 11,285 bp and 22,923 bp from 237 transcription initiation sites in murine and rat genes, respectively. Therefore, the first intron in the rat 238 gene, while incompletely sequenced at this point, appears to be much larger (22,862 bp vs 11,147 bp). 239 Once cloned, the rat promoter was placed upstream of codon-optimized iCre with a HA tag and subcloned 240 into a *Sleeping Beauty* transposon vector for generation of transgenic rats (Figure 1D), as described in the 241 Material and Methods. Founders were bred and backcrossed into the parental strain to establish the 242 colony. tdTomato fluorescence was tested among the progeny from the colony to assess for rat CDH5 243 promoter efficacy.

244 tdTomato fluorescence is limited to the endothelium within the conduit vessel wall. A spectral 245 library containing the unique spectra of each endmember was compiled using image data from single 246 label control samples. Controls for tdTomato and NucBlue were prepared using HEK 293 cells that were 247 either transfected with DNA encoding tdTomato or labeled with NucBlue. Controls for autofluorescence 248 were prepared in an organ and tissue-specific manner. Tissues and vessels from control Sprague Dawley 249 wild-type rats were harvested. Tissues and vessels were prepared and mounted as described in Materials 250 and Methods. Spectral images of each single-label control and tissue type were acquired, including 251 tdTomato (Figure 2A), NucBlue (Figure 2B) and tissue-specific autofluorescence (Figure 2C- example 252 from jugular vein autofluorescence). Spectral libraries (Figure 2D) containing the unique spectra of 253 tdTomato, NucBlue, and the autofluorescence of the tissue of interest were constructed by extracting the 254 pixel-averaged spectrum from regions of interest in images of control specimens (shown as red, blue and

255 yellow regions in Figure 2A-C). Spectral libraries were then used for spectral analysis to unmix 256 respective signals from each fluorescence signature.

257 A pixel-by-pixel analysis was performed using custom non-negatively constrained linear spectral 258 unmixing algorithms in the MATLAB programming environment (Figure 2D). Raw spectral image data 259 (Figure 2E) were unmixed into individual spectral components, called endmembers: tdTomato (Figure 260 2F), NucBlue (Figure 2G), and tissue autofluorescence (for example, jugular vein autofluorescence 261 shown in Figure 2H) using a spectral library. To examine the distribution of tdTomato signal in 3 262 dimensions, a projection was created through the unmixed z-stack images (Figure 2J). With this 3-263 dimensional analysis different nuclear orientations were apparent, where overlying endothelial cell nuclei 264 are round and underlying smooth muscle cell nuclei are oblong and oriented at 90 degree angles relative 265 to endothelial nuclei. To assess the total tdTomato signal in the sample, a maximum intensity projection 266 was also created (Figure 2K includes autofluorescence and Figure 2L does not include 267 autofluorescence). The maximum intensity projection was utilized to visualize the distribution and 268 expression of tdTomato in subsequent figures. For mounted vascular specimens, a rotated 3-dimensional 269 view (Figure 2M) was used to confirm that the distribution and expression of tdTomato was confined to 270 the endothelium. As seen in the image, tdTomato fluorescence is limited to the apical side of the vessel 271 wall, along with endothelial cell nuclei, and is not associated with underlying smooth muscle cell nuclei, 272 indicating the rat CDH5 promoter possesses the necessary gene regulatory elements to suppress 273 expression in smooth muscle cells. To better resolve tdTomato fluorescence on a single cell level, high 274 magnification images are shown in Figure 3. Here, tdTomato-positive cells are seen adjoining -negative 275 cells. Endothelial nuclei are shown relative to the orientation of blood flow, and the perpendicular 276 orientation of the underlying smooth muscle cell nuclei are visible.

tdTomato fluorescence in the conduit endothelium decreases as animals age. We examined agedependent distribution of endothelial-restricted tdTomato expression in carotid arteries (Figure 4) and jugular veins (Figure 5). Similar lookup tables were applied across all unmixed images so that tdTomato intensity was comparable across different image panels. When unmixed overlay images of vessel preparations obtained at young age (panels **D**, **G**, and **J** in **Figure 4** and **Figure 5**) were compared to images obtained at middle and old age, it was readily observable that the intensity of tdTomato fluorescence in both the carotid artery and jugular vein decreased with age.

284 tdTomato fluorescence reveals an endothelial cell microheterogeneity within the conduit vessel wall. 285 Images in Figures 2-5 illustrate a heterogeneous tdTomato fluorescence pattern among endothelial cells 286 within the vessel wall. Examples in Figures 3, 4J and 5J highlight this point, where immediately adjacent 287 cells either did, or did not, display fluorescence. This differential fluorescence pattern signifies an 288 endothelial microheterogeneity based upon the cell's ability to drive iCre expression sufficient to activate 289 the tdTomato reporter. Summary data from 4-6 rats across 3 ages, including young (6-7.5 months), 290 middle (14-16.5 -months) and old (17-19.5-month) age groups, revealed that $\approx 50\%$ of the vascular 291 surface area was covered by red fluorescence in young animals, and further, that this relative expression 292 pattern decreased significantly in middle- and old-age groups (Figure 6). A similar expression pattern, 293 and a similar age-related decrease in tdTomato fluorescence, was observed in both the carotid arteries 294 (Figure 6A) and jugular veins (Figure 6B). We noted similar tdTomato fluorescence patterns in both 295 male and female subjects. A similar expression of the tdTomato fluorescence signal was observed in other 296 vessel preparations, including the basilar artery, inferior vena cava, and aorta (see Supplemental Figure

297 1 at https://doi.org/10.6084/m9.figshare.12461894.

298 Organ microcirculations exhibit uniform tdTomato fluorescence. We screened various organs for 299 tdTomato fluorescence in the microcirculation, including the mesentery, liver, and spleen (Figure 7). In 300 all cases red fluorescence was observed, signifying widespread expression, especially prominent in the 301 microcirculation. This uniform capillary endothelial expression was also visualized in lung capillaries. 302 Here, to better visualize capillaries the circulation was filled with gelatin, the airways were filled with 303 agarose, and lung was cut into $\approx 300 \ \mu m$ sections prior to imaging. Fluorescence intensity is shown in 304 each channel (Figure 8A) and composites reveal extensive tdTomato fluorescence in the alveolar 305 capillaries (Figure 8B). In Figure 8B, a precapillary arteriole adjacent to the capillary network is 306 negative for tdTomato fluorescence, highlighting endothelial heterogeneity within the microcirculation.

Discussion

309 Here, we report development of a novel CDH5-iCre driven transgenic reporter rat that exhibits 310 exceptional endothelial-selective tdTomato fluorescence. In conduit arteries and veins the reporter is 311 restricted to the endothelium and is not seen in the underlying smooth muscle layer. Endothelium from 312 more than 50% of the vascular surface area possesses reporter fluorescence in the conduit vessels of both 313 males and females. The endothelial microheterogeneity visible within the intimal layer is notable; the 314 reason for such heterogeneity has not been established. Prominent tdTomato fluorescence is seen in the 315 parenchyma of various organs, especially in the lung's microcirculation where occlusive neointimal 316 lesions arise. Yet, in both the conduit vessels and the microcirculations, reporter intensity decreases 317 significantly with age.

318 While a majority of endothelial cells in conduit arteries and veins express the reporter, tdTomato 319 fluorescence was absent in cell clusters throughout the vessel wall. This differential expression pattern 320 supports the notion of endothelial microheterogeneity, where immediately adjacent cells differ in the 321 tdTomato fluorescence. Clonal niches have been previously reported in conduit blood vessels like the 322 aorta. For example, Schwarz and Benditt identified endothelial cell clonal niches based on thymidine 323 uptake as an estimate of replication rates(33, 34). They found that not all endothelial cells in the vessel 324 wall are replication competent. Rather, proliferation relies on the relatively high replication rates of few 325 cells within clusters. A similar heterogeneity of endothelial cell proliferation was reported in pulmonary 326 arteries and arterioles, especially during the development of pulmonary arterial hypertension. Ki-67 327 positive endothelial cells are seen in vascular locations that are remodeling to form occlusive lesions(9, 328 28). A hierarchy of single endothelial cell growth potentials exists among cells isolated from the vessel 329 wall. Single cell cloning of endothelial cells isolated from conduit vessels reveals that few cells are highly 330 replication competent(5, 20, 39). In contrast, endothelial cells isolated from the lung microcirculation 331 exhibit a high proportion of replication competent clones, suggesting it is an enriched endothelial 332 progenitor niche(3); a molecular basis for these proliferative lung capillary endothelial cells has recently 333 been identified(25). It is uncertain whether the presence or absence of tdTomato relates to endothelial cell

proliferative potential, yet this reporter animal provides a way to track endothelial heterogeneity both *in vivo* and *in vitro*.

336 Endothelial cell dysfunction is a cardinal feature of aging. We report a significant loss of endothelial 337 cell tdTomato fluorescence as the animal ages. Whether this decrease in fluorescence reflects a silencing 338 of tdTomato transcription, an increase in tdTomato turnover or some impairment in the fluorescent 339 property of tdTomato remains to be determined. Nonetheless, it is noteworthy that this decrease in 340 reporter efficacy tracks with the age-dependent loss of VE-cadherin within adherens junctions(8). The 341 loss of VE-cadherin in the adherens junction contributes to both impairment of endothelium-dependent 342 vasodilation(8) and increased permeability(6, 11, 26). Thus, the tracking tdTomato fluorescence as a 343 function of age may provide insight into vascular dysfunction that accompanies the aging process.

347 In conclusion, we report a first-in-kind endothelial-restricted transgenic reporter rat. Endothelial 348 expression of the tdTomato was ubiquitous in all circulations tested. Whereas a microheterogeneity in 349 tdTomato fluorescence was seen in conduit vessels, the fluorescence pattern was uniform in the 350 microcirculations of various organs, most prominently in the lungs. This transgenic reporter rat represents 351 an ideal model for assessing endothelial remodeling in various chronic vascular disease states, like 352 atherosclerosis and pulmonary arterial hypertension. The model will be useful for acute injury studies, 353 such as ischemic and hemorrhagic stroke, where adherens and tight junctions are disrupted and where 354 blood brain permeability is compromised. It allows for assessment of endothelial injury and repair in the 355 lung, as occurs during infection, where loss of capillary density and angiogenesis or vasculogenesis can 356 be tracked fluorescently. tdTomato fluorescence can be measured alongside other fluorescent 357 macromolecular tracers to assess sites of permeability within the vessel wall. However, tdTomato 358 fluorescence decreases as the animals age, which represents a limitation for its use in aging models. 359 Nonetheless, we anticipate this unique animal resource will have widespread application to physiological 360 studies in vascular biology.

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362	Acknowledgements
363	This work was supported by HL60024 (T.S., M.A., S.L., T.R., M.T., R.B., D.F.A.), HL66299 (T.S. and
364	M.A.), HL140182 (M.L. and T.S.), HL114474 (A.M.G.), HL116264 (A.M.G.), OD026560 (A.M.G.),
365	HL118334 (D.F.A.), and HL136869 (C.M.F. and T.S.). We would like to thank Dr. Michele Schuler and
366	Leigh Ann Wiggins for their assistance in veterinary care, and Dr. Madhuri Mulekar for her assistance
367	with statistical analysis.
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471

Figure Legends

472 Figure 1. The rat CDH5 promoter was cloned from pulmonary microvascular endothelial cells. 473 Organization of the murine (A) and rat (B) CDH5 genes. The cloned murine and rat CDH5 promoters 474 extend 86 and 79 bp, respectively, beyond corresponding transcription start sites. Translation initiation 475 sites are 11,285 bases and 22,923 bp downstream from the transcription initiation sites in murine and rat 476 primary transcripts, respectively. [C] Alignment of the mouse and rat promoter reveals moderate 477 similarity (from Clustal Omega multiple sequence alignment). [D] Schematic of the piggyback transposon 478 construct. ITR, piggyback inverted terminal repeats; PCDH5, rat CDH5 promoter; attP, PhiC31 479 recombinase sites; iCre, codon-optimized Cre recombinase; HA, influenza virus hemagglutinin (HA) tag; 480 pA, bovine growth hormone polyadenylation signal.

481 Figure 2. Spectral library construction and linear spectral unmixing to distinguish tdTomato signal 482 from tissue autofluorescence. False colored images of single label control samples of tdTomato (A), 483 NucBlue (B), and autofluorescence of tissue specimens for each tissue type from Sprague Dawley wild-484 type rats; a jugular vein is shown as a representative example (C). Spectral libraries (D) were constructed 485 using pure spectra of each endmember that were obtained from selected regions of interest shown in 486 panels A, B, and C. A Representative image from a 3-dimensional image stack (z-stack) of jugular vein 487 acquired at 20X magnification and false-colored by wavelength (E). Spectrally unmixed (non-negatively 488 constrained linear unmixing) images of tdTomato (F), Nuclei (G), and tissue autofluorescence (H) for a 489 given slice in the z-stack. 3-dimensional projections of the raw spectral image stack (I) and false colored 490 (red = tdTomato, blue = NucBlue, green = autofluorescence) unmixed images (J). Maximum intensity 491 projections of unmixed images with (K) and without autofluorescence signals (L). 3-dimensional project 492 highlighting the intima (upper panel of M) and lumen (lower panel of M) of an open vessel. Scale bar = 493 50 µm.

494 Figure 3. Single cell tdTomato fluorescence. [A] Unmixed false colored image of the jugular vein
495 vessel preparation (blue = nuclei, green = autofluorescence, and red = tdTomato). White lines indicate the

496 axis of blood flow through the vessel. The arrowhead highlights the orientation of the oblong endothelial 497 cell nuclei in the foreground that is perpendicular to the underlying and elongated smooth muscle cell 498 nuclei. [B and C] regions of interests shown on panel A (white boxes) are selected to visualize the 499 expression pattern of the tdTomato fluorescence signal in single cells. Scale bar on A, B, and C = 10 μ m.

500 Figure 4. Age-dependent loss of tdTomato fluorescence in the carotid artery. A representative 501 maximum intensity projection of a raw spectral z-stack image acquired using a 4X objective at young (A), 502 middle (B), and old (C) ages. Raw spectral images were unmixed to identify 3 spectral endmembers – 503 tdTomato in red, NucBlue in blue, and autofluorescence in green – and a false-colored merged image is 504 created (D-F). This procedure was also applied for image data acquired with a 20X (G-I) and a 60X 505 objective (J-L). Similar color look-up-tables were used such that the expression intensity is comparable 506 across the images. It is readily observable that the tdTomato fluorescence signal is decreased with aging 507 (compare D to E and F, G to H and I, and J to K and L). White lines in panels A, B and C indicate the axis 508 of blood flow through the vessel. n = 4, 6, and 6 animals for young, middle, and old age groups, 509 respectively.

Figure 5. Age-dependent loss of tdTomato fluorescence in the jugular vein. Maximum intensity projections of the raw spectral image data revealed that the total fluorescence intensity of the tdTomato signal is decreased over age (A, B and C). These results are easily visible by examining the unmixed merged images at 4X magnification (D-F). Similar expression and distribution trends of tdTomato fluorescence were observed when visualized with 20X (G, H, and I) and 60X (J, K, and L) objectives. White lines in panels A, B and C indicate the axis of blood flow through the vessel. n = 4, 6, and 6 animals for young, middle, and old age groups, respectively.

Figure 6. Quantitative assessment of the tdTomato fluorescence signal reveals a decrease with age in both males and females. Fraction area occupied by the tdTomato fluorescence signal is quantified in the carotid artery (A) and jugular vein (B) harvested from young (open circles, male, n = 2, and closed circles, female, n = 2), middle (open squares, male, n = 2, and closed squares, female, n = 4), and old (open triangles, male, n = 2, and closed triangles, female, n = 4) age groups. The percentage of the area 522 occupied by the tdTomato fluorescence signal (expression) decreased with age in both carotid arteries and 523 jugular veins. * denotes p < 0.05 by one-way ANOVA with a Newman-Keuls Multiple Comparison Test.

524 **Figure 7. tdTomato fluorescence is observed among organs.** tdTomato fluorescence was seen in the 525 mesentery, hepatic, and spleen microcirculations. In each case detection of the fluorescence was 526 decreased with age.

Figure 8. The pulmonary circulation displays prominent tdTomato fluorescence, particularly in the microcirculation. [A] Fluorescence signals in each of the tdTomato, autofluorescence, and NucBlue channels is shown, with a composite image from young-, middle-, and old-rat age groups. Robust tdTomato fluorescence is seen in the alveolar-capillary endothelium. Fluorescence intensity decreases with age. [B] A composite image reveals uniform tdTomato fluorescence in alveolar-capillary endothelium, although a precapillary arteriole is negative for fluorescence, illustrating heterogeneous cell phenotypes. Scale bar = 50 μm. [A]





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Figure 1

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Young

Middle

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Figure 8

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[B]