In Vivo Brain Delivery of v-myc Overproduced Human Neural Stem Cells via the Intranasal Pathway: Tumor Characteristics in the Lung of a Nude Mouse

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Abstract

We aimed to monitor the successful brain delivery of stem cells via the intranasalroute and to observe the long-term consequence of the immortalized human neural stem cells in the lungs of a nude mouse model. Stably immortalized HB1.F3 human neural stem cells with firefly luciferase gene (F3-effluc) were intranasally delivered to BALB/c nude mice. Bioluminescence images were serially acquired until 41 days in vivo and at 4 hours and 41 days ex vivo after intranasal delivery. Lungs were evaluated by histopathology. After intranasal delivery of F3-effluc cells, the intense in vivo signals were detected in the nasal area, migrated toward the brain areas at 4 hours (4 of 13, 30.8%), and gradually decreased for 2 days. The brain signals were confirmed by ex vivo imaging (2 of 4, 50%). In the mice with initial lung signals (4 of 9, 44.4%), the lung signals disappeared for 5 days but reappeared 2 weeks later. The intense lung signals were confirmed to originate from the tumors in the lungs formed by F3-effluc cells by ex vivo imaging and histopathology. We propose that intranasal delivery of immortalized stem cells should be monitored for their successful delivery to the brain and their tumorigenicity longitudinally.

In Vivo Delivery of therapeutic cells as well as nanoparticles or small molecules has been done in a variety of ways, such as direct or systemic administration. To treat neurologic disorders, stem cell–based therapy has been regarded as a promising therapeutic strategy.1,2 An important issue in stem cell therapy is to determine the safe and efficient way for optimal delivery to the brain. Direct transplantation of stem cells to the brain is confirmative but invasive, with possible unexpected side effects. Thus, intranasal delivery (IND) is regarded as a good alternative with favorable delivery efficiency and safety.

In a previous study, successful delivery of stem cells to the brain bypassing the blood-brain barrier (BBB) was reported via the nasal pathway for the first time.3 Following this report, several studies demonstrated successful IND of stem cells to the brain in the diseased animal model with substantial therapeutic efficacy.3–6 Molecular imaging modalities including bioluminescence optical imaging, magnetic resonance (MR) imaging, and radionuclide imaging have different characteristics in terms of their signal sensitivity and contrast. In particular, the bioluminescence imaging using luciferase enzyme has shown great potential for elucidating numerous biological phenomena noninvasively with a high signal to background ratio. IND of various drug or delivery carriers can simply be evaluated using specific imaging probes and in vivo bioluminescence imaging techniques. In a previous report, intranasally delivered semiconductor quantum dots (QDs) were used as a potential drug vehicle and monitored successfully by fluorescence image guidance.7 QDs were

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widely delivered to the brain areas, including the olfactory bulb, cortex, and caudate-putamen, at 3 hours after delivery. In another report, \(^{18}\)F-labeled nanogel was delivered efficiently to the brain via IND as a therapeutic vaccine, which was monitored successfully using positron emission tomography (PET).\(^8\) We report the in vivo imaging of the IND of neural stem cells into the nude mouse brain using a bioluminescence imaging technique.

In addition to the efficiency of IND, safety is another important issue that we should be concerned about in stem cell implantation because stem cells have the characteristics of self-renewal and homing/migration. Oncogene-immortalized stem cells are widely considered to be due to their (stem cell) genetic uniformity and easy access. Intracerebral transplantation of human neural stem cells immortalized with \(v\)-myc showed improved functional recovery from the Rotarod test and limb placement behavioral test in an intracerebral hemorrhage stroke mouse model.\(^9\) Although these oncogene-transduced stem cells showed therapeutic effect with continuous proliferation, these cells might cause unexpected outcomes in vivo.

Image-guided stem cell tracking was proposed to be useful for evaluating the in vivo distribution of stem cells delivered via the nasal route. In this study, we monitored longitudinally the intranasally delivered human neural stem cells in a noninvasive way using a bioluminescence imaging technique and examined their tumorigenic characteristics in the lungs when the cells migrated to the lungs via the respiratory tract.

**Materials and Methods**

**Human Neural Stem Cell Culture**

A stably immortalized human neural stem cell line, HB1.F3 (F3), was generated via transfection of fetal brain cells with a retroviral vector encoding the \(v\)-myc oncogene as reported previously.\(^{10}\) F3 human neural stem cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 5% fetal bovine serum, 5% horse serum (Invitrogen), 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin. A retroviral vector containing codon-optimized firefly luciferase complementary DNA, Thy1.1 (CD90.1), and an internal ribosomal entry site (IRES) was constructed for enhanced bioluminescence cell imaging (Figure 1A).\(^9\) F3 cells were transduced with the recombinant retroviral vector encoding luciferase gene (F3-effluc). To see whether the luciferase gene in established F3-effluc cells is constantly expressed during cell growth, \(0.5 \times 10^5\) F3-effluc cells were seeded into a six-well plate, and in vitro luciferase assays were done serially for 6 days using a luminometer (TR717, Applied Biosystems, Grand Island, NY). The detailed transduction process and in vitro luciferase assay were described in our previous report.\(^9\)

![Figure 1. Representation of effluc construct and in vitro luciferase assay of F3-effluc cells in a time-dependent manner. A, Retroviral vector contains engineered luciferase gene and Thy1.1 (CD90.1) with an internal ribosomal entry site (IRES). B, Bioluminescence signals of seeded F3-effluc cells were increased serially in a time-dependent manner. LTR = long terminal repeat.](image-url)
IND of F3-effluc Neural Stem Cells in Nude Mouse

Nineteen BALB/c nude mice were housed under the standard laboratory conditions and were used according to the approved guidelines of the Institutional Animal Care and Use Committee (IACUC) of Seoul National University College of Medicine. Animals were kept in the facilities for at least 1 week before the experiment. Nineteen mice are divided into three groups: (1) IND of F3-effluc cells (n = 13), (2) IND of F3 cells (n = 3), and (3) intravenous (IV) delivery of F3-effluc cells (n = 3). The optimized protocol for IND of cells to the brain was adopted from previously reported studies. Before cell delivery, mice were anesthetized with an intramuscular injection of 10 μL of 5% zoletil 50 (Virbac, Carros, France) and 2% xylazine (Rompun, Bayer, Leverkusen, Germany) solution (2:1) and placed in a supine position with rolled-up tissue under the neck so that the head was kept flat. Hyaluronidase (Sigma-Aldrich, St. Louis, MO) was dissolved in phosphate-buffered saline (PBS), and 5 μL of 100 U hyaluronidase was intranasally delivered into each nostril alternately every 2 minutes (total 20 μL per each mouse) to promote the efficiency of stem cell penetration as previously described. Mice were left in the same position for 30 minutes, and then neural stem cells (F3-effluc cells or F3 cells) were intranasally delivered in the same position. Five microliters of F3-effluc or F3 cells was delivered alternately into each nostril every 2 minutes (total 5 × 10⁶ cells/20 μL PBS per each mouse). Mice were kept in the same position for another 60 minutes after IND. IND of F3-effluc cells (total 5 × 10⁶ cells/200 μL PBS per each mouse) was done via the tail vein under the same anesthesia method as IND.

In Vivo and Ex Vivo Bioluminescence Imaging

Of 13 mice receiving IND of F3-effluc cells, 4 were randomly selected and sacrificed at 4 hours after IND and the other 9 mice were monitored using in vivo bioluminescence imaging until 41 days after IND. Among the 9 mice, 4 expired unexpectedly 26 days after IND. Thus, ex vivo bioluminescence images could be taken in 4 mice, which were sacrificed at 4 hours after IND, and 5 mice, which survived for 41 days. Of 3 mice receiving IND of F3 cells, 1 was sacrificed at 4 hours after IND and the other 2 mice were used as control in vivo bioluminescence imaging and sacrificed 41 days after IND. Lastly, among 3 mice that were delivered F3-effluc intravenously, 1 mouse expired unexpectedly and the other 2 mice were sacrificed at 8 days after F3-effluc IV delivery because the mice met the criteria of the humane euthanasia guidelines of the IACUC of our institute. The detailed timeline is described in Figure S1 (online version only).

Mice were anesthetized with intramuscular injection of zoletil-xylazine solution and had intraperitoneal injection of 100 μL of 30 mg/mL D-luciferin (Caliper Life Sciences, Waltham, MA) per each mouse 5 minutes before every bioluminescence imaging. Anesthetized mice were placed in the prone position in the chamber of the IVIS 100 imaging system equipped with a cooled charge-coupled device camera (Caliper Life Sciences). Bioluminescence images were acquired by integration for 5 minutes (binning: med, f stop: 1) twice as follows: (1) whole body and (2) brain area after covering the nose and body with black paper to reduce the influence of other signals. Each serial time point for image acquisition was 1 hour, 4 hours, 1 day, 2 days, 5 days, 8 days, 12 days, 19 days, 26 days, and 41 days after IND of F3-effluc or F3 cells. To quantify the signal intensity, regions of interest (ROI) were defined over the nasal, head, and lung areas. The same size and shape of ROI were used between the animals. For ex vivo imaging, mice were sacrificed immediately after the last in vivo bioluminescence image acquisition. The harvested organs were placed inside the chamber of the IVIS 100 and the images were taken for 1 minute (binning: med, f stop: 1). Signal intensities were displayed in pseudocolor scale. The signal was quantified in units of photons per second per square centimeter per steradian (photons/s/cm²/sr).

Histopathologic Examination

The harvested organs of all the mice were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, and section slides of lung were prepared onto the slide glass. Immunohistochemistry was performed in lung tumor using rabbit anti-Ki67 antibody (AB9260, 1:300, Millipore, Billerica, MA), rabbit antinestin antibody (N5413, 1:100, Sigma-Aldrich), and rabbit anti-MAP2 antibody (M3696, 1:300, Sigma-Aldrich). Tissue sections were incubated with the primary antibody for 1 hour followed by secondary antibodies for 30 minutes, which were Dako polyclonal rabbit antigoat immunoglobulins/biotinylated for goat primary antibody (LSAB system, Dako Corp., Carpinteria, CA) and UltraMap anti-rabbit IgG conjugated with horseradish peroxidase (research use only) for rabbit primary antibody (Ventana Medical Systems, Tucson, AZ). Antibody binding was visualized with a DAB Map kit for goat primary antibody and a ChromoMap kit for rabbit primary antibody (Ventana Medical Systems).
Statistical Analysis
Statistical analysis was performed using SPSS version 17.0 (IBM Corp., Armonk, NY), and \( p < .05 \) was considered significant. A Mann-Whitney test was done to evaluate the difference between the bioluminescence signals of the lungs of groups A and B at each time point.

Results

F3-effluc Neural Stem Cells Showing Bioluminescence Signal
In serial in vitro luciferase assay, a continuous increase in the bioluminescence signal of F3-effluc cells was found as the cell population increased, suggesting that F3-effluc did not show any luciferase gene silencing over time (Figure 1B).

In Vivo Bioluminescence Imaging of Intranasal Brain Delivery of Neural Stem Cells
IND of F3-effluc cells was successfully conducted in all mice without mortality. Among 13 mice with intranasally delivered F3-effluc cells (\( n = 13 \)), bioluminescence signals in the brain areas were found in 4 (30.8%), and the signals were prominent from 4 hours after IND and gradually disappeared for 2 days (Figure 2A). In contrast, no signal was found in the mice with intranasally delivered nonlabeled F3 cells (\( n = 3 \); see Figure 2A). In addition, there was no signal in the brains of mice (0 of 3, 0%) with intravenously delivered F3-effluc cells for 8 days after IV delivery; however, intense bioluminescence signals were observed in the lungs (3 of 3, 100%; Figure S2, online version only).

Longitudinal In Vivo Bioluminescence Imaging of Mice with Intranasally Delivered Neural Stem Cells
Among 13 mice with intranasally delivered F3-effluc cells, 4 were randomly selected and sacrificed at 4 hours to observe early ex vivo findings, and the other 9 mice were monitored for the long-term in vivo imaging. The 9 mice were divided into two groups by visual inspection of the bioluminescence imaging: mice with presence (group A) and mice with absence (group B) of intense bioluminescence signals in the lung fields during a day after IND (Figure 2B). In group A (\( n = 4 \)), the signals of delivered F3-effluc cells were found in the lung fields during a day after IND. In contrast, other mice (group B, \( n = 5 \)) showed that most of the F3-effluc cells remained in the nasal cavity area without migrating to the lung fields during the first day. The bioluminescence signal in the lung fields disappeared in the group A mice until 5 days after IND, but the signal reappeared at 26 days after IND. The signals in the lung increased vigorously until 41 days after IND.

Ex Vivo Imaging of Brains and Lungs after IND of Neural Stem Cells
Among randomly selected four mice that were sacrificed immediately after in vivo imaging at 4 hours after IND of F3-effluc, one mouse showed increased bioluminescence signals in the brain in in vivo and ex vivo bioluminescence imaging. In addition, one more mouse showed increased bioluminescence signal in the brain in ex vivo bioluminescence imaging, which was not evident in in vivo imaging, probably due to the limited sensitivity of in vivo imaging.\(^{11}\) On the other hand, there was no bioluminescence signal in nonlabeled F3-delivered mice (Figure 2C). The signals emitted from the resected brain suggested that F3-effluc cells could reach the brain area within 4 hours (2 of 4, 50%). In addition to the brain, bioluminescence signals were also found in the lungs of all F3-effluc–delivered mice at 4 hours in ex vivo imaging (see Figure 2C). In ex vivo imaging at 41 days after IND (\( n = 5 \)), there was a positive bioluminescence signal in the brain of one mouse (1 of 5, 20%), although no signal had been detected in in vivo imaging. In addition, two mice showed intense lung signals that had shown intense bioluminescence signal in in vivo imaging (see Figure 2, B and D).

Quantitative Monitoring of Intranasally Delivered Neural Stem Cells in the Lungs
Intense bioluminescence signals were found in the nasal areas of all nine mice immediately after IND (mean ± SD 7.1 ± 6.5 \( \times \) \( 10^4 \) photons/s/cm\(^2\)/sr at 1 hour postdelivery). Besides bioluminescence signals in the nasal and head areas, signals in the lung fields were observed with variable intensity from \( 3.9 \times 10^2 \) to \( 3.4 \times 10^4 \) photons/s/cm\(^2\)/sr in the nine mice within 1 hour after IND (mean ± SD 0.7 ± 1.1 \( \times \) \( 10^4 \) photons/s/cm\(^2\)/sr, \( n = 9 \)) (Figure 3A). At every time point within 5 days after IND, the bioluminescence signals in the lung fields of all nine mice were higher than those of the control mice with nonlabeled F3 cells. In four of the nine mice, which are classified as group A in Figure 2B, the signal intensities in the lung fields were higher over \( 10^4 \) photons/s/cm\(^2\)/sr within 5 days after IND. In addition, the signal intensities in the lung fields were significantly higher in group A than in of group B at 4 hours after IND (\( p < .05 \); Figure 3B). After the initial peak, the signal...
Figure 2. Representative in vivo and ex vivo images of a nude mouse after intranasal delivery (IND) of F3-effluc or nonlabeled F3 cells. A, The bioluminescence signal of the brain area was detected from 4 hours and gradually disappeared 2 days after IND of F3-effluc cells. However, no signal was detected in the nonlabeled F3-delivered mouse. The nasal and body area were covered with black paper to reduce the influence of high bioluminescence signal from the other part. B, Representative in vivo whole body bioluminescence serial images of group A and group B from F3-effluc cell–delivered mice. The mouse in group A shows initial and reappearing high bioluminescence signal in the lungs, and the mouse in group B shows no bioluminescence signal in the lungs. C, In vivo and ex vivo images at 4 hours after IND of F3-effluc or F3 cells. Bioluminescence signal was observed in the brain area of the F3-effluc–delivered mouse. Also, isolated brain and lung tissue showed a clear bioluminescence signal in the ex vivo image (upper row, left to right: brain, lung, liver, kidneys, heart). No bioluminescence signal was observed in the F3 cell–delivered mouse, both in vivo and ex vivo images (lower row). D, Ex vivo images of the brains and lungs of five mice that survived for 41 days after IND of F3-effluc cells. One mouse showed a bioluminescence signal in the brain, and two mice showed a positive signal in the lungs (organs from the same mouse in each row).
Intensities of the lungs of all mice decreased until 5 days after IND (mean ± SD 1.1 ± 0.7 × 10^3 photons/s/cm^2/sr, n = 9). Around 4 weeks after IND, lung signals increased over 10^6 photons/s/cm^2/sr in group A, whereas the signals of group B remained low (below 10^4 photons/s/cm^2/sr); thus, the lung signals of group A were significantly higher than those of group B from 12 to 26 days after IND (p < .05; see Figure 3B).

Histopathologic Findings of Lung Tumors

In vivo images were followed up for a maximum of 41 days, and the five mice that survived were sacrificed immediately after in vivo image acquisition for the ex vivo validation study. When the lungs were dissected, no gross tumor was found in the three mice in which lung signal was not detected in in vivo imaging. In contrast, multiple masses in the lung were found in the other mice (2 of 5, 40%) that had shown high bioluminescence signals in the lung fields at 41 days after IND. Also, there were multiple lung masses in one mouse that received IND of nonlabeled F3 cells at 41 days after IND (1 of 2, 50%). The histologic findings of the lungs revealed mass-forming F3-effluc cells with central necrosis (Figure 4A). Tubule-like structures with high mitosis were observed, which reflected the characteristics of the neural stem cells (Figure 4, B and C). Similar histologic findings were observed in the lung masses of the mouse that received the intranasally delivered nonlabeled F3 cells (data not shown). Immunohistochemistry of lung masses showed a positive stain for anti-Ki67, indicating the high mitosis (Figure 5A). In addition, the mass-forming F3-effluc cells were positive for nestin but negative for MAP2, suggesting that the delivered cells maintained their neural stem cell property and were not differentiated (Figure 5, B and C).

Discussion

The aims of this study were to investigate the feasibility of the use of optical image–based monitoring to examine stem cell distribution after IND, to evaluate stem cell delivery and survival with long-term follow-up in vivo in a nude mouse, and to demonstrate the possible use of the same technique to disclose tumor formation in the lungs after IND of immortalized human neural stem cells. After the first report of Danielyan and colleagues indicating that intranasally delivered stem cells could penetrate the BBB and enter the brain parenchyma,3 other investigators recapitulated the findings in various disease animal models.4–6 Recent studies developed an MR- or fluorescence-based in vivo imaging system for tracking intranasally delivered stem cells in a disease animal model.6,10 However, none of them paid attention to the whereabouts and fate of stem cells in the long-term follow-up after IND.

In this study, we could clearly examine the in vivo distribution of the delivered neural stem cells via the nasal route using in vivo bioluminescence imaging techniques. IND of neural stem cells showed the in vivo distribution partially in the mouse head or sometimes in the lungs at...
initial time points, and those with initial lung signals showed highly intense bioluminescence signals in the lungs 41 days after IND. Consistent with the results in the literature for QD nanoparticle-based or mesenchymal stem cell-based IND, at around 3 to 6 hours after IND of stem cells, we could also find that F3-effluc cells were taken up highly in the brain area. However, the signals of brain-delivered F3-effluc cells were decreased after 2 days post-IND, indicating that the majority of brain-delivered cells migrated out from the brain or did not survive in the brain tissue. These results are in line with our previous report showing that transplanted F3-effluc cells decreased until 10 days after transplantation. In the present study, the signals vanished at an earlier time point than that of the previous report (2 days vs 10 days). This is probably because of a different delivery method. In the previous report, we transplanted F3-effluc cells directly to the striatum. Thus, the numbers of brain-delivered cells were

Figure 4. Histology of mass-forming F3-effluc cells in the lung of the nude mouse, sacrificed at 41 days after intranasal delivery (hematoxylin-eosin stain). A, A lung section with gross masses containing central necrosis is shown (arrow). B and C, The primitive cell population with tubule-like structures (arrow) is found in mass-forming F3-effluc cells. A: original magnification X12.5, B: original magnification X100, C: original magnification X400.

Figure 5. Immunohistochemistry results of the lung masses in the nude mouse, sacrificed at 41 days after intranasal delivery. The tumor was positively stained for anti-Ki67 (A) and anti-nestin (B) but negative for anti-MAP2 (C). Upper panels: original magnification X40, Lower panels: original magnification X200.
smaller in the present study than in the previous report, inferred from the in vivo bioluminescence signal (IND in the present study vs direct transplantation in the previous report, $5.2 \times 10^3 \pm 4.1 \times 10^3$ vs $1.9 \times 10^4 \pm 8.6 \times 10^3$ photons/s/cm$^2$/sr). Moreover, intranasally delivered cells are not localized in the same area, so it might be more difficult to survive in brain tissue than direct transplantation. Although brain areas showed no bioluminescence signal in in vivo imaging until 41 days after IND, one brain showed positive signal in ex vivo imaging at 41 days after IND. This discrepancy could be explained by limited sensitivity of in vivo bioluminescence imaging. In concordance with the present study, long-term survival of intranasally delivered stem cells was reported in previous studies. 

Immunohistologic analysis showed mass-forming F3-effluc cells with a highly mitotic pattern in the lung tissues. There were several reports regarding tumor formation after transplantation of embryonic stem (ES) cells in vivo. Although the human neural stem cells are usually considered to be safer regarding tumorigenic risk than ES cells, the transformation into tumorigenic cell type from mesenchymal stem cell or neuronal stem cell was reported when the cells were cultured for a long period of time in vitro. However, there has been no such report that tumor was formed in vivo in the lungs except IND of v-myc-expressing F3 cells. This report raises the concern.

A plausible explanation of the tumorigenic characteristics of the immortalized neural stem cells in the present study might be that we used the cells immortalized via infection with a retroviral vector encoding the v-myc oncogene. However, there was no report that transplantation of v-myc-immortalized F3 cells caused tumor formation. In a recent study, the tumorigenic properties of F3 cells were investigated and in vivo brain inoculation of F3 cells expressing both v-myc and h-ras oncogenes caused tumor formation, but not with F3 cells expressing only v-myc. Another study proposed tumorigenesis from neuronal stem cells when abnormally expressed c-myc and RE1-Silencing Transcription Factor (REST)/Neuron-Restrictive Silencer Factor (NRSF), which is a global transcriptional repressor for maintenance of stemness, act together. Accordingly, other unknown factor(s) might have caused the tumor formation in the lungs by immortalized F3 cells in the present study.

Following are the possible attributable factors that explain the tumorigenesis in the lungs. First, unlike the other investigations using v-myc-immortalized F3 cells in the literature, we used IND, and some cells could escape to unwanted areas, such as the lungs, which had an abundant blood supply and a humid environment. Second, the number of transplanted cells ($5 \times 10^6$ cells) was greater than in previous IND studies with mouse ($3 \times 10^5 - 5 \times 10^5$ cells). Lastly, the animals used were nude immunocompromised mice, whose immunosurveillance was not sufficient to eliminate the human-originated neural stem cells.

For clinical translation of IND as an alternative method for brain delivery of stem cells instead of invasive methods such as intracerebral or intrathecal delivery, first, an effective way to make stem cells directed solely to the target areas of the brain should be developed. Second, the number of stem cells should be optimized, not only to obtain a sufficient number of brain-delivered cells for treatment efficacy but also to avoid the risk of tumor formation by grafted stem cells.

**Conclusion**

Using bioluminescence imaging, the IND of v-myc-transduced human neural stem cells was successfully monitored, regarding their survival and migration serially. Intranasally delivered neural stem cells could be naturally delivered to the lung and form tumor. Therefore, IND of stem cell should be optimized in a safe way, and image-guided in vivo tracking of the delivered stem cells is necessary for successful cell therapy.

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**References**

Figure S1. Schematic display of the study design. Among 13 mice receiving intranasal delivery (IND) of F3-effluc cells, 4 were sacrificed at 4 hours after IND, 4 expired unexpectedly, and the other 5 were sacrificed after 41 days after IND. Among 3 mice receiving IND of F3 cells, 1 was sacrificed at 4 hours after IND and the other 2 were sacrificed at 41 days after IND. Among 3 mice receiving intravenous (IV) delivery of F3-effluc cells, 1 died unexpectedly and the other 2 were sacrificed at 5 days after IV delivery. In vivo imaging was done serially, and ex vivo imaging was done after the last in vivo imaging of the mice. Lastly, histopathologic examinations were done.

Figure S2. A. Representative serial in vivo images after intravenous delivery of F3-effluc cells. Intense lung signals were found at every time point. B, Ex vivo image of the mouse sacrificed at 8 days after intranasal delivery of F3-effluc cells. Intense signals were found in the lungs, liver, and heart.