Identification of differentially expressed proteins involved in fetal scarless wound healing using a rat model of cleft lip

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Abstract. In early pregnancy, fetal skin wounds can heal quickly and undergo a transition period from scarless healing to scar formation. The aim of the present study was to identify potential biomarkers associated with scarless repair of cleft lips, in order to determine the intrinsic factors leading to scar formation in embryonic tissue. A stable model of cleft lip was established using microsurgery by constructing a wedge-shaped cleft lip-like defect in fetal rats at gestational age (Ga) 16.5 and Ga18.5. The Ga16.5 and Ga18.5 groups were used to model scarless healing and scar formation, respectively. The fetuses were returned to the uterus following surgery, then removed 72 h after the procedure. Macroscopic observation of the cleft defect and histological examination were carried out. Reverse transcription-quantitative (RT-qPCR) and parallel reaction monitoring (PRM) were used to detect mRNA and protein expression levels, respectively. The upper-left lip completely healed 72 h after surgery in the Ga16.5 group of fetal rats. However, this was not the case in the Ga18.5 group. Histological examination indicated new follicles visible under the epidermis of the scarless group (GA16.5). Scarring was visible on the upper-left cleft lip wound of the fetal rats in the GA18.5 group. The expression of some growth and pro-inflammatory factors, including TNF-α, were also different between two groups. Label-free quantification was used to identified differentially expressed proteins and five differentially expressed proteins (Smad4, Fabp5, S100a4, S100a8 and S100a9) were identified. The relative expression of these molecules at the mRNA and protein levels were measured using RT-qPCR and PRM. These molecules may represent potential biomarkers for the scarless repair of fetal rat cleft lip wounds.

Introduction

The cleft lip is a very common congenital oral and maxillofacial malformation, often accompanied by cleft palate and alveolar cleft. Although surgical repair techniques are continuously being improved, numerous patients still experience inevitable secondary scar formation after surgery. In recent years, with the development of prenatal diagnosis and treatment technology (1), intrauterine surgery has made it possible to correct developmental deformities, such as a cleft lip.

The concept of scarless healing was first proposed by Burrington (2) in 1971. It was later observed that fetal skin wounds that occur during early pregnancy can heal quickly and restore intact skin barrier functions. In contrast, fetal skin damage that occurs in the third trimester of pregnancy can result in the formation of scar tissue similar to that of an adult (3). Therefore, the different manifestations of scarless healing of mammalian fetal wounds are related to the gestational age of the fetus (4). Dang et al (5) and Longaker et al (6) demonstrated that this transition period from scarless healing to scar formation occurred between day 16.5 of gestational age (GA) and GA18.5 in rats and mice, which have a gestation period of ~21.5 days. Lorenz et al (7) and Cass et al (8) suggested that when 1-2 mm incisions are inflicted on fetal rats, the transition...
period of scarless healing to healing with scar formation was still between 16.5 (GA16.5) and 18.5 days (GA18.5).

This phenotypic difference in fetal wounds has inspired further examination of the specific underlying mechanisms. Initially, it was hypothesized that the reason for early scar repair was that the fetus developed in amniotic fluid, which is rich in growth factors and extracellular matrix (ECM) components (9,10). Previous studies typically utilized large animal models to study the presence of scars following repair (11,12). However, only a few studies have reported the use of a fetal rat cleft lip wound model to establish the effectiveness of surgical repair at different gestational ages. Moreover, due to the short gestation period of rats, the experimental cycle can be shortened, and the experiment can therefore be repeated.

Given the importance of this process, the present study, screened out several specific markers of early fetal scarless repair. The present study aimed to gain insight into the occurrence and mechanisms of scarless repair, and to identify new clinical targets for the prevention and treatment of scars.

Materials and methods

Animals. A total of 36 SPF-grade adult Sprague-Dawley (SD) rats (female; mean weight, 250 g; age, 12 weeks) were obtained from the Third Xiangya Hospital of Central South University Animal Experiment Center (Hunan, China) and divided into two groups that received surgery once their fetuses reached GA16.5 or GA18.5, respectively (n=18 each). The following housing conditions were implemented: a temperature between 25±2°C, relative humidity of 55±15%, ventilation rate of 10-20 times per hour, time-controlled artificial lighting (12-h day-night cycle) and ad libitum access to food and water. The experiments were supervised throughout and were performed in accordance with animal experimentation ethics.

Preliminary study on different repair modes applicable to fetal rats with artificial cleft lip wounds. Fetal rats located away from the uterine horn were selected to prevent subsequent abortion, as described previously (13). In the current study, rats were anesthetized with 30 mg/kg pentobarbital sodium intraperitoneally before surgery. A wedge-shaped cleft-like defect was created on the upper-left lip of the fetal rats. The upper-right lip did not receive any treatment and was used as a control condition. The fetal rats were then removed three days post-surgery as previously described (4) (i.e., at GA19.5 or GA21.5, respectively). All fetuses and rats were euthanized using carbon dioxide (30% volume displaced). The upper-left lip tissue samples from the GA16.5 group and the upper-right lip from the GA18.5 group were defined as group 2. In addition, the upper-left lip tissue samples from the GA18.5 group were defined as group 3, whereas the upper-right lip tissue samples from the GA18.5 group were defined as group 4. Each subgroup included 27 samples. Protein expression was compared between group 1 and 2, group 3 and 4, as well as group 5 and 1. Label-free quantification PRM was performed as previously described (17) and was used to detect the differentially expressed proteins among the different groups. MaxQuant 1.5.6 (https://www.maxquant.org) and Perseus 1.4 (https://www.maxquant.org/perseus/) were used to analyze the results of label-free quantification PRM: Volcano plots were generated for differentially expressed proteins: Y-axis, -log10(P-value); x-axis: log2(ratio). The points distributed outside the two vertical borders and above the horizontal border represented the proteins with significant differences; proteins with at least a 1.5-fold change in expression and P<0.05 were considered significant. Subsequently, bioinformatics analysis, including GO and KEGG pathway analysis, was performed to identify differentially expressed proteins (18).

Experimental verification of tissue repair proteins in fetal rats with artificial cleft lip wounds. The mRNA levels of the differentially expressed molecules were assessed using reverse transcription-quantitative (RT-q) PCR, as previously described (19). Differentially expressed levels of proteins were detected by label-free quantification PRM as previously described (17).

Statistical analysis. GraphPad Prism 8.0 (GraphPad Software, Inc.) and SPSS 22.0 (IBM Corp.) were used to perform calculations and carry out statistical analysis. Student’s t-test was used to compare differences between two groups. The experimental data from each group were analyzed for congruence of variance before the t-test were applied. The FDR values were within 0.01 in the comparisons. Mixed ANOVA followed by Sidak's post hoc test was used to analyze the differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Gross observation. All fetal rats were observed before delivery. The nasolabial cleft was first observed before surgery and images were captured to facilitate the observation of changes in the fetal rats from the GA16.5 and GA18.5 groups. We observed the same area again 72 h post-surgery to identify differences. The cuneiform tissue of the upper-left lip was removed by microsurgery to create a cleft lip wound. The changes in the fetal rats were observed macroscopically. In the GA16.5 group, the upper-left cleft lip wound completely healed 72 h after surgery (i.e., GA19.5) and the continuity of the upper lip tissue was restored. Only a slight depression was observed in the surgical area. The upper-left lip tissue was nearly symmetrical with that of the right side. However, in the GA18.5 group, the cleft lip wound was not completely healed 72 h after surgery (i.e., GA21.5); a clear scar was observed in the surgical area, and the upper lip was asymmetrical on both sides due to wound contracture (Fig. 1).
Histological analysis. In the GA16.5 group 72 h after surgery, the tissue of the upper-left lip wound demonstrated complete regeneration when observed under the microscope (Figs. 2-4). The results of H&E staining demonstrated complete epithelialization of the upper-left lip, and the structure of new follicles was detected under the epidermis. Compared with the normal skin of the upper-right lip, a slight depression in the cleft part of the upper-left lip and thickening of the skin was noted, whereas inflammatory cell infiltration and neovascularization were not apparent (Fig. 2). Masson's Trichrome staining revealed collagen fibers under the epidermis, demonstrating a fine reticular and emerging follicular structure (Fig. 3). Immunohistochemical analysis indicated no obvious difference in the amount of type-I collagen in the upper-left cleft lip area and the rest of the upper lip (Fig. 4).
In the GA18.5 group, the position of the wound was easily identified by a distinct scar on the upper lip. H&E staining demonstrated that partial epithelialization occurred in the upper-left cleft lip area. Compared with the normal skin of the upper-right lip, the upper-left lip displayed a clear scar, new capillary formation around the wound and increased fibroblast proliferation and ECM volume, whereas structural components of hair follicles were not observed under the epidermis (Fig. 5). Masson’s Trichrome staining demonstrated the absence of new follicular structure and the presence of dense collagen fibers under the epidermis (Fig. 6). Immunohistochemical analysis in the upper-left cleft lip wound demonstrated an increase in type-I collagen expression and fiber density, as well as a more compact structure and absence of adnexal skin (Fig. 7), compared with normal upper lip tissue.

Immunohistochemical analysis of cell proliferation markers was also carried out. Compared with GA16.5 fetal rats, the expression of Ki67 and CD31 slightly increased in the GA18.5 group following surgery. By contrast, the expression of CK10 decreased in the GA18.5 group, compared with the GA16.5 group (Fig. 8).

RT-qPCR analysis of inflammatory factors. The relative mRNA expression levels of the pro-inflammatory factors...
TNF-α, IL-10 and TGF-β were evaluated in the two groups of fetal rats. The mRNA levels of TNF-α and IL-10 were significantly higher in GA18.5 rats, compared with GA16.5 rats. Furthermore, the mRNA expression levels of TGF-β were significantly reduced in the GA18.5 group (Fig. 9).

Protein identification and differential protein screening. Compared with group 1, 57 differentially expressed proteins were identified in group 2, of which 37 were upregulated and 20 were downregulated. A comparison of groups 3 and 4 revealed 312 differentially expressed proteins, of which 171 were upregulated and 141 were downregulated. Lastly, compared with group 1, 1,289 differentially expressed proteins were identified in group 3, of which 151 were upregulated and 138 were downregulated. Only 50 differentially expressed proteins and their multiple variations were upregulated or downregulated between all groups (Tables I-IV). The distribution of the differentially expressed proteins among the selected samples is presented as volcano plots (Figs. 10-12).

Bioinformatics analysis. Gene ontology (GO) enrichment analysis was performed on the differentially expressed proteins, and their properties were generally described as
biological process (BP), molecular function (MF) or cellular component (CC). The first 10 GO enrichment results from each group are displayed in Fig. 13. The results demonstrated that 73, 542 and 376 differentially expressed proteins were significantly enriched between groups 1 and 2, 3 and 4 and 3 and 1, respectively. The results of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis identified the possible pathway related to the differentially expressed proteins between groups (Fig. 14).

In addition, the interaction network of the differentially expressed proteins that regulate wound repair were analyzed. Examples of the interaction networks of the differentially expressed proteins involved in wound repair are as follows:

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**Figure 7.** IHC staining 72 h after model establishment in the GA18.5 group. (A) IHC staining of the upper lip tissue. A clear depression in the upper-left lip identifies the location where the cleft lip-like defect was created. Magnification, x40. (B) Normal subepidermal structure of the upper-right lip. Magnification, x400. (C) The expression of type-I collagen in the upper-left lip is higher compared with the upper-right lip. Magnification, x400. GA, gestational age; IHC, immunohistochemistry.

**Figure 8.** Immunohistochemical analysis 72 h after model establishment in the GA16.5 and GA18.5 groups. (A and B) Ki67 staining of upper lip tissue from (A) the GA16.5 group and (B) the GA18.5 group. (C and D) CD31 staining of upper lip tissue from (C) the GA16.5 group and (D) the GA18.5 group. (E and F) CK10 staining of upper lip tissue from (E) the GA16.5 group and (F) the GA18.5 group. (G) IOD values for Ki67, CD31 and CK10 staining in both groups. *P<0.05; **P<0.001; ***P<0.0001. GA, gestational age; IOD, integral optical density.

**Figure 9.** Relative mRNA expression levels of TNF-α, IL-10 and TGF-β. **P<0.001; ****P<0.0001. GA, gestational age.
### Table I. Comparison of differentially expressed protein numbers between samples.

| Sample                        | Differentially expressed proteins, n | Upregulated proteins, n | Downregulated protein, n |
|-------------------------------|--------------------------------------|-------------------------|--------------------------|
| Group 1 vs. Group 2           | 57                                   | 37                      | 20                       |
| Group 3 vs. Group 4           | 312                                  | 171                     | 141                      |
| Group 3 vs. Group 1           | 312                                  | 151                     | 138                      |

Group 1, upper-left lip without scar repair group at 72 h after modeling in GA16.5 rats; Group 2, upper-right lip normal group at 72 h after modeling in GA16.5 rats; Group 3, upper-left lip scar repair group at 72 h after modeling in GA18.5 rats; Group 4, upper-right lip normal group at 72 h after modeling in GA18.5 rats.

### Table II. Differential protein expression in group 1 and group 2.

| Protein ID | Gene name | Protein name                                      | P-value | Fold-change |
|------------|-----------|---------------------------------------------------|---------|-------------|
| G3V8R3     | Hbz       | Hemoglobin, zeta                                   | 0.004   | 7.788       |
| B2RY8S     | Ndufb8    | NADH dehydrogenase                                 | 0.000   | 5.024       |
| Q920P6     | Ada       | Adenosine deaminase                                | 0.038   | 4.831       |
| O88752     | Hbe1      | Hemoglobin, epsilon 1                              | 0.003   | 4.801       |
| Q499N7     | Ptpn6     | Tyrosine-protein phosphatase non-receptor type 6   | 0.003   | 4.700       |
| Q4FZU2     | Krt6a     | Keratin 6A                                         | 0.001   | 4.044       |
| P06762     | Hmox1     | Heme oxygenase 1                                   | 0.011   | 3.902       |
| Q6IFU9     | Krt16     | Keratin, type I cytoskeletal 16                    | 0.006   | 3.613       |
| Q99PD6     | Tgfb1i1   | Transforming growth factor beta-1-induced transcript 1 protein | 0.004   | 3.590       |
| Q6F7S1     | Asah1     | Acid ceramidase                                    | 0.029   | 3.167       |
| Q63066     | Hbg1      | Hemoglobin, gamma A                                | 0.002   | 3.082       |
| Q10758     | Krt8      | Keratin, type II cytoskeletal 8                    | 0.004   | 2.901       |
| Q6AYQ4     | Tmem109   | Transmembrane protein 109                          | 0.048   | 2.710       |
| Q9Z2Q7     | Stx8      | Syntaxin-8                                         | 0.035   | 2.660       |
| G3V9M8     | Fam50a    | Protein fam50a                                     | 0.034   | 2.515       |
| M0R9Y3     | Nup43     | Nucleoporin 43                                     | 0.005   | 2.461       |
| B2GV89     | Fermt3    | Fermitin family homolog 3                          | 0.018   | 2.425       |
| G3V8H      | Olfm3     | Olfactomedin-like protein 3 precursor              | 0.045   | 2.383       |
| D4A531     | Polr2i    | Rna polymerase ii subunit i                        | 0.049   | 2.290       |
| Q6FS1      | Nubp2     | Cytosolic Fe-S cluster assembly factor             | 0.028   | 2.221       |
| D3ZLS5     | Hectd1    | Hect domain e3 ubiquitin protein ligase 1          | 0.033   | 2.112       |
| D4A0M2     | Nxn       | Nucleoredoxin                                      | 0.033   | 2.056       |
| Q6IE17     | Stfa212   | Stefin-3                                           | 0.007   | 1.969       |
| P27139     | Ca2       | Carbonic anhydrase 2                               | 0.032   | 1.957       |
| D3ZF44     | LOC684499 | Protein LOC684499                                 | 0.015   | 1.940       |
| Q6LDZ3     | Ptprc     | Receptor-type tyrosine-protein phosphatase C       | 0.007   | 1.878       |
| Q5X138     | Lcp1      | Plastin-2                                          | 0.018   | 1.843       |
| Q5PPG2     | Lgmn      | Legumain precursor                                 | 0.015   | 1.820       |
| P06765     | Pf4       | Platelet factor 4                                  | 0.050   | 1.708       |
| Q9RT3      | Ctsz      | Cathepsin Z                                        | 0.011   | 1.707       |
| Q5U1Y2     | Rac2      | Ras-related C3 botulinum toxin substrate 2        | 0.021   | 1.669       |
| Q5U2V4     | Pld1      | Phospholipase B-like 1                             | 0.028   | 1.630       |
| Q9EPX0     | Hspb8     | Heat shock protein beta-8                          | 0.005   | 1.603       |
| O35532     | Msomo1    | Methylsterol monoxygenase 1                        | 0.037   | 1.592       |
| Q91Z1N1    | Coro1a    | Coronin-1A                                         | 0.013   | 1.586       |
| O88201     | Clec11a   | C-type lectin domain family 11 member A            | 0.025   | 1.547       |
| Q5U329     | Slec4a1   | Band 3 anion transport protein                     | 0.027   | 1.512       |
| Q496Z5     | Prph      | Peripherin                                         | 0.041   | 0.626       |
| P19527     | Nefl      | Neurofilament light polypeptide                    | 0.040   | 0.608       |
Table II. Continued.

| Protein ID  | Gene name | Protein name                                         | P-value | Fold-change |
|------------|-----------|------------------------------------------------------|---------|-------------|
| Q9ESI7     | Dcx       | Neuronal migration protein doublecortin              | 0.003   | 0.597       |
| Q6AY98     | Ube2e2    | Ubiquitin conjugating enzyme e2 e2                   | 0.046   | 0.577       |
| Q7T5X7     | Nr3c1:gr  | Glucocorticoid receptor                              | 0.026   | 0.560       |
| O70437     | Smad4     | Mothers against decapentaplegic homolog 4            | 0.043   | 0.557       |
| F1M754     | Map4k4    | Mitogen-activated protein kinase kinase kinase kinase 4 | 0.022   | 0.526       |
| D4A2Z8     | Dhx36     | Probable ATP-dependent RNA helicase DHX36             | 0.009   | 0.522       |
| P31430     | Dpep1     | Dipeptide 1                                          | 0.010   | 0.513       |
| Q6AXY8     | Dhrs1     | Dehydrogenase/reductase SDR family member 1          | 0.019   | 0.495       |
| D4A414     | Cox15     | COX15 homolog                                        | 0.031   | 0.476       |
| D4A5B5     | C4m1      | Calmodulin 1                                         | 0.012   | 0.473       |
| D3ZRN3     | Actb12    | Beta-actin-like protein 2                             | 0.048   | 0.413       |
| Q8CGS4     | Chmp3     | Charged multivesicular body protein 3                | 0.022   | 0.410       |
| D3ZHA7     | Myl6b     | Myosin light chain 6b                                | 0.011   | 0.390       |
| P70541     | Eif2b3    | Translation initiation factor eif-2B subunit gamma    | 0.002   | 0.324       |
| D3ZX0      | Krtap11-1 | Uncharacterized protein                               | 0.037   | 0.287       |
| D3ZD07     | Fmo9      | Flavin containing monooxygenase 9 pseudogene         | 0.007   | 0.277       |
| Q6FX1      | Krt24     | Keratin, type I cytoskeletal 24                       | 0.012   | 0.050       |
| Q6G02      | Krt2      | Keratin, type II cytoskeletal 2                       | 0.008   | 0.021       |

Group 1: Upper-left lip of fetus at 72 h after modeling in GA16.5 rats; Group 2, upper-right lip of fetus at 72 h after modeling in GA16.5 rats. GA, gestational age.

Figure 10. Volcano plot of the differentially expressed proteins in group 1 and group 2. Group 1, upper-left lip of fetus at 72 h after modeling in GA16.5 rats; Group 2, upper-right lip of fetus at 72 h after modeling in GA16.5 rats. GA, gestational age.

- i) Smad4, Tgfl11, Ptpn6 and Hmox1 in group 1 and 2; ii) S100a9, Fgg, Anxa1, Fgb, Plg and S100a8 in group 3 and 4; and iii) CD36, S100a9, S100a8, Cd9Fgg, Anxa1, Fgb, Plg and S100a8 in group 3 and 1 (Fig. 15).
Table III. Differential protein expression in group 3 and group 4.

| Protein ID     | Gene name          | Protein name                                  | P-value | Fold change |
|----------------|--------------------|-----------------------------------------------|---------|-------------|
| D3GE2          | Mpo                | Myeloperoxidase                               | 0.000   | 377.923     |
| Q62714         | Np4                | Neutrophil antibiotic peptide NP-4            | 0.000   | 231.771     |
| D3ZY96         | Ngp                | Neutrophilic granule protein precursor        | 0.010   | 226.724     |
| P50115         | S100a8             | S100 Calcium Binding Protein A8               | 0.001   | 92.828      |
| Q7TP54         | Fam65b             | Protein FAM65B                                | 0.000   | 77.718      |
| D3ZM16         | Olfm4              | Olfactomedin-4 precursor                      | 0.001   | 63.833      |
| D4A081         | Setdb1             | Histone-lysine N-methyltransferase SETDB1     | 0.000   | 47.032      |
| Q9JH30         | Itgam              | Integrin alpha-M precursor                   | 0.011   | 39.489      |
| B2RYB8         | Itgb2              | Integrin beta 2 precursor                     | 0.003   | 34.443      |
| P50116         | S100a9             | S100 Calcium Binding Protein A9               | 0.000   | 30.191      |
| Q920P6         | Ada                | Adenosine deaminase                           | 0.028   | 27.433      |
| Q499N7         | Ptpn6              | Tyrosine-protein phosphatase non-receptor type 6 | 0.003   | 23.157      |
| Q9ERL1         | Cybb               | Cytochrome b-245, beta polypeptide            | 0.002   | 21.484      |
| Q9JKB7         | Gda                | Guanine deaminase                             | 0.019   | 20.202      |
| Q5U1Y2         | Rac2               | Ras-related C3 botulinum toxin substrate 2   | 0.007   | 19.291      |
| Q6IFU9         | Krt16              | Keratin, type I cytoskeletal 16               | 0.001   | 13.524      |
| O54854         | Klk6               | Kallikrein-6 precursor                        | 0.000   | 11.605      |
| B2GV9B         | Fermt3             | Fermin family homolog 3                       | 0.012   | 11.176      |
| Q5PQW8         | Gbp2               | Interferon-induced guanylate-binding protein 2 | 0.019   | 10.854      |
| Q6LDZ3         | Ptprc              | Receptor-type tyrosine-protein phosphatase C  | 0.015   | 10.626      |
| Q4G075         | Serpinb1a          | Leukocyte elastase inhibitor A                | 0.001   | 9.051       |
| Q6PDV1         | Lyz1               | Lysozyme C-1 precursor                        | 0.002   | 9.049       |
| Q6IE17         | Stfa2L             | Stefin-3                                      | 0.000   | 8.930       |
| Q5U2V4         | Plbd1              | Phospholipase B-like 1                        | 0.003   | 8.669       |
| Q9IZN1         | Coro1a             | Coronin-1A                                    | 0.001   | 8.199       |
| P14669         | Anxa3              | Annexin A3                                    | 0.008   | 8.100       |
| Q9R0D6         | Tcn2               | Transcobalamin-2 precursor                   | 0.014   | 7.286       |
| Q4QQV6         | Lsp1               | Lymphocyte specific 1                         | 0.004   | 6.785       |
| P06768         | Rbp2               | Retinol-binding protein 2                     | 0.006   | 6.051       |
| Q5X138         | Lcp1               | Plastin-2                                     | 0.001   | 5.841       |
| Q91W30         | Akib1b             | Aldo-Keto Reductase Family 1 Member B8        | 0.001   | 5.492       |
| Q63015         | Csap1              | Common salivary protein 1 precursor           | 0.001   | 5.454       |
| P31720         | C1qa               | Complement C1q subcomponent subunit A         | 0.008   | 5.339       |
| Q3V904         | Pld4               | Phospholipase D4                              | 0.005   | 4.857       |
| D4ADD7         | Glrx5              | Glutaredoxin-related protein 5                 | 0.002   | 4.782       |
| P22985         | Xdh                | Xanthine dehydrogenase/oxidase               | 0.003   | 4.221       |
| P06866         | Hp                 | Haptoglobin Haptoglobin alpha chain Haptoglobin beta chain | 0.002 | 3.945       |
| B2RYS9         | Trmt112            | Uncharacterized protein                       | 0.016   | 3.827       |
| P23640         | Rab27a             | Ras-related protein Rab-27A                  | 0.017   | 3.774       |
| P06762         | Hmox1              | Heme oxygenase 1                              | 0.008   | 3.769       |
| Q9WUQ4         | Slpi               | Secretory leukocyte peptidase inhibitor precursor | 0.015 | 3.710       |
| P07150         | Anxa1              | Annexin A1                                   | 0.003   | 3.449       |
| D3ZX79         | L66g6c             | Lymphocyte antigen 6 complex G6C precursor    | 0.018   | 3.236       |
| O88752         | Hbe1               | Hemoglobin, epsilon 1                         | 0.029   | 3.211       |
| Q9R1T3         | Ctsz               | Cathepsin Z                                   | 0.002   | 3.195       |
| D3ZH9H         | Me2                | NAD-dependent malic enzyme, mitochondrial     | 0.034   | 2.926       |
| P05942         | S100a4             | S100 Calcium Binding Protein A4               | 0.002   | 2.897       |
| Q5XW6          | Cfh                | Complement factor H precursor                 | 0.008   | 2.891       |
| O54892         | Hk2                | Hexokinase-2                                  | 0.007   | 2.876       |
| Q6P7D4         | Cyp20a1            | Cytochrome P450 20A1                          | 0.013   | 0.490       |
| D3ZW6C         | Snb1               | beta-1-syntrophin                             | 0.025   | 0.490       |
| Q62997         | Gfra1              | GDNF family receptor alpha-1                 | 0.043   | 0.488       |
Table III. Continued.

| Protein ID | Gene name | Protein name | P-value | Fold change |
|------------|-----------|--------------|---------|-------------|
| P02600     | Myl1      | Myosin light chain 1/3 | 0.011   | 0.482       |
| O35878     | Hspb2     | Heat shock protein beta-2 | 0.005   | 0.481       |
| P17209     | Myl4      | Myosin light chain 4    | 0.004   | 0.475       |
| D4A8H3     | Uba6      | Ubiquitin-like modifier-activating enzyme 6 | 0.031   | 0.471       |
| A1L1K3     | Anapc5    | Anaphase-promoting complex subunit 5 | 0.046   | 0.470       |
| D3ZTW9     | Exog      | Nuclease EXOG           | 0.028   | 0.467       |
| D4A3D2     | Smyd1     | SET and MYND domain-containing protein 1 | 0.004   | 0.465       |
| P04466     | Mylpf     | Myosin regulatory light chain 2 | 0.009   | 0.464       |
| P12847     | Myh3      | Myosin-3               | 0.007   | 0.461       |
| P13413     | Tnni1     | Troponin I             | 0.001   | 0.460       |
| D4A4Y2     | Hsd17b14  | 17-beta-hydroxysteroid dehydrogenase 14 | 0.033   | 0.455       |
| P23928     | Cryab     | Alpha-crystallin B chain | 0.020   | 0.454       |
| Q7TNB2     | Tnnt1     | Troponin T             | 0.002   | 0.451       |
| D3ZCD7     | Tp53rk    | TP53-regulating kinase | 0.004   | 0.450       |
| P00564     | Ckm       | Creatine kinase M-type | 0.037   | 0.450       |
| Q80W95     | Hrc       | Sarcoplasmic reticulum histidine-rich calcium-binding protein precursor | 0.019   | 0.445       |
| P50463     | Csrp3     | Cysteine and glycine-rich protein 3 | 0.013   | 0.444       |
| Q5XIG1     | Ldb3      | Ldb3 protein           | 0.017   | 0.443       |
| D3ZUB7     | Anapc4    | Anaphase-promoting complex subunit 4 | 0.030   | 0.442       |
| Q64578     | Atp2a1    | ATPase, Ca++ transporting, cardiac muscle, fast twitch 1 | 0.032   | 0.442       |
| Q6P792     | Fhl1      | Four and a half LIM domains protein 1 | 0.013   | 0.431       |
| Q8K4F2     | Alox15b   | Arachidonate 15-lipoxygenase B | 0.025   | 0.428       |
| M0RBL8     | Tceal6    | Protein LOC679974       | 0.003   | 0.427       |
| P51868     | Casq2     | Calsequestrin-2 precursor | 0.008   | 0.425       |
| B4F789     | Apobec2   | Probable C->U-editing enzyme APOBEC-2 | 0.008   | 0.421       |
| P16290     | Pgam2     | Phosphoglycerate mutase 2 | 0.014   | 0.418       |
| Q9Z2J4     | Nexn      | Nexilin                | 0.002   | 0.412       |
| Q9QYU4     | Crym      | Thiomorpholine-carboxylate dehydrogenase | 0.017   | 0.411       |
| D3ZUQ0     | Rilpl1    | RILP-like protein 1     | 0.006   | 0.409       |
| D4A2H6     | Rbfox3    | Fox-1 homolog C         | 0.037   | 0.408       |
| D3ZVM5     | Hspa12b   | Heat shock 70 kDa protein 12B | 0.038   | 0.406       |
| O54747     | Pold1     | DNA polymerase delta catalytic subunit | 0.001   | 0.403       |
| P52481     | Cap2      | Adenylyl cyclase-associated protein 2 | 0.007   | 0.396       |
| Q63544     | Sncg      | Gamma-synuclein         | 0.004   | 0.381       |
| Q496Z5     | Prph      | Peripherin              | 0.001   | 0.376       |
| P07483     | Fabp3     | Fatty acid-binding protein, heart | 0.011   | 0.357       |
| P23565     | Ina       | Alpha-internexin        | 0.005   | 0.332       |
| D4AD54     | Mgst3     | Microsomal glutathione S-transferase 3 | 0.024   | 0.328       |
| P19527     | Nefl      | Neurofilament light polypeptide | 0.006   | 0.326       |
| P12839     | Nefm      | Neurofilament medium polypeptide | 0.004   | 0.326       |
| B2RZ77     | Dpt       | Dermatopontin precursor | 0.024   | 0.320       |
| Q6AYG3     | Prune     | Prune homolog           | 0.017   | 0.320       |
| G3V7K1     | Myom2     | Myomesin 2              | 0.025   | 0.299       |
| G3V6V5     | Atplb4    | Protein ATP1B4          | 0.005   | 0.272       |
| Q9Z2Z8     | Dhcr7     | 7-dehydrocholesterol reductase | 0.000   | 0.270       |
| P19633     | Casq1     | Calsequestrin-1          | 0.021   | 0.201       |
| D3ZX18     | Myoz2     | Myozenin-2              | 0.001   | 0.198       |
| Q812D3     | Ppil3     | Peptidyl-prolyl cis-trans isomerase-like 3 | 0.000   | 0.179       |

Group 3, upper-left lip of fetus at 72 h after modeling in GA18.5 rats; Group 4, upper-right lip of fetus at 72 h after modeling in GA18.5 rats. GA, gestational age.
Table IV. Differential protein expression in group 3 and group 1.

| Protein ID | Gene name | Protein name | P-value | Fold change |
|------------|-----------|--------------|---------|-------------|
| Q6jh3      | Smgc      | Submandibular gland protein c precursor | 0.001   | 753.286     |
| D3ge2      | Mpo       | Myeloperoxidase precursor | 0.000   | 271.832     |
| Q62714     | Np4       | Neutrophil antibiotic peptide np-4 | 0.000   | 244.647     |
| D3zm6      | Olfm4     | Olfactomedin-4 precursor | 0.000   | 189.639     |
| D3zy96     | Ngp       | Neutrophilic granule protein precursor | 0.014   | 130.459     |
| B2yb8      | Itgb2     | Integrin beta 2 precursor | 0.002   | 44.874      |
| G3v817     | Itgam     | Integrin alpha-m precursor | 0.012   | 32.024      |
| Q6ig02     | Krt2      | Keratin, type ii cytoskeletal 2 epidermal | 0.011   | 26.577      |
| Q63015     | Csap1     | Common salivary protein 1 precursor | 0.003   | 26.571      |
| P50115     | S100a8    | S100 calcium binding protein a8 | 0.002   | 25.933      |
| P50116     | S100a9    | S100 calcium binding protein a9 | 0.001   | 19.538      |
| Q9jb7      | Gda       | Guanine deaminase | 0.023   | 19.076      |
| D3zd07     | Fmo9      | Flavin containing monoxygenase 9 pseudogene | 0.003   | 17.428      |
| Q5u1y2     | Rac2      | Ras-related c3 botulinum toxin substrate 2 | 0.011   | 12.859      |
| O54854     | Klk6      | Kallikrein-6 precursor | 0.000   | 10.614      |
| Q5u2v4     | Plbd1     | Phospholipase b-like 1 | 0.002   | 10.508      |
| Q6pdv1     | Lyz1      | Lysozyme c-1 precursor | 0.002   | 8.631       |
| Q4g075     | Serpinb1a | Leukocyte elastase inhibitor a | 0.001   | 8.260       |
| Q6ldz3     | Ptpc      | Receptor-type tyrosine-protein phosphatase c | 0.023   | 8.077       |
| Q9wuq4     | Slpi      | Secretory leukocyte peptidase inhibitor precursor | 0.007   | 7.093       |
| Q9er1l     | Cybb      | Cytochrome b-245, beta polypeptide | 0.009   | 7.056       |
| E0a3n4     | Serpina3n | Serine protease inhibitor a3n | 0.013   | 7.049       |
| G3v6k1     | Tcn2      | Transcobalamin-2 precursor | 0.017   | 6.577       |
| P14669     | Anxa3     | Annexin a3 | 0.010   | 5.005       |
| P22985     | Xdh       | Xanthine dehydrogenase/oxidase | 0.003   | 4.802       |
| Q91zn1     | Coro1a    | Coronin-1a | 0.001   | 4.694       |
| P05982     | Nqo1      | Nad(p)h quinone dehydrogenase 1 | 0.001   | 4.334       |
| P23640     | Rab27a    | Ras-related protein rab-27a | 0.019   | 3.830       |
| Q6fu9      | Krt16     | Keratin, type i cytoskeletal 16 | 0.001   | 3.797       |
| Q62894     | Ecm1      | Extracellular matrix protein 1 | 0.039   | 3.655       |
| Q5xi38     | Lcp1      | Plastin-2 | 0.003   | 3.469       |
| P07150     | Anxa1     | Annexin a1 | 0.003   | 3.446       |
| Q78rz5     | Hopx      | Homeodomain-only protein | 0.002   | 3.376       |
| P01015     | Agt       | Angiotensinogen angiotensin-1 angiotensin-2 angiotensin-3 | 0.010   | 3.300       |
| Q6axy8     | Dhrs1     | Dehydrogenase/reductase sdr family member 1 | 0.006   | 3.287       |
| Q91w30     | Akr1b8    | Aldose reductase-related protein 2 | 0.007   | 3.244       |
| P32755     | Hpd       | 4-hydroxyphenylpyruvate dioxygenase | 0.024   | 3.149       |
| Q499n7     | Ptn6      | Tyrosine-protein phosphatase non-receptor type 6 | 0.038   | 3.025       |
| G3v755     | Spr1a     | Cornilin-a | 0.002   | 3.006       |
| Q5xfv4     | Fabp4     | Fatty acid-binding protein, adipocyte | 0.024   | 2.910       |
| B1wbv8     | Pltd4     | Phospholipase d4 | 0.011   | 2.909       |
| D3zp9      | Serpinb12 | Serpin b12 | 0.038   | 2.880       |
| Q4qy6      | Lsp1      | Lymphocyte specific 1 | 0.001   | 2.665       |
| P29524     | Serpinb2  | Plasminogen activator inhibitor 2 type a | 0.001   | 2.653       |
| O55162     | Lypd3     | Ly6/plaur domain-containing protein 3 | 0.004   | 2.551       |
| D4a5u3     | Tgm3      | Protein-glutamine gamma-glutamyltransferase e protein | 0.033   | 2.547       |
| D3zh7      | Col17a1   | Collagen alpha-1(xvii) chain | 0.002   | 2.485       |
| D3zk2      | Serpinb3a | Protein serpinb3a | 0.038   | 2.445       |
| Q6ie17     | Stfa2l2   | Stefin-3 | 0.005   | 2.439       |
| Q5u206     | Calml3    | Calmodulin-like protein 3 | 0.013   | 2.429       |
| Q4v885     | Colec12   | Collectin-12 | 0.017   | 0.547       |
| D3zqi1     | Gpx7      | Glutathione peroxidase 7 precursor | 0.036   | 0.541       |
Table IV. Continued.

| Protein ID   | Gene name | Protein name                                             | P-value | Fold change |
|--------------|-----------|----------------------------------------------------------|---------|-------------|
| D3z9m5       | Fkbp7     | Peptidyl-prolyl cis-trans isomerase fkbp7 precursor      | 0.014   | 0.530       |
| O88201       | Clec11a   | C-type lectin domain family 11 member a                  | 0.047   | 0.529       |
| D3zrd3       | Pde6d     | Retinal rod rhodopsin-sensitive cgmp 3',5'-cyclic        | 0.009   | 0.528       |
|              |           | phosphodiesterase subunit delta                          |         |             |
| P21807       | Prph      | Peripherin                                               | 0.008   | 0.527       |
| G3v6m4       | Capn6     | Calpain-6                                                | 0.024   | 0.514       |
| D3zg88       | Sscsa1    | Sjogren syndrome/scleroderma autoantigen 1 homolog       | 0.034   | 0.502       |
| Q2eja0       | Yap1      | Yorkie homolog                                           | 0.002   | 0.494       |
| Q5b7u1       | Maged2    | Melanoma-associated antigen d2                           | 0.003   | 0.492       |
| O35276       | Nrp2      | Neurropilin-2                                            | 0.015   | 0.491       |
| D3zun5       | Pofut2    | Gdp-fucose protein o-fucosyltransferase 2 precursor      | 0.018   | 0.490       |
| P70583; d4a6v3 | Dut     | Deoxyuridine 5'-triphosphate nucleotidohydrolase         | 0.021   | 0.489       |
| P19527       | Nefl      | Neurofilament medium polypeptide                          | 0.026   | 0.479       |
| M0r649       | Exoc4     | Exocyst complex component 4                              | 0.031   | 0.466       |
| Q99pd6       | Tgfb1i1   | Transforming growth factor beta-1-induced transcript 1 protein | 0.048   | 0.466       |
| P54001       | P4ha1     | Prolyl 4-hydroxylase subunit alpha-1                     | 0.019   | 0.461       |
| D3zto7       | Sept5     | Septin-5                                                 | 0.046   | 0.452       |
| P12839; g3v7s2 | Nefm     | Neurofilament medium polypeptide                          | 0.020   | 0.444       |
| B5d5f0       | Ga1nt2    | Polypeptide n-acetylgalactosaminyltransferase 2           | 0.038   | 0.436       |
| D3zuq0       | Rilpl1    | Rilp-like protein 1                                       | 0.041   | 0.418       |
| D4a8h3       | Uba6      | Ubiquitin-like modifier-activating enzyme 6              | 0.024   | 0.415       |
| D4a9u4       | Eln       | Elastin                                                  | 0.041   | 0.409       |
| D4ad75       | Dpy191l   | Protein dpy-19 homolog 1                                  | 0.014   | 0.408       |
| Q6p7d4       | Cyp20a1   | Cytochrome p450 20a1                                      | 0.007   | 0.406       |
| Q5x28        | Raver1    | Ribonucleoprotein ptb-binding 1                          | 0.045   | 0.398       |
| P09117       | Aldoc     | Fructose-bisphosphate aldolase c                         | 0.004   | 0.396       |
| D3zct5       | Pald1     | Paladin                                                  | 0.004   | 0.395       |
| A111k3       | Anapc5    | Anaphase-promoting complex subunit 5                     | 0.028   | 0.392       |
| P62966       | Crabp1    | Cellular retinoic acid-binding protein 1                  | 0.016   | 0.384       |
| Q569b7       | Rwd4      | Rwd domain-containing protein 4                           | 0.040   | 0.384       |
| Q5hze4       | Mr1       | Methylthioribose-1-phosphate isomerase                    | 0.011   | 0.376       |
| F11q23       | Kif3a     | Kinesin family member 3a                                  | 0.011   | 0.376       |
| O88752       | Hbe1      | Hemoglobin, epsilon 1                                     | 0.033   | 0.375       |
| Q5u120       | Rab3gap2  | Rab3 gtpase-activating protein non-catalytic subunit      | 0.003   | 0.373       |
| A1a5r1       | Rbfox1    | Fox-1 homolog c                                           | 0.033   | 0.366       |
| D4a845       | Rpa3      | Replication protein a 14 kda subunit                      | 0.021   | 0.366       |
| D3zwc6       | Sntb1     | Beta-1-syntrophin                                         | 0.003   | 0.365       |
| G3v8m1       | Pold1     | DNA polymerase delta catalytic subunit                    | 0.003   | 0.353       |
| P23565       | Ina       | Alpha-internexin                                          | 0.039   | 0.352       |
| Q4klk9       | Ssu72     | RNA polymerase ii subunit a c-terminal domain phosphatase ssu72 | 0.025   | 0.349       |
| F1mah6       | Cdhl1     | Cadherin 13                                               | 0.007   | 0.326       |
| Q6ayg3       | Prune     | Prune homolog (drosophila) (ec:3.6.1.1)                   | 0.001   | 0.278       |
| P04638       | Apoa2     | Apolipoprotein a-ii                                       | 0.010   | 0.270       |
| Q9z2x8       | Dhc7      | 7-dehydrocholesterol reductase                           | 0.000   | 0.221       |
| Q10758       | Krt8      | Keratin, type ii cytoskeletal 8                           | 0.002   | 0.214       |
| Q812d3       | Ppil3     | Peptidyl-prolyl cis-trans isomerase-like 3                | 0.000   | 0.188       |
| G3v8r3       | Hbz       | Hemoglobin, zeta                                          | 0.004   | 0.128       |
| B5d9f9       | Sestd1    | Sec14 and spectrin domains 1                              | 0.000   | 0.065       |
| Q9eph1       | A1bg      | Alpha-1b-glycoprotein                                    | 0.016   | 0.033       |

Group 1, upper-left lip of fetus at 72 h after modeling in GA16.5 rats; Group 3, upper-left lip of fetus at 72 h after modeling in GA18.5 rats. GA, gestational age.
RT-qPCR analysis of possible target protein in cleft lip repair. RNA was extracted from tissue samples with TRIzol® reagent and the quality was checked using gel electrophoresis. Relative mRNA levels were analyzed using RT-qPCR (Fig. 16). The relative mRNA expression levels of Smad4 were significantly higher in group 2, compared with group 1 (P<0.05). Moreover, the relative mRNA expression levels of Fabp5 were significantly lower in groups 4 and 1, compared with group 3 (P<0.05). Additionally, the relative mRNA expression levels of S100a4 were significantly lower in group 4, compared with group 3 (P<0.05). S100a8 and S100a9 were significantly higher in group 3, compared with in groups 1 and 4 (P<0.05).

Immunofluorescence results. Immunofluorescence staining of Smad4, Fabp5, S100a4, S100a8 and S100a9 was performed on samples from both the GA16.5 and GA18.5 groups 72 h post-surgery. The expression levels of all five proteins increased in GA18.5 compared to GA16.5, and the differences were statistically significant (P<0.05; Fig. 17).
PRM analysis of differential protein expression. The differences in multiple variations of Smad4 expression were compared between groups 1 and 2. The panel reaction monitoring calculated this difference as 0.557, indicating downregulation in group 1 compared with in group 2 (P=0.043) (Table II). In contrast, no statistically significant differences were observed between groups 3 and 4. The difference in multiple variations of Fabp5 between groups 3 and 4 was calculated as 2.91, indicating upregulation in group 3 compared with in group 4 (P=0.024) (Table III). Additionally, the expression levels of Fabp5 were upregulated (P=0.01) in group 3 compared with in group 1; however, the difference between the variations present in groups 1 and 2 was not statistically significant. The difference in the multiple variations of S100a4 and S100a8 between groups 3 and 4 was calculated as 2.897 and 92.828, respectively, indicating an upregulation of the expression levels of both proteins in group 3 (P=0.001 and P=0.002, respectively) (Table III). Furthermore, the difference in the multiple variations of S100a8 between groups 3 and 1 was 25.933, which indicates upregulation in group 3 (P=0.002) (Table IV). However, the differences were not statistically significant between groups 1 and 2. The difference in the multiple variations of S100a9 was 30.191 and 19.538 between groups 3 and 4 and groups 3 and 1, respectively, suggesting upregulation in group 3 (P=0.0004 and P=0.001, respectively) (Tables III and IV). In contrast, the difference in the multiple variations of S100a9 between groups 1 and 2 was not statistically significant (Tables II-IV).

Discussion
In recent decades, various animal models of congenital cleft lip have been successfully established through surgical induction (4,20). It has been suggested that intrauterine cleft
lip repair can effectively improve this defect and reduce the impact of scars on normal facial development after birth. Thus, it also provides a new way for the effective repair of congenital cleft lips. In the present study, pregnant SD rats were used to establish a fetal rat model of cleft lip wound at two time points, Ga16.5 and Ga18.5. The different pregnancy models were induced by using two different repair methods of a cleft lip wound of the fetus (21,22). The exact gestational age is particularly important for the results of the repair of cleft lip in the fetal rats. Thus, the use of a rat model provides an added advantage in that the exact time of conception can be replicated, thereby minimizing differences between groups.

The present findings confirmed the hypothesis that fetal rat defects can be regenerated during early pregnancy without scar formation (23). It was also demonstrated that fetal rat defects could not be completely regenerated in late pregnancy and resulted in scarring (24). Furthermore, the expression of pro-inflammatory factors was different between the two groups. However, these observations were only made at one time point (72 h) after constructing cleft lip wounds in fetal rats. Future studies are needed to examine samples collected at different time points following surgery. Another shortcoming of this study entails the lack of comparison between the cleft lip wound repairs of fetal rats at different ages, such as the fetus in the early stages of pregnancy, or in newborn and/or adult rats. Label-free quantitative proteomics were used to examine proteins that play important roles in the postoperative repair process of fetal cleft lip. Protein expression was examined in four groups of samples. In addition, bioinformatics analysis was conducted to identify potential
biological markers, providing a theoretical reference and methodological basis for the examination of relevant mechanisms underlying fetal intrauterine scar repair. However, further studies are required to determine whether any one protein or several proteins, plays a key role in wound healing.

Smad4 belongs to the family of Smad proteins and is a common mediator in the signal transduction processes of the TGF-β family (25). TGF-β expression can lead to fibroblast proliferation and ECM deposition (26,27). The present findings indicated that the mRNA and protein expression levels of Smad4 were downregulated in the scar-free repair group. Furthermore, the mRNA and protein expression levels of Fabp5 were upregulated in the scar formation group. Therefore, it may be hypothesized that Fabp5 could be involved in the fibrosis of the fetal cleft lip wound, which may be mediated by the TGF-β signaling pathway (28-30).

S100a4 is a member of the S100 calcium-binding protein family, and its expression is associated with various non-neoplastic diseases, such as chronic obstructive pulmonary disease and cardiac hypertrophy (31-35). The present study demonstrated that the mRNA and protein expression levels of S100a4 were upregulated in the scar formation group, which may be associated with scar repair of fetal cleft lip wounds.

S100a8 is also a member of the S100 calcium-binding protein family (36-42). mRNA and protein expression levels of S100a8 were significantly upregulated in the scar repair group in the present study, indicating a potential role for S100a8 in the process of fetal cleft lip wound healing.

Current reports frequently associate S100a9, a member of the calcium-binding protein family S100, with infectious diseases, immune diseases and tumors, such as non-small cell lung adenocarcinoma (43-45). mRNA and protein expression levels of S100a9 were significantly upregulated in the scar formation group. Therefore, we speculated that S100a9 may play an important role in the process of fetal wound healing. However, whether the reduced expression levels of Fabp5, S100a4, S100a8 and S100a9 in the third trimester of pregnancy would reduce or worsen scar formation remains unclear. Further functional testing and regulatory studies are required to confirm the role of these five differentially expressed proteins in fetal wound repair.

The cleft lip is a very common congenital condition that often leaves life-long scarring. The present study identified five differentially expressed proteins, namely Smad4, Fabp5, S100a4, S100a8 and S100a9, that may be potential biomarkers of the scarless repair process in fetal rat cleft lip wounds. These findings may facilitate the discovery of new clinical targets for the prevention and treatment of scars. However, the role of these proteins in fetal wound repair and potential underlying mechanisms require further examination.

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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

YY, FH and JZh conceived and designed research. YY and FH performed animal experiments and staining. YY, HL, PJ, FH, JY, KG, SH and JZh performed PCR and label-free quantification PRM. YY, FH, JC, JY, ZC, AW and JZh analyzed data. YY and FH prepared figures. YY drafted the manuscript. FH and JZh edited and revised the manuscript. YY, FH, HL AW, PJ and JZh approved the final version of the manuscript. FH and JZh confirmed the authenticity of all of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study followed the regulations stipulated by the People's Republic of China regarding the Management of Experimental Animals and was approved by The Animal Experiment Management and Medical Ethics Sub-committee of The Third Xiangya Hospital of Central South University, Hunan, China (approval no. 2014-S168).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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