·Basic Research·

# *In vivo* bioluminescence imaging of hyperglycemia exacerbating stem cells on choroidal neovascularization in mice

*Xiang Gao[1], Yu Wang[1], Hui-Yuan Hou[1], Yang Lyu[1], Hai-Yan Wang[1], Li-Bo Yao[2], Jian Zhang[2], Feng Cao[3], Yu-Sheng Wang[1]*

[1]Department of Ophthalmology, Xijing Hospital, Eye Institute of Chinese PLA, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
[2]State Key Laboratory of Cancer Biology, Department of Biochemistry and Molecular Biology, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
[3]Department of Cardiology, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
**Co-first authors:** Xiang Gao and Yu Wang
**Correspondence to:** Yu-Sheng Wang. Department of Ophthalmology, Xijing Hospital, Eye Institute of Chinese PLA, Fourth Military Medical University, No.15 West Changle Road, Xi'an 710032, Shaanxi Province, China. wangys003@126.com. Feng Cao. Department of Cardiology, Xijing Hospital, Fourth Military Medical University No.15 West Changle Road, Xi'an 710032, Shaanxi Province, China. fengcao8828@163.com. Jian Zhang. State Key Laboratory of Cancer Biology, Department of Biochemistry and Molecular Biology, Fourth Military Medical University No.15 West Changle Road, Xi'an 710032, Shaanxi Province, China. biozhangj@yahoo.com.cn
Received: 2015-10-08 Accepted: 2015-11-20

## Abstract

**• AIM: To investigate the influence of hyperglycemia on the severity of choroidal neovascularization (CNV), especially the involvement of bone marrow-derived cells (BMCs) and underlying mechanisms.**

**• METHODS: BMCs from firefly luciferase (Fluc)/green fluorescent protein (GFP) double transgenic mice were transplanted into C57BL/6J wide-type mice. The recipient mice were injected intraperitoneally with streptozotocin (STZ) daily for 5 consecutive days to induce diabetes mellitus (DM), followed by CNV laser photocoagulation. The BMCs recruitment in CNV exposed to hyperglycemia was firstly examined in Fluc/GFP chimeric mice by *in vivo* optical bioluminescence imaging (BLI) and *in vitro* Fluc assays. The CNV severity was evaluated by H&E staining and choroidal flatmount. The expression of vascular endothelial growth factor (VEGF) and stromal cell derived factor-1 (SDF-1) was detected by Western blot.**

**• RESULTS: BLI showed that the BMCs exerted dynamic effects in CNV model in Fluc/GFP chimeric mice exposed to hyperglycemia. The signal intensity of transplanted Fluc$^+$GFP$^+$ BMCs in the DM chimeric mice was significantly higher than that in the control chimeric mice with CNV induction at days 5, 7, 14 and 21 (121861.67±9948.81 *vs* 144998.33±13787.13 photons/second/cm$^2$/sr for control and DM mice, $P_{5d}$<0.05; 178791.67±30350.8 *vs* 240166.67±22605.3, $P_{7d}$<0.05; 124176.67±16253.52 *vs* 196376.67±18556.79, $P_{14d}$<0.05; 97951.60±10343.09 *vs* 119510.00±14383.76, $P_{21d}$<0.05), which was consistent with *in vitro* Fluc assay at day 7 [relative light units of Fluc (RLU1)], 215.00±52.05 *vs* 707.33±88.65, $P$<0.05; RLU1/relative light units of renilla luciferase (RLU2), 0.90±0.17 *vs* 1.83±0.17, $P$<0.05]. The CNVs in the DM mice were wider than those in the control group at days 5, 7, 14 and 21 (147.83±17.36 *vs* 220.33±20.17 μm, $P_{5d}$<0.05; 212.17±24.63 *vs* 326.83±19.49, $P_{7d}$<0.05; 163.17±18.24 *vs* 265.17±20.55, $P_{14d}$<0.05; 132.00±10.88 *vs* 205.33±12.98, $P_{21d}$<0.05). The average area of CNV in the DM group was larger at 7d (20688.67±3644.96 *vs* 32218.00±4132.69 μm$^2$, $P$<0.05). The expression of VEGF and SDF-1 was enhanced in the DM mice.**

**• CONCLUSION: Hyperglycemia promots the vasculogenesis of CNV, especially the contribution of BMCs, which might be triggered by VEGF and SDF-1 production.**

**• KEYWORDS:** hyperglycemia; choroidal neovascularization; bone marrow-derived cells; molecular imaging; *in vivo* optical bioluminescence imaging
**DOI:10.18240/ijo.2016.04.07**

Gao X, Wang Y, Hou HY, Lyu Y, Wang HY, Yao LB, Zhang J, Cao F, Wang YS. *In vivo* bioluminescence imaging of hyperglycemia exacerbating stem cells on choroidal neovascularization in mice. *Int J Ophthalmol* 2016;9(4):519-527

## INTRODUCTION

Age-related macular degeneration (AMD) is a common and devastating disease resulting in irreversible visual loss [1]. The hallmark of neovascular AMD is choroidal neovascularization (CNV), which is characterized by naturally occurring new blood vessels in the choroid to grow

aberrantly into the subretinal space through breaking the RPE and Bruch's membrane, thus causing exudative or hemorrhagic retinal detachments [2]. The pathogenesis of CNV is clearly multifactorial, involving both angiogenesis (development of new blood vessels from resident adjacent preexisting capillaries) and postnatal vasculogenesis (the new vessel complex derived from bone marrow-derived circulating vascular progenitors) [3-4]. Previous studies have shown that bone marrow-derived cells (BMCs) are recruited into CNV by vascular endothelial growth factor (VEGF), stromal cell derived factor (SDF)-1 and differentiated into endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), incorporating into the new blood vessel wall and forming vascular tubes[5-7].

Diabetes mellitus (DM), a condition characterized by micro- and macroangiopathy, is a global health problem. Vascular complications in diabetes are major causes of human morbidity and mortality, affecting multiple organs and persisting despite tight glucose control [8]. Epidemiological studies have verified that diabetes is a risk factor for AMD[9-10]. Growing evidence has shown that diabetes exacerbated the development of laser-induced CNV in mice but the underlying mechanism for this enhancement remained unsolved [11]. Our previous studies have demonstrated that hyperglycemia promoted the progression of CNV in diabetic mice, by enhancing the expression of VEGF [induced by oxidative stress and activation of signal transducer and activator of transcription 3 (STAT3) signalling] and SDF-1 in RPE cells, promoting the recruitment and incorporation of BMCs, and affecting the differentiation of BMCs in CNV[11-12]. By taking advantage of this recruitment potential, a therapeutic strategy for CNV due to hyperglycemia has been described, using BMCs as a delivery vehicle carrying antiangiogenic factors, thereby inhibiting the growth of CNVs and stimulating regressive features [13]. However, it seemed contrary to the findings in several studies revealing that diabetes led to multiple bone marrow microenvironmental defects, such as impaired stem cell mobilization (mobilopathy)[8,14]. Therefore, it is necessary to explore a method real-time monitoring BMCs' cellular kinetics after their transplantation into diabetic mice with CNV model and ultimately to verify our recent conclusion that hyperglycemia aggravated the severity of CNV by recruiting more BMCs in CNV.

*In vivo* bioluminescence imaging (BLI) is now the most sensitive optical technique for longitudinally tracking cell behavior *in vivo*, based on detection of light emission (photoproteins and luciferases) from cells or tissues [15]. This molecular imaging modality has several advantages over traditional screening methods, not least the ability to analyse ongoing biological processes and quantitatively monitor pharmacodynamic changes at the cellular and molecular level in living animals non-invasively in real time[16-18]. As the most conventional technique for BLI, the firefly luciferin-luciferase system is exceptionally functional *in vitro* and *in vivo* [19]. When the engineered stem cells labeled with firefly luciferase (Fluc) are injected into the mice, BLI provides valuable information about their dissemination and functional status using the location and intensity of the light signal, which is released by Fluc-luciferin reaction with limited loss or attenuation, resulting in consecutive images obtained from the same mice, instead of point data from conventional approaches [20-21]. Our previous study suggested that the noninvasive BLI analysis has been extensively used to provide detailed cellular and molecular characterization of the CNV pathology and engrafted stem cells[17].

In the present study, we firstly applied molecular reporter gene imaging techniques in conjunction with conventional histological methods, to dynamically verify the positive effect of hyperglycemia on the recruitment and participation of BMCs in laser-induced CNV mice model. Further, we also investigated the role of local VEGF and SDF-1 expression in engrafted BMCs in CNV under high glucose.

## MATERIALS AND METHODS

**Animals, Diabetes Induction and Grouping** The animal experiments were performed in accordance with the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research. The animal protocol was approved by the Animal Care Committee of the Fourth Military Medical University. Eight week-old female C57BL/6J mice, expressing Fluc and green fluorescent protein (GFP) reporter genes induced by β-actin promoter (Department of Cardiology, Xijing Hospital, Fourth Military Medical University, Xi'an, China) and age matched female congenic wide-type (WT) mice (Experimental Animal Center, Fourth Military Medical University, Xi'an, China) were used. The animals were randomly divided into five groups ($n$ = 6/group): 1) control chimeric mice (normal); 2) diabetic chimeric mice (DM); 3) control chimeric mice with CNV (normal+CNV); 4) diabetic chimeric mice with CNV (DM+CNV); 5) control WT mice.

In the normal and normal+CNV groups, mice were injected intraperitoneally with sodium citrate buffer (0.05 mol/L, pH 4.5) only daily for five consecutive days. In the DM and DM+CNV groups, mice were injected intraperitoneally with 60 mg/kg streptozotocin (STZ; Sigma Chemical, St. Louis, MO, USA) in sodium citrate buffer (0.05 mol/L, pH 4.5) daily for 5 consecutive days to induce diabetes. Seven days after the fifth injection, blood glucose levels from tail vein were measured using a glucomonitor. Mice with glucose levels >300 mg/dL were included in the study [22-23]. Three weeks after the fifth injection, all of mice underwent laser-induction of CNV as described below.

**Choroidal Neovascularization Model** CNV was induced by laser photocoagulation as previously reported[24-25]. Briefly, mice were anesthetized, and their pupils were dilated. Laser (532 nm wavelength, 75 mm spot size, 0.1s duration, and 90 mW intensity) was delivered into the eyes using a slit lamp and a cornea contact lens. The laser burns were performed on the fundus 1.5-2 disc diameters away from the optic nerve. Only laser spots where Bruch's membrane was ruptured (confirmed *via* the presence of a vaporization bubble and the absence of hemorrhage) were considered effective and included in the study.

**Bone Marrow Transplantation** The bone marrow transplantation was conducted as previously described, with slight modifications [3]. Fluc/GFP double transgenic mice (8 weeks old, Department of Cardiology, Xijing Hospital, Fourth Military Medical University, Xi'an, China), as donor mice, were sacrificed by cervical dislocation, and were placed in 75% ethanol water for 5min. The femurs and tibias of donor mice were obtained with skin and muscles away, then washed in PBS and immersed in DMEM culture medium (Gibco, Grand Island, NY, USA) with 1% penicillin and 1% streptomycin. The BMCs were isolated from the femurs and tibias of donor mice by slowly flushing DMEM culture medium into the diaphyseal channel, and erythrocytes were schizolysed. The isolated cells were centrifuged at 800 rpm for 5min and resuspended into PBS. The chimeric mice were injected with $4\times10^6$ cells into the tail vein within 10h after irradiation (8.0 Gy). The level of chimerism in all recipients for subsequent use in this study was above the standard by flow cytometry. So the engraftment of BMCs was similar between the diabetic group and control group.

***In Vivo* Optical Bioluminescence Imaging** The recipient mice were anesthetized with 2% isoflurane and injected with D-luciferin (375 mg/kg body weight; Xenogen, USA) intraperitoneally. The mice were placed in a prone position in the IVIS kinetic system (Xenogen, USA) and their mouths were covered with black papers in order to prevent interference [26]. The mice were imaged at days 1, 3, 5, 7, 14, 21 and 28 after CNV induction. Photon emissions from predefined regions of interest (ROIs) were normalized as photons per second per square centimeter per steradian. The ROIs in every chimeric mice had fixed shape and area, with two eyes covered in and avoiding signals from ears and nose. Peak signals of ROIs were evaluated using Living Image 4.0 software (Xenogen, USA).

***In Vitro* Firefly Luciferase Assays** The recipient mice were sacrificed at 7d after CNV induction. The choroidal organization of each mouse was dissected and a hand-held pestle was used to homogenize the organizations into cell suspensions. The suspensions were lysed using 200 μL 1× passive lysis buffer (Promega, Madison, Wisc., USA) and centrifuged at 1200 rpm for 2min. Each sample was placed into luminometer tubes and consisted of 20 μL supernatant in 100 μL Fluc [relative light units of Fluc (RLU1)] activitiy assay reagent (LAR II; Promega) and 100 μL renilla luciferase [relative light units of renilla luciferase (RLU2)] activitiy assay reagent (Stop & Glo® ; Promega). Both Fluc and renilla luciferase activities were measured by using luciferase photometer.

**Evaluation of Choroidal Neovascularization Severity** Choroidal flatmount was prepared at 7d after CNV induction (6 eyes in each group, 6 spots per eye) in accordance with a previously described protocol [13]. Anesthetized mice were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde. The entire eye globes were enucleated, followed by the anterior segment and the neural retina removed. The remained RPE-choroid-sclera complex was flatmounted with six radial cuts or more, permeabilized in 0.2% Triton X-100 for 24h, then transferred into 1:1000 rhodamine-conjugated Ricinus communisagglutinin (Vector Laboratories, Burlingame, CA, USA) for 24h, and washed in 0.01 mol Tris-buffered saline Tween-20 (TBST) for 24h. The flatmounts were examined and photographed by a confocal laser scanning microscopy (Fv1000, Olympus Corporation, Tokyo, Japan) and CNV area was assessed automatically with image pro plus 6.0 software (IPP 6.0). For measuring CNV surface area, the agglutinin-positive red area at the laser spots in the flatmounts was measured and expressed in $\mu m^2$. Individual lesions with surface areas more than 0.50 disc areas (DAs) were defined as having CNV[13,27-28]. Histopathological analysis was performed as described previously [7] (3 eyes in each group, 6 spots per eye). The mice were killed at days 1, 3, 5, 7, 14, 21 and 28 after photocoagulation and eyes were enucleated, then eyecup preparations were fixed in Bouin's fixative (Zhongshan Biotechnology Company, Beijing, China) at 4℃ for 24h. The fixed tissues were embedded in paraffin, serially sectioned at 3 μm, and stained with hematoxylin and eosin (H&E). Serial slices of each CNV were examined and digitized using a light microscope (BX51, Olympus Corporation, Tokyo, Japan). CNV thickness was measured vertically from the adjacent RPE layer to the top of the CNV and CNV length was measured as horizontal maximize distance of CNV using IPP 6.0 software, which expressed in μm.

**Western Blot Assay** The RPE-choroid-sclera complex was collected and lysed, and the expression levels of VEGF and SDF-1 were measured using standard techniques [11]. The monoclonal primary antibodies to VEGF (1:200, Abcam Biotechnology, USA) and SDF-1 (1:200, Abcam Biotechnology, USA) were used. The β-actin levels were used to normalize the quantity of the proteins and all experiments were repeated at least three times.

**Statistical Analysis** Statistical analyses were performed using SPSS 19.0 software. The data from several

experiments were pooled and then presented as the mean ± SEM. Analysis of variance followed by LSD-$t$ test was used to compare two groups. Student's $t$-test was used for the rest statistical analyses. All experimental data sets were scrutinized for normal distribution of variance, and nonparametric tests were appropriately applied when necessary. A two-tailed value of $P$ <0.05 was considered significant.

**RESULTS**

**Kinetic Observation of Hyperglycemia Promoting the Transplanted Stem Cells´ Participation in Choroidal Neovascularization by Bioluminescence Imaging** To confirm the hypotheses that hyperglycemia promotes the participation of stem cells in CNV formation, we conducted the bone marrow transplantation, STZ intraperitoneal injection and laser photocoagulation to induce the chimeric mice, DM and CNV model, respectively. By using *in vivo* optical BLI, the recruitment of $Fluc^+GFP^+$ BMCs was detected at 1d after laser photocoagulation, with the peak signals occupied at 7d, then weakened at subsequent time points, indicating the donor cell death. The fact that the DM+CNV group showed a stronger signal than the Normal+CNV group indicated that hyperglycemia promoted the transplanted BMCs to participate in CNV (Figure 1A). Quantitative analysis of ROIs showed that the radiance (photons per second per square centimeter per steradian) of $Fluc^+GFP^+$ BMCs increased from 1d to 7d; after reaching a maximum intensity at 7d, the radiance decreased in the following days. At each time point, the radiance in the CNV groups were stronger than that in the control groups no matter the blood glucose concentration was normal or high, and the radiance in the DM+CNV group was stronger than that in the Normal+CNV group at days 5, 7, 14 and 21 (121861.67±9948.81 *vs* 144998.33±13787.13 photons/second/$cm^2$/sr for control and DM mice, $P_{5d}$<0.05; 178791.67±30350.8 *vs* 240166.67±22605.3, $P_{7d}$<0.05; 124176.67±16253.52 *vs* 196376.67 ±18556.79, $P_{14d}$<0.05; 97951.60 ±10343.09 *vs* 119510.00±14383.76, $P_{21d}$<0.05; Figure 1B), consistent with the BLI signals mentioned above.

**Histopathological Analysis of Hyperglycemia Promoting the Development of Choroidal Neovascularization in Mice** Vascular complexes that formed after the damage to Bruch's membrane extended from the choroid to the subretinal space. To confirm the effects of hyperglycemia on the progression of CNV, we used the histopathological assay at days 1, 3, 5, 7, 14, 21 and 28 after CNV induction (Figure 2A) and found that the CNVs in the cross-sectional slices stained with H&E from the hyperglycemic mice were wider than those in the control group at days 5, 7, 14 and 21, with the maximum at 7d (147.83±17.36 *vs* 220.33±20.17 μm for control and DM mice, $P_{5d}$<0.05; 212.17±24.63 *vs* 326.83±19.49, $P_{7d}$<0.05; 163.17±18.24 *vs* 265.17±20.55, $P_{14d}$<0.05; 132.00±10.88 *vs* 205.33±12.98, $P_{21d}$<0.05; Figure 2B), but the average thickness of CNVs between the two groups had no significant difference (Figure 2C).

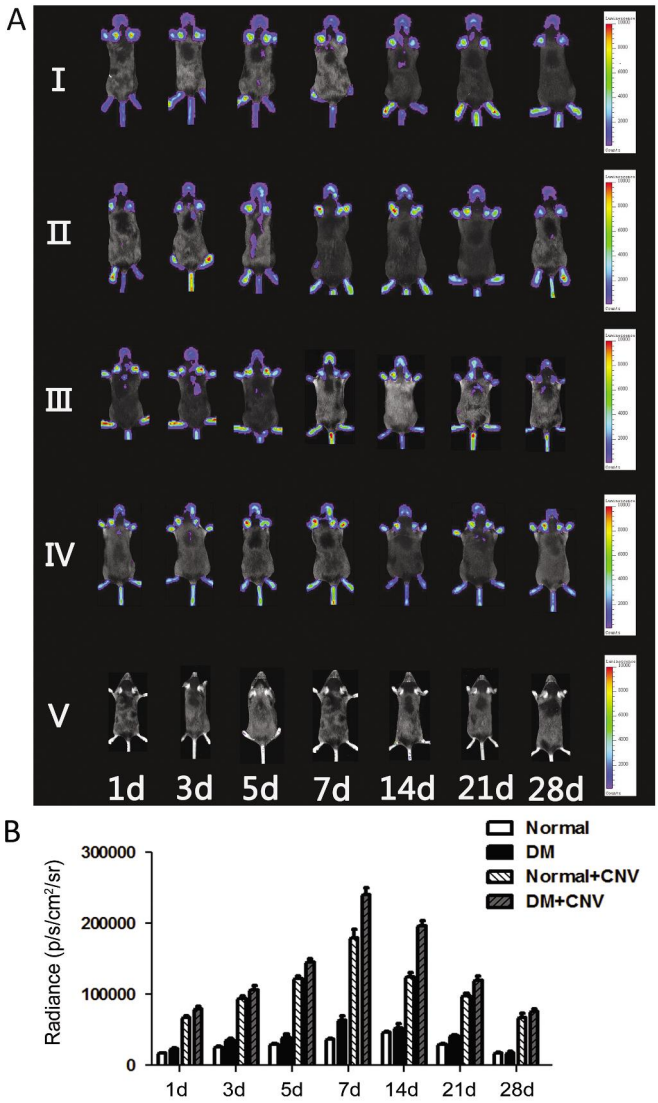


**Figure 1 Hyperglycemia promoted the participation of BMCs in CNV after laser photocoagulation** A: *In vivo* optical BLI signals of transplanted $Fluc^+GFP^+$ BMCs in living mice among five groups at days 1, 3, 5, 7, 14, 21 and 28 after laser photocoagulation. The BLI signals were observed at 1d after laser photocoagulation, with the peak signals occupied at 7d, then decreased at the subsequent time points. Ⅰ: control chimeric mice (Normal); Ⅱ: diabetic chimeric mice (DM); Ⅲ: control chimeric mice with CNV (Normal+CNV); Ⅳ: diabetic chimeric mice with CNV (DM+CNV); Ⅴ: control WT mice. Scale bars represent BLI signals in photons per second per square centimeter per steradian (p/s/$cm^2$/sr). B: Quantitative analysis of the BLI signals in ROIs. The radiance in the Normal+CNV and DM+CNV groups were stronger than that in the Normal and DM control groups, respectively, and the radiance in the DM+CNV group was stronger than that in the Normal+CNV group at days 5, 7, 14 and 21 after laser induction ($P$<0.05). There was no signal in ROIs in the control WT mice, and the control WT group was not included in the analyses.

***In Vivo* Flatmount and *in Vitro* Fluc Assay of Hyperglycemia Exacerbating the Choroidal Neovascularization at 7d After Laser Photocoagulation** Based on

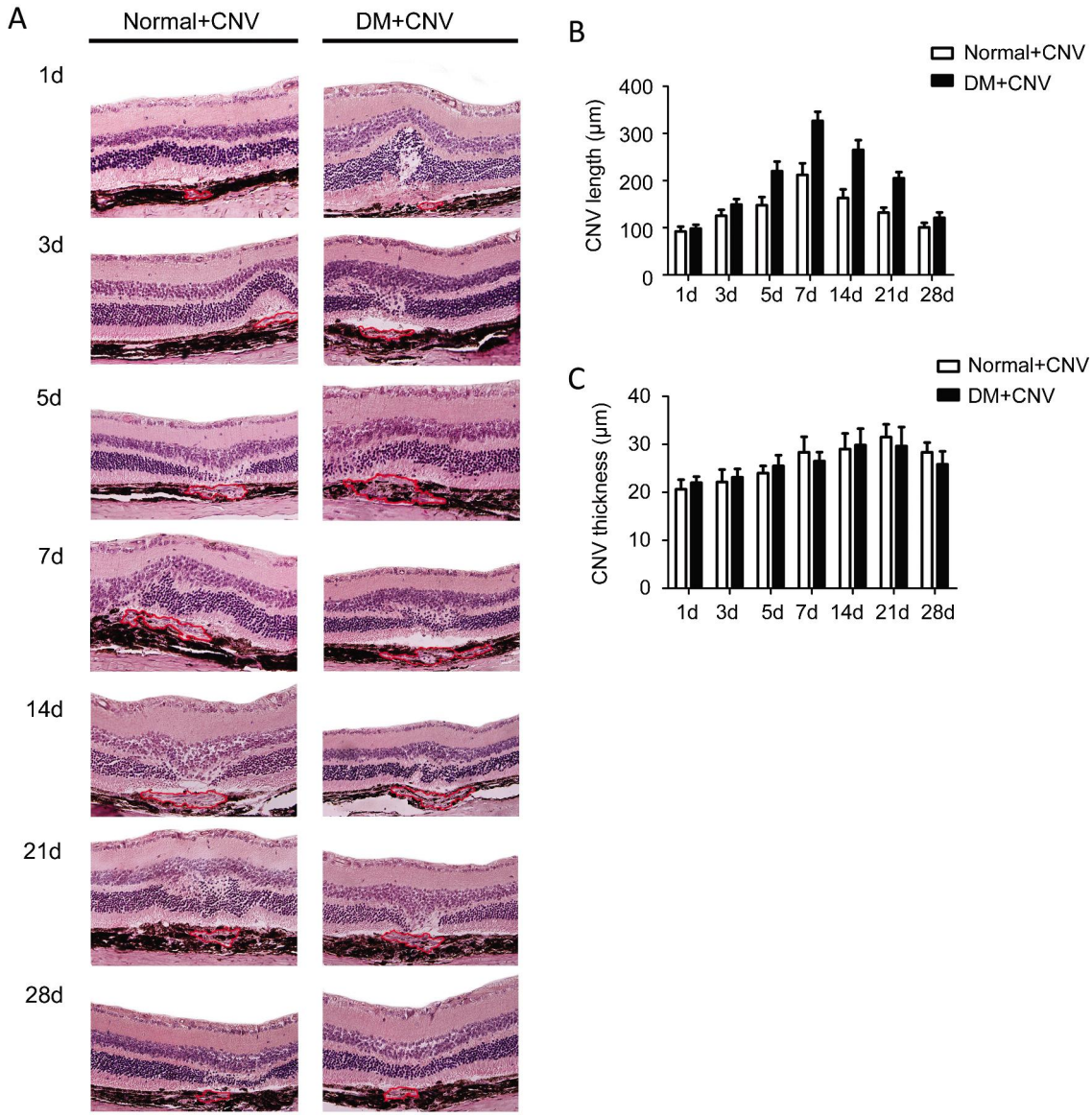


**Figure 2 Hyperglycemia aggravated the development of CNV after laser photocoagulation** A: Representative images of the H&E-stained serial cross-sections of the eyecups from control (left) and diabetic (right) mice at days 1, 3, 5, 7, 14, 21 and 28 after laser photocoagulation (Red lines: area of CNV). B: Statistical analysis of the average length of CNV between the control and diabetic groups in A. The CNVs in the hyperglycemic mice were wider than those in the control group at days 5, 7, 14 and 21 after laser photocoagulation ($P$< 0.05), with the maximum at 7d. C: Statistical analysis of the average thickness of CNV between the two groups in A. There was no significant difference.

the fact that the largest CNV and the maximum amount of transplanted stem cells were appeared at 7d after laser photocoagulation, we conducted the flatmount assay *in vivo* and the quantitative analysis of Fluc reporter enzyme *in vitro* at 7d. Compared with the control group, the average area of CNV in the diabetic group was notably larger at 7d (20688.67±3644.96 *vs* 32218.00±4132.69 μm$^2$ for control and diabetic mice, respectively; $P$<0.05; Figure 3A, 3B). In view of the BLI data only showing the differential recruitment and participation of BMCs in CNV but unable to afford accurate quantitative analysis, a Fluc assay was used to quantify the Fluc reporter enzyme in the diabetic and control mice with CNV model. Three sets of data were obtained using a luminometer for each group: RLU1, RLU2 and the ratio of RLU1 and RLU2. RLU1 and ratio are standard for the comparisons of Fluc reporter enzyme. Compared with the control group, RLU1 and ratio were markedly higher at 7d in the diabetic group (215.00±52.05 *vs* 707.33±88.65, $P$<0.05; 0.90±0.17 *vs* 1.83±0.17, $P$<0.05; for control and diabetic mice, respectively; Figure 3C, 3D).

**Mechanism of Hyperglycemia Promoting the Participation of Bone Marrow -derived Cells in Choroidal Neovascularization After Laser Photocoagulation** To explore the underlying mechanism that hyperglycemia exacerbated the recruitment and participation of BMCs in CNV after laser photocoagulation, we investigated the levels of VEGF and SDF-1 production in the diabetic and control mice with CNV model by Western Blot examination (Figure 4A)

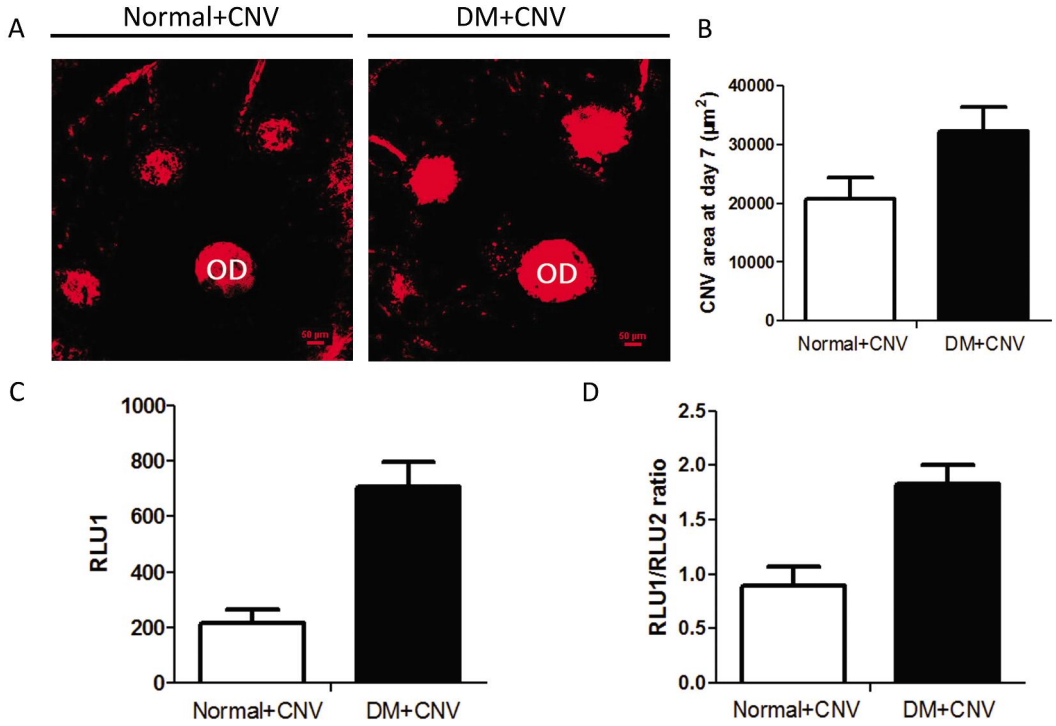


**Figure 3 Hyperglycemia exacerbated the CNV lesion at 7d after laser photocoagulation** A: Representative flatmount preparations of the eyecups from the control (left) and diabetic (right) mice at 7d after laser photocoagulation (OD: Optic disc). B: Statistical analysis of CNV area in A. The average area of CNV in the diabetic mice was markedly larger than that in the control group at 7d ($P$<0.05). C, D: *In vitro* characterization of reporter gene expression at 7d. RLU1 and the RLU1/RLU2 ratio in the diabetic group were significantly higher than those in the control group ($P$<0.05), determined using the luminometer assay.

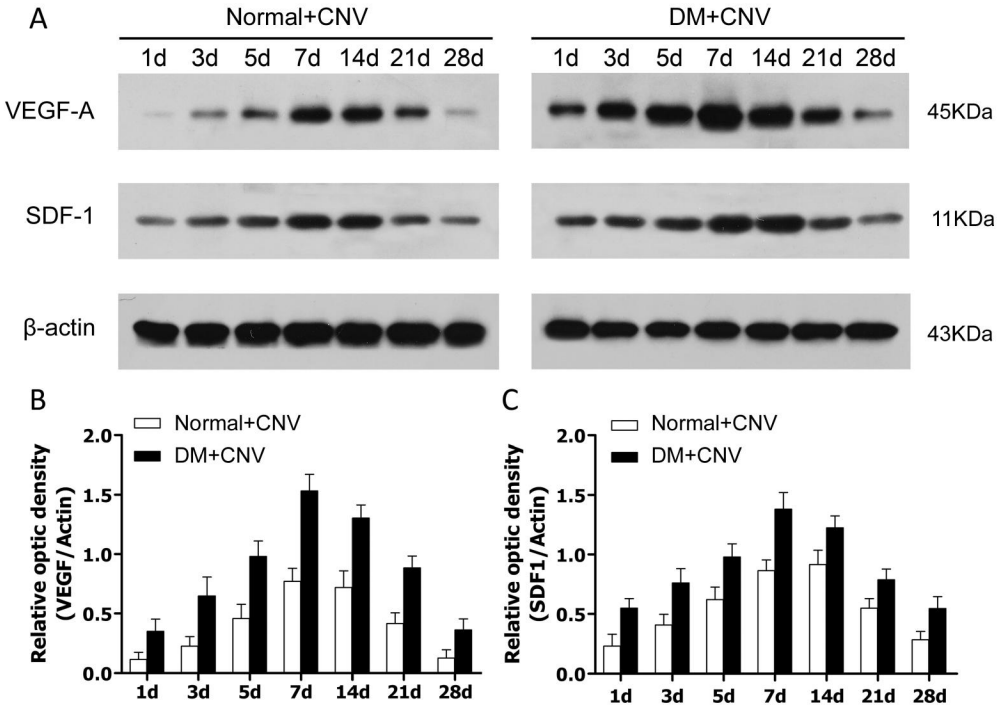


**Figure 4 Mechanism of hyperglycemia exacerbating the participation of BMCs in CNV after laser photocoagulation** A: Expression of VEGF and SDF-1 in the control (left) and diabetic (right) mice at days 1, 3, 5, 7, 14, 21 and 28 after laser photocoagulation by Western Blot examination. B, C: Statistical analysis of the data in A. The level of VEGF and SDF-1 protein expression in hyperglycemic mice significantly increased in a time-dependent manner, with the peak value occupied at 7d ($P$<0.05).

and found that the level of VEGF protein expression significantly increased from 1d to 7d; after reaching a maximum at 7d, it decreased subsequently, and at each time point, the amount of VEGF protein in the diabetic group was higher than that in control group (Figure 4B, $P$<0.05). In addition, the amount of SDF-1 protein was also up-regulated in the diabetic group, and the changing timecourse of the up-regulation was similar to the VEGF protein expression

(Figure 4C, $P<0.05$).

## DISCUSSION

Neovascular AMD, the primary cause of blindness among elderly people in developed countries, is characterized by the presence of CNV under the macula [29]. Because diabetes functions on vascular systems, most epidemiological studies have focused on the relationship between diabetes and AMD. Diabetes-related changes have been considered as risk factors for developing AMD and the underlying mechanism needs to be further carried out[30].

In previous studies of our research group, we found that BMCs (GFP-positive) recruited to CNV expressed the mature vascular ECs marker CD31, VSMCs marker α-SMA and the macrophage marker F4/80, indicating BMCs recruited to CNV differentiated into these three types of cells and actually integrated into the CNV structures, that is the vasculogenesis of CNV [6,13,24]. Furthermore, hyperglycemia significantly increased the constituent ratio of vascular ECs and macrophages labelled GFP, up-regulated the expression of VEGF and SDF-1 in diabetic group, suggesting that hyperglycemia promoted recruitment, differentiation and incorporation of BMCs in CNV [12]. In addition, hyperglycemia promoted the development of CNV by inducing oxidative stress, which in turn activated STAT3-regulated VEGF expression in RPE cells [11]. In this study, molecular imaging techniques, including *in vivo* BLI and *in vitro* Fluc assay, were used to observe the *in vivo* behaviors and migration of BMCs implanted into diabetic mice with CNV model for the first time and to verify our previous conclusion that hyperglycemia exacerbated the vasculogenesis of CNV due to more BMCs recruited. In combination with conventional histological methods, we demonstrated that hyperglycemia enhanced the progression of CNV and the recruitment of BMCs in CNV associated with elevation of VEGF and SDF-1 in the eye; molecular imaging using the Fluc reporter enzyme was a reliable method for monitoring stem cell survival *in vivo*.

In previous studies, *in vivo* stem cell research has been predominantly relied on postmortem histological analysis and molecular biology examination, which are inappropriate to reveal the time scale of the dynamic interplay between the stem cell graft, the CNV lesion and the endogenous related mechanisms [31-33]. In the present study, the noninvasive molecular imaging technique using the Fluc reporter gene and D-luciferin reporter probe provided complementary *in vivo* information and showed great potential for *in vivo* monitoring stem cell events in CNV lesions in a diabetic mouse model. This *in vivo* optical BLI method might avoid sample biases caused by the sacrifice of multiple animals at different time points, permit simple animal preparation, repetitive experimental conditions and relatively medium-cost instrumentation, and be performed under mild anesthesia, thus nearly under physiological conditions [34]. Furthermore, molecular imaging technology is a valuable, unpolluted tool for the analysis of disease processes at the molecular level in living intact animals, which would extend our understanding of the basis of optimal cell administration system and dose, and would greatly influence our views on the efficacy of future cell-based therapies[20].

Although cells in situ play an angiogenetic role in CNV, BMCs-mediated postnatal vasculogenesis has been reported as the main responsible for the regulation of CNV progression [7]. We demonstrated that hyperglycemia exacerbated the severity of CNV in diabetic mice by choroidal flatmount and histological sections as reported before, detected that more BMCs were recruited to CNV lesions in diabetic chimeric mice by *in vivo* BLI monitoring and *in vitro* Fluc assay for the first time, and further revealed that more severe CNV and the increased number of BMCs in CNV exposed to high glucose were associated with up-regulation expression of VEGF and SDF-1 by RT-PCR and Western blot, consistent with our previous studies. However, the results seemed contrary to the conclusion that BMCs were decreased and disordered in diabetes as reported. Hyperglycemia was related to the dysfunction of bone marrow-derived endothelial progenitor cells (EPCs) at each step of their lifespan (bone marrow mobilization, trafficking into the bloodstream, survival, differentiation into ECs, and homing in damaged tissues/organs) [35-36]. Hyperglycemia alone, through the mitochondrial overproduction of reactive oxygen species (ROS), induced changes in gene expression and cellular behavior, would explain the impairments in vasculogenesis, the process by which circulating EPCs contributed to new vessel formation in diabetes [14]. EPCs were functionally impaired in hyperglycemia through the p38 MAPK signaling pathway[37]. These conflicts might be due to the different animal models being used. The CNV model has been used in most studies on CNV, but still exists some defects such as its relative short disease duration (about 4wk), which might be insufficient to produce significant reduction and dysfunction of BMCs in diabetic mice, forcing us optimize the CNV model in the future. In addition, BMCs consist of mesenchymal stem cells, hematopoietic stem cells, EPCs and various other cell subtypes, all of which need further detection using molecular imaging technologies in order to ascertain which components of BMCs predominantly participate in CNV development in diabetic mice[38].

In conclusion, the present study used dynamic molecular imaging techniques for the first time to observe the *in vivo* behavior of stem cells exposed to hyperglycemia in CNV mice and demonstrated the diabetes-aggravated vasculogenesis of CNV, suggesting the potential of BMCs as powerful delivery vehicles carrying antiangiogenic agents

targeting VEGF in CNV. The underlying mechanisms might be the increased levels of VEGF and SDF-1 and needs further investigation. Our findings reveal that diabetes is a risk factor for disorders involving the vasculogenesis of CNV, and cell-based protocol may serve as a therapeutic strategy for the treatment and prevention of these diseases.

## ACKNOWLEDGEMENTS

**Foundations:** Supported by the National Natural Science Foundation of China (No. 81070748; No. 81200708); National Basic Research Program of China (973 Program).

**Conflicts of Interest: Gao X,** None; **Wang Y,** None; **Hou HY,** None; **Lyu Y,** None; **Wang HY,** None; **Yao LB,** None; **Zhang J,** None; **Cao F,** None; **Wang YS,** None.

## REFERENCES

1 Whitmore SS, Sohn EH, Chirco KR, Drack AV, Stone EM, Tucker BA, Mullins RF. Complement activation and choriocapillaris loss in early AMD: Implications for pathophysiology and therapy. *Prog Retin Eye Res* 2015; 45:1-29.

2 Solomon SD, Lindsley K, Vedula SS, Krzystolik MG, Hawkins BS. Anti-vascular endothelial growth factor for neovascular age-related macular degeneration. *Cochrane Database Syst Rev* 2014;8:CD005139.

3 Zhang ZX, Wang YS, Shi YY, Hou HY, Zhang C, Cai Y, Dou GR, Yao LB, Li FY. Hypoxia specific SDF-1 expression by retinal pigment epithelium initiates bone marrow-derived cells to participate in choroidal neovascularization in a laser-induced mouse model. *Curr Eye Res* 2011; 36(9):838-849.

4 Espinosa-Heidmann DG, Caicedo A, Hernandez EP, Csaky KG, Cousins SW. Bone marrow-derived progenitor cells contribute to experimental choroidal neovascularization. *Invest Ophthalmol Vis Sci* 2003;44 (11): 4914-4919.

5 Csaky KG, Baffi JZ, Byrnes GA, Wolfe JD, Hilmer SC, Flippin J, Cousins SW. Recruitment of marrow-derived endothelial cells to experimental choroidal neovascularization by local expression of vascular endothelial growth factor. *Exp Eye Res* 2004;78(6):1107-1116.

6 Hou HY, Wang YS, Xu JF, Wang BR. Nicotine promotes contribution of bone marrow-derived cells to experimental choroidal neovascularization in mice. *Exp Eye Res* 2008;86(6):983-990.

7 Shi YY, Wang YS, Zhang ZX, Cai Y, Zhou J, Hou HY, van Rooijen N. Monocyte/macrophages promote vasculogenesis in choroidal neovascularization in mice by stimulating SDF-1 expression in RPE cells. *Graefes Arch Clin Exp Ophthalmol* 2011;249(11):1667-1679.

8 Fadini GP, Ferraro F, Quaini F, Asahara T, Madeddu P. Concise review: diabetes, the bone marrow niche, and impaired vascular regeneration. *Stem Cells Transl Med* 2014;3(8):949-957.

9 Klein R, Deng Y, Klein BE, Hyman L, Seddon J, Frank RN, Wallace RB, Hendrix SL, Kuppermann BD, Langer RD, Kuller L, Brunner R, Johnson KC, Thomas AM, Haan M. Cardiovascular disease, its risk factors and treatment, and age-related macular degeneration: Women's Health Initiative Sight Exam ancillary study. *Am J Ophthalmol* 2007;143 (3): 473-483.

10 Duan Y, Mo J, Klein R, Scott IU, Lin HM, Caulfield J, Patel M, Liao D. Age-related macular degeneration is associated with incident myocardial infarction among elderly Americans. *Ophthalmology* 2007;114(4):732-737.

11 Li X, Cai Y, Wang YS, Shi YY, Hou W, Xu CS, Wang HY, Ye Z, Yao LB, Zhang J. Hyperglycaemia exacerbates choroidal neovascularisation in mice via the oxidative stress-induced activation of STAT3 signalling in RPE cells. *PLoS One* 2012;7(10):e47600.

12 Cai Y, Li X, Wang YS, Shi YY, Ye Z, Yang GD, Dou GR, Hou HY, Yang N, Cao XR, Lu ZF. Hyperglycemia promotes vasculogenesis in choroidal neovascularization in diabetic mice by stimulating VEGF and SDF-1 expression in retinal pigment epithelial cells. *Exp Eye Res* 2014; 123:87-96.

13 Hou HY, Liang HL, Wang YS, Zhang ZX, Wang BR, Shi YY, Dong X, Cai Y. A therapeutic strategy for choroidal neovascularization based on recruitment of mesenchymal stem cells to the sites of lesions. *Mol Ther* 2010;18(10):1837-1845.

14 Callaghan MJ, Ceradini DJ, Gurtner GC. Hyperglycemia-induced reactive oxygen species and impaired endothelial progenitor cell function. *Antioxid Redox Signal* 2005;7(11-12):1476-1482.

15 Mezzanotte L, Blankevoort V, Lowik CW, Kaijzel EL. A novel luciferase fusion protein for highly sensitive optical imaging: from single-cell analysis to *in vivo* whole-body bioluminescence imaging. *Anal Bioanal Chem* 2014; 406(23):5727-5734.

16 Wang H, Cao F, De A, Cao Y, Contag C, Gambhir SS, Wu JC, Chen X. Trafficking mesenchymal stem cell engraftment and differentiation in tumor-bearing mice by bioluminescence imaging. *Stem Cells* 2009;27(7): 1548-1558.

17 Li H, Wang Y, Cao F. *In vivo* bioluminescence imaging monitoring of stem cells' participation in choroidal neovascularization. *Ophthalmic Res* 2013;50(1):19-26.

18 O'Farrell AC, Shnyder SD, Marston G, Coletta PL, Gill JH. Non-invasive molecular imaging for preclinical cancer therapeutic development. *Br J Pharmacol* 2013;169(4):719-735.

19 Li J, Chen L, Du L, Li M. Cage the firefly luciferin! - a strategy for developing bioluminescent probes. *Chem Soc Rev* 2013;42(2):662-676.

20 Roura S, Galvez-Monton C, Bayes-Genis A. Bioluminescence imaging: a shining future for cardiac regeneration. *J Cell Mol Med* 2013;17 (6): 693-703.

21 Sato A, Klaunberg B, Tolwani R. *In vivo* bioluminescence imaging. *Comp Med* 2004;54(6):631-634.

22 Ebrahimian TG, Heymes C, You D, Blanc-Brude O, Mees B, Waeckel L, Duriez M, Vilar J, Brandes RP, Levy BI, Shah AM, Silvestre JS. NADPH oxidase-derived overproduction of reactive oxygen species impairs postischemic neovascularization in mice with type 1 diabetes. *Am J Pathol* 2006;169(2):719-728.

23 Gallagher KA, Liu ZJ, Xiao M, Chen H, Goldstein LJ, Buerk DG, Nedeau A, Thom SR, Velazquez OC. Diabetic impairments in NO-mediated endothelial progenitor cell mobilization and homing are reversed by hyperoxia and SDF-1 alpha. *J Clin Invest* 2007;117 (5): 1249-1259.

24 Hou HY, Wang YS, Xu JF, Wang YC, Liu JP. The dynamic conduct of bone marrow-derived cells in the choroidal neovascularization microenvironment. *Curr Eye Res* 2006;31(12):1051-1061.

25 Ryan SJ. The development of an experimental model of subretinal neovascularization in disciform macular degeneration. *Trans Am Ophthalmol Soc* 1979;77:707-745.

26 Li H, Li JZ, Helm GA, Pan D. Non-invasive imaging of firefly luciferase reporter gene expression using bioluminescence imaging in human prostate cancer models. *Biotechnol Appl Biochem* 2007;46:179-184.

27 Espinosa-Heidmann DG, Suner I, Hernandez EP, Frazier WD, Csaky KG, Cousins SW. Age as an independent risk factor for severity of experimental choroidal neovascularization. *Invest Ophthalmol Vis Sci* 2002;43(5):1567-1573.

28 Dou GR, Wang YC, Hu XB, Hou LH, Wang CM, Xu JF, Wang YS, Liang YM, Yao LB, Yang AG, Han H. RBP-J, the transcription factor downstream of Notch receptors, is essential for the maintenance of vascular homeostasis in adult mice. *FASEB J* 2008;22 (5): 1606-1617.

29 Campa C, Harding SP. Anti-VEGF compounds in the treatment of neovascular age related macular degeneration. *Curr Drug Targets* 2011;12 (2):173-181.

30 Choi JK, Lym YL, Moon JW, Shin HJ, Cho B. Diabetes mellitus and early age-related macular degeneration. *Arch Ophthalmol* 2011;129 (2): 196-199.

31 Lecomte J, Louis K, Detry B, Blacher S, Lambert V, Bekaert S, Munaut C, Paupert J, Blaise P, Foidart JM, Rakic JM, Krane SM, Noel A. Bone marrow-derived mesenchymal cells and MMP13 contribute to experimental choroidal neovascularization. *Cell Mol Life Sci* 2011;68(4):677-686.

32 Sasahara M, Otani A, Yodoi Y, Yoshimura N. Circulating hematopoietic stem cells in patients with idiopathic choroidal neovascularization. *Invest Ophthalmol Vis Sci* 2009;50(4):1575-1579.

33 Aswendt M, Adamczak J, Tennstaedt A. A review of novel optical imaging strategies of the stroke pathology and stem cell therapy in stroke. *Front Cell Neurosci* 2014;8:226.

34 Chang GY, Xie X, Wu JC. Overview of stem cells and imaging modalities for cardiovascular diseases. *J Nucl Cardiol* 2006;13(4):554-569.

35 Albiero M, Menegazzo L, Boscaro E, Agostini C, Avogaro A, Fadini GP. Defective recruitment, survival and proliferation of bone marrow-derived progenitor cells at sites of delayed diabetic wound healing in mice. *Diabetologia* 2011;54(4):945-953.

36 Petrelli A, Di Fenza R, Carvello M, Gatti F, Secchi A, Fiorina P. Strategies to reverse endothelial progenitor cell dysfunction in diabetes. *Exp Diabetes Res* 2012;2012:471823.

37 Bai YY, Wang L, Peng XG, Wang YC, Chang D, Zheng S, Ding J, Li C, Ju S. Non-invasive monitoring of transplanted endothelial progenitor cells in diabetic ischemic stroke models. *Biomaterials* 2015;40:43-50.

38 Sengupta N, Caballero S, Mames RN, Butler JM, Scott EW, Grant MB. The role of adult bone marrow-derived stem cells in choroidal neovascularization. *Invest Ophthalmol Vis Sci* 2003;44(11):4908-4913.