Dickkopf-1 is downregulated early and universally in the skin of patients with systemic sclerosis despite normal circulating levels

D. Daoussis[1], D.J. Papachristou[2], T. Dimitroulas[3], T. Sidiropoulos[4], I. Antonopoulos[1], A.P. Andonopoulos[1], S.-N. Liossis[1]

[1]Department of Rheumatology, Patras University Hospital, University of Patras Medical School, Greece;
[2]Department of Anatomy-Histology-Embryology, Laboratory of Bone and Soft Tissue Studies, University of Patras Medical School, Greece;
[3]4th Department of Internal Medicine Hippokration Hospital, Medical School, Aristotle University of Thessaloniki, Greece;
[4]Hospital of Skin and Venereal Diseases, Thessaloniki, Greece.

Dimitrios Daoussis, MD
Dionysios J. Papachristou, MD
Theodoros Dimitroulas, MD
Theodoros Sidiropoulos, MD
Ioannis Antonopoulos, MD
Andrew P. Andonopoulos, MD
Stamatis-Nick Liossis, MD

Please address correspondence to:
Dr D. Daoussis,
Department of Internal Medicine,
Division of Rheumatology,
Patras University Hospital,
26504 Rion, Patras, Greece.
E-mail: jimdaoussis@hotmail.com

Received on May 23, 2018; accepted in revised form on August 28, 2018.

Clin Exp Rheumatol 2018; 36 (Suppl. 113): S45-S49.

Key words: systemic sclerosis, scleroderma, Wnt, Dkk-1, fibrosis

Funding: This study was funded by the Hellenic Rheumatology Society and Professional Organisation for Rheumatologists.

Competing interests: none declared.

ABSTRACT

Objective. *The activity of the Wnt pathway, a critical mediator of fibrosis, is regulated by Dickkopf-1 (Dkk-1). Dkk-1 is absent from scleroderma skin in contrast to skin from healthy subjects where it is clearly expressed. There are no data on circulating levels and function of Dkk-1 in patients with systemic sclerosis (SSc).*

Our objectives are to assess: i) circulating and functional levels of Dkk-1 in patients with SSc and ii) whether the striking lack of Dkk-1 skin expression is also evident in a) clinically uninvolved skin from patients with SSc and b) very early disease prior to skin thickening.

Methods. *Circulating Dkk-1 levels were measured in 50 patients with SSc and 50 controls. Skin biopsies were obtained from SSc patients from a) clinically involved skin b) clinically uninvolved skin, c) oedematous skin prior to skin thickening.*

Results. *Circulating and functional Dkk-1 levels were similar in patients with SSc and controls. Healthy skin displayed a high Dkk-1 immuno-expression in the epidermis and dermal fibroblasts in contrast to clinically involved scleroderma skin where Dkk-1 was totally absent. In all biopsies of clinically uninvolved skin Dkk-1 was only moderately expressed whereas skin from very early disease displayed only a weak Dkk-1 immunoreactivity.*

Conclusion. *The downregulation of Dkk-1 at the oedematous phase of the disease indicates that the Wnt pathway is involved early in the disease process and may play a role in driving fibrosis. The decrease in Dkk-1 expression in clinically uninvolved scleroderma skin indicates that skin in SSc is universally affected.*

Introduction

SSc is a prototype multisystem fibrotic disease that associates with substantial morbidity and mortality (1). In SSc fibroblasts are hyperactivated and produce increased amounts of collagen that eventually leads to fibrosis and organ dysfunction. The question of what drives fibroblast activation remains unanswered. Recently, research has focused on developmental pathways; several lines of experimental evidence suggest that these pathways are crucial mediators of the fibrotic process (2-5). The developmental pathway that has received most attention is the canonical Wnt pathway where β-catenin serves as the central mediator. The activity of the pathway is tightly regulated by several soluble inhibitors such as Dickkopf-1 (Dkk-1) (6, 7). Dkk-1 is critically involved in the process of new bone formation in both animal models (8) and patients with ankylosing spondylitis (AS) (9). We have previously shown that in patients with AS, Dkk-1 is dysfunctional something that may lead to aberrant Wnt pathway activation and eventually new bone formation (10). Even though Dkk-1 was thought to be a bone specific molecule, it is now apparent that it is expressed in other tissues as well, such as the skin. Recently, Dkk-1 has been implicated in the pathophysiology of fibrosis. We, among others, have reported that Dkk-1 has virtually no expression in scleroderma skin in sharp contrast to normal skin where Dkk-1 was clearly expressed (11, 12). Interestingly, overexpression of Dkk-1 ameliorated fibrosis in several animal models, highlighting the key role of this molecule in the fibrotic process (12). It is currently not known whether decreased Dkk-1 skin

expression in patients with SSc associates with altered systemic levels. Up until now, Dkk-1 skin expression has only been assessed in established fibrosis, in biopsies obtained from clinically involved areas. It is not known whether the downregulation of Dkk-1 in scleroderma skin is an early event appearing prior to fibrosis and whether Dkk-1 is also absent from clinically uninvolved skin from patients with SSc. In this study we aimed at exploring whether circulating levels and function of Dkk-1 are altered in patients with SSc and investigating potential correlations with clinical and demographic characteristics. Moreover, we aimed to assess whether the striking lack of Dkk-1 skin expression is also evident in a) clinically uninvolved skin from patients with SSc and b) very early disease at the puffy/oedematous phase, prior to skin thickening. We report herein that downregulation of Dkk-1 in scleroderma skin occurs early in the disease process, prior to the establishment of fibrosis and is a universal finding affecting clinically uninvolved skin as well. In sharp contrast, circulating levels and function of Dkk-1 are not impaired in patients with SSc despite the striking lack of skin expression. These data indicate that local profibrotic factors suppress Dkk-1 expression in scleroderma skin.

Table I. Demographic and clinical characteristics of patients with SSc.

	Cohort 1 (n=50)	Cohort 2 (n=12)
Age (years) mean±SEM	57.3 ± 2.1	51.2 ± 3.23
Gender female n (%)	42 (84%)	10 (83.3)
Disease duration (years) mean±SEM	8 ±1.3	7.4 ± 0.7
Diffuse disease n(%)	32 (64%)	9 (75)
Anti-Scl70 positive n(%)	28 (56%)	8 (66.6)
Anti-centromere positive n(%)	17 (34%)	4 (33.3)
MRSS mean±SEM	17.1±1.2	16.17 ± 1.5
Interstitial lung disease (ILD)	26 (52%)	7 (58.3%)
Pulmonary arterial hypertension (PAH)	3 (6)	1 (8.3%)
Digital ulcers (DU)	18 (36%)	3 (25%)

ILD as indicated by findings in PFTs and HRCT. PAH diagnosed by right heart catheterisation. DU either present or reported in their medical records.

Patients and methods

Patients

Assessment of circulating levels and function of Dkk-1 was performed in 50 patients with SSc, fulfilling the 2013 criteria for the classification of the disease (13), that were recruited from routine outpatient clinics at the Rheumatology Department of Patras University Hospital, Greece. Basic clinical and demographic characteristics of study subjects are presented in Table I (Cohort 1). Most patients had diffuse disease (64%). Interstitial lung disease (ILD) was more common among patients with diffuse disease compared to patients with limited disease (62.5% *vs*. 33.3%, respectively). On the other hand, digital ulcers were less common in the diffuse *vs*. the limited subtype (25% *vs*. 55.5%), as expected. All patients underwent an extensive baseline evaluation including a full review of their medical records, a complete physical examination, including an assessment of skin thickening using the MRSS tool and calculation of the Scleroderma Health Assessment Questionnaire (S-HAQ). Laboratory workup consisted of full blood count, inflammatory markers, routine biochemistry tests, full serologic profile and pulmonary function tests (PFTs). Fifty healthy volunteers, age and sex matched, were used as controls. A local (Patras University Hospital, Patras, Greece) Ethics Committee approved the study protocol, which fulfilled the requirements of the Declaration of Helsinki, and a written informed consent was obtained from all participating individuals.

Skin histology

Skin biopsies (5mm punch) were obtained from: a) 12 patients with SSc from lesional skin of the forearm. Basic clinical and demographic characteristics of these patients are presented in Table I (Cohort 2); b) clinically uninvolved skin was obtained from 5 patients with SSc (4 diffuse-1 limited) with a median age of 50 (46-72) and disease duration of 6 (2-13) years from the upper back which was not affected clinically; c) 2 patients with systemic sclerosis (SSc) with very early disease (<12 months). The first patient was a 45-year old female with a 2-year history of Raynaud's, positive anti-Scl70, scleroderma pattern in capillaroscopy and puffy fingers. She had normal PFTs and no clinical evidence of internal organ involvement. The second patient was a 65-year old female with a 3-year history of Raynaud's, positive anti-Scl70, telangiectasias and puffy fingers. She had normal PFTs but reported mild gastroesophageal reflux. Both these patients had puffy fingers but no skin thickening; the biopsy was obtained from the distal part of the forearm, in close proximity to the oedematous area and prior to initiation of any kind of immunomodulatory therapy d) 5 healthy subjects. Dkk-1 expression was immunohistochemically assessed using a mouse anti-human monoclonal antibody (R&D Systems) as previously described (11) by a semi-quantitative method (high/moderate/weak/no expression).

Assessment of circulating and functional levels of Dkk-1

Serum samples were obtained from all patients and were stored at -20°C. Circulating Dkk-1 levels were measured using an established solid phase immunoassay, according to the manufacturer's instructions (R&D Systems). The functional integrity of Dkk-1 was assessed by a functional enzyme linked immunoabsorbent assay (ELISA), as previously described (10). Briefly, this functional assay measures only Dkk-1 bound to low density lipoprotein receptor-related protein 6 (LRP6). All measurements were performed in triplicates for each sample and the mean value was calculated.

Statistical analysis

Statistical analysis was performed using the SPSS software (SPSS Inc, Chicago, Illinois), v. 20. Data are presented as mean ± SEM, median (25-75th

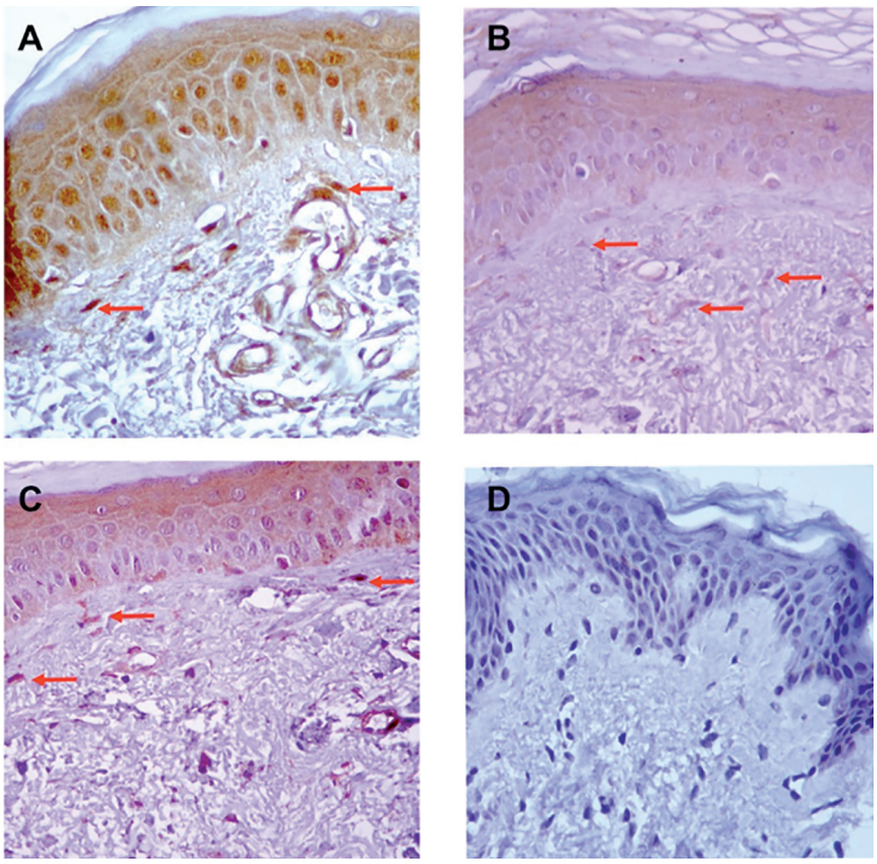

Fig. 1. Dkk-1 skin expression.
(**A**) Normal skin displaying strong Dkk-1 immunoreactivity in the epidermis and the fibroblasts of dermis (red arrows).
(**B**) In a section from skin of early scleroderma (puffy hands/oedematous stage) Dkk-1 shows only weak immunoexpression in both the epidermis and the fibroblasts of the dermis (red arrows).
(**C**) In this section of clinically uninvolved skin from a scleroderma patient, Dkk-1 exhibited moderate immunoreactivity, in both the epidermis and the fibroblasts of the dermis (red arrows) .
(**D**) In sharp contrast, clinically involved scleroderma skin (epidermis and dermal fibroblasts) was negative for Dkk-1.

percentile values) or percentages as appropriate. Correlations between Dkk-1 and other variables were analysed by Pearson or Spearman test, as appropriate. Significance was defined as $p<0.05$ (two-tailed).

Results

Downregulation of Dkk-1 skin expression in SSc is an early event, occurring independently of fibrosis

Healthy skin displayed a high Dkk-1 immunoexpression in basal cells of the epidermis as well as in the fibroblasts of the dermis in sharp contrast to clinically involved scleroderma skin that displayed no Dkk-1 immunoexpression. In clinically uninvolved skin obtained from patients with SSc Dkk-1 was only moderately expressed in basal cells of the epidermis and dermal fibroblasts. Clinically uninvolved scleroderma skin could be differentiated by immunohistochemical means from both skin from healthy subjects (high Dkk-1 expression) and clinically involved scleroderma skin (no Dkk-1 expression). Skin from very early disease at the oedematous phase, prior to skin thickening, exhibited only a weak Dkk-1 immunoreactivity in basal cells of the epidermis as well as in the fibroblasts of the dermis. Representative histology is depicted in Figure 1.

Despite the lack of skin expression circulating levels of Dkk-1 are normal in patients with SSc

We next assessed whether the striking differences in Dkk-1 skin expression in patients with SSc and healthy subjects coincided with differences in circulating Dkk-1 levels. Circulating levels of Dkk-1 did not differ significantly in patients with SSc compared to healthy subjects (mean ± SEM: 1603±154 pg/ml *vs*. 1889±95 pg/ml for patients with SSc and healthy subjects, respectively, p=ns). These are diagrammatically depicted in Figure 2A. We next assessed whether circulating Dkk-1 levels correlated with basic clinical and demographic characteristics in patients with SSc. We found no correlation of circulating Dkk-1 levels with age, gender, disease duration or disease type (diffuse *vs*. limited). More detailed analysis also revealed no significant correlations with MRSS, SHAQ, inflammatory markers (ESR, CRP), PFT's or the presence of auto-abs (anti-Scl70 or anti-centromere Abs).

Binding of Dkk-1 to LRP6 receptor is not impaired in SSc

We next assessed the functional integrity of Dkk-1 in patients with SSc. We applied a functional ELISA which measures only Dkk-1 bound to LRP6 receptor. Patients with SSc had similar functional Dkk-1 levels compared to healthy subjects (mean ± SEM: 257.8±89.5 pg/ml *vs*. 325.3±74.9 pg/ml for patients with SSc and healthy subjects, respectively, p=ns, Fig. 2B).

Discussion

A substantial amount of experimental data suggests that the Wnt pathway is a pivotal mediator of fibrosis. Fibroblast specific stabilisation of β-catenin leads to spontaneous fibrosis in animal models; on the other hand, fibroblast specific deletion of β-catenin prevents fibrosis (14). In humans with SSc, the Wnt pathway has been found to be highly activated in the skin (12, 14). It is not entirely known what drives aberrant Wnt signalling in scleroderma skin. Recent data indicate that enhanced Wnt pathway activation in scleroderma skin is linked to downregulation of the Wnt pathway inhibitor Dkk-1. It has been shown that Dkk-1 has almost no expression in scleroderma skin in sharp contrast to normal skin where Dkk-1 is strongly expressed (11, 12).

Akhmetshina *et al*. have shown that transforming growth factor β (TGFβ),

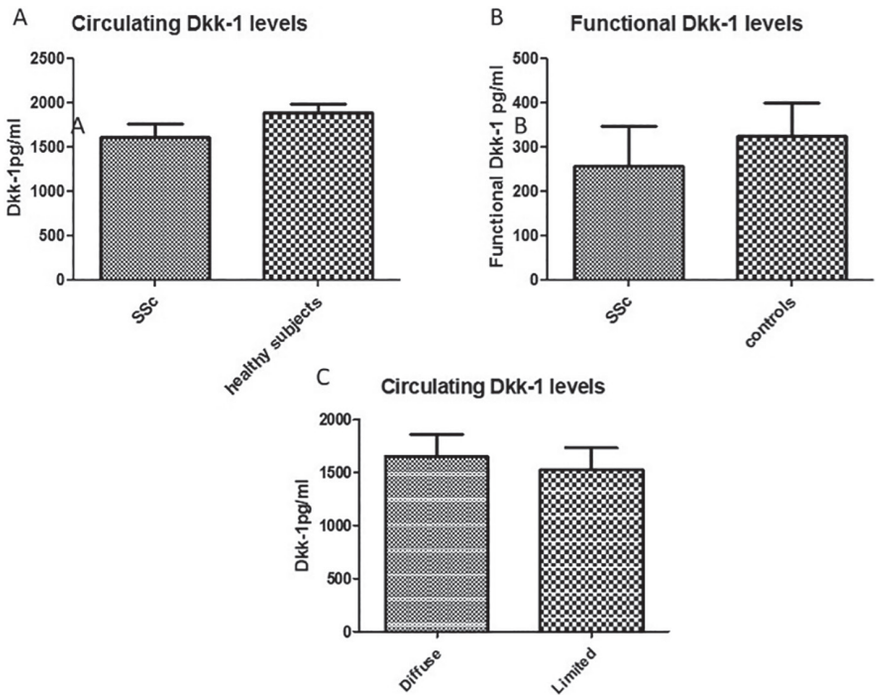

Fig. 2. Circulating and functional Dkk-1 levels in patients with SSc.
Circulating Dkk-1 levels (**A**) and functional Dkk-1 levels (**B**) in patients with SSc and healthy subjects are similar. There are no differences in circulating Dkk-1 levels in patients with diffuse *vs*. limited disease (**C**).

a master regulator of fibrosis, is able to potentiate Wnt signalling by down regulating the expression of Dkk-1, thus providing evidence of an intriguing link between TGFβ, Dkk-1 and Wnt signalling (12). Moreover, the promoter of DKK1 appears to be hypermethylated, a process known to attenuate gene expression (15). These data indicate that epigenetic mechanisms may also be involved in the down regulation of Dkk-1 in scleroderma skin. It is of interest that decreased Dkk-1 expression and aberrant Wnt signalling is not solely observed in scleroderma skin but is a universal finding in all forms of fibrosis such as liver cirrhosis and idiopathic lung fibrosis (12).

So far, Dkk-1 has only been assessed in cases of established fibrosis. Therefore, it is not known whether the downregulation of Dkk-1 precedes the development of fibrosis or occurs late in the fibrotic process. Our data indicate that the downregulation of Dkk-1 skin expression is a very early event occurring in the oedematous phase of the disease pointing to the direction that the Wnt pathway is involved early in the disease process, prior to establishment of fibrosis. This is a finding with potential pathogenetic implications; the fact that Dkk-1 is downregulated prior to the development of skin thickening suggests that the Wnt pathway is a driver of fibrosis.

In this study we also assessed Dkk-1 expression in clinically uninvolved skin of patients with SSc. Interestingly, clinically uninvolved skin from patients with SSc displayed a moderate Dkk-1 immunoreactivity and could be differentiated from both normal skin (high Dkk-1 expression) and clinically involved scleroderma skin (no Dkk-1 expression). The decrease in Dkk-1 expression in clinically uninvolved scleroderma skin substantiates previous evidence that the skin in SSc is universally affected underlining the systemic nature of the disease (16). Gene expression studies have shown a similar gene expression profile in both clinically involved and clinically uninvolved scleroderma skin (17).

The Wnt pathway is a significant orchestrator of embryogenesis; moreover, it is crucially involved in many homeostatic functions such as osteoblastogenesis, among others (18). Dkk-1 has been previously considered as a bone specific molecule and its role in the regulation of bone mass and joint remodelling has been extensively studied. In animal models of inflammatory arthritis, increased Dkk-1 expression has been linked to bone resorption whereas blockade of Dkk-1 to osteophyte formation, indicating that Dkk-1 is a master regulator of joint remodelling (8).

Our data, alongside with previous reports, clearly show that Dkk-1 is not a bone specific molecule as previously thought, since it is clearly expressed in other tissues such as the skin. Experimental data inversely linking Dkk-1 expression with bone mass (19, 20) have spurred enthusiasm related to the use of Dkk-1 blockers as a mean of treating osteoporosis. Even though data from animal models are supportive, in light of recent findings that Dkk-1 expression is not restricted to the bone, this approach should be rather discouraged; Dkk-1 blockade may enhance Wnt signalling in other tissues and lead to fibrosis. Indeed, a trend towards increased skin thickening was found in healthy mice treated with a mAb against Dkk-1 (12).

In this study we have shown that Dkk-1 is strikingly absent from scleroderma skin and is downregulated early in the disease process. However, despite the lack of skin expression, circulating and functional levels are not impaired. This is the first study that provides data indicating that there is no systemic defect of the molecule; therefore local factors (potentially transforming growth factor β-TGFβ) suppress Dkk-1 expression in the skin. Our data indicate that Dkk-1 is functionally intact in SSc in contrast to other diseases such as AS where it is dysfunctional (10).

A limitation of our study is the low number of biopsies assessed; this was due to obvious difficulties in recruiting patients with very early disease prior to skin thickening and obtaining biopsies from sites not clinically involved. Our findings should be confirmed in larger scale studies before definite conclusions can be drawn.

In conclusion, circulating and functional levels of Dkk-1 are normal in SSc despite the striking lack of skin expression. Our data indicate that local profibrotic factors may suppress Dkk-1 expression in scleroderma skin early in

the disease process and further substantiate existing evidence pointing to the direction of a critical role of this molecule in the pathogenesis of SSc.

References

1. VARGA J, TROJANOWSKA M, KUWANA M: Pathogenesis of systemic sclerosis: recent insights of molecular and cellular mechanisms and therapeutic opportunities. *J Scleroderma Relat Disord* 2017; 2: 137-52.
2. BERGMANN C, AKHMETSHINA A, DEES C *et al*.: Inhibition of glycogen synthase kinase 3beta induces dermal fibrosis by activation of the canonical Wnt pathway. *Ann Rheum Dis* 2011; 70: 2191-8.
3. WEI J, BHATTACHARYYA S, TOURTELLOTTE WG, VARGA J: Fibrosis in systemic sclerosis: emerging concepts and implications for targeted therapy. *Autoimmun Rev* 2011; 10: 267-75.
4. BEYER C, REICHERT H, AKAN H *et al*.: Blockade of canonical Wnt signalling ameliorates experimental dermal fibrosis. *Ann Rheum Dis* 2013; 72: 1255-8.
5. CHEON SS, WEI Q, GURUNG A *et al*.: Beta-catenin regulates wound size and mediates the effect of TGF-beta in cutaneous healing. *FASEB J* 2006; 20: 692-701.
6. DAOUSSIS D, ANDONOPOULOS AP: The emerging role of Dickkopf-1 in bone biology: is it the main switch controlling bone and joint remodeling? *Semin Arthritis Rheum* 2011; 41: 170-7.
7. DAOUSSIS D, ANDONOPOULOS AP, LIOSSIS SN: Wnt Pathway and IL-17: Novel Regulators of Joint Remodeling in Rheumatic Diseases. Looking Beyond the RANK-RANKL-OPG Axis. *Semin Arthritis Rheum* 2010; 39: 369-83.
8. DIARRA D, STOLINA M, POLZER K *et al*.: Dickkopf-1 is a master regulator of joint remodeling 1. *Nat Med* 2007; 13: 156-63.
9. HEILAND GR, APPEL H, PODDUBNYY D *et al*.: High level of functional dickkopf-1 predicts protection from syndesmophyte formation in patients with ankylosing spondylitis. *Ann Rheum Dis* 2012; 71: 572-4.
10. DAOUSSIS D, LIOSSIS S-NC, SOLOMOU EE *et al*.: Evidence that Dkk-1 is dysfunctional in ankylosing spondylitis. *Arthritis Rheum* 2010; 62: 150-8.
11. DAOUSSIS D, TSAMANDAS A, ANTONOPOULOS I *et al*.: B cell depletion therapy upregulates Dkk-1 skin expression in patients with systemic sclerosis: Association with enhanced resolution of skin fibrosis. *Arthritis Res Ther* 2016; 18: 118.
12. AKHMETSHINA A, PALUMBO K, DEES C *et al*.: Activation of canonical Wnt signalling is required for TGF-β-mediated fibrosis. *Nat Commun* 2012; 3: 735.
13. VAN DEN HOOGEN F, KHANNA D, FRANSEN J *et al*.: 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. *Arthritis Rheum* 2013; 65: 2737-47.
14. BEYER C, SCHRAMM A, AKHMETSHINA A *et al*.: beta-catenin is a central mediator of profibrotic Wnt signaling in systemic sclerosis. *Ann Rheum Dis* 2012; 71: 761-7.
15. DEES C, SCHLOTTMANN I, FUNKE R *et al*.: The Wnt antagonists DKK1 and SFRP1 are downregulated by promoter hypermethylation in systemic sclerosis. *Ann Rheum Dis* 2014; 73: 1232-9.
16. WHITFIELD ML, FINLAY DR, MURRAY JI *et al*.: Systemic and cell type-specific gene expression patterns in scleroderma skin. *Proc Natl Acad Sci USA* 2003; 100: 12319-24.
17. SARGENT JL, WHITFIELD ML: Capturing the heterogeneity in systemic sclerosis with genome-wide expression profiling. *Expert Rev Clin Immunol* 2011; 7: 463-73.
18. GOLDRING SR, GOLDRING MB: Eating bone or adding it: the Wnt pathway decides 6. *Nat Med* 2007; 13: 133-4.
19. LI J, SAROSI I, CATTLEY RC *et al*.: Dkk1-mediated inhibition of Wnt signaling in bone results in osteopenia. *Bone* 2006; 39: 754-66.
20. MACDONALD BT, JOINER DM, OYSERMAN SM *et al*.: Bone mass is inversely proportional to Dkk1 levels in mice. *Bone* 2007; 41: 331-9.