# TSG-6 as a biomarker to predict efficacy of human mesenchymal stem/progenitor cells (hMSCs) in modulating sterile inflammation in vivo

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Human mesenchymal stem/progenitor cells (hMSCs) from bone marrow and other tissues are currently being administered to large numbers of patients even though there are no biomarkers that accurately predict their efficacy in vivo. Using a mouse model of chemical injury of the cornea, we found that bone-marrow–derived hMSCs isolated from different donors varied widely in their efficacy in modulating sterile inflammation. Importantly, RT-PCR assays of hMSCs for the inflammation-modulating protein TSG-6 expressed by the TNFα-stimulated gene 6 (*TSG-6* or *TNFAIP6*) predicted their efficacy in sterile inflammation models for corneal injury, sterile peritonitis, and bleomycin-induced lung injury. In contrast, the levels of TSG-6 mRNA were negatively correlated with their potential for osteogenic differentiation in vitro and poorly correlated with other criteria for evaluating hMSCs. Also, a survey of a small cohort suggested that hMSCs from female donors compared with male donors more effectively suppressed sterile inflammation, expressed higher levels of *TSG-6*, and had slightly less osteogenic potential.

neutrophils | gender | myeloperoxidase | MPO

**H**uman mesenchymal stem/progenitor cells (hMSCs) from bone marrow, adipose tissues, placenta, umbilical cord, and other tissues are currently being administered to large numbers of patients. Over 80 clinical trials with hMSCs have been registered (clinicaltrials.gov), and 5 have reached the Phase II or III stage of development (1). The trials are proceeding even though cultures of the cells are heterogeneous, and there is large variability in quality of hMSCs dependent on conditions, such as differences among donors, conditions used to expand the cells in culture, and random sampling in harvesting the cells from bone marrow and other tissues (2–5). Accounting for the variability among preparations is confounded by the lack of definitive markers for the cells. In addition, there are no biomarkers to predict the efficacy of hMSC samples in vivo. Therefore, the value of the data obtained from different clinical trials may well be compromised by variations in the quality of the hMSCs used.

Recent data suggest that the therapeutic effects of the cells were explained in part by their paracrine effects, such as expression of factors that modulate inflammatory and immune responses, that limit growth of cancers, or that enhance tissue repair (2, 6–8). We recently observed that i.v.-infused hMSCs modulated excessive sterile inflammation and thereby improved mouse models for myocardial infarction (9), corneal injury (10), or peritonitis (11), in part because the hMSCs were activated to secrete TNFα-stimulated gene 6 (TSG-6), a protein that is a natural modulator of inflammation (12–14). Of special interest was that a well-known model of chemical injury of the cornea made it possible to obtain quantitative dose–response data for the effectiveness of recombinant TSG-6 on both neutrophil infiltration and the functional integrity of the tissue (15). Using this model and two other models for sterile inflammation, here, we demonstrated that bone-marrow–derived hMSCs isolated from

different donors showed wide variations in their efficacy in modulating inflammation. Also, we identified a biomarker of mRNA levels for TSG-6 that predicts the in vivo efficacy of different donor-derived hMSCs in suppressing inflammation. The biomarker should prove useful in selecting preparations of hMSCs for therapy of patients.

## Results

We previously demonstrated (10) that, after ethanol injury and scraping of the cornea, i.v. infusion of bone-marrow hMSCs decreased sterile inflammation and thereby decreased opacity of the tissue. The hMSCs were effective even though quantitative PCR assays of mRNA for human GAPDH indicated that few if any of the cells reached the cornea after i.v. infusion of 10 million hMSCs in a similar rat model of ethanol injury of the cornea (10). As indicated in Fig. 1A, the decrease in opacity was proportional to the decrease in the cornea of myeloperoxidase (MPO), a semiquantitative measure of activated neutrophils. We used the same model to compare the efficacy of different preparations of hMSCs, here defined by the donors who provided the bone-marrow aspirates and expanded under essentially the same conditions, and found that they varied in their efficacy in suppressing opacity and MPO levels (Fig. 1 B and C). Some MSC preparations (from donors 235, 6015, and 269 of marrow aspirates) were highly effective, preventing opacity and decreasing MPO, and others (donors 7052, 7074, and 7075) provided little protection.

To identify a biomarker that predicted efficacy of hMSCs in the model, we assayed the same cells with conventional assays

**Significance**

The clinical trials with human mesenchymal stem/progenitor cells (hMSCs) from bone marrow and other tissues are proceeding even though cultures of the cells are heterogeneous and there is large variability among preparations of hMSCs due to differences among donors, culture conditions, and inconsistent tissue sampling. However, there is currently no in vitro bioassay for the evaluation of hMSC efficacy in vivo. Therefore, the value of the data obtained from current clinical trials may well be compromised by variations in the quality of the hMSCs used. This study provides, to our knowledge, the first biomarker that can predict the efficacy of hMSCs in suppressing sterile inflammation in vivo.

**Fig. 1.** hMSCs from different donors exhibit variations in therapeutic efficacy in suppressing sterile inflammation in the chemical burn-injured cornea. (A) Correlation between opacity grades and myeloperoxidase (MPO) levels in the cornea 3 d after ethanol injury and scraping of the epithelium. The values were data from 15 mice that were each treated with different numbers of hMSCs (0.01–1 × 10^6 cells) from donor 6015 or HBSS. (B) Representative corneal photographs on day 7 after injury and i.v. administration of vehicle (HBSS 1) or 1 × 10^6 hMSCs from different donors of bone-marrow aspirates. (C) Inflammation of the cornea as measured by MPO levels in the cornea on day 1 after injury. Values are means ± SD ($n$ = 3–5 for each of 13 donor hMSCs; $n$ = 6 for HBSS, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; N.S., no significant difference; assessed by one-way ANOVA with Dunnett's multiple comparison test).

used to characterize MSCs in culture (16, 17). Surprisingly, there were no significant correlations with the efficacy of the cells in suppressing MPO in the model (Fig. 2A). In fact, there was a negative correlation with the potential of the hMSCs for osteogenic differentiation in vitro (Fig. 2A). Also, there was no significant correlation with adipogenic potential, rate of proliferation, and colony forming units-fibroblastoid (CFU-f). Similarly, there was no apparent relationship to the spindle-shaped morphology of cells (Fig. S1) that has been used to identify early progenitors in the cultures (18, 19), and there were no significant

differences in expression of standard surface markers between the effective and ineffective hMSCs (Table S1).

To search further for informative biomarkers, we performed RT-PCR assays on the hMSCs for the expression of genes that have been suggested as responsible for anti-inflammation and immune-suppressive effects of hMSCs (6, 9–11, 20–24). As expected from previous observations in the cornea model (10, 15), there was a highly significant correlation between the levels of mRNA for TSG-6 in the hMSCs and their efficacy in reducing MPO levels in the cornea model (Fig. 2B). The correlation was essentially the same if based on assays of hMSCs that were freshly isolated from culture or on assays of the same cells after expression of *TSG-6* was increased by incubation with TNF-α for 16 h (9). There was a less-significant positive correlation with the levels of heme oxygenase 1 (HMOX1) in hMSCs that were freshly isolated from culture, but no significant correlation with hMSCs that were incubated with TNF-α. In addition, there were no significant correlations with the levels of mRNA for cyclooxygenase 2 (COX2), a key enzyme of synthesis of Prostaglandin E2, IL-1 receptor antagonist (IL-1Ra), transforming growth factor-β1 (TGF-β1), or indoleamine 2,3-dioxygenase 1 (IDO1).

The role of TSG-6 in the efficacy of the cells was confirmed by experiments (Fig. 3) in which *TSG-6* was overexpressed in hMSCs that initially expressed a low level of *TSG-6* (donor 7052). Overexpression of *TSG-6* decreased the potential of the cells to differentiate to mineralizing cells (Fig. 3B) and increased the efficacy of the hMSCs in the model for injury of the cornea (Fig. 3 C–E).

However, as hMSCs expressing high levels of *TSG-6* were expanded, there was a marked decrease in expression of *TSG-6* between 10 and 20 population doublings (Fig. S2). The results were consistent with previous observations that cultures of hMSCs lose many of their biological properties as they are expanded beyond about 20 population doublings in culture (17, 25).

To evaluate further the predictive value of *TSG-6* expression as a biomarker, we compared the efficacy of the hMSCs in two additional murine models: sterile inflammation of the peritoneum induced by zymosan (11) and lung injury induced by bleomycin (26). We tested six different donor preparations of hMSCs (Fig. 4A): three with the highest levels of *TSG-6* expression (defined as TSG-6^hi hMSCs) and three with the lowest levels (defined as TSG-6^low hMSCs). The efficacy of each of the six samples was then tested separately in the models.

As expected, the three preparations of TSG-6^hi hMSCs were more effective than the three preparations of TSG-6^low hMSCs in preventing opacity and decreasing MPO in the cornea model

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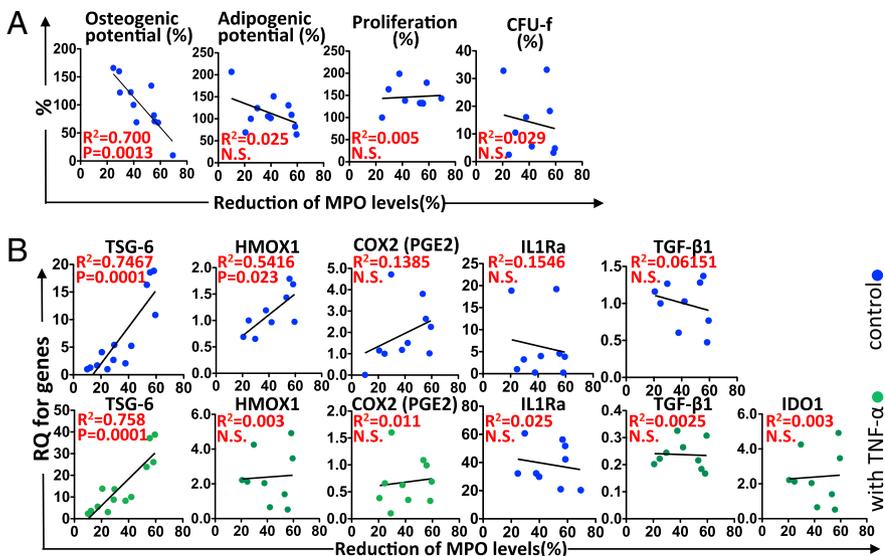




**Fig. 2.** Correlation between potential biomarkers and effectiveness in reducing MPO levels in the injured cornea. (A) Correlations between efficacy of hMSCs in reducing MPO levels in the cornea model and conventional assays in vitro for MSCs. (B) Correlations between efficacy of hMSCs in reducing MPO levels in the cornea model and expression by RT-PCR of genes previously linked to the therapeutic benefits of hMSCs. (Upper and Lower) Values obtained with (Lower) or without (Upper) stimulation of the cells with TNF-α. IDO1 was not detected in some hMSCs that were not stimulated with TNF-α.
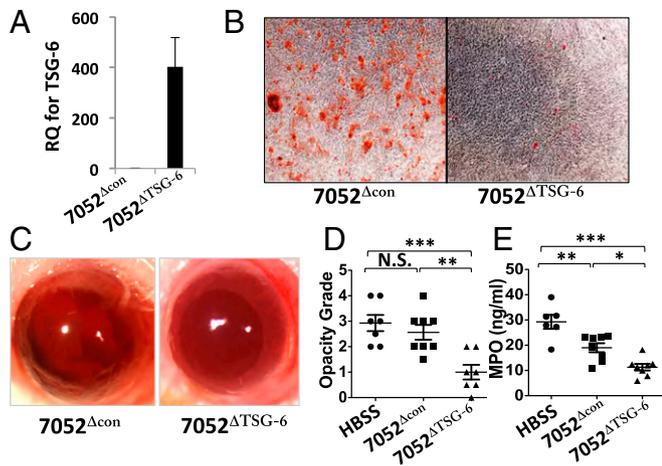
**Fig. 3.** Overexpression of *TSG-6* in hMSCs decreases osteogenic capacity in vitro and increases anti-inflammatory effects in vivo. (*A*) Real-time RT-PCR for the levels of *TSG-6* in hMSCs (donor 7052) transfected with control vector (7052^Δcont) or TSG-6 construct (7052^ΔTSG-6) after 24 h. Values are means ± SD. (*B*) Representative photos of cultures of hMSCs stained with Alizarin Red after osteogenic differentiation. (*C*) Photographs of corneas of mice that received i.v. injections of $1 \times 10^6$ cells of 7052^Δcont or 7052^ΔTSG-6 after ethanol injury to cornea. (*D*) Quantification of corneal opacity on day 3 using clinical grading system on a 0–4 scale (n = 7 or 8 mice for each group, **P < 0.01; ***P < 0.001; N.S., no significant difference; data evaluated by one-way ANOVA). (*E*) Inflammation of the cornea as assayed by MPO levels 1 d after the injury. The 7052 control MSCs were more effective in suppressing MPO levels than in Fig. 1*C*, apparently because transfection with control vector itself increased expression of *TSG-6* (not shown) (n = 6–8 for each group, *P < 0.05, **P < 0.01, ***P < 0.001; assessed by one-way ANOVA).

(Fig. 4 *B* and *C*). They were also more effective in reducing proinflammatory cytokines in the peritonitis model (Fig. 4*D*). In addition, in the bleomycin model for lung injury, in which fibrosis is detected by histology at 8 d (26), they were more effective in improving survival (Fig. 4*E*) and preserving body weight (Fig. S3*A*) in the treated mice compared with a control group. However, the differences in survival between mice that received TSG-6^hi hMSCs and TSG-6^low hMSCs were not significant because one (donors 265 and 7075) of each group showed moderate survival (Fig. S3*B*).

The negative correlation between efficacy in the cornea model and osteogenic potential of the hMSCs (Fig. 2*A*) raised the possibility that efficacy of the cells was linked to increased NF-κB signaling because a recent report indicated that increased NF-κB signaling inhibited osteogenesis (27). As expected, there was a negative correlation between osteogenic potential and expression of *TSG-6* in the hMSCs (Fig. 5*A*). Also, there was a weak correlation between expression of *TSG-6* and expression of *TNFRSF1A*, a tumor necrosis factor receptor superfamily member 1A, that may reflect the potential of TNF-α and other proinflammatory factors to up-regulate expression of *TSG-6* (28). More importantly, NF-κB binding activity was higher in hMSCs expressing high levels of *TSG-6* (Fig. 5*C*), and inhibition of NF-κB signaling with SN50 (29) both decreased expression of *TSG-6* (Fig. 5*D*) and increased osteogenic differentiation of hMSCs in culture (Fig. 5 *E* and *F*).

To search for additional useful markers for hMSCs, we tested for correlations with the anonymous personal data available on the donors of the bone-marrow aspirates from which the hMSCs were obtained. Of special interest was that, in the small cohort available, expression of *TSG-6* has higher in hMSCs from female donors, especially after the cells were stimulated with TNF-α (Fig. 6*A*). Also, as expected, hMSCs from the female donors were more effective in suppressing MPO levels in the cornea model and had slightly less osteogenic potential in culture (Fig. 6*B*). There were negative correlations with height and weight
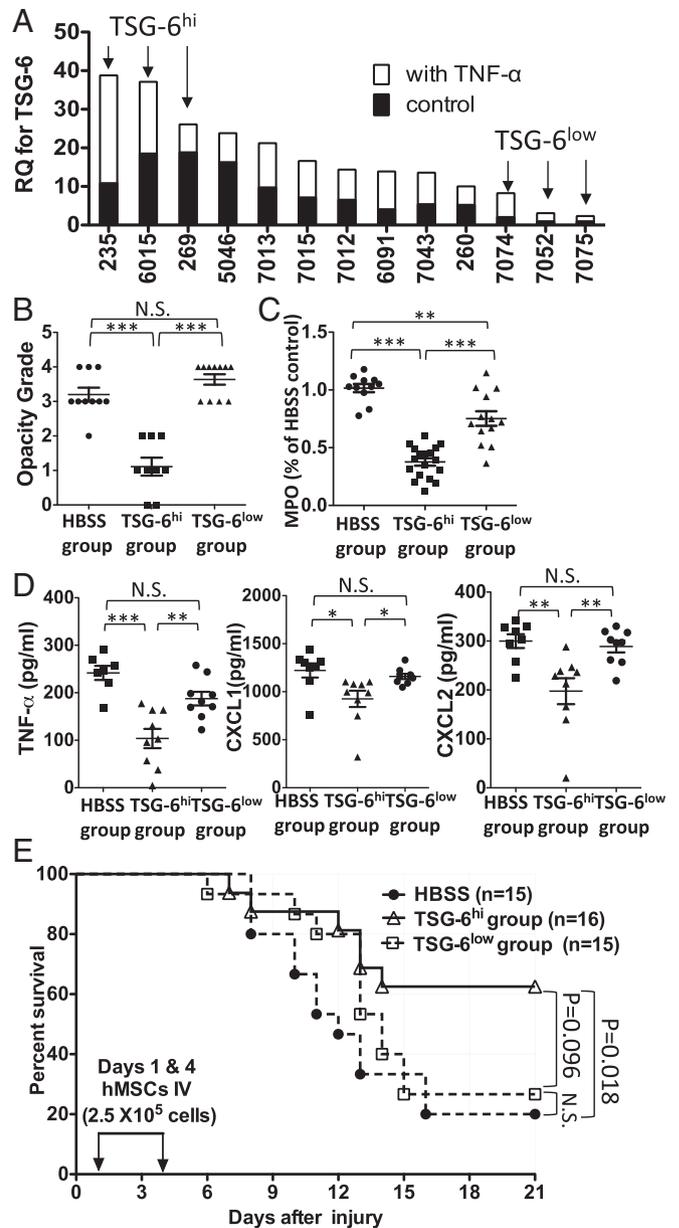


**Fig. 4.** The TSG-6^hi hMSCs are more effective in suppressing sterile inflammation in three in vivo models. (*A*) RT-PCR assays for *TSG-6* expression in hMSCs from a series of donors of bone-marrow aspirates with and without TNF-α stimulation. Values are relative to express level in donor 7075 that was set as 1.0. To simplify comparisons of the data from experiments in vivo, three preparations with the highest levels were designated as TSG-6^hi, and three with the lowest levels were designated as TSG-6^low. (*B*) Opacity of the cornea on day 7 after ethanol injury. The values are data from three to five mice that were each treated with hMSCs from the same six donors, of which three were TSG-6^hi hMSCs and three were TSG-6^low hMSCs (***P < 0.001; N.S., no significant difference; assessed by one-way ANOVA). (*C*) Inflammation of the cornea 1 d after injury as assayed by MPO levels. (n = 4 or 5 mice that were treated with hMSCs from the same donor and n = 12 for HBSS control; **P < 0.01, ***P < 0.001; assessed by one-way ANOVA). (*D*) Assays of the efficacy of the hMSCs in the mouse model for zymosan-induced peritonitis. Values are from ELISAs for mouse TNF-α, CXCL1, and CXCL2 in peritoneal lavage collected 4 h after i.p. injection of zymosan followed by injection of $1.5 \times 10^6$ hMSCs (n = 3 mice treated with the same donor MSCs and n = 7 for HBSS control; *P < 0.05, **P < 0.01, ***P < 0.001; N.S., no significant difference; assessed by one-way ANOVA). (*E*) Survival proportions after bleomycin injury of lung in mice followed by i.v. administration of $2.5 \times 10^5$ hMSCs [n = 5 or 6 mice treated with hMSCs from the same of six donors and n = 15 for HBSS controls; assessed by log-rank (Mantel-Cox) test].
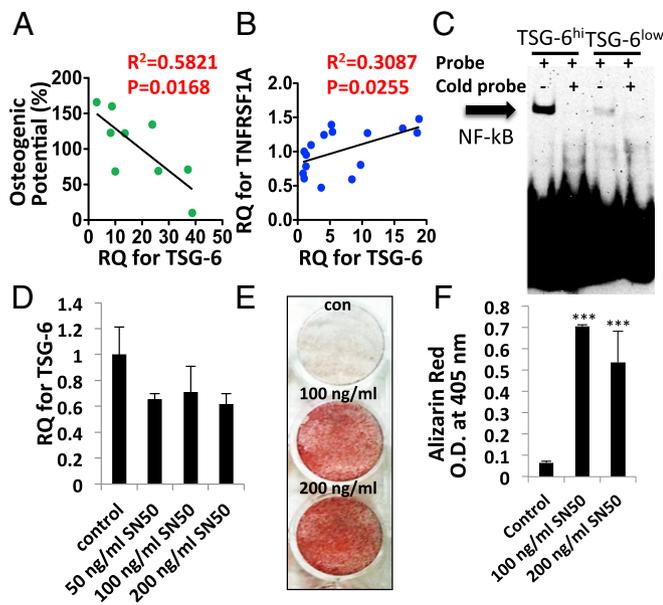
**Fig. 5.** Negative correlation between osteogenic differentiation potential and increased NF-κB signaling. (A) Correlation between osteogenic differentiation potential in vitro and the levels of mRNA for TSG-6 in hMSCs after stimulation with 5 ng/mL TNF-α stimulation for 16 h. (B) Correlation between the levels of mRNA for TSG-6 and TNFRSF1A in hMSCs. (C) Nuclear extracts from TSG-6hi and TSG-6low hMSCs were assayed for NF-κB DNA-binding activity by EMSA. The specific DNA-binding activity of NF-κB complex is indicated by an arrow. (D) Real-time RT-PCR for the levels of TSG-6 in hMSCs (TSG-6hi donor 6015) after 24-h treatment of SN50, an inhibitor of NF-κB signaling. Values are means ± SD. (E) Representative photo of cultures of TSG-6hi hMSCs (donor 6015) treated with SN50, incubated in osteogenic medium, and stained with Alizarin Red. (F) Quantification of Alizarin Red staining of cultures treated as in E. Values are means ± SD (n = 3; ***P < 0.001; assessed by one-way ANOVA).

(Fig. 6C), but no correlation with age in the small cohort that was clustered in the younger ages (Fig. 6C). Adjusting for height and weight via a body mass index, analyses of the data with three subsequent multiple linear-regression models incorporating all of the above measures indicated that the most parsimonious models for TSG-6 levels in hMSCs (with and without TNF-α stimulation) included a sex coefficient as the common predictive item. The MPO-level difference between females and males is

highly significant ($P = 0.0009$); the 95% confidence interval for this difference indicated that male donors showed decreases in MPO levels that ranged from 7.06% to 40.07%, compared with female donors.

We also detected a negative correlation between date of collection of the bone-marrow aspirates from which hMSCs were immediately prepared and the efficacy of the cells in the cornea model (Fig. S4): i.e., the older samples were more efficacious. This correlation was probably explained by the fact that most of the older samples were from female donors and the more recent from male donors (Table S2).

To explore further the apparent sex bias, we examined the effects of incubating hMSCs with estradiol, the female hormone that reaches the highest peak values in serum (up to 1.6 nM) during the menstrual cycle (30). One-day exposure of hMSCs to low doses (10–100 pM) of estradiol decreased TSG-6 levels in hMSCs whereas a high dose (100 nM) of estradiol increased TSG-6 expression in hMSCs (Fig. S5A). The effects of the high dose persisted after incubation with 100 nM for 2 d, in that the increased levels of TSG-6 mRNA were observed after the cells were incubated without estradiol for an additional 2 d (Fig. S5B). Furthermore, pretreatment of low doses of estradiol in hMSCs for 4 d promoted osteogenic differentiation whereas pretreatment with a high dose of 400 nM for 4 d suppressed osteogenic differentiation (Fig. S5 D and E), without affecting cell viability (Fig. S5C).

## Discussion

The results presented here may overcome a major barrier to research with hMSCs: they provide, to our knowledge, the first biomarker that can predict the efficacy of the human cells in reducing sterile inflammation in in vivo models. Assays in the model for ethanol injury of the cornea demonstrated marked differences in the inflammation-suppressive efficacy of different preparations of hMSCs. The efficacy of the hMSCs, in turn, was proportional to their mRNA levels for the inflammation-modulating protein TSG-6. The levels of TSG-6 expression predicted the efficacy of the cells not only in the model for chemical injury of the cornea but also for sterile inflammation produced by i.p. injection of the yeast extract zymosan in mice and, with somewhat less accuracy, in a more complex murine model of bleomycin-induced lung injury. The more variable results in the bleomycin model probably reflect the complexity of this model in which bleomycin triggers apoptosis and releases oxidants, and this injury followed first by a phase marked by invasion of inflammatory and immune cells and then by a fibrotic phase (31).

The RT-PCR assay for TSG-6 expression that was used is robust, and it can be performed in about 4 h. As reported
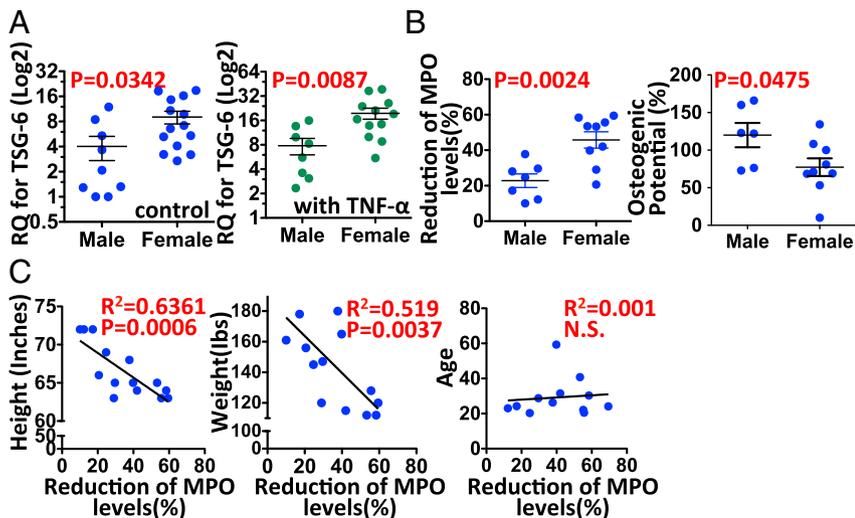


**Fig. 6.** Correlations with sex and other variables. (A) RT-PCR assays of TSG-6 in hMSCs from male and female donors of bone-marrow aspirates, with and without stimulation of the cells with TNF-α. (B) Comparison of hMSCs from male and female donors in reducing MPO in the model for ethanol injury of the cornea and in osteogenic potential in vitro. (C) Correlation of the efficacy of hMSCs in reducing MPO in the cornea model with height, weight, and age of donors.

previously (9), there was a close correlation between levels of mRNA for TSG-6 in MSCs and secretion of the protein in naive hMSCs, hMSCs stimulated with TNF-α, and knock-down experiments with siRNAs. Therefore, the levels of *TSG-6* expression with this assay, or some modified assay for *TSG-6* expression, should be useful in selecting hMSCs to reduce the variability in experiments and clinical trials with MSCs for the large number of diseases in which sterile inflammation is now recognized to play a critical role (3, 9, 32, 33). hMSCs, however, are highly sensitive to their microenvironments (3, 4, 9, 17, 25), and the levels of TSG-6 in a given preparation of MSCs may vary under different culture conditions to prepare the cells before administration in vivo.

One of the critical observations was that the conventional assays used to characterize hMSCs did not predict the efficacy of the cells in suppressing inflammation in vivo. Also, with the exception of heme oxygenase 1, there was no significant correlation with expression of several other genes previously linked to the therapeutic potentials of the cells (6, 9–11, 20–24). Of special interest was the negative correlation between the effectiveness of the cells in suppressing inflammation in the cornea model and their potential for osteogenic differentiation in culture because this negative correlation was linked to increased NF-κB signaling in the more efficacious cells and provided an independent marker for such cells. Also, the negative correlation with osteogenic differentiation suggests that hMSCs optimal for one application, such as suppression of inflammation, may be suboptimal for other applications, such as bone engineering. The decrease in *TSG-6* expression between 10 and 20 populations doubling suggests that the extensively expanded MSCs from a single "universal donor" may decrease the effectiveness of the cells in suppressing sterile inflammation.

The use of *TSG-6* expression as a biomarker for the efficacy of hMSCs in suppressing inflammation in vivo is consistent with previous observations on the protein (10, 12–15). TSG-6 is a naturally occurring protein of 35 kDa that is secreted by most cells in response to proinflammatory cytokines, and it has multiple actions that are linked to modulation of inflammation and stabilization of the extracellular matrix (12–14). Among its multiple actions is that TSG-6, either directly or through a complex with hyaluronan, binds to CD44 on resident macrophages in a manner that decreases TLR/NF-κB signaling and modulates the initial phase of the inflammatory response of most tissues (11, 20). hMSCs were observed to lose their effectiveness in several animal models for human diseases after siRNAs were used to knock down expression of *TSG-6* (9–11, 20, 34). Also, administration of recombinant TSG-6 reproduced most of the beneficial effects of the hMSCs (9–11, 20, 34). The role of TSG-6 in the cornea model was further validated here by the demonstration that overexpression of *TSG-6* greatly enhanced the effectiveness of hMSCs. However, the data to date have not established that TSG-6 is the only paracrine factor secreted by MSCs that suppresses inflammation, and it is possible that genes expressed upstream of *TSG-6* may prove to be useful biomarkers.

Surprisingly, comparison of hMSCs from a small cohort suggested that hMSCs from female donors compared with male donors were more effective in suppressing inflammation in the cornea model, expressed higher levels of *TSG-6*, and had slightly less potential for osteogenic differentiation. The data suggesting a sex bias in donors of hMSCs is consistent with a large body of literature demonstrating marked differences in susceptibility to diseases between men and women (35) and the effects of menstrual cycles on women (36, 37).

The use of *TSG-6* expression as a biomarker should improve selection of the most effective MSCs for clinical trials and overcome some of the variations in patient responses.

## Materials and Methods

**Cell Preparations.** hMSCs were prepared from bone-marrow aspirates under the Institutional Biosafety Committee as previously described (9, 11). The aspirates were obtained under institutional review board approved protocols over several years from normal volunteers who responded to local postings in an academic setting (Table S2) and who were screened beforehand with blood assays for infectious agents. In brief, mononuclear cells were isolated by ficoll gradient separation of bone-marrow aspirates from the iliac crest of normal volunteers, incubated in complete culture medium (CCM) [α-MEM (Life Technologies) containing 17% (vol/vol) FBS (Atlanta Biologicals), 2 mM L-glutamine, and 1% (vol/vol) penicillin–streptomycin (Life Technologies)] at high density to obtain adherent cells (P0 cells), replated at low density (60–100 cells per cm$^2$), incubated to about 70% confluency (cell density about 10,000 cells per cm$^2$ at harvest), and frozen (P1 cells, $1 \times 10^6$ cells per vial). Frozen vials of P1 cells were thawed and incubated at high density for 24 h to obtain adherent viable cells, replated at low density (200 cells per cm$^2$), and incubated to about 70% confluency (cell density about 10,000 cells per cm$^2$ at harvest) to obtain P2 hMSCs that we used for the experiments. Population doublings were estimated as an average of five doublings (50-fold increase in cell number) per passage.

To activate the cells to express TSG-6, P2 hMSCs were incubated with 5 ng/mL TNF-α (R&D Systems) in α-MEM containing 2% FBS for 16 h (9). Similar results were obtained with two or more vials of P1 hMSCs from the same master bank prepared from the same donor. The FBS used for the experiments was selected by screening four to five lots for rapid growth of hMSCs. Different lots standardized to provide about the same propagation rate of hMSCs were used to prepare P0 hMSCs, but the same lot was used to expand P1-to-P2 hMSCs for the experiments here.

**RNA Extraction from Cultured Cells and Real-Time RT-PCR Analysis.** Total RNA from monolayer cells was extracted (RNeasy Mini kit; Qiagen), and about 0.1–1 μg of total RNA per sample was used to synthesize double-stranded cDNA by reverse transcription (SuperScript III; Life Technologies). Real-time RT-PCR was performed in triplicate for human *GAPDH*, *TSG-6 (TNFAIP6)*, *HMOX1*, *COX2*, *IL1Ra*, *TGF-β1*, *IDO1*, and *TNFRSF1A*, using TaqMan Gene Expression Assays (Life Technologies). Real-time amplification was performed with TaqMan Universal PCR Master Mix (Life Technologies) and analyzed on a 7900HT fast real-time PCR system (Life Technologies). For assays, reactions were incubated at 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles at 95 °C for 15 s, followed by 60 °C for 1 min. Data were analyzed with Sequence Detection Software, version 2.3 (Life Technologies), and relative quantities (RQs) were calculated with the comparative CT method using RQ Manager, version 1.2 (Life Technologies).

**Animals.** The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Texas A&M Health Science Center. Six- to seven-week-old male BALB/c mice (BALB/cAnNCrl; Charles River Laboratories International) were used in all experiments.

**Animal Model of Corneal Injury and Treatment.** Chemical burned corneal injury was produced as described previously (15). Mice were anesthetized by isoflurane inhalation. To create the chemical burn, 100% ethanol (Sigma-Aldrich) was applied to the whole cornea, including the limbus, for 30 s, followed by rinsing with 1 mL of PBS (Life Technologies). Then, the epithelium over the whole corneal and limbal region was mechanically scraped using a surgical blade. Upon completion of the procedure, the eyelids of a mice were closed with one 8-0 silk suture at the lateral third of the lid margin. Immediately after injury, mice received i.v. injection of hMSCs ($1 \times 10^6$) in 0.1 mL of Hanks' balanced salt solution (HBSS; Life Technologies).

**Ocular Surface Evaluation.** After injury and treatment, the mouse corneas were examined for corneal opacity and photographed at 3 d or 7 d. Corneal opacity was assessed and graded as described previously from the photographs by an ophthalmologist who was not aware of the treatment of the mice (15).

**Protein Extraction from Cornea.** Corneas were excised by cutting just beyond the limbus, the interior surface was scraped with a small sponge to remove endothelial cells and iris, and then the corneas were cooled with liquid nitrogen. A 3-mm punch was used to obtain a sample from the center of each cornea that was lysed in 150 μL of tissue-extraction reagent containing protease inhibitors (Life Technologies). The samples were sonicated on ice and centrifuged at $15,000 \times g$ at 4 °C for 15 min. The supernatant was used for MPO ELISAs.

**Mouse Model of Peritonitis and Measurements of Inflammation.** To induce inflammation in male BALB/c mice, 1 mL of zymosan solution (1 mg/mL; Sigma-Aldrich) was administered by i.p, followed by i.p. injection of $1.5 \times 10^6$ each donor-derived hMSCs 15 min later (11). After 4 h, inflammatory exudates

were collected by peritoneal lavage, and the cell-free supernatant was used to measure levels of the proinflammatory molecules [mouse Tumor necrosis factor alpha (mTNFα), mouse chemokine (C-X-C motif) ligand 1 (mCXCL1), mouse Chemokine (C-X-C motif) ligand 2 (mCXCL2)] by ELISAs.

**Mouse Model of Lung Injury Induced with Bleomycin.** Lung injury was induced in female C57BL/6J mice anesthetized with isoflurane by administration of bleomycin sulfate (Sigma-Aldrich) at 2.25 U/kg of body weight in 0.9% sodium chloride via an intubation technique (26). Sham animals were given 0.9% sodium chloride alone. Intravenous administration of each donor-derived hMSCs ($2.5 \times 10^5$ cells in 150 μL) was performed on days 1 and 4 postinjury. A portable mouse pulse oximeter (STARR Life Sciences Corp.) was used to monitor arterial blood oxygen saturation ($SpO_2$) in free-roaming nonanesthetized mice. Weight and $SpO_2$ measurements were recorded every other day for the entire duration of the 21-d survival study.

**ELISAs.** Mouse MPO (mouse MPO ELISA kit; HyCult Biotech), TNF-α, CXCL1, and CXCL2 (R&D Systems) were detected with commercially available ELISA kits following procedures described by the manufacturers.

**TSG-6 Overexpression.** To prepare TSG-6 cDNA, total RNA was isolated from hMSCs that were stimulated by incubation with 5 ng/mL TNF-α in α-MEM containing 2% FBS overnight (9). About 1 μg of total RNA was used to produce the first-strand cDNA pool by Reverse Transcriptase (Superscript II/oligo dT12-18; Life Technologies). cDNAs encoding human *TSG-6* (GenBank accession no. NM_007115) was amplified by PCR using the following primers: 5′-CGGGGTACCATGATCATCTTAATTTACTT-3′ (sense for hTSG-6), and 5′-GGTGATCAGTGGCTAAATCTTCCA-3′ (antisense for hTSG-6-WT). The PCR products were subcloned into the BamHI and EcoRI sites in multicloning sites of a pEF4-Myc/His plasmid (Life Technologies), and the plasmids were amplified in *Escherichia coli* DH5α cells (Life Technologies).

The TSG-6 or control plasmid (0.1 μg per well in six wells) was transfected into hMSC with Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were harvested for assays.

**Differentiation Assay.** For details on the differentiation assay, see *SI Materials and Methods*.

**Nuclear Extraction and NF-κB Electrophoretic Mobility-Shift Assay.** Cells were harvested at a density of 10,000 cells per cm², nuclear fraction was extracted (Nuclear Extraction Kit; Signosis), and assays for nuclear NF-κB were performed using EMSA Kit (Signosis) according to the manufacturer's instructions.

**Inhibition of NF-κB Signaling.** hMSCs were plated at 10,000 per well in CCM in six-well plates. To inhibit NF-κB signaling, cells were treated with the NF-κB inhibitor SN50 (EMD Millipore) in CCM every 2 d for 4 d. Then, the medium was changed to osteogenic differentiation media. For RT-PCR, cells were treated with SN50 (50–200 ng/mL) for 24 h in CCM and harvested for RNA extraction.

**Statistical Analyses.** Comparisons between two groups were made with the use of unpaired and two-tailed Student *t* tests. Comparisons of more than two groups were evaluated by ANOVA. Survival of mice between groups was compared using a log-rank (Mantel–Cox) test. $P < 0.05$ was considered significant.

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# Supporting Information

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### SI Materials and Methods

For differentiation assay, hMSCs were plated at 10,000 cells per cm$^2$ in a six-well plate. To induce adipogenesis, hMSCs were cultured in CCM supplemented with 500 nM dexamethasone (Sigma-Aldrich), 500 nM isobutylmethylxanthine (Sigma-Aldrich), and 50 µM indomethacin (Sigma-Aldrich) for 14 d, with medium changes every 2–3 d. To induce osteogenesis, hMSCs were cultured in CCM supplemented with 10 nM dexamethasone, 10 mM β-glycerolphosphate (Sigma-Aldrich), and 50 µM ascorbate-2-phosphate (Sigma-Aldrich) for 18 d, with medium changes every 2–3 d. For quantitative assays of adipogenic differentiation, the monolayer cells were fixed in 10% formalin for 10 min, washed three times with PBS, and stained with fresh Oil Red-O solution in 60% (vol/vol) isopropyl alcohol in PBS for 20 min. The samples were washed extensively with PBS to remove unbound dye, and then 1 mL of isopropyl alcohol was added to the stained culture dish. After 5 min, the absorbance of the extract was assayed by a spectrophotometer (Fluostar Optima; BMG Labtechnologies) at 485 nm. For quantitative assay osteogenic differentiation, the cellular aggregates were washed in PBS and fixed in formalin for 30 min. The cells were stained with 40 mM Alizarin Red S for 30 min and washed with distilled water. The stained cells were transferred to a 2-mL screw-top microcentrifuge tube and incubated at 85 °C for 15 min in 1 mL of 10% (vol/vol) acetic acid (1). The extract was cooled on ice and centrifuged at 21,000 × $g$ for 5 min. About 0.5 mL of the supernatant was transferred to a fresh tube containing 0.2 mL of 10% (vol/vol) ammonium hydroxide. The red solution was transferred to a 96-well plate and read at 485 nm on a spectrophotometer.

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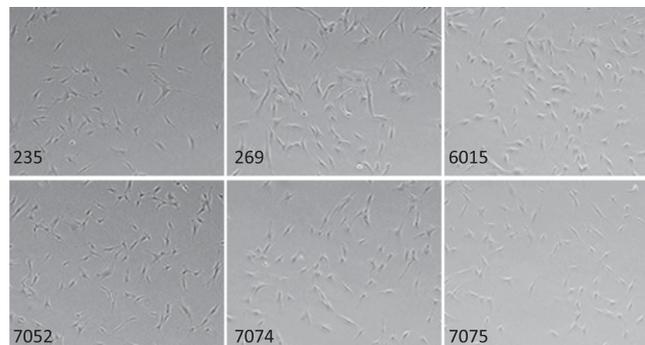
**Fig. S1.** hMSC morphology from different donors. Representative hMSC photographs at day 4 after plating at 200 cells per cm$^2$ in six wells. One of the TSG-6$^{hi}$ hMSCs (donor 269) consisted primarily of large cells whereas the TSG-6$^{low}$ hMSCs (donors 7052, 7074, and 7075) had small spindle-shaped morphology.
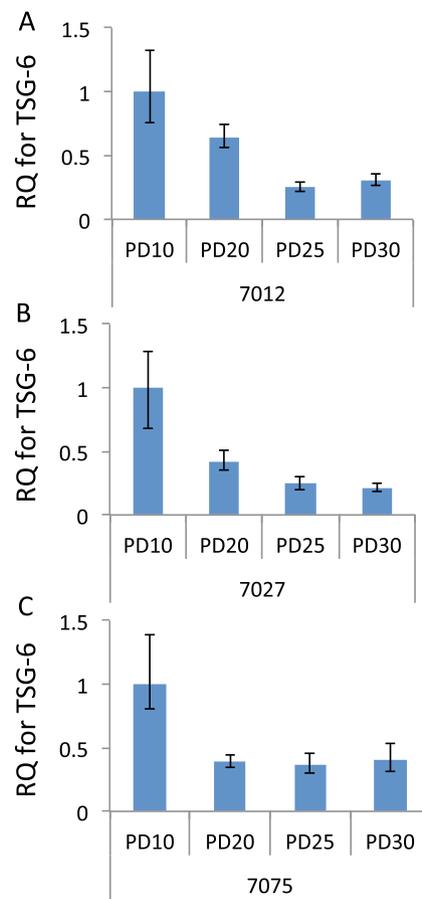
**Fig. S2.** Decrease in *TSG-6* expression in hMSCs (*A*, donor 7012; *B*, donor 7027; *C*, donor 7075) after expansion in culture through 30 population doublings (PDs). The cells were repeatedly passed by plating at 200 cells per cm² and cultured 5 d before lifting. Values are means ± SD.

**Fig. S3.** Survival proportions and relative weight changes after bleomycin injury followed by treatment with hMSC. (*A*) Relative weight changes before death or end point were expressed as a percentage of preinjury weight (data assessed by one-way ANOVA). (*B*) The survival proportions of mice that received hMSCs (*n* = 5 or 6) from the same donor of which three were TSG-6^{hi} MSCs (*Left*) and three were TSG-6^{low} MSCs (*Right*) (as shown in Fig. 4*A*) or HBSS control (*n* = 15).



**Fig. S4.** Negative correlation between reduction of MPO levels in the ethanol injury of cornea model and the date of collection of bone-marrow aspirates from which MSCs were immediately isolated. Pearson correlation analysis indicated that there was a very significant correlation between date of collection and the MPO levels (*r* = −0.760, *P* = 0.007), TSG-6 in control hMSCs (*r* = −0.628, *P* = 0.002), and TSC-6 in TNF-α–stimulated hMSCs (*r* = −0.709, *P* < 0.001). As indicated in Table S1, most of the older samples were from female donors, and the more recent samples were from male donors.

**Fig. S5.** Effects of estradiol on hMSCs from a male donor. (*A*) hMSCs (male donor 7052) were plated at 1,000 cells per cm², incubated with estradiol for 24 h, and assayed by real-time RT-PCR for *TSG-6* expression. Low concentrations of estradiol decreased TSG-6 levels, but a high concentration increased them. (*B*) hMSCs (donor 7052) plated at 1,000 cells per cm² were treated with 100 nM estradiol for 2 d and then incubated without estradiol for an additional 2 d. The increased levels of TSG-6 mRNA persisted. (*C*) Representative photographs of MSC cultures after estradiol treatment for 4 d. There were no apparent effects on morphology or viability. (*D*) hMSCs (donor 7052) were plated at 1000 cells per cm² and incubated in medium containing estradiol for 4 d with a change of medium on day 2. The cells were then incubated in osteogenic medium for 2 wk with a change of medium every 2–3 d. Representative photographs of cultures stained with Alizarin Red. Low concentrations of estradiol increased mineralization, but a high concentration decreased the level. (*E*) Quantification of Alizarin Red staining from experiment in *D* (n = 3; ***P < 0.001; assessed by one-way ANOVA). Because of variations in the efficacy of two different preparations of β-estradiol from the same supplier (Sigma-Aldrich), a maximum dose of 100 nM of one preparation (catalog no. E2758) was used for the experiments in *A* and *B*, and 400 nM of a second preparation (catalog no. E8875) was used for the experiments in *C–E*.

**Table S1. Expression of cell-surface markers (CSMs) in hMSCs**

| | Donor number | | | | | | | | | | | |
| | 7075 | | 7052 | | 7074 | | 6015 | | 235 | | 269 | |
| SCM | % of (+) cells | X-mean of (+) cells | % of (+) cells | X-mean of (+) cells | % of (+) cells | X-mean of (+) cells | % of (+) cells | X-mean of (+) cells | % of (+) cells | X-mean of (+) cells | % of (+) cells | X-mean of (+) cells |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| CD73 | 99.9 | 166 | 99.9 | 143 | 100 | 149 | 100 | 145 | 99.9 | 158 | 99.9 | 113 |
| CD90 | 100 | 2,190 | 91.9 | 2,070 | 100 | 1,950 | 91.9 | 2,310 | 100 | 1,500 | 100 | 2,050 |
| CD105 | 100 | 311 | 99.9 | 270 | 99.9 | 288 | 99.9 | 289 | 100 | 332 | 99.9 | 299 |
| CD146 | 99.9 | 206 | 99.9 | 238 | 100 | 283 | 100 | 217 | 100 | 221 | 99.9 | 588 |
| CD147 | 100 | 285 | 99.9 | 235 | 100 | 253 | 99.9 | 302 | 100 | 276 | 99.9 | 308 |
| CD29 | 100 | 303 | 100 | 256 | 100 | 222 | 100 | 285 | 100 | 212 | 100 | 242 |
| CD166 | 100 | 489 | 91.9 | 536 | 100 | 484 | 100 | 558 | 100 | 461 | 100 | 450 |
| HLA a,b,c | 99.9 | 126 | 99.9 | 78.4 | 99.9 | 114 | 100 | 160 | 99.9 | 112 | 99.8 | 113 |
| HLA II | 0.03 | 12.1 | 0.03 | 10.6 | 0.04 | 22.5 | 0.04 | 14 | 0.04 | 12.4 | 0.07 | 26.7 |

Symbols: (+) cells, positive cells; X-mean, mean fluorescence intensity.

**Table S2. Information for hMSC donors**

| Bone-marrow sample number | Sample date | Sex | Age | Weight, pounds | Height, inches | Aspirate volume (left; right) |
|---|---|---|---|---|---|---|
| 235 | 6 Jan 2004 | F | 24.10 | 120 | 63 | 3 |
| 260 | 6 Apr 2004 | F | 30.30 | 115 | 64 | 2 |
| 269 | 11 May 2004 | F | 31.50 | 112 | 64 | 3 |
| 5046 | 30 Nov 2004 | F | 40.80 | 112 | 65 | 3 |
| 5062 | 4 Jan 2005 | F | 21.00 | 156 | 65 | 3; 4 |
| 6015 | 22 Mar 2005 | F | 20.50 | 128 | 63 | 2.5 |
| 6091 | 28 Jun 2005 | F | 22.10 | 156 | 66 | 3 |
| 7012 | 19 Jul 2006 | F | 26.70 | 172 | 64 | 2; 2 |
| 7013 | 19 Jul 2006 | F | 33.00 | 135 | 70 | 2 |
| 7015 | 9 Aug 2006 | F | 29.70 | 125 | 65 | 2 |
| 7027 | 31 Jan 2007 | M | 47.00 | 175 | 75 | 2; 2 |
| 7043 | 5 Jul 2007 | M | 28.75 | 147 | 65 | 3 |
| 7049 | 13 Sep 2007 | F | 25.70 | 145 | 62 | 2; 2 |
| 7052 | 30 Oct 2007 | M | 20.30 | 126 | 69 | 2 |
| 7055 | 6 Nov 2007 | F | 59.33 | 165 | 65 | 3 |
| 7064 | 2 Jan 2008 | M | 24.20 | 178 | 72 | 2; 2 |
| 7068 | 4 Mar 2008 | M | 37.20 | 230 | 72 | 1 |
| 7073 | 26 Mar 2008 | M | 21.60 | 175 | 73 | 2 |
| 7074 | 1 Apr 2008 | M | 26.30 | 180 | 68 | 2 |
| 7075 | 15 Apr 2008 | M | 24.20 | 161 | 72 | 2; 2 |
| 8006 | 25 Jan 2012 | M | 23.00 | 200 | 72 | 2; 4 |