**Comparison of the homogeneity of mRNAs encoding SFRP5, FZD4,
and Fosl1 in post-injury intervals: Subcellular localization of
markers may influence wound age estimation**

Xi-yan ZHU^{a,1}. Qiu-xiang DU^{a,1}. San-qiang LI^a. Jun-hong SUN^{a,*}

^a Department of Forensic Pathology, Shanxi Medical University, Taiyuan,
China.

*** Corresponding author:** Jun-hong SUN. Department of Forensic
Pathology, Shanxi Medical University, 56 South Xinjian Road, Taiyuan
030001, Shanxi, PR China. Tel./fax +86 03 514135175.

E-mail addresses: junhong.sun@sxmu.edu.cn

¹ These authors contributed equally to the study.

Abstract

The inter-group heterogeneity and intra-group homogeneity of relative expression are very necessary when the mRNA were used to determine wound age accurately in forensic medicine. The aim of this study was to assess the intra-group homogeneity of SFRP5, FZD4 and Fos11 mRNAs in post-injury intervals. The corresponding proteins show different subcellular locations. A total of 78 Sprague–Dawley rats were divided into control and contusion groups. At 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, or 48 h (n = 6 per group) after contusion (under anesthesia by chloral hydrate intraperitoneally), the rats were sacrificed using a lethal dose of pentobarbital, and samples of the injured muscles were collected. The raw Ct values of SFRP5, FZD4, and Fos11 mRNAs were obtained using real-time PCR. After normalized to RPL13 mRNA levels, the coefficient of variation (CV) and the relative average deviation (d%) of each normalized Ct, and their relative expression levels, were calculated in each post-injury interval. Two methods were applied to compare the homogeneity of the three genes. First, each gene was given a score based on its CV value in each post-injury interval. Then, the sum of the 13 scores was calculated; a low sum indicated high homogeneity. Second, the 13 calculated CVs or d%s were used as raw data, which was described as the mean $\pm$ SD. Based on this mean $\pm$ SD, a CV of the CVs and a d% of the d%s were calculated to represent the variation; a low value indicated high homogeneity. The sum of the variability of FZD4 mRNA was lower than those of the SFRP5 and Fos11 mRNAs, consistent with the results that the FZD4 mRNA had the lowest mean, the smallest CV of all CVs, and the smallest d% of all d%s, among the three genes. In conclusion, these data indicated that mRNA encoding membranous FZD4 was likely to be more homogeneous than those encoding SFRP5 and Fos11 within post-injury intervals.

Keywords

Introduction

Wound age estimation is one of the most important (and difficult) tasks in forensic medicine. Forensic pathologists must estimate wound age daily in cases of murder, manslaughter, bodily harm with fatal consequences, and accidents (usually involving survivors)¹. Sometimes, wound age must be estimated as accurately as possible. As molecular biology techniques have evolved, various biological factors involved in coagulation or inflammation have been identified as useful markers. Especially, cytokines and growth factors, such as TNF- α , IL-1- β , and IL-6, are considered to be useful candidate markers for determination of wound vitality or age, because their sensitivity, specificity, and positive and negative predictive values are high²⁻⁵. However, although a great deal of data are available, no marker has yet proven to be both efficient and reliable⁵.

Generally, both the central tendency and the dispersion (arithmetic mean $\pm$ standard deviation, mean $\pm$ SD) are required to describe the expression of a marker when exploring whether the marker is suitable for wound age determination. In most cases, researchers tend to pay more attention to the central tendency, but do not attach importance to the dispersion⁶⁻⁸. However, to some degree, the dispersion could influence the accuracy of wound age, as the estimated interval would be rather wide if the dispersion is significantly high. The dispersion reflects the intra-group variability, and a marker with low dispersion or intra-group variability is termed as homogeneous marker in present study.

A relatively ideal marker should show statistically low intra-group variability or high homogeneity, though biological variation in vital processes and wound repair is unavoidable¹. Interestingly, it has been reported that subcellular location is important in terms of protein stability. The binding of extracellular proteins to subcellular structures may stabilize the proteins, whereas the stability of

intracellular proteins was determined mainly by their own structures⁹. Thus, we wondered whether the expression of particular genes showed intra-group homogeneity in the process of expression during wound healing.

In the present study, the intra-group homogeneity of frizzled-related protein 5 (SFRP5), frizzled class receptor 4 (FZD4), and Fos-like antigen 1 (Fos11) were evaluated and compared; these proteins are members of the canonical Wnt signaling pathway, which could regulate myogenic lineage progression and terminal differentiation during injured muscle repair¹⁰. In skeletal muscle cells, SFRP5, FZD4 and Fos11 have different subcellular locations, as shown in Figure 1. SFRP5 is an extracellular protein, Fos11 is intranuclear, and FZD4 is located on the cell membrane. In the canonical Wnt signaling pathway, binding of the Wnt ligand to its cell surface receptor, which consists of Frizzled (Fz) proteins such as FZD4 and low-density lipoprotein receptor-related protein (LRP), results in an increase in cytoplasmic β -catenin levels and translocation into the nucleus to regulate gene expression, including that of *Fos11*^{11, 12}.

In summary, the aim of the current study was to assess the intra-group homogeneity of SFRP5, FZD4, and Fos11 mRNAs during wound repair, and to further analyze the relationship between the intra-group homogeneity and subcellular location of markers.

Materials and Methods

Animal model and tissue preparation

This animal study was performed in strict accordance with the "Guiding Principles in the Use and Care of Animals" published by the National Institutes of Health (NIH Publication No 85–23, Revised 1996). All animals received humane care in accordance with the principles of the Guide for the Care and Use of Laboratory Animals protocol, published by the Ministry of the People's Republic of China

(issued on 3rd June, 2004), and approved by the Institutional Animal Care and Use Committee of Shanxi Medical University of China [Batch number of rats: SCXK (Jin) 2009–0001)]. The laboratory personnel have attended and finished the training about how to use experimental animal ethically and were allowed and supported to conduct this study by the mentioned Committee.

A total of 78 Sprague–Dawley male rats, 10–12 weeks old, weighing 250–300 g, were purchased from the Animal Center of Shanxi Medical University. All animals were kept pair-wise in stainless steel wire cages under a 12-h light-dark cycle at 22–24°C at a relatively humidity of 40–60%. They had free access to standard rat chow and water. The rats were divided randomly into a control group (n = 6) and the 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 h (n = 6/group) contusion groups.

Animals were kept under a 12-h light-dark cycle with free access to food and water. After anesthesia was induced by intraperitoneal injection of 10% chloral hydrate (300 mg/kg), the right posterior limbs were shaved, and residual hair was removed using a depilatory agent (Nair: Carter-Wallace, New York, NY, USA), as in our previous studies¹³. Subsequently, each rat was laid on a foam bed, and a 100-g counterpoise was allowed to fall freely through a clear Lucite guide tube from a height of 200 cm onto the thigh muscles of the right posterior limb, which resulted in skeletal muscle contusion^{14, 15}. The rats were transferred to clean cages and given commercial rat chow and tap water *ad libitum*. The rats were sacrificed with a lethal dose of pentobarbital (350 mg/kg body weight, intraperitoneal injection) at 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 h post-contusion. Approximately 100 mg muscle were dissected from each injured skeletal muscle, divided into two parts, and frozen immediately in liquid nitrogen. Muscle specimens were also dissected from the control group.

Total RNA preparation

Total RNA was extracted from skeletal muscle specimens (approximately 50-mg samples) using TRIzol Reagent (CoWin Biotech Co., Ltd., Beijing, China), following the manufacturer's instructions. The concentration (ng/ μ L) and integrity of freshly extracted total RNA were evaluated with the aid of a UV/visible spectrophotometer (UITrospec 4300 Pro: Biochrom, Cambridge, UK), and only RNAs with OD260/OD280 ratios of 1.8–2.2 were used in the following experiments.

Real-time fluorescent quantitative PCR

Primer pairs and Taqman probes were designed using AlleleID 6 software (Premier Biosoft International) and validated by BLAST search of the sequences obtained from GenBank. The specific primers and probes were synthesized by Invitrogen Trading Co. and are listed in Table 1.

First-strand cDNA was synthesized using the Prime Script RT-PCR Kit (Takara Biotechnology Co. Ltd., Dalian, China) following the standard protocol. For reverse transcription, 0.4 μ g total RNA was used in a reaction volume of 10 μ L.

Real-time PCR amplification of target and reference genes was performed synchronously in 25- μ L reaction mixtures containing 12.5 μ L Premix Ex Taq (Probe qPCR), 5 μ L dH₂O, 0.5 μ L (10 μ M) each primer, 0.5 μ L (10 μ M) each TaqMan fluorogenic probe, 2.5 μ L 10% DMSO, and 2 μ L cDNA. This was in accordance with the manufacturer's protocol provided using the Premix Ex Taq™ (Probe qPCR) kit (Takara Biotechnology Co. Ltd., Dalian, China). After initial denaturation at 95°C for 10 s, amplification consisted of 40 cycles of denaturation at 95°C for 5 s, followed by annealing and extension at 60°C for 40 s. The fluorescent signal was measured at the end of each cycle. All reactions were run on the Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA). All PCRs were repeated twice. To normalize the amounts of different cDNAs, mRNA encoding ribosomal protein L13 (RPL13) was used as an internal standard. The fluorescence curves of PCR products were evaluated

using Stratagene MxPro (Stratagene).

The amplification efficiencies of target and reference genes were calculated from the standard curves for SFRP5, FZD4, and Fos11, each of which featured five-fold serial dilutions (1, 1:5¹, 1:5², 1:5³, and 1:5⁴) of the template. Sterile ultrapure water was used as a negative control.

Data analysis

After threshold values were manually equalized among the several experiments on the same gene, the raw Ct values of each sample were obtained. Then, the normalized Ct value (thus, normalized to the RPL13 level) and the relative expression level of each sample were calculated. Subsequently, coefficients of variation (CVs) were calculated for each group of animals at 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 h post-contusion and for the control group, yielding a series of 13 CVs for each gene, using the formula: $CV = (SD/mean) \times 100$, where SD is the standard deviation.

Two methods were used to evaluate the homogeneity of the three mRNAs. First, the SFRP5, FZD4, and Fos11 genes were scored based on their CV values (score: 1 = minimal, 2 = moderate, 3 = maximum) in each post-injury interval. Next, all 13 obtained scores were amalgamated to form a variability value for each gene; a low value indicated high homogeneity. Second, for each gene, the 13 obtained CVs were used as raw data, which was described as the mean $\pm$ SD; based on the mean $\pm$ SD, a CV, that is CV of CVs, was further calculated as a value of variation using the formula: $CV = (SD/mean) \times 100$.

To confirm these results, the homogeneity of each gene was also evaluated using the relative average deviation (d%) in place of the CV. The formula was $d\% = (MD/mean) \times 100$, where MD was the mean deviation.

Statistical significance was evaluated by one-way analysis of variance (ANOVA), followed by

post-hoc least significant difference (LSD) testing; a *P* value less than 0.05 was considered significant.

Results

Amplification efficiency of the SFRP5, FZD4, Fosl1, and RPL13 mRNAs

The amplification curves for the SFRP5, FZD4, Fosl1, and RPL13 genes are shown in Figure 2. The amplification efficiencies (Eff. values) of the three test mRNAs were 110.5%, 103.4%, and 104.5%, respectively, similar to that of RPL13 mRNA. All R squared (Rsq) values were close to 1.00. The results indicated that the RPL13 mRNA was suitable for normalization of the SFRP5, FZD4, and Fosl1 mRNA data.

Expression of the SFRP5, FZD4, and Fosl1 mRNAs

The expression of SFRP5, FZD4, and Fosl1 mRNAs were detected during wound healing. As shown in Figure 3, heterogeneity was observed in different post-injury intervals for each gene. The level of SFRP5 mRNA decreased initially and then increased gradually towards to basal level, with lowest expression observed at 20 hours post contusion. The level of FZD4 mRNA was increasing to a peak at 12 hours and then decreasing to a valley at 28 hours, after which its expression increasing to another peak at 40 hours followed by a basal level. Fosl1 mRNA showed increase expression until 24 hours, with a peak noted at 12 hours, after which its expression remains basal level until 44 hours followed by an increase at 48 hour after injury.

Intra-group homogeneity of the SFRP5, FZD4, and Fosl1 mRNAs

For each gene, the CV values varied among samples collected at 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 h, but the FZD4 data varied the least, as shown in Figure 4.

Based on the CV values of the normalized Ct data, the sum of the 13 values for SFRP5 was 26,

that for FZD4 was 23, and that for FosI1 was 29. This indicated that the variability of FZD4 was the lowest among the three genes, as shown in Table 2.

To further evaluate the intra-group homogeneity of the three genes, the means, SDs, and CVs of the obtained CVs were calculated, as shown in Table 3. FZD4 had the lowest mean among the three mRNAs, though this did not differ significantly from those of the other genes by ANOVA ($P>0.05$). Moreover, compared with SFRP5 or FosI1 mRNA, the FZD4 mRNA had a lower CV. The results were similar when the d% values were used to calculate homogeneity.

To further explore and compare the intra-group homogeneity among the SFRP5, FZD4, and FosI1 genes, the homogeneity of the RPL13-normalized Ct values was also analyzed. Similar to what was found when the final relative quantities were examined, the raw normalized Ct value for FZD4 also had the lowest mean, the smallest CV of all CVs, and the smallest d% of all d%s, among the three genes, as shown in Table 4.

Discussion

Intra-group homogeneity is one of the most important characteristics of a valuable marker. As individual variability (or dispersion) is an unavoidable biological phenomenon, a marker can provide only limited information in terms of forensic wound age estimation if the intra-group variability is significantly high¹⁶⁻¹⁸. On the contrary, low intra-group dispersion not only renders experimental data credible statistically but also increases the accuracy of the estimated wound age. Therefore, it is necessary to explore intra-group homogeneous markers, which should show intra-group homogeneity not merely in a single post-injury interval, but in a series of intervals.

The intra-group homogeneity of the transcription and translation of a gene were probably controlled by multiple mechanisms. It was reported that the expression of gene, with translated protein

located on cell membrane, may exhibit relatively high stability, because the cell membrane protects proteins from degradation⁹. Moreover, as the molecule is located on the cell membrane, the expression level of that molecule is not likely to change greatly in physiological terms, as the bio-effect of a receptor is related to its affinity and the proportion of the receptor in the active versus inactive state. In contrast, non-membranous molecules probably execute their bio-functions mainly via up- or down-regulation, resulting in high variations in level. Therefore, our main hypothesis was that the subcellular location of a protein may indicate the intra-group homogeneity of its corresponding gene in the process of expression, and the membranous proteins implicate high intra-group homogeneity.

To estimate wound age as accurately as possible, forensic pathologists have made various attempts. In addition to traditional morphological techniques (immunofluorescence, immunohistochemistry), methods involving the study of mRNA (real-time PCR, in situ hybridization) and proteins (ELISA, western blotting) have also been used in wound age estimation^{5, 19, 20}. Even a multiplexed bead-based immunoassay²¹, electric impedance spectroscopy²², and an advanced scoring system²³ have been explored. Among these methods, real-time PCR technology has a high degree of automation which making the experimental data objective and accuracy, which is optimal for our aim to explore the homogeneity of marker. Besides, it is more appropriate to detect mRNA in early wound age estimation as various proteins were synthesized after the induction of mRNA²⁰. Therefore, we detected the intra-group homogeneity of the transcription of several genes using real-time PCR in the present study.

We evaluated the intra-group heterogeneity and intra-group homogeneity of SFRP5, FZD4, and Fos11 mRNAs. FZD4, as a receptor for Wnt ligands, is anchored to the cell membrane and plays a role in canonical Wnt signaling pathway²⁴. SFRP5 and Fos11 are non-membranous molecules (free proteins). Fos11 is the product of a Wnt signaling downstream target gene, and its expression is regulated by

several factors. As a proto-oncogene, Fos11 performs essential functions in regulating proliferation, differentiation, apoptosis, cell motility, invasion, and metastasis, as reported in many studies^{25, 26}. SFRP5 is an extracellular secretory protein. The SFRPs are not always Wnt antagonists and can serve as agonists sometimes beyond previous expectation²⁷⁻²⁹.

In the present study, the time-dependent expression of SFRP5, FZD4 and Fos11 mRNAs was confirmed by real-time PCR (Fig. 3), which implied their potential for skeletal muscle wound age determination. As their different expression pattern, combination of the expression of SFRP5, FZD4 and Fos11 mRNAs can accurately determine the wound intervals.

Besides, the intra-group homogeneity of SFRP5, FZD4 and Fos11 mRNAs were also evaluated. In the present study, both the CV and d% were calculated as measures of dispersion, as the fact that SD does not represent dispersion reliably if the means are rather different in the compared groups. However, the CV and d% values are commonly used as measures of dispersion when a new method is being explored or a novel instrument evaluated³⁰⁻³². We found that the variability of FZD4 mRNA expression was lower than those of SFRP5 and Fos11 mRNAs (Table 2). Furthermore, of the three genes, FZD4 had the lowest mean, the smallest CV of all CVs, and the smallest d% of all d%s (Table 3). Similar results were obtained when the raw normalized Ct values were further analyzed (Table 4). Taken together, the data probably indicate that FZD4 was the most homogeneous marker within post-injury interval among the three evaluated genes, supporting our hypothesis.

Limitations

Our study had several limitations, including a relatively low number of time points at which gene levels were measured, and the lack of an accepted statistical model for comparing intra-group homogeneity, either of these limitations may explain why we found a trend only, and not a statistically

significant difference ($P>0.05$). In addition, only mRNA was evaluated in the present study; it would be interesting to also measure protein expression levels in future work. Despite these drawbacks, our study underscores the potential importance of the subcellular location may influence the homogeneity of a mRNA marker.

Conclusions

In the present study, the inter-group heterogeneity and intra-group homogeneity of SFRP5, FZD4 and FosI1 mRNAs were evaluated. We confirmed that FZD4 was more homogeneous than SFRP5 or FosI1 within post-injury interval, proving our hypothesis. Thus, subcellular location should be taken into account when selecting homogeneous markers for wound age estimation. Prior selection of homogeneous markers, which may avoid unnecessary studies, combination of several markers, and the application of various methods will probably promote practical knowledge transfer in forensic practice. Therefore, our work aids in the selection of markers useful in wound age determination, and membranous proteins are recommended as homogeneous markers to estimate wound age accurately.

References

1. Grellner W, Madea B. Demands on scientific studies: vitality of wounds and wound age estimation. *Forensic Sci Int* 2007;**165**(2-3):150-4.
2. Ohshima T. Forensic wound examination. *Forensic Sci Int* 2000;**113**(1-3):153-64.
3. Kondo T, Ishida Y. Molecular pathology of wound healing. *Forensic Sci Int* 2010;**203**(1-3):93-8.
4. Cecchi R. Estimating wound age: looking into the future. *Int J Legal Med* 2010;**124**(6):523-36.
5. Casse JM, Martrille L, Vignaud JM, Gauchotte G. Skin wounds vitality markers in forensic pathology: An updated review. *Med Sci Law* 2015.
6. Sadler AJ, Rossello FJ, Yu L, Deane JA, Yuan X, Wang D, et al. BTB-ZF transcriptional regulator PLZF modifies chromatin to restrain inflammatory signaling programs. *Proc Natl Acad Sci U S A* 2015;**112**(5):1535-40.
7. Fronczek J, Lulf R, Korkmaz HI, Witte BI, van de Goot FR, Begieneman MP, et al. Analysis of inflammatory cells and mediators in skin wound biopsies to determine wound age in living subjects in forensic medicine. *Forensic Sci Int* 2015;**247**:7-13.
8. Kubo H, Hayashi T, Ago K, Ago M, Kanekura T, Ogata M. Temporal expression of wound healing-related genes in skin burn injury. *Leg Med (Tokyo)* 2014;**16**(1):8-13.
9. Rogers SW, Rechsteiner M. Degradation of structurally characterized proteins injected into HeLa cells. Effects of intracellular location and the involvement of lysosomes. *J Biol Chem* 1988;**263**(36):19843-9.

10. Jones AE, Price FD, Le Grand F, Soleimani VD, Dick SA, Megeney LA, et al. Wnt/beta-catenin controls follistatin signalling to regulate satellite cell myogenic potential. *Skelet Muscle* 2015;**5**:14.
11. Kurimoto S, Jung J, Tapadia M, Lengfeld J, Agalliu D, Waterman M, et al. Activation of the Wnt/beta-catenin signaling cascade after traumatic nerve injury. *Neuroscience* 2015;**294**:101-8.
12. Lee HK, Laug D, Zhu W, Patel JM, Ung K, Arenkiel BR, et al. Apcdd1 stimulates oligodendrocyte differentiation after white matter injury. *Glia* 2015.
13. Du QX, Sun JH, Zhang LY, Liang XH, Guo XJ, Gao CR, et al. Time-dependent expression of SNAT2 mRNA in the contused skeletal muscle of rats: a possible marker for wound age estimation. *Forensic Sci Med Pathol* 2013;**9**(4):528-33.
14. Iino M, Nakatome M, Ogura Y, Fujimura H, Kuroki H, Inoue H, et al. Real-time PCR quantitation of FE65 a beta-amyloid precursor protein-binding protein after traumatic brain injury in rats. *Int J Legal Med* 2003;**117**(3):153-9.
15. McBrier NM, Lekan JM, Druhan LJ, Devor ST, Merrick MA. Therapeutic ultrasound decreases mechano-growth factor messenger ribonucleic acid expression after muscle contusion injury. *Arch Phys Med Rehabil* 2007;**88**(7):936-40.
16. Hausmann R, Nerlich A, Betz P. The time-related expression of p53 protein in human skin wounds--a quantitative immunohistochemical analysis. *Int J Legal Med* 1998;**111**(4):169-72.
17. Betz P, Nerlich A, Tubel J, Wiest I, Hausmann R. Detection of cell death in human skin wounds of various ages by an in situ end labeling of nuclear DNA fragments. *Int J Legal Med* 1997;**110**(5):240-3.
18. Hausmann R, Biermann T, Wiest I, Tubel J, Betz P. Neuronal apoptosis following human brain injury. *Int J Legal Med* 2004;**118**(1):32-6.
19. Sato Y, Ohshima T. The expression of mRNA of proinflammatory cytokines during skin wound healing in mice: a preliminary study for forensic wound age estimation (II). *Int J Legal Med* 2000;**113**(3):140-5.
20. Fan YY, Zhang ST, Yu LS, Ye GH, Lin KZ, Wu SZ, et al. The time-dependent expression of alpha7nAChR during skeletal muscle wound healing in rats. *Int J Legal Med* 2014;**128**(5):779-86.
21. Takamiya M, Fujita S, Saigusa K, Aoki Y. Simultaneous detections of 27 cytokines during cerebral wound healing by multiplexed bead-based immunoassay for wound age estimation. *J Neurotrauma* 2007;**24**(12):1833-44.
22. Mao S, Fu F, Dong X, Wang Z. Supplementary pathway for vitality of wounds and wound age estimation in bruises using the electric impedance spectroscopy technique. *J Forensic Sci* 2011;**56**(4):925-9.
23. van de Goot FR, Korkmaz HI, Fronczek J, Witte BI, Visser R, Ulrich MM, et al. A new method to determine wound age in early vital skin injuries: a probability scoring system using expression levels of Fibronectin, CD62p and Factor VIII in wound hemorrhage. *Forensic Sci Int* 2014;**244**:128-35.
24. Birdsey GM, Shah AV, Dufton N, Reynolds LE, Osuna Almagro L, Yang Y, et al. The endothelial transcription factor ERG promotes vascular stability and growth through Wnt/beta-catenin signaling. *Dev Cell* 2015;**32**(1):82-96.
25. Baker EK, Taylor S, Gupte A, Sharp PP, Walia M, Walsh NC, et al. BET inhibitors induce apoptosis through a MYC independent mechanism and synergise with CDK inhibitors to kill osteosarcoma cells. *Sci Rep* 2015;**5**:10120.
26. Kumar K, Raza SS, Knab LM, Chow CR, Kwok B, Bentrem DJ, et al. GLI2-dependent c-MYC upregulation mediates resistance of pancreatic cancer cells to the BET bromodomain inhibitor JQ1. *Sci Rep* 2015;**5**:9489.
27. Canivell S, Rebuffat S, E GR, Kostov B, Siso-Almirall A, Novials A, et al. Circulating SFRP5 levels are elevated in drug-naïve recently diagnosed type 2 diabetic patients as compared with prediabetic subjects and controls. *Diabetes Metab Res Rev* 2015;**31**(2):212-9.
28. Xu CQ, de la Monte SM, Tong M, Huang CK, Kim M. Chronic Ethanol-Induced Impairment of Wnt/beta-Catenin Signaling is Attenuated by PPAR-delta Agonist. *Alcohol Clin Exp Res* 2015;**39**(6):969-79.
29. Yokota T, Oritani K, Sudo T, Ishibashi T, Doi Y, Habuchi Y, et al. Estrogen-inducible sFRP5 inhibits early B-lymphopoiesis in vivo, but not during pregnancy. *Eur J Immunol* 2015;**45**(5):1390-401.
30. Tonelli de Oliveira AC, Martinez D, Vasconcelos LF, Goncalves SC, Lenz MC, Fuchs SC, et al. Diagnosis of obstructive sleep apnea syndrome and its outcomes with home portable monitoring. *Chest* 2009;**135**(2):330-6.

31. Almeida AE, Stein R, Gus M, Nascimento JA, Arevalo JR, Fuchs FD, et al. Improved diagnostic accuracy of a 3-day protocol of home blood pressure monitoring for the diagnosis of arterial hypertension. *Blood Press Monit* 2013;**18**(2):119-26.
32. Wang YF, Liu YN, Xiong W, Yan DM, Zhu Y, Gao XM, et al. A UPLC-MS/MS method for in vivo and in vitro pharmacokinetic studies of psoralenoside, isopsoralenoside, psoralen and isopsoralen from *Psoralea corylifolia* extract. *J Ethnopharmacol* 2014;**151**(1):609-17.

Fig 1. Canonical Wnt signaling pathway.

a

Standard Curve

Fig 2b. Amplification efficiencies of FZD4 mRNA

b

Fig 2c. Amplification efficiencies of Fos11 mRNA

C

Amplification Plots of Fos11

Fig 3a. Expression of the SFRP5 mRNA

Fig 3b. Expression of the FZD4 mRNA

Fig 3c. Expression of the Fos1 mRNA

Fig 4a. Dispersion of SFRP5 mRNA

Fig 4b. Dispersion of FZD4 mRNA

Fig 4c. Dispersion of Fos11 mRNA

Captions

Fig 1. Canonical Wnt signaling pathway. SFRP5, FZD4, and Fosl1 are members of the canonical Wnt signaling pathway; SFRP5 is an extracellular protein, Fosl1 is intranuclear, and FZD4 is located on the cell membrane.

Fig 2. Amplification efficiencies of the SFRP5, FZD4, Fosl1, and RPL13 mRNAs. Standard curves were plotted using threshold fluorescence values obtained upon amplification of serially diluted template cDNAs. The amplification efficiencies (Eff. values) of the SFRP5 and RPL13 mRNAs were 110.5% and 120.2%, respectively (a); those of the FZD4 and RPL13 mRNAs were 103.4% and 100.5% respectively (b); and those of the Fosl1 and RPL13 mRNAs were 104.5% and 103.4% , respectively (c).

Fig 3. Expression of the SFRP5, FZD4, and Fosl1 mRNAs. Compared with the control, SFRP5 expression decreased at 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 h post-contusion (a); FZD4 expression increased at 8, 12, 36, and 40 h post-contusion (b); and Fosl1 expression increased at 4, 8, 12, 16, 20, 24, and 48 h post-injury (c). * $P < 0.05$.

Fig 4. Dispersion of SFRP5, FZD4 and Fosl1 mRNAs. The CV% values varied over time. The CV% of SFRP5 mRNA ranged from 40.1 to 170.0% (a), that of FZD4 from 39.3 to 93.1% (b), and that of Fosl1 from 34.5 to 158.8% (c).

Table 1 Primers and probes used for real-time fluorescent quantitative PCR

Gene symbol	GenBank accession no.	Strand	Nucleotide sequence	Location	Reporter	Quencher	Size of PCR products (bp)
SFRP5	NM_001107591.1	Probe	TCCATGAGGCCGTCAGCACTGTGC	793-816	HEX	BQH	140
		Sense	CCTCTGGACAACGACCTCTG	698-717			
		Antisense	AAGTCGCTGGAGCACATCTG	818-837			
FZD4	NM_022623.2	Probe	TGAAAGACACATGCCACCGCACGG	525-548	HEX	BQH	84
		Sense	GCACAGAGAAGATCAACATCCC	496-517			
		Antisense	CTTTCAGGACTGGTTCACATCG	558-579			
FosI1	NM_012953.1	Probe	CCAAGCATCAACGCTGTCAGTGGC	346-369	HEX	BQH	117
		Sense	GCAGCAGAAGTTCCACCTTG	324-344			
		Antisense	TAGGTCAGAGGTCGGGGATAG	420-440			
RPL13	NM_031101.1	Probe	CACACCAAGGTCCGGGCTGGCAG	238-260	FAM	BQH	107
		Sense	TCGTGAGGTGCCCTACAGTTAG	212-233			
		Antisense	GGTGCGTGCCATTTCTTGTG	298-318			

Table 2 Variability values for the SFRP5, FZD4, and FosI1 mRNAs

Group	SFRP5			FZD4			FosI1		
	CV (%)	d%	Score	CV (%)	d%	Score	CV (%)	d%	Score
Control	40.1*	28.9*	1	50.1	39.0	2	146.8	107.5	3
4 h	71.7	56.2	3	60.9	47.9	2	56.0	44.6	1
8 h	106.8	83.2	3	93.1#	61.4#	2	67.0	53.3	1
12 h	55.3	46.5	1	66.5	50.5	2	74.9	53.4	3
16 h	58.8	39.2	1	66.4	50.2	3	59.1	46.3	2
20 h	52.9	38.9	1	64.0	44.3	2	147.6	99.6	3
24 h	170.0#	115.0#	3	59.8	39.7	2	34.5*	22.6*	1
28 h	127.7	86.7	3	65.1	46.6	1	73.2	54.2	2
32 h	44.0	31.1	1	57.5	44.7	2	121.5	80.5	3
36 h	68.5	54.9	2	39.3*	31.2*	1	76.9	68.2	3
40 h	84.5	73.8	3	78.6	70.1	2	60.1	39.9	1
44 h	124.6	86.8	2	51.9	41.4	1	158.8#	107.3#	3
48 h	74.9	51.5	2	60.6	48.4	1	89.5	60.0	3
	26			23			29		

* Minimal value; # Maximum value.

Table 3 Homogeneity of the SFRP5, FZD4, and Fos11 expression levels

Gene	CVs		d%s	
	mean±SD (%)	CV (%)	mean±SD (%)	d%
SFRP5	83.06±38.61	46.49	60.98±26.05	35.47
FZD4	62.60±13.15*	21.01	47.34±9.90*	14.44
Fos11	89.69±40.38	45.02	64.42±26.85	33.69
P value	<i>P</i> =0.11		<i>P</i> =0.13	

* The minimal values were calculated as: CV = (SD/mean)*100; d% = (MD/mean)*100.

Table 4 Homogeneity of the normalized Ct values of the SFRP5, FZD4, and Fos11 mRNAs

Gene	CVs		d%s	
	mean±SD (%)	CV(%)	mean±SD (%)	d%
SFRP5	4.30±1.76	40.86	3.20±1.30	36.11
FZD4	3.60±0.91*	25.26	2.66±0.82*	19.63
Fos11	4.94±2.10	42.53	3.68±1.64	36.79
<i>P</i> value	<i>P</i> =0.14		<i>P</i> =0.15	

* The minimal values were calculated as CV = (SD/ mean)*100; d% = (MD/ mean)*100.