Gremlin Enhances the Determined Path to Cardiomyogenesis

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Abstract

Background: The critical event in heart formation is commitment of mesodermal cells to a cardiomyogenic fate, and cardiac fate determination is regulated by a series of cytokines. Bone morphogenetic proteins (BMPs) and fibroblast growth factors have been shown to be involved in this process, however additional factors needs to be identified for the fate determination, especially at the early stage of cardiomyogenic development.

Methodology/Principal Findings: Global gene expression analysis using a series of human cells with a cardiomyogenic potential suggested Gremlin (Grem1) is a candidate gene responsible for in vitro cardiomyogenic differentiation. Grem1, a known BMP antagonist, enhanced DMSO-induced cardiomyogenesis of P19CL6 embryonal carcinoma cells (CL6 cells) 10–35 fold in an area of beating differentiated cardiomyocytes. The Grem1 action was most effective at the early differentiation stage when CL6 cells were destined to cardiomyogenesis, and was mediated through inhibition of BMP2. Furthermore, BMP2 inhibited Wnt/β-catenin signaling that promoted CL6 cardiomyogenesis.

Conclusions/Significance: Grem1 enhances the determined path to cardiomyogenesis in a stage-specific manner, and inhibition of the BMP signaling pathway is involved in initial determination of Grem1-promoted cardiomyogenesis. Our results shed new light on renewal of the cardiovascular system using Grem1 in human.

Introduction

The critical event in heart formation is commitment of mesodermal cells to a cardiomyogenic fate and their migration into anterolateral regions of the embryo during late gastrulation. In this process, morphogenetic movements and cardiac fate determination are regulated by cytokines such as bone morphogenetic proteins (BMPs) [1–3], and fibroblast growth factors (FGFs) [4–7]. These secreted proteins from neighboring endoderm, ectoderm, and the mesoderm itself, play important roles in induction of cardiac transcription factors [8] and differentiation of cardiomyocytes in amphibians [9] and avians [4]. Cardiomyogenic signals, such as BMPs and FGFs, indeed activate expression of cardiac specific transcription factors (Csx/Nkx2.5, Gata4, Mef2c), and these transcriptional factors activate expression of circulating hormones (atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP)), and cardiac specific proteins (myosin heavy chain (MyHC), myosin light chain (MyLC)). Wnt family proteins, cysteine-rich, and secreted glycoproteins, have also been implicated in embryonic development [10,11], and cardiomyogenesis [12,13]. In Drosophila, ‘wingless’, a homologue of vertebrate Wnt is involved in expression of ‘tinman’, a Drosophila homologue of Csx/Nkx2.5, through ‘amnialdo’, a Drosophila ortholog of β-catenin, and drives heart development [14]. In vertebrates, however, Wnt1/3a, which activates the canonical Wnt/β-catenin signaling pathway leading to stabilization of β-catenin as a downstream molecule through inactivation of glycogen synthase kinase-3β, inhibits cardiomyocytic differentiation from cardiac mesoderm [15–18]. Wnt11 promotes cardiac differentiation via the non-canonical pathway in Xenopus [12] and murine embryonic cell lines [19]. The secretion of Wnt inhibitors such as ‘Cerberus’, ‘Dickkopf’ and ‘Crescent’ by the anterior endoderm prevents Wnt3a secreted by the neural tube from inhibiting heart formation [15–17].

In this study, we performed GeneChip analysis to identify multiple extracellular determinants, such as cytokines, cell
RT-PCR of differentiated or undifferentiated CL6 cells

GeneChip and statistical analysis

To identify cytokines and transcription factors responsible for cardiomyogenic differentiation, 69 human cells were analyzed, depending on gene expression levels, by GeneSpringGX software, and clustered into 30 groups (Fig. 1A, Table 1). Among the 30 groups, 21 groups included cells with a cardiomyogenic potential (Fig. 1B; red numbers). To identify genes specific for these groups, hierarchical clustering was employed, using the average distance method. Genes with the lowest average expression E(G1) within the cluster that can differentiate into cardiomyocytes and genes with the highest average expression E(G2) outside the cluster were identified, as previously described [20–22]. Genes which have E(G1)>E(G2) were estimated, using the False Discovery Rate (FDR<0.05). Grem1 was nominated as a cluster-specific cardiomyocyte-promoting gene in cells that could differentiate into cardiomyocytes following NIA array analysis (Fig. 1B). The gene expression profile reported in this paper has been deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo: accession no. GSE8481, GSM41342-GSM41344, and GSM201137- GSM201145).

Cardiomyogenic differentiation of CL6 cells with Grem1 and DMSO

To investigate cardiomyogenic activity of Grem1, P19CL6 embryonal carcinoma cells (CL6 cells) were used for assessment of in vitro cardiomyogenic differentiation, since CL6 cells are reproducibly and stably induced into beating cardiomyocytes by DMSO (Fig. 2Aa) [23]. CL6 cells did not differentiate following exposure to Grem1 alone at concentrations of 63 or 125 ng/ml for 14 days (Fig. 2B). However, Grem1 dramatically promotes DMSO-induced cardiomyogenic differentiation at a concentration of 63 and 125 ng/ml; Grem1 (125 ng/ml) especially increased DMSO-induced cardiomyogenic differentiation of CL6 cells as assessed by beating area (Fig. 2Ab and B) (Movie S1 and S2, http://1954.jukuin.keio.ac.jp/umezawa/kami/index.html).

RT-PCR of differentiated or undifferentiated CL6 cells

To investigate gene expression as well as morphological analysis, i.e. beating, during cardiomyogenic differentiation, RT-PCR analysis was performed to detect expression of cardiomyocyte-specific/associate transcription factors, and structural genes (Fig. 2C). Genes encoding Gata4, Hand2, MyoD, ANP, MyLC-2v, MyLC-2a, and β-MyHC were up-regulated during cardiomyogenic differentiation of CL6 cells treated with Grem1 and DMSO (Fig. 2C: lanes 6, 7 versus lane 3). Triplicate independent experiments confirmed the concentration-dependent Grem1 action on cardiomyogenic differentiation. The cardiomyocyte-specific genes (Gata4, Hand2, MyoD, ANP, MyLC-2a, MyLC-2v) expression level of CL6 cells treated with DMSO and Grem1 (63 and 125 ng/ml) were also the same as or higher than that of DMSO-induced CL6 cells by semi-quantitative RT-PCR (Figure S1).

Immunocytochemistry of differentiated or undifferentiated CL6 cells

To examine CL6 cells for expression of cardiomyocytic protein, immunocytochemical analysis was performed. CL6 treated with Grem1 (125 ng/ml) and DMSO exhibited clear striation with immunostain using anti-cTnT or anti-α-actinin (Fig. 2Da and b). The MF20- and cTnT-positive cells after exposure to Grem1 and DMSO formed clusters (Fig. 2Ea), compared with the cells after exposure to DMSO alone (Fig. 2Eb). CL6 cells treated with Grem1 alone were negative for MF20 and cTnT, but became positive for both markers following exposure to Grem1 (63 and 125 ng/ml) and DMSO (Fig. 2F). The beating area (Fig. 2B) showed a tendency similar to the MF20- and cTnT-positive area (Fig. 2F), thus there were positive correlations between them.

Cardiomyogenic differentiation

genes.

inhibition of the BMP signaling pathway is, at least in part, involved in initial determination of Grem1-promoted cardiomyogenesis.
**Table 1.** 69 human cells clustered into 30 groups

| Group | Title | Description | GSM |
|-------|-------|-------------|-----|
| 1 | Normal epithelial cell, primary | NHEK-Neo1 Normal epidermal keratinocyte, neonate, primary | GSM210361 |
| | | NHEBE-1 Normal bronchial epithelial cell, primary | GSM210362 |
| 2 | Pulmonary epithelial cell line | A549 Pulmonary epithelial cell line | GSM210363 |
| | | BEAS-2B control (6hr) | GSM210364 |
| 3 | Lymphocyte | RPMI8226 control (6hr) B cell line | GSM210365 |
| | | Raji-1 B cell line | GSM210366 |
| | | NK92 NK cell line | GSM210367 |
| 4 | Myelomonocytic leukemia | U937c U937 control | GSM210368 |
| | | U937h U937+HRF | GSM210369 |
| | | U937ha U937+HRF+antibody | GSM210370 |
| | | U937a U937+antibody | GSM210371 |
| 5 | Embryonal carcinoma, cancer | NCR-G3 Embryonal carcinoma, NCR-G3, non-adherent | GSM201141 |
| | | NCR-G2NAd Embryonal carcinoma, NCR-G2, non-adherent | GSM201373 |
| | | NCR-G4Ad Embryonal carcinoma, NCR-G4, adherent | GSM201142 |
| | | NCR-G3Ad Embryonal carcinoma, NCR-G3, adherent | GSM201375 |
| 6 | ES cell | H1_P43 Undifferentiated hES | GSM41342 |
| | | H1-P46 Undifferentiated hES | GSM41343 |
| | | H1-P41 Undifferentiated hES | GSM41344 |
| 7 | Embryonal carcinoma, cancer | NCR-G2Ad Embryonal carcinoma, NCR-G2, adherent | GSM201140 |
| | | NCR-G1 Embryonal carcinoma, NCR-G3, non-adherent | GSM201139 |
| 8 | Ewing, cancer | NCR-EW2 Ewing, cancer | GSM210378 |
| | | NCR-EW3 Ewing, ET4, cancer | GSM210379 |
| 9 | Ewing, cancer | GST6 Ewing, POUSF1, cancer | GSM201137 |
| | | GST6-extra Ewing, POUSF1, cancer | GSM201381 |
| 10 | Ewing, cancer | GST6-5az Ewing, POUSF1, SaazC, cancer | GSM201138 |
| | | GST6-5az-extra Ewing, POUSF1, SaazC, cancer | GSM201383 |
| 11 | Bone marrow cell, primary | H4-1 Bone marrow cell, primary | GSM201143 |
| | | UBT5 Bmi-1, hTERT, bone marrow cell | GSM201385 |
| | | UBT7 Bmi-1, E6, hTERT, bone marrow cell | GSM201386 |
| 12 | Ligament-derived cells | #10 Ligament, primary | GSM201387 |
| | | Marrow stromal cells | H10-2Vec Vector, bone marrow cell | GSM201388 |
| | | | H10-2TERT hTERT, bone marrow cell | GSM201389 |
| | | | H10-2Bmi1 Bmi-1, bone marrow cell | GSM201390 |
| 13 | Placenta, primary | PL90 Placenta, primary | GSM201391 |
| 14 | De-differentiated chondrocyte | TdhC1 E6, E7, hTERT, de-differentiated chondrocyte | GSM201392 |
| 15 | Neural differentiated marrow stromal cell | UET13 Neural differentiation | E7, hTERT, neural differentiation, bone marrow cell | GSM201393 |
| | | UET13 Neural differentiation1 | E7, hTERT, neural differentiation, bone marrow cell | GSM201394 |
| | | UET13 Neural differentiation4 | E7, hTERT, neural differentiation, bone marrow cell | GSM201395 |
| | | UET13 Neural differentiation5 | E7, hTERT, neural differentiation, bone marrow cell | GSM201396 |
| 16 | Cord blood-derived cells | UET13 E7, hTERT, bone marrow cell | GSM201397 |
| | | UCB408 Cord blood, primary | GSM201398 |
| | | UCB408E6E7-31 E6, E7, umbilical cord blood | GSM201399 |
| | | Adipocyte cell, primary | HAdPC1(S2/21) HAdpc1E6E7TERT28 | GSM201400 |
| 18 | Marrow mesenchymal cell, primary | UEE16 E6, E7, hTERT, bone marrow cell | GSM201401 |
| | | UEE16 E6, E7, bone marrow cell | GSM201402 |
| | | EPC hTERT+1 E6, E7, hTERT, endometrial cell | GSM201144 |
| 19 | Cord blood, primary | UCB302 Cord blood, primary | GSM201382 |
| | | UCB302-D7 Cord blood, primary | GSM201405 |
| | | UCB302TERT hTERT, cord blood | GSM201406 |
| | | UET9 E7, hTERT, bone marrow cell | GSM201407 |
Grem1 and DMSO were most effective at the early stage (days 1–3) of CL6 differentiation.

To determine if Grem1 (125 ng/ml) functions during the early or the late stage of differentiation, CL6 cells were treated with Grem1 for different time periods (Fig. 3A). Grem1 and DMSO were most effective on CL6 differentiation at 1–3 days (Fig. 3B, C) as assessed by percentages of MF20-positive area and beating area. Since Grem1 inhibits BMPs through direct binding [24], we hypothesized that BMP signaling is inhibitory to CL6 cardiomyogenesis during days 1–3. To confirm this hypothesis, RT-PCR analysis was performed to determine expression of the early mesodermal marker (BrachyuryT and Tbx6), cardiomyocyte-specific transcription factors (Cdx/Nkx2.5, structural genes β-MycHC), and Gapdh (Fig. 4A). DMSO induced the BrachyuryT and Tbx6 genes, and their expressions peaked at 3 days and then decreased; BMP2 down-regulated expression of these genes from days 3–7. The Cdx/Nkx2.5 and β-MycHC genes started to be expressed at days 3 and 5, respectively, and their expression increased up to 14 days, at which time the timeframe analysis was terminated. BMP2 clearly inhibited expression of the Cdx/Nkx2.5 and β-MycHC genes (Fig. 4A, lanes 1–7 versus lanes 8–14).

To examine cardiomyogenic differentiation, immunocytochemical analysis was performed on CL6 cells treated with the inducers. CL6 cells treated with DMSO and BMP2 for 3 days were negative for sarcomeric myosin (MF20) at 14 days, but became positive for sarcomeric myosin, following exposure to DMSO alone during days 1–3 (Fig. 4B). To determine if DMSO induces BMP production in CL6 cells, expression levels of Bmp2 and Bmp4 were determined by quantitative real-time RT-PCR analysis (Fig. 4C). DMSO clearly induced the Bmp2 and Bmp4 genes, and DMSO-induction was inhibited by BMP2 protein. The expression level of Bmp2 was highest during days 7–10 (Fig. 4C: Bmp2) in DMSO-induced CL6 cells, and that of Bmp4 was highest during days 5–7 (Fig. 4C: Bmp4).

To investigate BMP signaling on cardiomyogenic differentiation, we used the Id1 promoter-Lux plasmid that includes the luciferase gene driven by the Id1 promoter, known as a BMP target promoter (Fig. 4D). BMP2 protein increased BMP signaling activity at 3 days (Fig. 4D, open square); BMP2 protein increased BMP signaling activity at 3 days (Fig. 4D, open square), but lost BMP signaling activity at 5 days and later, implying that this loss of BMP signaling leads to lack of cardiomyogenic induction.

Since Wnt/β-catenin signaling is involved in CL6 cardiomyogenesis [23,25], we hypothesized that the BMP effect on CL6 cardiomyogenesis is mediated through Wnt/β-catenin signaling. Expression of Wnt3a, an activator of canonical Wnt signaling, was indeed detected in CL6 cells exposed to DMSO, and BMP2 significantly down-regulated Wnt3a expression at day 3 (Fig. 4E). Using the TOPflash plasmid [23] which includes the luciferase gene driven by two sets of three copies of the TCF recognition site, Wnt/β-catenin signaling was assessed to investigate the effect of BMP2. Wnt/β-catenin signaling activity increased at 48 h after treatment with DMSO. Activity was increased by DMSO treatment but decreased by BMP2 (Fig. 4F). Time course analysis revealed that Wnt/β-catenin activity peaked at 5 days after DMSO treatment, and decreased thereafter (Fig. 4G). BMP2 inhibited DMSO-induced Wnt/β-catenin activity throughout the experimental period (up to 14 days). These results imply that BMP signaling inhibits CL6 cardiomyogenesis at the early stage through inhibition of Wnt/β-catenin signaling.

### Table 1. cont.

| Group | Title | Description | GSM |
|-------|-------|-------------|-----|
| 20    | Cord blood, primary | UCB408E7-32 | E7, hTERT, cord blood GSM210408 |
| 21    | Fetal fibroblast, primary | HFDP cont. | Normal follicular dermal papillar cell, primary GSM210409 |
| 22    | Bone marrow cell, primary | 3F0664 | Bone marrow cell (commercial item), primary GSM201114 |
| 23    | ES cell-derived mesenchymal cell | H1 clone 2 | ES cell-derived mesenchymal precursor GSM38628 |
| 24    | Endometrial cell | EPC100 | E6, E7, hTERT, endometrial cell GSM210413 |
| 25    | Bone marrow cell, primary | Yub10F | Bone marrow cell, primary GSM210414 |
| 26    | Endometrial cell | EPC hTERT+2 | E6, E7, hTERT, endometrial cell GSM210415 |
| 27    | Endometrial cell | EPC214 | E6, E7, hTERT, endometrial cell GSM210416 |
| 28    | Menstruation blood-derived mesenchymal cell, primary | #E4 | Menstruation blood, primary GSM210418 |
| 29    | Menstruation blood-derived mesenchymal cell, primary | #E4HRF | Menstruation blood, HRF treatment, primary GSM210419 |
| 30    | Menstruation blood-derived mesenchymal cell, primary | #E6 | Menstruation blood, primary GSM210420 |

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Figure 2. Grem1 enhanced cardiomyogenic differentiation in DMSO-induced CL6 cells. (A) Phase contrast micrograph of CL6 cells with exposure to DMSO alone (a), Grem1 (125 ng/ml) and DMSO (b) for 14 days. The medium, including Grem1 and DMSO, was changed every day. CL6 cells exhibited apparent spontaneous beating between days 9–11. Beating CL6 cell colonies are outlined by white lines. (B) Percentage of beating area in differentiated CL6 cells. CL6 cell treated with Grem1 (125 ng/ml) and DMSO exhibited the strongest contraction. (C) RT-PCR analysis of the genes encoding cardiac-specific transcriptional factors (Csx/Nkx2.5, Gata4, Me2c, Hand2), circulating hormone (ANP, BNP), cardiac-specific proteins (MyLC-2a, MyLC-2v, β-MyHC), cytokines (Bmp2, Bmp4, Fgf8, Grem1, Wnt1, Wnt3a, Wnt5a, Wnt7a, Wnt11), SM-MyHC, and Gapdh (From top to bottom). Mouse total heart RNA for the Csx/Nkx2.5, Gata4, Me2c, Hand2, ANP, BNP, MyLC-2a, MyLC-2v, β-MyHC, Bmp2, Bmp4, Grem1, Wnt11, SM-MyHC, and Gapdh genes, mouse embryonic stem cell RNA for the Fgf8 gene, and mouse total skeletal muscle RNA for the Wnt1, Wnt3a, Wnt5a, and Wnt7a genes were used for positive controls. H2O (without RNA) served as a negative control. (D) Immunocytochemistry of CL6 cells 14 days after exposure to Grem1 (125 ng/ml) and DMSO with MF20 and cTnT (a), and α-actinin (b). Cell nuclei are stained with DAPI. Clear striations are evident. (E) Immunocytochemistry of CL6 cells 14 days after exposure to Grem1 and DMSO with cardiac troponin T (cTnT) and sarcomeric myosin (MF20). CL6 cells treated with Grem1 (125 ng/ml) and DMSO (a), and DMSO alone (b) stained positive for cTnT and MF20. Untreated CL6 cells, i.e. not exposed to Grem1(125 ng/ml) or DMSO, stained negative for cTnT and MF20. Cell nuclei were stained with DAPI. (F) Percentage of MF20- and cTnT-double positive area.

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Discussion

Our bioinformatics study using the results from the global gene expression analysis of human cells (GSM412342-41344 and GSM201137-201145 at http://www.ncbi.nlm.nih.gov/geo) nominated Grem1 as a candidate gene that may participate in cardiomyogenesis. By using CL6 embryonic cells as a model of cardiomyogenesis, we obtained two major findings: the first is that Grem1 enhanced cardiomyogenic differentiation of DMSO-induced CL6 cells at the early stage; the second is that Wnt/β-catenin and BMP signaling activity had developmental stage-specific effects on cardiomyogenesis (Fig. 5). Wnt/β-catenin activity at the early stage enhanced embryonic cell differentiation into cardiomyocytes, while suppressing this activity by BMP2 or BMP4 proteins as reported in the avian embryo [26]. In contrast, BMP signaling activity in the late stage enhanced cardiomyocytic differentiation. Grem1 regulated the stage-specific Wnt/β-catenin and BMP signaling activity on cardiomyogenesis.

Many studies have indicated that Grem1 is involved in cell differentiation and development, such as osteogenesis [27], lung morphogenesis [28], myogenesis [29], and limb formation [30], through inhibition of BMP2 and BMP4. Grem1-null mice show intact heart development, despite impairment of lung and kidney [31], and therefore Grem1 is considered not to be involved in cardiogenesis, or supplementary factors such as Noggin [32], with a similar function, may compensate Grem1 during development. Grem1 had an enhancing or promoting activity in in vitro cardiomyogenesis, as is the case with platelet-derived growth factor as a promoter of cell growth [33]. In this study, Grem1 was involved in cardiomyocyte differentiation. However Grem1 alone could not induce cardiomyocytic differentiation of CL6 cells in the absence of DMSO (Fig. 2C and F), suggesting that Grem1 is solely
Figure 4. Cardiomyogenic differentiation in CL6 cells (days 1–3) is inhibited by BMP2. (A) RT-PCR analysis of the gene encoding BrachyuryT, Tbx6, cardiac-specific transcriptional factor (Csx/Nkx2.5), cardiac-specific protein (β-MyHC), and Gapdh (From top to bottom) of CL6 cells treated with DMSO alone, or DMSO and BMP2 (100 ng/ml) for the first 3 days (days 1–3). The medium, including BMP2 and DMSO, was changed every day. (B) Percentage of MF20-positive area. Immunocytochemistry was carried out on CL6 cells 14 days after cells had been exposed to DMSO and BMP2 (100 ng/ml) for the first 3 days (days 1–3). The asterisk indicates a significant statistical difference (P<0.05). (C) Quantitative real-time RT-PCR analysis of the gene encoding Bmp2 (left), and Bmp4 (right) in CL6 cells treated with DMSO alone (open square), or DMSO and BMP2 (100 ng/ml) for the first 3 days (days 1–3). (D) BMP signaling activity of CL6 cells treated with DMSO alone (open square), or DMSO and BMP2 (100 ng/ml) for the first 3 days (days 1–3) were determined by luciferase activity analysis using Id1 promoter-Lux (a firefly luciferase reporter plasmid driven by the Id1 binding sites), pRL-CMV as co-transfected control, and Dual luciferase reporter assay system. Relative luciferase unit of the CL6 cells untreated with inducers at day 3 is regarded as 0.1 (data not shown). (E) Quantitative real-time RT-PCR analysis of the gene encoding Wnt3a in CL6 cells treated with DMSO alone (open square), or DMSO and BMP2 (100 ng/ml) for the first 3 days (days 1–3). (F) Wnt/β-catenin signaling activity of CL6 cells 48 h after exposure to DMSO, or DMSO and BMP2 (100 ng/ml) was determined by luciferase activity analysis using TOPflash (a firefly luciferase reporter plasmid driven by two sets of three copies of the TCF binding site and herpes simple virus thymidine kinase minimal promoter), pRL-CMV as co-transfected control, and Dual luciferase reporter assay system. Relative luciferase unit of the CL6 cells untreated with inducers is regarded as 0.1. The asterisk indicates a significant statistical difference (P<0.05). (G) Timeframe of Wnt/β-catenin signaling activity in CL6 cells treated with DMSO alone (open square), or DMSO and BMP2 (100 ng/ml) for the first 3 days (days 1–3). Relative luciferase unit of the CL6 cells untreated with inducers at day 3 is regarded as 0.1 (data not shown).

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a promoter of cardiomogenic differentiation. One of the possible mechanisms for Grem1-enhanced cardiomogenesis at the early stage is inhibition of the BMP signaling pathway [3]. Alternatively, Grem1-enhanced cardiomogenesis may be mediated through proliferation of cardiac progenitor cells, as is the case of myogenic Grem1-enhanced cardiomyogenesis may be mediated through inhibition of the BMP signaling pathway. One of the possible regulation of BMP- and Wnt/\(\beta\)-catenin-signaling pathways. CL6 embryonic cells start to differentiate into mesodermal cells through Wnt/\(\beta\)-catenin signaling pathway at the early stage (days 1–3), and mesodermal CL6 cells differentiate into mature cardiomyocytes by BMP pathway at the late stage (days 4–14). Grem1 accelerates DMSO-induced cardiomyogenesis through inhibition of the BMP-signaling pathway. doi:10.1371/journal.pone.0002407.g005

Figure 5. Grem1-accelerated CL6 cardiomyogenesis through regulation of BMP- and Wnt/\(\beta\)-catenin-signaling pathways. CL6 embryonic cells start to differentiate into mesodermal cells through Wnt/\(\beta\)-catenin signaling pathway at the early stage (days 1–3), and mesodermal CL6 cells differentiate into mature cardiomyocytes by BMP pathway at the late stage (days 4–14). Grem1 accelerates DMSO-induced cardiomyogenesis through inhibition of the BMP-signaling pathway.

Materials and Methods

GeneChip analysis

GeneChip analysis was performed (Fig. 1A, Table 1) as previously described [38]. Human genome-wide gene expression was examined with the Human Genome U133A Probe array (GeneChip, Affymetrix), which contains the oligonucleotide probe set for approximately 23,000 full-length genes and expressed sequence tags, according to the manufacturer’s protocol (Expression Analysis technical manual and GenChip Small Sample Target Labeling Assay version 2 technical note [http://www. affymetrix.com/support/technical/index.aff]). Data analysis was performed by the GeneChip Operation System (Affymetrix) and GeneSpringGX software (Silicon Genetics). To normalize the staining intensity variations between chips, the average difference values for all genes on a given chip were divided by the median of all measurements on that chip. Hierarchical-clustering analysis was performed using a minimum distance value of 0.001, a separation ratio of 0.5, and the standard definition of the correlation distance.

Cell culture and differentiation

CL6 cells were grown on 100 mm dishes (Becton Dickinson) in \(\sigma\)-MEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (JRH Bioscience, Inc.), penicillin, and streptomycin, and were maintained in a 5% CO\(_2\) atmosphere at 37°C. To induce differentiation, CL6 cells were plated at a density of 1.8×10\(^5\) cells in a 6-well plate (Becton Dickinson) or gelatin-coated 35 mm glass base dishes (IWAKI) with \(\sigma\)-MEM containing Grem1 (63 or 125 ng/ml; R&K system) and/or 1% dimethyl sulfoxide (DMSO) for 14 days. Recombinant human bone morphogenetic protein-2 (BMP2) was purchased from R&D systems.

Reverse transcriptase-PCR (RT-PCR) and quantitative real-time RT-PCR analysis

Total RNAs were extracted from differentiated and undifferentiated CL6 cells and mouse embryonic stem (ES) cells with RNaseq minikit and DNase I treatment (QIAGEN). Mouse ES cell (129 strains) RNA, mouse heart total RNA (Clontech) and mouse skeletal muscle/total RNA (UNITECH. Co., Ltd.) were used as a positive control for each primer. Total RNA (2.0 \(\mu\)g each) for RT-PCR was converted to cDNA with Superscript\textsuperscript{TM} III RNA H\(_{-}\)-reverse transcriptase (Invitrogen), according to the manufacturer’s manual. PCR conditions were optimized and linear amplification range was determined for each primer by varying annealing temperature and cycle number. PCR products were identified by positive control size. RT-PCR was performed using the primers of the genes of cardiac specific transcription factors: Csx/Nkx2.5, Gata4, Mef2c, Hand2; circulating hormone: ANP, BNP; cardiac structural proteins: \(\beta\)-MyHC, MyLC-2a, MyLC-2c, cytokines: Bmp2, Bmp4, Fgf8, Grem1, Wnt1, Wnt3a, Wnt5a, Wnt7a, Wnt11; smooth muscle structural protein: smooth muscle-myosin heavy chain (SM-MyHC); the early mesodermal marker: Brachyury, T-box6 (T-box6); and Gapdh as control. PCR was performed with exTaq DNA polymerase and exTaq PCR buffer (Takara) or LA Taq DNA polymerase and GC buffer I (Takara) for 25 or 30 cycles, with each cycle consisting of 95°C for 30 s, 55°C, 60°C or 65°C for 45 s, and 72°C for 45 s, with an additional 5 min incubation at 72°C after completion of the final cycle. PCR primers for the genes of Csx/Nkx2.5, Gata4, Mef2c, Hand2, ANP, BNP, \(\beta\)-MyHC, MyLC-2a, MyLC-2c, Bmp2, Bmp4, Fgf8, Grem1, Wnt1, Wnt3a, Wnt5a, Wnt7a, Wnt11, SM-MyHC, Brachury, T-box6, and Gapdh (Table S1a) were obtained from Mouse Genome
Immunocytochemistry

A laser confocal microscope (LSM510, Zeiss) was used for immunocytochemical analysis. Differentiated and undifferentiated CL6 cells were fixed with 4% paraformaldehyde (Wako) for 5 min at 4°C and treated with 0.1% triton X-100 (Sigma) in PBS for 20 min at room temperature, then incubated for 20 min at room temperature in a protein-blocking solution consisting of PBS supplemented with 5% normal goat serum (DakoCytomation). These CL6 cells were then incubated overnight with primary antibody monoclonal anti-sarcomeric myosin antibody (MF20, mouse IgG2b, isotype, 1 mg/ml, University of Iowa Hybridoma Bank) and Troponin T, and Cardiac Isofrom Ab-1 clone 13-11 (cTnT, mouse IgG1, isotype, 1:300, Lab Vision Corp), or the monoclonal anti-α-actinin (SARCOMERIC) CLONE EA-53 (α-actinin, mouse IgG1, isotype, 1:300, Sigma) in PBS at 4°C. The cells were extensively washed in PBS and incubated at room temperature with Alexa Fluor 568-conjugated goat anti-Mouse IgG2b (anti-MF20) (Molecular Probe; diluted 1:300), Alexa Fluor 488-conjugated goat anti-mouse IgG1 (anti-cTnT) (Molecular Probe; diluted 1:300), Alexa Fluor 546-conjugated goat anti-mouse IgG1 (anti-α-actinin) (Molecular Probe; diluted 1:300), and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Wako; diluted 1:300) for 45 min. To prevent fading, cells were then mounted in DakoCytomation Fluorescent Mounting Medium (DakoCytomation).

Transfection and luciferase assays

Cells (8.0 x 10^4) seeded and cultured in 60 mm dishes (Becton Dickinson) were transfected 18 h after plating using Lipofectamine 2000 (Invitrogen) and PLUS reagent (Invitrogen) in Opti-MEM medium supplemented with 5% normal goat serum (DakoCytomation). Cells were grown in KnockOut DMEM (Gibco) supplemented with 15% fetal bovine serum (Cell Culture Technologies), 2 mM glutamine (Gibco), 0.1 mM non-essential amino acid (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), penicillin, streptomycin, and 2,000 U/ml mouse leukemia inhibitory factor (LIF) (Chemicon). For cardiomyogenic differentiation, ES cells were exposed to 125 ng/ml Grem1 (R&D Systems) and myogenic differentiation of P19CL6 cells at a concentration of 125 ng/ml. Grem1 dramatically promotes DMSO-induced cardiomyogenesis when used at a concentration of 125 ng/ml.

Results, shown as the mean±SE, were compared by ANOVA followed by Scheffé’s test, with p<0.05 considered significant.

Supporting Information

Figure S1 A semi-quantitative RT-PCR of cardiomyocyte-specific genes. To investigate expression level of cardiomyocyte-specific genes (Csx/Nkx2.5, Gata4, MyLC-2a, and MyLC-2v), a semi-quantitative RT-PCR was performed from CL6 cells treated with 1% DMSO and the indicated concentration of Grem1 for 14 days. Each RT-PCR product was electrophoresed in 2% agarose gel, and was measured using ImageJ software (http://rsb.info.nih.gov/ij/) to calculate the ratio of each gene to Gapdh. The expression level for each gene is determined relative to that of Gapdh, and expression level in CL6 cells treated with DMSO alone was regarded as 1.0. The relative expression levels were averaged from at least three independent experiments.

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Figure S2 Grem1 enhanced cardiomyogenic differentiation of mouse ES cells. Mouse ES cells (NCH1.5, C57BL/6J×129ter/Sv) were cultured on a mouse embryonic fibroblast feeder layer inactivated with 30 Gy γ-irradiation in gelatin-coated 60 mm dishes (Becton, Dickinson). Cells were grown in KnockOut DMEM (Gibco) supplemented with 15% fetal bovine serum (Cell Culture Technologies), 2 mM glutamine (Gibco), 0.1 mM non-essential amino acid (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), penicillin, streptomycin, and 2,000 U/ml mouse leukemia inhibitory factor (LIF) (Chemicon). For cardiomyogenic differentiation, ES cells were exposed to 125 ng/ml Grem1 (R&D Systems) for the three days. The cells were then trypsinized and cultured to form embryonic bodies (EBs) from a single cell using a three-dimensional culture system (without LIF) on low cell binding dishes (96-well plate round bottom). This represented day 0 of EB formation. On the next day, the medium was replaced with the same medium without LIF. EBs were re-seeded on gelatin-coated 48-well plates with one EB per well, on day 8 after the start of EB formation. The cardiomyogenic induction was estimated by the beating EB number per total EB number, measured on day 12 under a phase-contrast microscope. Grem1 increased the percentage of beating EBs to 69.2%, as compared with 26.7% in EBs without Grem1 treatment. The numbers in parentheses indicate the EB numbers counted.

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Table S1 Primer sequences.

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Movie S1 CL6 cells treated with DMSO alone. P19CL6 cells are reproducibly and stably induced into beating cardiomyocytes with DMSO.

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Movie S2 CL6 cells treated with Grem1 (125 ng/ml) and DMSO. Grem1 dramatically promotes DMSO-induced cardiomyogenic differentiation of P19CL6 cells at a concentration of 125 ng/ml.

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Author Contributions
Conceived and designed the experiments: AU DK. Performed the experiments: DK HM RI KM. Analyzed the data: AU AN DK YT RI

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