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ORIGINAL PAPER

Impacts of age and gender on bone marrow profiles of BMP7, BMPRs and Stro-1$^+$ cells in patients with total hip replacement

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Abstract
Purpose A successful osseointegration relies on the interplay of implant surface and surrounding bone marrow cells. This study was undertaken to investigate the impact of age and gender on the bone marrow composition.
Methods Bone marrow aspirates were obtained from the discarded metaphysis region of the femoral head in 24 patients with total hip replacement. Flow cytometry was used to measure the expression of Stro-1$^+$ cells and BMP receptors (BMPRs)-expressing cells. ELISA was used to measure bone marrow aspirate bone morphology protein7 (BMP7) concentration.
Results Our data demonstrates that there are diverse bone marrow profiles (Stro-1$^+$ cell and BMPRs$^+$ cells). There are no differences of Stro-1$^+$ cells, BMPRs$^+$ cells, and BMP7 concentration between male and female patients. Though there are slight increases in the number of Stro-1$^+$ cells and BMPRs$^+$ cells in younger patients (<70 years old) than those of old patients (≥70 years old), the difference is not statistically significant. However, we found a close association between the Stro-1$^+$ cells, BMPR1a cells and BMP7 concentration. In addition, a correlation exists between the number of Stro-1$^+$ cells and BMIs of these patients.
Conclusion Our data suggests that the age and gender of THR patients have little impact on their bone marrow osteogenic potential. The significance of the number of the Stro-1$^+$ with BMPRs expression on the implant fixation and osseointegration warrants further investigation.

Abbreviations

BMSC	Bone marrow stromal cells
BMP	Bone morphology protein
BMPR	BMP receptors

Introduction

Cementless total hip replacement (THR) is rapidly being accepted as the surgery for arthritic diseases of the hip joint [1]. THR patients must undergo initial stabilisation to obtain long-term durability. Implant stability and a long-term survival of THR require efficient osseointegration (a direct anchorage of an implant by bone formation at the bone–implant surface). Osseointegration is a process requiring the recruitment of bone marrow mesenchymal stromal cells (BMSC) to the prosthetic surface. The commitment of BMSC cells to an osteoblastic differentiation pathway is apparently under the control of both systemic and local growth factors, such as bone morphogenic proteins (BMP) [2, 3]. A favourable bone marrow microenvironment should have a sufficient amount of osteoprogenitor cells being able to differentiate to functional osteoblasts in response to growth factors. The differences in patient bone marrow microenvironments and the association of their bone marrow osseointegration potentials with post-THR outcomes have been long overlooked [4, 5].

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Human bone marrow cells contain two populations of bone marrow stem cells: mesenchymal stem cells (MSCs also called bone marrow stromal cells) and hematopoietic stem cells (HSCs). Stro-1^+ is a cell surface antigen expressed by BMSCs [6, 7]. A cell population that is positive for the anti Stro-1^+ antibody has been shown to contain BMSC stem cells. The bone marrow Stro-1^+ is capable of differentiating into multiple mesenchymal lineages including adipocytes, osteoblasts, and chondrocytes [6, 8]. The variation of Stro-1^+ cells can be observed between human subjects [9]. However, the impact of age and gender on the population of Stro-1^+ cells has not been well studied.

BMPs are a group of growth factors known for their ability to induce bone formation [10, 11]. To date, over 20 BMP family members have been isolated and characterised [10, 11]. BMPs activate target cells by binding to type-Ia, -Ib and -II BMP receptors (BMPRs). BMP signals are mediated by type I and type II serine/threonine kinase receptors. These transmembrane receptors recruit and phosphorylate cytoplasmic proteins, especially the receptor-regulated signal transducers Smads 1, 5 and 8 [12]. It has been demonstrated that BMP7 treatment is sufficient to induce all of the genetic markers of osteoblast differentiation in many cell types [13]. The response of human BMSCs to BMP7 is highly diversified, and current clinical studies continue to show a variable success rate of recombinant BMP7 in the treatment of fracture repair and nonunion [14–17]. It is unknown whether the expression of BMP and BMPRs in human BMSCs are diversified in THR patients and whether the expression of these BMPRs is influenced by patient's age and gender.

The purpose of this study was to investigate the impact of age and gender on the bone marrow osteogenetic profiles (Stro-1^+, BMP7 and BMPRs) in a group of THR patients.

Materials and methods

Patients

From March 2009 to March 2011, a total of 24 primary total hip replacement (THR) patients were consecutively enrolled in this study (12 men and 12 women, 52–87 years old, average age 66.7±11.0 years old; see Table 1). Body mass index (BMI) ranged from 17.3 to 33.5 (average 23.99±3.73). Of them, 13 subjects were classified for convenience as younger (<70 years, mean 57.8± 4.2 years) and 11 were classified as older (≥70 years, mean 77.3±5.6 years). In order to minimise any possible impact of the operation, the THR operations were accomplished and standardised by the same team. Informed consent was obtained from all patients, and this study was approved by the Institutional Ethics Committee at Renji Hospital, Shanghai JiaoTong University School of Medicine. Criteria for exclusion were symptoms or signs of inflammation and infection, rheumatoid arthritis and other autoimmune disease. Also excluded were patients taking nonsteroidal anti-inflammatory drugs (NSAIDs) within one month before examination.

Table 1 Patient characteristics

Patient number	Gender	Age	Height (cm)	Weight (kg)	BMI
1	Male	65	170	88	30.45
2	Male	63	172	58	19.61
3	Male	70	168	65	23.03
4	Female	59	158	60	24.03
5	Male	56	170	62	21.45
6	Male	70	170	70	24.22
7	Female	77	159	62	24.52
8	Female	56	154	61	25.72
9	Female	52	159	61	24.13
10	Female	77	158	61	24.44
11	Female	87	152	60	25.97
12	Male	77	164	60	22.31
13	Male	52	170	73	25.26
14	Male	52	174	63	20.81
15	Female	72	160	77	30.08
16	Female	58	162	88	33.53
17	Male	63	170	65	22.49
18	Female	58	160	50	19.53
19	Female	58	160	47	18.36
20	Male	86	170	50	17.30
21	Female	59	165	67.5	24.79
22	Male	78	159	64	25.32
23	Female	79	153	55	23.50
24	Male	77	168	70	24.80

Human bone marrow stromal cells (BMSC)

Bone marrow aspirates (5 mL) were obtained from the discarded metaphysis region of the femoral head during total hip replacement (THR) for osteoarthritis. The bone marrow aspirates were diluted 1:4 with phosphate buffered saline (PBS) and layered on Histopaques (Sigma Aldrich, St. Louis, MO, USA) density gradient. Mononuclear cells were isolated by density gradient centrifugation at 600 g for 30 minutes and washed in PBS. The supernatant (bone marrow aspirate washout) was collected and stored frozen at −70°C for the measurement of BMP7. BMSC collected were then culture-expanded in alpha-modified Eagle's medium (α-MEM)/10% foetal bovine serum (FBS) medium. The medium was changed initially at day four

and then every other day thereafter until the cultures reached confluence. At day 14, the cell was digested with TrypLE Express (Gibco, Grand Island, NY, USA) and collected by centrifugation at 200 g for ten minutes. Harvested BMSCs were fixed in 2% (w/v) paraformaldehyde (in PBS) for 15 minutes, and then used for the following analysis.

Flow cytometry analysis

Detection of cell surface markers, Strol-1$^+$, BMPR1A, and BMPR2 was performed by trained technicians blinded to patient identity using Becton Dickinson FACS Calibur Flow Cytometry System (Becton Dickinson, Beckman Coulter, Brea, CA, USA) equipped with Cell Quest software (Beckman Coulter). BMSC cell suspension was incubated with primary antibodies for one hour at 4°C. Unbound antibodies were removed by washing with PBS. The following primary monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoertythrin (PE), or allophycocyanin (APC) were used to detect BMPR-1A, BMPR-2, and Stro-1$^+$ (BD Pharmingen, San Diego, CA, USA) (1:100 dilution). After incubation, cells were washed and resuspended in 500 μl of wash buffer and measure by FACS. The signals corresponding to debris and cell aggregates were first gated out by using the forward light scatter (FSC) and side light scatter (SSC) display. Blank samples without staining of any antibody were used to establish positivity. For each sample, at least 10,000 cells were calculated for the positive signal rate of each antigen and emission data were analysed using Cell Quest software. Furthermore, absolute counts of Stro-1$^+$ positive cells in BMSC were determined using BD TruCOUNT Tubes (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. During analysis, the absolute number of Stro-1$^+$ positive cells in cultured BMSC was manually calculated using the following equation: (events of Stro-1$^+$ positive cells/events of beads) × (number of beads per test/test volume).

Enzyme Linked Immunosorbent Assay (ELISA)

Concentrations of BMP7 in the samples of bone marrow aspirates were determined by commercial ELISA kits (R&D System, Minneapolis, MN, USA), following the manufacturer's instructions. A standard curve was generated and the BMP7 concentration (pg/mL) of samples was calculated from the standard curve.

Statistical analysis

Statistical analysis was performed using the SPSS (SPSS, Chicago, IL, USA) software package. Data were expressed as mean ± SD. The data were also summarised by age group (<70 vs. ≥70 years old) and gender. Group mean values were compared using one-way analysis of variance with Newman–Keuls test if a normal model could be set up; if not, the Mann–Whitney U test was used. The Spearman's test was used for correlation analysis, with a P values less than 0.05 considered to be statistically significant.

Results

Cell culture and flow cytometry analysis

Cells isolated from bone marrow aspirates were cultured and cell colonies were formed at day nine (Fig. 1a). At day 14, cells were digested and collected. After fixation, cell surface markers, Strol-1$^+$, BMPR1a, and BMPR2, were stained with immunofluorescence and examined using flow cytometry (Fig. 1).

Yield of Stro-1$^+$ cells from human bone marrow stromal cells (BMSC)

Culture-expanded cells of bone marrow aspirate from 24 samples were sorted for Stro-1$^+$ cells by flow cytometry. The mean percent yield of Stro-1$^+$ cells was 17.77%± 13.88% (from 3.07% to 48.89%) indicating a great deal of variability between patients (Fig. 2).

Effects of age and gender on the percentage of Stro-1$^+$ cells from bone marrow stromal cells (BMSCs)

As shown in Fig. 3, there was no significant difference in the % yield of Stro-1$^+$ cells with gender (13.51±10.91 vs. 22.03±15.63, p=0.14). We also compared the value of the % yield of Stro-1$^+$ cells between younger and older patients. We found that the younger patients (<70 years old) had a slightly higher % yield of Stro-1$^+$ cells (21.13± 16.73%, n=13) than the older patients (≥70 years old, 13.81±8.67%, n=11), but the difference was not statistically significant (p=0.19).

Effects of gender and age on the expression of BMPR1a and BMPR2 from BMSCs

We observed that the expression of BMPR1a and BMPR2 by flow cytometry analysis was highly diversified. The percent of BMPR1a cells ranged from 1.79 to 91.36% (average 21.24±21.35%). The percent of BMPR2 cells ranged from 0.95 to 95.99% (average 28.22±24.66%). As shown in Fig. 4, there was no significant difference for both BMPR1a (25.38±27.27 vs. 17.11±13.88, p=0.95) and BMPR2 (28.79±26.13 vs. 27.65±24.81, p=0.19) with

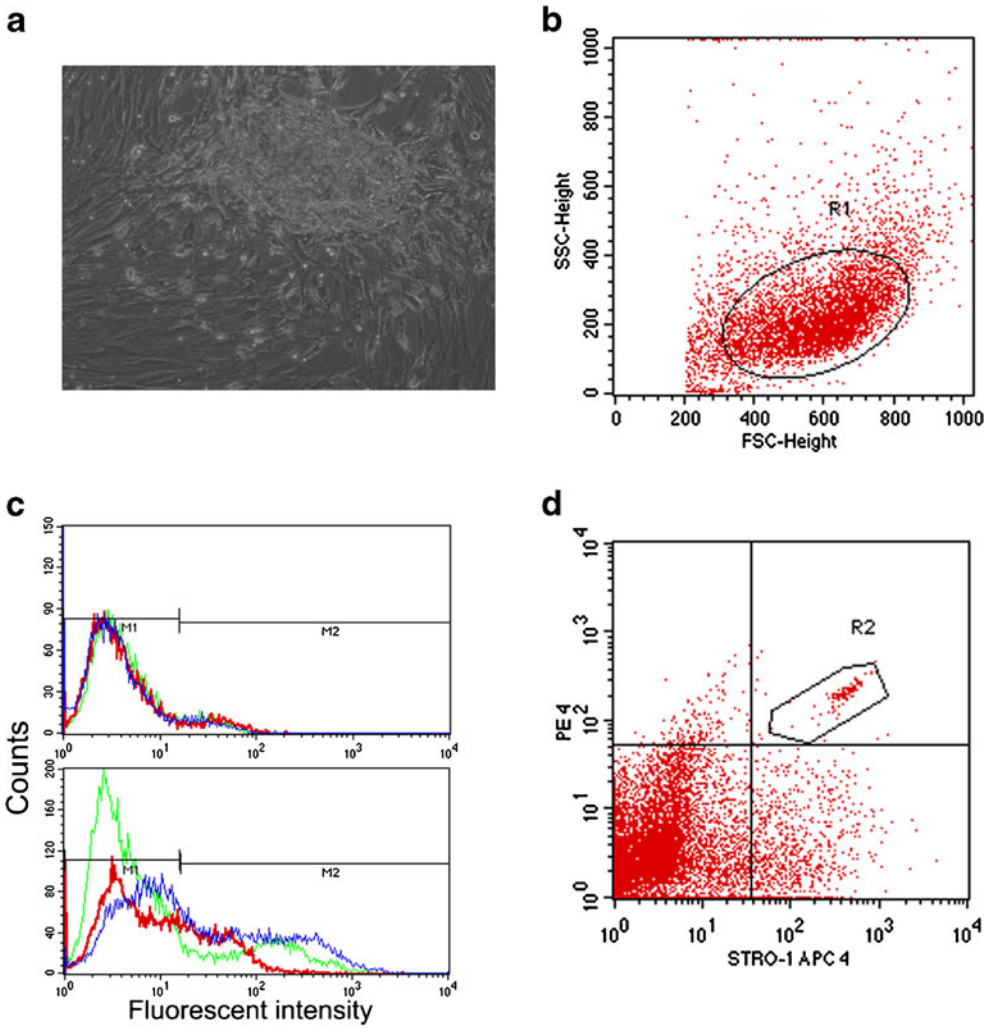

Fig. 1 Cell culture and flow cytometry analysis. **a** Cell colony formed after culture at day 9. **b** Flow cytometry gated cell by FSC and SSC display. **c** Curve charts showed Stro-1$^+$ (*blue curve*), BMPR1a (*red curve*) and BMPR2 (*green curve*) positive cell rate detected by flow cytometry. The M1 region was considered as a negative signal and the M2 region as a positive signal. The up chart showed the negative control for deciding the positive threshold and the down chart showed one of the stained samples. **d** A scatter plot showed the calculation of Stro-1$^+$ positive cell number. The R2 region was gated for calculating the bead events

gender. Similarly, the difference of both BMPR1a (25.22± 25.36% vs. 16.55±15.97%, p=0.09) and BMPR2 (34.90± 29.05% vs. 20.32±17.02%, p=0.19) expressed in BMSC from different age sections was also not statistically significant.

Effects of age and gender on the BMP7 expression

We measured bone marrow aspirate BMP7 protein concentrations. The mean concentration was 185.71± 118.64 (pg/ml), ranging from 10 pg/ml to 551.42 pg/ml.

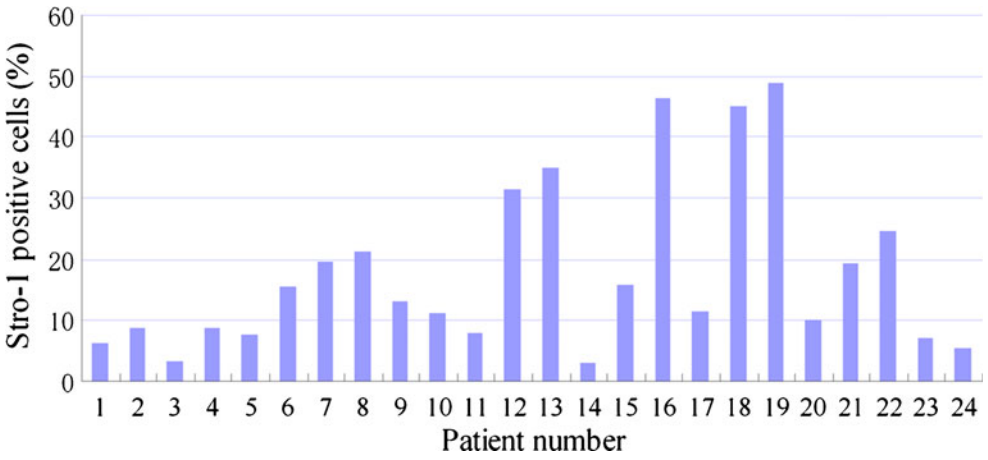

Fig. 2 The number of Stro-1$^+$ cells of human bone marrow cells were measured by flow cytometry in 24 patients with total hip replacement

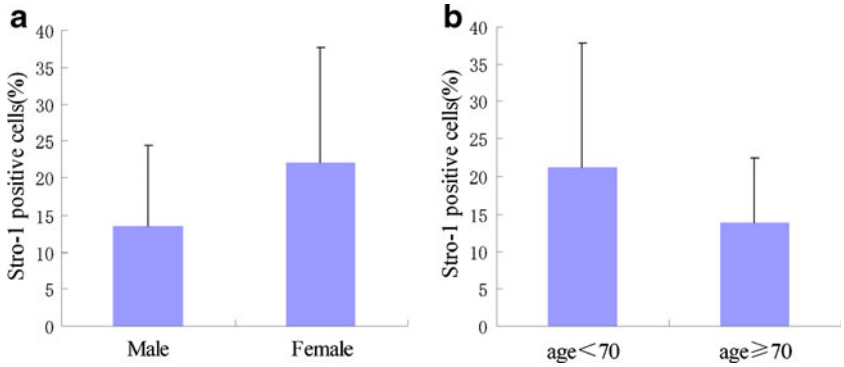

Fig. 3 Age and gender-related differences in the number of Stro-1$^+$ cells of human bone marrow cells. **a** No significant difference was found between the young group (<70 years, n=13) and old group (≥70 years, n=11). **b** No difference was found between female (n=12) and male (n=12) patients

As shown in Fig. 5, no correlation was observed between BMP7 concentration with gender (p=0.46). There was no significant difference in BMP7 concentration between younger and old patients (p=0.21).

Association of Stro-1$^+$, BMPR1a, BMPR2 expression and BMP7 concentration

A correlation analysis was performed to determine any potential linkage between the level of Stro-1$^+$ cells, BMPR1a, BMPR2, BMP7 concentration and BMI. As shown in Fig. 6, we found a strong association exists between the rate of Stro-1$^+$ cells and BMPR1a (r=0.4957, P=0.01), as well as the rate of Stro-1$^+$ cell and BMP7 concentration (r=−0.4671, P=0.02). Additionally, Stro-1$^+$ cell number was correlated with patient's BMI (r=0.4642, P=0.02). No significant association was found between the other targets.

Discussion

A successful osseointegration relies on the interplay of implant surface and periprosthetic bone marrow composition. A favourable bone marrow environment should have sufficient osteoprogenitor cells able to differentiate into osteoblasts in response to systemic or local growth factors. There are conflicting results regarding the association of THR success rate with age, gender and body weight [18–21]. The explanation for these conflicting results include the influences of ethnic status, general disease background, activity level [21] and surgical variables. A better understanding of the association of patient's age and gender with their bone marrow composition is critical in predicting the outcomes of post-THR implant fixation and implant longevity.

This study demonstrated that there are diverse bone marrow profiles (Stro-1$^+$ cell and BMPRs) in a group of 24 THR patients. There are no differences of Stro-1$^+$ cell, BMPRs-bearing cells, and BMP7 concentration between male and female patients. Though there was a slight increase in the number of Stro-1$^+$ cells and BMPR-bearing cells in younger patients (<70 years old) than those of older patients (>70 years old), the difference was not statistically significant. However, we found a close association of the Stro-1$^+$ cells with BMPR1a cells and BMP7 concentration. In addition, a correlation exists between the number of Stro-1$^+$ cells and BMIs of these patients. Our data suggested that the age and gender of THR patients have little impact on their bone marrow osteogenic potential. The significance of the number of the Stro-1$^+$ with BMPRs expression on the implant fixation and osseointegration warrants further investigation.

To avoid the possible blood contamination present in the bone marrow aspirate samples, we collected the samples from discarded femur head samples using a standardised 2-ml aspiration volume. Therefore, the variation of bone marrow composition was found to relate to variation between subjects, indicating that the individual subjects differed significantly from one another with respect to the number of Stro-1$^+$ cells and BMPRs expression on BMSC cells. Stro-1$^+$ is a cell surface antigen expressed in human

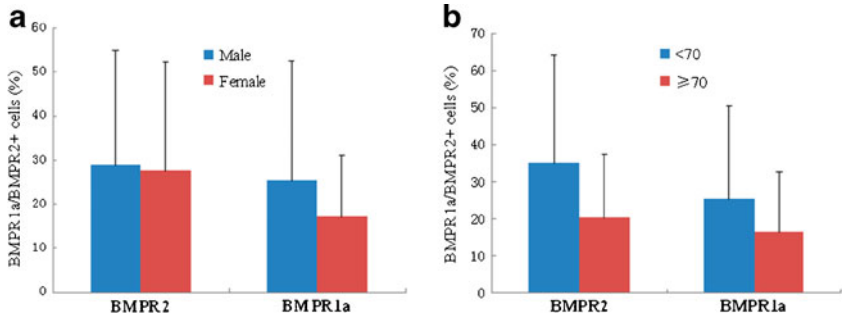

Fig. 4 Age and gender-related differences in the number of BMPR2$^+$ and BMPR1a$^+$ cells of human bone marrow cells. **a** No significant difference of both BMPRs was found between the young group (<70 years, n=13) and old group (≥70 years, n=11). **b** No difference was found between female (n=12) and male (n=12) patients

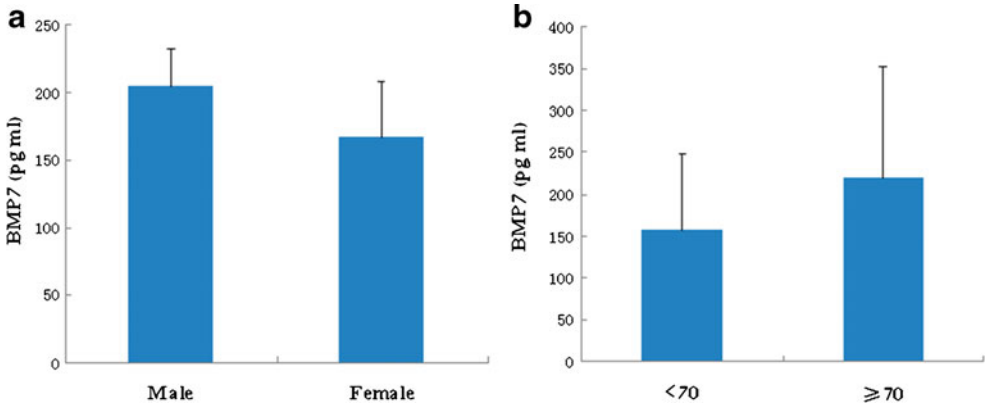

Fig. 5 Age and gender-related differences in the concentration of BMP7 from bone marrow aspirates. **a** No significant difference was found between the young group (<70 years, $n=13$) and old group (≥70 years, $n=11$). **b** No difference was found between female ($n=12$) and male ($n=12$) patients

bone marrow [22]. A Stro-1$^+$enriched subset of BMSCs is capable of differentiating into multiple mesenchymal lineages including adipocytes, osteoblasts and chondrocytes. There is a lower number of BMSC Stro-1$^+$ cells in old rats than that of younger rats [23]. Many clinical variables might contribute to the variations in the number of BMSC Stro-1$^+$ cells, including the age and gender of the subjects [8, 9]. In addition, one cannot exclude the possibility that some of the age-related changes we have found could relate to BMSC Stro-1$^+$ expression, which is associated with osteoarthritis, and this should be considered [24, 25].

It is recognised that osteoarthritis patients are always subject to a sustained loss of bone with aging, in part due to an increased volume of marrow adipose tissue [26]. Additional studies are required to define whether the changes of BMSC Stro-1$^+$ cells represent one of the biomarkers indicating the BMSC osteogenesis potential, which has been described as either age dependent [27, 28] or age independent [29, 30].

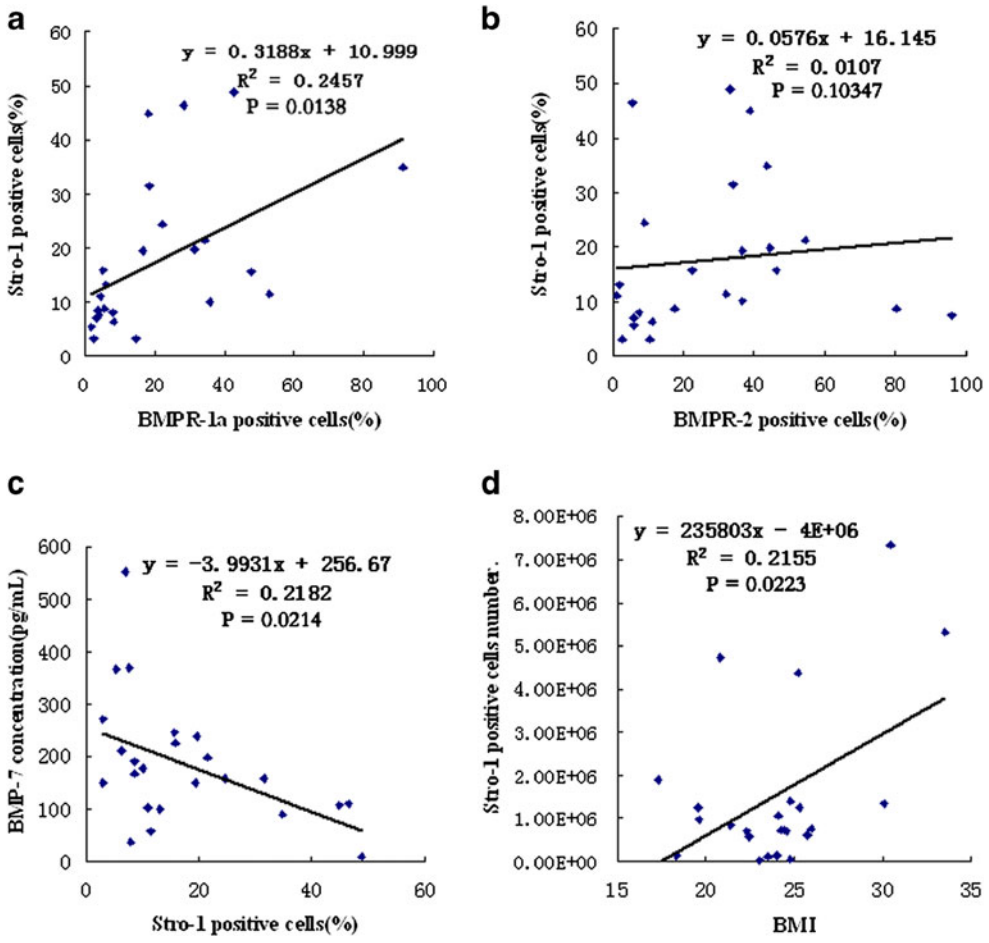

Fig. 6 Correlation between the osteogenetic factors. **a** A significant association of Stro-1$^+$ cells with BMPR1a. **b** The correlation between Stro-1$^+$ cells and BMPR2 was not significant. **c** A negative correlation was observed between Stro-1$^+$ cells and BMP7 concentration. **d** A significant association of Stro-1$^+$ cell number with BMI

BMPs are a group of growth factors known for their ability to induce the bone formation, and over 20 BMP family members have been isolated and characterised so far [11, 31]. BMPs interact with specific receptors on the cell surface, referred to as BMP receptors (BMPRs) [32]. BMP signals are mediated by type I and type II serine/threonine kinase receptors. Two type I receptors have been identified: BMPR1a (ALK3) and BPMR1b (ALK6). BMPR1a is necessary for the extracellular matrix deposition by osteoblasts [12, 33]. The type II receptor (BMPR2) binds BMPs, and the signalling begins with the binding of a BMP to the BMPR2. This causes the recruitment of a BMP type I receptor, which it phosphorylates. BMP7 plays a key role in the transformation of BMSC into bone and cartilage [13, 34]. It has been demonstrated that BMP7 treatment is sufficient to induce all of the genetic markers of osteoblast differentiation in many cell types. BMP7 has been used in clinical applications to accelerate fracture healing, to treat established nonunions, to enhance primary spine fusions, and to treat large bone-loss defects [13, 35]. The responses of human BMSC to BMP2 are highly diversified and current clinical studies continue to show a variable success rate of recombinant BMP7 in the treatment of fracture repair and nonunion [14, 36]. BMPs play a critical role in controlling implant osseointegration [2, 3, 37].

Few studies have been performed to understand the impact of age and gender on the bone marrow contents of BMPs and BMPR-expressing cells, and the potential influence of local changes of BMP and BMPRs on osseointegration. Our data showed that the percentage of BMPR1a (1.79–91.36%) and BMPR2 (0.95–95.99%) cells was differed widely. The expression of BMPR1a and BMPR2 in BMSCs from patients of different gender and age had no significant difference (Fig. 4).

The concentration of BMP7 protein in bone marrow aspirate is also highly diverse among patients. No correlation was observed between BMP7 concentration with either gender or age (Fig. 5). It is still unclear whether the changes of bone marrow BMP and BMPRs play a role in the efficiency of osseointegration after THR. Additional efforts are required to define the local or systemic factors that are responsible for the changes of BMP and BMPRs expression. In addition to the quantitative changes, the impact of age and gender on the response of BMSCs to BMPs is still uncertain [38].

Another interesting finding is that a strong association exists between the Stro-1^+ cells, BMPR1a cells and BMP7 (Fig. 6). It is unclear whether some Stro-1^+ cells have co-expression of BMPRs and higher osteogenitic potential through binding BMPs, such as BMP7, through either autocrine or paracrine pathways. However, a negative correction was found between the Stro-1^+ cells and BMP7 concentration in bone marrow aspirates, which means the high expression of BMP7 would cause a rise in osteoblastogenesis and a potential decrease of Stro-1^+ cells. It is uncertain whether BMP7 could reduce the Stro-1 expression as reported [39] or if BMP7 could decrease Stro-1^+ cells through inducing osteoblast differentiation. Besides, the results also showed that Stro-1^+ cell number in BMSC exhibited a positive effect, significantly connected with BMI, which was considered highly predictive of the number of BMSC according to Roseleen [40]. Our finding strongly supported this new hypothesis.

In conclusion, this study demonstrates the potential impacts of age and gender on the human bone marrow composition of THR patients. The highly diverse bone marrow profiles of these patients might be useful for the prediction of osseointegration efficiency after THR. The potential impacts of the BMMSC profiles on the long-term outcome of THR patients warrants further investigation. A better understanding of the patient's bone marrow composition in individual THR patients will help clinicians to predict and prevent post-THR implant failure due to the disruption of osseointegration.

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