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Critical molecular regulators, histomorphometric indices and their correlations in the trabecular bone in primary hip osteoarthritis

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SUMMARY

Objective: This study examined differential gene expression, histomorphometric indices and relationships between these, in femoral trabecular bone from osteoarthritis (OA) patients and control (CTL) subjects, with the aim of identifying potential molecular drivers consistent with changes in structural and remodelling indices in the OA pathology.
Materials and methods: Bone samples from the intertrochanteric (IT) region were obtained from age and sex-matched cohorts of 23 primary hip OA patients and 21 CTL subjects. Real-time polymerase chain reaction (PCR) and histomorphometric analysis were performed on each sample and correlations between gene expression and histomorphometric variables determined.
Results: Alterations in gene expression, structural indices and correlations between these were found in OA bone compared to CTL. In OA bone, expression of critical regulators of osteoblast differentiation (TWIST1) and function (PTEN, TIMP4) were decreased, while genes associated with inflammation (SMAD3, CD14) were increased. Bone structural and formation indices (BV/TV, Tb.N, OS/BS) were increased, whereas resorption indices (ES/BS, ES/BV) were decreased. Importantly, significant correlations in CTL bone between CTNNB1 expression and formation indices (OS/BS, OS/BV, OV/BV) were absent in OA bone, indicating altered WNT/β-catenin signalling. TWIST1 expression and BV/TV were correlated in CTL bone, but not in OA bone, consistent with altered osteoblastogenesis in OA. Matrix metalloproteinase 25 (MMP25) expression and remodelling indices (ES/BS, ES/BV, ES/TV) were correlated only in OA pointing to aberrant bone remodelling in this pathology.
Conclusions: These findings indicate an altered state of osteoblast differentiation and function in OA driven by several key molecular regulators. In association with this differential gene expression, an altered state of both trabecular bone remodelling and resulting microarchitecture were also observed, further characterising the pathogenesis of primary hip OA.

Introduction

Osteoarthritis (OA) is a musculoskeletal disease known to cause significant disability, worldwide figures suggest it is becoming one of the greatest health care problems in the developed world[1]. The impact of OA will increase considerably as the population of developed nations age. Primary OA is the most prevalent form and develops in the absence of any known cause and commonly occurs in the hip joint[2].

The pathology of hip OA involves the whole joint with focal and progressive hyaline articular cartilage loss and concomitant changes in the subchondral bone, including development of marginal bone outgrowths, osteophytes, and increased thickness of the bony envelope[3]. *In vitro* and *in vivo* observations of subchondral plate changes preceding articular cartilage damage suggest that primary OA may be a bone disease, in addition to a cartilage condition[4–6]. Early changes in OA are at the very least concomitant in the bone and cartilage and may initiate in the bone[4].

It is estimated that at least 60% of the susceptibility to hip OA is attributable to genetic factors, and many potential candidates with bone associated functions have been identified in familial studies[5]. Linkage and association studies have identified genetic candidates from the wingless-type MMTV integration site (WNT) signalling

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pathway, transforming growth factor-β (TGFβ), and tumour necrosis factor-α (TNFα) superfamilies, as involved in primary OA of the hip[5–7].

In a recent study utilising gene microarray technology, a number of differentially expressed genes were identified with potential roles in the pathology, from the trabecular bone of end-stage OA patients consisting of both males and females[8]. Among the molecules identified include genes involved in osteoblast differentiation, phosphatase and tensin homolog (PTEN), β-catenin (CTNNB1), twist homolog 1 (TWIST1), SMAD family member 3 (SMAD3) and S100 calcium binding protein A4 (S100A4). Other molecules identified relate to adipocyte differentiation, osteoclastogenesis and bone remodelling, including adipocyte differentiation-related protein (ADFP), CD14 antigen (CD14), interleukin-10 (IL10), matrix metalloproteinase 25 (MMP25) and TIMP metallopeptidase inhibitor 4 (TIMP4).

Alongside the molecular changes occurring in the femoral trabecular bone of OA patients, there are also microarchitectural changes associated with the earliest symptomatic manifestations of the disease[9]. Observations in the hard tissue include increased bone volume fraction (BV/TV) and hypomineralisation[9,10]. Among other histomorphometric parameters, trabecular number (Tb.N) and osteoid surface (OS/TV) measured in the subchondral trabecular bone of hip OA patients were found to be increased against controls, while trabecular separation (Tb.Sp) was found to be decreased[3,14]. Bone turnover also appears to be deregulated: significant associations between remodelling indices such as erosion surface (ES/BS) and osteoid surface (OS/BS) and osteoclastogenic regulators are also uncoupled in the late stages of the disease, suggesting that resorption in OA is regulated differently compared to controls[11].

Significant changes in gene expression and histomorphometric indices in trabecular bone suggest an altered remodelling state in OA[8,12]. Although differences in gene expression and histomorphometry have been established in previous studies, as yet no study has comprehensively examined the relationships between these parameters and the possible contribution of these interactions to the pathology[13].

In this study, human intertrochanteric (IT) trabecular bone from primary hip OA cases and non-OA controls (CTLs) was examined. This was done to identify systemic changes in the bony compartment in proximity of the disease site, away from the diseased joint surface. The first aim of the study was to investigate differential expression of bone regulatory molecules between OA patients and CTL individuals, as well as differences in histomorphometric parameters, to explore structural and remodelling changes between the two cohorts. The second aim was to assess correlations between the expression of molecular regulators, as well as correlations between the structural histomorphometric parameters. The third aim was to evaluate correlations between the molecular regulators and the histomorphometric indices. The overall aim was to identify associations amongst molecular drivers consistent with changes in structural and remodelling indices within this complex pathology.

Methods

Human bone samples

Twenty-three primary end-stage OA trabecular bone samples were obtained from patients undergoing hip arthroplasty, these comprised 13 females and 10 males (mean age ± standard deviation (SD); 70 ± 10 years and 67 ± 10 years, respectively). These OA cases were macroscopically graded for OA by assessment of the degree of fibrillation and degeneration[14]. At surgery all OA cases were either Grade III or Grade IV, characterised by cartilage loss, eburnation of bone, osteophytes, and remodelling of the articular contour. Twenty-one CTL samples were obtained post-mortem within 24 h, an approach validated by numerous previous studies[8,11,15–21]. These comprised 11 females and 10 males (mean age ± SD; 73 ± 12 years and 67 ± 10 years, respectively). None of the CTL cases had worse than Grade II OA, these cases were known to be free of any extenuating bone pathologies, as described previously[8,21]. No significant difference in age was found between OA and CTL, and between sexes (Student's t test, $P < 0.05$). The trabecular bone samples were obtained as cylindrical tube saw biopsies from the IT region of the proximal femur. Both OA and CTL samples were approximately 50 mm in length and 10 mm in diameter. Each sample was divided into two equal parts, one for histomorphometric analysis and the other for RNA extraction. Informed consent was obtained for the collection of these specimens, with approval from the Royal Adelaide Hospital Research Ethics Committee.

RNA extraction and real-time polymerase chain reaction (PCR)

The extraction of RNA and real-time PCR was conducted as described previously[8]. Trabecular bone samples were rinsed briefly in diethylpyrocarbonate-treated water and then separated into small fragments, containing bone and marrow, using sterile instruments. Total RNA was extracted as described previously[8,18]. First-strand reverse transcription cDNA synthesis was performed on 1 μg amplified RNA from each sample using a first-strand cDNA synthesis kit with Superscript II (Invitrogen, Carlsbad, CA, USA) and 250 ng random hexamer primer (Geneworks, Adelaide, SA, Australia), in accordance with the manufacturer's instructions. Template cDNA (1 μl of 1/100 dilution of cDNA) was amplified using iQ SYBR Green Supermix (BioRad, Hercules, CA, USA) on a Rotor-Gene thermocycler (Corbett Research, Mortlake, NSW, Australia)[8]. The reactions were incubated at a temperature of 94 °C for 10 min for 1 cycle, and then at 94 °C (20 s), at 60 °C (20 s), or at 65 °C (MMP25 only) and at 72 °C (30 s) for 40 cycles. This set of cycles was followed by an additional extension step at 72 °C for 5 min. All PCR reactions were validated by the presence of a single peak in the melt curve analysis, and further confirmed by electrophoresis on a 2.5% weight/vol agarose gel. Primers were designed as described previously (Geneworks, Adelaide, SA, Australia)[8]. Real-time PCR validation was carried out using the $2^{-\Delta\Delta CT}$ method[22]. Reactions were performed in triplicate. Gene expression cycle threshold values were normalised to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The genes amplified were PTEN, CTNNB1, TWIST1, SMAD3, S100A4, ADFP, CD14, IL10, MMP25 and TIMP4[8].

Histomorphometric analysis

Both the OA and CTL specimens for histomorphometry were fixed, infiltrated and embedded in methylmethacrylate as described previously[23]. All bone blocks were trimmed and sectioned on a microtome (Polycut-E, Leica SP 2600, Cambridge Instruments). Sections, 5 μm thick, were stained by the von Kossa method and counterstained with hematoxylin and eosin to distinguish between the mineralized bone, the cellular components of the marrow and the osteoid. Analysis was performed on a microscope (Leica DM6000B, Leica Microsystems) using an ocular mounted 10 × 10 graticule at a magnification of ×100. Measurements were made of the following structural parameters: bone volume fraction (BV/TV, %), trabecular separation (Tb.Sp, μm), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, #/mm) and osteoid thickness (O.Th, μm). The remodelling parameters analysed include percent eroded specific surface (ES/BS, %), eroded surface (ES/BV, mm^2/

mm^3), eroded surface in the bone tissue volume (ES/TV, mm^2/mm^3), percent osteoid specific surface (OS/BS, %), osteoid surface (OS/BV, mm^2/mm^3), percent osteoid volume (OV/BV, %) and percent osteoid surface to eroded surface (OS/ES, %)[24].

Data analysis

The quantified gene expression and histomorphometry data were tested for normality using the Shapiro–Wilks statistic (Graphpad Prism v5.00 San Deigo, CA, USA). The parametrically distributed datasets were expressed as mean ± SD, the non-parametric datasets were expressed as median (25th percentile, 75th percentile). Subsequently, as applicable, a Student's *t* test or the Mann–Whitney *U* test was used to test for significant differences between the OA and CTL groups for each gene and histomorphometric parameter. Differences between the sexes within each cohort were examined for each parameter (data not shown); these findings confirmed the observations from the sex-pooled cohorts. Therefore the results of the pooled datasets are presented, consistent with previous studies[20,21]. Pearson product moment (*r*) statistics were applied within the OA and CTL cohorts respectively, to examine correlations between PCR product/GAPDH ratios and between structural histomorphometric variables. Finally, correlations between gene expression and the histomorphometric parameters were assessed. The critical value for significance was chosen as $P = 0.05$.

Results

In this study, 23 OA and 21 CTL trabecular bone samples were collected and all samples underwent total RNA extraction, target gene real-time PCR and histomorphometric analysis. As we have described previously, this approach allows analysis of gene expression contributions from all of the cellular elements of bone, including the bone marrow[8]. This provides a 'snapshot' of the gene expression in both the OA and CTL bone microenvironment. Although the specific cell type(s) contributing to the altered gene expression cannot readily be identified, osteoblasts and osteocytes, which represent the most abundant cells in the trabecular bone, would be expected to contribute significantly to the gene expression measured[8,25]. The comparison in gene expression and histomorphometric parameters, as well as correlations among these measures, is presented below. Photomicrographs of an OA and CTL case are presented as an example (Fig. 1).

Comparison in gene expression and histomorphometric indices

The mRNA of a number of critical signalling molecules was found to be differentially expressed between the OA and CTL cohorts (Table I). Of the molecules assessed, PTEN, TWIST1 (Fig. 2a), ADFP, IL10 and TIMP4 were significantly down-regulated in OA, whereas SMAD3 and CD14 were up-regulated ($P < 0.005$).

Assessment of the histomorphometric parameters between OA and CTL indicated that among the structural indices, BV/TV (Fig. 2b) and Tb.N were significantly increased in OA, whereas Tb.Sp was significantly decreased. Among the remodelling indices measured, ES/BS and ES/BV were significantly lower in OA ($P < 0.05$). There was no statistically significant difference between the groups for the other genes and histomorphometric indices measured.

Correlations within gene expression and histomorphometry

Correlations of gene expression were examined within the OA and CTL cohorts (Table II). A statistically significant positive correlation between PTEN and S100A4 expression was observed only in the OA cohort. Exclusively in the CTL cohort, PTEN correlated positively with TIMP4. Significant positive correlations between CTNNB1 and MMP25 were observed in both the OA and CTL cohorts. Negative correlation between CTNNB1 and IL10 was found in the OA samples but not in CTL. TWIST1 expression correlated negatively with CTNNB1 and positively with S100A4, PTEN and TIMP4, all exclusively in the OA cohort. Similarly, SMAD3 expression correlated negatively with CTNNB1 and MMP25 only in the OA cohort. SMAD3 and CD14 expression correlated positively in both cohorts. S100A4 expression correlated positively with IL10 and negatively with CTNNB1 in the OA cohort alone. MMP25 and IL10 expression correlated positively only in the CTL cohort.

The quantified histomorphometric indices were examined by correlation within the OA and CTL cohorts, respectively (Table II). In both cohorts, concurrent increases in BV/TV and Tb.N and a negative correlation between BV/TV and Tb.Sp were observed. BV/TV and Tb.Th correlated positively exclusively in the OA cohort. Conversely, only in the CTL cohort negative correlations between Tb.N and Tb.Sp and between Tb.N and O.Th were observed, alongside positive correlations between Tb.Sp and O.Th and between Tb.Th and O.Th.

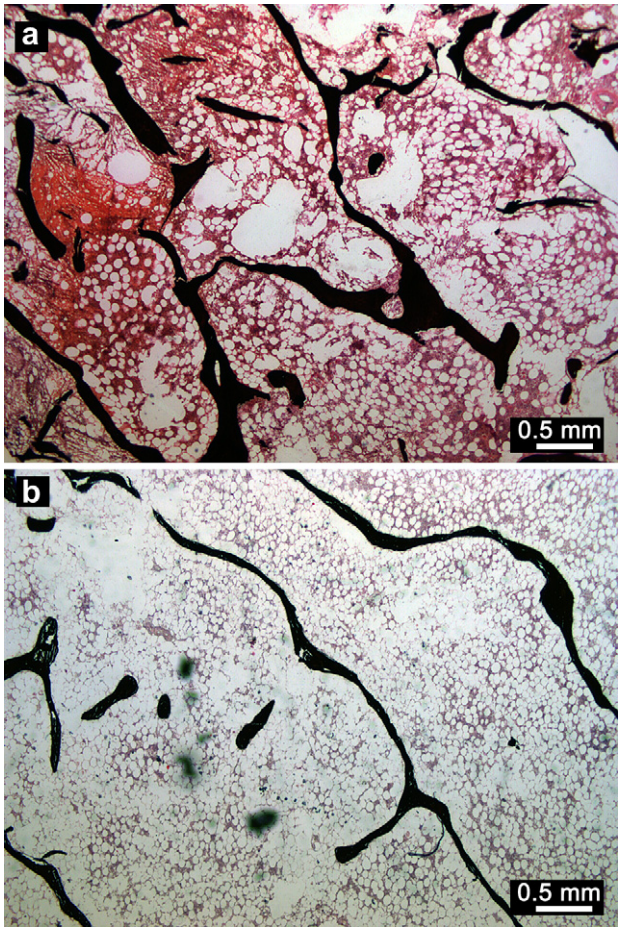

Fig. 1. a) Photomicrograph of a 5 μm section of trabecular bone section from the IT region of the proximal femur of a primary hip OA case (×2.5 magnification). (b) Photomicrograph of a 5 μm section of trabecular bone section from the IT region of the proximal femur of a CTL case (×2.5 magnification).

Correlations between gene expression and histomorphometry

Statistically significant correlations of bone gene expression and histomorphometric parameters were found between a number of

Table I
Comparison of the expression of molecular regulators and structural and remodelling indices between OA patients and CTL cases*

	OA	CTL	*P*-value
Gene			
PTEN	0.042 (0.034, 0.051)	30 (0.077, 0.17)	<0.001
CTNNB1	1.6×10^{-3} (7.2×10^{-4}, 7.1×10^{-3})	7.8×10^{-4} (3.5×10^{-4}, 2.0×10^{-3})	0.091
TWIST1	1.8×10^{-3} (1.0×10^{-3}, 3.1×10^{-3})	3.4×10^{-3} (2.4×10^{-3}, 6.3×10^{-3})	0.002
SMAD3	$8.9 \times 10^{-4} \pm 4.3 \times 10^{-4}$	$4.7 \times 10^{-4} \pm 2.7 \times 10^{-4}$	<0.001
S100A4	0.33 (0.16, 0.50)	0.33 (0.13, 0.45)	0.638
ADFP	0.012 (0.010, 0.018)	0.30 (0.014, 0.076)	0.002
CD14	1.7×10^{-2} (1.4×10^{-2}, 2.8×10^{-2})	4.0×10^{-3} (2.7×10^{-3}, 9.3×10^{-3})	<0.001
IL10	$6.9 \times 10^{-5} \pm 3.8 \times 10^{-5}$	$1.3 \times 10^{-4} \pm 7.0 \times 10^{-5}$	0.005
MMP25	4.2×10^{-5} (6.7×10^{-6}, 1.1×10^{-4})	2.2×10^{-5} (9.7×10^{-6}, 6.9×10^{-5})	0.913
TIMP4	6.3×10^{-3} (3.0×10^{-3}, 1.1×10^{-2})	2.4×10^{-2} (1.0×10^{-2}, 5.6×10^{-2})	0.001
Histomorphometry			
BV/TV (%)	8.60 (6.20, 12.10)	5.40 (4.10, 8.30)	0.009
Tb.Sp (mm)	1.04 ± 0.40	1.87 ± 0.90	0.007
Tb.Th (mm)	0.09 (0.07, 0.12)	0.09 (0.08, 0.11)	1.000
Tb.N (#/mm)	1.06 ± 0.38	0.60 ± 0.35	0.001
O.Th (μm)	3.75 (0.02, 12.46)	6.76 (0.01, 12.44)	1.000
ES/BS (%)	2.50 (1.10, 6.90)	6.80 (3.90, 9.30)	0.009
ES/BV (mm^2/mm^3)	0.47 (0.19, 0.97)	1.03 (0.65, 2.45)	0.018
ES/TV (mm^2/mm^3)	0.04 (0.02, 0.11)	0.06 (0.04, 0.18)	0.145
OS/BS (%)	6.50 (2.70, 10.60)	8.80 (4.90, 15.50)	0.097
OS/BV (mm^2/mm^3)	1.04 (0.60, 2.40)	1.86 (0.89, 4.97)	0.202
OV/BV (%)	1.00 (0.65, 2.47)	1.23 (0.65, 3.93)	0.572
OS/ES (%)	1.77 (0.38, 2.66)	1.04 (0.70, 4.24)	0.903

* Sampled from the IT region of the proximal femur (trabecular bone). Data are expressed as parametric mean ± SD or non-parametric median and inter-quartile range, statistical significance was set at $P < 0.05$. Gene expression data are expressed as mean expression normalized to GAPDH.

molecular regulators and remodelling and structural indices. CTNNB1 expression was positively correlated with OS/BS, OS/BV and OV/BV in the CTL but not in the OA cohort (Table III). TWIST1 and BV/TV were found to be positively correlated among CTLs only (Fig. 2c). ADFP and Tb.Sp correlated negatively exclusively in OA, while ADFP expression and OS/ES correlated positively only in the CTL cohort. Similarly, IL10 expression also correlated positively with BV/TV only among the CTLs. Conversely, MMP25 expression correlated positively with ES/BS, ES/BV and ES/TV in the OA cohort, but not in the CTL. A positive correlation between TIMP4 expression and O.Th was observed exclusively in the CTL cohort. All the remaining correlations were not statistically significant in either cohort.

Discussion

In this study, gene expression and histomorphometric indices of trabecular bone from 23 OA and 21 CTL cases were measured and compared. Subsequently, correlations were assessed in the OA cohort and in CTLs, to identify critical relationships between molecular regulators and structural and remodelling indices.

Comparison in gene expression and histomorphometric indices

Gene expression in femoral trabecular bone indicated significantly lower levels of PTEN expression in the OA cohort compared with CTL, consistent with previous studies[8]. PTEN is a known indirect inhibitor of osteoblast proliferation, differentiation and mineralisation, an action confirmed in another study using an osteoblastic cell line[26]. TWIST1 expression was also lower in OA than in CTL samples. TWIST1 is a critical regulator of downstream transcription, whose timely expression is vital for the normal differentiation of osteoblasts and collagen matrix mineralisation[27,28]. Lower expression of TWIST1 suggests deregulation of downstream factors in the OA bone microenvironment, leading to aberrant osteoblast differentiation and potentially immature osteoblasts in OA[27]. SMAD3 was expressed significantly higher in OA compared to CTL. Increased SMAD3 expression could inhibit mature osteoblast differentiation and maintain an immature osteoblast phenotype. This is consistent with previous findings in OA, ultimately contributing to the observed increase in bone volume fraction[24–26]. ADFP is a known marker of adipocyte differentiation and currently has no established profile in human bone. ADFP expression in this study was found to be significantly lower in OA. This finding is consistent with other reports, which have found lower adipogenic potential in the bone of hip OA cases[29]. However, this does not exclude other genes involved in adipogenesis from being up-regulated in circulation and the joint, consistent with the increased obesity associated with the pathology[30]. CD14 expression was found to be significantly higher in OA compared to CTL. This gene is a pan-marker of the monocyte/macrophage phenotype, but is also expressed by granulocytes including neutrophils[31]. This cell type is known to be increased in number in OA, thus increased CD14 expression may indicate increased recruitment of inflammatory cells to the IT region in OA bone[32,33]. IL10 is known to reduce osteoclastogenesis, and in this study was found to be significantly lower in OA[34]. It is thus likely that the decreased resorption observed in the OA cohort (significantly lower ES/BS and ES/BV as discussed later), maybe driven by other regulators. For example, previous studies have found that in OA, the critical molecular triad of OPG/RANK/RANKL may have a more profound role in the regulation of resorption in the pathology[35]. TIMP4 was significantly down-regulated in OA when compared to non-OA CTLs. A known inhibitor of MMPs, lower expression within the pathology indicates increased breakdown of the extracellular matrix in the bone microenvironment[36,37].

Histoquantification of IT trabecular bone structural and remodelling indices indicated significant differences in BV/TV, Tb.Sp, Tb.N, ES/BS and ES/BV between the two cohorts (Table I). In general, the observed differences in structural and remodelling indices are consistent with previous changes found in OA[12,38]. These include increased bone volume fraction, increased trabecular number and decreased trabecular separation, alongside significantly decreased erosion surface. These differences confirm the altered microarchitectural state in OA bone compared to CTL[10,36,37]. This suggests that primary hip OA is a disease characterised by altered expression of critical molecular regulators in the bone

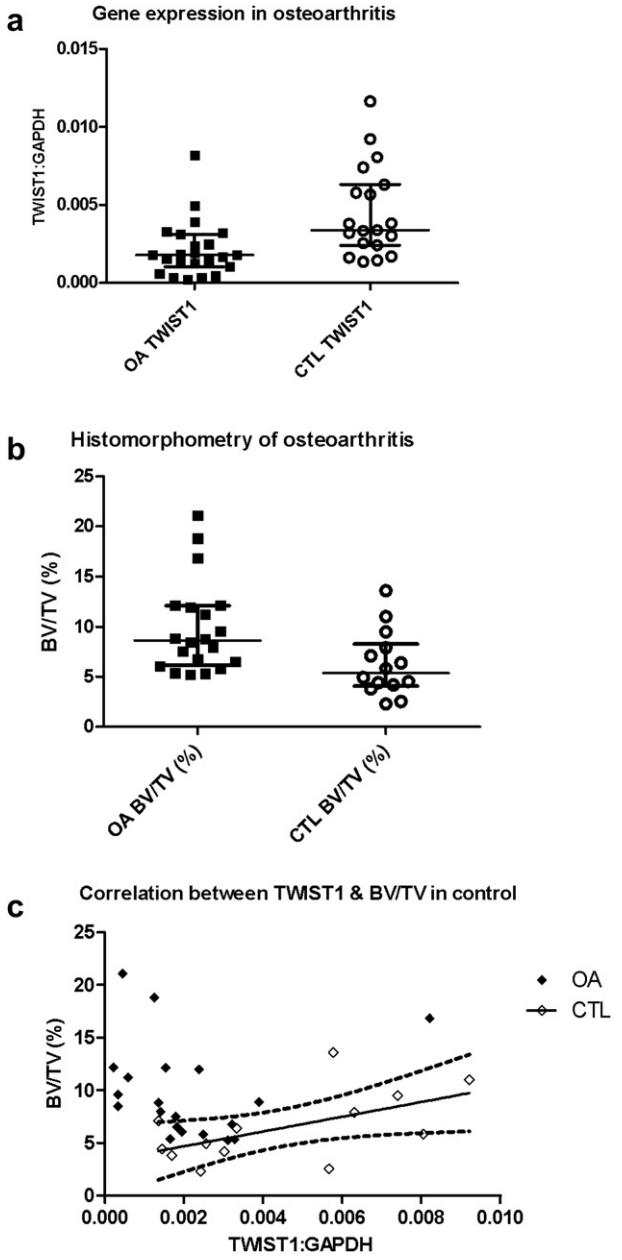

Fig. 2. a) TWIST1 mRNA expression from the IT region of the proximal femur of OA patients ($n = 23$) and CTL cases ($n = 21$), data are expressed as non-parametric median and inter-quartile range ($P = 0.002$). (b) Percent bone volume fraction (BV/TV) from the IT region of the proximal femur of OA patients and CTL cases, data are expressed as median and inter-quartile range ($P = 0.009$). (c) Significant positive correlation with 95% confidence interval ($r = +0.567$) between TWIST1 and BV/TV from the IT region of the proximal femur in CTL ($P = 0.043$), no significant correlation was observed in OA.

microenvironment, which associates with an aberrant state of remodelling and thus differences in structural parameters, consistent with previous reports[8,11,18,39].

Correlations in gene expression

PTEN and S100A4 mRNA expression correlated positively in OA. Since both molecules are associated with inhibition of osteoblast differentiation and mineralisation, this suggests that both may contribute to the dysfunctional osteoblast differentiation and mineralisation exhibited in OA[26,40]. Conversely, the positive correlation between PTEN and TIMP4, an inhibitor of MMPs, was

Table II
Correlations in gene expression amongst molecular regulators and between structural indices in OA patients and CTL cases*

	OA	CTL
Genetic correlations		
PTEN vs S100A4	$r = +0.519$, $P = 0.011$	$r = +0.202$, $P = 0.381$
PTEN vs TIMP4	$r = +0.374$, $P = 0.126$	$r = +0.559$, $P = 0.024$
CTNNB1 vs MMP25	$r = +0.599$, $P = 0.003$	$r = +0.500$, $P = 0.021$
CTNNB1 vs IL10	$r = -0.534$, $P = 0.011$	$r = +0.246$, $P = 0.325$
TWIST1 vs CTNNB1	$r = -0.447$, $P = 0.032$	$r = +0.024$, $P = 0.923$
TWIST1 vs S100A4	$r = +0.712$, $P < 0.001$	$r = -0.305$, $P = 0.204$
TWIST1 vs PTEN	$r = +0.581$, $P = 0.004$	$r = +0.239$, $P = 0.325$
TWIST1 vs TIMP4	$r = +0.799$, $P < 0.001$	$r = +0.050$, $P = 0.861$
SMAD3 vs CTNNB1	$r = -0.463$, $P = 0.026$	$r = -0.205$, $P = 0.386$
SMAD3 vs CD14	$r = +0.564$, $P = 0.005$	$r = +0.602$, $P = 0.005$
SMAD3 vs MMP25	$r = -0.468$, $P = 0.028$	$r = -0.346$, $P = 0.136$
S100A4 vs IL10	$r = +0.720$, $P < 0.001$	$r = -0.033$, $P = 0.897$
S100A4 vs CTNNB1	$r = -0.472$, $P = 0.023$	$r = -0.188$, $P = 0.414$
MMP25 vs IL10	$r = -0.340$, $P = 0.131$	$r = +0.540$, $P = 0.021$
Histomorphometric correlations		
BV/TV vs Tb.N	$r = +0.535$, $P = 0.015$	$r = +0.610$, $P = 0.021$
BV/TV vs Tb.Sp	$r = -0.501$, $P = 0.024$	$r = -0.710$, $P = 0.006$
BV/TV vs Tb.Th	$r = +0.783$, $P < 0.001$	$r = +0.004$, $P = 0.669$
Tb.N vs Tb.Sp	$r = -0.352$, $P = 0.128$	$r = -0.891$, $P < 0.001$
Tb.N vs O.Th	$r = -0.406$, $P = 0.094$	$r = -0.665$, $P = 0.009$
Tb.Sp vs O.Th	$r = +0.189$, $P = 0.452$	$r = +0.711$, $P = 0.007$
Tb.Th vs 0.Th	$r = -0.196$, $P = 0.452$	$r = +0.673$, $P = 0.011$

* Sampled from the IT region of the proximal femur (trabecular bone). Correlations are expressed as r values, statistical significance was set at $P < 0.05$. Gene expression data were normalized to GAPDH. Only correlations significant in one or both cohorts are presented, all other correlations were not significant.

observed in CTL bone but not in the OA cohort. This suggests that normal regulatory mechanisms controlling bone turnover are not conserved in OA bone[26,36]. Positive correlations in gene expression were observed between the key osteoblast transcription factor, CTNNB1, and the matrix modifying enzyme, MMP25, in both the OA and CTL cohorts (Table III)[39–41]. Furthermore, expression of both genes was not different between the two cohorts. This relationship may represent a level of balance in bone remodelling that is conserved in OA, despite the dysfunction between other lineage allocation markers and matrix resorption. However, CTNNB1 expression in OA bone was associated unusually with other important regulatory genes. For example, CTNNB1 and IL10 correlated negatively only in the OA cohort, this association between an osteoblastogenic regulator and an osteoclastogenic inhibitor also contributes to the altered state of remodelling observed in OA[34,41]. TWIST1 correlated negatively with CTNNB1 and positively with S100A4, PTEN and TIMP4 exclusively in the OA cohort. Negative correlation between these two critical transcription factors, suggests the emergence of a dysfunctional relationship leading to abnormal osteoblast differentiation, based on the known roles of these molecules in the bone microenvironment[8]. S100A4 is a negative regulator of mineralisation that maybe downstream of TWIST1[40]. In the present study, primary hip OA patients expressed TWIST1 in positive correlation with S100A4, whereas no significant correlation was observed in CTLs. This suggests that although lower levels of TWIST1 are observed in the OA trabecular bone, its regulatory action is associated with the inhibition of normal mineralisation in OA bone. Also, TWIST1 mRNA expression correlated positively with that of PTEN in OA, consistent with abnormal osteoblast differentiation and reduced calcium apposition in OA[26]. TIMP4 mRNA expression also increased with that of TWIST1 in OA. This relationship is also consistent with the increased BV/TV, Tb.N and decreased Tb.Sp observed in the OA trabecular bone. Negative correlations between SMAD3 and CTNNB1 expression, as well as MMP25 in OA suggest the emergence of further dependencies not seen in the CTL cohort. Based on the roles of SMAD3 and CTNNB1 in

Table III
Correlations in gene expression amongst molecular regulators and structural and remodelling indices from OA and CTL cases*

Gene-histomorphometry correlations	OA	CTL
CTNNB1 vs OS/BS	$r=-0.069$, $P=0.772$	$r=+0.655$, $P=0.011$
CTNNB1 vs OS/BV	$r=-0.239$, $P=0.310$	$r=+0.652$, $P=0.012$
CTNNB1 vs OV/BV	$r=+0.060$, $P=0.810$	$r=+0.607$, $P=0.021$
TWIST1 vs BV/TV	$r=+0.011$, $P=0.964$	$r=+0.567$, $P=0.043$
ADFP vs Tb.Sp	$r=-0.511$, $P=0.021$	$r=+0.181$, $P=0.554$
ADFP vs OS/ES	$r=-0.397$, $P=0.083$	$r=+0.542$, $P=0.045$
IL10 vs BV/TV	$r=-0.436$, $P=0.062$	$r=+0.679$, $P=0.015$
MMP25 vs ES/BS	$r=+0.491$, $P=0.033$	$r=-0.049$, $P=0.869$
MMP25 vs ES/BV	$r=+0.464$, $P=0.046$	$r=+0.250$, $P=0.410$
MMP25 vs ES/TV	$r=+0.583$, $P=0.011$	$r=-0.250$, $P=0.389$
TIMP4 vs O.Th	$r=-0.233$, $P=0.423$	$r=+0.660$, $P=0.027$

* Sampled from the IT region of the proximal femur (trabecular bone). Correlations are expressed as r values, statistical significance was set at $P<0.05$. Gene expression data were normalized to GAPDH. Only correlations significant in one or both cohorts are presented, all other correlations were not significant.

signal transduction during osteoblastogenesis, this relationship also indicates irregular activity within the WNT and TGFβ pathways in the OA trabecular bone[41,42]. The negative correlation between MMP25 and SMAD3 expression may also contribute to the higher bone volume fraction observed in OA, as the inhibitory action of SMAD3 may be further augmented by a decrease in the turnover of the bone matrix[43,44]. The positive correlations observed between SMAD3 and CD14 in both the OA and CTL cohorts may represent conserved signalling pathways in cells of the granulocyte and monocytic lineages in both cohorts. The elevated expression of both these genes in OA is consistent with increased inflammatory cell activity in OA bone[31]. The negative correlation between S100A4 and CTNNB1 is consistent with abnormal differentiation of osteoblasts and bone formation in OA[10,40]. The positive correlation between S100A4 and IL10 in OA suggests that, as normal differentiation and mineralisation is inhibited, osteoclastogenesis may also be inhibited within the trabecular bone of the pathology, consistent with previous observations in OA[34,45]. MMP25 and IL10 correlated positively exclusively in the CTL cohort, suggesting that as degradation of the extracellular matrix increases, osteoclastogenesis is inhibited in the CTL cohort[34,43,46]. This relationship is not present in the OA cohort, consistent with the disruption of the bone remodelling process in this pathology. Together, these findings indicate that the altered state of remodelling found in OA bone may be driven by a complex aberrant program of gene expression among molecular regulators of osteoblast differentiation, mineralisation and bone turnover. This may ultimately lead to the altered bone structure in OA, indicated by the observed changes in the bone histomorphometric indices.

Correlations among histomorphometric indices

Among the histomorphometric indices assessed in this study, a positive correlation between BV/TV and Tb.N, and a negative correlation between BV/TV and Tb.Sp were observed, as found in previous studies. Conservation of these correlations in both cohorts confirms that although there is higher bone volume fraction in OA, increases in BV/TV are concomitant with increases in Tb.N and decreases in Tb.Sp, similar to CTLs[15,47,48]. In the CTL cohort, the negative correlation between Tb.N and Tb.Sp is consistent with previous findings[20]. However, these did not correlate in OA. Tb.N and O.Th also correlated negatively, and Tb.Sp and O.Th correlated positively, just in CTL. Thus, alongside the conservation of the correlations between BV/TV and Tb.Sp and between BV/TV and Tb.N, the absence of other correlations may indicate partially altered structural relationships in the OA bone.

Correlations between gene expression and histomorphometry

In this study the significant correlations found in CTL bone, between CTNNB1 expression and the three formation indices (OS/BS, OS/BV and OV/BV) were not observed in OA bone. This suggests that WNT/β-catenin control of osteoblast differentiation and bone formation is fundamentally altered in OA and is consistent with the altered bone formation and decreased mineralisation state observed in this pathology[10,41]. In CTL bone the correlation between TWIST1 and BV/TV is consistent with the role of this transcription factor in osteoblast differentiation, contributing to the process of bone formation[28]. This relationship is not maintained in OA, suggesting the dysfunctional differentiation of the osteoblast in OA may also in part be driven by altered TWIST1 activity. ADFP correlated positively with OS/ES in the CTL cohort indicating an increase in adipocytic cells alongside the increased osteoid surface. This relationship is not maintained in the OA cohort, consistent with aberrant lineage allocation in OA[8]. IL10 and BV/TV correlated positively in CTL but not in OA. IL10 expression inhibits osteoclastogenesis; as such, positive correlation with bone volume in the CTL cohort is consistent with the known roles of this gene[34]. The loss of this relationship in OA may add to an altered state of remodelling in the pathology. The significantly lower resorption indices observed in OA (ES/BS and ES/BV), as well as ES/TV, correlate positively with MMP25 expression, exclusively in the pathology. Secretion of this molecule can indirectly activate serine proteinases capable of damaging the extracellular matrix[43]. These associations indicate that although lower resorption indices are observed in OA, the resorption that does occur, maybe in part mediated by MMP25. TIMP4 is a known inhibitor of MMPs, and correlates positively with O.Th in CTL bone. Increased inhibition of degradative proteinases is consistent with increases in O.Th[37]. The loss of this correlation in OA is consistent with increased MMP activity as found in other studies, and the abnormal process of formation observed among the previous correlations in this study[30].

Notably the expression of some key factors did not show overt differences between OA patients and CTL, such as CTNNB1, S100A4 and MMP25, whereas their link with histomorphometric parameters showed different correlations between OA and CTL. These differences indicate that other factors might be important or masked herein, in order to understand the links established here. Further studies are needed to investigate these links. Also, further investigation of sex-related differences within OA and CTL maybe undertaken, with a specifically designed study for this purpose to further examine the differences and correlations established here.

In the present study the sample size consisted of 23 OA and 21 CTL cases, these two cohorts were similar in number to other studies (24 OA and 21 CTL, or 19 OA and 25 CTL) and were also age and sex-matched[8,12]. The examination was undertaken in the IT region, in order to evaluate systemic changes in hip OA in proximity of the bony compartment of the disease, away from the diseased surface[8,12]. A characteristic of this study is that it combined two analytical techniques, both real-time PCR and histology, on the same bone biopsy, enabling complementary assessment of both gene expression and histomorphometry on each case.

In summary, this study investigated changes in gene expression and histomorphometric indices, as well as the correlations between these measures in OA and CTL from human IT trabecular bone. The findings suggest that OA is characterised by an altered state of osteoblast differentiation and function, driven by differential expression of critical molecular regulators. This differential

expression was found to associate with an altered state of remodelling, resulting in increased bone volume fraction and altered trabecular microarchitecture in the pathology.

Contributions

All the authors have made substantial contributions to all three sections of the study as outlined below.

1) D. Kumarasinghe, B. Hopwood, G. Atkins and N. Fazzalari contributed to the conception and design of the study. H. Tsangari, L. Truong, J. Kuliwaba and B. Hopwood contributed to the acquisition of data. D. Kumarasinghe, E. Perilli, J. Kuliwaba, G. Atkins and N. Fazzalari contributed to the analysis and interpretation.
2) D. Kumarasinghe drafted the article. E. Perilli, H. Tsangari, L. Truong, J. Kuliwaba, B. Hopwood, G. Atkins and N. Fazzalari provided critical revision of the intellectual content.
3) All the authors, D. Kumarasinghe, E. Perilli, H. Tsangari, L. Truong, J. Kuliwaba, B. Hopwood, G. Atkins and N. Fazzalari provided final approval of the version to be submitted.

D. Kumarasinghe (Duminda.Kumarasinghe@health.sa.gov.au) and N. Fazzalari (Nick.Fazzalari@health.sa.gov.au) take responsibility for the work as a whole.

Conflicts of interest

None of the authors have any conflict of interest related to this work.

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