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Original Article

Quantitative assessment of human uterine fibroid xenograft using sequential in vivo bioluminescence imaging

Yihong Pan[1], Tongqian Xiao[2], Yuanshuai Zhou[2], Zhihong Chai[3], Zhongjuan Xu[2], Meizhen Dai[4], Xuejiao Chen[4], Guangli Suo[2], Deyou Tao[5]

[1]Gynecology of Taizhou Enze Medical Center (Group) Enze Hospital, Taizhou, Zhejiang, China; [2]CAS Key Laboratory of Nano-Bio Interface, Suzhou Institute of Nano-Tech and Nano-Bionics, Chinese Academy of Sciences, Suzhou, Jiangsu, China; [3]Gynecology of Taizhou Municipal Hospital, Taizhou, Zhejiang, China; [4]Central Laboratory of Taizhou Hospital, Taizhou, Zhejiang, China; [5]Surgical Oncology of Taizhou Enze Medical Center (Group) Enze Hospital, Taizhou, Zhejiang, China

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Abstract: Uterine fibroid is one of the most common solid tumors occurring in reproductive age women. Lack of accurate methods for *In vivo* quantitative assessment of uterine fibroid progression severely impedes the basic research and drug screen of this disease. To solve this problem, the correlation between bioluminescence imaging (BLI) and initial cell number used to form xenograft was investigated in this study. The results showed that both subcutaneous (SC) and intraperitoneal (IP) D-luciferin administration led to fast increase of bioluminescence signal (BLS) intensity and caused large variation of peak signal intensity of xenografts through the analysis of BLI kinetic curves. We found that a distinct linear stage appeared in xenograft BLI curve for each mouse subjected to IP-injection of D-luciferin. Moreover, a high positive correlation was found between linear slope and the initial number of human uterine fibroid smooth muscle cells (fSMCs) used for xenograft formation. Our research indicates that the slope of linear stage in BLI curve is more appropriate for *in vivo* quantitative assessment of human uterine fibroid xenograft.

Keywords: Bioluminescence imaging, *in vivo* quantitative assessment, human uterine fibroid, xenograft

Introduction

Uterine fibroid, one of the most common solid tumors, have a harmful influence on reproductive age women [1]. Uterine fibroids cause some symptoms such as heavy menstrual bleeding, subfertility, uterine pain and pressure, which results in hysterectomy and myomectomy [1-3]. Although uterine fibroid is prevailing, it is still not effectively treated nowadays [1]. Lack of the approaches for accurate *in vivo* quantitative assessment hindered the basic research and drug screen for uterine fibroid.

Bioluminescence imaging (BLI), as a noninvasive and sensitive optical approach, can evaluate the magnitude and distribution of the inoculated cells in intact small animals, and provides the longitudinal assessment and real time monitoring of disease progression. So far, BLI has been widely used for monitoring tumor [4-6] and tracking stem cells *in vivo* [7, 8], and monitoring genes [9, 10] and evaluating protein stability and interaction *in vitro* [9].

In vitro BLI quantitative assessment has proven to accurately exhibit the positive correlation between bioluminescent intensity and cell number [11]. However, *in vivo* BLI quantitative assessment faces more challenges for the complicated drug delivery system. D-luciferin systemically injected will travel through the blood stream and pass various membranes to reach the inoculated cells in animal. Therefore, the signal intensity often exhibits difference among animal individuals due to inoculated cells location, animal weight, physical condition, and different injection routes of D-luciferin. Generally, three injection routes of D-luciferin are selected for *in vivo* BLI, they are intraperitoneal (IP), intravenous (IV) and subcutaneous (SC) injections [12, 13]. Their pros and cons have been

widely discussed in some reports [14, 15]. The administration route can influence biodistribution and local concentration of D-liciferin, leading to the distortion of quantitative assessment. IP injection route is the most widely used although with some shortcomings such as failed injections into the intestine [16, 17] and variations in the D-luciferin absorption rate through the peritoneum [18]. Notably, SC injection was regarded to be able to attenuate these drawbacks in several previous studies [16, 17].

The appropriate image acquisition windows and analysis methods for *in vivo* BLI after D-luciferin administration were previously discussed [16, 18, 19]. Maximal photon emission, area under the curve (AUC), or photon emission at a predefined time point were used as parameters to quantify *in vivo* BLI [15]. Full AUC analysis was likely not appropriate for *in vivo* BLI analysis due to the prolonged acquisition time with more than 1 hour. Baba et al. [16] reported that the best correlation between cell number and AUC value was showed at 20-minute time point after IP injection of substrate in their mouse model. Additionally, it was reported that BLS surrounding the theoretical time of peak photon emission was the most appropriate to the reproducibility of the technique [15, 19]. But the error was still caused by different injection time of D-luciferin among the animal individuals who were performed the synchronous BLI examination, which was not carefully considered.

We have developed a murine xenograft model for human uterine fibroid based on *in vivo* BLI [20]. Here, using the this xenograft model, we studied the behavior of sequential BIL curves of xenografts in mice subjected to IP or SC D-luciferin administration, and then analyzed the correlation between initial fSMC number for xenograft formation and BLI value of corresponding xenograft. We found that a distinct linear stage from 5 to 23 minutes time point stably existed in the BLI curve for most mice only subjected to IP injection of D-luciferin. Moreover, there was a high positive correlation between the slope of linear stage and the initial fSMC number for xenograft formation. These results suggested that the linear stage slope in BLI curve is more appropriate for *in vivo* quantitative assessment of human uterine fibroid xenograft.

Materials and methods

Uterine tissues

Women with symptomatic fibroids were recruited in study subject and underwent hysterectomy at Obstetrics and Gynecology Hospital of Fudan University. The uterine fibroid tissue (1-3 g) was collected at the time of hysterectomy, transported to the Ob-Gyn research lab, and cut into small pieces. These specimens were shipped directly to the Medical Center of Suzhou Institute of Nano-Tech and Nano-Bionics (SINANO) for in vitro analysis. Transport medium was Dulbecco's modified Eagle's (DMEM) medium (GIBCO). All procedures performed in studies involving human participants were complied with ethical standards of the institutional committee and informed consent was obtained from the patients.

Primary culture of uterine fSMCs and lentiviral transduction

Primary human uterine fSMCs were generated, identified and cultured as described in our previous report [20, 21]. For lentiviral transduction, the primary cultures were cultured for 2-4 passages and 4×10^6 fSMCs were seeded in one 10 cm culture plate. After one day of culture, the cells were infected with GFP-LUC (green fluorescent protein-luciferase) lentiviral stock at 20 MOI (Multiplicity of Infection) and cultured in a humidified CO_2 (5%) incubator at 37°C. After 24 hours of incubation, the medium was replaced with fresh complete DMEM containing 10% fetal bovine serum (FBS, GIBCO), 1% penicillin/streptomycin (P/S) and the cells were cultured for 3 more days before inoculation into mice. The methods of lentivirus production and transduction into fSMCs were according to the previous description [20, 21]. The GFP-LUC-transduced fSMCs (GFP-LUC-fSMCs) were verified by GFP and LCU expression examined by fluorescence microscope and BLI.

In vitro quantitative assay of uterine fSMCs by BLI method

For quantitative assay by BLI test, the primary GFP-LUC-fSMCs were seeded in 24-well plates with a series of number of cells (0, 500, 1000, 2000, 5000, 8000, 10,000, 20,000, 50,000,

100,000, 150,000 and 200,000 cells/per well) and cultured in 400 μL DMEM containing 10% FBS and cultured at 37°C, 5% CO_2 for 4 hours. To detect the BLS of LUC, D-Luciferin (Thermo Fisher Scientific) was added to the complete medium in 24-well plates with a final concentration of 150 μg/mL at 5 minutes before BLI using the In Vivo Imaging System (IVIS) (IVIS 200 series, Caliper Inc.). Images were acquired at exposure time 20 seconds and analyzed through total photon flux emission (photons/second) in the ROI (the whole well of 24-well plate) by Living Image Software (Xenogen). The Pearson correlation analysis was performed to determine the correlation between the true cell number and total photon flux emission value from BLI.

Mice for transplantation

RAG2$^{-/-}\gamma_c^{-/-}$ double knock out female mice [21], 6 to 9 weeks old, were bred and maintained on sulfamethoxazole water under specific pathogen-free conditions in the animal care facility at the Suzhou Institute of Nano-Tech and Nano-Bionics. The animal study protocol was approved by the Review Committee for animal use and welfares, Suzhou institute of Nano-tech and Nano-bionics, Chinese Academy of Sciences.

The transplantation of human uterine fSMCs, in vivo BLI and matrigel plug BLI

The GFP-LUC-fSMCs in 10 cm culture plate were washed once with 10 mL of phosphate-buffered saline (PBS) and trypsinized with 2 mL 0.25% Trypsin-EDTA (Invitrogen) for 2 minutes. Afterwards, the trypsin solution was removed and the cells were collected with 1 mL complete DMEM medium moved to 2 mL eppendorf tube. The cells were spun down in Microfuge 22R centrifuge (Beckman Coulter) at 3000 rpm, 4°C for 1 minute. The cell pellet in each eppendorf tube was resuspended by gentle mixing with ice-cold PBS by different densities (8×10^4, 4×10^5, 1×10^6, 2×10^6 cells per 150 μL) and then mixed with 150 μL ice-cold matrigel (BD Biosciences) solution. Each of the 300 μL cell-matrigel mixture was filled into a pre-chilled 1 mL syringe with a 18 gauge needle, and then inoculated the mixture into the indicated body parts of RAG2$^{-/-}\gamma_c^{-/-}$/DKO female mice. To avoid of mixture out-flow, a heat lamp was used to warm up mice skin and to facilitate the rapid solidification of the matrigel-cell mixture. At the second day, the first photon flux image was taken with IVIS.

To take BLI, the whole body bioluminescence of inoculated mice was detected using the IVIS 200 series (Caliper Inc). Mice were anesthetized with isoflurane using a Gas Anesthesia System, and then first injected subcutaneously (SC) near the scapula with D-luciferin (75 mg/kg body weight, Caliper Inc.) in 100 μL of PBS at 4 minutes before imaging. BLS was acquired at a fixed time point (20 minutes after D-luciferin injection), or every 2 minutes at exposure time 20 seconds to generate a kinetic time-intensity curve for LUC expression in xenografts. Based on our observation, the BLS of fSMCs xenografts (1 - 2 × 10^6 cells) will disappear to undetectable level 5 hours after D-luciferin administration. Here, 12 hours after SC injection of D-luciferin and BLI in the same day, the same mice were IP injected with same amount of D-luciferin and performed BLI again; merely, the exposure time was changed to 40 seconds. Before IP D-luciferin injection, the mice were performed BLI detection without D-luciferin administration and compared with control mice only inoculated with matrigel implant to ensure no residual BLS interference caused by previous SC D-luciferin administration. Mice were conducted with BLI analysis on indicated day after inoculation of fSMCs. Dorsal images were taken and the total photon flux emission (photons (p)/second) in the region of interest (ROI) was calculated using the Living Image Software (Xenogen).

For BLI of matrigel plug, the mice were sacrificed by CO_2 asphyxiation at the indicated days after inoculation. The matrigel plugs were excised and placed into a 6 cm plate and then immersed in complete medium with final concentration of 150 ug/mL of D-Luciferin and incubated for 5 minutes before imaging with the IVIS system. Total photon flux emission (photons/second) in the ROI for each plug was determined by Living Image Software (Xenogen).

Immunofluorescence of xenograft-frozen sections

The matrigel plugs were excised from the sacrificed mice and placed into plastic molds, and

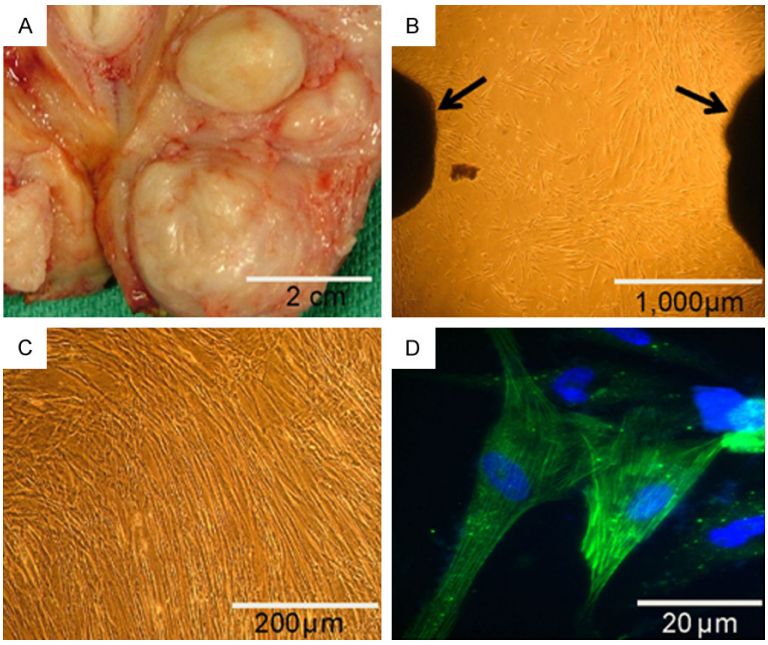

Figure 1. Primary culture and identification of human uterine fibroid smooth muscle cells (fSMCs). A. Fibroid tumor from hysterectomy patient, scale bar = 2 cm. B. Smooth muscle cells grew out from human uterine fibroid explants, scale bar = 1,000 μm. C. Human uterine fSMCs cultured to 100% confluence, scale bar = 200 μm. D. ACTA2 staining for identifying fSMCs, green: ACTA2, blue: DAPI, scale bar = 20 μm.

then filled with optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA). These plastic molds were placed into dry ice/isopentane slurry until OCT turned white. Frozen matrigel sections of 4 to 6 μm were cut from blocks and directly observed GFP signal under fluorescence microscope after DAPI staining. Immunofluorescence (IF) assay followed the method described previously [20]. Briefly, after fixation with 10% formalin and permeabilization with 0.25% Triton X-100, the xenograft sections were blocked in 1% (w/v) bovine serum albumin (BSA) in PBS for 30 minutes. The slides were incubated with mixture of primary antibodies (1:100 dilution of GFP rabbit polyclonal antibody from Santa Cruz, CA and 1:50 dilution of ACTA2 mouse monoclonal antibody from Abcam) in a moist chamber at 4°C for 18 hours. After 3 times of wash with PBS, the slides were then incubated with a mixture of secondary antibodies (Texas Red conjugated anti-mouse and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit from Abcam at 1:300 dilution) for 2 hours at room temperature in the dark. The mixture of the secondary antibody solution was decanted and the slides were washed 3 times with PBS in the dark. The 0.1 mg/mL Hoechst 33342 was used to stain the nuclei. The fluorescent signals were captured under fluorescence microscope.

Statistical analysis

To determine the correlation between BLI intensity and fSMC number, the Pearson correlation analysis was performed using GraphPad Prism 4 package (GraphPad Software). Here, determination coefficient, namely R^2, rang from 0-1, which 1 means a total positive and 0 means no correlation between the variables BLI intensity and fSMC number. All the data were expressed as average value ± standard deviation (S.D.) from three biological replicates.

Results

The primary culture of fSMCs

Figure 1 showed the primary culture of fSMCs derived from human uterine fibroid tumor. The solid fibroid tumor were minced into small explants and spread in the culture dish (**Figure 1A** and **1B**). The fSMCs grew out from the explants after 3 weeks of culture (**Figure 1B**). They were performed passage when cells reached to 100% confluence (**Figure 1C**). We found that more than 90% cells from human fibroid tissues expressed SMCs marker ACTA2 (α-actin) (**Figure 1D**), suggesting that these cells are qualified for following experiments as fSMCs.

The evaluation of in vitro and in vivo quantitative assessment of GFP-LUC-fSMCs by BLI

We constructed primary fSMCs stably expressing GFP-LUC fusion protein (**Figure 2A**) via lentiviral-mediated gene transfer. The expression of GFP and LUC (luciferase) were readily detected by fluorescence microscopy (**Figure 2B**) and BLI (**Figure 2C**) *in vitro*. We found that there was a strong positive correlation between cell number and the corresponding BLS intensity with a wide range from 500 to 2000,000 cells (**Figure 2D** and **2E**). These results demonstrated that

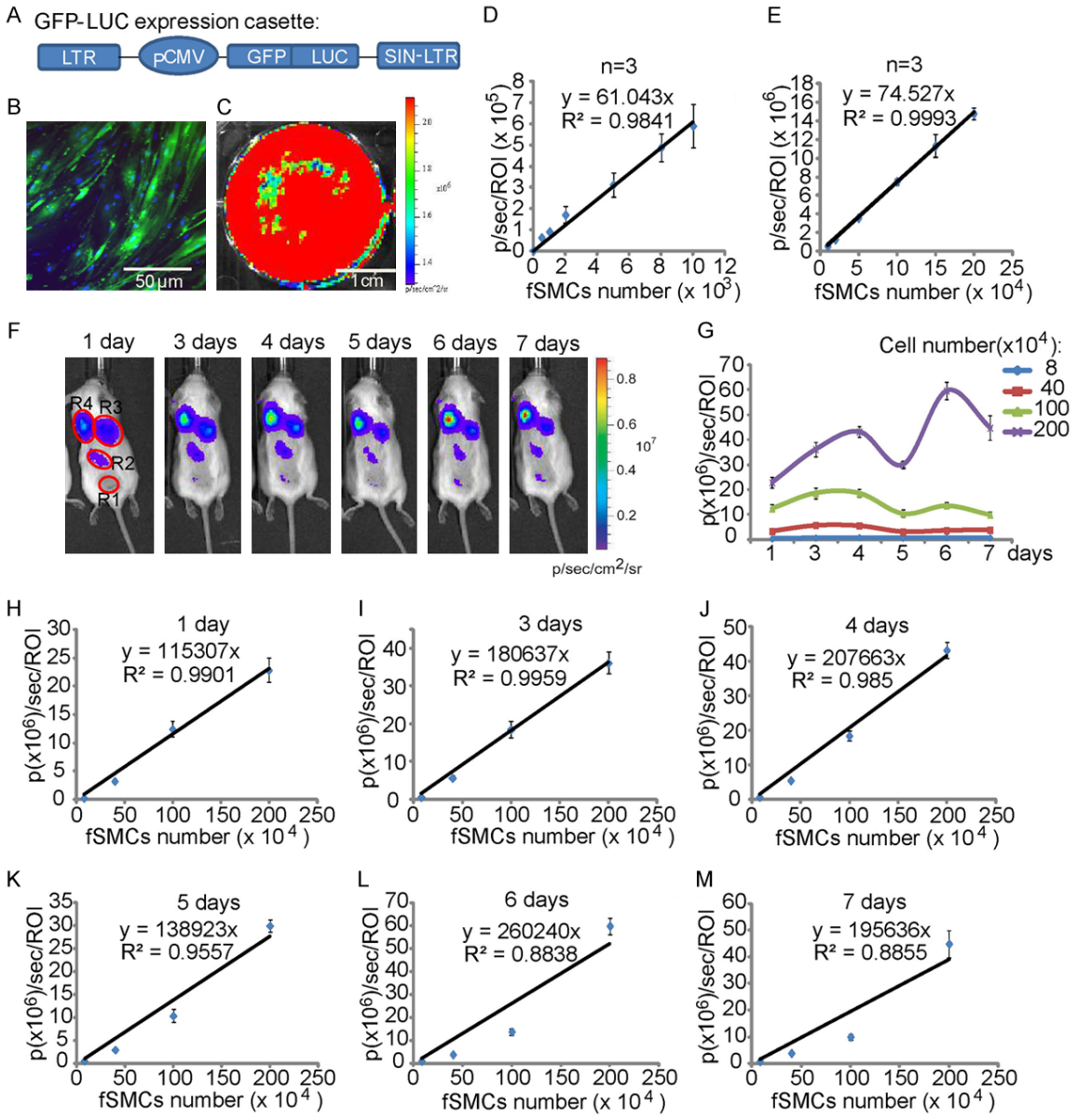

Figure 2. Quantitative evaluation of GFP-LUC-fSMCs *in vitro* and *in vivo*. (A) Schematic summary of the lentiviral constructs carrying the GFP-LUC fusion expression cassette. (B) Detection of GFP expression and (C) LUC (luciferase) expression in GFP-LUC-fSMCs, scale bar = 50 μm in (B) and = 1 cm in (C-E). Pearson correlation between fSMC number and corresponding BLI intensity (photo flux) for a large range number of cells from 500 to 200,000. The x-axis indicated cell numbers. (F) Representative BLIs of one single RAG2$^{-/-}\gamma_c^{-/-}$ mouse on the indicated day post-SC inoculation with different number of fSMCs into indicated regions of dorsal body (8×10^4, 40×10^4, 100×10^4 and 200×10^4 fSMCs were inoculated into R1, R2, R3 and R4 regions respectively). (G) The BLI signal curves of different exnografts on day 7. The x-axis indicated time. (H-M) Correlation between initial fSMC number for different xenografts and their BLS intensity (photo flux) detected on indicated day. $R^2 > 0.8$ means high correlation. The x-axis indicated cell numbers. Error bars represent S.D. from average value of three biological replicates.

BLI was an accurate approach for *in vitro* quantitative assessment of GFP-LUC-fSMCs we generated.

To prove that BLS of GFP-LUC-fSMCs is sensitive enough for quantitative assessment *in vivo*, different amounts of GFP-LUC-fSMCs (8×10^4, 4×10^5, 1×10^6 and 2×10^6) were SC inoculated in different dorsal regions (R1, R2, R3 and R4) of one mouse to form 4 xenografts (**Figure 2F**). Here, we postulate that the environmental conditions for 4 xenografts in differ-

ent regions of one mouse body are same. The BLI was taken at a fixed time point (20 minutes post-D-Luciferin administration) on day 1, 3, 4, 5, 6 and 7 post-fSMCs inoculation. The expansion and regression of xenografs was determined by BLS intensities in ROI (**Figure 2G**). Pearson correlation analysis showed highly positive correlation between BLS intensity and initial inoculated cell number on day 1, 3, 4 and 5 with R-squared values of more than 0.95 (**Figure 2H-K**). Even on the day 6 and 7, the correlation was still significant with R-squared values more than 0.88 (**Figure 2L** and **2M**). These results indicated that GFP-LUC-fSMCs were qualified for quantitative assessment *in vitro* and *in vivo*.

The sequential BLI of fSMC xenografts in mice subjected to IP or SC administration of D-luciferin

To investigate the growth curve of fibroid xenograft *in vivo*, 1×10^6 GFP-LUC-fSMCs mixed with matrigel were inoculated in the dorsal flank of mice to form one xenograft (one xenograft per mouse). Two groups of mice (ten mice in each group) were subjected to IP or SC administration of D-luciferin. The BLI was taken at a fixed time point (20 minutes post-D-Luciferin administration) on day 1, 3, 5, 7, 9 and 11. *In vivo* expansion or regression of GFP-LUC fSMCs in matrigel implants as a function of days was determined by BLS intensity (**Figure 3A**). The growth curves of xenografts from IP or SC administration mice showed similar trend. The peak of BLS curve was from 3 to 7 days post-fSMCs inoculation and the BLS was faded away gradually after 10 days. The BLI of matrigel plugs and GFP fluorescence of plug frozen section was consistent with *in vivo* results (**Figure 3B**). The signals of BLI and GFP, standing for cell viability, were remarkably attenuated on days 12 as compared with those on days 7 (**Figure 3B**). This further confirmed by immunofluorescence of GFP and smooth muscle cell specific marker ACTA2 in plugs at 7 or 12 days post- fSMCs inoculation (**Figure 3C**).

We assessed the time course of BLS from fibroid xenograft in mice after IP or SC D-luciferin administration and investigated the effect of imaging timing on quantitative xenograft. Here, 1×10^6 GFP-LUC-fSMCs mixed with matrigel was inoculated in the dorsal flank of mouse (one xenograft per mouse). Sequential BLI was performed on day 3 post-fSMCs inoculation. Nine and ten mice were conducted with D-luciferin IP and SC injection, respectively. BLS was acquired for total 26 times with the interval of 2 minutes. The sequential curve of BLI was generated by a series of BLS data as a function of duration time from the first time point (**Figure 3D** and **3E**). Although same number of fSMCs was inoculated to form one implant at same region in each mouse, the kinetic time-intensity curves were far different among the mice either in IP or SC D-luciferin injection groups. SC group caused stronger BLS than IP injection at each same time point (**Figure 3D** and **3E**). In IP group, there was no obvious difference in BLS at the first time point, but the duration time from start point to peak was markedly different among the mice. For most mice in IP group, the duration time from start point to peak was about 30 minutes, and there was no obvious dropping even to 51 min time point. Notably, the linear stage from 5 to 23 minutes time point apparently existed in the curve for most mice (**Figure 3E**). However, in SC group, the BLS intensity at the first time point was different among the mice. For most mice in this group, the BLS increased fast after SC administration of D-luciferin. The duration time from start point to peak and the peak duration were shorter compared to the mice in IP administration group, which was consistent with the observations from other studies [13]. Although there were linear stages in most mice subjected to SC administration of D-luciferin, the duration of these stages was varied greatly, suggesting that it was not appropriate to adopt the BLS value at predetermined peak or fixed time point for accurate *in vivo* quantitative assessment of exnografts.

The slope values of sequential BLS were qualified for in vivo quantitative assessment of xenografts

To find a relative accurate method for *in vivo* quantitative assessment of xenografts, we designed 3 groups to acquire BLS data of xenograft for Pearson correlation analysis: 1) fixed time point (15 minutes) post-SC D-luciferin administration (SC+fixed time), 2) fixed time point (15 minutes) post-IP D-luciferin administration (IP+fixed time) and 3) linear stage slope of sequential BLI post-IP D-luciferin administra-

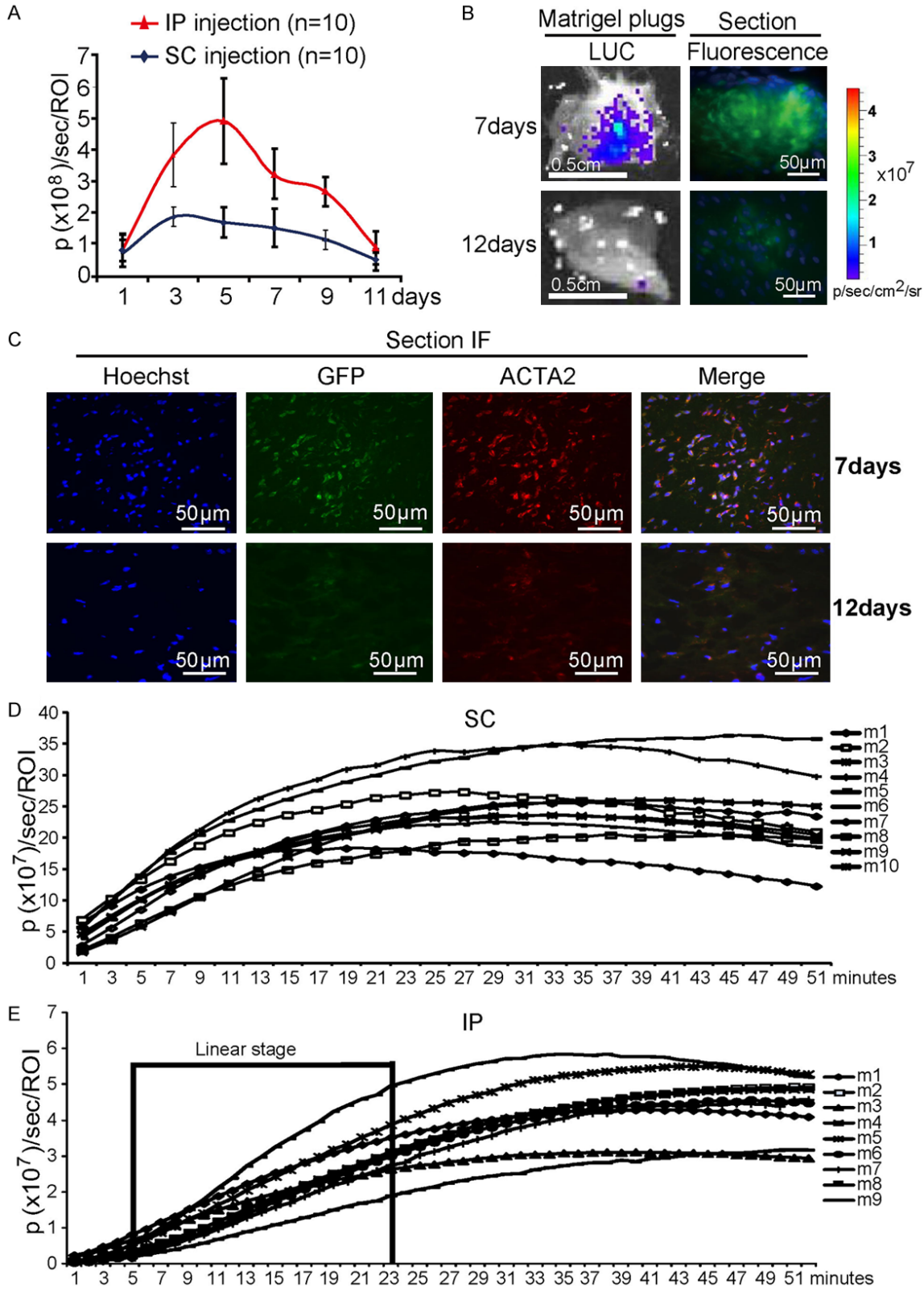

Figure 3. *In vivo* BLI of fSMC xenografts subjected to IP or SC administration of D-luciferin. (A) Typical growth curve of human uterine fibroid xenografts subjected to IP (red line) and SC (blue line) administration of D-luciferin for indicated days. The x-axis indicated time. (B) BLI of Matrigel plugs and GFP fluorescence of their corresponding frozen sections on day 7 and 12 post-fSMCs inoculation, scale bar = 0.5 cm and 50 μm, respectively. (C) Representative

immunofluorescence of matrigel plugs on day 7 and 12 post-fSMCs inoculation. Green: GFP, red: ACTA2, blue: DAPI, scale bar = 50 μm. (D) The kinetic curve of BLI intensity for xenografts from different mice subjected to SC administration of D-luciferin and (E) IP administration of D-luciferin. The "m1-10" means mice No. The linear stage was shown in (E) from 5 to 23 second time points. The x-axis indicated time.

tion (IP+slope). Here, slop of sequential BLI post-SC administration was not considered because there was no identical duration of linear stage for a group of mice.

Different amounts of GFP-LUC-fSMCs (8×10^4, 4×10^5, 1×10^6, and 2×10^6) were SC inoculated in indicated dorsal regions (R1, R2, R3 and R4) of different mice (**Figure 4A** and **4D**). Here, 8×10^4 and 1×10^6 cells were inoculated in mouse 1 (M1) and mouse 3 (M3) respectively; 4×10^5 and 2×10^6 cells were inoculated in different dorsal regions (R2 and R4) in mouse 2 (M2). The *in vivo* BLI of SC+fixed time was first performed, and after 12 hours, the *in vivo* BLI of IP+fixed time and IP+slope were performed at same day. We repeated the same operation on days 3, 4, 5, 6 and 7 post-fSMCs inoculation and then sacrificed mice for plugs BLI. Seven sequential BLS intensities (from 6 to 20 minutes, the interval time is 2 seconds) for each xenograft in the mice subjected to IP D-luciferin administration were used to generate the linear trend lines of time-intensity kinetic curve. The slope stands for the increasing rate of BLS per minute with the unit photon/sec/min/ROI. We supposed that these slope values might represent the expansion or regression of the implants.

For analysis of xenograft growth, *in vivo* expansion and regression xenografts as a function of time (days) were determined by BLS intensity acquired in the 3 groups (SC+fixed time, IP+fixed time and IP+slope). The growth curves from xenografts formed by same number of cells were varied widely among the 3 groups even if the BLS derived from the same xenograft in the same mice (**Figure 4B**, **4E** and **4M**). However, the growth curves from IP+slope mice were most consistent with typical growth curves of xenografts showed in **Figure 3A**.

To evaluate which group was the most accurate method for *in vivo* quantitative assessment, we performed the Pearson correlation analysis between the BLS intensity of xenografts *in vitro* or *in vivo* at the day 7 and their initial fSMC number. We found that there was a highly positive correlation between BLS intensity of matrigel plugs *in vitro* and initial cell number with R-squared value 0.99 (**Figure 4G-L**), group suggesting that BLS intensity precisely reflected the initial fSMC number in xenograft on day 7 without the obvious disturbance from individual differences of mice. Among the 3 groups, we also compared the BLS intensity of xenografts *in vivo* with their corresponding initial fSMC number on day 7. The highest correlation, with R-squared value of 0.8778, just occurred in IP+slope group (**Figure 4N**), not in SC+fixed time or IP+fixed time groups (**Figure 4C** and **4F**).

We further investigated the Pearson correlation between BLS intensity of xenografts and their corresponding initial fSMC number on other days in the 3 groups, including day 1, days 3, 4, 5 and 6 (Figure S1). We found that high correlations occurred on these days just in IP+slope group (Figure S1K-O), but not in SC+fixed time and IP+fixed time groups (Figure S1A-J). Aparently, our results indicate that the linear stage slope of sequential BLS is qualified for quantitative assessment of xenografts *in vivo.*

Discussion

Owing to high sensitivity and extraordinary signal-to-noise ratios in animals, BLI has been widely used for *in vivo* experiments over the past decade. However, BLI analysis *in vivo* is still limited owing to the biased readout frequently occurring in quantitative assessment. Although many approaches for *in vivo* quantitative assessment were intensively discussed [15], there was not available method for murine xenograft model of human uterine fibroid. Here, we attempted to explore a relatively accurate and convenient way for quantitative assessment of fibroid xenograft *in vivo*.

We first constructed GFP-LUC-fSMCs and evaluated if they were qualified for the study of *in vivo* quantitative assessment. *In vitro* BLI results manifested that these fSMCs were sensitive enough for BLI through a large range of cell number; moreover, high positive correlation was found between BLS intensity and cell number. To investigate if these cells were sensitive enough for *in vivo* quantitative assessment, we

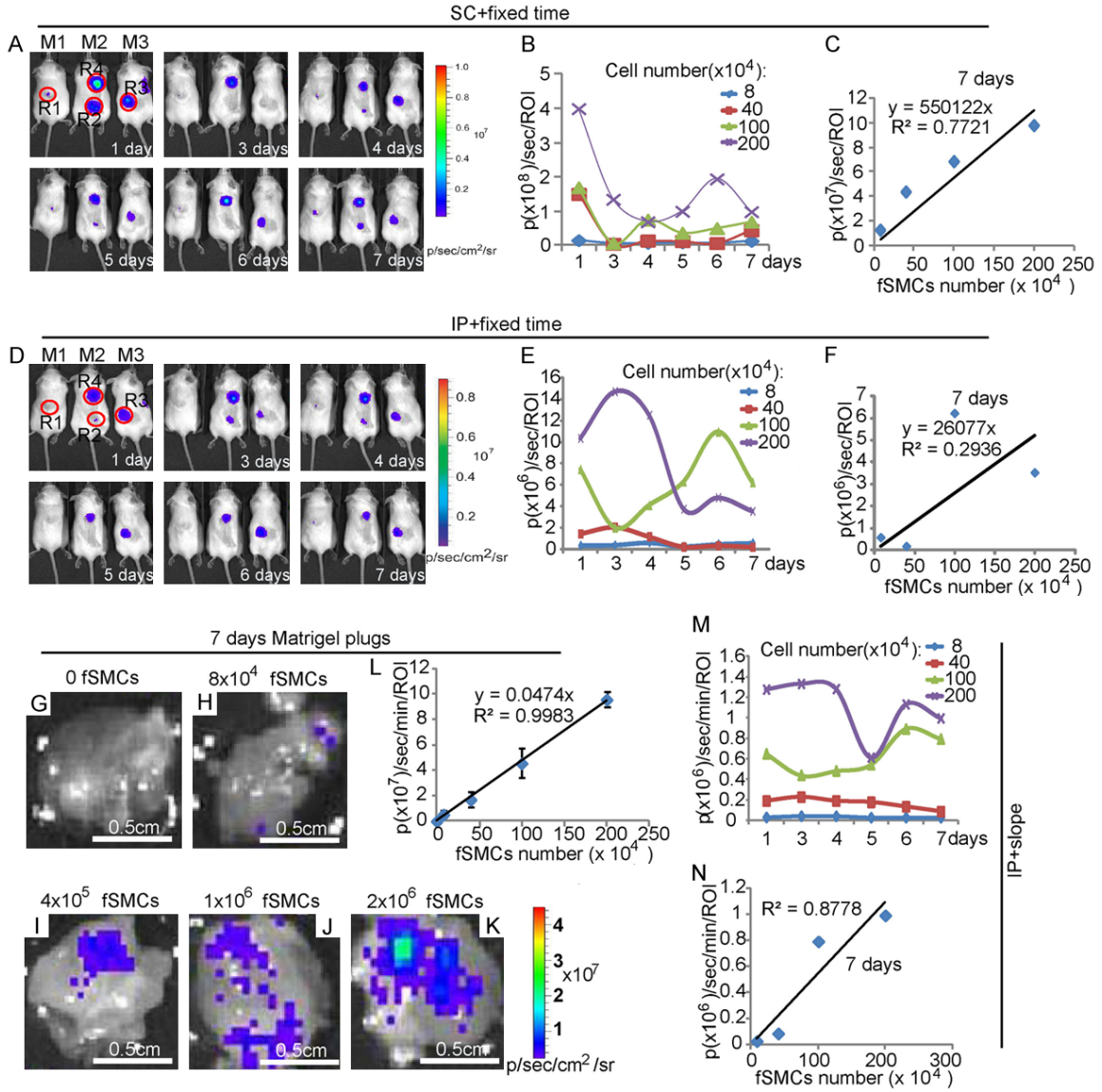

Figure 4. Quantitative analysis of BLI signals in xenografts formed with different amounts of human fSMCs. (A) Representative BLI on the indicated day post-fSMCs inoculation in mice subjected to SC administration of D-luciferin. Different numbers of fSMCs were inoculated into the dorsum of different mice. Here, 8×10^4 and 1×10^6 cells were inoculated in mouse 1 (M1) and mouse 3 (M3) respectively; 4×10^5 and 2×10^6 cells were inoculated in different dorsal regions (R2 and R4) in mouse 2 (M2). (B) The 7-day growth curves of BLI intensity for different xenografts in SC+fixed time group. The x-axis indicated time. (C) Correlation between initial fSMC number for different xenografts and corresponding BLI intensity detected on day 7 in SC+fixed time group. The x-axis indicated cell numbers. (D) Representative BLI on the indicated day post-fSMCs inoculation in mice subjected to IP administration of D-luciferin. (E) The 7-day growth curves of BLI intensity for different xenografts in IP+fixed time group and (M) in IP+slope group. The x-axis indicated time. (F) Correlation between initial fSMC number for different xenografts and corresponding BLI intensity detected on day 7 in IP+fixed time group and (N) in IP+slope group. The x-axis indicated cell numbers. (G-K) BLI of matrigel plugs formed by different initial numbers of fSMCs were acquired on day 7 post-fSMC inoculation and (L) correlation between initial fSMC number for different plugs and corresponding BLI intensity. The x-axis indicated cell numbers. R^2 means coefficient of determination. Error bars represent S.D. from average value of three biological replicates.

inoculated different numbers of fSMCs in different regions of one mouse. That could eliminate the disturbance from individual difference. The highly positive correlation between the BLS intensity of xenograft and initial cell number for xenograft formation indicated that these fSMCs were appropriate for the study of *in vivo* quantitative assessment.

Subsequently, we investigated the time course of BLS of fibroid xenograft in the mice subjected to IP or SC D-luciferin administration and evaluated the effect of imaging timing on quantitative assessment of xenograft *in vivo*. After analyzing the behavior of BLS kinetic curves for the mice subjected to IP and SC D-luciferin administration, we found that there was a large variation of BLS intensity at peak-point and first time point, and large variation of duration time to peak-point among individuals. The heterogeneity of mouse individual may mainly contribute to this variation. Notably, biased readout was caused because all mice were not subjected to D-luciferin administration at exactly same time but were performed BLI examination synchronously. At initial stage of sequential BLI, the BLS intensity increased faster for mice in SC injection group than that in IP injection group. Compared with IP D-luciferin administration, SC administration allowed excess D-luciferin to attain the xenograft rapidly and triggers enzyme reaction sufficiently. So, SC D-luciferin administration was more convenient and effective for BLI *in vivo* compared to IP administration [12]. However, among the mice, it could magnify the biased error of BLS at fixed time point and of duration time to peak-point. But, the stable linear stage from 5 to 23 min time point just occurred in BLS curves of mice subjected to IP administration of D-luciferin. For most mice subjected to SC administration of D-luciferin, the duration time of BLS linear stages was so short and variant that it was difficult to define an identical period of time for linear slope calculation.

The linear stage slope of BLI curve could partly eliminate the biased error of BLS among the mice caused by different injection time of D-luciferin. Therefore, we postulated that the stable slope in the BLS curve from mice subjected to IP D-luciferin administration is eligible for quantitative assessment of fSMC xenograft *in vivo*. To verify this hypothesis, we compared the xenograft BLS values acquired from the 3 groups (SC+fixed time, IP+fixed time and IP+slope) with initial number of fSMCs inoculated in different mice. We finished the evaluation of these 3 groups on day 7 post-fSMCs inoculation, when xenografts were still in the expansion sage. This evaluation system was validated by the high correlation between BLS intensity of xenograft matrigel plug and initial fSMC number forming xenograft. Eventually, the result demonstrated that *in vivo* BLS from IP+slope group exhibited the most positive correlation with the initial fSMC number compared to other two groups. Although, the IP injection failure has been reported owing to the fail injection of D-luciferin into the bowel [16, 17], in our practice, we substantially decreased the IP injection failure rate to below 1% by simply injecting D-luciferin with 18 gauge needle that could readily pierce the mouse abdominal cavity but not the bowel.

Taken together, we described a convenient and accurate way for *in vivo* quantitative assessment of human fibroid xenograft. Followed by IP injection of D-luciferin and short period of sequential BLI, the linear stage slope of BLI curve can be generated and calculated, and this slope is qualified for *in vivo* quantitative assessment of human uterine fibroid xenografts.

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Disclosure of conflict of interest

None.

Address correspondence to: Deyou Tao, Surgical Oncology of Taizhou Enze Medical Center (Group) Enze Hospital, No. 1 Tongyang Road East, Luqiao District, Taizhou 318050, Zhejiang, China. Tel: 86-0576-89218839; Fax: 860576-89218765; E-mail: taody@enzemed.com; Dr. Guangli Suo, CAS Key Laboratory of Nano-Bio Interface, Suzhou Institute of Nano-Tech and Nano-Bionics, Chinese Academy of Sciences, Suzhou, Jiangsu, China. Tel: 86-512-62872823; Fax: 86-512-62872569; E-mail: glsuo2013@sinano.ac.cn

References

[1] Istre O. Management of symptomatic fibroids: conservative surgical treatment modalities other than abdominal or laparoscopic myomectomy. Best Pract Res Clin Obstet Gynaecol 2008; 22: 735-747.

[2] Donnez J and Dolmans MM. Uterine fibroid management: from the present to the future. Hum Reprod Update 2016; 22: 665-686.

[3] Newton JN, Briggs AD, Murray CJ, Dicker D, Foreman KJ, Wang H, Naghavi M, Forouzanfar MH, Ohno SL, Barber RM, Vos T, Stanaway JD, Schmidt JC, Hughes AJ, Fay DF, Ecob R, Gresser C, McKee M, Rutter H, Abubakar I, Ali R, Anderson HR, Banerjee A, Bennett DA, Bernabe E, Bhui KS, Biryukov SM, Bourne RR, Brayne CE, Bruce NG, Brugha TS, Burch M, Capewell S, Casey D, Chowdhury R, Coates MM, Cooper C, Critchley JA, Dargan PI, Dherani MK, Elliott P, Ezzati M, Fenton KA, Fraser MS, Furst T, Greaves F, Green MA, Gunnell DJ, Hannigan BM, Hay RJ, Hay SI, Hemingway H, Larson HJ, Looker KJ, Lunevicius R, Lyons RA, Marcenes W, Mason-Jones AJ, Matthews FE, Moller H, Murdoch ME, Newton CR, Pearce N, Piel FB, Pope D, Rahimi K, Rodriguez A, Scarborough P, Schumacher AE, Shiue I, Smeeth L, Tedstone A, Valabhji J, Williams HC, Wolfe CD, Woolf AD and Davis AC. Changes in health in England, with analysis by English regions and areas of deprivation, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet 2015; 386: 2257-2274.

[4] Guo K, Huang P, Xu N, Xu P, Kaku H, Zheng S, Xu A, Matsuura E, Liu C and Kumon H. A combination of YM-155, a small molecule survivin inhibitor, and IL-2 potently suppresses renal cell carcinoma in murine model. Oncotarget 2015; 6: 21137-21147.

[5] Kuroda S, Kubota T, Aoyama K, Kikuchi S, Tazawa H, Nishizaki M, Kagawa S and Fujiwara T. Establishment of a non-invasive semi-quantitative bioluminescent imaging method for monitoring of an orthotopic esophageal cancer mouse model. PLoS One 2014; 9: e114562.

[6] Wu X, Song M, Yang X, Liu X, Liu K, Jiao C, Wang J, Bai C, Su G, Liu X and Li G. Establishment of bovine embryonic stem cells after knockdown of CDX2. Sci Rep 2016; 6: 28343.

[7] Barberi T, Bradbury M, Dincer Z, Panagiotakos G, Socci ND and Studer L. Derivation of engraftable skeletal myoblasts from human embryonic stem cells. Nat Med 2007; 13: 642-648.

[8] Sacco A, Doyonnas R, Kraft P, Vitorovic S and Blau HM. Self-renewal and expansion of single transplanted muscle stem cells. Nature 2008; 456: 502-506.

[9] Lehmann S, Stiehl DP, Honer M, Dominietto M, Keist R, Kotevic I, Wollenick K, Ametamey S, Wenger RH and Rudin M. Longitudinal and multimodal in vivo imaging of tumor hypoxia and its downstream molecular events. Proc Natl Acad Sci U S A 2009; 106: 14004-14009.

[10] Korpal M, Yan J, Lu X, Xu S, Lerit DA and Kang Y. Imaging transforming growth factor-beta signaling dynamics and therapeutic response in breast cancer bone metastasis. Nat Med 2009; 15: 960-966.

[11] Suo G, Sadarangani A, Tang W, Cowan BD and Wang JY. Telomerase expression abrogates rapamycin-induced irreversible growth arrest of uterine fibroid smooth muscle cells. Reprod Sci 2014; 21: 1161-1170.

[12] Khalil AA, Jameson MJ, Broaddus WC, Chung TD, Golding SE, Dever SM, Rosenberg E and Valerie K. Subcutaneous administration of D-luciferin is an effective alternative to intraperitoneal injection in bioluminescence imaging of xenograft tumors in nude mice. ISRN Mol Imaging 2013; 2013.

[13] Inoue Y, Kiryu S, Izawa K, Watanabe M, Tojo A and Ohtomo K. Comparison of subcutaneous and intraperitoneal injection of D-luciferin for in vivo bioluminescence imaging. Eur J Nucl Med Mol Imaging 2009; 36: 771-779.

[14] Wu H, Teng PN, Jayaraman T, Onishi S, Li J, Bannon L, Huang H, Close J and Sfeir C. Dentin matrix protein 1 (DMP1) signals via cell surface integrin. J Biol Chem 2011; 286: 29462-29469.

[15] Keyaerts M, Caveliers V and Lahoutte T. Bioluminescence imaging: looking beyond the light. Trends Mol Med 2012; 18: 164-172.

[16] Babaie Y, Herwig R, Greber B, Brink TC, Wruck W, Groth D, Lehrach H, Burdon T and Adjaye J. Analysis of Oct4-dependent transcriptional networks regulating self-renewal and pluripotency in human embryonic stem cells. Stem Cells 2007; 25: 500-510.

[17] Paroo Z, Bollinger RA, Braasch DA, Richer E, Corey DR, Antich PP and Mason RP. Validating bioluminescence imaging as a high-throughput, quantitative modality for assessing tumor burden. Mol Imaging 2004; 3: 117-124.

[18] Keyaerts M, Verschueren J, Bos TJ, Tchouate-Gainkam LO, Peleman C, Breckpot K, Vanhove C, Caveliers V, Bossuyt A and Lahoutte T. Dynamic bioluminescence imaging for quantitative tumour burden assessment using IV or IP administration of D: -luciferin: effect on intensity, time kinetics and repeatability of photon emission. Eur J Nucl Med Mol Imaging 2008; 35: 999-1007.

[19] Inoue H. Neurodegenerative disease-specific induced pluripotent stem cell research. Exp Cell Res 2010; 316: 2560-2564.

[20] Suo G, Sadarangani A, Lamarca B, Cowan B and Wang JY. Murine xenograft model for human uterine fibroids: an in vivo imaging approach. Reprod Sci 2009; 16: 827-842.

[21] Suo G, Jiang Y, Cowan B and Wang JY. Platelet-derived growth factor C is upregulated in human uterine fibroids and regulates uterine smooth muscle cell growth. Biol Reprod 2009; 81: 749-758.

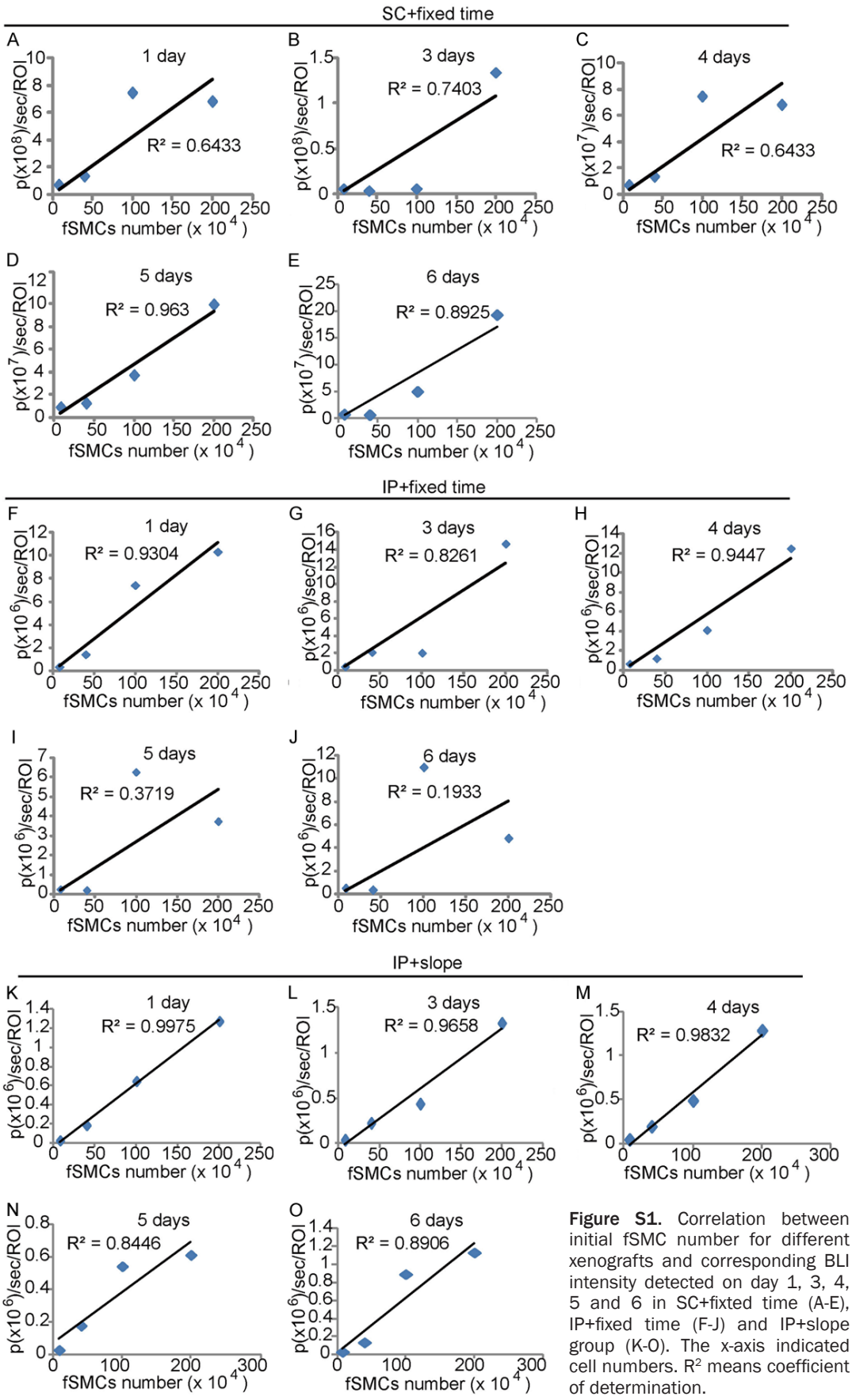

Figure S1. Correlation between initial fSMC number for different xenografts and corresponding BLI intensity detected on day 1, 3, 4, 5 and 6 in SC+fixted time (A-E), IP+fixed time (F-J) and IP+slope group (K-O). The x-axis indicated cell numbers. R^2 means coefficient of determination.