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Supplemental Information

Enhancing the Therapeutic Efficacy of Bone Marrow-Derived Mononuclear Cells with Growth Factor-Expressing Mesenchymal Stem Cells for ALS in Mice

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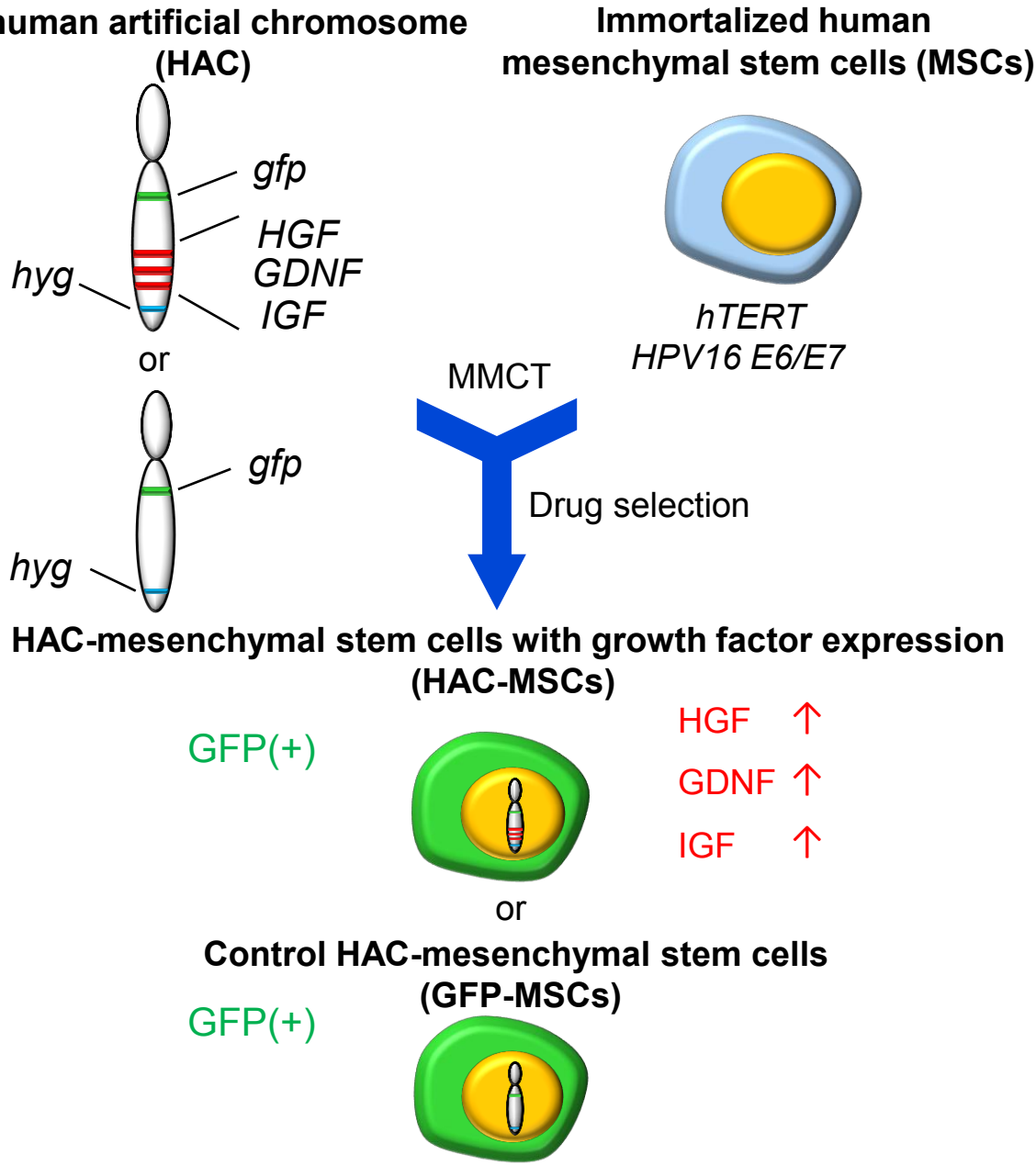

Figure S1. Scheme for generating HAC-MSCs with growth factor-expression and GFP-MSCs, related to Figure 1.

Green fluorescent protein (GFP), hepatocyte growth factor (HGF), glial cell line-derived neurotrophic factor (GDNF), insulin-like growth factor (IGF) genes and *hygromycin resistance gene (hyg)* were cloned into 21st human artificial chromosome vector (HAC) (Katoh et al., 2004, Watanabe et al., 2015). Immortalized human mesenchymal stem cells (MSCs) were prepared by insertion of *human telomerase reverse transcriptase (hTERT)* and *human papillomavirus (HPV)16 E6/E7* genes. After the HAC was transferred into the immortalized MSCs by microcell mediated chromosome transfer (MMCT), the line of growth factor-expressing human artificial chromosome-mesenchymal stem cells (HAC-MSCs) was generated with hygromycin selection. As an experimental control cells, control MSCs (GFP-MSCs) were generated by insertion of 21st human artificial chromosome vector including *gfp* gene and without growth factor genes into the immortalized MSCs (Watanabe et al., 2015).

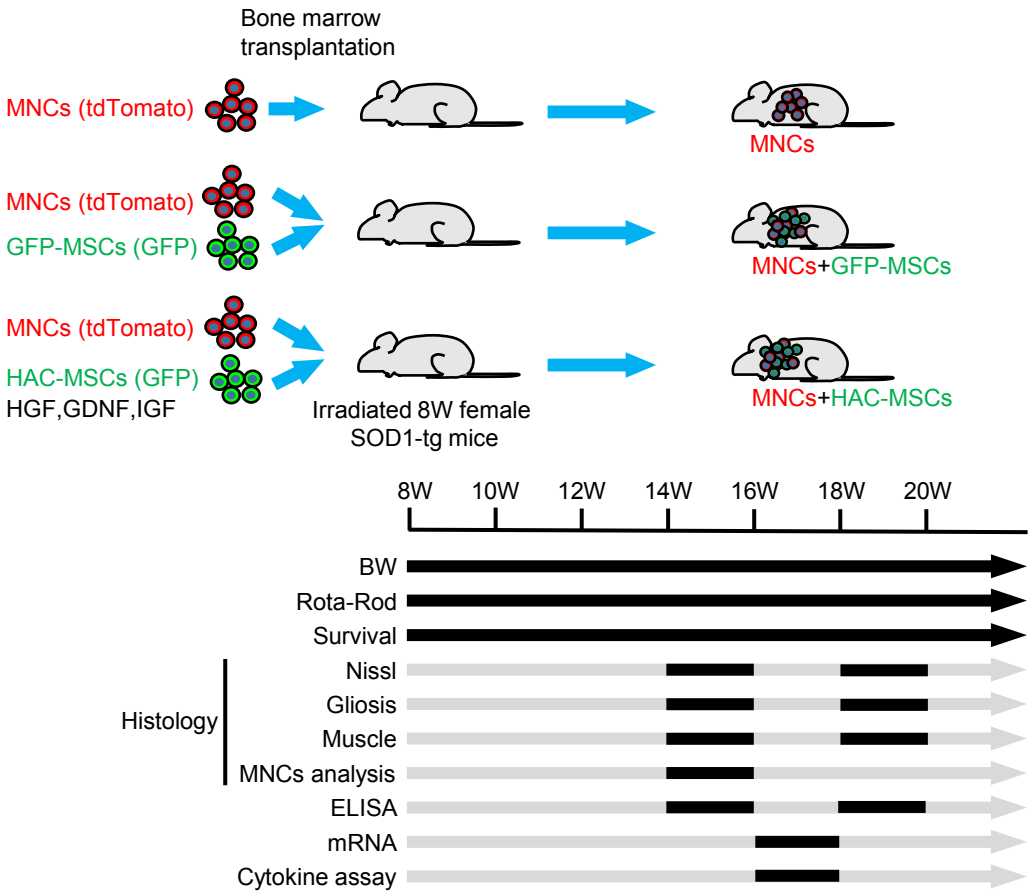

Figure S2. Schematic experimental design for transplantation of MNCs and HAC-MSCs with growth factor-expression, related to Figure 2-7. Primary MNCs from tdTomato transgenic mice and cultured HAC-MSCs were mixed and transplanted into irradiated 8-week-old female SOD1 G93A transgenic mice for the cell therapy of motor neuron diseases. Therapeutic effects were compared to only MNCs and MNCs + GFP-MSCs transplantation groups. SOD1-tg mice after three kinds of cell therapy were monitored body weight (BW), Rota-rod test and survival rate. In addition, histological and biological analysis were performed at the time point of black bar in above time course. ELISA; enzyme-linked immuno-sorbent assay, GDNF; glial cell line-derived neurotrophic factor, GFP; green fluorescent protein, HGF; hepatic growth factor, IGF; insulin-like growth factor, MNCs; mononuclear cells, MSCs; mesenchymal stem cells.

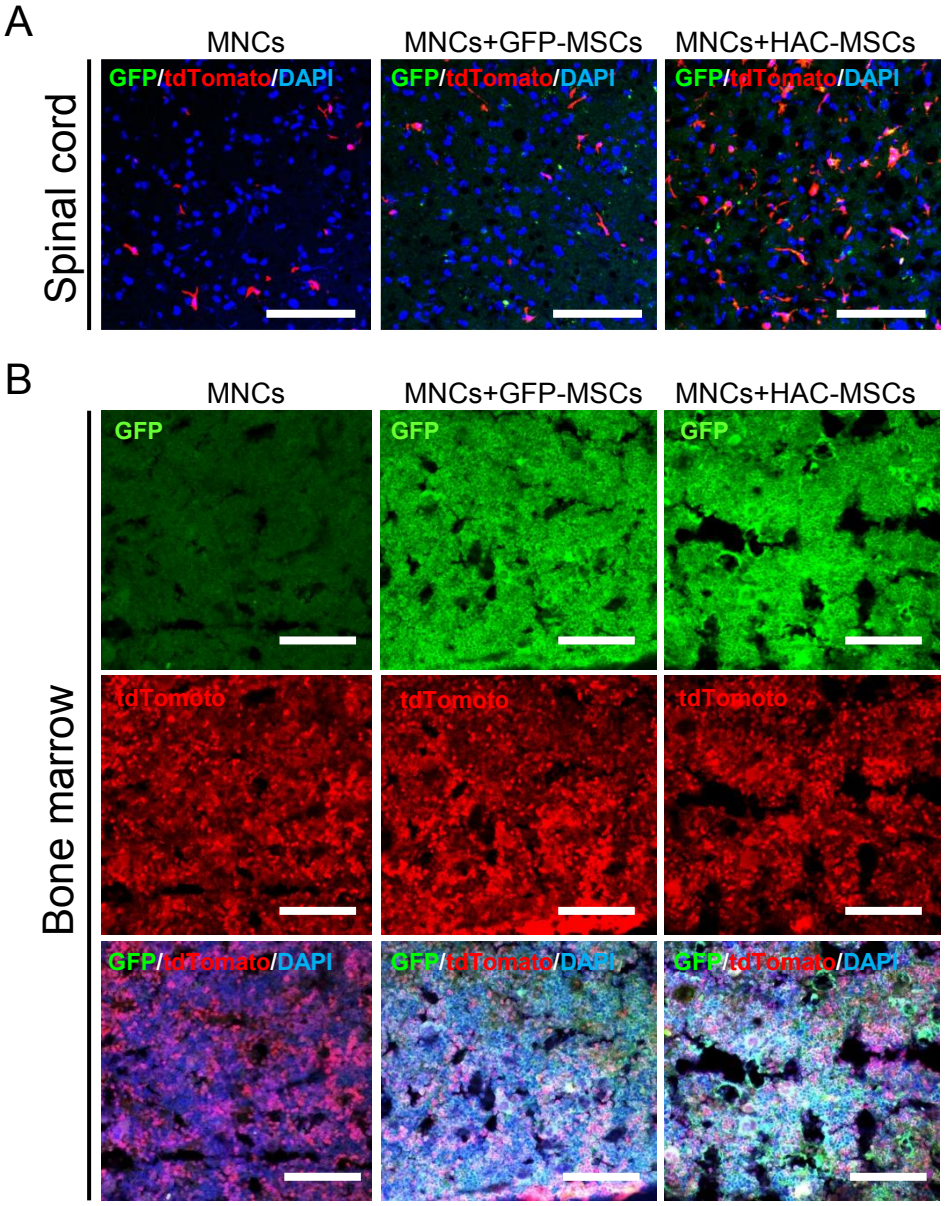

Figure S3. Histological analysis of spinal cord and bone marrow in SOD1-tg after cell therapy, related to Figure 5. (A) GFP (GFP-MSCs or HAC-MSCs; green), tdTomato (MNCs; red) signals and nuclear stain (DAPI, blue) in the sections of spinal cords in MNCs, MNCs + GFP-MSCs and MNCs + HAC-MSCs groups at 16 weeks old. Scale bar = 100 μm. **(B)** GFP (GFP-MSCs or HAC-MSCs; green), tdTomato (MNCs; red) and nuclear stain (DAPI, blue) in the sections of bone marrow tissues in MNCs, MNCs + GFP-MSCs and MNCs + HAC-MSCs groups. Scale bar = 100 μm.

Transparent Methods

Animals

C57BL/6 (WT), B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J and B6SJL-Tg(SOD1*G93A)1Gur/J (SOD1-tg) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Female SOD1-tg mice were used for the experimental study and male SOD1-tg mice were used only for the breeding. tdTomato-systemic expressing transgenic mice were generated by breeding B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J and Ayu1 promoter-driven Cre recombinase-expressing mice (Ayu1-Cre, Niwa et al., 1993), kindly gifted by Dr. Niwa. All animals were housed and provided with water and mouse chow ad libitum and maintained under a 12-hr light and 12-hr dark cycle. All animal experimental protocols were approved by the Institutional Animal Care and Usage Committee (IACUC) of Shiga University of Medical Science and were performed according to the guidelines of the IACUC of Shiga University of Medical Science.

HAC-MSC preparation

Human mesenchymal stem cells (MSCs) were immortalized by the combination of *human telomerase reverse transcriptase (hTERT)* and *human papillomavirus 16 E6* and *E7 (HPV16E6/E7)* genes (**Figure S1**) (Okamoto et al., 2002). The MSCs were maintained in the Dulbecco's modified Eagle medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (Thermo Fisher Scientific), 100 U/ml penicillin and 100 mg/ml streptomycin. Growth factor-expressing MSCs (HAC-MSCs) were generated by 21st human artificial chromosome (HAC) (Katoh et al., 2004) vector-containing cDNAs for HGF, GDNF, IGF and GFP as per methods described previously (Watanabe et al, 2015). Control MSCs expressing only GFP by human artificial chromosome vector (GFP-MSCs) were prepared to compare with the effects of HAC-MSCs (**Figure S1**) (Watanabe et al., 2015).

MNC preparation

Total bone marrow cells were collected from tdTomato transgenic mice expressing red fluorescence, systemically. Mononuclear cells (MNCs) were isolated from the total bone marrow cells by using Ficoll-Paque Plus gradient separation (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Bone marrow transplantation therapy with MNCs and HAC-MSCs for SOD1-tg mice

HAC-MSCs and MNCs were prepared at 1 x 10^6 cells for each mouse as enough cell number for reconstitution of bone marrow. After irradiation of the recipient female SOD1-tg mice with 9 Gy, bone marrow transplantation (BMT) therapy with both MNCs and HAC-MSCs was performed for SOD1-tg mice at 8 weeks of age via the injection of tail vein. A week prior to the BMT (7 weeks old), an immunosuppressive agent FK506 (3 mg/kg/day, AdooQ Bioscience, Irvine, CA) was administered orally to mice until the mice showed physiological death (17-24 weeks old). This was because the MSCs were of a human origin.

Behavior test

Rota-Rod tests (Ugo Basile, Comerio-Varese, Italy) were demonstrated once per week for all treatment mice from 8 weeks of age until their physiological death (If the result of Rota-Rod tests is zero second, the condition of the mice is recognized as physiologically dead). Rota-Rod tests were performed at a range from 5rpm/min to a maximum of 50 rpm/min for 5 min (acceleration was 9 rpm/min^2) as previously described (Terashima et al., 2014). Five times trial for each mouse with an interval of at least 3 min were performed and the averages of three medians were calculated for analysis. The number of survival mice was counted according to the definition of physiological death

until all mice were recognized as such for the Kaplan-Meier survival curve.

Histological analysis

For histological analysis of tissues from the treated mice at 14-16- and 18-20-week-old, transcardiac perfusion and fixation of 4% paraformaldehyde were performed. For immunostaining, sections of the spinal cord were prepared with a cryostat and incubated with a primary antibody (rabbit anti-β3-tubulin [Cell Signaling Technology, MA, USA], rabbit anti-Iba-1 [Wako, Osaka, Japan], or rabbit anti-GFAP [Cell Signaling Technology, MA, USA]) at 4 °C overnight. Then, the sections were incubated with a secondary antibody (goat anti-rabbit Alexa 633 [Life Technologies, Carlsbad, CA, USA]) at room temperature for 4 hours and mounted with Vectashield without nuclear stain. Some sections of spinal cords were performed Nissl stain with NeuroTrace 435/455 (Thermo Fisher Scientific) for the analysis of degeneration of motor neuron. Other sections of the spinal cord and bone marrow from each mouse were mounted with the Vectashield mounting medium with DNA staining using 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). These sections were observed under a confocal laser microscope (C1si; Nikon, Tokyo, Japan) with EZC1 3.90 software (Nikon). For quantitative analysis, Nissl-positive staining and GFAP-positive immunostaining were converted to the black white image and the intensity was measured in over 10 scenes per each mouse by ImageJ software version 1.51 (National Institutes of Health, Bethesda, MD). The numbers of tdTomato-positive MNCs were counted and calculated per unit area, and determined by sampling from eight to 10 scenes for each individual. To analyze the muscle degeneration, anterior tibial muscle was isolated from the treated mice after transcardiac perfusion fixation by 4% paraformaldehyde. Frozen sections were prepared after the muscle was embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan), and

were stained with hematoxylin-eosin. The area of muscle fibers was measured in over 10 scenes per each mouse by ImageJ software version 1.51 (National Institutes of Health).

Quantitative PCR of mRNA

Total RNA in spinal cord from each mouse was extracted by using the RNeasy Kit (Qiagen, Valencia, CA) and digested with DNase I (Thermo Fisher Scientific). After reverse transcription using the oligo dT primer (Thermo Fisher Scientific), each mRNA expression level was analyzed by quantitative PCR using a LightCycler 480 (Roche Diagnostics, Manheim, Germany) with the SYBR green method. The following primers were used: human HGF, forward primer 5-GAAGGATCAGATCTGGTTTTAATGA-3 and reverse primer 5-TGCATCCATAATTAGGTAAATCAATC-3; human GDNF, forward primer 5-GTCTGCCTGGTGCTGCTC-3 and reverse primer 5-GGATAATCCTCTGGCATATTTGAG-3; human IGF, forward primer 5-TGTGGAGACAGGGGCTTTTA-3 and reverse primer 5-ATCCACGATGCCTGTCTGA-3; tdTomato, forward primer 5-GCCACTACCTGGTGGAGTTC-3 and reverse primer 5-TGGTGTAGTCCTCGTTGTGG-3; GFP, forward primer 5-TCATGGCCGACAAGCAGA-3 and reverse primer 5-TCAGGTAGTGGTTGTCGGGCA-3; and ß-actin, forward primer 5-CGTGCGTGACATCAAAGAGAA-3 and reverse primer 5-TGGATGCCACAGGATTCCAT-3. Total RNA with DNase I digestion before reverse transcription was used as a negative control for each quantitative PCR and no amplification product originated from the HAC vectors was observed. The results were analyzed with LightCycler 480 software, version 1.5 (Roche Diagnostics). All data were normalized to ß-actin expression. The results were compared among all groups.

ELISA analysis for HGF, GDNF and IGF

For in vitro analysis of protein expression of growth factors, MNCs, GFP-MSCs and HAC-MSCs were prepared in 12 well-culture dishes at the cell density of 1 X 10^5 cells / well. After three days culture, the supernatant was collected from each cell culture and used for ELISA analysis of HGF, GDNF and IGF according to the manufactural protocol.

For in vivo analysis of protein expression of growth factors, spinal cords were isolated from SOD1-tg mice in no treatment, MNCs, GFP-MSCs and HAC-MSCs groups at middle stage (14-16 W) and at end stage (18-20 W) of disease status. The isolated spinal cords were homogenized into RIPA buffer (150mM Sodium Chloride, 2mM EDTA, 1% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulfate, 1.0% NP-40 substitute, 20mM Tris-HCl, pH7.4) with protease inhibitor cocktail (1tablet / 50ml buffer, Merck, Darmstadt, Germany). The supernatant was used for each ELISA analysis of HGF (Quantikine ELISA human HGF kit, R & D systems, Minneapolis, MN), GDNF (GDNF Human ELISA kit, Abcam, Cambridge, UK) and IGF (Quantikine ELISA human IGF-1 kit, R & D systems) according to the manufactural protocol.

Cytokine assay in spinal cord tissues

Spinal cord tissues were collected from SOD1-tg mice. After homogenization, the tissues were centrifuged at 15,000rpm. The supernatant of the spinal cord tissues was combined from over three mice in each group. The 32-cytokine assay in the supernatant was outsourced to GeneticLab (Sapporo, Japan). The sample was used for running a multiplex assay, and the concentration of the 32 cytokines was measured with a Milliplex MAP kit HCYTMAG-70K-PX32 (Millipore, Burlington, MA) and a Luminex®200™ System (Luminex Corp, Austin, TX) using ELISA. The procedure was performed according to the assay protocols and guidelines provided by the

manufacture. The cytokines included in the kit were as follows; G-CSF, Eotaxin, granulocyte macrophage-colony stimulating factor, interferon-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, leukemia inhibitory factor, IL-13, LPS-induced CXC chemokine, IL-15, IL-17, interferon-γ induced protein-10, keratinocyte-derived chemokines, monocyte chemoattractant protein-1, MIP-1α, MIP-1β, macrophage-colony stimulating factor, MIP-2, MIG, RANTES, tumor necrosis factor-α, and VEGF. The results were calculated and analyzed by using the MasterPlex® software (Hitachi Solutions America, Ltd, Irvine, CA).

Statistical analysis

For multiple data sets, one-way ANOVA and the Scheffe's test were used. The log rank test was used for statistical analysis of the Kaplan-Meier curve. Data were considered to be significantly different at $p < 0.05$.

Supplemental References

Niwa, H., Araki, K., Kimura, S., Taniguchi, S.I., Wakasugi, S., and Yamamura, K.I. (1993). An efficient gene-trap method using poly a trap vectors and characterization of gene-trap events. J. Biochem. *113*, 343–349.

Okamoto, T., Aoyama, T., Nakayama, T., Nakamata, T., Hosaka, T., Nishijo, K,. Nakamura, T., Kiyono, T., and Toguchida, J. (2002). Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. Biochem. Biophys. Res. Commun. 295, 354–361.