Dynamic Regulation of *TWIST1* Expression during Chondrogenic Differentiation of Human Bone-Marrow Derived Mesenchymal Stem Cells

Mairéad A. Cleary[1,2], Roberto Narcisi[2], Anna Albiero[2], Florien Jenner[3], Laurie M.G. de Kroon[2,4], Wendy J.L.M. Koevoet[5], Pieter A.J. Brama[1*], Gerjo J.V.M. van Osch[2,5*]

1 School of Veterinary Medicine, University College Dublin, Section Veterinary Clinical Sciences, Dublin, Ireland
2 Erasmus MC, University Medical Center, Department of Orthopaedics, Rotterdam, The Netherlands
3 University of Veterinary Medicine Vienna, Equine Hospital, Vienna, Austria
4 Experimental Rheumatology, Radboud University Medical Center, Nijmegen, The Netherlands
5 Erasmus MC, University Medical Center, Department of Otorhinolaryngology, Rotterdam, The Netherlands
*Both authors contributed equally to this manuscript

ABSTRACT

Mesenchymal stem cells (MSCs) are clinically promising to repair and regenerate damaged articular cartilage. However, an incomplete understanding of the mechanisms that regulate chondrogenic differentiation impedes clinical progress. This study investigated *TWIST1*, an important transcriptional regulator in mesenchymal lineages, in adult human bone marrow-derived MSC (BMSC) chondrogenesis. We hypothesized that downregulation of *TWIST1* expression is required for *in vitro* chondrogenic differentiation. Indeed, significant downregulation of *TWIST1* was observed in murine skeletal progenitor cells during limb development (N=3 embryos), and during chondrogenic differentiation of culture expanded human articular chondrocytes (N=3 donors) and isolated adult human BMSCs (N=6 donors), consistent with an inhibitory effect of *TWIST1* expression on chondrogenic differentiation. Silencing of *TWIST1* expression in BMSCs by siRNA, however, did not improve chondrogenic differentiation potential, measured by thionine staining of glycosaminoglycan content and *COL2A1* and *SOX9* gene expression. Interestingly, additional investigation of *TWIST1* expression in BMSCs revealed that downregulation of *TWIST1* in chondrogenic BMSCs is preceded by initial upregulation at day 1 of pellet culture. Similar upregulation is observed in non-chondrogenic BMSCs (N=4 donors), however, they fail to downregulate *TWIST1* expression thereafter, preventing their chondrogenic differentiation. This study, therefore, describes for the first time endogenous *TWIST1* expression during *in vitro* chondrogenic differentiation of human BMSCs, demonstrating dynamic regulation of *TWIST1* expression whereby upregulation and then downregulation of *TWIST1* expression is required for chondrogenic differentiation of BMSCs. Elucidation of the molecular regulation of, and by *TWIST1*, will provide targets for optimization of BMSC chondrogenic differentiation culture for clinical application.

Keywords: TWIST1; Mesenchymal Stem Cells; Chondrogenesis; Cartilage

Financial Support: This research was financially supported by Science Foundation Ireland (SFI; 11/RFP/BMT/3150), the Netherlands Institute for Regenerative Medicine (NIRM; FES0908) and the Netherlands Organisation for Health Research and Development (ZonMw) Translational Adult Stem Cell Research programme (TAS; 116005009). R.N. was further supported by a VENI grant from the Dutch Technology Foundation (STW, 13659). None of aforementioned had any involvement in the design or interpretation of data of this study.

INTRODUCTION

Formation of cartilage tissue *in vitro* from mesenchymal stem cells (MSCs) has emerged as a leading strategy for replacement and repair of damaged articular cartilage. Although lineage specific factors have been identified and induced *in vitro*, development of clinically useful constructs has not yet been realized. A broader understanding of the molecular mechanisms governing *in vitro* chondrogenesis is still required.

The twist family basic helix-loop-helix transcription factor 1 (*TWIST1)* is expressed in developing skeletal mesenchyme, where it regulates mesenchymal cell fate [1-5]. Consistent with this, some skeletal dysplasia, characterized by abnormal limb development and premature osteogenesis, occur as a result of *TWIST1* haploinsufficiency [6-9]. This latter observation, differentiation of osteoprogenitor cells triggered by decreased TWIST1, has led to investigation of the potential of TWIST1 as a target to facilitate osteogenic differentiation of mesenchymal stem cells (MSCs) for clinical application [10]. Notably, the initiation of osteoblast differentiation is determined by removal of direct TWIST1 inhibition on RUNX2, the master transcriptional regulator of osteogenic differentiation [11]. That TWIST1 is similarly capable of directly binding to and inhibiting SOX9 [12], the master transcriptional regulator of chondrogenesis, poses the question of whether TWIST1 could likewise be an *in vitro* target for optimization of MSC chondrogenic differentiation.

Several studies have previously demonstrated TWIST1 repression of chondrogenesis and chondrocyte maturation downstream of β-CATENIN and TGF-β signalling in murine and chick models [13-15]. Little is known, however, of the role of TWIST1 during human chondrogenic processes; a single study demonstrating significant downregulation of *SOX9* expression and glycosaminoglycan production in human MSCs overexpressing *TWIST1* [16], is, to our knowledge, the only available data. The aim of this study was, therefore, to analyze the endogenous expression profile of *TWIST1* during chondrogenesis. We hypothesized that downregulation of *TWIST1* expression is necessary and sufficient for chondrogenic differentiation of adult human bone marrow-derived MSCs (BMSCs). In this study we demonstrate dynamic regulation of *TWIST1* expression, with both upregulation and downregulation of *TWIST1* necessary for *in vitro* BMSC chondrogenic differentiation.

MATERIAL & METHODS

Isolation and culture

Primary human articular chondrocytes (HACs) from 3 donors (67–83 years) undergoing total knee arthroplasty were harvested from the femoral condyles

(MEC2004-322: Erasmus Medical Centre, Rotterdam). A single cell suspension was obtained following digestion with 2 mg/ml protease XIV (Sigma-Aldrich, St. Louis, Mo, USA), followed by 1.5 mg/ml collagenase B (Roche Diagnostics, Mannheim, Germany). HACs were seeded (4,000 cells/cm^2) and expanded (de-differentiated) in Coon's modified Ham's-F12 (Biochrom A.G., Berlin, Germany) containing 10 % fetal calf serum (FCS; Invitrogen, Paisley, Scotland, UK). Medium was renewed three times per week and HACs were used at passage 2 for all experiments.

After obtaining informed consent, bone marrow aspirates from the iliac crest were collected from 10 patients (15-74 years) undergoing total hip replacement (MEC 2004-142: Erasmus Medical Center, Rotterdam; MEC 2011.07: Albert Schweitzer Hospital, Dordrecht). Primary human adult BMSCs were selected by plastic adherence and cultured at a density of 2,300 cells/cm^2 at 37°C, 5 % CO_2, 21 % O_2 in α-MEM (Gibco, Carlsbad, CA, USA) containing 10 % FCS (Lonza, Verviers, Belgium), 1 ng/ml fibroblast growth factor-2 (FGF2; AbD Serotec Kidlington, United Kingdom), 10^{-4} M ascorbic acid-2-phosphate (Sigma-Aldrich), 1.5 μg/ml fungizone and 50 μg/ml gentamicin (Gibco). Medium was renewed twice weekly and BMSCs were used at passage 3-5 for experiments throughout this study.

To assess the effect of transforming growth factor-β1 (TGFβ1) on TWIST1 expression in monolayer, normal expansion medium was replaced 24 h after seeding (22,000 cells/cm^2) with α-MEM containing 10^{-4} M ascorbic acid-2-phosphate and either 1 ng/ml or 10 ng/ml TGFβ1 (R&D systems, Minneapolis, MN, USA) for 24 h.

TWIST1 silencing

Cells were seeded (4,000 – 5,700 cells/cm^2; Day0) and cultured for 24 h in the above mentioned expansion medium. Medium was then replaced (Day1) with expansion medium containing either 3.5 nM siRNA against *TWIST1* (siTWIST1; Ambion) or 3.5 nM non-targeting negative control siRNA (scramble, scTWIST1; Ambion). Treatment was repeated again after 72 h (Day4). All conditions, including control conditions (Ctrl), were also treated with Lipofectamine RNAiMAX carrier (1:2300; Invitrogen, California, USA) and OptiMEM (Gibco) to improve transfection efficiency. After 48 h (Day6), cells were harvested for chondrogenic culture. Medium of cells for mRNA and protein analysis was replaced with expansion medium only, 24 h before harvesting.

Chondrogenic differentiation

Following expansion or 48 h after the second silencing treatment, HAC pellets and BMSC pellets containing 2x10^5 cells were formed by centrifugation at 250 g and cultured for 1 or 21 days in chondrogenic induction medium consisting of DMEM-HG supplemented with Glutamax, ITS+1 (B&D Bioscience, Bedford, MA, USA), 40

µg/ml L-proline (Sigma-Aldrich), 1 mM sodium-pyruvate (Gibco, Carlsbad, CA, USA), 10 ng/ml TGFβ1, 10^{-4} M ascorbic acid-2-phophate, 5 µg/ml fungizone, 50 µg/ml gentamicin and 10^{-7} M dexamethasone (Sigma-Aldrich). Medium was renewed twice per week.

Gene expression analysis

For mRNA analysis, both HAC (1 well/donor 24 h after last refresh) and BMSC monolayer cells (3 wells/donor 24 last refresh) were washed with PBS and treated on ice with RLT Plus or RLT lysis buffer (Qiagen GmbH, Hilden, Germany) containing 1% β-mercaptoethanol. HAC pellets (2 pellets/donor 24 h after last refresh) and MSC pellets (2-3 pellets/donor 24 – 96 h after last refresh) were manually homogenized in RLT Plus lysis buffer, or RNA-Bee (TEL-TEST, Friendswood, TX, USA) with RNA extracted by addition of 20 % chloroform. Further RNA isolation and purification was performed using the RNeasy PLUS or RNeasy MicroKit (Qiagen). cDNA was prepared using the RevertAid First-Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany) according to manufacturer's instructions and qRT-PCR performed with TaqMan Universal PCR MasterMix (Applied Biosystems, Capelle a/d Ijssel, The Netherlands) or SybrGreen (Eurogentec, Seraing, Belgium). Primer sequences (Applied Biosystems) are listed in Table 1. After comparison of housekeeping genes (*GAPDH*, *RPS27a*, *HPRT1*, *B2M*, *18S*), *GAPDH* for chondrocytes and *RSP27A* for BMSCs were chosen for their stability across all conditions. Relative gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ formula [17].

Table 1. Primer sequences

Gene name	*Sequence*	*Company*
TWIST1	Assay on Demand: Hs01675818_s1	Applied Biosystems Capelle a/d IJssel, The Netherlands
COL2A1	F: 5′-GGCAATAGCAGGTTCACGTACA-3′	Eurogentec
	R: 5′-CGATAACAGTCTTGCCCCACTT-3′	
SOX9	F: 5′-TCCACGAAGGGCCGC-3′	Eurogentec
	R: 5′-CAACGCCGAGCTCAGCA-3′	
RUNX2	F: 5′-ACGTCCCCGTCCATCCA-3′	Eurogentec
	R: 5′-TGGCAGTGTCATCATCTGAAATG-3′	
MYC	F: 5′-GGCCTCCTGGCAAAAGGTC-3′	Erasmus MC (Internal)
	R: 5′-CTGCGTAGTTGTGCTGATGT-3′	
GAPDH	F: 5′-GTCAACGGATTTGGTCGTATTGGG-3′	Eurogentec
	R: 5′-TGCCATGGGTGGAATCATATTGG-3′	
RPS27A	F: 5′-TGGCTGTCCTGAAATATTATAAGGT-3′	Biolegio BV Nijmegen, The Netherlands
	R: 5′-CCCCAGCACCACATTCATCA-3′	

Western blot

Cells were washed with PBS, and on ice, incubated in M-PER containing 1% Halt™ Protease Inhibitor Cocktail and 1% Halt™ Phosphatase Inhibitor (Thermo Scientific). Cell lysate was harvested and total protein content determined using a BCA assay (Thermo Scientific). For western blot analysis, 8 µg of total protein was electrophoresed on an 8-16 % SDS-polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). After blocking with TBS-0.1 % Tween 20 (TBS-T) and 5-8 % bovine serum albumin (BSA) for 2 h, the membrane was probed with one of the following antibodies: mouse anti-human TWIST1 (1:400; Santa Cruz Biotechnology, CA, USA) or mouse anti-human α-tubulin (1:1000; Santa Cruz Biotechnology) in 5 % BSA. After washing with TBS-T, the membranes were incubated with peroxidase-conjugated anti-mouse antibody (1:2000; Dako, Glostrup, Denmark) in 2.5 % nonfat dried milk.

Quantification of blots was carried out using free ImageJ software with measurements recorded by 2 independent researchers.

(Immuno)Histochemistry analysis and quantification

After chondrogenic differentiation for 1 day or 3 weeks as described above, 3 pellets/donor were collected for histochemical analysis. Pellets were fixed for 24 h in 4 % formalin before paraffin-embedding. Sections (6 µm) were stained to assess glycosaminoglycan (GAG) content with 0.04% thionine solution. To determine proliferative activity, antigen retrieval was performed at 95°C in 10 mM citrate buffer (pH6) followed by incubation with a rabbit anti-human KI67 primary antibody (1:75; Novus Biologics, LLC, Littleton, CO, USA). Staining was developed using biotinylated anti-rabbit Ig (LINK; Biogenex, HK-325-UK) and alkaline phosphatase-conjugated streptavidin (LABEL; Biogenex, HK-321-UK) in combination with Neu Fuchsine substrate. Negative controls were treated with an isotype IgG rabbit antibody. Quantification of KI67 staining was performed by manual counting of cells (> 1400 cells/condition; 2-3 pelelts/donor) across multiple randomly taken images.

Murine articular cartilage development

Microarray analysis was performed as previously described [18]. Briefly, embryos were recovered on gestational day 11.5 (E11.5) and E13.5 from three timed pregnant outbred CD-1 IGS mice (Charles River Laboratories, Sulzbach, Germany & Margate, UK; AREC-P-10-47). Gestational stage E0.5 was considered as noon of the day the vaginal plug was first detected. Cryosections of hindlimbs stained with Cresyl Violet were prepared for harvesting of the intermediate layer (II) and the outer layer (OI) of the femorotibial interzone (IZ) that gives rise to the stable articular cartilage, and the femoral and tibial transient embryonic cartilage (EC) that is the cartilage template for future long bones, by laser capture microdissection (Carl Zeiss

Microscopy GmbH). Three biological replicates, each from a different litter, were harvested. Total RNA was extracted, amplified and labelled using the Agilent Low Input Quick Amp Labelling Kit (Agilent Technologies) and cRNA was hybridized to Agilent Whole Mouse Genome Oligo Microarrays (Agilent SurePrint G3 Mouse 8x60L Microarray, Agilent Technologies). Fluorescence signals of the hybridized microarrays were detected using Agilent's Microarray Scanner System, which were read and processed by Agilent Feature Extraction Software (FES).

Data analysis

Data were analyzed with PSAW statistics 20 software (SPSS Inc., Chicago, IL, USA). Normality and variance were determined by the Shapiro-Wilks test and Levene's Test of Homogeneity. In the case of normally distributed data with equal variance, a Student's t-test or one-way ANOVA with Bonferroni post hoc analysis was performed. A Mann-Whitney U test or Kruskal-Wallis test with pairwise comparisons was performed in the case of non-normally distributed data. In cases where the assumption of variance assumptions was not met, a one way ANOVA (Welch's F test) with Games-Howell post hoc analysis was performed. Data was deemed statistically significant for $p < 0.05$.

RESULTS

Downregulation of *TWIST1* expression occurs with chondrogenic differentiation

To understand whether *TWIST1* is involved in cartilage development and formation, we investigated the pattern of *Twist1* gene expression in the mesenchyme of developing embryonic murine limbs. Comparing *Twist1* expression in mesenchymal cells of the joint interzone (13.5II and 13.5OI) and transient epiphyseal cartilage (13.5EC) with cells of the mesenchymal condensation from which they arise (11.5M; Figure 1a), a significant downregulation in *Twist1* expression was observed in all tissues (Figure 1b). Despite this, and consistent with its hypertrophic phenotype (Figure 1a), only 13.5EC mesenchymal cells displayed a significant corresponding upregulation of all chondrogenic differentiation markers examined: *Col2a1* ($p=0.001$), *Sox9* ($p=0.001$) and *Runx2* ($p=0.013$; Figure 1b). Of the interzone layers from which the articular cartilage arises, mesenchymal cells of 13.5II failed to significantly upregulate any of the same differentiation markers while mesenchymal cells of 13.5OI upregulated *Col2a1* ($p=0.007$) and *Sox9* ($p<0.001$). This led to investigation of the involvement of *TWIST1* expression in the chondrogenic program of stable articular cartilage chondrocytes (Figure 2). After 3 weeks of HAC redifferentiation, significantly lower *TWIST1* expression was observed than

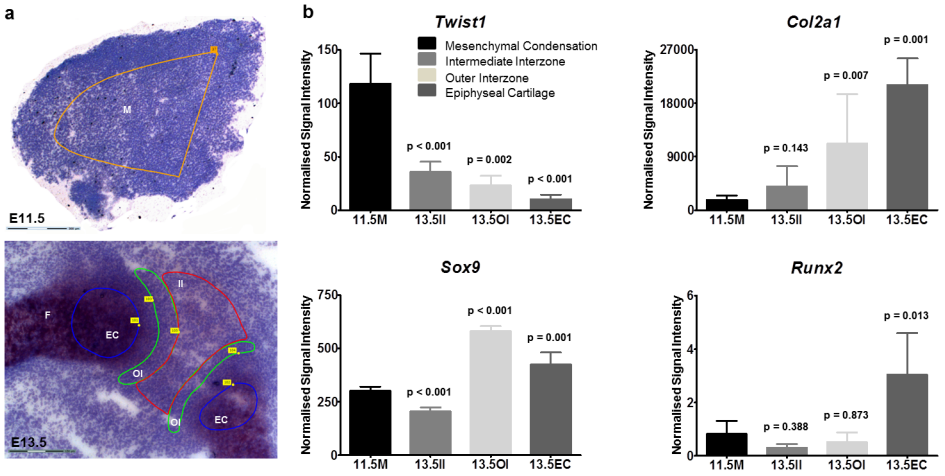

Figure 1. *Twist1* expression decreases during embryonic cartilage development.

(a) Cresyl violet stained sagittal sections of embryonic hindlimbs prepared for laser capture microdissection, **(b)** Normalized signal intensity of *Twist1*, *Col2a1*, *Sox9* and *Runx2* during murine embryonic cartilage development, gestational stage 11.5 and 13.5. M – mesenchymal condensation, II – intermediate interzone, OI – outer interzone, EC – transient epiphyseal cartilage. Mean + 95% CI; N=3 embryos, n=1-10 probes/embryo; comparison vs. 11.5M. (M – mesenchymal condensation, II – intermediate interzone, OI – outer interzone, EC – transient epiphyseal cartilage, F – femur, T – tibia.

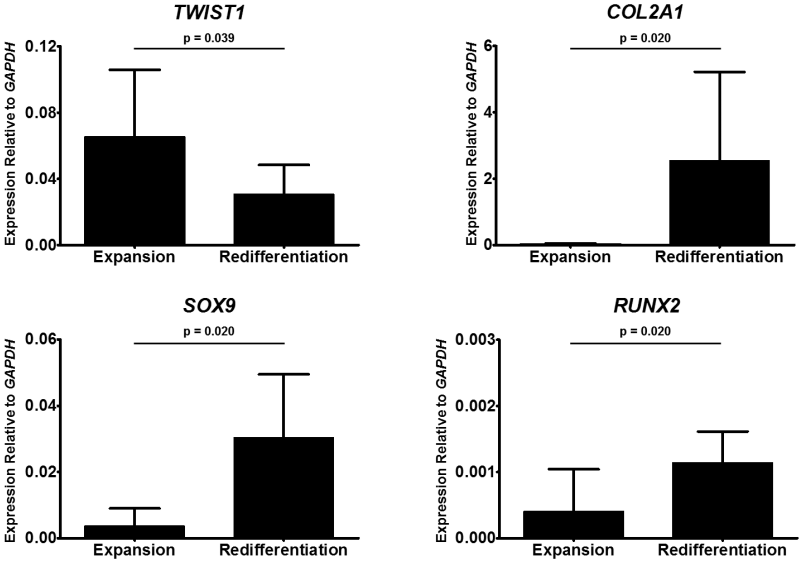

Figure 2. *TWIST1* expression decreases in redifferentiating HACs.

Relative *TWIST1*, *COL2A1*, *SOX9* and *RUNX2* gene expression levels in HACs after expansion and 21 days of redifferentiation. Mean + 95% CI; N=3 donors, n=1-2 replicates/donor.

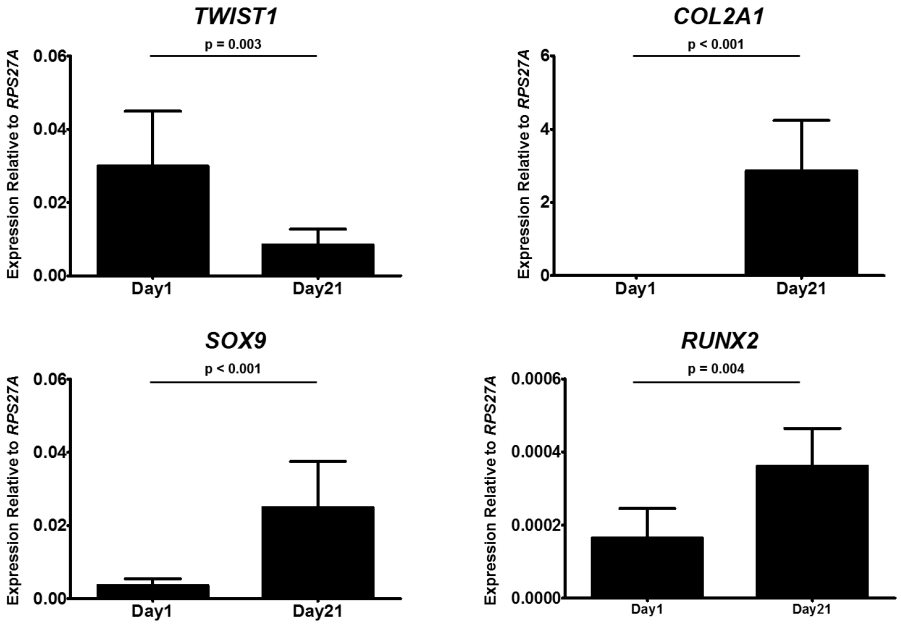

Figure 3. *TWIST1* expression decreases in chondrogenically differentiating BMSCs.

Relative *TWIST1*, *COL2A1*, *SOX9* and *RUNX2* gene expression levels in BMSCs after expansion and 21 days of chondrogenic differentiation. Mean + 95% CI; N=6 donors, n=1-3 replicates/donor.

in HACs following expansion (p=0.039), along with a significant upregulation of *COL2A1* (P=0.020), *SOX9* (P=0.020) and *RUNX2* (p=0.020).

Encouraged by this, *TWIST1* expression levels were then investigated in BMSCs in chondrogenic culture. As hypothesized, BMSCs undergoing chondrogenic differentiation, confirmed by *COL2A1* (p<0.001), *SOX9* (p<0.001), and *RUNX2* (p=0.004) expression, significantly downregulated *TWIST1* expression (p=0.003; Figure 3). These data are therefore supportive of a role for *TWIST1* in inhibition of chondrogenic differentiation of mesenchymal cells.

Silencing of *TWIST1* expression in BMSCs does not improve chondrogenic differentiation

Having observed downregulation of *TWIST1* with chondrogenic differentiation, we attempted to improve *in vitro* chondrogenic differentiation of BMSCs through inhibition of *TWIST1* expression using siRNA technology (Figure 4a). Despite lower *TWIST1* expression levels (p=0.004; Figure 4b) and protein levels (Figure 4c) in *TWIST1* silenced BMSCs, which appeared morphologically distinct (Figure 4d), no improvement in BMSC chondrogenic differentiation was observed (Figure 4e and Figure 4f). Indeed, following 21 days of chondrogenic culture, the GAG content (Figure 4e) and *COL2A1* expression (Figure 4f) of *TWIST1* silenced BMSCs appeared to be

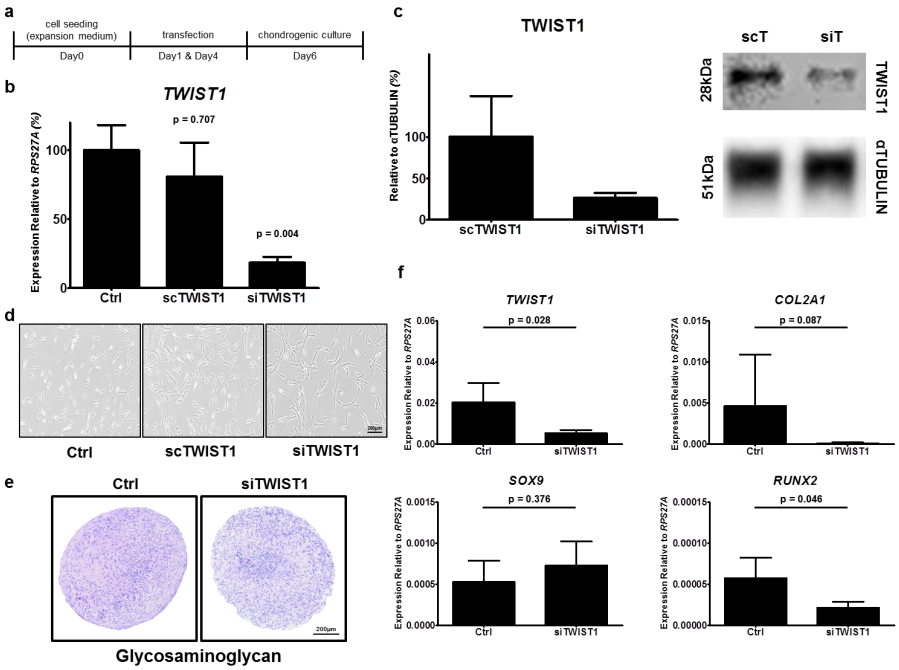

Figure 4. *TWIST1* silenced BMSCs display no improvement in chondrogenic differentiation potential.

(a) Schematic overview of experimental protocol, **(b)** Relative *TWIST1* gene expression levels in *TWIST1* silenced BMSCs after 5 days of treatment. Mean + SD; N=1 donor, n=3 replicates; comparison vs. Ctrl, **(c)** Western blot and quantified TWIST1 protein expression levels in *TWIST1* silenced BMSCs after 3 days of treatment (Ctrl = 100 %). Mean + SD; N=1 donor, n=2 independent measurements, **(d)** Morphology of *TWIST1* silenced BMSCs after 5 days of treatment, **(e)** Glycosaminoglycan content of *TWIST1* silenced BMSCs after 21 days of chondrogenic culture (thionine staining; representative image of 3 replicates), **(f)** Relative *TWIST1*, *COL2A1*, *SOX9* and *RUNX2* gene expression levels in *TWIST1* silenced BMSCs after 21 days of chondrogenic differentiation. Mean + SD; N=1 donor, n=2-3 replicates. Ctrl – Control, scT/scTWIST1 – scramble *TWIST1*, siT/siTWIST1 – silenced *TWIST1*.

less, with no improvement in *SOX9* expression (Figure 4f). These findings suggest an important role for *TWIST1* in not only inhibition of chondrogenic differentiation, but also in promotion of BMSC chondrogenic differentiation.

Initial upregulation of *TWIST1* expression required for chondrogenic differentiation of BMSCs

To gain an understanding of why *TWIST1* silencing impacted negatively upon BMSC chondrogenic differentiation, we further investigated *TWIST1* expression during *in vitro* culture, including non-chondrogenic BMSCs. Unexpectedly, between the end of monolayer expansion and Day1 of chondrogenic pellet culture, both chondrogenic (p=0.002) and non-chondrogenic (p=0.004) BMSCs upregulated

TWIST1 expression (Figure 5a). In non-chondrogenic BMSCs, however, upregulation was not followed by a downregulation of expression during chondrogenic differentiation culture (Day1 vs. Day21; p=1.000). To evaluate whether the observed upregulation of *TWIST1* expression occurred as a direct result of exposure to TGFβ1 in the chondrogenic induction medium, we assessed the effect of the presence or absence of TGFβ1 on BMSCs in monolayer (Figure 5b) and in chondrogenic pellet culture (Figure 5c). After 24 h in monolayer, TGFβ1 significantly downregulated *TWIST1* expression levels (p<0.001; Figure 5b), while comparison of *TWIST1* expression levels in BMSCs after 1 day of chondrogenic pellet culture revealed no direct effect of TGFβ1 (Figure 5c), implying initial upregulation in chondrogenic culture is not a consequence of TGFβ1 exposure. We, therefore, next investigated whether this upregulation in *TWIST1* expression and failure of *TWIST1* silenced BMSCs to undergo chondrogenesis were linked to proliferation. Indeed, significantly less

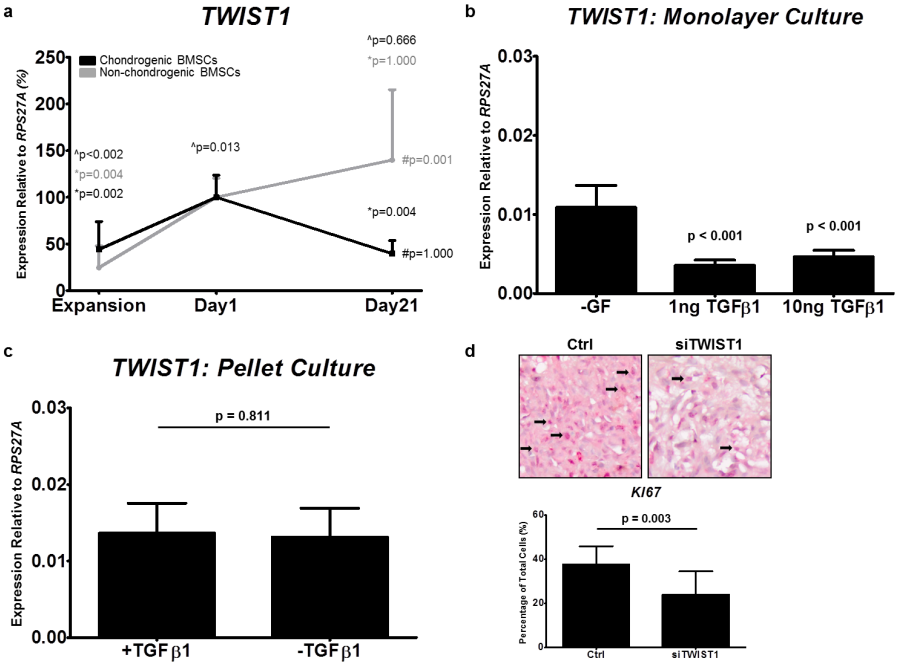

Figure 5. *TWIST1* expression increases during BMSC chondrogenic culture.

(a) Regulation of *TWIST1* gene expression in BMSCs after expansion and 1 and 21 days of chondrogenic differentiation culture. Mean + 95% CI; N=4-6 donors, n=2-3 replicates/donor; *vs. Day1, #vs. Expansion, and ^comparison of absolute *TWIST1* expression levels by timepoint, **(b)** Relative *TWIST1* gene expression levels in TGFβ1 treated (1 and 10 ng/ml) BMSCs after 24 h of monolayer culture. Mean + 95% CI; N=2 donors, n=4-5 replicates/donor; comparison vs. –GF. –GF – No Growth Factors, **(c)** Relative *TWIST1* gene expression levels in BMSCs after 1 day of chondrogenic differentiation culture. Mean + 95% CI; N=2 donors, n=3 replicates/donor, **(d)** KI67 protein and relative gene expression levels in BMSCs after 1 day of chondrogenic differentiation culture. Mean + SD; N=1 donor, n=3 replicates

proliferating cells ($p < 0.003$) were observed in pellets formed from *TWIST1* silenced BMSCs after 1 day of chondrogenic culture (Figure 5d), suggesting failure of *TWIST1* silenced BMSCs to chondrogenically differentiate may be due to a lack of proliferation during chondrogenic induction. In summary, these data demonstrate for the first time that during *in vitro* BMSC chondrogenic differentiation, an initial upregulation of *TWIST1* expression followed by downregulation of expression is required for chondrogenesis (Figure 6).

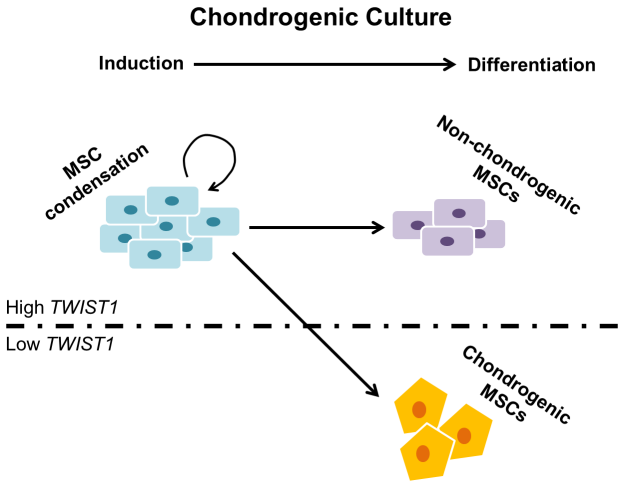

Figure 6. Proposed model of *TWIST1* expression during BMSC chondrogenic differentiation. *TWIST1* is expressed during chondrogenic induction where it is involved in proliferation. Thereafter, chondrogenic BMSCs downregulate *TWIST1* expression while non-chondrogenic BMSCs fail to do so, preventing differentiation.

DISCUSSION

The TWIST1 transcription factor is a recognized inhibitor of mesenchymal cell differentiation and is a novel target for improvement of *in vitro* engineering of MSC-based bone tissue [10, 11, 14, 19]. A similar target function of TWIST1 for *in vitro* MSC chondrogenic differentiation is envisioned; however, while it has been established that TWIST1 inhibits chondrogenic processes in murine and chick mesenchymal cells [12, 14, 15], a role for TWIST1 in human chondrogenic differentiation processes still requires establishment. In this study we provide the first evidence of dynamic regulation of *TWIST1* expression during *in vitro* human BMSC chondrogenic differentiation.

During skeletal development *Twist1* expression is present and restricted to limb bud mesenchyme from initiation through to the early stages of chondrogenesis [20, 21]. In our embryonic model of chondrogenesis, *Twist1* expression was present and

observed to decrease as mesenchymal cells progressed in development. Similarly, HACs and BMSCs undergoing chondrogenic differentiation downregulated *TWIST1* expression, whereas non-chondrogenic BMSCs failed to do so. Consequently, we considered *TWIST1* an important inhibitor of chondrogenesis and chondrocyte gene expression, and believed removal of *TWIST1* inhibition would improve *in vitro* BMSC chondrogenic differentiation. We, however, failed to foresee an upregulation of *TWIST1* expression in the first days of our chondrogenic culture set-up and subsequently, *TWIST1* silenced BMSCs actually displayed worse chondrogenic differentiation potential.

That *TWIST1* expression increases during this initial phase of chondrogenic culture is not completely unexpected. One of the first steps of cartilage formation *in vivo* is condensation of mesenchymal cells, a step which is equally important in *in vitro* chondrogenesis [22, 23]. *Twist1* expression is known to correlate with regions under the influence of FGF signalling, and *TWIST1* itself has been shown to be active during FGF/FGFR signaling [4, 5, 7, 24-26]. Previously we demonstrated that FGFR2, important for the condensation phase during development [27-29], is present in BMSCs during chondrogenic induction [30]. Furthermore, extensive work by the Tuan group has revealed condensation of adult human MSCs occurs downstream of TGFβ1 induced *β-CATENIN* expression, both known upstream signalling pathways of TWIST1 [22]. Therefore, upregulation of *TWIST1* in our culture may occur as a result of condensation processes. Absence of TGFβ1 signalling, however, had no direct effect on *TWIST1* expression levels, and *TWIST1* silenced BMSCs appeared to condense normally during chondrogenic induction. Thus, *TWIST1* expression does not appear crucial to this process and was not likely the cause of impaired chondrogenic differentiation of these cells.

Indeed, we believe that failure of *TWIST1* silencing to improve chondrogenic differentiation may be linked to proliferation. Higher proliferation rate in monolayer expansion culture is often linked to better chondrogenic differentiation potential [31]. Moreover, Dexheimer et al. demonstrated a requirement for proliferation during chondrogenic induction culture [32]. Freshly isolated adult BMSCs express high levels of *TWIST1*, a known promoter of MSC proliferation [16], which is downregulated during expansion culture [16, 31], as too is the chondrogenic differentiation potential of BMSCs [31]. In our system, *TWIST1* silencing could impact both monolayer expansion proliferation and pellet culture proliferation. Indeed, *TWIST1* silenced BMSCs seemed slower to reach confluence during expansion culture compared to control or scramble treated BMSCs [data not shown]. Additionally, TWIST1 silenced BMSCs displayed fewer proliferating cells after 1 day of chondrogenic pellet culture. It is, therefore, possible that decreased proliferation in our *TWIST1* silenced BMSCs resulted in their failure to undergo chondrogenesis. More extensive studies interfering with proliferation are required to confirm this.

Investigation of endogenous *TWIST1* expression during chondrogenic differentiation culture raised another interesting question; why do non-chondrogenic BMSCs, unlike chondrogenic BMSCs, fail to downregulate *TWIST1* expression following this initial upregulation? WNT signalling is a known upstream regulator of *TWIST1* expression that promotes MSC proliferation [14, 15, 31], that is transiently upregulated during chondrogenic induction [22], and which must be downregulated during chondrogenic differentiation [31]. Hence, its potential expression profile, upregulation followed by downregulation, mirrors that observed for *TWIST1* in chondrogenic BMSCs. It is possible, therefore, that failure of WNT signalling downregulation in these cells is responsible for their undesired differentiation outcome. Additionally, non-chondrogenic BMSCs had lower absolute levels of *TWIST1* following expansion. Failure of MSCs to undergo differentiation is often proposed to occur as a result of these cells being more committed and less able to respond to differentiation stimuli. That *TWIST1* expression is associated with an uncommitted state in adult MSCs [16, 31] lends support to this concept.

In our proposed model (Figure 6), an upregulation of *TWIST1* during chondrogenic induction followed by downregulation during differentiation is required to promote chondrogenic differentiation of adult human BMSCs. Upregulation of *TWIST1* expression during induction likely occurs in association with proliferation and its expression throughout may be modulated by *WNT* signaling. As an upregulation of *TWIST1* occurs, silencing of *TWIST1* expression before this induction phase may not prove a successful strategy for improvement of *in vitro* BMSC chondrogenic differentiation due to its interference with BMSC proliferation, as was demonstrated in this study. In actuality, *Twist1* expression is required by immature chondrocytes to prevent their progression toward hypertrophy, through inhibition of *Runx2* expression [15]. Chondrogenically differentiated BMSCs, redifferentiated HACs and mesenchymal cells of the transient embryonic cartilage all fail to sustain a stable articular chondrocyte phenotype, but show signs of hypertrophy and share a similar expression profile; decreased *TWIST1* expression with increased *RUNX2* expression. Thus, maintaining moderate levels of *TWIST1* expression during differentiation may help in maintaining a more stable articular chondrocyte phenotype, while complete silencing of *TWIST1* may prove more useful for endochondral ossification.

CONCLUSION

In this study we demonstrate for the first time the dynamic regulation of *TWIST1* expression during chondrogenic differentiation of BMSCs. Furthermore, we highlight a distinct difference between chondrogenic and non-chondrogenic BMSCs during chondrogenic culture; their regulation of *TWIST1* expression. Following

initial upregulation of *TWIST1* expression during induction of chondrogenic differentiation, non-chondrogenic BMSCs fail to downregulate *TWIST1* expression, unlike chondrogenic BMSCs. Elucidation of *TWIST1* upstream and downstream molecular activity will further identify targets to facilitate optimization of *in vitro* BMSC chondrogenic differentiation for clinical purposes.

ACKNOWLEDGEMENTS

We would like to acknowledge Andrea Lolli and Prof. Roberta Piva (University of Ferrara) for assistance in setting up and optimizing the transfection protocol.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare that there are no potential conflicts of interest associated with this study

REFERENCES

1. Gitelman I. Twist protein in mouse embryogenesis. **Dev Biol.** 1997;189:205-214.
2. Wolf C, Thisse C, Stoetzel C et al. The M-twist gene of Mus is expressed in subsets of mesodermal cells and is closely related to the Xenopus X-twi and the Drosophila twist genes. **Dev Biol.** 1991;143:363-373.
3. Chen ZF, Behringer RR. twist is required in head mesenchyme for cranial neural tube morphogenesis. **Genes Dev.** 1995;9:686-699.
4. Tavares AT, Izpisuja-Belmonte JC, Rodriguez-Leon J. Developmental expression of chick twist and its regulation during limb patterning. **Int J Dev Biol.** 2001;45:707-713.
5. O'Rourke MP, Soo K, Behringer RR et al. Twist plays an essential role in FGF and SHH signal transduction during mouse limb development. **Dev Biol.** 2002;248:143-156.
6. el Ghouzzi V, Le Merrer M, Perrin-Schmitt F et al. Mutations of the TWIST gene in the Saethre-Chotzen syndrome. **Nat Genet.** 1997;15:42-46.
7. Howard TD, Paznekas WA, Green ED et al. Mutations in TWIST, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome. **Nat Genet.** 1997;15:36-41.
8. Johnson D, Horsley SW, Moloney DM et al. A comprehensive screen for TWIST mutations in patients with craniosynostosis identifies a new microdeletion syndrome of chromosome band 7p21.1. **Am J Hum Genet.** 1998;63:1282-1293.
9. Rose CS, Patel P, Reardon W et al. The TWIST gene, although not disrupted in Saethre-Chotzen patients with apparently balanced translocations of 7p21, is mutated in familial and sporadic cases. **Hum Mol Genet.** 1997;6:1369-1373.
10. Quarto N, Senarath-Yapa K, Renda A et al. TWIST1 silencing enhances in vitro and in vivo osteogenic differentiation of human adipose-derived stem cells by triggering activation of BMP-ERK/FGF signaling and TAZ upregulation. **Stem Cells.** 2015;33:833-847.
11. Bialek P, Kern B, Yang X et al. A twist code determines the onset of osteoblast differentiation. **Dev Cell.** 2004;6:423-435.

12. Gu S, Boyer TG, Naski MC. Basic helix-loop-helix transcription factor Twist1 inhibits transactivator function of master chondrogenic regulator Sox9. **J Biol Chem.** 2012;287: 21082-21092.
13. Goodnough LH, Chang AT, Treloar C et al. Twist1 mediates repression of chondrogenesis by beta-catenin to promote cranial bone progenitor specification. **Development.** 2012; 139:4428-4438.
14. Reinhold MI, Kapadia RM, Liao Z et al. The Wnt-inducible transcription factor Twist1 inhibits chondrogenesis. **J Biol Chem.** 2006;281:1381-1388.
15. Dong YF, Soung do Y, Chang Y et al. Transforming growth factor-beta and Wnt signals regulate chondrocyte differentiation through Twist1 in a stage-specific manner. **Mol Endocrinol.** 2007;21:2805-2820.
16. Isenmann S, Arthur A, Zannettino AC et al. TWIST family of basic helix-loop-helix transcription factors mediate human mesenchymal stem cell growth and commitment. **Stem Cells.** 2009;27:2457-2468.
17. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. **Nat Protoc.** 2008;3:1101-1108.
18. Jenner F, A IJ, Cleary M et al. Differential Gene Expression of the Intermediate and Outer Interzone Layers of Developing Articular Cartilage in Murine Embryos. **Stem Cells Dev.** 2014.
19. Hebrok M, Wertz K, Fuchtbauer EM. M-twist is an inhibitor of muscle differentiation. **Dev Biol.** 1994;165:537-544.
20. Stoetzel C, Weber B, Bourgeois P et al. Dorso-ventral and rostro-caudal sequential expression of M-twist in the postimplantation murine embryo. **Mech Dev.** 1995;51:251-263.
21. Fuchtbauer EM. Expression of M-twist during postimplantation development of the mouse. **Dev Dyn.** 1995;204:316-322.
22. Tuli R, Tuli S, Nandi S et al. Transforming growth factor-beta-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk. **J Biol Chem.** 2003;278:41227-41236.
23. Delise AM, Tuan RS. Analysis of N-cadherin function in limb mesenchymal chondrogenesis in vitro. **Developmental Dynamics.** 2002;225:195-204.
24. Rice DP, Aberg T, Chan Y et al. Integration of FGF and TWIST in calvarial bone and suture development. **Development.** 2000;127:1845-1855.
25. Huang Y, Meng T, Wang S et al. Twist1- and Twist2-haploinsufficiency results in reduced bone formation. **PLoS One.** 2014;9:e99331.
26. Connerney J, Andreeva V, Leshem Y et al. Twist1 homodimers enhance FGF responsiveness of the cranial sutures and promote suture closure. **Dev Biol.** 2008;318:323-334.
27. Ornitz DM. FGF signaling in the developing endochondral skeleton. **Cytokine Growth Factor Rev.** 2005;16:205-213.
28. Delezoide AL, Benoist-Lasselin C, Legeai-Mallet L et al. Spatio-temporal expression of FGFR 1, 2 and 3 genes during human embryo-fetal ossification. **Mech Dev.** 1998;77: 19-30.
29. Peters KG, Werner S, Chen G et al. Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. **Development.** 1992;114:233-243.

30. Hellingman CA, Koevoet W, Kops N et al. Fibroblast growth factor receptors in in vitro and in vivo chondrogenesis: relating tissue engineering using adult mesenchymal stem cells to embryonic development. **Tissue Eng Part A.** 2010;16:545-556.
31. Narcisi R, Cleary MA, Brama PA et al. Long-term expansion, enhanced chondrogenic potential, and suppression of endochondral ossification of adult human MSCs via WNT signaling modulation. **Stem Cell Reports.** 2015;4:459-472.
32. Dexheimer V, Frank S, Richter W. Proliferation as a requirement for in vitro chondrogenesis of human mesenchymal stem cells. **Stem Cells Dev.** 2012;21:2160-2169.