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# Snail2 promotes osteosarcoma cell motility through remodelling of the actin cytoskeleton and regulates tumor development

Amir-Shaya Sharili [a,d], Steve Allen [a], Ken Smith [b], Joanna Price [c], Imelda M. McGonnell [a,*]

[a] Department of Comparative Biomedical Sciences, Royal Veterinary College, London, UK
[b] Department of Pathology and Pathogen Biology, Royal Veterinary College, London, UK
[c] Department of Clinical Veterinary Sciences, University of Bristol, Langford House, Langford, North Somerset, UK
[d] Barts and the London School of Medicine and Dentistry, Blizard Institute, 4 Newark Street, London E1 2AR, UK

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ABSTRACT

The function of Snail2 in mesenchymal tumors is, to date unknown. Using knockdown and overexpression studies, we show that Snail2 regulates migration and invasion of osteosarcoma cells. Knockdown resulted in significantly decreased motility, remodelling of the actin cytoskeleton, and loss of cellular protrusions. Over-expression increased motility, formation of actin-rich cellular protrusions, and altered expression of some non-canonical Wnt pathway components whilst decreasing expression of the adhesion molecule OB-cadherin. Unexpectedly, knockdown also resulted in significantly smaller tumors in an *in vivo* CAM assay. Therefore Snail2 may be a potential therapeutic target for clinical intervention of osteosarcoma.

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## 1. Introduction

In epithelial tumor types (e.g. breast, lung and ovarian) the functions of the Snail zinc finger transcriptional repressors have been extensively studied [1]. In this context, the key function of Snail2 is similar to its function in embryonic epithelial tissues, namely the promotion of epithelial-to-mesenchymal transition (EMT) [2]. The mechanism of action is also similar, utilizing transcriptional repression of epithelial cellular adhesion molecules, including E-cadherin, thus allowing cells to break their cell to cell contacts [3–5], which is an early step in the process of EMT.

During embryonic development Snail2 is present in one tissue of mesenchymal origin, namely the developing long bone [6]. However its functions in this tissue are largely unknown. Interestingly expression is lost with age and in post-natal bone *in vivo*, Snail2 is absent (unpublished observations).

In a recent study, we demonstrated that Snail2 is expressed in long bone canine osteosarcomas; tumors of mesenchymal origin [7]. Furthermore our study also showed that there was a strong correlation between levels of Snail2 and grade (malignancy) of these osteosarcomas. This suggests that the re-expression of high levels of Snail2 in this tumor type may, in part, be responsible for increasing malignancy. Since osteosarcomas are mesenchymal tumors the function of Snail2 cannot be to drive changes in epithelial cell adhesion during EMT, suggesting that it most likely has other unknown functions in these, and possibly other, mesenchymally derived tumor types.

In order to investigate the function of Snail2 in osteosarcoma, we generated stable cell lines in which loss of Snail2 function was achieved using small interfering RNA and gain of function using CMV promoter driven over-expression. The motility of these tumor cells in vitro was assessed using a scratch assay and tumor forming ability together with vascular invasion determined in an *in vivo* model. Knockdown of Snail2 resulted in reduced motility while over expression of Snail2 resulted in increased motility. These changes in motility were associated with changes in the polymerization of the actin cytoskeleton and in focal adhesions as well as altered expression of Wnt5a, sFRP2 and osteoblast cadherin (OB-Cad). Reduction of Snail2 expression also resulted in reduced tumor forming ability in an *in vivo* assay. These data indicate a role for Snail2 in both motility and tumor formation.

## 2. Materials and methods

### 2.1. Establishment of stable osteosarcoma cell lines

Stable Snail2 cell lines were derived from canine D-17 and human Saos-2 osteosarcoma cell lines. Knock-down cell lines were produced by stable integration of

* Corresponding author. Address: Department of Comparative Biomedical Sciences, The Royal Veterinary College, Royal College Street, Camden, London NW1 0TU, UK. Tel.: +44 020 7468 1223; fax: +44 020 7468 5204.
*E-mail addresses:* a.s.sharili@qmul.ac.uk (A.-S. Sharili), sallen@rvc.ac.uk (S. Allen), ksmith@rvc.co.uk (K. Smith), jo.price@bristol.ac.uk (J. Price), imcgonnell@rvc.ac.uk (I.M. McGonnell).

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shRNA producing plasmid vector, pLVX-shRNA2 (Clontech), using a previously characterized human Snail2 sequence (5′-GGACCACAGTGGCTCAGAA-3′) [8], also present in dog. Control vector contained a target sequence for eGFP. For Snail2 overexpression, the coding sequence of human Snail2, minus the stop codon, was inserted into pcDNA3.1 (Invitrogen) in frame with eGFP, producing a Snail2-eGFP fusion protein. Control vector contained the eGFP coding sequence. Cells were transfected with construct, plated in 100 mm culture dishes and selected with G418 and presence of GFP. Clonal colonies (2–3) of positive cells were ring cloned and individually amplified. A representative clone from each cell line was included. Overexpression and down-regulation of Snail2 was confirmed by immunofluorescent and qRT-PCR analysis. Cells from passages 4–10 were used in subsequent experiments.

### 2.2. Cell culture

Cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin. For scratch assays and qRT-PCR, $2.5 \times 10^5$ cells were plated onto 12-well plates and 60 mm culture dishes respectively. For immunofluorescence $5 \times 10^4$ cells were plated onto 13 mm diameter thermanox coverslips (Nunc, Rochester, NY). For Wnt5a blocking experiments *in vitro*, Saos-2 cells overexpressing Snail2 were plated onto 13 mm diameter coverslips and treated for either 4 or 24 h with anti-Wnt5a antibody (2 μg/ml; R&D Systems, [9]. Cells were fixed in 4% paraformaldehyde and their actin cytoskeleton visualized using Rhodamine pahalloidin (Invitrogen Molecular Probes, UK)

### 2.3. Scratch assays

Confluent cell monolayers were wounded with a pipette tip to obtain two perpendicular wounds, forming a cross shape. Wounds were photographed at 0, 24 and 72 h using an inverted microscope (Leica, Solms, Germany). Average distances between wound edges were calculated by measuring the uncovered wound area and dividing by the width of the field of view. Distance migrated was calculated by subtracting the average distance between wound edges from that at time 0. For each experiment a total of 12 wounds were measured per group, and each experiment was repeated three times.

### 2.4. In vivo cell invasion assays

Fertilized white leghorn chicken eggs (Henry Stewart, UK) were incubated at 37 °C. On day 9 of development [10] the chorio-allantoic membrane (CAM) surface was gently lacerated with filter paper, and a plastic ring (6 mm inner diameter) placed on this region. 25 μl of medium containing $3 \times 10^5$ control or Snail2 knockdown cells was added to the ring and the eggs re-incubated for a further 7 days before CAMs were excised and fixed in 4% PFA. Tumor size and cell motility were assessed using a Nikon SMZ1500 microscope and DS-2Mv digital fluorescent camera (Nikon Instruments Inc., Japan). Tumor areas from two separate experiments were measured using ImageJ software (NIH, Maryland, US).

### 2.5. Immunofluorescence

Cells were fixed with 4% PFA for 10 min, and incubated on ice for 10 min in permeabilization buffer (20 mM Hepes, 300 mM Sucrose, 50 mM NaCl, 0.5% TX-100, 3 mM $MgCl_2$, 0.05% Sodium Azide, pH 7.0). Cells were blocked in 10% Calf Serum and incubated with primary antibody overnight at 4 °C. Primary antibodies used were rabbit anti-Snail2 (4 mg/ml; Santa Cruz, Inc., USA.) and rabbit anti-Paxillin (4 μg/ml; Santa Cruz). Coverslips were washed and incubated for 45 min with biotin conjugated goat anti-rabbit secondary antibody (Dako, Glostrup, Denmark) followed by Strepavidin, Alexa Fluor® 555 or 633 (Invitrogen Molecular Probes, UK). Rhodamine–phalloidin staining was used to visualize actin (Invitrogen Molecular Probes, UK). Imaging was performed with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Inc., Thornwood, NY) or Leica DM4000B light microscope (Leica, Solms, Germany).

### 2.6. Quantitative real-time RT-PCR

Total RNA was isolated from cells using an RNeasy Plus Mini Kit (Qiagen). cDNA was synthesized using Superscript II Reverse transcriptase (Invitrogen Ltd., Paisley, Scotland, UK) and random hexamer primers. Quantitative real time RT-PCR (qRT-PCR) was carried out as previously described [11] using QuantiTect SYBR Green PCR kit and Opticon 2 LightCycler (MJ Research, Waltham, MA). Primers used were against Snail2, OB-Cadherin, Wnt5A, sFRP2 and the housekeeping genes β-actin, GAPDH and 18S (sequences and conditions in supplemental information). A relative standard curve was constructed for Snail2, OB-cadherin, Wnt5A, sFRP2 and the housekeeping genes using serial dilutions of their amplicons, and these standard curves were included in each run. Standards were run in duplicate and samples in triplicate. The expression levels for all the genes analyzed were normalized to β-actin, GAPDH and 18S.

### 2.7. Statistical analysis

Data are presented as mean ± standard deviation. Statistical comparison of each Snail2 modified cell line and their appropriate control cell line was performed using the Student's *t* test in Microsoft Excel. In all cases, $P < 0.05$ was considered significant.

## 3. Results

### 3.1. Generation of stable Snail2 over-expressing/knock-down osteosarcoma cell lines

To investigate the functional role of Snail2 in osteosarcoma tumorigenesis, stable cell lines were produced which either overexpressed or had reduced levels of Snail2. Overexpression and knock-down were confirmed by immunohistochemistry and qRT-PCR. Antibody labeling showed that levels of nuclear Snail2 protein were increased in both D-17 and Saos-2 overexpressing cells compared to controls (Fig. 1a). In contrast, knockdown cells showed reduced levels of nuclear Snail2 expression compared to controls in both cell lines (Fig. 1a). Analysis of Snail2 expression by qRT-PCR in D-17 cell lines matched the results seen for immunostaining (Fig. 1b and c). Snail2 transcript levels were increased and decreased for overexpression and knockdown lines respectively, and these changes were maintained over time in culture (Fig. 1b and c). In Saos-2 Snail2 overexpressing cells, increase in levels of Snail2 transcripts were only evident at later passage numbers (Fig. 1d), even though both low and high passage cells produced exogenous GFP tagged Snail2 from the inserted vector. Equally while immunostaining showed a decrease in Snail2 protein levels in shRNA cells (a decrease similar to that seen in another study using the same target sequence in an ovarian cell line [8]), qRT-PCR analysis showed no decrease (Fig. 1e). This may be due to an imperfect match between target and sequence which can produce inhibition without mRNA cleavage. Cell lines are known to possess SNPs and other genetic changes that may affect perfect matching and cleavage [12–14] and thus result in the apparent discrepancy between the mRNA and protein levels.

### 3.2. Snail2 modifies osteosarcoma cell morphology

Control cell lines maintained the same osteoblast-like phenotype as parental Saos-2 (human Fig. 2a: A and B and E and F) and D-17 (canine Fig. 2b: A and B and E and F) osteosarcoma cell lines. However, D-17 Snail2 shRNA cells showed a loss of normal osteoblast morphology, losing their characteristic spindle shape and becoming more polygonal or stellate (compare Fig. 2b: E and G), while Saos-2 Snail2 shRNA cells appeared relatively unchanged (compare Fig. 2a: E and G). Cell morphology was largely normal in D-17 cells overexpressing Snail2 (compare Fig. 2b: A and C), however Saos-2 cells overexpressing Snail2 showed an abnormal amoeboid appearance (compare Fig. 2a: A and C).

### 3.3. Snail2 regulates osteosarcoma cell motility

Scratch assays were performed to investigate changes in cell motility in Snail2 overexpressing and siRNA osteosarcoma cell lines. Knock-down of Snail2 in both canine and human osteosarcoma cells resulted in a significant reduction in motility from the wound edge compared to control cells (Fig. 3a and b). Overexpression of Snail2 significantly increased motility in Saos-2 but not D-17 osteosarcoma cells (Fig. 3a and b). Actin staining of Snail2 modified cells at the wound edge revealed morphological modifications. Control cells at the leading edge showed prominent cytoplasmic protrusions into which actin stress fibers extended

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