NR4A1 Promotes PDGF-BB-Induced Cell Colony Formation in Soft Agar

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Abstract

The fibroblast mitogen platelet-derived growth factor -BB (PDGF-BB) induces a transient expression of the orphan nuclear receptor NR4A1 (also named Nur77, TR3 or NGF1B). The aim of the present study was to investigate the pathways through which NR4A1 is induced by PDGF-BB and its functional role. We demonstrate that in PDGF-BB stimulated NIH3T3 cells, the MEK1/2 inhibitor CI-1040 strongly represses NR4A1 expression, whereas Erk5 downregulation delays the expression, but does not block it. Moreover, we report that treatment with the NF-κB inhibitor BAY11-7082 suppresses NR4A1 mRNA and protein expression. The majority of NR4A1 in NIH3T3 was found to be localized in the cytoplasm and only a fraction was translocated to the nucleus after continued PDGF-BB treatment. Silencing NR4A1 slightly increased the proliferation rate of NIH3T3 cells and the glioblastoma cell lines U-105MG and U-251MG. Thus, whereas NR4A1, induced by PDGF-BB, suppresses cell growth on a solid surface, it increases anchorage-independent growth.

Introduction

Platelet-derived growth factor (PDGF) is a key mitogen for cells of mesenchymal origin, with important functions during embryonic development and wound healing. The biologically active isoforms of PDGF are disulfide-bonded dimers of A, B, C or D polypeptide chains, i.e. PDGF-AA, -BB, -AB, -CC and -DD, which bind to structurally related α- and β-tyrosine kinase receptors (PDGFRα and PDGFRβ, respectively). The two PDGFRs have different ligand binding specificities; PDGFRα binds PDGF A-, B- and C-chains, whereas PDGFRβ binds B- and D-chains [1]. Binding of the dimeric PDGF isoforms results in homo- or hetero-dimerization of the receptors and subsequent autophosphorylation of tyrosine residues in their intracellular parts. The autophosphorylation activates the kinase activity of the receptors and the phosphorylated tyrosine residues serve as interaction sites for SH2-domain-containing signal transduction proteins, which relay or modulate several signaling pathways. Examples include GRB2/SOS1 which activates extracellular signal-regulated kinase (ERK)- and the protein tyrosine phosphatase SHP-2 [1] [2]. These signaling pathways promote cell proliferation, migration and survival. Overactivity of PDGF pathways is implicated in diseases involving excessive cell growth, including malignancies, cardiovascular disease and fibrosis [3].

The MAP-kinase pathways activated by PDGF include Erk1/2, Erk5, c-Jun N-terminal kinase (JNK), and p38 [4] [5]. Erk5, unlike the other MAP-kinases, has an extended, unique C-terminal region with a bipartite nuclear localization signal (NLS) [6], and a transcriptional activation domain [7], suggesting that Erk5 may function both as a kinase and as a transcription factor. Activated MAP-kinases phosphorylate several substrates, including cytosolic signaling proteins and transcription factors afecting cell proliferation, survival and migration.

Nuclear receptors function as ligand-activated transcription factors; however, there are several examples of so called orphan nuclear receptors for which no ligand has been identified. The function of orphan nuclear receptors can be regulated by expression levels and/or post-translational modifications, such as phosphorylation. NR4A1 (Nur77, TR3, NGF1B) is an example of an orphan nuclear receptor that can be phosphorylated by Erk1/2, Erk5 and JNK MAP-kinases, as well as other kinases such as Akt, Rsk, GSK3β and DNA-PK [8]. NR4A1 belongs to a family which also encompasses NR4A2 (NURR1) and NR4A3 (NOR-1) characterized by a conserved DNA binding domain that suggests redundancy among them. Notably, members of the NR4A1 family is frequently found to be induced by growth factors [11] [9]. Both phosphorylation and acetylation have been shown to control NR4A1 stability and/or subcellular localization [10] [11] [12] [13]. Multiple and sometimes opposing functions of NR4A1 have described in different cell types which may be related to differences in subcellular localization. Overexpression of NR4A1 resulted in...
increased survival and proliferation of human umbilical vein endothelial cells [14]. On the other hand, an apoptotic effect was associated with a mitochondrial localization of NR4A1, where it converted BCL-2 from an anti- to a pro-apoptotic protein [15] [16] [17]. Moreover, it has been shown that NR4A1 is involved in T cell receptors-mediated apoptosis in immature thymocytes [18] [19] and roles for NR4A1 has also been described in metabolism [20], steroidogenesis [21] [22], as well as in suppression of smooth muscle cells proliferation by upregulating p27kip1 [23] [24] [25]. NR4A1 has been found both to promote and inhibit tumorigenesis [26]. On one hand, NR4A1 behaves as a tumor suppressor by inhibiting growth of pancreatic cancer cells [27], and a double knock-out of NR4A1 and NR4A3 in mice was found to lead to the development of acute myeloid leukemia (AML); consistently, low expression of NR4A1 and NR4A3 has frequently been found in human AML [28] [29]. On the other hand, NR4A1 is commonly overexpressed in lung cancer patients and correlates to poor prognosis [30], and it has been shown to confer a proliferative advantage to colon cancer cells as well as increasing the invasive behavior of breast cancer by enhancing TGFβ signaling [31] [32]. Despite the well-established role of NR4A1 in apoptosis, its overexpression has also been reported to protect cells from apoptosis [33]. It is possible that the opposing effects of NR4A1 expression in different cancers may be explained by differences in post-translational modifications of NR4A1 and thereby its subcellular localization. Other aspects of NR4A1 functionality that may contribute to tumor development is its ability to promote cell migration, invasion through by promoting MMP-9 function, inflammation, repair of DNA double-strand breaks and VEGF-induced angiogenesis [34] [35] [36] [37]. Recently, yet another way that NR4A1 can impact tumorigenesis was discovered where NR4A1 regulate the expression of stemness-related genes Oct-4 and Nanog in gastric cancers cells [37].

In the present work, we elucidated the signaling mechanisms by which PDGF-BB promotes NR4A1 expression and the role of NR4A1 in PDGF-mediated responses and tumorigenicity.

### Materials and Methods

**Cell culture**

NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% bovine serum (BS). The human glioblastoma cell lines, U-105MG and U-251MG [38], were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 25 mM L-glutamine. For serum-starvation, cells were washed once and incubated in medium containing 0.1% bovine serum albumin (BSA). Recombinant human PDGF-BB was generously provided by Amgen (Thousand Oaks, CA). The inhibitors JNK Inhibitor II and BAY 11-7082 were from Calbiochem (San Diego, CA), CI-1040 (PD184352) and BIX02189 were from Sellek, and MG132 was from Sigma-Aldrich. For selection, 1 mg/ml of the antibiotic G418 Sulfate from Calbiochem (San Diego, CA) was added to the growth medium.

**Transfections**

Downregulation of NR4A1 and Erk5 was performed by using 80 nM of specific Silencer Selected pre designed for NR4A1 (UUUCUGUACUGUGCGCUUGaa and UACCCGUCCAU-GAAGGUGCtg) or for Erk5, both purchased from Ambion Life Technology. The inhibitors JNK Inhibitor II and BAY 11-7082 were from Calbiochem (San Diego, CA), CI-1040 (PD184352) and BIX02189 were from Sellek, and MG132 was from Sigma-Aldrich. For selection, 1 mg/ml of the antibiotic G418 Sulfate from Calbiochem (San Diego, CA) was added to the growth medium.

**Figure 1. PDGF-BB induces NR4A1 via Erk1/2 and Erk5.** NIH3T3 cells were serum-starved overnight in 0.1% BSA and treated with inhibitors starting 1 h before stimulation with PDGF-BB (20 ng/ml) for the indicated time periods. Total cell lysate (TCL) (A and C) were analyzed by immunoblotting (lb) using NR4A1, Erk2, phospho-Erk1/2 and Erk5 antibodies. NR4A1 mRNA was measured with quantitative RT-PCR and panel B shows one out of three independent experiments performed; error bars indicate the standard deviation between three replicates. An asterisk (*) indicate a p-value $<$0.05; with two (**) when it is $<$0.01 and with three (***) when it is $<$0.001.

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of knockdown were analyzed after 48 h by immunoblot or by qPCR.

Three µl polyethyleneimine (2.5 mg/ml) diluted in 100 µl of serum-free DMEM was added to 2 µg plasmid DNA (Myc-DKK-Tagged ORF clone of mouse NR4A1 [MR209316] or TrueClone Pmmt-Kan/Neo Vector from Origene), diluted in 100 µl of medium. The transfection mixture was incubated at room temperature for 20 min. Cells were washed and the medium was replaced with 2 ml/well of complete medium. The transfection mixture (200 µl) was added into the cell culture dish to give a total volume of 1.2 ml/well. For stable transfection, culture
medium was replaced with selection medium containing 1 mg/ml G-418, after 48 h and culturing continued for one week.

Real time PCR
Total DNA-free cellular RNA was extracted from cells treated with PDGF-BB for indicated periods of time with the RNeasy kit (Qiagen), and was reverse-transcribed (SuperScript II RNase; Invitrogen) to create cDNA templates. The PCR was performed by the CFX Manager (Bio-Rad) according to the manufacturer’s instructions. Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control for the relative quantification of the target message. Specific primers were as follows: NR4A1, CTCGCCATCAGACCAACT (forward) and CTTAGGCACCTGCCTGTCC (reverse); glyceraldehyde-3-phosphate dehydrogenase, CCCCATTGAAGCCTCAGCAT (forward) and GGGATTTCAGTGGATGACAG (reverse).

Immunoblotting
Subconfluent cells were starved and incubated with vehicle or inhibitors at the indicated concentrations and thereafter stimulated with PDGF-BB (20 ng/ml); total cell lysates (TCL) were prepared and subjected to immunoblotting (lb).

Figure 2. NR4A1 expression does not decrease after inhibition of p38, JNK and Src pathways. NIH3T3 cells were serum-starved overnight in 0.1% BSA and then pretreated for 1 h with DMSO or inhibitors SB203580 10 μM (A), SP600125 10 μM (B) and SU6656 2 μM (C), and then stimulated for indicated time periods with PDGF-BB (20 ng/ml); total cell lysates (TCL) were prepared and subjected to immunoblotting (lb).

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Mouse NR4A1 antibody (#554088) was purchased from BD Biosciences. Antibodies against human NR4A1 (#3960), phosphorylated Erk1/2 (#9106), phosphorylated AKT (#9271), phosphorylated Erk5 (#3371) and total Erk5 (#3372) were purchased from Cell Signaling Technology (Beverly, MA). a-Tubulin antibody was purchased from Sigma (St. Louis, MI). A rabbit antiserum recognizing Erk was raised against a peptide corresponding to the carboxyl-terminal sequence EETARFQPYR conjugated to KLH. A rabbit polyclonal antiserum against PDGFRβ was raised against a glutathione S-transferase fusion protein containing the COOH-terminal amino acid residues of PDGFRβ [39].

Preparation of cytoplasmic and nuclear fractions

Cells were washed with ice-cold PBS twice, scraped in lysis buffer containing 10 mM MES, pH 6.2, 1.5 mM MgCl2, 10 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, and 1% Triton X-100, supplemented with protease inhibitors (1 mM Pefabloc, 1% Trasylol, 1 mM sodium orthovanadate). After centrifugation at 3000 x g, the supernatant was collected as the cytoplasmic fraction.

Cell viability assay

Proliferation was evaluated in NIH3T3 cells. Ten thousand cells were plated in 96-well plates, serum-starved overnight and treated as indicated for 24 h. The assay was performed using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to manufacturer’s instructions. 20 µl/well of the CellTiter 96 AQueous One Solution Reagent containing tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES) were added to 100 µl of medium. The MTS tetrazolium compound is bioreduced by metabolically active cells into a colored formazan product that is soluble in tissue culture medium. After 3 h at 37°C in a humidified 5% CO2 atmosphere, the absorbance at 490 nm was recorded with an ELISA plate reader.

MTS proliferation assays were validated by manual counting of cells visualised with proliferation marker Ki-67 and nuclear marker DAPI. NIH3T3 cells grown on glass cover slips were fixed with washed in PBS, fixed in 100% acetone for 10 minutes. The cells were then incubated with Protein block Serum free solution (Dako) for 2 h. The cover slips were incubated overnight at 4°C in a primary rabbit anti-Ki-67 antibody solution (Cell Signaling; 1:400), washed and incubated with a secondary Alexa-Fluor594 donkey anti-rabbit antibody (Life technology; 1:1000) for 1 h at room temperature. After washing cover slips were mounted in DAPI-containing mountant (Vector Laboratories) and fluorescence was visualized under a microscope.

Apoptosis assay

Subconfluent cell cultures were serum-starved and then incubated, in triplicates, for 48 h with or without 20 ng/ml of PDGF-BB; cells cultured in growth media were used as negative controls. The pellet, enriched in nuclear proteins, was washed twice in lysis buffer supplemented with 1% NP-40 and then lysed in buffer containing 0.5% Triton X-100, 25 mM Tris-HCl, pH 10.5, 1 mM EDTA, 0.5 M NaCl, 5 mM β-mercaptoethanol, and supplemented with protease inhibitors. The supernatants from the two fractions were collected by centrifugation at 15,000 x g for 30 min.
control. Cells were harvested, and the extent of apoptosis was determined by quantification of nucleosomes released into the cytoplasm using the Cell Death Detection ELISA Plus kit (Roche Applied Science) according to the manufacturer's instructions.

Cell migration assays

Ninety-six-well ChemoTX (Neuroprobe, Gaithersburg, MD) cell migration microplate filters were coated with 50 μg/ml human fibronectin (#354008, BD Biosciences) for 1 h at room temperature. Cells were transfected with control or NR4A1 siRNA for 48 h, serum-starved overnight and then trypsinized into single cells. The wells of the ChemoTX microplate were filled with DMEM containing the indicated PDGF-BB concentrations. The filters were placed over the wells and 5 × 10^4 cells were added on top of each filter. The chamber was incubated for 4 h at 37°C, 5%
CO₂. Non-migratory cells on the upper membrane surface were mechanically removed, and cells that had moved through the pores and adhered to the bottom of the filter were fixed by 3 min incubation in 96% ethanol, Giemsa (Sigma) stained and quantified by a CCD camera (Fuji). Quantifications were performed using Quantity One software.

Soft agar colony formation assay
The bottom layer of a 6-well plate was prepared by pouring 800 µL of medium containing 0.8% low-melting-temperature agarose (Seaplaque), 3% serum, 100 µg/ml penicillin and 100 µg/ml streptomycin into each well, whereafter the agar was allowed to solidify. Cells were trypsinized and 2 × 10⁴ cells/ml were resuspended in medium containing 0.3% low-melting-temperature agarose, 3% serum, 100 µg/ml penicillin and streptomycin; 400 µL of this solution was poured as a top layer in each well. The cells were cultured for 5 days at 37 °C, 5% CO₂ in presence or absence of 50 ng/ml PDGF-BB and, after 10 days, the number and size of colonies were monitored manually using an Axiovert 40 CFL microscope.

Results

PDGF-BB induces NR4A1 expression through Erk1/2 and Erk5 MAP kinases and NF-κB
Previous studies have implicated MAP kinases in the regulation of NR4A1 expression [25] [40] [41], and we originally identified NR4A1 as a gene requiring Erk5 for its expression in response to PDGF-BB stimulation in a microarray analysis (unpublished data) comparing Erk5⁻/⁻ mouse embryonic fibroblasts transduced with empty virus or reconstituted with Erk5 [42]. To investigate the effect of the Erk5 MAP-kinase downstream of the PDGFR on the induction of NR4A1, we utilized the low molecular weight Erk5 inhibitor BIX02189. Treating cells with BIX02189 led to a decreased expression of NR4A1 after 1 h of PDGF-BB stimulation (Figure 1A). Since we saw a slight inhibition also of Erk1/2 phosphorylation, we also used a siRNA targeting Erk5; in cells where Erk5 was silenced, NR4A1 induction was delayed (Figure 1B and C). Inhibition of Erk1/2 MAP kinase by the MEK1/2 inhibitor CI-1040 repressed NR4A1 expression, and the combination of Erk5 siRNA and CI-1040 had the strongest effect with almost complete suppression of NR4A1 mRNA (Figure 1B). To verify that the changes observed on the mRNA level also translated to protein levels, we performed immunoblotting against NR4A1 in the absence of PDGF-BB stimulation in NIH3T3 cells NR4A1 is localized both in the cytoplasmic and in the nuclear compartments suggests a complex regulation of the proliferation-apoptosis balance and that integration of other pathways may determine the final outcome.

NR4A1 knock-down in NIH3T3 cells increases PDGF-BB-induced proliferation without affecting survival or migration
Previous reports have indicated roles for NR4A1 in survival, proliferation and cell migration [23] [45] [46]. To investigate the functional role of NR4A1 in PDGF-BB-stimulated cells, we silenced NR4A1 expression and performed functional assays. We observed that cells depleted of NR4A1 display an increased proliferation rate measured by MTS assay (Figure 5A). To rule out the possibility that the difference observed in the MTS assay was due to an increased metabolic activity, we validated the data by immunofluorescence; silencing of NR4A1 led to an increased number of cells positive for the proliferation marker Ki-67 (data not shown).

To elucidate the chemotactic response to PDGF-BB, we evaluated transwell migration of NR4A1 knock-down cells. We did not find any significant change in chemotaxis toward PDGF-BB in cells depleted of NR4A1 compared to control cells (Figure 5B).

There are reports claiming that NR4A1 may interact with proteins in the mitochodria and thereby promote apoptosis [15] [16] [17] [47], however, we did not observe any change in the ability of PDGF-BB to protect cells from serum starvation-induced apoptosis when comparing control cells to those with reduced NR4A1 expression (Figure 5C). In addition, we did not find any alteration of apoptosis in the serum-starved cells depleted of NR4A1 in the absence of PDGF-BB.

NR4A1 expression is important for the ability of PDGF-BB to promote anchorage-independent growth in soft agar
NR4A1 has been found to be overexpressed in many tumors. We therefore evaluated the role of NR4A1 for in vitro tumorigenicity, i.e. colony formation in soft agar. NIH3T3 cells do not form colonies in soft agar in the absence of PDGF-BB, however, PDGF-BB stimulation induced colony formation in soft agar and the effect was enhanced after overexpression of NR4A1 (Figure 5D). We further treated cells with the proteasomal inhibitor MG132 that blocks the degradation of, among other things, the NF-κB inhibitor protein IkB, and measured PDGF-BB-induced NR4A1. A strong suppression of both NR4A1 mRNA and protein levels was seen after proteasomal inhibition (Data not shown).

In summary, Erk5, Erk1/2 and NF-κB contributed to PDGF-BB-induced increase in NR4A1 expression, whereas inhibition of other kinases activated by PDGFR, i.e. p38, JNK and Src, did not decrease NR4A1 protein expression.

NR4A1 localizes to both cytoplasm and nucleus in PDGF-BB stimulated cells
It has been shown that the function of NR4A1 depends on its subcellular localization; nuclear NR4A1 regulates cell proliferation, whereas cytoplasmic NR4A1 affects survival [9] [43] [44]. To elucidate the localization of NR4A1 in cells treated with PDGF-BB, we performed biochemical nuclear and cytoplasmic fractionation and analyzed the samples to immunoblotting for NR4A1. We found that the majority of NR4A1 appeared in the cytoplasm, whereas a fraction was translocated to the nucleus after continued PDGF-BB treatment (Figure 4). The observation that in PDGF-BB-stimulated NIH3T3 cells NR4A1 is localized both in the cytoplasmic and in the nuclear compartments suggests a complex regulation of the proliferation-apoptosis balance and that integration of other pathways may determine the final outcome.
A strong enhancement of PDGF-BB stimulated colony growth was seen by overexpression of NR4A1 also in the glioblastoma cell line U-251MG (Figure 6B) and U-105MG exhibits a similar trend (Figure 6C). The U-251MG cells formed large colonies in soft agar, therefore we reported only those with a diameter larger than 100 μm.

Discussion

In the present study, we found that PDGF-BB stimulation of NIH3T3 cells resulted in a robust upregulation of NR4A1 mRNA and protein. In addition, the other two NR4A family members NR4A2 and NR4A3 were also upregulated, at least at the mRNA level (Figure S1). Furthermore, we found that NR4A1 expression relied to a large extent on Erk1/2 and NF-κB signaling, whereas Erk5 contributed to the rapid kinetics of upregulation. Initially the newly synthesized NR4A1 had a cytoplasmic localization, whereas prolonged PDGF-BB treatment dispatched part of the NR4A1 population to the nucleus. Functionally, we observed that NR4A1 downregulation increased proliferation promoted by mitogenic factors, such as PDGF-BB and serum, both using MTS assay and Ki-67 expression, whereas no effects on chemotaxis or apoptosis of NIH3T3 fibroblasts were observed. However, PDGF-BB-driven soft agar colony formation of NIH3T3 and glioma cells was strongly enhanced by NR4A1 expression.

The central role of Erk1/2 and NF-κB for NR4A1 induction that emerged from our work is consistent with previous studies that implicated MAP-kinases or NF-κB signaling in regulation of expression of NR4A family members [25] [40] [41] [48] [49] [50] [51]. It is possible that activation of PDGFReceptors, via activation of Erk1/2 and Erk5, promotes the nuclear localization of NF-κB, which increases the transcription of NR4A1. The finding that in amyloid plaques Erk1/2 is activated and directly interacts with 1kB kinase promoting NF-κB activation [52], is consistent with this possibility.

Consistent with studies in smooth muscle cells [53] [54], we found that NR4A1 reduces proliferation. However, in other studies, e.g. using endothelial cells and lung cancer cells, NR4A1 promotes cell proliferation [34] [25] [30]. It is possible that the different effects of NR4A1 on cell proliferation are related to the balance between nuclear and cytoplasmatic localization of NR4A1 which may influence its function, as well as the presence of other pathways integrating with NR4A1 in regulating these processes.

NR4A1 promotes mesenchymal stromal cell migration [55]. Since PDGF-BB has a robust chemotactic effect in fibroblasts, we investigated whether manipulation of NR4A1 expression affected cell migration. We did not see any effect of NR4A1 silence on PDGF-BB-induced cell migration. Similarly, we did not observe any NR4A1-dependence in the ability of PDGF-BB to protect cells against serum-induced apoptosis, despite several studies pointing to a critical role for NR4A1 in regulating cell survival [56] [57]. One possible reason for the lack of effect in NIH3T3 cells is redundancy with the two other NR4A family members NR4A2 and NR4A3, both of which, at least at the mRNA level, are induced by PDGF-BB stimulation in NIH3T3 cells (Figure S1), and may overlap in function with NR4A1.

Overexpression of NR4A1 has been seen in several types of tumors and NR4A1 overexpression can protect cells from apoptosis [17] [56]. The effect of NR4A1 on apoptosis is context-dependent since in other systems, such as TCR-mediated apoptosis, NR4A1 promotes this process [18] [19]. It is possible that the subcellular localization of NR4A1 impacts its effect on cell survival. Consistent with the observation that NR4A1 is overexpressed in tumor cells, we show that NR4A1 expression is essential for glioblastoma cell colony formation in soft agar. This is consistent with the observation that in different tumor types, including glioblastoma, migration and invasion are associated with Erk1/2 activation and NR4A1 expression [58] [59].

This study clarifies the regulation of NR4A1 in NIH3T3 and demonstrates its role in PDGF-BB-mediated cell transformation both in NIH3T3 and in glioblastoma-derived cell lines; our findings suggest that NR4A1 may be a target in cancer treatment.

Supporting Information

Figure S1 NR4A2 (Nurr1) and NR4A3 (NOR-1) mRNA are induced by PDGF-BB. NIH3T3 cells were treated with Erk5 siRNA or control siRNA and then stimulated by PDGF-BB (20 ng/ml) for indicated time periods. NR4A2 (A) and NR4A3 (B) mRNA levels were measured by quantitative RT-PCR. (TIF)

Author Contributions

Conceived and designed the experiments: GE. JL. CHH. Performed the experiments: GE. NP. Analyzed the data: GE. NP. JL. CHH. Contributed to the writing of the manuscript: GE. JL. CHH.

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