ORIGINAL RESEARCH

Christina N. Raykha, BMSc[1,2,3]
Justin D. Crawford, BMSc[1,2,3]
Andrea F. Burry, MSc, MD [1,2,3]
Darren S. Drosdowech, MD, FRCTC [1,4]
Kenneth J. Faber MD, MHPE, FRCTC [1,3,4]
Bing Siang Gan MD, PhD, FRCTC, FACS [1,2,3,4,5]
David B. O'Gorman PhD[1,2,3,4,6]

Roth|McFarlane Hand and Upper Limb Centre[1], Cell and Molecular Biology Laboratory[2]
St. Joseph's Health Centre, Lawson Health Research Institute[3]
Departments of Surgery[4], Medical Biophysics[5] and Biochemistry[6], University of Western Ontario, London, Ontario, Canada

IGF2 expression and β-catenin levels are increased in Frozen Shoulder Syndrome

Abstract

Purpose: Frozen Shoulder Syndrome is a fibrosis of the shoulder joint capsule that is clinically associated with Dupuytren's disease, a fibrosis of the palmar fascia. Little is known about any commonalities in the pathophysiology of these connective tissue fibroses. β-catenin, a protein that transactivates gene expression, and levels of *IGF2* mRNA, encoding insulin-like growth factor-II, are elevated in Dupuytren's disease. The aim of this study was to determine if correlating changes in β-catenin levels and *IGF2* expression are evident in Frozen Shoulder Syndrome.

Methods: Tissue from patients with Frozen Shoulder Syndrome and rotator cuff tear were obtained during shoulder arthroscopies. Total protein extracts were prepared from tissue aliquots and β-catenin immunoreactivity was assessed by Western immunoblotting. In parallel, primary fibroblasts were derived from these tissues and assessed for *IGF2* expression by quantitative PCR.

Results: β-catenin levels were significantly increased in Frozen Shoulder Syndrome relative to rotator cuff tear when assessed by Western immunoblotting analyses. *IGF2* mRNA levels were significantly increased in primary fibroblasts derived from frozen shoulder syndrome tissues relative to fibroblasts derived from rotator cuff tissues.

Conclusions: As in Dupuytren's disease, β-catenin levels and *IGF2* expression are elevated in Frozen Shoulder Syndrome. These findings support the hypothesis that these connective tissue fibroses share a common pathophysiology.

Manuscript submitted 23rd May, 2014
Manuscript accepted 30th June, 2014

Clin Invest Med 2014; 37 (4): E262-E267.

Correspondence to:
Dr. David B. O'Gorman,
Roth|McFarlane Hand and Upper Limb Centre
268 Grosvenor Street. London, Ontario
Canada N6A 4V2
Email: dogorman@uwo.ca

Frozen Shoulder Syndrome (FSS) is a chronic fibrotic disorder of the shoulder joint capsule characterized by painful and restricted shoulder motion. This disorder affects an estimated 2% of adults, is frequently bilateral and can be resistant to therapeutic interventions. Despite its common occurrence, the underlying pathophysiology of this disorder remains ill-defined [1, 2]. Previous studies have suggested that FSS shares a similar patho-mechanism with Dupuytren's disease (DD) [1], and that these two connective tissue disorders are clinically associated [3-13]. Levels of β-catenin, an established signaling component of the Wnt/frizzled pathway [14-16], have been shown to be increased in DD [17]. More recently, expression of *IGF2*, encoding insulin-like growth factor-II (IGF-II), has been shown to be increased in DD [18]. In this report, β-catenin levels and *IGF2* expression were assessed in tissues and cells derived from the rotator cuff interval of patients with FSS and Rotator Cuff Tear (RCT) to determine if disease-associated changes in these molecules are also evident in FSS and RCT.

Materials and Methods

Tissue collection

Tissue sections were collected with approval of the Human Subjects Research Ethics Board (HSREB) at Western University from surgical specimens of patients undergoing shoulder arthroscopy for the treatment of either FSS or subacromial decompression for RCT. An arthroscopic punch was used to obtain tissue specimens from the rotator cuff interval immediately adjacent to the antero-superior arthroscopic portal from patients with FSS and RCT. Representative samples of these tissues were removed at the time of surgery and immediately transported to the laboratory. The tissues were either snap frozen in liquid nitrogen for total protein extraction or processed for primary fibroblast derivation.

Western immunoblotting

Total protein extracts were prepared from snap frozen tissue using modified RIPA buffer. Tissue lysate (25 μg) was subjected to Western blot analysis and β-catenin levels were assessed using an anti-β-catenin monoclonal antibody (clone 14, Transduction Laboratories, Lexington, KY). β-actin levels were assessed in parallel using an anti-β-actin antibody (Sigma, St Louis, MO) to normalize for variability in total protein loading. Antibody specific bands were visualized using enhanced chemiluminescence (ECL) and Kodak XLS film. Densitometry analysis was carried out using Scion Image software (Scion Corporation, Beta 4.0.2, Frederick, MD). Normalized measurements of β-catenin were plotted as the sample mean (β-catenin /actin) ratio ± standard error of the mean (SEM). Statistical analysis was performed using a paired t-test.

Primary fibroblast derivation and culture

Primary fibroblasts were isolated from FSS and RCT tissues using the same techniques as previously described for primary DD fibroblasts [19]. Cultures were maintained in α-MEM-medium supplemented with 10% fetal bovine serum (FBS, Invitrogen Corporation, Carlsbad, CA) and 1% antibiotic-antimycotic solution (Sigma-Aldrich, St Louis, MO). All primary fibroblast cultures were assessed at the lowest passage number achievable up to a maximum of six passages, after which the cells were discarded. No changes in cell morphology, growth and viability attributable to serial passage were evident in these cells (data not shown).

Real Time PCR

Total RNA samples from primary fibroblasts were assessed for quality on an Agilent 2100 Bioanalyzer. High quality total RNA (2 μg) was reverse transcribed into cDNA first strand using the High-Capacity cDNA Archive Kit (Applied Biosystems, city, state) in accordance with the manufacturer's instructions. TaqMan gene expression assays were used to measure *IGF2* expression (Hs01005963_m1) relative to the *RPLP0* (Hs99999902_m1) as an endogenous control gene. The ΔΔCt method was employed after confirmation of parallel PCR amplification efficiencies of the target and endogenous control on a Real-Time PCR ABI Prism 7500. PCR reactions were carried out under the following conditions: initial denaturation at 95°C for 5 min followed by cycles of denaturation (95°C for 15 sec), primer annealing (60°C for 1 min) and transcript extension (50°C for 2 min) for 45 cycles.

Results

β-catenin levels are increased in FSS relative to RCT

Western immunoblotting analyses for β-catenin and β-actin immunoreactivity were performed on total protein lysates of FSS and RCT tissues as described in the methods. The majority of samples derived from FSS exhibited increased levels of β-catenin relative to RCT tissues, which mostly expressed very low to undetectable levels of β-catenin, when normalized to β-actin levels as controls. Densitometry analysis confirmed a significant difference in β-catenin levels between FSS and RCT samples ($p<0.01$, Fig. 1).

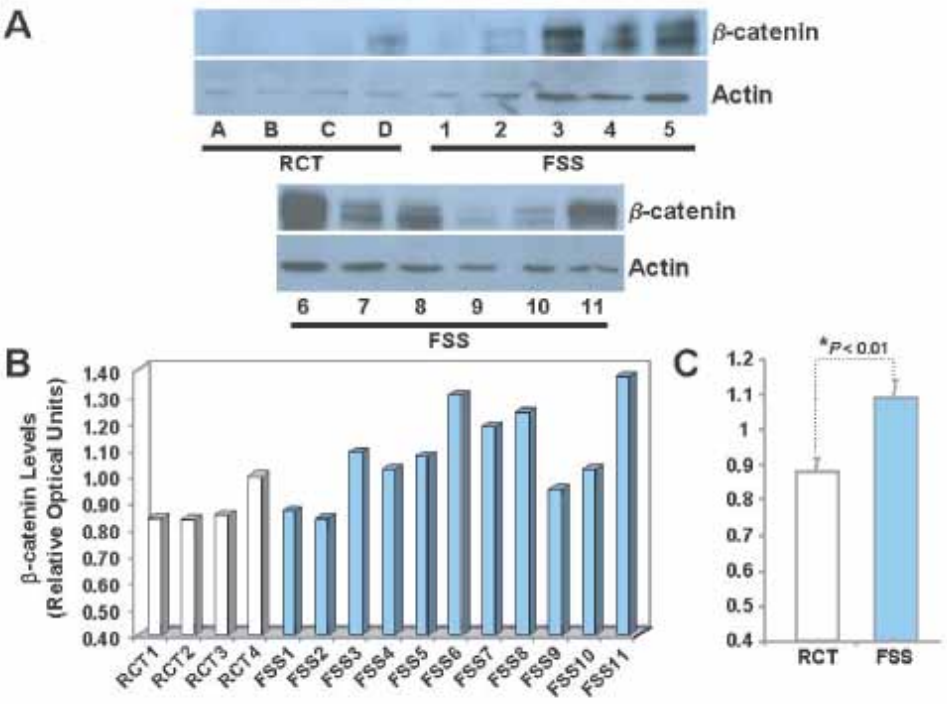

FIGURE 1. ***β-catenin accumulation in Frozen Shoulder Syndrome.*** Tissue extracts were prepared from surgical samples of patients with either Frozen Shoulder Syndrome (FSS), or rotator cuff tear (RCT). (**A**) Tissue lysates (25 μg) were subjected to Western immunoblotting and total β-catenin levels were assessed using an anti-β-catenin monoclonal antibody. β-actin levels were assessed to normalize protein loading between samples. Antibody specific bands were visualized by ECL on Kodak XLS film. (**B**) Densitometric analysis of the ECL exposed film was carried out using Scion Image software and normalized measurements of β-catenin were plotted as the sample mean (β-catenin /actin) ratio ± standard error of the mean (SEM). (**C**) Statistical analysis using a paired t-test ($p<0.01$).

IGF2 mRNA levels are increased in primary FSS fibroblasts relative to primary RCT fibroblasts

Quantitative (real time) PCR analyses for *IGF2* mRNA levels and *RPLP0* mRNA levels were performed on total RNA isolated from primary fibroblasts derived from FSS and RCT tissues as described in the methods. As shown in Fig 2, a significant ($p<0.05$) increase in *IGF2* mRNA levels was evident in the primary fibroblasts derived from FSS tissues relative to the primary fibroblasts derived from RCT tissues when normalized to *RPLP0* mRNA levels as controls.

Discussion

Increased β-catenin accumulation [17, 19] and *IGF2* expression [18] have been previously identified in DD tissue and primary fibroblasts compared with controls from adjacent, phenotypically non-fibrotic palmar fascia. As FSS and DD are clinically associated [3, 20], we were interested to see if these characteristics were common to both connective tissue fibroses. Our findings indicate that increased *IGF2* expression and β-catenin accumulation are also evident in FSS, supporting the hypothesis that these connective tissue fibroses share a common pathophysiology.

While β-catenin is best recognised as a component of the Wnt/frizzled signalling pathway, [15, 21], additional roles for β-catenin in the transforming growth factor (TGF)-β1 [22-24] and oxidative stress-activated [21] pathways have been described. β-catenin is also an integral component of adherens junctions [15, 25]; cell-membrane-associated structures that myofibroblasts use during cell to cell interactions to promote extra-cellular matrix contraction and remodelling during fibrosis development [26]. Increased β-catenin levels have been causatively linked to increased fibroblast proliferation in aggressive fibromatosis (desmoid tumor) [27, 28] and hypertrophic scar formation [22]. As these conditions share many molecular similarities with FSS and DD [18, 29], β-catenin is likely to play analogous fibroproliferative roles in these diseases. Western immunoblotting also revealed the presence of multiple molecular weight forms of β-catenin in FSS tissues, correlating with similar observations in DD tissues [17]. Whether these

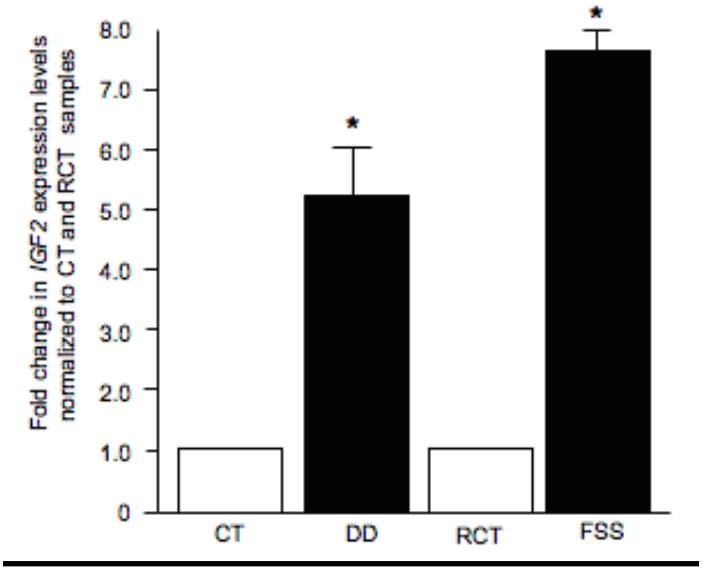

FIGURE 2. ***IGF2 expression in Dupuytren's Disease and Frozen Shoulder Syndrome.*** **A**) Quantitative (Real time) PCR analysis of *IGF2* mRNA levels in primary fibroblasts derived from normal palmar fascia (CT), Dupuytren's disease cord tissue (DD), rotator cuff tear (RCT) and Frozen Shoulder Syndrome (FSS) tissue. *IGF2* mRNA levels are increased in DD *vs.* CT and FS *vs.* RC (* $p<0.05$, mean ± SEM, paired t-test).

multiple forms of β-catenin in FSS indicate disease-associated changes in cytoplasmic β-catenin degradation and/or ubiquitination remains to be investigated.

IGF2 encodes insulin-like growth factor-II (IGF-II); a peptide growth factor with structural and functional similarities to insulin. In contrast to its well-established roles in promoting cancer growth [30-33], relatively little is known about the roles of IGF-II in benign fibroproliferative diseases like FFS and DD. In addition to DD [18], *IGF2* expression and IGF-II levels are reported to be increased in systemic sclerosis-associated pulmonary fibrosis [34]. IGF-II can act in combination with TGF-β1 signalling intermediates to promote myofibroblast development *in vitro* [35] and both IGF-II and TGF-β1 can independently induce β-catenin translocation to the nucleus during epithelial mesenchymal transition (EMT) [36]; a process implicated in both cancer and fibrosis development [37-39]. It is possible, therefore, that the increases in *IGF2* expression and β-catenin accumulation in FSS may represent components of the same or overlapping signalling pathways.

When assessed from this perspective, IGF-II and β-catenin may have potential as targets for therapeutic interventions to prevent FSS development. Humanized monoclonal antibodies that bind and inactivate the type-I IGF receptor (IGFRI), the primary IGF-II signaling receptor, are currently undergoing clinical trials to prevent IGF-II signaling in cancers [40-42]. These or similar interventions, potentially combined with TGF-β1 signaling suppressors [43], may have utility for preventing fibrosis development in FSS, DD and other fibroproliferative diseases that exhibit increased *IGF2* expression and β-catenin accumulation. A detailed understanding of their roles in fibrosis may also allow us to harness these factors to enhance tissue repair in conditions characterized by a loss of cell proliferation and/or viability, such as rotator cuff disease [44, 45].

Conclusion

This is first report of increased *IGF2* expression and β-catenin accumulation in FSS. These findings correlate with our previous reports in DD and support the hypothesis that these connective tissue fibroses have a common molecular pathophysiology. A greater understanding of these molecular pathways may enhance therapeutic interventions for FSS and may provide further insights into disorders that are characterized by abnormal healing.

Acknowledgments

This study was supported by the Canadian Orthopedic Foundation (KJF), and by grants from the Plastic Surgery Education Fund (235927) and the Canadian Institutes of Health Research (Operating Grant MOP 84247, BSG, DBO). The authors declare that they have no financial or other conflicts of interest to disclose in relation to this report.

References

1. Bunker TD, Reilly J, Baird KS, Hamblen DL. Expression of growth factors, cytokines and matrix metalloproteinases in frozen shoulder. J Bone Joint Surg Br 2000;**82**:768-773.
2. Rodeo SA, Suzuki K, Yamauchi M, Bhargava M, Warren RF. Analysis of collagen and elastic fibers in shoulder capsule in patients with shoulder instability. Am J Sports Med 1998;**26**:634-643.
3. Smith SP, Devaraj VS, Bunker TD. The association between frozen shoulder and Dupuytren's disease. J Shoulder Elbow Surg 2001;**10**:149-151.
4. Arkkila PE, Koskinen PJ, Kantola IM, Ronnemaa T, Seppanen E, Viikari JS. Dupuytren's disease in type I diabetic subjects: investigation of biochemical markers of type III and I collagen. Clin Exp Rheumatol 2000;**18**:215-219.
5. Noble J, Heathcote JG, Cohen H. Diabetes mellitus in the aetiology of Dupuytren's disease. J Bone Joint Surg [Br] 1984;**66**:322-325.

6. Hart MG, Hooper G. Clinical associations of Dupuytren's disease. Postgrad Med J 2005;**81**:425-428.
7. Cagliero E, Apruzzese W, Perlmutter GS, Nathan DM. Musculoskeletal disorders of the hand and shoulder in patients with diabetes mellitus. Am J Med 2002;**112**:487-490.
8. Arkkila PE, Kantola IM, Viikari JS. Dupuytren's disease: association with chronic diabetic complications. J Rheumatol 1997;**24**:153-159.
9. Arkkila PE, Kantola IM, Viikari JS, Ronnemaa T, Vahatalo MA. Dupuytren's disease in type 1 diabetic patients: a five-year prospective study. Clin Exp Rheumatol 1996;**14**:59-65.
10. Ravindran Rajendran S, Bhansali A, Walia R, Dutta P, Bansal V, Shanmugasundar G. Prevalence and pattern of hand soft-tissue changes in type 2 diabetes mellitus. Diabetes Metab 2012;**37**:312-317.
11. Redmond CL, Bain GI, Laslett LL, McNeil JD. Hand syndromes associated with diabetes: impairments and obesity predict disability. J Rheumatol 2009;**36**:2766-2771.
12. Cederlund RI, Thomsen N, Thrainsdottir S, Eriksson KF, Sundkvist G, Dahlin LB. Hand disorders, hand function, and activities of daily living in elderly men with type 2 diabetes. J Diabetes Complications 2009;**23**:32-39.
13. Akyol A, Kiylioglu N, Copcu E, Guney E, Aydeniz A. Is diabetes mellitus type 2 a risk factor for Dupuytren's contracture in the Mediterranean region? Plast Reconstr Surg 2006;**117**:2105-2106.
14. Widelitz R. Wnt signaling through canonical and non-canonical pathways: recent progress. Growth Factors 2005;**23**:111-116.
15. Bowley E, O'Gorman DB, Gan BS. Beta-catenin signaling in fibroproliferative disease. J Surg Res 2007;**138**:141-150.
16. Dolmans GH, Werker PM, Hennies HC, Furniss D, Festen EA, Franke L *et al.* Wnt Signaling and Dupuytren's Disease. N Engl J Med 2011.
17. Varallo VM, Gan BS, Seney S, Ross DC, Roth JH, Richards RS *et al.* Beta-catenin expression in Dupuytren's disease: potential role for cell-matrix interactions in modulating beta-catenin levels in vivo and in vitro. Oncogene 2003;**22**:3680-3684.
18. Raykha C, Crawford J, Gan BS, Fu P, Bach LA, O'Gorman DB. IGF-II and IGFBP-6 regulate cellular contractility and proliferation in Dupuytren's disease. Biochim Biophys Acta 2013;**1832**:1511-1519.
19. Howard JC, Varallo VM, Ross DC, Roth JH, Faber KJ, Alman B *et al.* Elevated levels of beta-catenin and fibronectin in three-dimensional collagen cultures of Dupuytren's disease cells are regulated by tension in vitro. BMC Musculoskelet Disord 2003;**4**:16.
20. Bunker TD, Anthony PP. The pathology of frozen shoulder. A Dupuytren-like disease. J Bone Joint Surg Br 1995;77:677-683.
21. Jin T, George Fantus I, Sun J. Wnt and beyond Wnt: multiple mechanisms control the transcriptional property of beta-catenin. Cell Signal 2008;**20**:1697-1704.
22. Cheon SS, Wei Q, Gurung A, Youn A, Bright T, Poon R *et al.* Beta-catenin regulates wound size and mediates the effect of TGF-beta in cutaneous healing. Faseb J 2006;**20**:692-701.
23. Amini Nik S, Ebrahim RP, Van Dam K, Cassiman JJ, Tejpar S. TGF-beta modulates beta-Catenin stability and signaling in mesenchymal proliferations. Exp Cell Res 2007;**313**:2887-2895.
24. Caraci F, Gili E, Calafiore M, Failla M, La Rosa C, Crimi N *et al.* TGF-beta1 targets the GSK-3beta/beta-catenin pathway via ERK activation in the transition of human lung fibroblasts into myofibroblasts. Pharmacol Res 2008;**57**:274-282.
25. Castano J, Raurell I, Piedra JA, Miravet S, Dunach M, Garcia de Herreros A. Beta-catenin N- and C-terminal tails modulate the coordinated binding of adherens junction proteins to beta-catenin. J Biol Chem 2002;**277**:31541-31550.
26. Hinz B, Pittet P, Smith-Clerc J, Chaponnier C, Meister JJ. Myofibroblast development is characterized by specific cell-cell adherens junctions. Mol Biol Cell 2004;**15**:4310-4320.
27. Alman BA, Li C, Pajerski ME, Diaz-Cano S, Wolfe HJ. Increased beta-catenin protein and somatic APC mutations in sporadic aggressive fibromatoses (desmoid tumors). Am J Pathol 1997;**151**:329-334.
28. Cheon SS, Cheah AY, Turley S, Nadesan P, Poon R, Clevers H *et al.* beta -Catenin stabilization dysregulates mesenchymal cell proliferation, motility, and invasiveness and causes aggressive fibromatosis and hyperplastic cutaneous wounds. Proc Natl Acad Sci U S A 2002;**30**:30.
29. Denys H, Jadidizadeh A, Amini Nik S, Van Dam K, Aerts S, Alman BA *et al.* Identification of IGFBP-6 as a significantly downregulated gene by beta-catenin in desmoid tumors. Oncogene 2004;**23**:654-664.
30. Lu L, Katsaros D, Wiley A, Rigault de la Longrais IA, Puopolo M, Schwartz P *et al.* Promoter-specific transcription of insulin-like growth factor-II in epithelial ovarian cancer. Gynecol Oncol 2006;**103**:990-995.
31. Zhang S, Wu X, Jiang T, Lu Y, Ma L, Liang M *et al.* The up-regulation of KCC1 gene expression in cervical cancer cells by IGF-II through the ERK1/2MAPK and PI3K/AKT pathways and its significance. Eur J Gynaecol Oncol 2009;**30**:29-34.
32. Kalla Singh S, Tan QW, Brito C, De Leon M, Garberoglio C, De Leon D. Differential insulin-like growth factor II (IGF-II) expression: A potential role for breast cancer survival disparity. Growth Horm IGF Res 2012;**20**:162-170.
33. Hwang PH, Kim SY, Lee JC, Kim SJ, Yi HK, Lee DY. PTEN/MMAC1 enhances the growth inhibition by anticancer drugs with downregulation of IGF-II expression in gastric cancer cells. Exp Mol Med 2005;**37**:391-398.
34. Hsu E, Feghali-Bostwick CA. Insulin-like growth factor-II is increased in systemic sclerosis-associated pulmonary fibrosis and contributes to the fibrotic process via Jun N-terminal kinase- and phosphatidylinositol-3 kinase-dependent pathways. Am J Pathol 2008;**172**:1580-1590.

35. Grotendorst GR, Rahmanie H, Duncan MR. Combinatorial signaling pathways determine fibroblast proliferation and myofibroblast differentiation. Faseb J 2004;**18**:469-479.
36. Morali OG, Delmas V, Moore R, Jeanney C, Thiery JP, Larue L. IGF-II induces rapid beta-catenin relocation to the nucleus during epithelium to mesenchyme transition. Oncogene 2001;**20**:4942-4950.
37. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. Evidence that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest 2002;**110**:341-350.
38. Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest 2003;**112**:1776-1784.
39. Radisky DC, Kenny PA, Bissell MJ. Fibrosis and cancer: do myofibroblasts come also from epithelial cells via EMT? J Cell Biochem 2007;**101**:830-839.
40. Riedemann J, Macaulay VM. IGF1R signalling and its inhibition. Endocr Relat Cancer 2006;**13 Suppl 1**:S33-43.
41. Schmitz S, Kaminsky-Forrett MC, Henry S, Zanetta S, Geoffrois L, Bompas E *et al.* Phase II study of figitumumab in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck: clinical activity and molecular response (GORTEC 2008-02). Ann Oncol 2012;**23**:2153-2161.
42. Olmos D, Postel-Vinay S, Molife LR, Okuno SH, Schuetze SM, Paccagnella ML *et al.* Safety, pharmacokinetics, and preliminary activity of the anti-IGF-1R antibody figitumumab (CP-751,871) in patients with sarcoma and Ewing's sarcoma: a phase 1 expansion cohort study. Lancet Oncol 2013;**11**:129-135.
43. Katz LH, Li Y, Chen JS, Munoz NM, Majumdar A, Chen J *et al.* Targeting TGF-beta signaling in cancer. Expert Opin Ther Targets 2013;**17**:743-760.
44. Nho SJ, Delos D, Yadav H, Pensak M, Romeo AA, Warren RF *et al.* Biomechanical and biologic augmentation for the treatment of massive rotator cuff tears. Am J Sports Med 2013;**38**:619-629.
45. Longo UG, Rizzello G, Berton A, Maltese L, Fumo C, Khan WS *et al.* Biological Strategies to Enhance Rotator Cuff Healing. Curr Stem Cell Res Ther 2013.

