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Murine Neonatal Intravascular Injections: Modeling Newborn Disease

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The ability to perform murine neonatal intravascular injections likely will prove useful in studying many newborn-specific disease states that are modeled in mice. Unfortunately, effective intravascular injection in the neonatal mouse has been limited by developmental immaturity and small size. To establish a mouse model of neonatal intravascular injection, C57Bl/6 pups between birth and 6 d of age were injected with a buffered solution containing cells or vehicle alone. For both external jugular and superficial temporal vein injections, a 2-member team was used to position the pup, insert the needle, and perfuse the injectate. For superficial temporal vein injections, the vascular anatomy was visualized by using transillumination. After injection into the jugular or superficial temporal vein, the survival rate to adulthood was 100% (n = 30 pups per group), with no long-term complications. Occasional extravasation of injectate was well tolerated, allowing for serial injections (n = 40 pups). Intravascular access was confirmed by using fluorescent dye perfusion studies and cellular engraftment analysis. The 2 techniques are safe and reproducible methods of obtaining intravascular access via the external jugular and superficial temporal veins in newborn mice. These methods provide a mechanism for delivering a wide variety of substances, ranging from aqueous solutions to suspensions.

Abbreviations: GFP, green fluorescent protein

The mouse is widely accepted as a practical model to study human disease. Genetic malleability, along with relatively short reproductive and life cycles, makes the mouse model particularly useful compared with models in other, larger animals such as rabbits, pigs or sheep. However, for developmental and neonatal studies, the mouse pups' small size poses considerable challenges, particularly when intravascular injection is needed.

The ability to successfully perform murine neonatal intravascular injections likely will prove useful in studying many disease states unique to the newborn animal, such as respiratory distress syndrome, retinopathy of prematurity, and necrotizing enterocolitis. Unfortunately, effective intravascular injection in a neonatal mouse has been limited by both developmental immaturity and the small size of the pups. Typically, intravascular access in the adult mouse involves injection via the retroorbital or tail vein. In the murine neonate, persistent fusion of the eyelids after birth prevents retroorbital venous access, and the tail vein is prohibitively small for injection.

We describe 2 techniques for intravascular injection in neonatal mice from birth to 6 d of age. Both allow the injection of a variety of substances, including cells, medications, toxins, and cytokines, and both permit serial injections. Compared with a previously described technique using the superficial temporal vein,[2,8] this new technique allows alternate and multiple sites of injection, a smaller and less traumatic needle and syringe set-up, improved visualization through the use of transillumination, and, most importantly, an extended time period for serial injections (from birth until 6 d of life).

Material and Methods

Animals. All animal studies were carried out according to the guidelines set forth by the Baylor College of Medicine Institutional Animal Care and Use Committee. Animals were housed at the Baylor College of Medicine animal facility with a 12:12-h light:dark cycle and free access to food and water. We chose 1- to 6-d-old male and female C57Bl/6 mice (The Jackson Laboratory, Bar Harbor, ME) as a representative mouse model. Pregnant dams were obtained and monitored closely until delivery. The pups were housed with their mothers for routine maternal care and continued suckling.

Injection of external jugular vein. Pups were sedated for injection of the external jugular vein by using mild hypothermia.[7] Materials included a 33-gauge, 1/4-in. syringe with luer hub (Popper and Sons, New Hyde Park, NY), T-connector extension set (4 3/4 in., 0.25 ml; Braun, Bethlehem, PA), 1-ml tuberculin syringe, and gloves. Pups were injected with 100 µl of either Hanks Balanced Salt Solution alone (vehicle) or containing 1×10^7 unfractionated bone marrow cells. Bone marrow cells were isolated from the femurs and tibias of adult C57Bl/6 mice, as described previously.[3]

Injection of superficial temporal vein. Pups were sedated by using mild hypothermia. In addition to the materials described for external jugular injections, we used a handheld transilluminator (WeeSight, Respironics, Murrysville, PA), and a hands-free 2.75× magnification lens (MagEyes, Kerrville, TX). The injectates used were the same as described for external jugular vein injections.

We performed 4 additional experiments to assess the feasibility of serial injections of the external jugular and superficial temporal veins. After the first injection, pups (age, 3 to 6 d; 4 independent experiments, total n = 40) were left with the dam for 24 h, at which time they were reinjected, either in the same vessel or the contralateral one.

Intravascular injection of fluorescent dextran. Using the described injection methods, we introduced 20 µl of fluorescent Texas Red dextran (molecular weight, 10,000 Da; 50 mg/ml, Invitrogen-Molecular Probes, Eugene, OR) into the newborn vasculature. Immediately after the injection, the distribution

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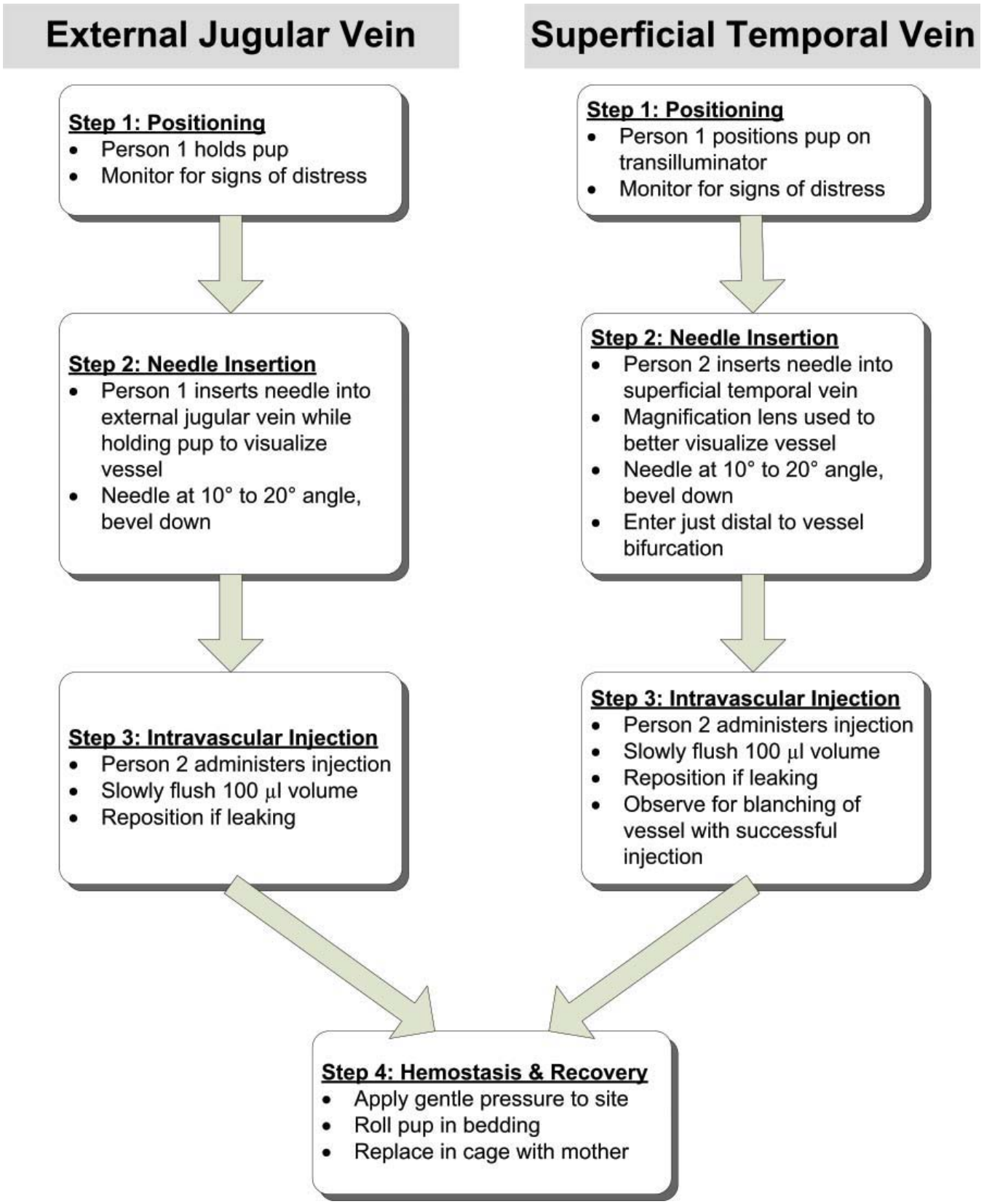

Figure 1. Overview. Strategy used for injection of external jugular and superficial temporal veins.

of dye was visualized by using a Zeiss fluorescent dissecting microscope equipped with AxioVision software (Carl Zeiss MicroImaging, Thornwood, NY).

Lung immunohistochemistry. In additional studies, pups were injected with unfractionated bone marrow cells isolated from the femurs and tibias of adult C57BL/6-Tg(ACTB-EGFP)1Osb/J mice (The Jackson Laboratory), which carry the gene for green fluorescent protein (GFP). After 28 d, lungs were harvested, airways perfused with sucrose-OCT (Tissue-Tek, Sakura Finetek, Torrance, CA) at a pressure of 20 cm, and frozen in OCT. Frozen

sections (14 µm) of experimental and control lung tissue were immunostained with rat antimouse CD45 antibody (dilution, 1:100; BD Biosciences, San Jose, CA) followed by goat antirat AlexaFluor 594 secondary antibody (dilution, 1:500; Invitrogen-Molecular Probes) to identify hematopoietic cells. Slides were mounted in mounting medium (VectaShield Mounting Medium with 4′,6-diamidino-2-phenylindole, Vector Laboratories, Burlingame, CA) for microscopic imaging and analysis. Tissues were analyzed using a Zeiss Axiovert 200M fluorescent microscope equipped with AxioVision (release 4.6) software (Carl Zeiss MicroImaging).

Results

Injection of external jugular vein. The external jugular veins in mice closely resemble those in humans, with only minor differences. Mice have 2 superior vena cava: the right external jugular vein drains into the right superior vena cava and then into the right atrium, whereas the left external jugular drains into the left superior vena cava, into the inferior vena cava, and then into the right atrium. This pattern contrasts with that in humans, in whom the left brachiocephalic vein carries blood from the left side of the head and neck and left arm into the single (right) superior vena cava.[4,9]

The injectate, either vehicle only or cellular suspension, was drawn into a 1-ml syringe and then used to prime the T connector and needle. Bubbles were eliminated to prevent potentially lethal air emboli. The selection of total volume and cellular concentration were based on estimates of total blood volume and data from previous reports.[5,8,10] With newborn mouse weights ranging from 1 to 3 g in the first 6 d of life,[9] the total blood volume was estimated to be 90 to 270 µl. We therefore were able to inject 100% to 33% of the total blood volume from 1 to 6 d of life, respectively. Pups were injected by a 2-person team: 1 person to position the pup and insert the needle, and the other to administer the syringe volume (Figure 1).

Step 1: Positioning. Person 1 held the pup in his or her nondominant hand, with the head and trunk stabilized between thumb and index finger and the neck extended slightly and rotated away from the vein to be injected so that the external jugular became more prominent and visible (Figure 2). Pups were closely monitored for signs of airway impedance, such as pallor, poor perfusion, decreased activity, and irregular respirations.

Step 2: Needle insertion. While holding the pup, person 1 inserted the 33-gauge needle, bevel down and at a 10° to 20° angle with the skin surface, into the external jugular vein just deep enough to observe insertion of the bevel into the vessel.

Step 3: Intravascular injection. Once the needle was inserted, person 2 slowly (over 10 to 30 s) infused 100 µl from the syringe. We found that a gentle tapping motion on the syringe plunger allowed a sufficiently slow infusion. A slow infusion rate was necessary to prevent extravasation of fluid through the vessel wall as well as to avoid lysis of the cellular injectate by the small gauge of the needle. The T connector provided flexible positioning of the needle and syringe, thereby reducing the risk of dislodging the needle from a small vessel during the injection. However, if the additional priming volume (0.25 ml) had been prohibitive, the T-connector tubing could have been omitted from the set-up. In the event of extravasation from the vessel, which was readily apparent in the thin, translucent skin of the neonatal mouse after injection of approximately 10 µl, the needle could have been repositioned or the contralateral external jugular vein used.

Step 4: Hemostasis and recovery. After injection, the needle was withdrawn, and gentle pressure was applied at the site of injection for approximately 30 to 60 s to prevent hematoma formation. The use of a cotton-tipped swab provided localized pressure and limited the risk of airway obstruction. The pups were rolled in cage bedding to re-establish their normal scent and then replaced in the cage with the nursing mother.

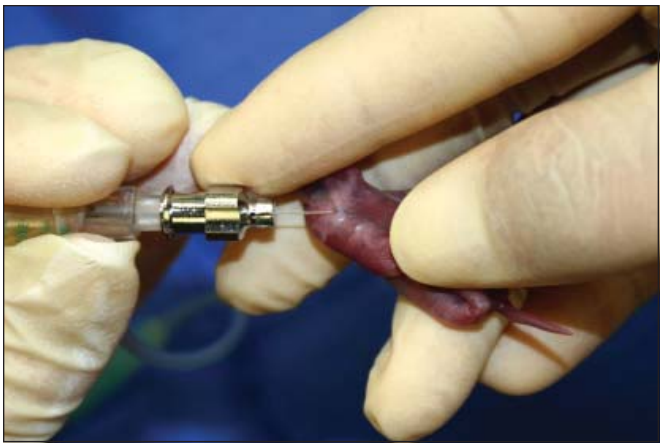

Figure 2. Positioning of pup for injection of external jugular vein. To visualize the external jugular vein, person 1 holds and positions the pup in the nondominant hand with the neck partially extended and rotated. Using the dominant hand, the needle is inserted for intravascular injection.

Injection of superficial temporal vein. Pups were injected between birth and 6 d of life. As with external jugular injections, accessing the superficial temporal vein required a 2-person team (Figure 1).

Step 1: Positioning. Person 1 positioned the pup over the transilluminator, using 1 hand to stabilize the trunk and exposed upper limb and the other to position the head and neck. The pup was positioned obliquely, between lateral and supine, with the neck extended and the head rotated to the side to provide optimal exposure of the superficial temporal vein (Figure 3). As with external jugular injections, pups were monitored for signs of airway obstruction or distress.

Step 2: Needle insertion. Person 2 used hands-free magnification lenses to improve visualization of the vessel and the injection procedure. Person 2 inserted a 33-gauge needle, bevel down and at a 10° to 20° angle, through the skin at a point just distal to the bifurcation of the vessel. The needle then was slowly advanced until it entered the vessel lumen. By gently lifting the needle, it was possible to determine whether the needle had not yet advanced through the vessel wall.

Step 3: Intravascular injection. While holding the needle in place, person 2 gently tapped the syringe plunger to begin infusion. If the needle was malpositioned outside of the vessel, the injected fluid was visible immediately (usually before 10 µl had been injected) in the extravascular space, and the needle was repositioned. When the fluid was injected into the intravascular space we observed blanching of the vessel, followed by pulsatile flow of injectate through the local vascular network. In the case of extravasation and hematoma formation, we moved proximally along the same vessel or to the contralateral side.

Step 4: Hemostasis and recovery. After injection, gentle pressure with a cotton-tipped swab was applied at the site of injection for 30 to 60 s to prevent hematoma formation. The pups were rolled in cage bedding to re-establish their normal scent and then replaced in the cage with the nursing mother.

Morbidity and mortality. Successful injections (that is, without extravasation) were accomplished into the external jugular or superficial temporal vein of all mice (n = 30 for each group). Experimental pups were evenly distributed throughout the range of 1 to 6 d of life. Additional experiments using serial

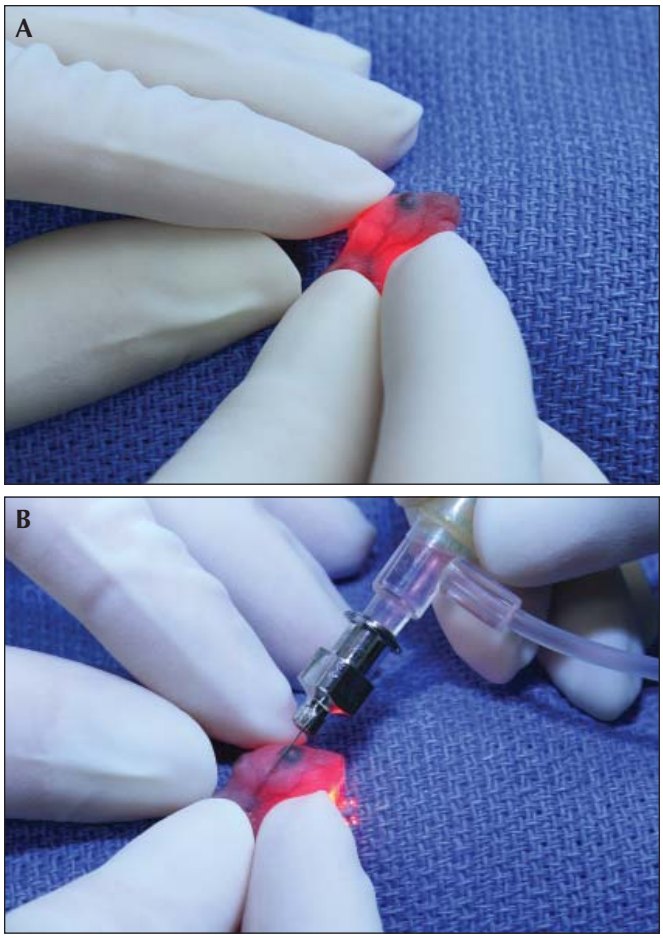

Figure 3. Positioning of pup for injection of superficial temporal vein. (A) Person 1 holds the pup obliquely, with the neck extended and head rotated to the side to expose the superficial temporal vein. Transillumination of the pup optimizes visualization of the vascular anatomy. (B) Person 2 inserts the needle into the superficial temporal vein for injection.

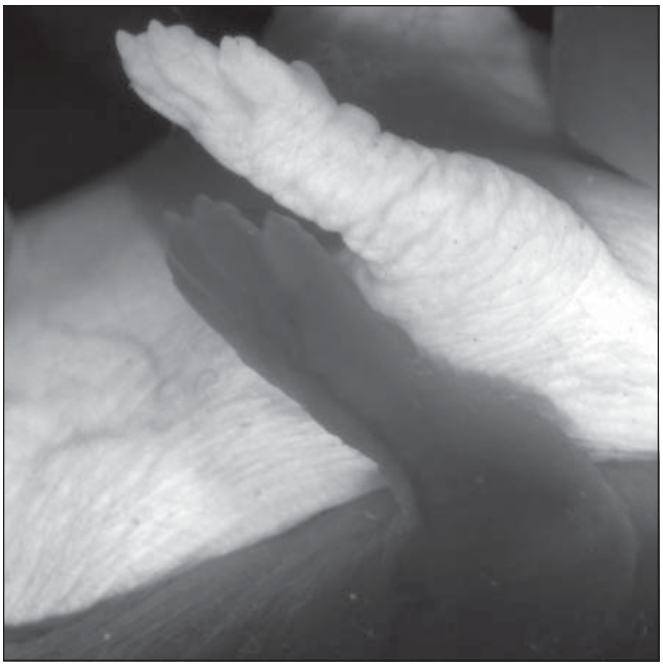

Figure 4. Intravascular injection of fluorescent dextran. After intravascular injection of Texas Red Dextran, the fluorescent dye was distributed rapidly throughout the pups' vasculature. The noninjected control pup showed no fluorescence. In these grayscale images, used to convey fluorescence data more accurately, darker shades represent progressively higher levels of red fluorescent signal, whereas white represents an absence of fluorescence.

injections were performed on pups (4 independent experiments, total n = 40) between days 3 to 6 of life, with successful injections in all of the animals. Pups were left to recover with the nursing dams. At 4 wk, all of the 100 pups were alive with no evident morbidity.

Evidence of successful intravascular access. Additional experiments were performed to confirm that the neonatal injections adequately delivered substrates into the intravascular space. Fluorescent-labeled dextran conjugates often are used to study the circulation and vasculature.[1,6] To document short-term vascular distribution, we injected Texas Red dextran into the mouse pups by using the described techniques. Immediately after injection, the dye was visible throughout the pup, most easily in the blood vessels of the limbs and tail. Distribution of the dye throughout the microvasculature was visualized by using a fluorescent dissecting microscope. A representative image is shown in Figure 4.

To demonstrate both long-term vascular distribution and the viability of cells after neonatal injection, we analyzed various tissues, including lung, 28 d after injection of the external jugular (n = 5) or superficial temporal (n = 9) vein for the presence of injected GFP-labeled bone marrow cells or progeny thereof. Anti-CD45 antibodies were used to identify hematopoietic cells. Colocalization of CD45 and GFP by using fluorescent microscopy represented hematopoietic cells derived from the injected bone marrow population. Both GFP-positive (injectate origin) and GFP-negative (host origin) hematopoietic cells were present in the lung tissue of all of the injected animals (n = 14; Figure 5). Saline-injected control animals showed no GFP-positive cells. Therefore, neonatal injections achieved successful intravascular delivery and maintained the viability of injected cells.

Discussion

The use of mice for neonatal research calls for intravascular access techniques that can be learned and applied easily. To that end, we developed 2 injection methods for neonatal mice: external jugular vein injection and superficial temporal vein injection through transillumination. Both are relatively simple techniques with steep learning curves and can be mastered readily by researchers with minimal experience in obtaining intravenous access. Both methods were safe, with high survival and success rates.

The benefits of external jugular injections are multifold. Because the external jugular vein is a large-caliber vessel, it is easily visible without magnification and is tolerant of concentrated injectates. Bilateral external jugular locations provide alternate access sites for use with serial injections or in cases of extravasation and hematoma formation. Most importantly, because the large external jugular vessel remains visible as skin pigmentation increases, use of the EJ vein extends the window for neonatal injections to 6 d of age.

Injections into the superficial temporal vein by using transillumination provide similar benefits. The use of both magnification and transillumination markedly improve visualization of the vessel, needle position, and the injection process and thereby facilitate consistently successful injections. Bilateral positions provide multiple injection sites. As with external jugular vein

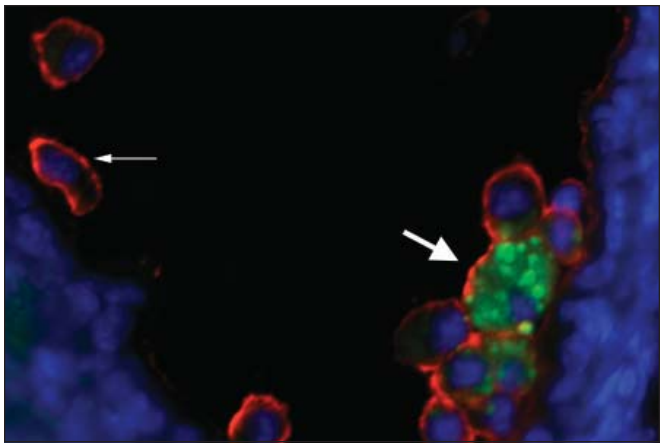

Figure 5. Engraftment of injected hematopoietic cells. To demonstrate both intravascular access and the viability of injected cells, lung tissues were analyzed 28 d after neonatal injection of GFP-labeled bone marrow cells (green). Sections were stained with anti-CD45 antibody (red) to identify hematopoietic cells. This section of a large blood vessel in the lung shows hematopoietic cells of both injectate origin ($CD45^+$ GFP^+, large arrow) and host origin ($CD45^+$ GFP^-, small arrow). Nuclei are stained with 4′,6-diamidino-2-phenylindole (blue).

injections, the enhanced visualization extends the use of the superficial temporal vein from birth to 6 d of age.

The ability to reliably achieve intravascular access in newborn mouse pups will allow researchers to further develop mouse models of human neonatal disease. Our newly developed injection techniques provide the mechanism to deliver cells, medications, chemokines, hormones, and other agents to neonatal animals. The availability of improved neonatal research methods will advance our knowledge of often devastating newborn diseases, from genetic disorders to complications of prematurity.

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