MTT assay

Cells were incubated with substrate for MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 h, and washed with PBS and lysed to release formazan from cells. Then cells were analyzed in a Safire microplate reader (BIO-RAD, Hercules, CA, USA) at 562 nm. Cyclopamine and tomatidine was purchased from Funakoshi (Tokyo, Japan). 143B cell were serum starved for 12 h, and then cultured with recombinant human sonic hedgehog (R&D Systems, Minneapolis, Japan).

SMO shRNA was purchased from SABiosciences (Maryland, USA). *SMO* and control shRNAs were cloned into pGeneClip™ Neomycin Vector, which express shRNA under the control of the U1 promoter. Lipofection of shRNA was performed every other day as recommended in the supplier's protocol using FuGENE 6 (Roche, Basel, Switzerland).

Colony formation assay

Colony formation assay was performed as previously described [68]. Briefly, cells were suspended in DMEM containing 0.33% agar and 10% fetal bovine serum and plated onto the bottom layer containing 0.5% agar. The cells were plated at a density of 5×10^3 per well in a 24-well plate, and colonies were counted 14 days later. Each condition was analyzed in triplicate, and all experiments were repeated three times.

Real-time PCR

All primer sets amplified 100- to 200-bp fragments. Total RNA was extracted using the miR-Vana RNA isolation system or TRIzol (Invitrogen, Carlsbad, CA, USA). Reactions were run using SYBR Green (BIO-RAD, Hercules, CA, USA) on a MiniOpticon™ machine (BIO-RAD, Hercules, CA, USA). The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *βII-microglobulin*, or *GAPDH*, or *ACTB*. Each sample was run at three concentrations in triplicate. The following primers were used. *Desert hedgehog*: 5-TGATGACCGAGCGTTGTAAG-3, 5-GCCAGCAACCCATACTTGTT-3; *Indian Hedgehog*: 5-ACTTCTGCCTGGTCCTGTTG-3, 5-AGCGATCTTGCCTTCATAGC-3; *Sonic hedgehog*: 5-ACCG AGGGCTGGGACGAAGA-3, 5-ATTTGGCCGCCACCGAGTT-3; *PATCHED*: 5-TAACGCTGCAACAACTCAGG-3, 5-GAAGGCTGTGACATTGCTGA-3; *SMOOTHENED*: 5-GGGAGGCTACTTCCTCATCC-3, 5-GGCA GCTGAAGGTAATGAGC-3; *GLI1*: 5-GTGCAAG TCAAGCCAGAACA-3, 5-ATAGGGGCCTGACTGGAGAT-3, *GLI2*: 5-CGACACCAGGAAGGAAGGTA-3, 5-AGAACGGAGGTAGTGCTCCA-3; *cyclin D1*: 5-ACAAA CAGATCATCCGCAAACAC-3, 5-TGTTGGGGCTCCT CAGGTTC-3; *cyclin E1*: 5-CCACACCTGACAAAGAAGATGATGAC-3, 5-GAGCCTCTGGATGGTGCAATAAT-3; *SKP2*: 5-TGGGAATCTTTTCCTGTCTG-3, 5-GAACACTGAGACAGTATGCC-3; *NMYC*: 5-CTTCGG TCCAGCTTTCTCAC-3, 5-GTCCGAGCGTGTTCA ATTTT-3; *βII-microgloblin*: 5-TCAATGTCGGATGGATGAAA-3, 5-GTGCTCGCGCTACTC TCTCT-3; *GAPDH*: 5-GAAGGTGAAGGTCGGAGTC-3, 5-GAAGATGGTGATGGGATTTC-3; *ACTB*: 5-AGAAAATCTGGCACCACACC-3, 5-AGAGGCGTACAGGGATAGCA-3.

Immunohistochemistry

The following primary antibodies were used; anti-SMO (diluted 1:200, Santa Cruz, CA. U.S.A), anti-GLI2 (diluted 1:200, Abcam, Cambridge, UK), and anti-ki67 (Zymed laboratories, San Francisco, USA). The following secondary antibodies were used:; fluorescein-conjugated goat anti-mouse IgG antibody (diluted 1:200; Jackson ImmunoResearch, PA, USA) and rhodamine-conjugated donkey anti-rabbit IgG antibody (diluted 1:200; Chemicon, Temecula, CA). The cells were counterstained with Hoechst 33258 (Molecular Probes, Carlsbad, CA, USA) to identify nuclei. Immunohistochemistry with each second antibody alone without primary antibody was performed as a control.

Western blot

Cells were lysed using NP40 lysis buffer (0.5% NP40, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 3 mM pAPMSF (Wako Chemicals, Kanagawa, Japan), 5 mg/ml aprotinin (Sigma, StLouis, USA), 2 mM sodium orthovanadate (Wako Chemicals, Kanagawa, Japan), and 5 mM EDTA). Lysates were subjected to SDS-PAGE and subsequent immunoblotting with antibodies to cyclin D1, E1, p21, SKP2, and pRb (Santa Cruz, CA. U.S.A). Detection was performed using the ECL detection system (Amersham, Giles, UK).

Animal experiments

143B cells (1×10^6) were mixed with a collagen gel in a 1:1 volume, and were inoculated subcutaneously in 5-week-old nude mice. The mice were randomly assigned to receive either cyclopamine (25 mg/kg-10 mg/kg) or an equal volume of DMSO as control. Cyclopamine and saline solution were administered by intraperitoneal injection. The treatment with cyclopamine was initiated 1 week after tumor inoculation when the tumors had grown to visible size. The injections were repeated every other day. Tumor size was measured with calipers weekly, and tumor volume was calculated using a formula of $LW^2/2$ (L and W representing the length and width of tumors). SMO shRNA (SABiosciences, Maryland, USA) transfected 143B cells (1×10^6) or control shRNA (1×10^6) cells were mixed with a collagen gel in a 1:1 volume, and were inoculated subcutaneously in 5-week-old nude mice. Tumor size was measured with calipers weekly, and tumor volume was calculated using a formula of $LW^2/2$ (L and W representing the length and width of tumors). All experimental procedures were performed in compliance with the guiding principles for the Care and Use of Animals described in the American Journal of Physiology and with the Guidelines established by the Institute of Laboratory Animal Sciences,

Faculty of Medicine, Kagoshima University. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize possible alternatives to in vivo techniques.

Cell cycle analysis

Cell cycle analysis was performed by Reprocell (Tokyo, Japan). At 48 h after cyclopamine treatment, cells were collected by trypsinization and washed with DPBS. Cells were fixed in 70% (v/v) ethanol at 4°C, washed with PBS, and resuspended with 500 μl of staining solution [PBS pH 7.4, 100 μg/ml DNase-free RNase, 1 mg/ml propidium iodide]. Cells were then analyzed by flow cytometry using a FACS Vantage SE (Becton Dickinson, Franklin Lakes, NJ). Data were gated using pulse width and pulse area to exclude doublets, and the percentage of cells present in each phase of the cell cycle was calculated using FlowJo software (Tree Star, Ashland, OR, USA).

Statistics and supplemental data

Each sample was analyzed in triplicate, and experiments were repeated three times. In all figures, error bars are standard divisions. All statistical analyses were performed using Microsoft Office Excel (Microsoft, Albuquerque, New Mexico, USA) and STASTISCA (StatSoft, Tulsa, OK, USA). Differences between mean values were evaluated by the unpaired *t*-test, and differences in frequencies by Fisher's exact test. Differences were considered significant at $P < 0.05$.

List of abbreviations

(Hh): Hedgehog; (SMO): SMOOTHENED; (PTCH1): PATCHED; (SHH): Sonic hedgehog; (DHH): Desert hedgehog.

Additional file 1: A, Immunohistochemical examination revealed that SMO was expressed on cytoplasm of 143B and GLI2 was localized in the nucleus of 143B. B, MTT assay showed that Sonic hedgehog promote proliferation of osteosarcoma cells. The experiment was triplicate with similar results.
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Additional file 2: Real-time PCR was performed to quantify mRNAs of cell cycle related genes. *SMO* shRNA reduced levels of *cyclin D1*, *cyclin E1*, *SKP2*, and *E2F1* transcription (error bar means S.D.). The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *ACTB*. The experiment was triplicate with similar results.
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Additional file 3: We performed real-time PCR using formed tumors. Real-time PCR revealed that transcription of *GLI1*, *GLI2*, and *PTCH1* was decreased in tumors formed by *SMO* shRNA-transfected 143B. In addition, *SMO* shRNA reduced levels of *Cyclin E1*, *SKP2*, and *E2F1* transcription. The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *ACTB*. The experiment was triplicate with similar results.
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Additional file 4: Cyclopamine prevents proliferation of osteosarcoma *in vivo*. Immunohistochemical examination of ki67 was performed in xenograft tumors. Ki67 staining revealed that proliferation of osteosarcoma cells was decreased by cyclopamine treatment. The numbers of Ki67-positive cells was decreased to 50% of control revel by cyclopamine administration at day 14 (error bar means S.D.).
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Authors' contributions

TS was involved in the design and execution of the experiments, drafted the manuscript and contributed to the overall experimental design. MH conducted most of the experiments. HS was conducted a most of experiments. HG was conducted a part of experiments. YM was conducted a part of experiments. HN was conducted a part of experiments. OK was conducted a part of experiments. SK contributed to the overall experimental design. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Tumour formation by single fibroblast growth factor receptor 3-positive rhabdomyosarcoma-initiating cells

M Hirotsu[1,3], T Setoguchi[*,1,3], Y Matsunoshita[1], H Sasaki[1], H Nagao[1], H Gao[1], K Sugimura[2] and S Komiya[1]

[1]Department of Orthopaedic Surgery, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan; [2]Faculty of Engineering, Department of Bioengineering, Kagoshima University, 1-21-40 Korimoto, Kagoshima 890-0065, Japan

BACKGROUND: The hypothesis that malignant tumours are generated by rare populations of cancer stem cells that are more tumourigenic than other cancer cells has gained increasing credence. The objective of this study was to identify and characterise a subpopulation of human sarcoma-initiating cells.

METHODS: We examined established rhabdomyosarcoma cell lines by flow cytometry. Tumourigenesis was examined by xenograft models. Real-time PCR and immunohistochemistry were performed to examine the gene expression using cell lines and biopsy specimens.

RESULTS: Rhabdomyosarcoma cell lines included small populations of fibroblast growth factor receptor 3 (FGFR3)-positive cells. FGFR3-positive KYM-1 and RD cells were more strongly tumourigenic than FGFR3-negative cells. In addition, xenoengraftment of 33% of single FGFR3-positive KYM-1 cells yielded tumour formation. Stem cell properties of FGFR3-positive cells were further established by real-time PCR, which demonstrated upregulation of undifferentiated cell markers and downregulation of differentiation markers. We showed that in the absence of serum, addition of basic fibroblast growth factor maintained and enriched FGFR3-positive cells. On the other hand, ciliary neurotrophic factor reduced the proportion of FGFR3-positive cells. Real-time PCR and immunohistochemical examination revealed that embryonal rhabdomyosarcoma patient biopsy specimens were found to over-express FGFR3.

CONCLUSIONS: Our findings suggest that rhabdomyosarcoma cell lines include a minor subpopulation of FGFR3-positive sarcoma-initiating cells, which can be maintained indefinitely in culture and which is crucial for their malignancy.

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Keywords: rhabdomyosarcoma; sarcoma-initiating cells; cancer stem cell; fibroblast growth factor receptor 3 (FGFR3)

The hypothesis that malignant tumours are generated by rare populations of tumour-initiating cells (TICs), also called cancer stem cells, that are more tumourigenic than other cancer cells has gained increasing credence (Clarke and Fuller, 2006). TICs were initially identified in acute myeloid leukaemia (AML), and were found capable of inducing AML in immunodeficient mice (Lapidot *et al*, 1994; Bonnet and Dick, 1997). TICs have since been identified in numerous other tumours, including melanoma, lung, head, neck, pancreatic, prostate, colon, squamous cell cancers, and benign tumours (Collins *et al*, 2005; Fang *et al*, 2005; Kim *et al*, 2005; Dalerba *et al*, 2007; Prince *et al*, 2007; Loebinger *et al*, 2008; Xu *et al*, 2009). Although the AML TICs resemble and probably originate from the transformation of a stem cell, it is possible that other TICs originate from transformation of early or late progenitor cells. Thus, the definition of a TIC is not related to the cell of origin for a tumour but rather to its ability to self-renew, initiate cancer, and give rise to more differentiated cells that have lost the capacity for self-renewal and tumourigenic potential. The notion that cancer is driven by TICs has obvious therapeutic implications (Al-Hajj *et al*, 2004; Raguz and Yague, 2008). The efficacy of tumour response to systemic therapy has traditionally been assessed based on the bulk of tumour cells by monitoring of changes in tumour size (Therasse *et al*, 2000). However, if only a small fraction of TICs are capable of initiating cancer, then curative therapy should be designed to target these rare TICs rather than the bulk of nontumourigenic cells. Analysis of TICs might thus yield novel therapeutic targets.

In this study, we attempted to identify rhabdomyosarcoma-initiating cells (RICs) using cell surface markers. We examined many markers of undifferentiated cells. We found that human rhabdomyosarcoma cell lines include a small proportion of fibroblast growth factor receptor 3 (FGFR3)-positive cells. Single FGFR3-positive cells have the potential for tumour formation *in vivo*. In addition, tumours formed by FGFR3-positive cells could be used for serial propagation of tumours in animals. Moreover, basic fibroblast growth factor (bFGF) could maintain and enrich FGFR3-positive RICs in the KYM-1 cell line in the absence of serum. The easy method of preparation we describe will be useful for the development of anti-RICs target therapy.

*Correspondence: Dr T Setoguchi;
E-mail: setoro@m2.kufm.kagoshima-u.ac.jp
[3]These authors contributed equally to this work.
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MATERIALS AND METHODS

Cell culture

KYM-1 and RD rhabdomyosarcoma cell lines were purchased from Health Sciences Research Resources Bank (Tokyo, Japan). The A204 cell line was purchased from ATCC (Manassas, VA, USA). These cells were cultured in DMEM, supplemented with 10% FCS, 100 units per ml penicillin G, and 100 $\mu g\,ml^{-1}$ streptomycin (Invitrogen, Carlsbad, CA, USA). In some experiments, KYM-1 cells were cultured in serum-free S-Clone (Sanko Junyaku, Japan) containing 10 $ng\,ml^{-1}$ bFGF, 10 $ng\,ml^{-1}$ epidermal growth factor (EGF), 2.5 $ng\,ml^{-1}$ transforming growth factor beta (TGF-β), 10 $ng\,ml^{-1}$ ciliary neurotrophic factor (CNTF), 10 $ng\,ml^{-1}$ platelet-derived growth factor (PDGF)-AA, 10 $ng\,ml^{-1}$ PDGF-BB, and 5 $\mu g\,ml^{-1}$ heparin. Normal human skeletal muscle cells (HSkMC) were purchased from TOYOBO (Osaka, Japan). HSkMC were cultured in skeletal muscle cell growth medium (Cell Applications Inc., San Diego, CA, USA) or DMEM, supplemented with 10% FCS, 100 units per ml penicillin G, and 100 $\mu g\,ml^{-1}$ streptomycin (Invitrogen). In all experiments, cells were maintained in 100 mm culture dishes (Nunc, New York, NY, USA) at 37°C in a humidified 5% CO_2 per 95% air atmosphere.

Flow cytometry analysis and sorting

Fluorescence-activated cell sorting analysis was performed using an Epics Altrta (Beckman Coulter, Fullerton, CA, USA). Cells were conjugated with anti-FGFR3 antibody (R&D, Minneapolis, MN, USA) for 30 min on ice. Cells were washed three times in PBS, re-suspended in the same buffer at 5×10^6 per ml, and kept on ice until analysis. Live single cells (fixed FSC-A/FSC-W ratio; PI negative) were gated for analysis.

Rhabdomyosarcoma patients' biopsy specimens

We obtained two biopsy specimens of human rhabdomyosarcoma from primary lesions. Pathological examination revealed that patient 1 had embryonal rhabdomyosarcoma and patient 2 had alveolar rhabdomyosarcoma. Biopsy was performed before chemotherapy or radiotherapy to make the diagnosis. Control muscle was obtained from surgery for scoliosis. The study protocol was approved by the institutional review board of the Kagoshima University. All patients and controls gave written informed consent.

Real-time PCR

Each sample was run minimally at three concentrations in triplicate. All primer sets amplified 100–200 bp fragments. Primers were designed by Primer3. Total RNA was extracted using the miR-Vana RNA isolation system (Ambion, Austin, TX, USA) or TRIzol (Invitrogen). Reactions were run using SYBR Green (Bio-Rad, Hercules, CA USA) on a MiniOpticon machine (Bio-Rad). The comparative Ct ($\Delta\Delta$Ct) method was used to determine fold change in expression using *βII-macroglobulin*, *ACTB*, or *GAPDH*. The following primers were used: *CD34*: 5- CACCCTGTGTCTCA ACATGG-3, 5-GGCTTCAAGGTTGTCTCTGG-3; *PAX3*: 5- GCCT GACGTGGAGAAGAAAA-3, 5-GCCTCCTCCTCTTCACCTTT-3; *PAX7*: 5-GAACCTGACCTCCCACTGAA-3, 5-CCTCTGTCAGCTTGGTCCTC-3; *MYF5*: 5-AATTTGGGGACGAGTTTGTG-3, 5- CATGGTGGTGGACTT CCTCT-3; *NANOG*: 5-AATACCTCAGCCTCCAGCAGATG-3, 5-TGCGT CACACCATTGCTATTCTTC-3; *OCT-4*: 5-GAGAACCGAGTGAGAGG CAACC-3, 5-CATAGTCGCTGCTTGATCGCTTG-3, *SOX2*: 5-AGAAC CCCAAGATGCACAAC-3, 5-CGGGGCCGGTATTTATAATC-3; *MYH1*: 5-GCTCATCGAGAAGCCTATGG-3, 5-CAAAGAGAAGTGGGCCTCAG -3; *desmin*: 5-CATCGCGGCTAAGAACATTT-3, 5-GCCTCATCAGGG AATCGTTA-3; *myogenin*: 5-TGGGCGTGTAAGGTGTGTAA-3, 5-CGA TGTACTGGATGGCACTG-3; *dystrophin*: 5-ACCACCTCTGACCCTAC ACG-3, 5-GCAATGTGTCCTCAGCAGAA-3; *β2-microglobulin*: 5-TCAA TGTCGGATGGATGAAA-3, 5-GTGCTCGCGCTACTCTCTCT-3; *ACTB*: 5-AGAAAATCTGGCACCACACC-3, 5-AGAGGCGTACAGGGATAGCA-3; *GAPDH*: 5-GAAGGTGAAGGTCGGAGTC-3, 5-GAAGATGGTGAT GGGATTTC-3.

Immunohistochemical examination

We obtained two biopsy specimens of human rhabdomyosarcoma from primary lesions. Pathological examination revealed that patient 1 had embryonal rhabdomyosarcoma and patient 2 had alveolar rhabdomyosarcoma. Anti-FGFR3 (diluted 1:200, R&D) was used as a primary antibody. Rhodamine-conjugated donkey anti-rabbit IgG antibody (diluted 1:200; Chemicon, Billerica, MA, USA) was used as a secondary antibody. The cells were counter-stained with Hoechst 33258 to identify nuclei. Immunohistochemistry with second antibody alone without primary antibody was performed as a control.

Animal experiments

KSN/SLC nude mice were purchased from SLC. FGFR3-positive cells were collected by magnetic sorting by MACS according to the manufacturer's recommendations (Miltenyi Biotec, Gladbach, Germany). The following antibodies were used: PE-conjugated anti-FGFR3 antibody (R&D) and anti-PE Microbeads (Miltenyi Biotec). Cell inoculation was performed as reported earlier (Tanaka *et al*, 2009). Cells were mixed with a collagen gel, and were inoculated subcutaneously in 5-week-old nude mice. Grafts were excised and small portions of tumour (20 mg) were serially inoculated into other nude mice. In addition, graft was excised and trypsinised. Each number of cells was serially inoculated into other mice. Grafts were fixed with 10% buffered formaldehyde and stained with hematoxylin and eosin. All experimental procedures were performed in compliance with the guiding principles for the Care and Use of Animals described in the American Journal of Physiology and with the Guidelines established by the Institute of Laboratory Animal Sciences, Faculty of Medicine, Kagoshima University. All efforts were made to minimise animal suffering, to reduce the number of animals used, and to use possible alternatives to *in vivo* techniques.

RESULTS

Rhabdomyosarcoma cell lines include a small portion of FGFR3-positive cells

To determine whether any of the established osteosarcoma and rhabdomyosarcoma cell lines included small portions of undifferentiated cell marker-positive cells, we performed flow cytometry. We examined many markers of undifferentiated cell, such as side population (SP), CD9, CD10, CD13, CD29, CD31, CD34, CD44, CD117, CD133, FLT3, LNGFR, and FGFR3 (Caligaris-Cappio *et al*, 1985; Robinson *et al*, 1999; Erices *et al*, 2000; Singh *et al*, 2003; Kondo *et al*, 2004; Bobis *et al*, 2006; Jones *et al*, 2006; Small, 2008). We found that three rhabdomyosarcoma cell lines, KYM-1, RD, and A204, each included a small proportion of FGFR3-positive cells (1.6–2.6%) (Figure 1).

The malignancy of KYM-1 and RD cells *in vivo* depends to a large extent on FGFR3-positive RICs

To determine whether the subset defined by FGFR3 was enriched for RICs, we compared the abilities of FGFR3+ and FGFR3− rhabdomyosarcoma cells to initiate tumour formation *in vivo*. After 8W, all mice inoculated with 100 KYM-1 cells had formed tumours. After 5W, in 5 out of 6 of 10 FGFR+ KYM-1 cells

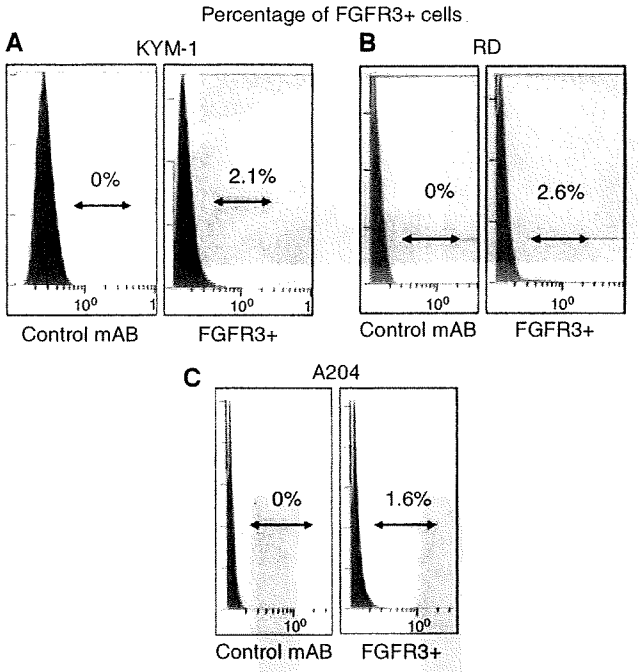

Figure 1 Rhabdomyosarcoma cell lines include a small portion of FGFR3-positive cells. Cells of human KYM-1 rhabdomyosarcoma (**A**), human RD rhabdomyosarcoma (**B**), and human A204 rhabdomyosarcoma (**C**) cell lines were labelled with anti-FGFR3 antibody and then analysed by flow cytometry. These three human rhabdomyosarcoma cell lines included small subpopulations of FGFR3-positive cells. These experiments were repeated at least three times with similar results.

inoculated mice, there was tumour formation. In contrast, in 1 out of 6 of 10 FGFR− KYM-1 cells inoculated mice, there was tumour formation. Surprisingly, 2 out of 6 of only single FGFR3+ KYM-1 cell inoculated mice also exhibited tumour formation. (Figure 2A). Next, we examined RD cells. After 6W, in 2 out of 3 of 100 FGFR+ RD cells inoculated mice, there was tumour formation. In contrast, in 0 out of 3 of 100 FGFR− RD cells inoculated mice, there was tumour formation after 12W inoculation. In addition, in 1 out of 3 of 10 FGFR+ RD cells inoculated mice, there was tumour formation. In contrast, in 0 out of 3 of 10 FGFR− RD cells inoculated mice, there was tumour formation (Figure 2C). We next performed serial transplantation. Small portions of formed tumour (20 mg) were excised and then inoculated into other nude mice. Six of six tumours formed by FGFR3+ cells inoculated into mice formed tumour. In contrast, none of six tumours were formed by FGFR− KYM-1 cells. In addition, 3 out of 3 of 1000 cells prepared from FGFR3+ tumour inoculated mice formed tumour (Figure 2D). In contrast, 0 out of 3 of 1000 cells prepared from FGFR− tumour formed tumour (Figure 2D). Immunohistochemical examination revealed that tumours formed by FGFR3+ KYM-1 cell contained both FGFR3+ cells and FGFR3− cells *in vivo* (Figure 2E).

RICs express undifferentiated cell markers

We next examined the expression of genes specific to skeletal muscle development or embryonic stem cells. RNA from FGFR3+ KYM-1 or FGFR3− KYM-1 cells was analysed by real-time PCR for *CD34*, *PAX3*, *PAX7*, *MYF5*, *NANOG*, *OCT4*, *SOX2*, *myosin heavy chain 1* (*MYH1*), *desmin*, *myogenin*, and *dystrophin*. Real-time PCR revealed that expression of *CD34* and *PAX3* in FGFR3+ cells was markedly increased by 7.23- and 2.47-fold, respectively. In addition, the expression of *PAX7*, *MYF5*, *NANOG*, *OCT3*, and *SOX2* was slightly increased by 1.15-, 1.13-, 1.35-, 1.56-, and 1.5-fold, respectively. On the other hand, the expression of the differentiated muscle markers *MYH1*, *desmin*, *myogenin*, and *dystrophin* was decreased to 0.85-, 0.91-, 0.81-, and 0.25-fold baseline levels, respectively (Figure 3).

RICs can be maintained and enriched by bFGF

We then examined which factor(s) can maintain KYM-1 FGFR3+ cells in serum-free culture media. We tested bFGF, EGF, TGF-β1, CNTF, PDGF-AA, and PDGF-BB as candidates. These mitogens are important factors in maintaining many types of progenitor cells (Marmur *et al*, 1998; Kondo *et al*, 2004; Vallier and Pedersen, 2005). We first cultured unfractionated KYM-1 cells in serum-free culture medium alone or with a mixture of bFGF, EGF, TGF-β, CNTF, PDGF-AA, and PDGF-BB. KYM-1 cells could not grow without growth factors. On the other hand, this growth factor cocktail promoted KYM-1 cell growth. We next examined which mitogen is essential for KYM-1 cell growth, by withdrawing each mitogen individually. All culture conditions promoted KYM-1 growth but bFGF withdrawal (Figure 4A). These findings suggested that bFGF is essential for KYM-1 survival and proliferation. We cultured 1000 KYM-1 cells in each condition and counted 20 days after culture. Addition of EGF to serum-free culture medium with bFGF appreciably stimulated KYM-1 cell growth approximately three-fold, the same as neural progenitor cells (Figure 4B) (Kitchens *et al*, 1994). On the other hand, when cultured in serum-free culture medium with EGF alone, KYM-1 cells could not survive. We then stained cells with anti-FGFR3 antibody and analysed them by flow cytometry. When cultured in serum-free medium with bFGF, FGFR3+ cells were maintained, and their proportion increased to 7.6–9.2%. In addition, when cultured in both bFGF and EGF, FGFR3+ cells increased to 4.2–6.0 % and total cell number increased three-fold (Figure 4C). We next examined which factor prevents expansion of FGFR3+ cells. When KYM-1 cells were cultured with bFGF plus CNTF, CNTF reduced the proportion of FGFR3+ cells by approximately 15% (Figure 4D), although CNTF did not affect the total number of KYM-1 cells. These findings suggest that RICs can be maintained and increased in bFGF alone and that a combination of bFGF and EGF can increase cell numbers. On the other hand, CNTF decreased the proportion of RICs.

FGFR3 was upregulated in rhabdomyosarcomapatient biopsy specimens

We next examined the expression of FGFR3 in patient biopsy specimens. Real-time PCR revealed that FGFR3 was upregulated in embyonal rhabdomyosarcoma patient biopsy specimens (Figure 5A). Immunohistochemical examination revealed that a portion of rhabdomyosarcoma cells expressed FGFR3. The intensity of FGFR3 expression differed among rhabdomyosarcoma cells (Figure 5B).

DISCUSSION

Although there is an expanding literature supporting the existence of cancer stem cells, important caveats of these studies continue to provoke debate. The current definitive test for a cancer stem cell is the capacity to propagate tumours as xenografts in immunocompromised mice (Clarke *et al*, 2006). We have described here the isolation of a highly tumourigenic subpopulation of cells from human rhabdomyosarcoma cell lines in accord with terminology. To our knowledge, this is the first isolation of

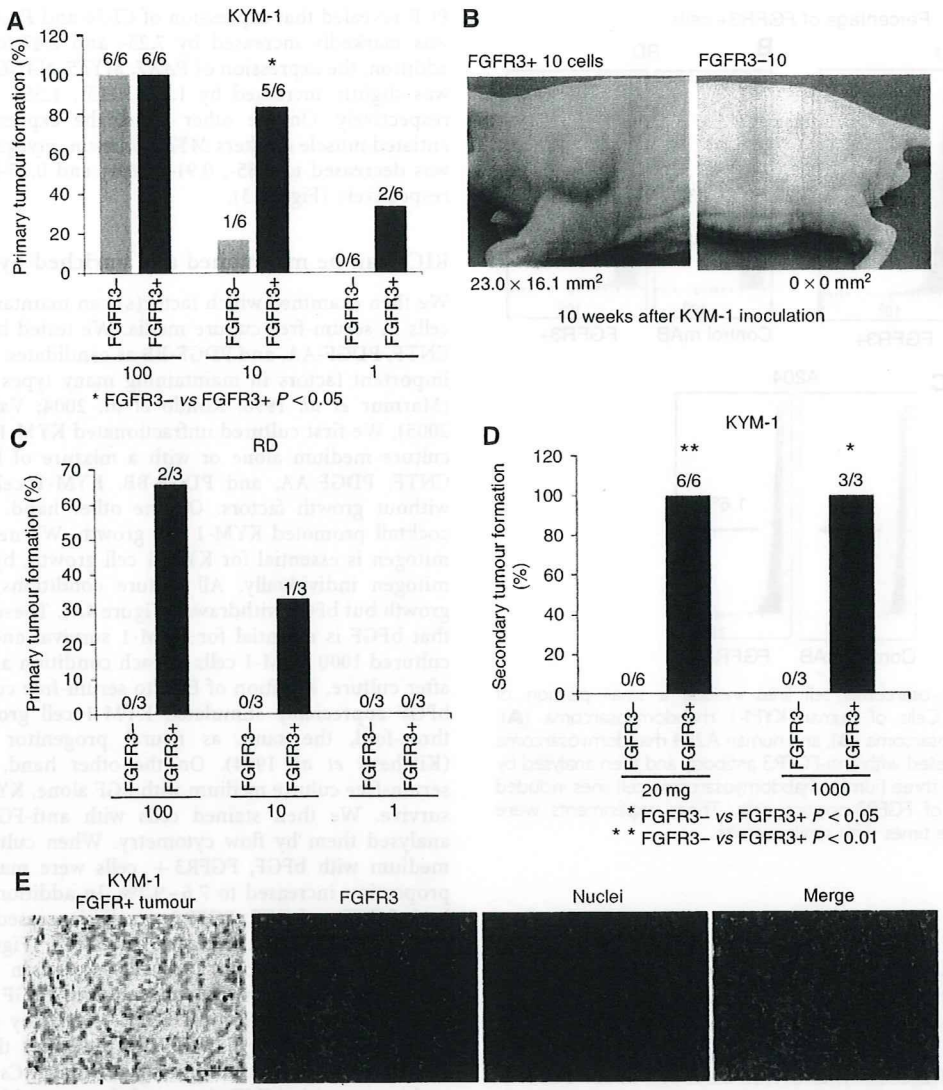

Figure 2 The malignancy of rhabdomyosarcoma cells *in vivo* depends to a large extent on the FGFR3-positive cells. FGFR3-dependent cell sorting was performed using immunomagnetic selection, with culture for one night to exclude dead cells by mechanical stress followed by inoculation of cells from either population interdermally into nude mice. (**A**) Primary tumour formation by KYM-1, FGFR3−, or FGFR3 + cells. After 8W, all mice inoculated with 100 KYM-1 cells had formed tumours. After 5W, in 5 out of 6 of 10 FGFR + KYM-1 cells inoculated mice, there was tumour formation. In contrast, in 1 out of 6 of 10 FGFR− KYM-1 cells inoculated mice, there was tumour formation after 12W inoculation. Surprisingly, 2 out of 6 of only single FGFR3 + KYM-1 cell inoculated mice also exhibited tumour formation. In contrast, 0 out of 6 of single KYM-1 FGFR cell inoculated mice formed tumour after 12W inoculation. (**B**). Ten FGFR3-positive cells form tumour 8 weeks after inoculation. (**C**). Primary tumour formation by RD, FGFR3−, or FGFR3 + cells. After 6W, in 2 out of 3 of 100 FGFR + RD cells inoculated mice, there was tumour formation. In contrast, in 0 out of 3 of 100 FGFR− RD cells inoculated mice, there was tumour formation after 12W inoculation. In addition, in 1 out of 3 of 10 FGFR + RD cells inoculated mice, there was tumour formation. In contrast, in 0 out of 3 of 10 FGFR− RD cells inoculated mice, there was tumour formation after 12W inoculation. (**D**) Secondary tumour formation by FGFR3− or FGFR3 + KYM-1 cells. We next performed serial transplantation. Small portions of formed tumour (20 mg) were excised and then inoculated into other nude mice. Six of six tumours formed by FGFR3 + KYM-1 cells inoculated into mice formed tumour. In contrast, none of six tumours formed by FGFR− KYM-1 cells inoculated into mice formed tumour after 12W inoculation. In addition, 3 out of 3 of 1000 cells prepared from FGFR3 + tumour inoculated mice formed tumour. In contrast, 0 out of 3 of 1000 cells prepared from FGFR− tumour inoculated mice formed tumour after 12W inoculation. (**E**) HE staining of tumour formed by FGFR3 + KYM-1cells. Immunohistochemical examination revealed that tumour formed by FGFR3 + KYM-1cells contains both FGFR3 + and FGFR3− cells (red: FGFR3, blue: Hoechst). * FGFR3− *vs* FGFR3 + $P<0.05$, * * FGFR3− *vs* FGFR3 + $P<0.01$.

malignant progenitors from human rhabdomyosarcoma to be described.

Initially, to identify candidate RICs, we used a side population method as reported earlier (Kondo *et al*, 2004; Setoguchi *et al*, 2004). We detected approximately 1–3% SP cells among KYM-1 cells. We sorted SP and non-SP cells and then inoculated them into nude mice subcutaneously, but could not detect differences between them in tumourigenisity (data not shown). We next examined CD133, which has been reported to be a cancer stem cell marker (Singh *et al*, 2003; Hermann *et al*, 2007; Ricci-Vitiani *et al*, 2007; Chearwae and Bright, 2008; Mizrak *et al*, 2008). KYM-1 cells also included a small proportion of CD133-positive cells. We sorted CD133 + and CD133− cells and then inoculated them into nude mice subcutaneously, but found no

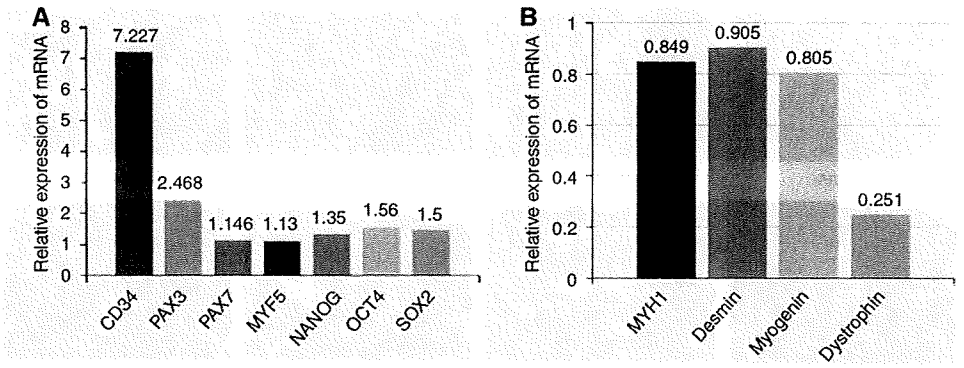

Figure 3 FGFR3-positive cells over-expressed undifferentiated cell genes. (**A**) As demonstrated by real-time PCR, FGFR3 + cells (RICs) over-express several undifferentiated cell marker genes compared with FGFR3− cells (**B**). Real-time PCR revealed that FGFR3 + cells (RICs) exhibited downregulation of several differentiated cell marker genes compared with FGFR3− cells. The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *GAPDH* or *βII-microglobulin*. Each sample was run minimally at three concentrations in triplicate. The experiment was triplicate with similar results.

differences between them in tumourigenisity (data not shown). In addition, we were unable to identify subpopulations by other undifferentiated cell markers. In our study, RICs were enriched in rhabdomyosarcoma subpopulations defined by FGFR3 alone. These findings suggest that sarcomas may differ from other epithelial malignancies, including cancers of the breast, head and neck, lung, pancreas, colon, and prostate (Al-Hajj *et al*, 2003; Collins *et al*, 2005; Fang *et al*, 2005; Kim *et al*, 2005; Dalerba *et al*, 2007; Li *et al*, 2007; Prince *et al*, 2007). It has been reported that FGFR3 is expressed in human muscle from 11 weeks of gestation and is decreased in adult muscle (Sogos *et al*, 1998). Muscle stem cells (muscle satellite cells) express FGFR3 whereas muscle-derived fibroblasts do not (Sheehan and Allen, 1999). These findings suggest that FGFR3 is expressed not only in RICs but also in muscle stem cells. In addition to exhibiting aggressive tumourigenisity, RICs expressed stem cell markers intensely with fewer markers of differentiation. These findings suggest that RICs have the characteristics of undifferentiated cells. In particular, RICs upregulated *CD34* and that downregulated *dystrophin*. These genes are muscle cell linage specific. These findings suggest that RICs are already to some extent committed to the muscle cell linage from more undifferentiated stages such as mesenchymal stem cells.

Rhabdomyosarcoma is composed of embryonal and alveolar subtypes. KYM-1 and RD is established from embryonal rhabdomyosarcoma (McAllister *et al*, 1969; Sekiguchi *et al*, 1985). The subtype of A204 was not described in article of cell line establishment (Giard *et al*, 1973). Embryonal rhabdomyosarcoma contain primitive undifferentiated round cells (Gallego Melcon and Sanchez de Toledo Codina, 2007). Consistent with these findings, we showed that embryonal rhabdomyosarcoma cell lines contain undifferentiated RICs. In addition, real-time PCR revealed that the amount of FGFR mRNA in the embryonal rhabdomyosarcoma biopsy sample was more than that in the normal skeletal muscle or alveolar rhabdomyosarcoma sample.

Mammals have four FGFR tyrosine kinase genes (FGFR1–4) (Eswarakumar *et al*, 2005). FGFRs are composed of an extracellular ligand-binding domain, a transmembrane domain, and a split cytoplasmic tyrosine kinase domain. In this study, we examined only FGFR3. Whether related members of the FGFR family are markers of RICs requires further study. We found that bFGF could maintain and expand RICs. It has been reported that bFGF promotes proliferation and inhibits differentiation of muscle satellite cells (Guthridge *et al*, 1992; Lefaucheur and Sebille, 1995). The bFGF binds to FGFR1, FGFR2, FGFR3, and FGFR4. The binding of bFGF to FGFR3 activate FGF signalling pathway (Ornitz and Leder, 1992; Maric *et al*, 2007). These data suggest that FGFR3 is not only a cell surface marker for RICs but also mediates signals important for RICs maintenance and proliferation. In addition, we found that CNTF reduced the proportion of RICs. There is increasing evidence that chemotherapy and radiation can each efficiently eradicate the majority of malignant cells within neoplastic lesions. However, these regimens frequently fail to eliminate a minor subpopulation of resistant cancer stem cells (Trumpp and Wiestler, 2008). Inhibition of FGFR3 signalling or activation of CNTF signalling might thus be a good candidate for anti-cancer stem cell therapy for rhabdomyosarcoma.

Recent studies have suggested that FGFR3 has a significant function in the pathogenesis and progression of some malignancies including thyroid carcinoma, bladder carcinoma, multiple myeloma, and peripheral T-cell lymphoma (Cappellen *et al*, 1999; Onose *et al*, 1999; Kastrinakis *et al*, 2000; Yagasaki *et al*, 2001; Wolff *et al*, 2005). Whether FGFR3 is a marker of TICs in these malignancies requires further study.

In our study, 33% of single KYM-1 RICs formed tumours. This TIC frequency is somewhat higher than previously reported for other TICs (Singh *et al*, 2003; Hermann *et al*, 2007; Ricci-Vitiani *et al*, 2007; Mizrak *et al*, 2008). These more strong tumourigenic RICs may be more useful than other TICs for examining the molecular mechanisms of tumour initiation, proliferation, anti-apoptotic capacity, and metastasis. Although RICs are enriched in the rhabdomyosarcoma subpopulations defined by FGFR3, not every FGFR3 + cell is an RIC, as 67% of purified single RICs did not form tumours. Quintana *et al* reported that frequency of tumourigenisity in mice depends to a large extent on the status of immunodeficiency (Quintana *et al*, 2008). When melanoma cells were transplanted into NOD/SCID mice, 1 in 111 000 cells formed tumour. When transplanted into highly immunocompromised NOD/SCID interleukin-2 receptor γ chain null mice, 27% of single cells formed tumours. The tumourigenisity of RICs might vary depending on the experimental conditions used, such as the tissue site of xenotransplantation, or differences among recipient immunodeficient mice.

In summary, we identified FGFR3-positive RICs in human rhabdomyosarcoma cell lines. RICs were more strongly tumourigenic than other previously reported TICs. Our easy method of preparing RICs may prove useful for further exploration of

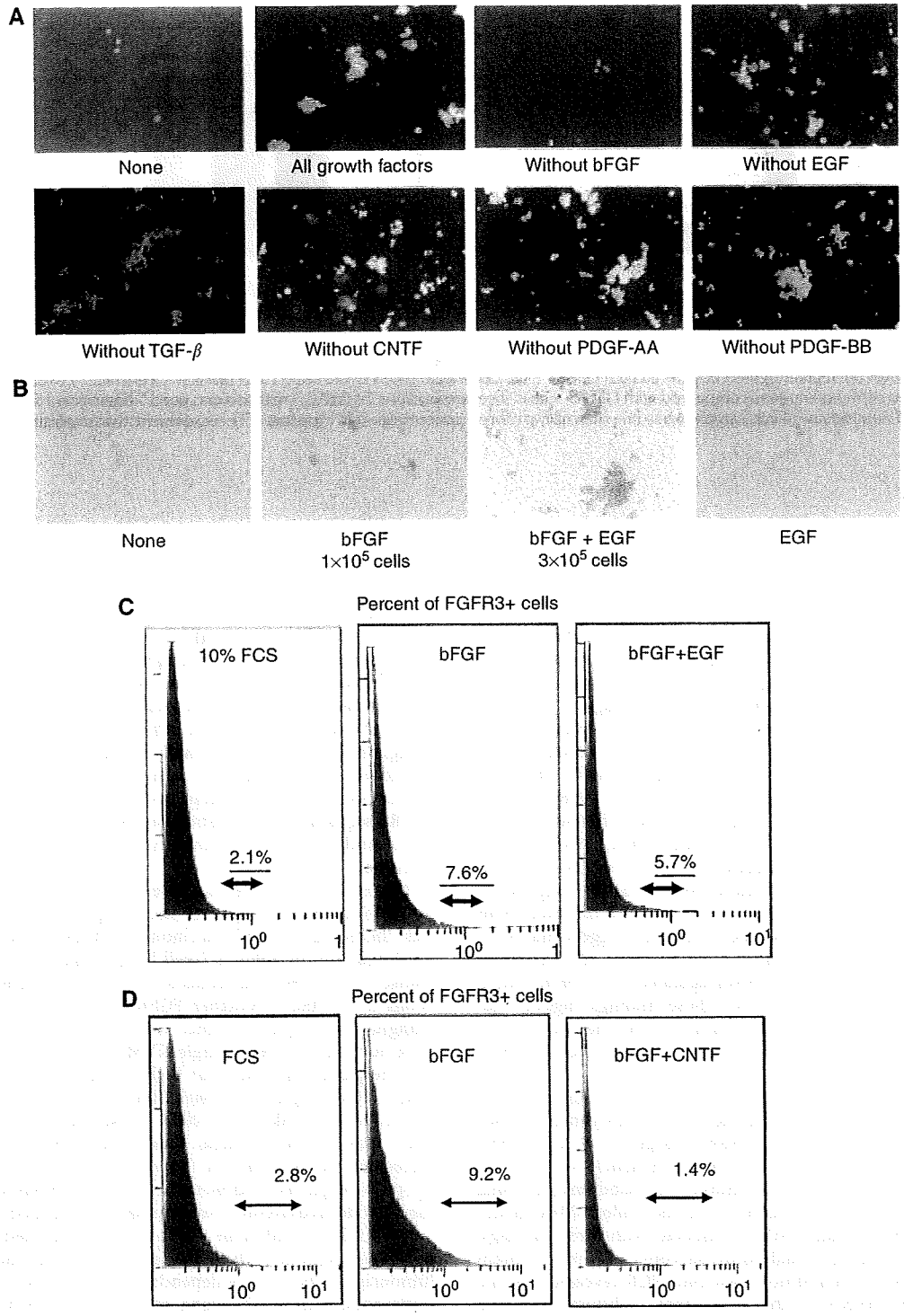

Figure 4 Increase in proportion of KYM-1 FGFR3-positive cells with bFGF. (**A**) KYM-1 cells were cultured in serum-free medium with or without growth factor cocktails. Cells were cultured for 3 weeks and then photographed. When cultured without bFGF, KYM-1 cells did not increase in proportion. (**B**) 1000 KYM-1 cells were cultured for 3 weeks in serum-free medium with bFGF, EGF, or both or neither and were then photographed. When cultured with bFGF, KYM-1 cells were maintained. Cell number was increased with bFGF plus EGF. (**C**) Proportion of FGFR3 + cells was analysed by flow cytometry. KYM-1 cells were cultured in FCS, bFGF, or bFGF plus EGF for 3 weeks. The proportion of FGFR3-positive RICs was increased by bFGF. When cultured with bFGF plus EGF, total number of cells was increased three-fold compared with bFGF alone. All experiments were repeated at least three times with similar results. (**D**) Proportion of FGFR3 + cells was analysed by flow cytometry. KYM-1 cells were cultured in FCS, bFGF, or bFGF plus CNTF for 3 weeks. When cultured with bFGF plus CNTF, the proportion of FGFR3 + cells was markedly decreased compared with bFGF alone or FCS. All experiments were repeated at least three times with similar results.

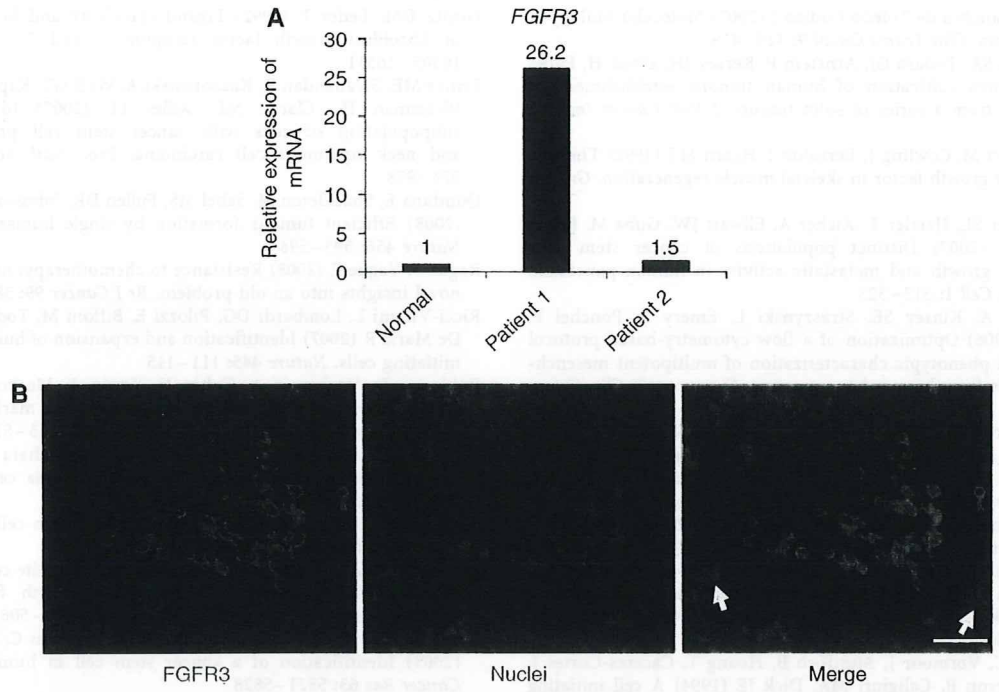

Figure 5 FGFR3 is over-expressed in rhabdomyosarcoma patient specimens. (**A**) We obtained two biopsy specimens of human rhabdomyosarcoma from primary lesions. Pathological examination revealed that patient 1 had embryonal rhabdomyosarcoma and patient 2 had alveolar rhabdomyosarcoma. Real-time PCR revealed that the amount of FGFR mRNA in the embryonal rhabdomyosarcoma biopsy sample was more than that in the normal skeletal muscle or alveolar rhabdomyosarcoma sample. (**B**) Immunohistochemistry revealed that FGFR3 was expressed in a portion of rhabdomyosarcoma patient 1 biopsy specimens. Arrows indicate FGFR3-negative cells. Immunohistochemical examination showed that 11.2 ± 2.8% cells were positive for FGFR3.

pathogenesis of rhabdomyosarcoma and molecular characterisation of cancer stem cells.

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Conflict of interest

The authors declare no conflict of interest.

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Chromatin protein HMGB2 regulates articular cartilage surface maintenance via β-catenin pathway

Noboru Taniguchi[a], Beatriz Caramés[a], Yasuhiko Kawakami[b], Brad A. Amendt[c], Setsuro Komiya[d], and Martin Lotz[a,1]

[a]Department of Arthritis Research, The Scripps Research Institute, La Jolla, CA 92037; [b]Stem Cell Institute and Department of Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN 55455; [c]Institute of Biosciences and Technology, Texas A&M University System Health Science Center, Houston, TX 77030-3303; and [d]Department of Orthopaedic Surgery, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

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The superficial zone (SZ) of articular cartilage is critical in maintaining tissue function and homeostasis and represents the site of the earliest changes in osteoarthritis. Mechanisms that regulate the unique phenotype of SZ chondrocytes and maintain SZ integrity are unknown. We recently demonstrated that expression of the chromatin protein high mobility group box (HMGB) protein 2 is restricted to the SZ in articular cartilage suggesting a transcriptional regulation involving HMGB2 in SZ. Here, we show that an interaction between HMGB2 and the Wnt/β-catenin pathway regulates the maintenance of the SZ. We found that the Wnt/β-catenin pathway is active specifically in the SZ in normal mouse knee joints and colocalizes with HMGB2. Both Wnt signaling and HMGB2 expression decrease with aging in mouse joints. Our molecular studies show that HMGB2 enhances the binding of Lef-1 to its target sequence and potentiates transcriptional activation of the Lef-1-β-catenin complex. The HMG domain within HMGB2 is crucial for interaction with Lef-1, suggesting that both HMGB2 and HMGB1 may be involved in this function. Furthermore, conditional deletion of β-catenin in cultured mouse chondrocytes induced apoptosis. These findings define a pathway where protein interactions of HMGB2 and Lef-1 enhance Wnt signaling and promote SZ chondrocyte survival. Loss of the HMGB2-Wnt signaling interaction is a new mechanism in aging-related cartilage pathology.

aging | osteoarthritis | apoptosis | superficial zone

Articular cartilage is a tissue that provides biomechanical properties that allow near frictionless joint movement and dispersion of mechanical loads. Cartilage is composed of a single cell lineage but differences in the organization, phenotype and function of cells in the various layers of cartilage have been recognized (1–4). The superficial zone (SZ) is the most unique. SZ cells produce lubricin, also termed proteoglycan-4 (PRG4) or superficial zone protein (SZP), an important joint lubricant (5–7), and are more responsive to stimulation by catabolic cytokines such as IL-1 (8). Recent studies also suggest that the SZ contains cells that express mesenchymal stem cell markers (9–11).

Articular cartilage is among the tissues that undergo profound aging-related changes and aging represents the major risk factor for osteoarthritis (OA), the most prevalent joint disease (12). Aging-related changes in cartilage include reduced cellularity, increased apoptosis and altered cellular responses to growth factors, cytokines and mechanical stress (13–15). Cartilage changes in aging and OA begin in the SZ and once the SZ is disrupted this is followed by progressive erosion of the remaining cartilage layers (16).

To address mechanisms that maintain the unique phenotype of SZ cells we performed gene expression analyses and observed that expression of the chromatin protein HMGB2 is restricted to the SZ (17). Joint aging in humans and mice leads to loss of HMGB2 expression and this is correlated with the onset of OA-like changes. Mice deficient in *Hmgb2* develop early onset and more severe OA, and this is associated with a reduction in cartilage cellularity attributable to increased cell death (17).

Wnt proteins are secreted factors that regulate cell proliferation and differentiation during early stages of chondrogenesis (18, 19). Overexpression of β-catenin in prechondrogenic cells inhibits overt chondrocytic differentiation (20) and overexpression in chick limb buds accelerates hypertrophic differentiation (21). In contrast, inhibition of β-catenin signaling by overexpression of Frzb-1, dominant negative Wnt receptors, results in delayed maturation (22). Homozygous deletion of β-catenin is embryonic lethal but conditional deletion in cartilage was associated with delayed chondrocyte hypertrophy and reduced chondrocyte proliferation in growth plates (23). Conditional mutant mice deficient in Wnt/β-catenin signaling displayed a defective flat cell layer normally abutting the synovial cavity and markedly reduced levels of PRG4/SZP (24). This supports the importance of Wnt signaling in skeletal development and early stages of chondrocyte differentiation.

Recent studies indicate that Wnt signaling has a role in adult articular cartilage. Increased Wnt signaling due to loss of sFRPS function represents a risk factor for OA (25). Similarly, overexpression of β-catenin in chondrocytes stimulates the expression of matrix degradation enzymes (26). However, Wnt signaling also contributes to differentiation and maintenance of articular cartilage chondrocytes. Inhibition of β-catenin signaling by transgenic overexpression of its intracellular antagonist ICAT results in progressive SZ degradation and development of OA (27). These studies suggest that the precise temporal and spatial activation of Wnt signaling in articular cartilage determines its homeostatic versus pathogenic effects.

Taken together, these reports on Wnt/β-catenin and our observations on HMGB2 suggest possible interactions in the maintenance of the SZ in adult cartilage. Here, we define a molecular mechanism by which HMGB2 and β-catenin regulate cartilage SZ integrity.

Results

β-Catenin Signaling Is Activated in the SZ of Articular Cartilage and Decreases with Aging. β-catenin is an important regulator of chondrocyte maturation in growth plate and its expression and function during skeletal development have been characterized (19, 21, 28). To analyze β-catenin in adult cartilage we used the TOPGAL transgenic mouse model where the β-galactosidase gene is under the control of a LEF/TCF and β-catenin inducible promoter and allows direct detection of cells and tissues with active Wnt signaling (29). Wnt/β-catenin signaling has been reported to be active at early stages during joint formation and to remain active and prominent at later stages in small and large joints (24). In 1-month-old TOPGAL mice, we detected β-galactosidase activity in all zones of articular cartilage. At 3 months with joint maturation it became

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[1]To whom correspondence should be addressed. E-mail: mlotz@scripps.edu.

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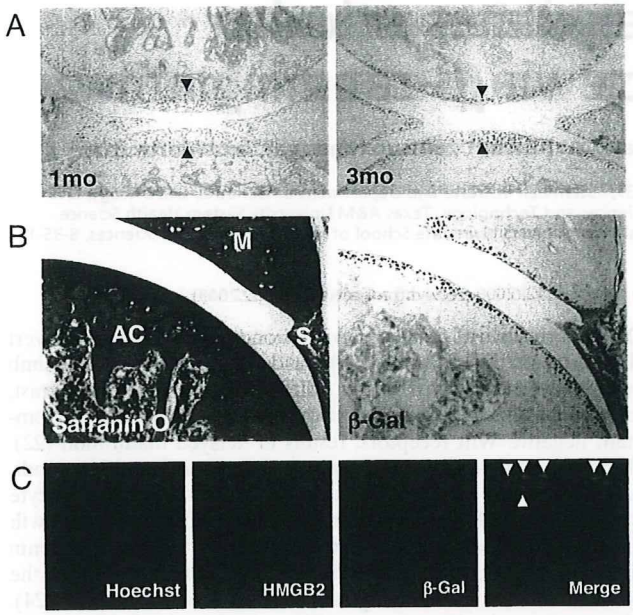

Fig. 1. Active Wnt signaling and correlation with HMGB2 expression in the articular cartilage SZ. (*A*) Immunohistochemistry was performed with β-galactosidase antibody on knee joint sections from 1 and 3-month-old TOPGAL mice. Between 1 and 3 months of age the β-galactosidase positive cells become more restricted to the superficial cell layers in articular cartilage. (*B*) β-galactosidase (β-Gal) positive cells are found in articular cartilage and meniscus, whereas synovium is negative. Safranin O staining of the adjacent section. AC, articular cartilage; M, meniscus; S, synovium. X100. (C) HMGB2 and β-galactosidase expression by immunofluorescence assay. Colocalization of HMGB2 and β-galactosidase (β-Gal) positive cells is found in the SZ in articular cartilage at 3 months of age (arrowheads). Hoechst dye 33258 was used to stain nuclei. (Magnification: ×400.)

more restricted to the cartilage surface (Fig. 1*A*). At this stage, β-galactosidase protein was expressed in the SZ of articular cartilage in meniscus but not in synovium (Fig. 1*B*). Because this pattern is similar to that of HMGB2 (17), we performed double immunofluorescence assay, and verified that most SZ cells express both HMGB2 and β-galactosidase (Fig. 1*C*).

Articular cartilage in C57BL/6J mice undergoes aging-related changes that are similar to osteoarthritis joint pathology (30), and this was also observed in TOPGAL mice on CD1 background (Fig. 2). At 6 months of age articular cartilage had normal appearance, and HMGB2 and β-galactosidase positive cells were present in the superficial cell layers. At 12 months of age there was a reduction in cartilage thickness and cellularity and surface irregularities were prominent in the central weight-bearing areas of the tibial plateau. At 12 months HMGB2 and β-galactosidase were both absent in the SZ in the weight bearing areas, and HMGB2 was completely absent in all regions of articular cartilage by 18 months (Fig. 2). In contrast, β-galactosidase was enhanced in the mid and deep zone, in calcified cartilage, subchondral bone and in osteophytes at 18 months. These findings demonstrate a correlated aging-related loss of HMGB2 and β-catenin signaling in the SZ of articular cartilage and this is associated with OA-like pathology.

Functional Interactions of β-Catenin and HMGB2. The in vivo colocalization of HMGB2 and Wnt/β-catenin activity (Fig. 1) and the correlation of their loss in OA-like pathology suggest interaction of HMGB2 and Wnt/β-catenin in the SZ. To study this in detail, we performed luciferase-reporter assays. Using cyclin D1 promoter (−962CD1) (31), β-catenin transfection caused the expected increase in luciferase activity in both SW1353 chondrosarcoma cells

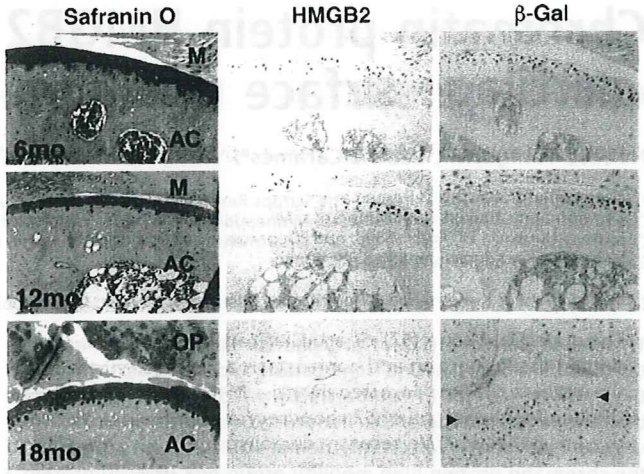

Fig. 2. HMGB2 and β-galactosidase expression during aging in TOPGAL mice. Safranin-O stained sections of joints from TOPGAL mice show normal cartilage at 6 months, reduced thickness and cellularity at 12 and 18 months. HMGB2 and β-galactosidase (β-Gal) are detected by immunohistochemistry at 6 months in the articular cartilage surface. At 12 months both are absent in the weight bearing areas, and HMGB2 is completely absent in the articular cartilage by 18 months. In contrast, at 18 months β-galactosidase becomes detectable in all other zones of articular cartilage except for the SZ (arrowheads). AC, articular cartilage; M, meniscus; OP, osteophyte. X100.

and 293T kidney epithelial cells (Fig. 3 *A* and *B*). Transfection of HMGB2 (32) did not change luciferase activity but cotransfection of HMGB2 and β-catenin resulted in synergistic enhancement in SW1353 chondrosarcoma cells (Fig. 3*A*); this synergy was not observed in 293T kidney epithelial cells (Fig. 3*B*). Similar differences between cell types were obtained using the TOPflash promoter, which contains multiple repeats of the β-catenin-TCF/LEF consensus sequences (33) (Fig. 3 *C* and *D*). The synergistic activity of HMGB2 and β-catenin was also seen in chondrogenic ATDC5 cells. Transfection of the FOPflash promoter with a mutated Lef-1 binding site showed no activity (Fig. 3*E*) but the activity of TOPflash promoter was enhanced by HMGB2 in a dose-dependent manner under β-catenin transfection (Fig. 3*F*). These experiments demonstrate synergistic interaction of β-catenin and HMGB2 in enhancing Lef-1 responsive promoters, specifically in chondrogenic cell types.

Physical Interactions of HMGB2 and Lef-1. To examine molecular interactions between β-catenin, Lef-1 and HMGB2, GST-pull down assays were performed using bacterially expressed GST-HMGB2, β-catenin and Lef-1 and in vitro-translated HMGB2, β catenin and Lef 1 (34). We observed that in vitro translated HMGB2 bound GST-Lef-1, but not GST-β-catenin (Fig. 4*A*). The results from the reverse experiment showed that in vitro-translated Lef-1 interacted with GST-HMGB2 and GST-β-catenin (Fig. 4*B*). When in vitro-translated β-catenin protein was incubated with GST-HMGB2 and GST-Lef-1, only GST-Lef-1 but not GST-HMGB2 was pulled down (Fig. S1). This indicates a specific interaction between HMGB2 and Lef-1, leading to enhanced transcriptional activation of the Lef-1-β-catenin complex.

Interaction Domains of HMGB2 and Lef-1. To define the Lef-1 interaction domain within HMGB2, in vitro GST pull-down assays were performed using GST-Lef-1 and HMGB2 deletion mutants (A-box, B-box, acidic tail) as shown in Fig. S2*A*. The results demonstrated that none of these 3 HMGB2 mutants interacts with Lef-1 (Fig. S2*B*). Then we constructed A-box and B-box domains with linker regions and found that both bind Lef-1 (Fig. S2*B*). Delta box, which contains linker and acidic tail but not A-box or B-box,

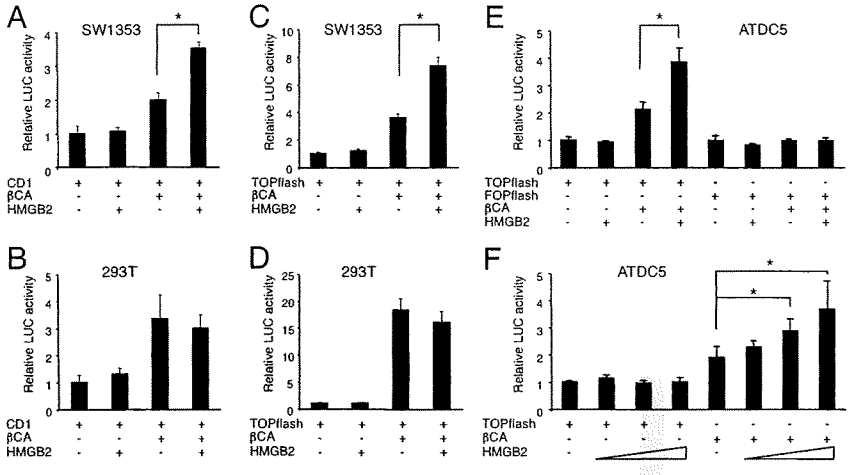

Fig. 3. Synergy of HMGB2 and β-catenin. HMGB2 and β-catenin (βCA) were cotransfected with the cyclin D1 (CD1) (*A* and *B*) and TOPflash reporter genes (*C–F*) in SW1353 cells (*A* and *C*) and 293T cells (*B* and *D*), and luciferase assay was performed after 24 h. In SW1353 cells, HMGB2 enhances luciferase activity when co-transfected with β-catenin (*A* and *C*), whereas this synergistic effect is not seen in 293T cells (*B* and *D*). This synergistic effect is also found with TOPflash in a dose-dependent manner (*F*), but not with FOPflash reporter genes in ATDC5 cells (*E*) (*, $P < 0.05$).

did not interact with Lef-1. These results indicate that A-box or B-box together with the linker region are required for HMGB2 binding to Lef-1.

Next we determined the domains in Lef-1 that are required for interaction with HMGB2. GST pull-down assays were performed with GST-HMGB2 and Lef-1 deletion mutant plasmids (FL, ΔN113, ΔN295, ΔN113-ΔC102) (35). The results showed that GST-HMGB2 could pull down Lef-1 FL (Fig. 4*B*), Lef-1 ΔN113 and Lef-1 ΔN295 but not Lef-1 ΔN113-ΔC102 (Fig. S2*C*), indicating that the HMG domain in Lef-1 is responsible for the physical interaction with HMGB2.

DNA Binding Interactions of HMGB2 and Lef-1. To understand how the HMGB2-Lef-1 interaction contributes to Wnt/β-catenin activity, we examined whether the interaction affects Lef-1 DNA binding. Gel shift assays were performed to determine binding specificity and interactions of HMGB2 and Lef-1. We prepared oligonucleotide probes with Lef-1 binding sites (cyclin D1 and TOP) and a probe with a mutated Lef-1 binding site (FOP) as described in ref. 36. Using nuclear extracts of Lef-1 transfected SW1353 chondrosarcoma cells, we detected binding of Lef-1 to both cyclin D1 and TOP probes, and this binding was enhanced by the addition of purified HMGB2 (Fig. 4*C*). In contrast, no binding was detected on the FOP probe. HMGB2 did not interact with cyclin D1 and TOP probes without overexpressed Lef-1.

c-Jun is a target gene for the β-catenin-Tcf/Lef transcriptional complex (37), and Wnt signaling induces c-Jun expression in chondrocytes (38). We also detected binding of Lef-1 to c-Jun probes in SW1353 cells in the presence of Lef-1, and this binding was potentiated by the addition of HMGB2 protein (Fig. S3). These results suggest that interaction between HMGB2 and Lef-1 enhances DNA binding affinity of Lef-1, to enhance Wnt/β-catenin signaling.

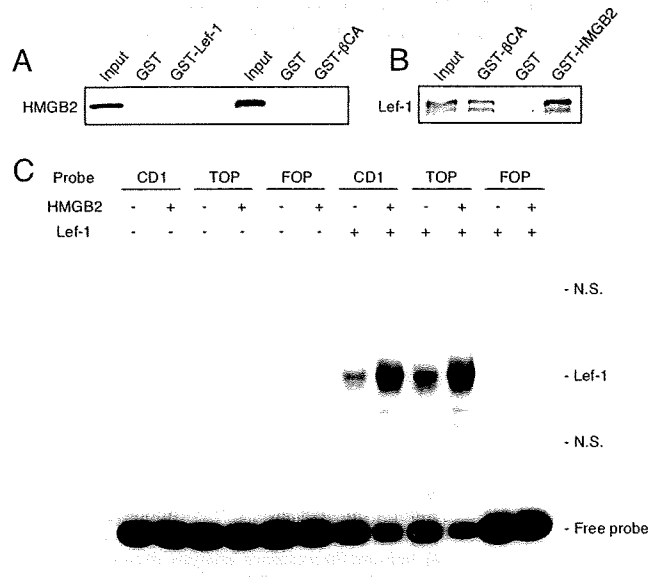

Fig. 4. GST-pull down assay for β-catenin (βCA), Lef-1 and HMGB2. (*A*) In vitro-translated HMGB2 interacts with GST-Lef-1, but not with GST-β-catenin. (*B*) In vitro-translated Lef-1 interacts with both GST-β-catenin and GST-HMGB2. (*C*) DNA binding interactions of HMGB2 and Lef-1 by EMSA. Using nuclear extracts of SW1353 cells transfected with Lef-1, binding of Lef-1 with both cyclin D1 (CD1) and TOP probes was detected. This was enhanced by the addition of HMGB2 protein (1 μg). In contrast, no binding was detected on the FOP probe. N.S., nonspecific.

HMGB2 and Wnt/β-Catenin Target Gene Expression. To examine whether the interaction between HMGB2 and Lef-1 potentiates Wnt/β-catenin signaling activity, we examined Wnt/β-catenin signaling in WT and *Hmgb2*$^{-/-}$ chondrocytes using SuperTOPflash, which contains 8 TCF/LEF binding sites and the corresponding negative control vector SuperFOPflash (39). We did not detect a difference in luciferase activity between two groups when the cells were unstimulated; however, in response to stimulation with recombinant Wnt3a luciferase activity was increased. Importantly, this activation was significantly lower in *Hmgb2*$^{-/-}$ chondrocytes than in WT chondrocytes (Fig. 5*A*).

To further examine this, we analyzed levels of Wnt/β-catenin targets genes. HMGB2 was reduced by siRNA in immature murine articular chondrocytes, which strongly express endogenous HMGB2 (17) (Fig. 5*B*). Quantitative PCR shows that HMGB2 siRNA reduced cyclin D1 mRNA expression. Additional Wnt/β-catenin target genes, Gli3 and Frizzled 2 (Fzd2), which are expressed in articular cartilage (26, 40), were also reduced by HMGB2 siRNA, whereas PRG4/SZP that is expressed in murine *Hmgb2*$^{-/-}$ chondrocytes and WT chondrocytes was unaffected (17) (Fig. 5*C*).

To verify that HMGB2 does bind to targets of Lef1/β-catenin genes, we used chromatin immunoprecipitation assay. We observed that HMGB2 can facilitate binding affinity of Lef-1 to the human Gli3 enhancer (R2) (Fig. 5 *D* and *E*), which shows strong activity among highly conserved noncoding DNA regions that contained Tcf/Lef binding sequence within human Gli3 locus (R1–4) (41). These results further support our notion that HMGB2 potentiates Wnt/β-catenin activity.

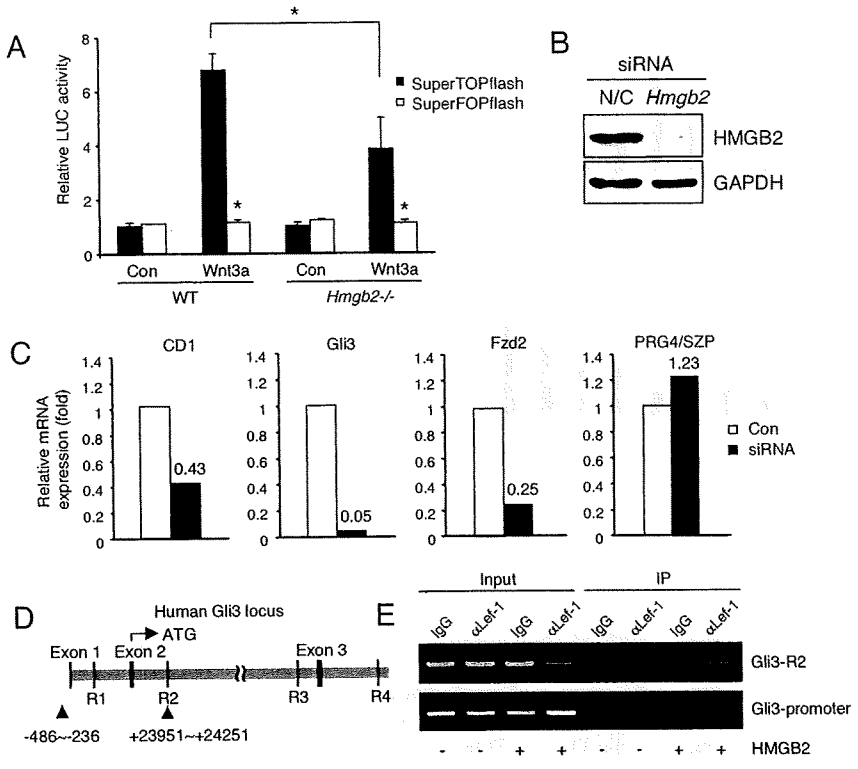

Fig. 5. Wnt/β-catenin signaling in WT and *Hmgb2*$^{-/-}$ chondrocytes. (*A*) SuperTOPflash or SuperFOPflash reporter genes were transfected into murine WT or *Hmgb2*$^{-/-}$ chondrocytes. Upon addition of recombinant Wnt3a, stronger luciferase activity was found in WT chondrocytes compared with *Hmgb2*$^{-/-}$ chondrocytes. SuperFOPflash vector was used as negative control (*, $P < 0.01$). HMGB2 siRNA reduces Wnt/β-catenin target genes in articular chondrocytes. (*B*) Murine chondrocytes were transfected by oligo-siRNA negative control (N/C) or HMGB2 and cultured for 48 h, followed by measurement of HMGB2 by Western blot analysis. HMGB2 siRNA specifically and efficiently down-regulated protein levels of HMGB2. (*C*) mRNA levels of Wnt/β-catenin target genes were measured by real-time PCR. The expression levels of cyclin D1 (CD1), Gli3 and Fzd2 but not PRG4/SZP were reduced in chondrocytes with HMGB2 siRNA. (*D*) Schematic representation of the human GLI3 locus. Gray indicates the Gli3 gene, and Tcf/Lef-binding sites as putative enhancers are depicted in red (R1–4). (*E*) Chromatin immunoprecipitation assay shows that HMGB2 potentiates binding affinity of Lef-1 on human Gli3 enhancer within R2 (*Upper*). Sequences not predicted to contain Tcf/Lef-1 binding sites upstream of the Exon 1 were not pulled down (*Lower*). IP, immunoprecipitation.

Loss of β-Catenin Signaling Results in Chondrocyte Apoptosis. To further test the functional significance of β-catenin signaling we conditionally inactivated β-catenin. Chondrocytes were isolated from knee and hip cartilage of β-catenin floxed mice (Ctnnb1$^{flox/flox}$), infected with adenovirus-GFP-Cre and cultured for 72 h. Immunofluorescence analysis of GFP demonstrated effective adenoviral transduction (Fig. 6*A*). β-catenin protein levels were reduced with increasing amounts of adenovirus-GFP-Cre in Ctnnb1$^{flox/flox}$ chondrocytes (Fig. 6*B*). Next, chondrocytes from Ctnnb1$^{flox/flox}$ mice with or without adenovirus-GFP-Cre infection were analyzed by flow cytometry for viability and the apoptosis marker Annexin V. Upon adenovirus-GFP-Cre infection there was a significant increase in apoptotic cells (Fig. 6*C*) without addition of apoptosis inducers. When the chondrocytes were stimulated with anti-Fas antibody CD95 and proteasome inhibitor MG132 (17), a higher percentage of apoptotic chondrocytes was found after

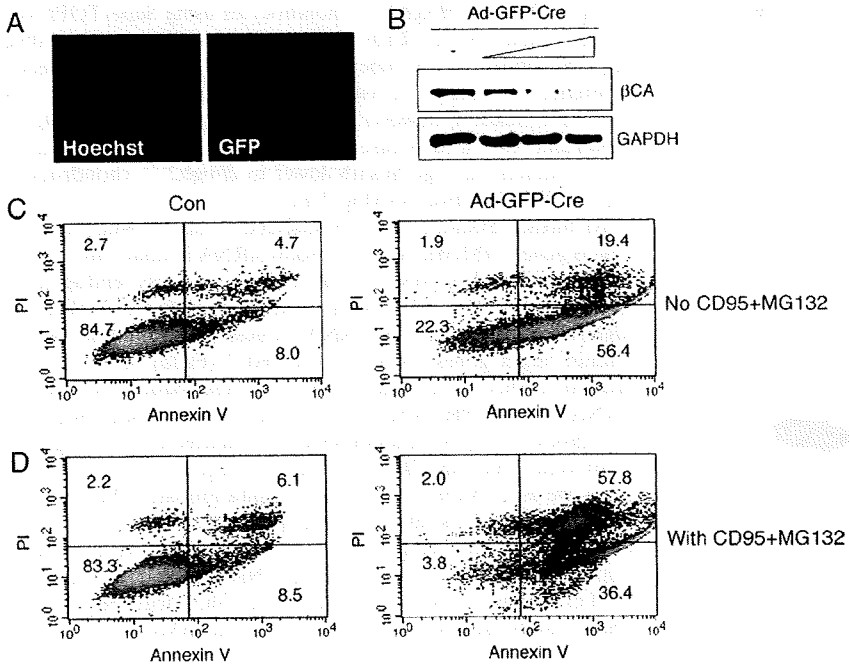

Fig. 6. Conditional inactivation of β-catenin and chondrocyte survival. βCA$^{flox/flox}$ chondrocytes were infected with GFP-Cre adenovirus and cultured for 72 h. (*A*) Immunofluorescence analysis shows GFP expression in infected cells. (*B*) Western blot shows reduction in β-catenin protein levels with increasing amounts of GFP-Cre adenovirus (Ad-GFP-Cre). (*C* and *D*) FACS analysis for annexin V and propidium iodide (PI) staining. GFP-Cre adenovirus infected cultures showed a higher percentage of apoptotic chondrocytes compared with noninfected (Con) cells in the absence or presence of anti-Fas antibody CD95 (1 μg/mL) and MG132 (20 μM).

adenovirus-GFP-Cre infection compared with control (Fig. 6*D*). Thus, β-catenin signaling promotes chondrocyte survival under basal conditions and in response to apoptosis inducers.

Discussion

Understanding mechanisms that control articular cartilage formation and maintenance is of significance to cartilage tissue engineering and the prevention and treatment of diseases affecting articular cartilage. In regards to cartilage tissue engineering a major unmet challenge is the generation of a tissue that recapitulates the zonal organization of normal cartilage. In regards to joint diseases, the major current deficit is in the lack of therapies for OA, the most prevalent form of arthritis. The initial lesions in OA are at the articular surface and once the SZ of cartilage is disrupted, the chronic cartilage remodeling and degradation process is initiated.

To begin elucidating molecular mechanisms that govern the SZ phenotype we showed that the chromatin protein HMGB2 is uniquely expressed in the SZ (17). Aging in humans and mice is associated with a loss of HMGB2 expression, which correlates with OA-like cartilage changes and mice with *Hmgb2* deletion show early onset and more severe OA (17). This observation presented a starting point to further characterize the signaling network in which HMGB2 operates to control SZ cell survival and function.

The Wnt/β-catenin pathway presented a candidate based on a series of recent observations. Most notably, loss of β-catenin signaling leads to OA-like pathology (27). The first observations in this study addressed β-catenin activation patterns in articular cartilage. Wnt/β-catenin signaling is active at multiple embryonic stages of joint formation (24). Postnatally, we observed remarkable similarities between localization of HMGB2 and β-catenin. HMGB2 expression and β-catenin activation were found in all zones of articular cartilage in newborn mice. With joint maturation both became more restricted to the SZ and both showed an aging-related loss in the SZ. Although HMGB2 eventually was completely absent, β-catenin was activated in the other cartilage zones.

To determine molecular mechanisms related to these similarities in expression patterns we analyzed functional and physical interactions. Our EMSA data showed that HMGB2 does not directly bind to regulatory DNA elements but it augments DNA binding of Lef-1. HMGB2 does not alter the electrophoretic mobility of Lef-1 complexed with oligonucleotides, suggesting that HMGB2 dissociates from the complex after having provided its architectural activity (42). Similar results were observed for HMGB1, which increased the affinity of p53 complexes with oligonucleotides (43).

Transfection of HMGB2 did not activate β-catenin responsive promoters but cotransfection of HMGB2 and β-catenin did result in synergistic activation of Lef-1 responsive promoters. This synergy was seen in two chondrogenic cell types, including SW1353 chondrosarcoma cells and ATDC5 prechondrogenic cells but not in lineages such as kidney epithelial cells, suggesting other lineage specific factors mediate this interaction or the difference in both HMGB2 and HMGB1 between chondrogenic cells and 293T cells is responsible (Fig. S4).

Physical interaction studies showed there is no direct binding of HMGB2 and β-catenin. However, HMGB2 binds to Lef-1 and the complex that contains HMGB2, β-catenin, Lef-1 and probably other components leads to enhanced expression of genes containing Lef-1 binding sites. Mapping of interaction domains revealed that the HMG domain in Lef-1 is required for HMGB2 binding. The HMG domain is also responsible for interaction with Notch intracellular domain (44). Notch1 is expressed in developing articular cartilage surface (45) in a pattern similar to HMGB2 (17), indicating that Notch might be involved in Lef1-HMGB2 complex formation in temporally and spatially specific patterns during cartilage formation. HMGB2 has been reported to interact with steroid receptors (46), p53 and p73 (47). Stros et al. reported that B-box within human HMGB1 required the TKKKFKD motif that is included in the linker for interaction with p73, whereas A-box itself can bind p73 (47). It has also been shown that A-box, which contains the linker region within HMGB1, can interact with p53 (48). Our results demonstrate that the A-box or B-box within HMGB2 contribute to binding with Lef-1 only when the linker is present, because A-box, B-box and Δbox deletion mutants did not bind with Lef-1. HMGB1 can also interact with Lef-1 (Fig. S5). Considering that HMGB1 is ubiquitously expressed in the nuclei throughout normal articular cartilage (Fig. S6), we cannot exclude the possibility that HMGB1 and HMGB2 may function cooperatively as coactivators for Wnt/β-catenin signaling in the SZ (17).

The findings on interactions between HMGB2 and the Wnt signaling pathway are similar to a report demonstrating that HMG-17 was responsive to Wnt/β-catenin signaling. HMG-17 forms a chromatin complex with PITX2 to repress PITX2 transcriptional activity. This complex is inactive and switched to an active transcriptional complex through the interaction of β-catenin with PITX2 (49).

To determine functional consequences of the HMGB2/Lef-1 interaction in a cellular context, we analyzed cell survival and expression of representative Lef-1 target genes. The conditional deletion of β-catenin by Cre adenovirus infection of chondrocytes from β-catenin floxed mice increased basal and in vitro induced apoptosis. This observation is consistent with our earlier findings that *Hmgb2* deficient cells are more susceptible to CD95/Fas mediated apoptosis (17). Inhibition of Wnt proteins promotes programmed cell death in different types of cancer cells (50, 51). In human OA cartilage, FrzB-2 is highly expressed and is associated with chondrocyte apoptosis (52). In Col2a1-ICAT-transgenic mice in which β-catenin signaling is selectively blocked in chondrocytes, apoptosis is increased (27).

Our results also show that promoters with Lef-1 binding sites were less responsive to Wnt3a treatment in *Hmgb2*$^{-/-}$ chondrocytes compared with WT chondrocytes. Then we analyzed cyclin D1, Gli3 and Fzd2, three representative and well characterized Lef-1 target genes in cartilage (26, 40, 53). The expression levels of these genes were reduced by HMGB2 siRNA in chondrocytes. Thus, it is possible that reduction of these three genes at least in part explains the increased apoptosis seen in both the *Hmgb2* deficient mice (17) and in chondrocytes with deficient β-catenin (27).

In conclusion, this study demonstrates similar expression and activation patterns of HMGB2 and β-catenin in articular cartilage and that a loss of these pathways in the SZ of articular cartilage may lead to altered gene expression, cell death and OA-like changes.

Materials and Methods

Mice. The β-catenin floxed mice (β-catenin$^{flox/flox}$) with loxP sites in introns 1 and 6 of the β-catenin gene (6.129-Ctnnb1tmKem/KnwJ line) and TOPGAL mice (Tg(Fos-lacZ)34Efu/J line) (29) were purchased from the Jackson Laboratory. Mice were used according to protocols approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute.

Plasmid Construction. The HMGB2 deletion constructs were prepared by PCR amplification of full-length murine HMGB2 cDNA and cloned into pcDNA3-flag vector after the mapping of A-box and B-box within human HMGB2 (54). pGEX-HMGB2 and pGEX-HMGB1 were constructed by subcloning of murine HMGB2 or human HMGB1 into pGEX (Promega), respectively. pGEX-β-catenin was provided by X. He (Harvard Medical School, Boston) and pGEX-Lef-1 by M.R. Stallcup (University of Southern California, Los Angeles).

GST Pull-Down Assay. The wild-type and deletion mutants of HMGB2 and Lef-1 were in vitro transcribed/translated with the TNT reticulocyte lysate kit (Promega) in the presence of [^{35}S]methionine. GST-null, GST-HMGB2, GST-Lef-1, or GST-β-catenin proteins were produced in *E. coli* and purified, and then incubated overnight at 4 °C rotating with the ^{35}S-met-labeled proteins in PC100+βME buffer (55). After extensive washes, we added SDS loading buffer to the beads, boiled them, and separated the supernatant on SDS/PAGE gels. As a positive control, the amount of ^{35}S-met-labeled protein loaded was 20% of the input. The gels were dried and then exposed to X-ray film.

Electrophoretic Mobility-Shift Assay (EMSA). Preparation of DNA for EMSA with ^{32}P-labeled duplex oligonucleotide probes for CD1, CD1TOP and CD1FOP was described earlier (36). We also generated synthetic duplex oligonucleotides encompassing regions evolutionarily conserved in the c-Jun promoter (37). The binding reaction contained 40,000 cpm of ^{32}P-labeled DNA that was incubated with nuclear extracts from SW1353 cells with or without transfection of HA-tagged Lef-1 expression vector (gift from P.K. Vogt, The Scripps Research Institute) in the presence of purified calf thymus HMGB2 protein (Shino-Test), following Gel Shift Assay Systems Protocol (Promega). DNA-protein complexes were electrophoresed in 6% DNA retardation gel (Invitrogen) and visualized by autoradiography.

Quantitative PCR. Total RNA was extracted and oligo(dT)-primed cDNA was prepared from 500 ng of total RNA by using SuperScript III (Invitrogen). The resulting cDNAs were analyzed by using the SYBR green system for quantitative analysis of specific transcripts as described in ref. 56. All mRNA expression data were normalized to GAPDH expression in the same sample. The primers used in real-time PCR are listed in *SI Text*.

Apoptosis Induction and Analysis in Vitro. Chondrocytes were prepared from 5-day-old β-catenin floxed mice (β-catenin$^{flox/flox}$) as described in ref. 57. The cells were plated in 6-well plates at semiconfluence, infected with adenovirus expressing both Cre recombinase and green fluorescent protein (GFP) (Adv-Cre-GFP) and cultured for 72 h. We used an E1/E3-deleted, replication-incompetent, serotype 5 adenovirus-expressing Cre recombinase and GFP under control of the cytomegalovirus (CMV) promoter. Medium was changed to DMEM/F12 with 0.5% FBS and chondrocytes were stimulated with NA/LE hamster anti-mouse CD95 antibody (BD PharMingen) and proteasome inhibitor MG132 (Sigma) for 12 h, which induces apoptosis in articular chondrocytes (58). Cells were incubated with FITC-labeled annexin V (BD PharMingen) or propidium iodide (Sigma) and analyzed on a BD FACSCalibur as described in ref. 17.

Statistical Analysis. Results are expressed as mean ± standard deviation. Statistical comparison between genotypes or treatment groups was performed with a two-tailed Student's *t* test. *P* values <0.05 were considered significant.

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Laboratory Investigation (2009) 89, 278–289

Intraocular expression and release of high-mobility group box 1 protein in retinal detachment

Noboru Arimura[1,2], Yuya Ki-i[1,2], Teruto Hashiguchi[2], Ko-ichi Kawahara[2], Kamal K Biswas[2], Makoto Nakamura[3], Yasushi Sonoda[1], Keita Yamakiri[1], Akiko Okubo[1], Taiji Sakamoto[1] and Ikuro Maruyama[2]

High-mobility group box 1 (HMGB1) protein is a multifunctional protein, which is mainly present in the nucleus and is released extracellularly by dying cells and/or activated immune cells. Although extracellular HMGB1 is thought to be a typical danger signal of tissue damage and is implicated in diverse diseases, its relevance to ocular diseases is mostly unknown. To determine whether HMGB1 contributes to the pathogenesis of retinal detachment (RD), which involves photoreceptor degeneration, we investigated the expression and release of HMGB1 both in a retinal cell death induced by excessive oxidative stress *in vitro* and in a rat model of RD-induced photoreceptor degeneration *in vivo*. In addition, we assessed the vitreous concentrations of HMGB1 and monocyte chemoattractant protein 1 (MCP-1) in human eyes with RD. We also explored the chemotactic activity of recombinant HMGB1 in a human retinal pigment epithelial (RPE) cell line. The results show that the nuclear HMGB1 in the retinal cell is augmented by death stress and upregulation appears to be required for cell survival, whereas extracellular release of HMGB1 is evident not only in retinal cell death *in vitro* but also in the rat model of RD *in vivo*. Furthermore, the vitreous level of HMGB1 is significantly increased and is correlated with that of MCP-1 in human eyes with RD. Recombinant HMGB1 induced RPE cell migration through an extracellular signal-regulated kinase-dependent mechanism *in vitro*. Our findings suggest that HMGB1 is a crucial nuclear protein and is released as a danger signal of retinal tissue damage. Extracellular HMGB1 might be an important mediator in RD, potentially acting as a chemotactic factor for RPE cell migration that would lead to an ocular pathological wound-healing response.

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KEYWORDS: danger signal; high-mobility group box 1 protein; photoreceptor degeneration; retinal detachment; tissue damage; wound healing

Cell death is the predominant event of degenerative tissue damage and can be a trigger that activates the immune system and repair program. Recently, there has been much interest in the pivotal role of endogenous danger signals released during cell death.[1] High-mobility group box 1 (HMGB1) protein is a prototypic innate danger signal, and appears to be crucial in this context because extracellular HMGB1 [2] can modulate inflammation, proliferation, and remodeling, which are involved in the wound-healing process.[3]

HMGB1 was originally described as an abundant and ubiquitous nuclear DNA-binding protein that had multiple functions dependent on its cellular location.[2,4] In the nucleus, HMGB1 binds to DNA and is critical for proper transcriptional regulation. It is also called amphoterin and accelerates cellular motility on the cell surface.[5] HMGB1 is reported to be passively released into the extracellular milieu by necrotic cells, but not by apoptotic cells,[6] or is exported actively by monocytes/macrophages[7] and neural cells[8] upon receiving appropriate stimuli. In damaged tissue, extracellular HMGB1 acts as a necrotic signal, which alerts the surrounding cells and the immune system.[2] Although extracellular HMGB1 can contribute to normal tissue development and repair, it is also implicated in the pathogenesis of several diseases (including lethal endotoxemia,[7] disseminated intravascular coagulation,[9] ischemic brain,[10] tumor,[11] atherosclerosis,[12] rheumatoid arthritis,[13] and periodontitis[14]).

[1]Department of Ophthalmology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan; [2]Department of Laboratory and Vascular Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan and [3]Division of Ophthalmology, Department of Surgery, Kobe University Graduate School of Medicine, Kobe, Japan
Correspondence: Professor T Sakamoto, MD, PhD, Department of Ophthalmology, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1, Sakuragaoka, Kagoshima 890-8520, Japan.
E-mail: tsakamot@m3.kufm.kagoshima-u.ac.jp

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Retinal detachment (RD), the physical separation of photoreceptors from the underlying retinal pigment epithelium (RPE), is one of the main causes of visual loss. Photoreceptor degeneration due to RD is thought to be executed by apoptosis[15,16] and necrosis,[17] which usually occur after tissue damage. Although retinal cell death and the following reactive responses must occur in almost all forms of retinal disease including RD,[18] data regarding the relationship among cell death, danger, and responses in the eye, have been very limited, especially in terms of danger signals. We previously reported that HMGB1 was significantly elevated in inflamed eyes with endophthalmitis, and suggested a possible link between HMGB1 and ocular inflammatory diseases.[19] On the other hand, considering the properties of HMGB1, we hypothesized that HMGB1 might have some roles in photoreceptor degeneration and subsequent damage-associated reactions in RD.

To investigate whether HMGB1 is involved in the pathogenesis of RD, we first examined the expression and release of HMGB1 both in a retinal cell death *in vitro* and in a rat model of RD-induced photoreceptor degeneration *in vivo*. To focus on human RD, we assessed the intravitreous concentrations of HMGB1 in human eyes affected by RD. Monocyte chemoattractant protein 1 (MCP-1), which was recently documented to be a potential proapoptotic mediator in RD,[20] was also measured in the same vitreous samples. We further analyzed the effects of recombinant HMGB1 (rHMGB1) on chemotactic activity in a RPE cell line *in vitro*. Our findings suggest that extracellular HMGB1 is evident in eyes with RD as a danger signal, potentially acting as a chemotactic factor for RPE cell migration that would lead to ocular pathological wound healing.

MATERIALS AND METHODS

Reagents

Full-length, LPS-free rat rHMGB1 protein, which is 99% identical to human HMGB1 and is fully functional on cells of mammalian origin,[21] was purchased from HMGBiotech (Milan, Italy). Human recombinant MCP-1 (rMCP-1) was purchased from Peprotec (Rocky Hill, NJ). Rabbit polyclonal antibody against HMGB1 was provided by Shino-Test Corporation (Kanagawa, Japan). Antibodies against phospho- and total extracellular signal-regulated kinase (ERK)-1/2 were obtained from Cell Signaling Technology (Beverly, MA). U0126 was obtained from Calbiochem (La Jolla, CA).

Human Vitreous Samples

This study was approved by our institutional ethical committee (Kagoshima University Hospital), and was performed in accordance with the Declaration of Helsinki. All surgeries were performed at Kagoshima University Hospital. All patients gave informed consent before treatment. The clinical histories of all patients were obtained from their medical records. Undiluted vitreous fluid samples (0.5–0.7 ml) were obtained by pars plana vitrectomy. Vitreous humor was collected in sterile tubes, placed immediately on ice, centrifuged to remove cells and debris, and stored at −80°C until analysis.

Animals

All animal experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research and the approval of our institutional animal care committee (Kagoshima University). Adult male Brown Norway rats (250–300 g; KBT Oriental, Saga, Japan) were housed in covered cages and kept at constant temperature and relative humidity with a regular 12-h light–dark schedule. Food and water were available *ad libitum*.

Surgical Induction of RD

Rat experimental RD was induced as described previously.[22] Briefly, the rats were anesthetized with an intramuscular injection of ketamine and xylazine, and their pupils were dilated with topical 1% tropicamide and 2.5% phenylephrine hydrochloride. The retinas were detached using a subretinal injection of 1% sodium hyaluronate (Opegan; Santen, Osaka, Japan) with an anterior chamber puncture to reduce intraocular pressure. Sodium hyaluronate (0.05 ml) was slowly injected through the sclera into the subretinal space to enlarge the RDs. These procedures were performed only in the right eye, with the left eye serving as a control. Eyes with lens injury, vitreous hemorrhage, infection, and spontaneous reattachment were excluded from the following analysis. The rats were killed at 3, 7, and 14 days after treatment, with six animals per each time point.

Cell Culture

The rat immortalized retinal precursor cell line R28, a kind gift from Dr GM Siegel (The State University of New York, Buffalo), was cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 10% fetal bovine serum (FBS), 10 mM non-essential amino acids, and 10 μg/ml gentamicin as described previously.[23] The human immortalized RPE cell line ARPE-19, obtained from American Type Culture Collection (Manassas, VA), was grown in DMEM/F12 supplemented with 10% FBS, 2% penicillin–streptomycin, and 1% fungizone (all products were obtained from Invitrogen-Gibco, Rockville, MD). Cells were incubated at 37°C in a 5% CO_2 incubator and subcultured with 0.05% trypsin-EDTA. Subconfluent cultures were trypsinized and seeded for the following experiments. ARPE-19 cells were obtained at passage 21 and used at passages 24–30. Increased passage did not alter the following experimental results up to this passage number.

Cell Viability Assay

Cell viability was analyzed by mitochondrial respiratory activity measured using MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenol tetrazolium bromide) assay (Wako Chemicals,