

Celecoxib induces adipogenic differentiation of hemangioma-derived mesenchymal stem cells through the PPAR- γ pathway *in vitro* and *in vivo*

YUAN WANG, LIANGLIANG KONG, BUHAO SUN, JIE CUI and WEIMIN SHEN

Department of Burns and Plastic Surgery, Children's Hospital of Nanjing Medical University, Nanjing, Jiangsu 210008, P.R. China

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Abstract. Infantile hemangioma (IH) is a benign tumor that produces a permanent scar or a mass of fibro-fatty tissue after involution in 40-80% of cases. Celecoxib is an inhibitor of cyclooxygenase-2 (COX-2), and can inhibit angiogenesis and fibrosis. The present study aimed to clarify whether celecoxib is able to induce tumor regression with minimal side effects. For that purpose, the regulation of celecoxib in the involution of IH was investigated in an IH model. Hemangioma-derived mesenchymal stem cells (Hem-MSCs) were isolated from proliferating specimens, and an IH model was established by injecting these cells into nude mice. Celecoxib was administered *in vitro* and *in vivo*. Oil Red O staining and reverse transcription-quantitative-PCR were used to detect the adipogenic differentiation of Hem-MSCs. Histologic analysis and immunohistochemical staining of the tumor xenografts were performed to investigate the pathological evolution of the tumor. The results showed that celecoxib inhibited the proliferation and induced the adipogenic differentiation of Hem-MSCs *in vitro*. *In vivo*, adipocytes were only present in the celecoxib group at week 4, while a larger number of fibroblasts and collagenous fibers could be observed in the basic fibroblast growth factor group. Therefore, celecoxib may be a potential agent used for IH treatment by inducing adipogenesis and inhibiting fibroblast formation.

Introduction

Infantile hemangioma (IH) is the most common benign tumor among children, and occurs in 1-12% of infants worldwide (1). There are three characteristic stages in this

disease, including the proliferating, involuting and involuted periods (2). The possible complications of IH include immediate side effects such as pain, purpura, edema, ulceration and crusting, as well as long-lasting complications such as hyperpigmentation, hypopigmentation, and atrophic or hypertrophic scarring (3). However, no specific treatment has been proposed for IH thus far, since the mechanisms behind the initiation and spontaneous regression of the tumor are unknown.

The isolation of hemangioma-derived mesenchymal stem cells (Hem-MSCs) has indicated that IH may originate from multipotential stem cells and may differentiate into endothelial cells, pericytes, adipocytes and fibroblasts (4). Adipocytes and fibroblasts are closely associated with the resident fibro-fatty tissues after the involution of the tumor. Multiple pathways are involved in this pathogenesis, including the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF) and peroxisome proliferator-activated receptor- γ (PPAR- γ) signaling pathways (5).

Celecoxib, a non-steroidal anti-inflammatory drug, has attracted considerable attention due to its preventive role in numerous cancer types (6). As a selective inhibitor of cyclooxygenase-2 (COX-2), it can inhibit angiogenesis by reducing the production of prostaglandin E2 (PGE2), which promotes tumor progression through different mechanisms (7). The potential mechanisms behind its action may be the inactivation of the VEGF signaling pathway, which is highly expressed in multiple types of cancer and is closely associated with angiogenesis (8). In addition, celecoxib can reduce fibroblast proliferation and decrease collagen expression (9). COX-2 inhibition can reduce the overall deposition of collagen in tumors and increase tendon healing (10,11).

Therefore, it was hypothesized that celecoxib can inhibit cell proliferation, induce adipogenesis and reduce fibroblast formation in IH. The present study was undertaken to investigate the effect of celecoxib on the pathogenesis of IH in an IH mouse model.

Materials and methods

Ethics approval. The present study was approved by the Ethics Board of The Children's Hospital of Nanjing Medical University

Correspondence to: Dr Weimin Shen, Department of Burns and Plastic Surgery, Children's Hospital of Nanjing Medical University, 72 Guangzhou Road, Nanjing, Jiangsu 210008, P.R. China
E-mail: swmswmswm@sina.com

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(China) (approval no. 201705097-1). Written informed consent was obtained regarding the handling of samples from the parents according to the Declaration of Helsinki. The experimental animal protocol was reviewed and approved by the Animal Ethics and Welfare Committee of Nanjing Medical University (China) (approval no. IACUC-1902023).

Preparation of hemangioma specimens. Five fresh IH samples from different patients in the proliferating phase were obtained between March 2019 to May 2019 from The Children's Hospital in Nanjing, China, under a protocol for human subjects that was approved by the Committee on Clinical Investigation of Nanjing Medical University (approval no. 201705097-1). Tissues were immediately used for cell isolation of Hem-MESs. The postoperative pathological evaluation of IH samples showed proliferative IH (CD34⁺/CD31⁺/SMA⁺/Glut-1⁺/D2-40).

Hem-MSC isolation and culture. Hem-MSCs were isolated from clinically resected specimens as previously described, with certain modifications (12). Briefly, the hemangioma samples were rinsed in PBS, cut into small pieces (1x1x1 mm³) and dispersed on the surface of a plastic culture dish. The cells were cultured in Dulbecco's modified Eagle's medium-low glucose (DMEM-L; HyClone; Cytiva), supplemented with 10% fetal bovine serum (FBS) (HyClone; Cytiva), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) for 4 days (with 5% CO₂ at 37°C). Next, the medium was changed every other day, and the cells were passaged when reaching 90% confluency. Generation 3-5 were used for the experiments. MSCs from all 5 specimens were used for the experiments as the amount of MSCs between generation 3-5 was limited. Cells were labeled with FITC- or phycoerythrin (PE)-conjugated isotype-matched control antibodies, FITC-conjugated antibodies against human CD45 (eBioscienceTM, cat. # 11-0459-42) and CD90 (eBioscienceTM), and/or PE-conjugated antibodies against human CD34 (eBioscienceTM, cat. # 12-0349-42) and CD105 (eBioscienceTM, cat. # 12-1057-42) (Thermo Fisher Scientific, Inc.). Samples were analyzed on a FACSVantage SE flow cytometer (BD Pharmingen; BD Biosciences) and analyzed with WinMDI software (v2.9).

In vitro cell proliferation assay with celecoxib. Celecoxib was purchased from Pfizer, Inc. and was dissolved in 100% dimethyl sulfoxide (DMSO), and then diluted with DMEM-L for subsequent experiments. The final concentration of DMSO for all treatments was maintained below 0.1%.

Hem-MSCs were counted by Neubauer Chamber under a microscope (Olympus, magnification x100) and then seeded into a 96-well plate at a concentration of 10⁴ cells per well. Next, the cells were cultured with different concentrations of b-FGF (0.1-0.0001 ng/ml; Peprotech[®]) and celecoxib (0.1-1,000 µg/ml) for 24 h. Subsequently, the culture medium was replaced with fresh medium containing 10 µl CCK-8 solution (Dojindo). After a 2-h incubation, the absorbance at 450 nm was detected by ELISA using a microplate reader to assess the cell counts. The data were analyzed by SPSS 21 (IBM Corp.).

In vitro adipogenic differentiation. Hem-MSCs were plated at the same density (10⁶/ml) in separate dishes and divided into four groups: (A) control group; (B) adipogenic induction group; (C) celecoxib group; (D) adipogenic induction + celecoxib group. After 24 h, the cells were induced to differentiate into adipocytes in an adipogenic induction medium for human bone marrow MSCs (BM-MSCs) (HUXMD-90031; Cyagen Biosciences). The medium was changed every 3 days. After induction for 7 or 14 days, the cells were stained with Oil Red O (O8010; Solarbio) to detect the adipocytes.

Gene expression was analyzed by reverse transcription-quantitative PCR (RT-qPCR). Total RNA from cells in the different groups was isolated and reverse transcribed into cDNA as previously described (13). According to the manufacturer's instruction, total RNA was extracted by Trizol total RNA isolation kit (TaKaRa Biotechnology). The reverse transcription was performed using PrimeScriptTM Reverse Transcriptase (TaKaRa Biotechnology). qPCR was carried out with SYBR Green Master Mix (Vazyme Biotech Co., Ltd.) on a QuantStudio 3 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling program consisted of a preliminary denaturation (95°C for 10 min), followed by 40 cycles (95°C for 15 sec and 60°C for 1 min). The relative mRNA levels were normalized to the levels of β-actin and calculated using the comparative cycle threshold (ΔΔCq) method (14). The primers used in qPCR were as follows: human-COX-2, forward 5'-ATGCTGACT ATGGCTACAAAAGC-3' and reverse 5'-TCGGGCAATCAT CAGGCAC-3'; human-PPAR-γ, forward 5'-GGGATCAGC TCCGTGGATCT-3' and reverse 5'-TGCACTTTGGTACTC TTGAAGTT-3'; human-CCAAT-enhancer-binding protein (CEBP)α forward 5'-AGGAGGATGAAGCCAAGCAGCT-3' and reverse 5'-AGTGC GCGATCTGGA ACTGCAG-3' and β-actin forward 5'-AGCGAGCATCCCCCAAAGTT-3' and reverse 5'-CGGCACGAAGGCTCATCATT-3'.

In vivo treatment with b-FGF and celecoxib. A total of 27 specific-pathogen-free, 6-week-old, male, BALB/C-nu/nu mice (16±2 g) were purchased from Nanjing Medical University (China). The mice were raised under controlled 12-h light-dark cycles, with a constant temperature (22-24°C) and humidity (55-60%) in a pathogen-free animal research center at Nanjing Medical University (Nanjing, China). The animals had continuous free access to sterilized food (γ-ray-irradiated food) and autoclaved water.

After a 1-week acclimation, the experiments were initiated. The mice were randomly separated into three groups: control, b-FGF and celecoxib groups. To generate xenograft tumors, Hem-MSCs were trypsinized, washed and re-suspended in Corning Matrigel (354277; Corning Life Sciences). Next, 1x10⁷ cells in 0.3 µl Matrigel were inoculated subcutaneously into the back of each mouse as previously described (15). In the b-FGF group, the cells were treated with 0.01 ng/ml b-FGF for 24 h before injection, while in the celecoxib treatment group, celecoxib (0.1 mg/g/day) was administered to the mice via oral intake for 4 weeks (16). The animal health and behavior were monitored every day. At the end of weeks 1, 2 and 4, three mice in each group were sacrificed and their tumors were harvested (3x3x3=27). The maximum allowed tumor dimension was ~1 cm. The mice were sacrificed by

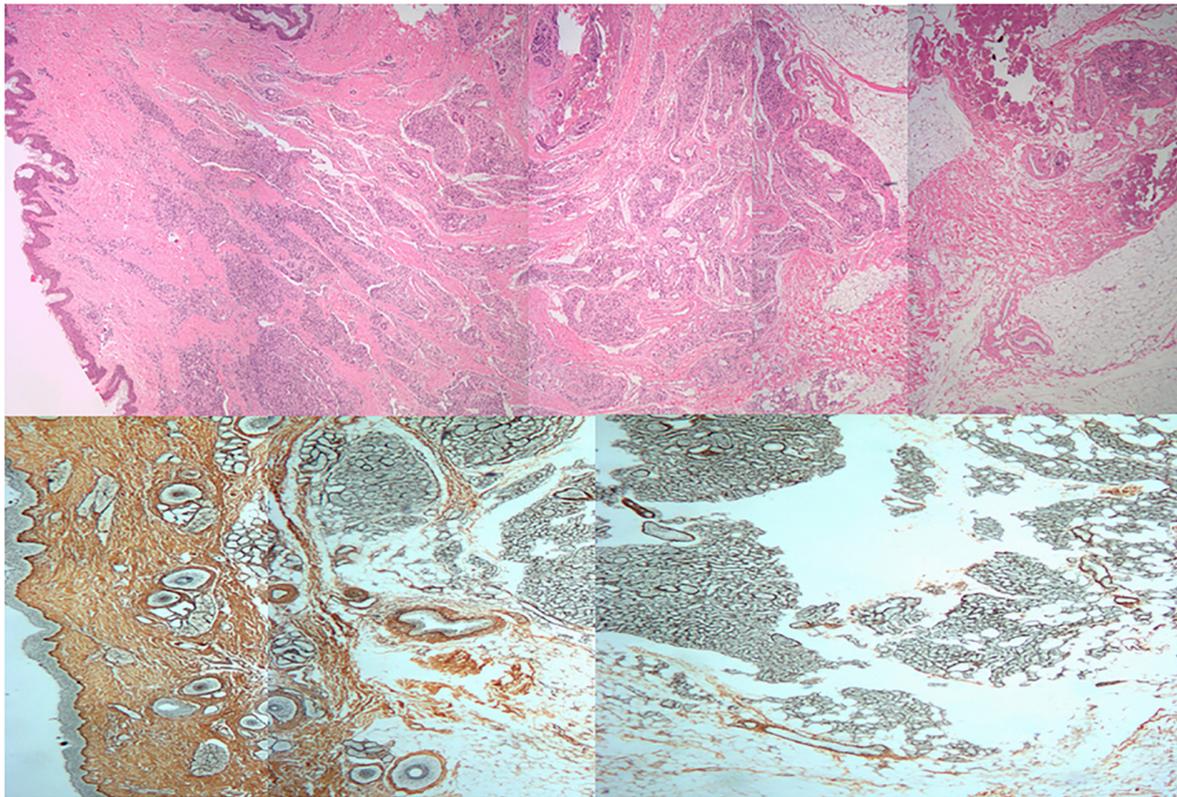


Figure 1. Hematoxylin and eosin (H&E) staining (top panel) and ammoniacal silver staining (bottom panel) of proliferative infantile hemangioma (magnification, x40). The proliferative phase is highly cellular with immature vessels. Capillaries are separated by fibers in which the number of collagenous fibers (yellow) is higher than that of reticular fibers (black).

injection of pentobarbital sodium (100 mg/kg). The death of the mice was indicated with no heart beat and breath detected after 10 min.

Histopathological analysis. Tumors were isolated from the sacrificed mice and immediately fixed in 10% buffered formalin for histopathology processing. Tissue samples were embedded in paraffin and 5- μ m sections were cut using a manual microtome. Sections were stained with hematoxylin and eosin (H&E). Silver staining (SBJ-0285; Gorden-Sweet protocol) was used to observe the fibers in the tumor as previously described (17).

Immunohistochemical analysis. Immunohistochemical staining with anti-fibroblast antibodies was performed to evaluate expression patterns of fibroblasts in the tumor. After retrieval by citric acid (10 mM citrate buffer, pH 6.0) (eBioscience; Thermo Fisher Scientific, Inc.) in a microwave, tissue sections from the IH model mice were incubated with a primary antibody against S100 calcium binding protein (dilution 1:300 in 10% normal goat serum) (Abcam, cat. no. ab242659), a marker of fibroblasts (18), overnight at 4°C. Next, the sections were incubated with an anti-rabbit IgG (biotin, cat. no. a0516, dilution 1:500) antibody, and detected using DAB with hematoxylin counterstaining. Images were obtained by microscopy (Olympus, x100 magnification).

Statistical analysis. Statistical analysis was performed using GraphPad 7.0 statistical software (GraphPad Software, Inc.).

Differences between two groups were evaluated using the Student's t-test. A value of $P < 0.05$ was considered significant.

Results

Histopathology of IH. Histopathological examination in the present study revealed that the proliferative phase IH is composed of well-defined, unencapsulated masses of capillaries lined by plump endothelial cells. These lesional capillaries are separated by fibers and collagen. H&E and ammoniacal silver staining showed that proliferative-phase IHs are complex cellular mixtures with a large number of collagen (Fig. 1).

Hem-MSCs isolated from clinically resected specimens were spindle-shaped, tightly packed and evenly distributed under an inverted microscope at x100 magnification. Flow cytometric analyses showed that these cells expressed the mesenchymal cell markers CD90 and CD103, but did not express markers for endothelial cells, such as CD34 or CD45 (Fig. 2). The cell-surface markers on Hem-MSCs indicate a mesenchymal cell phenotype.

Celecoxib inhibits cell proliferation. As shown in Fig. 3, the results of Cell Counting Kit-8 assay revealed that b-FGF significantly promoted Hem-MSC cell proliferation with a relatively low cell toxicity at a concentration of 0.01 ng/ml. Celecoxib had a cytotoxic effect on Hem-MSCs and inhibited cell proliferation at various concentrations. When the concentration of celecoxib was lower than 0.1 mg/ml, the difference

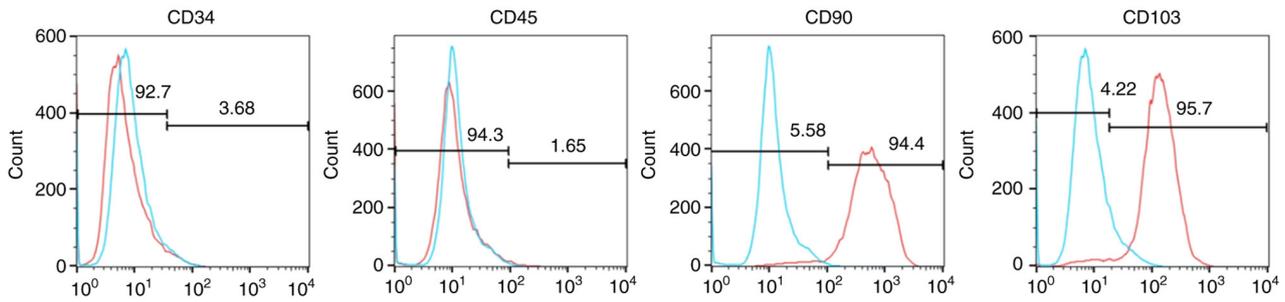


Figure 2. Hemangioma-derived mesenchymal stem cells (Hem-MSCs) express CD103 and CD90, but do not express CD34 or CD45, according to flow cytometry.

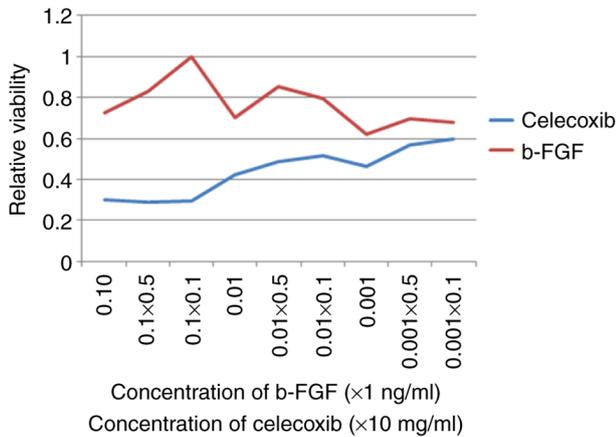


Figure 3. Treatment of hemangioma-derived mesenchymal stem cells (Hem-MSCs) with different concentrations of basic fibroblast growth factor (b-FGF) and celecoxib. When treated with b-FGF, the relative viability of the Hem-MSCs reached a peak at the concentration of 0.01 ng/ml. The cytotoxicity of celecoxib decreased with decreasing doses, and remained above that of the control at the lowest concentration.

in viability at the different concentrations was not very large. Thus, we selected 0.1 mg/ml for use in the following *in vitro* experiments. *In vivo*, the celecoxib was received via oral intake as previously described (16).

Celecoxib induces adipogenesis. *In vitro*, lipid droplets stained with Oil Red O were apparent on day 7 (week 1), and continued to accumulate through day 14 (week 2) in the celecoxib group (Fig. 4C) and the two induction groups [adipogenic induction group (Fig. 4B) and the adipogenic induction + celecoxib group (Fig. 4D)] when compared to the control group (Fig. 4A). Celecoxib was able to induce the formation of lipid drops without induction medium.

The expression levels of genes involved in adipogenic differentiation [cyclooxygenase-2 (COX-2), human-CCAAT-enhancer-binding protein (CEBP) α , and peroxisome proliferator-activated receptor- γ (PPAR- γ)] were increased in the celecoxib group (group C) (Fig. 5). Nude mouse models of IH were constructed successfully. H&E staining showed that, in the celecoxib group, a large number of adipocytes appeared in the plugs on week 4, while these fat-like tissues were rare in the other groups (Fig. 6). On weeks 1 and 2, no lipid droplet was observed in any of the xenografts, while lipid droplets could only be observed in the celecoxib group on week 4. This suggests that

celecoxib induced the adipogenic differentiation of Hem-MSCs. In addition, since part of the mass was adipose tissue, fewer collagen fibers were observed in the xenograft tumors.

Celecoxib reduces the number of fibroblasts and collagen fibers. As revealed by H&E staining, the transplanted tissue in all groups developed an increased number of collagen fibers surrounding the microvasculature. In the b-FGF group, the expression of collagen was greater than that of the control and celecoxib groups on the same week (Fig. 6).

Ammoniacal silver staining revealed the presence of fibers of various sizes. On week 1, the reticular fibers stained dark, and collagenous fibers, appearing yellow or brown in color, were present in all the groups. The number of fibers increased over time, reaching its peak on week 4. Compared with the findings in other groups, the b-FGF group had the largest number of fibers, particularly collagenous fibers, while the number of collagenous fibers in the other two groups showed no apparent difference (Fig. 7).

The number of fibroblasts was the largest in the b-FGF group compared with that in the other two groups according to the results of immunohistochemical analysis (Fig. 8). This is consistent with the presence of fibers in the different groups.

Discussion

The present results showed that celecoxib can inhibit the proliferation and induce the adipogenic differentiation of hemangioma-derived mesenchymal stem cells (Hem-MSCs) *in vitro* and *in vivo*. There may be two events involved in the self-regression of infantile hemangioma (IH), including apoptosis (19) and adipogenesis (20). This process is accompanied by tissue repair mediated by fibroblasts. Finally, the lesion may be replaced by fibro-fatty tissue. The present study focused on adipogenesis and cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE2) signaling, and investigated the effect of celecoxib on the involution of IH in an IH model constructed by Hem-MSCs. When treated with celecoxib, a COX-2 inhibitor, the IH model exhibited a characteristic pathological evolution.

Adipogenesis is the most essential pathological process during the involution of IH. Adipogenesis, lipid metabolism and associated factors play an important role in the survival, metastasis and invasion of numerous tumors. It has been reported that trans-differentiation of breast cancer cells into adipocytes can repress cancer metastasis, and overcome therapy resistance and cancer relapse (21). Fatty acid

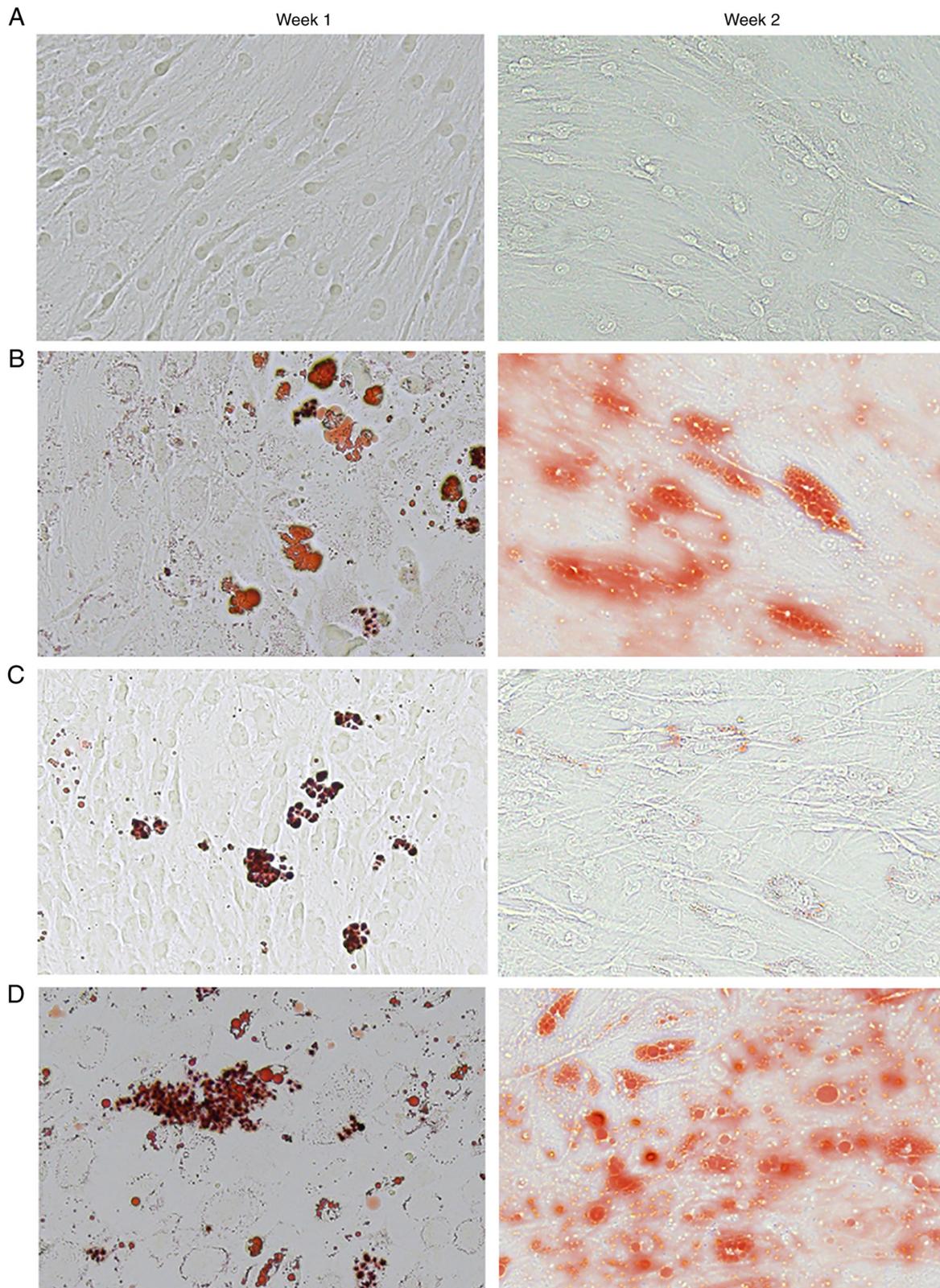


Figure 4. Adipogenic differentiation in the different groups, as revealed by the accumulation of cytoplasmic lipid vacuoles during 2 weeks (magnification, x400). (A) Oil Red O staining demonstrated absence of adipose droplets in the control group. Fewer adipose droplets were observed in the (C) celecoxib group compared with those in the (B) adipogenic induction group and the (D) adipogenic induction + celecoxib group. There was no obvious difference in the number of adipose droplets between panels B and D.

transport protein (FATP) is associated with tumor immunosuppression. Inhibition of FATP2 suppresses the progression of tumors through the reduction in arachidonic acid and

PGE2 levels (22). The effect of a COX-2 inhibitor on adipogenesis and expression of associated factors has been studied in non-alcoholic steatohepatitis (23), diabetes (24), breast

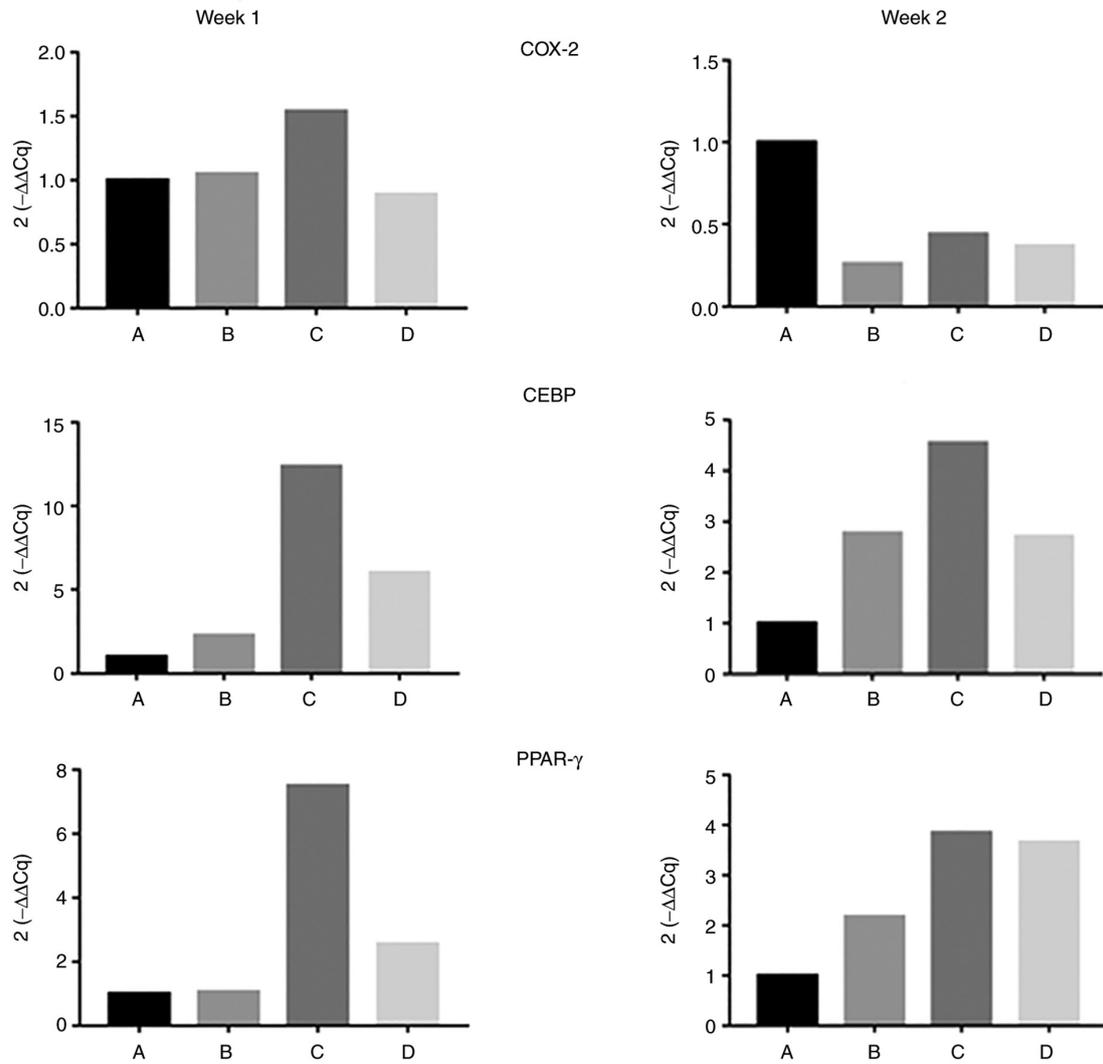


Figure 5. Expression of genes involved in adipogenic differentiation. Reverse transcription-quantitative PCR shows that cyclooxygenase-2 (COX-2) gene expression was similar among all groups on week 1, and was decreased in all groups on week 2, with the exception of the control group. CCAAT-enhancer-binding protein (CEBP) and proliferator-activated receptor (PPAR- γ) gene expression levels were highest in the celecoxib group compared with that in other groups, and particularly statistically significant on week 1. Groups: A, control group; B, adipogenic induction group; C, celecoxib group; D, adipogenic induction + celecoxib group.

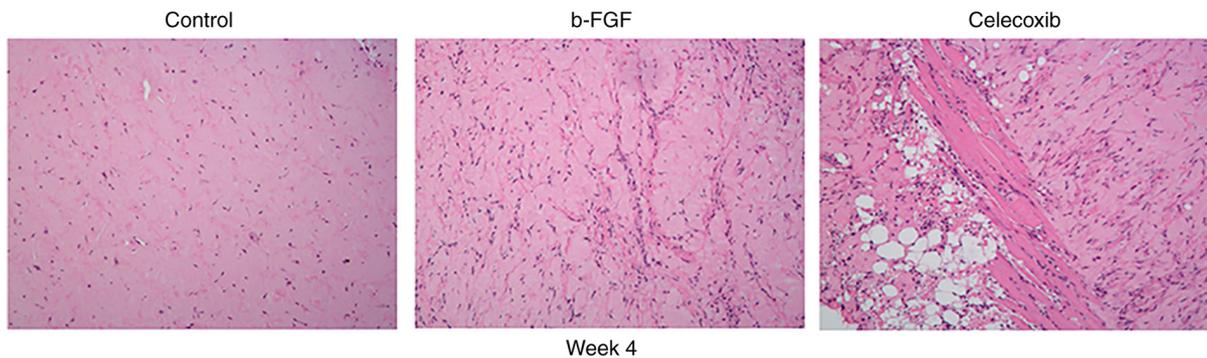


Figure 6. Hematoxylin and eosin (H&E) staining of the implanted tumors (magnification, $\times 100$). The number of microvessels in the basic fibroblast growth factor (b-FGF) group was shown to be higher than that in the other two groups. Adipose tissue was only observed in the celecoxib group on week 4.

cancer (21) and melanoma (25). Previous results showed that a COX-2 inhibitor can induce peroxisome proliferator-activated receptor- γ (PPAR- γ) expression and adipogenesis. Elevated

PPAR- γ level can inhibit the production of 15-deoxy- δ 12,14-prostaglandin J2 (26), a PPAR- γ agonist, by inhibiting COX-2 and preventing inflammation. Based on these studies

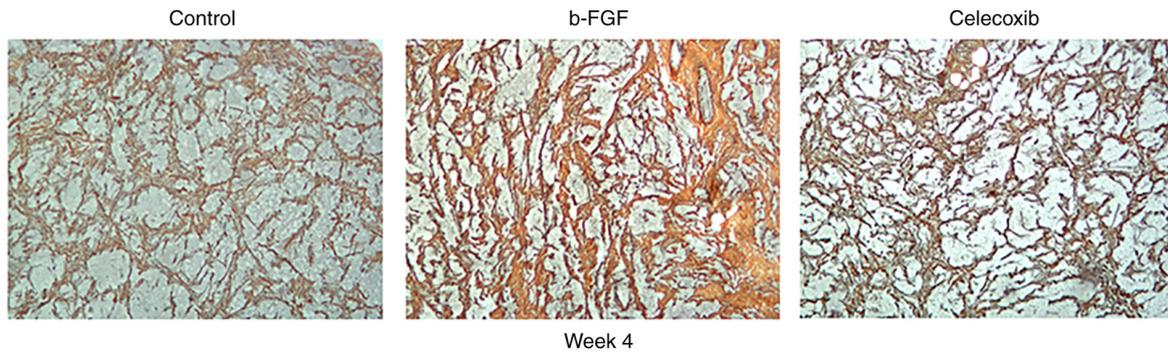


Figure 7. Ammoniacal silver staining of the implanted tumor (magnification, x100). The expression of collagenous fibers (yellow) in the basic fibroblast growth factor (b-FGF) group was greater than that in the other two groups on the same week. The expression of collagenous fibers (yellow) was highest on week 4.

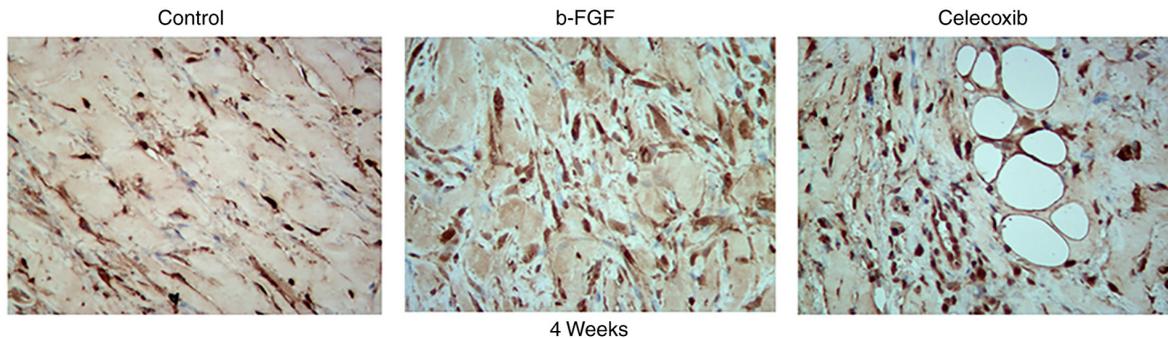


Figure 8. Immunohistochemical staining of the implanted tumor (magnification, x400). The cell nuclei in fibroblasts were counterstained with an anti-S100 calcium binding protein antibody. The expression of fibroblasts in the basic fibroblast growth factor (b-FGF) group was the highest.

and the characteristics of IH, it was hypothesized that effective treatment of IH may rely on inducing adipogenesis.

The fibro-fatty accumulation after involution of IH indicates adipogenesis during this period (27). Previous research has focused on this process with the aim of identifying improved treatments for IH (28). Propranolol, a widely used drug for treating IH, was reported to promote adipogenesis, which was associated with the PPAR- γ and CEBP α pathways (29). Furthermore, rosiglitazone, one of the agonists of PPAR- γ , can promote the adipogenic differentiation of Hem-MSCs and activate the PPAR- γ signaling pathway of endothelial cells in hemangioma (12). The present study showed that celecoxib can induce the adipogenic differentiation of Hem-MSCs and increase the expression of associated genes *in vitro*. *In vivo*, apparent adipose tissue was only present in the celecoxib group, and could not be observed in the other groups. It was hypothesized that COX-2 inhibitors have the potential to induce adipogenesis, which may be associated with the PPAR- γ /CEBP signaling pathway.

The potential mechanism behind the effect of celecoxib in IH involution is more complex than simply inducing adipogenesis. It was reported that PPAR- γ agonists exert an anti-angiogenic effect by inhibiting vascular endothelial growth factor (VEGF)-induced endothelial COX-2 expression and prostaglandin production (23). Celecoxib can be used to inhibit angiogenesis through blocking VEGF. It has been reported that COX-2 promoted the angiogenesis of several cancer types by upregulating angiogenic factors, including VEGF (30), which is also the most important factor in the pathogenesis of hemangioma. Celecoxib can reduce the microvessel density

within xenografts and the growth of several tumors (31). The present results showed that celecoxib can inhibit the proliferation of Hem-MSCs *in vitro*. However, the microvessel density of the xenografts showed no difference between the celecoxib and control groups. This may be caused by the indirect oral mechanism of action of celecoxib on IH xenografts.

Other essential components of the resident tissue in involuted IH lesions are collagen and extracellular matrix (ECM). Scars appear when the repaired tissue contains excessive fibroblasts with high expression of ECM, particularly excessive synthesis and deposition of collagen (32). There are numerous factors involved in this process, including basic fibroblast growth factor (b-FGF), which has multiple functions in mitogenesis, migration, morphogenesis, angiogenesis, organ development, organ regeneration and wound healing (33). b-FGF is able to enhance cell adhesion, cell proliferation and secretion of ECM (34). The present results showed that b-FGF enhances the proliferation of Hem-MSCs *in vitro*, while, *in vivo*, b-FGF promotes collagen deposition in xenografts.

By contrast, as a non-steroidal anti-inflammatory drug, celecoxib can reduce fibrogenesis and expression of collagen (35), thus delaying all types of wound healing (36), and reducing scar formation and adhesion (37). The results of histological analysis of the present IH model showed that there was a lower number of collagen fibers in the celecoxib group. This indicated that celecoxib accentuated adipogenesis while simultaneously reducing collagen production. Celecoxib could have a potential effect on IH regression by inhibiting the differentiation and proliferation of fibroblasts. The IH model

constructed by Hem-MSCs has the potential to generate fibers (including reticular and collagenous fibers) by fibroblasts differentiated from Hem-MSCs, according to the results of ammoniacal silver and immunohistochemical staining. It was previously reported that celecoxib can inhibit the proliferation and collagen expression of fibroblasts (38), which is consistent with the present results. Cell proliferation and collagen production play important roles in cancer formation and tissue fibrosis, which is associated with IH proliferation and the residual fibro-adipose tissue. Treatment with celecoxib may reduce the possibility of a remaining scar.

In conclusion, based on the aforementioned findings, it may be hypothesized that celecoxib may be used to induce the regression of IH by promoting adipogenesis with formation of fewer fibers, which may lead to a remaining scar of the tumor. In order to verify the mechanism behind the acceleration of adipogenesis, the expression of adipogenesis-related factors and proteins following treatment with celecoxib should be evaluated in future studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YW conducted the experimental operations and manuscript writing. LK was responsible for the research design. BS conducted the experimental operations. JC was involved in drafting the work and revising it critically for important intellectual content, and made substantial contributions to the conception of the research design. WS made substantial contributions to the conception of the research design and findings. JC and WS confirm the authenticity of all the raw data. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work (as well as the data provided) are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Board of The Children's Hospital of Nanjing Medical University (China) (approval no. 201705097-1). Written informed consent was obtained regarding the handling of samples from the parents according to the Declaration of Helsinki. The experimental animal protocol was reviewed and approved by the Animal Ethics and Welfare Committee of Nanjing Medical University (China) (approval no. IACUC-1902023).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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