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冠動脈疾患 上

—診断と治療の進歩—

VI. 冠動脈疾患における血管新生・心筋再生療法

心筋再生療法

エリスロポエチンを用いた心血管疾患の治療

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Application of erythropoietin to cardiovascular diseases

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Key words：血管新生，エリスロポエチン，下肢虚血，心筋梗塞

はじめに

そもそも心血管系は血液の輸送機関であり，循環器と血球は密接に関連しつつ発生する．尾索動物の段階で赤血球とともに毛細血管が系統発生し，開放循環系から閉鎖循環系に進化した．脊椎動物の成体型造血幹細胞は大動脈・生殖器・腎臓を形成するAGM領域で血球と血管内皮細胞の共通前駆細胞ヘマンジオブラストから発生する．単心室の魚類は全身酸素濃度のモニターに都合の良い心臓でエリスロポエチン(EPO)を分泌する．脊椎動物では造血のみならず心臓の発生においてもEPO/EPO受容体(EPOR)システムは必須であり[1]，胎児期における心筋細胞の増殖には心外膜から分泌されるEPOとレチノイン酸が必要である[2]．血管内皮にEPORが発現していることは間違いないが，EPOの心臓に対する作用はEPOR陽性の線維芽細胞を介した間接作用である可能性がある[3]．

1．エリスロポエチンの構造

EPOは熊本大学の宮家が純化精製に成功した赤血球造血ホルモンで[4]，腎性貧血治療薬として20年の歴史をもつ．EPORはG-CSF受容体やIL-6受容体と同様にType-Iサイトカン受容体家系に属し，成長ホルモン受容体を分子進化上のテンプレートとする．したがって，細胞内シグナルの基本はJAK/STAT系のリン酸化を利用するが，陽性シグナルは活性化したSHP-1によるJAKの脱リン酸化によって一過性で終息する．サイトカインとその受容体の多様性獲得に伴ってJAKおよびSTATも多様化し，細胞外情報を細胞内シグナルに翻訳する過程が複雑化したが，Type-Iサイトカイン受容体はtype-1～5の5群に分類される．

同じtypeに属する受容体同士は，細胞外ドメインも細胞内ドメインも類似した分子構造をもち，原則として同じ細胞内シグナル伝達系を利用するため作用が類似する．例えば成長ホルモン受容体とともにtype-1に属するEPORやトロンボポエチン(TPO)受容体はJAK2/STAT5/MGFボックス配列を利用して転写制御を行い，type-2に属するIL-6受容体のGP130はJAK1/STAT3/APRE配列を用いる．骨髄系造血前駆細胞はTPO受容体とIL-6受容体を同時に発現しているが，異なるJAK/STAT系を利用することで細胞内情報伝達の混乱を避けていると考えられる．実際に，EPOは骨髄系造血前駆細胞の細胞株UT-7に生存因子として作用するのに対し，IL-6は増殖作用のみを示し生存はさせない[5]．ちなみにEPOとTPOの作用は類似しており，EPOには血小板増加・活性化作用があ

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るため，高リスク患者へのEPO投与においては脳心血管イベントの合併に注意を払う必要が生じる．

EPOは165アミノ酸残基に1つのO-型糖鎖と3つのN-型糖鎖が結合した糖タンパクで，4本のα-ヘリックスが2往復しN末端とC末端がS-S結合して閉じたラグビーボール様の形態をとる．小胞体でのポリペプチド伸長中に付加された糖鎖はゴルジ体で複雑な構造変化を受けた後，非還元末端のガラクトースにシアル酸が付加(シアル酸キャッピング)されて成熟する．分泌されたEPOのシアル酸は時間経過とともに自然に脱落し，脱シアル化したEPOは肝臓のガラクトース受容体によって除去される．したがってアシアロEPO(AEPO)は血中濃度が上昇せず造血活性を欠く．腎臓以外の臓器でEPOを産生している細胞がα(2,3)シアル酸転移酵素を発現していなければ，最初からAEPOを分泌する可能性がある．EPOは組織親和性をもたないためエンドクリンに適しているが，AEPOは組織親和性をもつためパラクリンに適していると推測される．

2. エリスロポエチンの造血以外の作用

血液脳関門によりエンドクリンから独立した独自の情報伝達系をもつ中枢神経系内部ではガラクトース受容体による不活性化がなく，アシアロ体糖タンパクが細胞間情報伝達に重要な役割を果たしているとされている．EPO/EPORシステムの造血以外の作用が中枢神経系および心血管系で明らかとなってきた．脳の星状細胞や神経細胞は低酸素・虚血ストレスに反応してEPOを分泌し，EPORを発現している中枢神経系を各種のストレスから保護する[6]．また全身性に投与されたrhEPOは脳毛細血管内皮のEPORを介して中枢神経系に到達し，神経保護作用を発揮する．心血管系においてもEPO/EPORパラクリンシステムが存在し，外因性のrhEPOが心筋を虚血・再環流障害から保護することはよく知られているが[7]，心筋細胞と線維芽細胞をきれいに分離することは困難であり，細胞種を特定したうえでのEPOを介したクロストークの厳密な証明はなされていない．

赤血球系細胞に発現しているEPO受容体はEPORのホモ2量体である．これに対し，EPOポリペプチドのリシン残基をカルバミル化した人工EPO誘導体であるカルバミルEPO(CEPO)を用いた研究で，脳・心臓およびその他の臓器にEPOR/βcヘテロ2量体が発現していることが示された[8]．CEPOはEPORホモ2量体と結合せず造血能を欠くが，脳や心臓の虚血モデルに対してCEPO投与は臓器保護作用を示し，またβcノックアウトマウスでは造血が正常であるがCEPO投与による脳・心臓保護作用が消失した．βcはGM-CSF・IL-3・IL-5の受容体共通β鎖(cytokine receptor common β-chain)である．βcの細胞内シグナルはEPORと同様STAT5が中心となる．心臓からEPOR/βcヘテロ2量体が検出されたが，血管内皮はCEPOに反応せずEPORホモ2量体が発現していると思われるので，線維芽細胞がEPOR/βcヘテロ2量体を発現している可能性がある．

EPORの細胞内シグナル伝達系はJAK/STAT系のほかにPI3K/Akt系およびRas/MAPK系を介することが知られているが，家兎を用いた冠動脈虚血再環流モデルでEPO投与がこれら3つの細胞内伝達系を介して心筋保護的に作用することが示された[9]．その後ラットやマウスを用いた種々の動物モデルでEPOの心筋保護作用が追試されたが，EPO投与により心臓ではSTAT3とSTAT5の両者のリン酸化が観察される[10]．大型動物を用いた冠動脈虚血再環流モデルでもEPOの心筋保護作用が観察されたが，EPO投与による血管保護・血管新生が重要な役割を担うことが示されている[11]．*in vitro*で線維芽細胞と血管内皮を共培養して毛細血管の新生を再現すると，EPOによる血管新生作用が観察されるが，線維芽細胞は恒常的にIL-6を分泌しており，この内因性IL-6を抗体で中和するとEPOの血管新生作用が消失することから，EPOによる血管新生には低濃度の内因性IL-6の存在が必要であることがわかる(**図1**)．体内で線維芽細胞が存在しない組織はまれであり，したがってEPOの作用は直接作用なのか内因

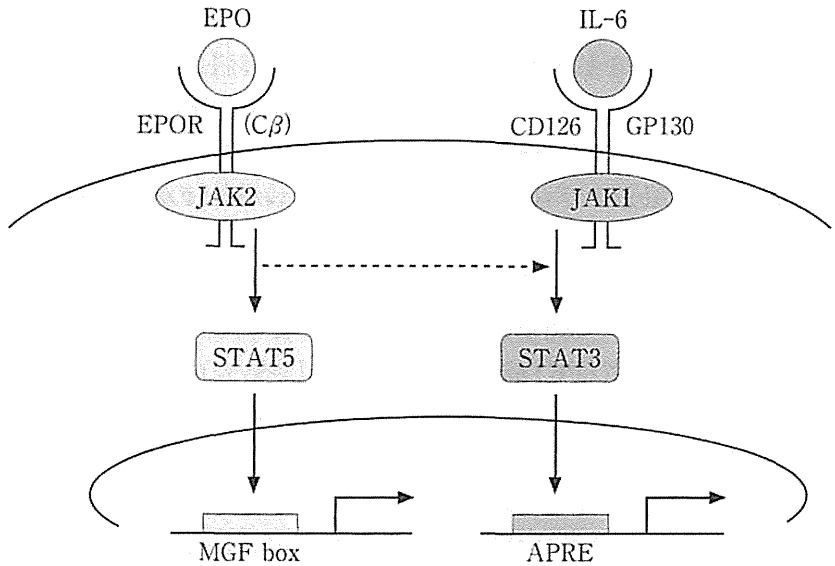

図1 EPOとIL-6の関連

EPORの細胞内ドメインの8つのチロシン残基がJAK2によってリン酸化される．これらのうち426YはSTAT5との結合に重要で，細胞膜に一番近い368YがSTAT5と相互作用・活性化する．454YはITIMの成分でSHP-1を介してJAK2の脱リン酸化にかかわる．一方456YはSTAT3の活性化に必須とされるが，EPOによるSTAT3のリン酸化には内因性IL-6の存在が必要である可能性がある．

性IL-6に依存した作用なのか判断が難しい．

3. エリスロポエチンの臨床応用

EPOの心血管系疾患への応用として，血栓性微小血管障害などに対する血管内皮保護治療，ASOやバージャー病などの慢性下肢虚血患者に対する血管新生療法，急性心筋梗塞患者に対する心筋保護治療，腎不全などが原因で起こる慢性心不全の治療などが考えられる．モノクロタリン誘発による実験的肺高血圧症モデルでEPOの投与が有効であった．経静脈的にEPOを投与したときの血管内皮保護作用は内皮がさらされている血中のEPO濃度に依存するため，AEPOは無効である．またCEPOが無効であることから，内皮はEPORホモ2量体を発現していることが推測される．

EPOの腫瘍血管新生作用やEPOR陽性腫瘍に対する増殖促進作用の有無についての議論が盛んであるが，結論は出ていない[12]．とはいえ，EPOそれ自体の血管新生作用はVEGFやIL-6に比しはるかに微弱である[13]．EPOの糖鎖末端のシアル酸はカルボキシル基が陰性に荷電し組織外マトリックスとの親和性を欠くが，AEPOの糖鎖末端のガラクトースは生体内で陽性荷電を有し組織外マトリックスの主成分であるグリコサミノグリカンの硫酸基と相互作用するため組織親和性を示し，強い血管新生作用を有する（特許第4200509号）．AEPOのパラクリンホルモンとしての生理的な意義を暗示している可能性がある．慢性下肢虚血患者に対するVEGFのnaked plasmid投与臨床試験では十分な有効性が得られず，現段階では骨髄単核細胞移植治療や末梢血単核細胞移植治療などの細胞療法が盛んに行われているが，AEPOを用いることで低侵襲の血管新生治療が実現可能である．血管新生治療の意外なオプションとして，患者から採取した比較的少量の造血幹細胞・前駆細胞を，EPOを含むサイトカインカクテルを用いて体外増幅培養し，得られた未熟赤芽球をEPOとともに患者の虚血下肢に移植する方法がある[14]．まだ実施症例数は少ないが，いまのところ重症例に対しても有効かつ安全に行われている．

前述のとおり，齧歯類や大型動物の虚血再環流を用いたモデルなどでEPOの心筋保護作用が認められたが[7,9]，障害組織でEPOが示す血管新生作用が大きな役割を果たしているようであ

る[10,11]. これらの中で興味深いことは, 貧血を合併した腎不全モデル動物でAEPOがEPOと同等の慢性期心不全改善効果を示していることである. 貧血の改善なしに慢性心不全を改善したこと, 血中濃度の上昇しないAEPOでも十分な心臓組織内濃度が得られていた可能性があることが特筆に値する.

EPOの心筋梗塞急性期に対する臨床試験が行われている. いずれもSTEMIのPCI成功後に投与されているが, ミュンヘンのグループによるREVIVAL-III試験では33,300国際単位の3日間連日投与(計約10万単位)を行い, MRIによる心筋梗塞サイズの有意な縮小を認めていない. オランダのHEBE-III試験では6万単位の1回投与を行い, 6週間後のTc-シンチでのEFの有意な改善はなかったものの, EPO投与群で心血管イベントの有意な減少とNT-proBNPの低下を観察している. 我が国のEPO/AMI-I試験では12,000単位の1回投与を行い, 6カ月後のTc-シンチでの心筋梗塞の縮小とEFの改善が観察され, また同じく我が国のEPOC-AMI試験では6,000単位の3日間連日投与(計1.8万単位)を行い, やはりTc-シンチでのEFの改善を報告している. EPOには至適投与量があって高用量EPOが必ずしも有効ではないのか, それとも比較する時期や指標の問題なのか不明であるが, ラットの虚血再環流治療モデルではEPOには至適投与量が存在することが示されている[15]. 我が国では多施設二重盲検臨床試験EPO/AMI-IIがまもなく開始される.

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A Crucial Role of Activin A-Mediated Growth Hormone Suppression in Mouse and Human Heart Failure

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Abstract

Infusion of bone marrow-derived mononuclear cells (BMMNC) has been reported to ameliorate cardiac dysfunction after acute myocardial infarction. In this study, we investigated whether infusion of BMMNC is also effective for non-ischemic heart failure model mice and the underlying mechanisms. Intravenous infusion of BMMNC showed transient cardioprotective effects on animal models with dilated cardiomyopathy (DCM) without their engraftment in heart, suggesting that BMMNC infusion improves cardiac function *via* humoral factors rather than their differentiation into cardiomyocytes. Using conditioned media from sorted BMMNC, we found that the cardioprotective effects were mediated by growth hormone (GH) secreted from myeloid (Gr-1(+)) cells and the effects was partially mediated by signal transducer and activator of transcription 3 in cardiomyocytes. On the other hand, the GH expression in Gr-1(+) cells was significantly downregulated in DCM mice compared with that in healthy control, suggesting that the environmental cue in heart failure might suppress the Gr-1(+) cells function. Activin A was upregulated in the serum of DCM models and induced downregulation of GH levels in Gr-1(+) cells and serum. Furthermore, humoral factors upregulated in heart failure including angiotensin II upregulated activin A in peripheral blood mononuclear cells (PBMNC) via activation of NFκB. Similarly, serum activin A levels were also significantly higher in DCM patients with heart failure than in healthy subjects and the GH levels in conditioned medium from PBMNC of DCM patients were lower than that in healthy subjects. Inhibition of activin A increased serum GH levels and improved cardiac function of DCM model mice. These results suggest that activin A causes heart failure by suppressing GH activity and that inhibition of activin A might become a novel strategy for the treatment of heart failure.

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Introduction

Heart failure is a major cause of mortality in many countries. Infusion of bone marrow-derived mononuclear cells (BMMNC) is expected as a novel treatment of heart failure. Animal experiments and clinical trials have shown that BMMNC infusion ameliorates cardiac dysfunction after acute myocardial infarction and chronic myocardial ischemia [1]–[4]. Although the outcomes vary among trials, recent meta-analyses revealed that cardiac function slightly improves following BMMNC infusion for ischemic heart diseases [5], [6]. Bone marrow cells were reported to be incorporated into the damaged myocardium and to differentiate into various cell types including cardiomyocytes [7]. However, whether bone marrow-derived stem cells can differentiate into many cardiomyocytes is still an open question [8]. There are many reports indicating that transplantation of various types of stem cells improves the cardiac function of ischemic hearts, mainly by paracrine factors which induce angiogenesis and cardioprotection [9]–[11]. Since the effects of BMMNC infusion for non-ischemic cardiomyopathy remain unknown, we examined whether BMMNC infusion also improves cardiac function of non-ischemic cardiomyopathy.

Results

Preparation of non-ischemic dilated cardiomyopathy (DCM) mice

Two kinds of non-ischemic DCM mice were used. The first model was generated by transgenic overexpression of a mutant epidermal growth factor receptor (EGFR) with C-terminal truncation (EGFRdn). The expression of mutant EGFRdn is

activated by the cardiomyocyte-specific α-myosin heavy chain (αMHC) promoter (Figure 1A, Figure S1). EGFRdn mice exhibited heart failure and died at 5–30 weeks of age (Figure 1B). Gross inspection of the EGFRdn hearts showed global chamber dilatation with marked wall thinning (Figure 1C). The heart/body weight ratio was approximately 1.5-fold higher at 6 weeks of age in EGFRdn mice than in wild-type mice (Figure 1D). Echocardiography showed a significant decrease in the fractional shortening (FS) together with chamber dilatation (Figure 1E). In the second model, cardiomyopathy was induced by intraperitoneal injection of doxorubicin in wild-type mice. Doxorubicin-induced cardiomyopathy (DOX) mice showed marked dilatations of the left ventricular diastolic and systolic dimensions, and reduction of cardiac function (Figure S2).

Intravenous infusion of BMMNC transiently improved the cardiac function in DCM mice

BMMNC (2.0×10^7 cells) were isolated from wild-type healthy mice and intravenously infused *via* the tail veins to 8-week-old EGFRdn mice and 11-week-old DOX mice. An equal volume of PBS was infused into control mice. Three days after infusion, echocardiography showed that the FS was significantly improved in BMMNC-treated EGFRdn (Figure 2A) and DOX (Figure 2A) mice, compared with the respective controls. However, these effects were lost by 14 d after infusion (Figure 2A). When the infusion was repeated every 2 weeks, cardiac function showed improvements for >50 d (Figure 2B).

Although infusion of BMMNC is not promising for the treatment of heart failure, we may be able to apply alternative treatment if we understand the underlying mechanisms of beneficial effects of BMNNC infusion. To elucidate the mechanisms, we infused BMMNC derived from GFP mice. Although many GFP-positive cells were observed in the peripheral blood and the spleen at 3 d after infusion (Figure 2C, D), none were found in the heart, lung, liver, kidney or skeletal muscle (Figure 2E). At day 14, few GFP-positive cells were observed even in the peripheral blood (Figure 2C). This was consistent with the observation that BMMNC infusion improved cardiac function at day 3, but not at day 14. These results suggest that BMMNC infusion improves the systolic function of DCM mice not by transdifferentiation of BMMNC into cardiomyocytes but probably by humoral factors secreted from BMMNC. Size of each cardiomyocyte was larger in BMMNC-infused EGFRdn mice than in PBS-infused EGFRdn mice when infusions were repeated every 2 weeks for 8 weeks (i.e.,

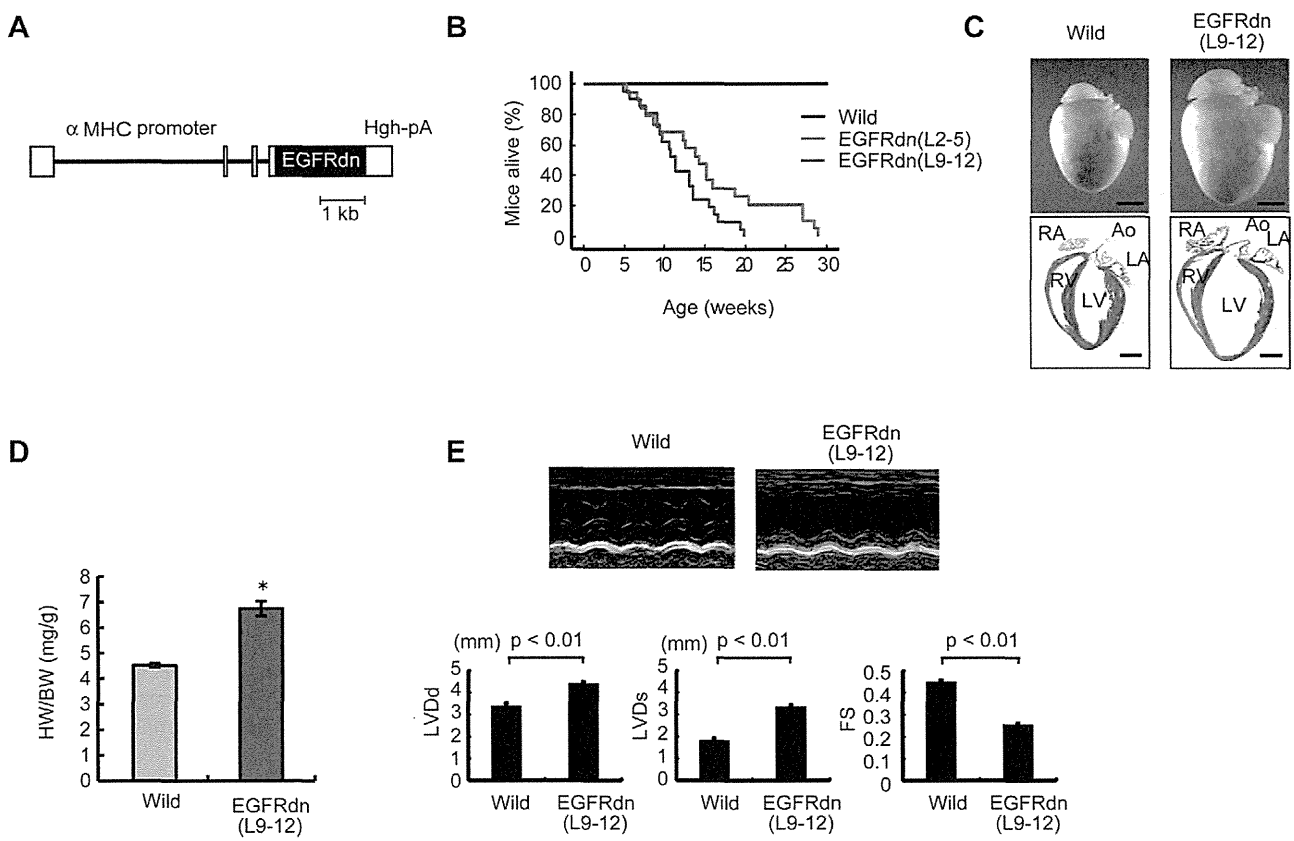

Figure 1. Transgenic overexpression of EGFRdn in the heart causes progressive heart failure. (A) Schematic representation of the cDNA construct used to generate EGFRdn mice. The construct contains an αMHC promoter, human EGFRdn cDNA and a human *growth hormone* polyadenylation signal (Hgh-pA). (B) Kaplan-Meier survival curves for wild-type ($n=62$) and EGFRdn (L2–5, $n=19$; L9–12, $n=21$) mice, showing a significant reduction in the survival rates in EGFRdn mice (log rank test, $P<0.0001$). (C) Gross morphology of whole hearts (upper panels) and longitudinal sections (lower panels) of hearts from wild-type and EGFRdn mice (L9–12) at 6 weeks of age. Ao, aorta; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. Scale bars: 2 mm. (D) Heart-to-body weight ratios (HW/BW) of wild-type ($n=9$) and EGFRdn (L9–12, $n=7$) mice at 6 weeks of age. *$P<0.01$. (E) Echocardiographic analysis. The upper photographs show representative M-mode images. The lower graphs show the left ventricular diastolic and systolic dimensions and FS of 8 week-old EGFRdn mice (L9–12) ($n=23$) and age-matched wild-type mice ($n=10$). LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension. Data are means ± s.e.m.
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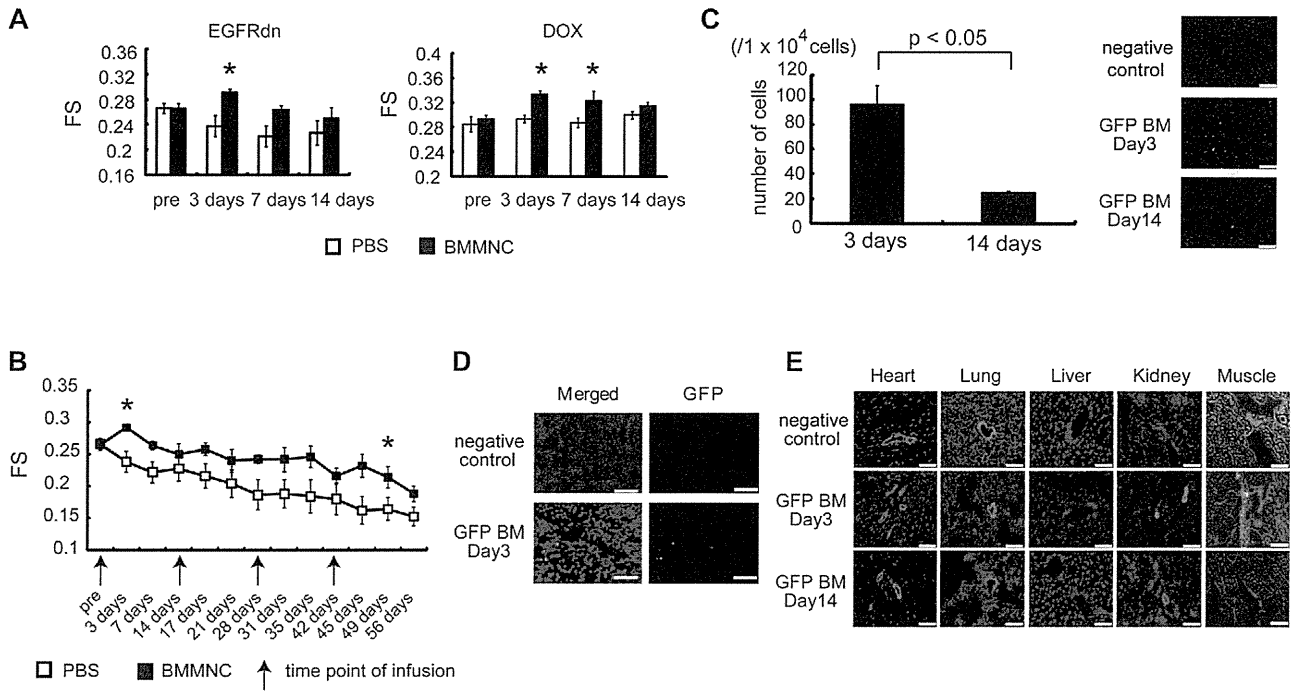

Figure 2. BMMNC infusion transiently improved the cardiac function of DCM mice. (A) Echocardiographic analysis. Transient improvements of FS were observed at 3 d in the BMMNC-treated group, but not the control (PBS) group, in EGFRdn mice (left), and at 3 and 7 d in DOX-treated mice (right). *$p<0.05$ versus PBS ($n=8$ per group). (B) Repeated-infusion experiments. BMMNC were infused every 2 weeks. A similar pattern of improvement in FS was observed after each infusion. *$p<0.05$ versus PBS ($n=8$ per group). (C–E) Immunohistochemical analysis. (C) Left, the number of GFP-positive BMMNC in peripheral blood ($n=3$). Right, photomicrographs of peripheral blood. Nuclei were stained with Hoechst 33258 (blue). Scale bars, 75 μm. (D) Images of the spleen 3 d after infusion. Many GFP-positive cells were observed in the spleen (lower photographs). Upper photographs, negative control. Nuclei were stained with Hoechst (blue color). Scale bars, 25 μm. (E) No GFP-positive cells were observed in any organs. Upper photographs, negative control. Middle and lower photographs, images taken at 3 and 14 d, respectively, after infusion. The vessels were stained with smooth muscle cell actin (red). Nuclei were stained with Hoechst 33258 (blue). The photographs of muscle are merged fluorescent and phase-contrast images. Scale bars, 75 μm. Data are means ± s.e.m.
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4 injections) (Figure S3). There were no changes in capillary density or the number of apoptotic cells in the heart between the BMMNC-infused group and the control group (data not shown).

BMMNC-derived conditioned medium (CM) improved cardiomyocyte contractility

To elucidate whether factors secreted from BMMNC were involved in their beneficial effects on cardiac function, we first examined the effects of CM from BMMNC on the contractility of cultured cardiomyocytes of neonatal rats. After serum starvation for 12 h, cardiomyocytes were challenged with culture medium conditioned by BMMNC. Cell shortening was significantly enhanced and beating rate was markedly increased at 30 min and at 12 h after starting culture with the CM, compared with those in untreated cells (Figure 3A), suggesting that BMMNC secrete factors that positively affect cardiomyocyte contractility. Flow cytometric analysis revealed that BMMNC consisted of several cell populations including myeloid (Gr-1(+) cells, ~40%), erythroid (TER119(+) cells, ~20%), and lymphoid cells (B220(+) cells, ~20%) (Figure S4). The individual cell populations, including the lineage-negative population of cells, were sorted by magnetic beads. The isolated cells were 0.8×10^7 Gr-1(+) cells, 0.4×10^7 B220(+) cells, 0.2×10^7 TER(+) cells, and 0.1×10^7 lineage-negative cells from 2.0×10^7 BMMNC. When CM was collected from each population and added to cardiomyocytes starved for 12 h, only the CM from Gr-1(+) cells significantly enhanced cell shortening and increased the beating rate (Figure 3B), suggesting that Gr-1(+) cells mainly contribute to BMMNC-mediated improvements in cardiomyocyte contractility. CM from Gr-1(+) cells or BMMNC isolated from wild-type mice also induced significant hypertrophy of cardiomyocytes (Figure S5). We next examined the effects of CM from Gr-1(+) cells on DOX mice. At 1 and 3 d after the infusion of CM from Gr-1(+) cells, FS was significantly improved, as with infusion of BMMNC (Figure 3C). Furthermore, +dp/dt, as determined by catheterization of the left ventricle, was also improved at 1 d after the infusion, as compared with the control group (Figure 3D). Collectively, these results indicate that factors secreted from Gr-1(+) cells are responsible for BMMNC-induced improvements in cardiac function in DCM mice.

Analysis of factors secreted from Gr-1(+) cells

The CM from wild-type Gr-1(+) cells significantly enhanced cell shortening and increased the beating rate, while CM from EGFRdn Gr-1(+) cells had marginal effects (Figure 4A). This suggests that the factors that improve cardiomyocyte contractility are more abundant in cells of wild-type mice than cells of EGFRdn mice. We next performed DNA microarray analysis to identify the factors involved in these effects. Twenty three genes showed enhanced expression in Gr-1(+) cells from wild-type mice compared with EGFRdn mice (Table 1). The gene which showed the largest difference between two types of mice was growth

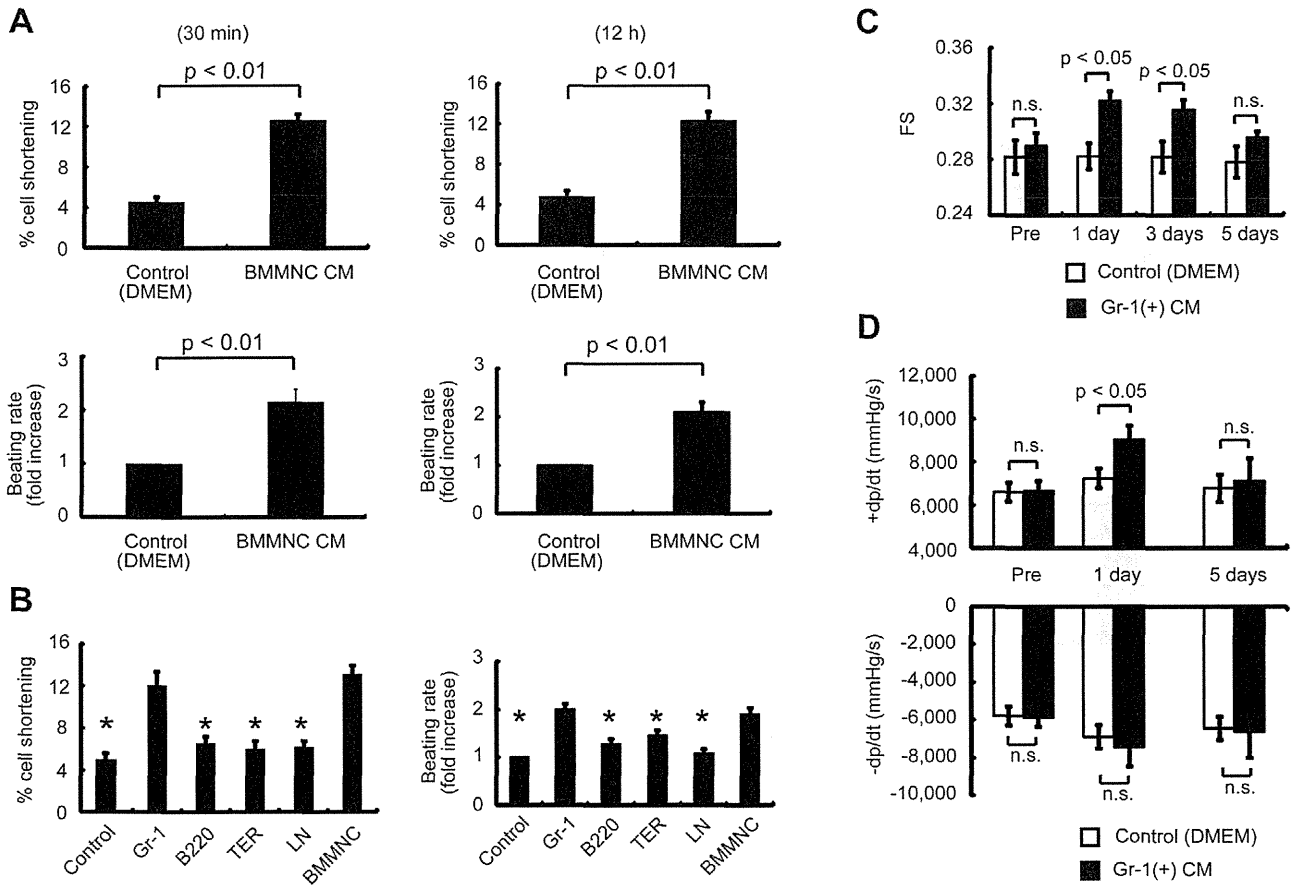

Figure 3. BMMNC-derived CM directly affects cardiomyocyte contractility. (A) Cell shortening and the beating rate of neonatal rat cardiomyocytes were significantly increased after exposure to CM from BMMNC compared with the control ($n = 26$ cells per group). The left and right graphs show the results at 30 min and at 12 h after treatment, respectively. Upper graph, cell shortening. Lower graph, beating rate. (B) CM from Gr-1(+) cells improved the cell shortening and increased the beating rate similar to that achieved by CM from BMMNC ($n = 27$ per group). (C, D) Effects of CM from Gr-1 cells on cardiac function *in vivo*. (C) Echocardiographic analysis ($n = 7$). The infusion of CM from Gr-1(+) cells significantly improved the FS of DOX mice at 1 and 3 d. (D) Infusion of CM from Gr-1(+) cells significantly improved the +dp/dt of DOX mice at 1 d, *in vivo* ($n = 7$). n.s., not significant. Data are means ± s.e.m.
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hormone (GH). The reduced expression of GH in Gr-1(+) cells from EGFRdn mice was confirmed by quantitative RT-PCR and ELISA (Figure 4B, C). GH levels were also lower in CM from Gr-1(+) cells isolated from old myocardial infarction (OMI) mice and DOX mice (Figure S6) than in CM from wild-type mice. Consistent with the downregulation of GH secretion from Gr-1(+) cells of heart failure mice, the serum GH levels were also lower in models of heart failure such as DOX, EGFRdn and OMI mice than in wild-type mice (Figure 4E).

Critical role of GH in Gr-1(+) cell-mediated cardioprotection

We examined the role of GH in the effects of Gr-1(+) cell-derived CM using pegvisomant, a specific inhibitor of the GH receptor [12]. Treatment with pegvisomant abolished the enhanced cell shortening and the increased beating rate induced by CM from Gr-1(+) cells (Figure 5A), while the anti-IGF-1 antibody had no effects (Figure 5B). These results suggest that Gr-1(+) cells improved the cardiomyocyte contractility *via* GH, but not *via* IGF-1 *in vitro*. CM from Gr-1(+) cells activated various signaling molecules, including Akt, extracellular signal-regulated kinase (Erk) 1/2, Janus kinase (Jak) 2, signal transducers and activators of transcription (Stat) 3/5 and protein kinase A (PKA) in cardiomyocytes (Figure 5C), and these effects were completely abolished by pegvisomant (Figure 5C). The addition of GH (500 pg/ml), a concentration equivalent to that in the CM from wild-type Gr-1(+) cells, activated the same signaling molecules (Figure 5C), suggesting that CM from Gr-1(+) cells activates Akt, Erk1/2, Jak2, Stat3/5 and PKA through the GH receptor signaling. Furthermore, the CM from Gr-1(+) cells, as well as GH, increased the amount of cyclic AMP (cAMP) in cardiomyocytes, which was also inhibited by pegvisomant (Figure 5D). The improvements in cardiac function induced by CM from Gr-1(+) cells were also abolished by treatment with the GH inhibitor (Figure 5E), whereas the anti-IGF-1 antibody had no effects (Figure 5F). Furthermore, the infusion of CM from Gr-1(+) cells increased the GH levels in serum of DCM mice (Figure 5G). These results suggest that Gr-1(+) cells improve the cardiac contractility *in vivo* also through GH. The BMMNC-mediated improvement in cardiac function of OMI mice was also affected by treatment with pegvisomant (Figure S7), suggesting that GH in BMMNC might have the therapeutic effects on heart failure caused by various etiologies.

Since Stat 3 is one of the important downstream targets of the GH receptor in cardiomyocytes (Figure 5C), we examined the

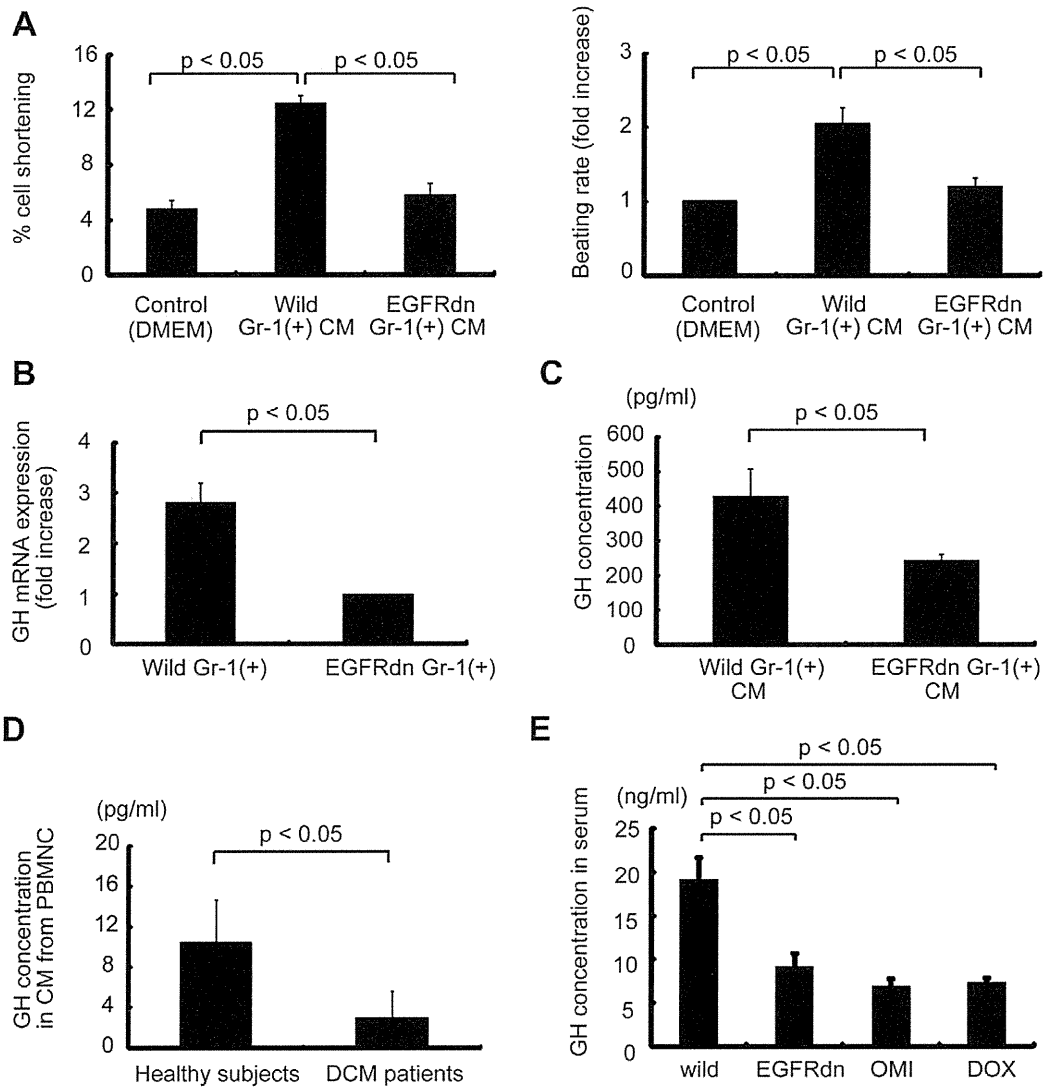

Figure 4. Analysis of secreted factors. (A) CM from Gr-1(+) cells from wild-type mice significantly improved the cell shortening and increased the beating rate in neonatal rat cardiomyocytes, as compared with CM from Gr-1(+) cells from EGFRdn mice. Left graph, cell shortening ($n = 24$ cells per group). Right graph, beating rate ($n = 24$ cells per group). (B) Quantitative RT-PCR analysis of GH mRNA in Gr-1(+) cells isolated from wild-type mice and EGFRdn mice ($n = 4$). (C, D) GH concentrations in (C) CM from Gr-1(+) cells isolated from wild-type mice and EGFRdn mice ($n = 4$) and (D) CM from PBMNC isolated from healthy ($n = 11$) and DCM subjects ($n = 10$). (E) GH concentration in serum from several mouse models of heart failure ($n = 4$). Data are means ± s.e.m.
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direct effects of GH in CM from Gr-1(+) cells on cardiomyocytes *in vivo* in transgenic mice overexpressing a dominant-negative mutant of STAT3 (STAT3dn) under the control of an αMHC promoter [13]. The Gr-1(+) cell CM-mediated improvements in cardiac function were not observed in DOX-treated STAT3dn mice (Figure S8), indicating that the CM improves cardiac function through activation of STAT3 in cardiomyocytes.

Upregulation of activin A in heart failure inhibits GH expression in Gr-1(+) cells

The expression of the GH gene has been reported to be regulated by transcription factors including pituitary transcription activator-1 (pit-1) [14], [15], and activin A has been reported to downregulate GH expression by reducing the stability of pit-1 [16]. Since activin A in the peripheral blood of heart failure patients has been reported to be upregulated compared with that in healthy controls [17], we investigated the role of activin A in the downregulation of GH in Gr-1(+) cells. Serum activin A levels were significantly higher in EGFRdn mice than in wild-type mice (Figure 6A), and were also elevated in other murine models of heart failure, including the OMI and DOX models (Figure S9). When Gr-1(+) cells were cultured with 400 pg/ml of activin A, a concentration equivalent to that in the peripheral blood of DCM mice, mRNA and protein levels of GH were significantly downregulated (Figure 6B), suggesting that activin A might be a key mediator of the reduced expression of GH in the Gr-1(+) cells of DCM mice. Furthermore, the serum activin A levels were remarkably higher in DCM patients (Table S1) than in healthy subjects (Figure 6A), while the GH levels in CM from peripheral blood mononuclear cells (PBMNC) of DCM patients was lower than that in healthy subjects (Figure 4D), suggesting that the

Table 1. DNA microarray analysis.

The fold increase	Gene symbol
4.9	Gh
4.3	Pdgfd
3.9	Figf
3.4	Tslp
3.2	Socs2
3.1	Lta
3.0	Bmp1
2.9	Il33
2.8	Ccl27a
2.7	Fgf20
2.6	Angpt1
2.5	Cxcl9
2.4	Il13
2.3	Fam3b
2.3	Il31
2.3	Gm6590
2.2	Spred1
2.2	Cmtm8
2.1	Kitl
2.1	Mif
2.1	Grem2
2.1	Il17d
2.1	Gdf10
2.0	Cxcl5

Each number indicates the fold-increase of gene expression in Gr-1(+) cells isolated from wild-type mice compared with those from EGFRdn mice.
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higher activin A levels might also inhibit GH expression in heart failure patients. A recent study showed that PBMNC are a major source of activin A in heart failure [17]. Since many humoral factors are known to contribute to the pathophysiology of heart failure [18], we examined whether humoral factors upregulated in heart failure might regulate activin A expression. Angiotensin II (AngII) (Figure 6C) and tissue necrosis factor-alpha (TNFα) (Figure S10A) increased the activin A levels in CM of PBMNC in a dose-dependent manner. Consistent with the previous reports [19], AngII and TNFα activated NFκB in the PBMNC (Figure 6D and Figure S10B) and AngII- and TNFα-induced upregulation of activin A in PBMNC were inhibited with a NFκB inhibitory peptide (Figure 6E and Figure S10C).

Inhibition of activin A in heart failure increases GH levels and improves cardiac function

To elucidate the role of activin A in EGFRdn mice, anti-activin A antibody was injected intraperitoneally for 2 weeks, with an alternate-day treatment regimen. Inhibition of activin A significantly increased GH protein levels in the CM from Gr-1(+) cells (Figure 6F). Furthermore, when neonatal rat cardiomyocytes were cultured with CM from Gr-1(+) cells isolated from anti-activin A antibody-treated EGFRdn mice, cell shortening was enhanced and the beating rate was increased significantly, as compared with CM from Gr-1(+) cells without antibody treatment (Figure 6G). Consistent with the upregulation of GH levels in Gr-1(+) cells by anti-activin A antibody treatment, the serum GH levels in EGFRdn mice were also increased (Figure 6H). Furthermore, FS and +dp/dt in EGFRdn mice treated with anti-activin A antibody were markedly improved compared with EGFRdn mice treated with isotype control (Figure 6H). Collectively, these results strongly suggest that inhibition of activin A improves cardiac function in non-ischemic DCM mice by restoring GH levels.

Discussion

Functional benefits of BMMNC infusion have been reported in human with ischemic heart diseases [2],[20]. Although we also observed the improvement of cardiac function of DCM model mice by BMMNC infusion, no engraftment of infused BMMNC was observed in the heart. At 3 d after infusion, BMMNC were only observed in the peripheral blood and spleen, but not in the heart, and very few GFP-positive cells were observed at 14 d even in the peripheral blood. This is consistent with the observations that BMMNC infusion only transiently improved cardiac function after infusion. These findings suggest that BMMNC improve cardiac function *via* humoral factors rather than *via* transdifferentiation into cardiomyocytes.

GH plays important roles in the protection of various tissues as well as the growth and development of many organs and whole body [21]. Serum GH levels have been reported to be low in patients with congestive heart failure [22]. Recent animal studies have demonstrated that GH treatment improves cardiac functions [23], [24]. The growth and protection of cardiomyocytes are regulated by various kinases such as Akt, Erk and Jak/Stat, and many studies have demonstrated that activation of Akt and Erk induces cardiac hypertrophy [25], [26] and prevents cardiomyocytes from stress-induced apoptosis [27]. Transgenic mice with cardiac-specific overexpression of the *stat3* gene were reported to show marked ventricular hypertrophy [28], while the cardioprotective effects of several cytokines including granulocyte colony-stimulating factor were reduced in mice with cardiac-specific expression of dominant-negative *stat3* [29]. In this study, we showed that GH produced by Gr-1(+) cells activated Akt, Erk, Jak2, Stat3/5 and PKA, and increased the levels of cAMP in neonatal rat cardiomyocytes (Figure 5C, D). GH has been reported to increase cAMP and activate PKA in reproductive organs by still-unknown mechanisms [30]. Here, we found that the beneficial effects of CM from Gr-1(+) cells on cardiac function were inhibited in cardiac-specific STAT3dn mice, suggesting that GH secreted by Gr-1(+) cells directly affects cardiomyocyte contractility. It has been reported that GH exerts some functions through the induction of IGF-1 expression [31], [32], and IGF-1 also promotes several cardioprotective effects in part by activating the Akt/phosphatidylinositol 3-kinase pathway [33], [34]. In the present study, the specific GH receptor inhibitor, but not anti-IGF-1 antibody, attenuated the improvements of cardiac contractility by the treatment of CM from Gr-1(+) cells in vitro (Figure 5A, B) and in vivo (Figure 5E, F). These findings suggest the effects of Gr-1(+) cells-derived CM on cardiac function of DCM mice mainly depend on GH rather than IGF-1.

It has been reported that the expression of GH gene is regulated by pit-1 at the transcriptional level [14], [15] and that activin A destabilizes pit-1 by phosphorylation [16]. Consistent with a previous report showing higher serum activin A levels in heart failure patients than in healthy controls [17], we found that serum levels of activin A were increased while GH levels in PBMNC CM were decreased in DCM patients. Similarly, the activin A levels were higher in the peripheral blood of DCM mice than in wild-type mice and activin A inhibited the production of GH in Gr-1(+)

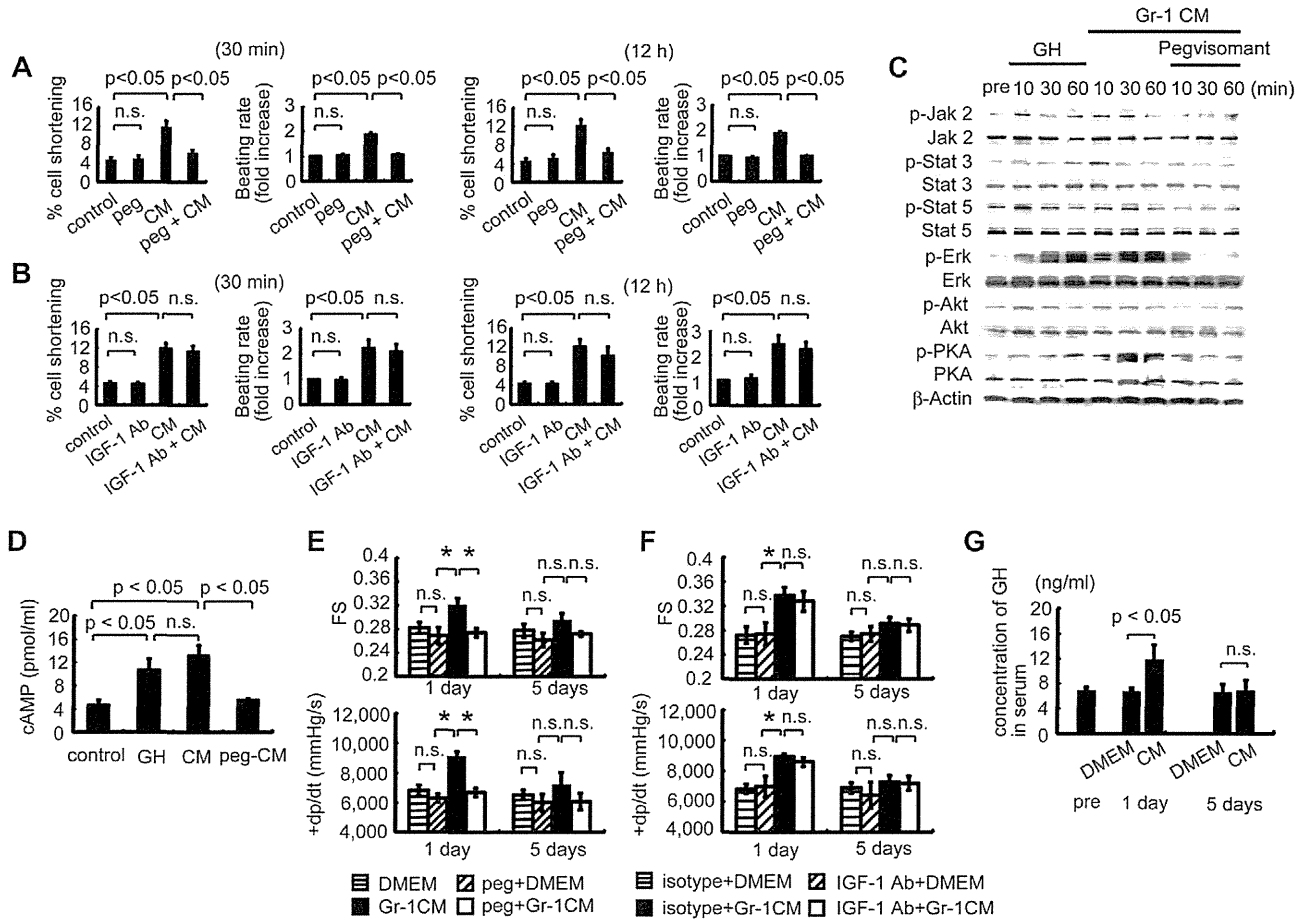

Figure 5. GH mediates the cardioprotective effects of Gr-1(+) cell-derived CM. (A) Pegvisomant (PEG) treatment inhibited the Gr-1(+) cell CM-mediated improvements cardiomyocyte cell shortening and beating rate at 30 min and at 12 h after treatment ($n = 27$ cells per group). Left graphs, cell shortening; right graphs, beating rate. (B) Anti-IGF-1 antibody failed to affect the Gr-1(+) cell CM-mediated improvements in cell shortening or beating rate at 30 min or at 12 h after treatment ($n = 23$ cells per group). Left graphs, cell shortening; right graphs, beating rate. (C) GH and CM from Gr-1(+) cells phosphorylated Akt, Erk, Jak2, Stat3/5 and PKA in cardiomyocytes ($n = 3$), which was inhibited by pegvisomant ($n = 3$). (D) GH (500 pg/ml) and CM from Gr-1(+) cells increased the cAMP concentration in cardiomyocytes ($n = 5$), which was inhibited by pegvisomant ($n = 5$). (E, F) Cardiac function analysis by echocardiography (upper graphs, $n = 8$) and catheterization (lower graphs, $n = 8$). Pegvisomant (E), but not anti-IGF-1 antibody (F), inhibited the improvements in FS and +dp/dt elicited by the infusion of CM from Gr-1(+) cells. *$p<0.05$ ($n = 8$). (G) Serum GH concentrations in DOX mice treated with CM from Gr-1(+) cells ($n = 4$ per group). The infusion of CM from Gr-1(+) cells from wild-type mice increased the serum GH concentration at 1 d, but not at 5 d. Data are means ± s.e.m.
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cells *in vitro*. These findings suggest that activin A, which is upregulated in heart failure, inhibits GH expression in various tissues/cells, including BMMNC. Treatment with anti-activin A antibody restored GH levels in Gr-1(+) cells and serum of EGFRdn mice and improved cardiac function, suggesting that normalizing the GH levels by inhibiting activin A is a novel therapeutic strategy for heart failure. Since many humoral factors such as AngII and TNFα are upregulated in heart failure and increased activin A expression by activating NFκB, the molecules that modulate NFκB activation might be also therapeutic targets to restore GH levels. On the other hand, anti-activin A treatment also increased expression levels of GH mRNA in the pituitary (N.F. K.M., unpublished data), suggesting that upregulation of activin A in heart failure might inhibit the expression of GH not only in Gr-1(+) cells but also in the pituitary, and that anti-activin A treatment might improve cardiac function of DCM mice in part by restoring GH expression in the pituitary.

The effects of GH on heart failure have been examined in many animal experiments and clinical trials [35]. A recent meta-analysis revealed that GH treatment improved several clinical parameters including left ventricular end-diastolic dimension, ejection fraction and New York Heart Association functional class [36]. Conversely, non-response to GH treatment for heart failure has been ascribed to GH resistance [37]. In patients with cardiac cachexia, GH levels were reported to be enhanced when compared with non-cachectic patients and normal subjects [38]. In this study, GH levels in heart failure mice and patients were significantly lower than those in healthy control subjects. Moreover, GH derived from Gr-1(+) cells improved cardiac function of heart failure animals, suggesting that our models were in a non-cachectic state and non-cachectic patients of heart failure might be suitable for GH treatment. Because of only temporary improvements in cardiac function (Figure 2A), bone marrow cell infusion might not be an appropriate treatment for heart failure, however inhibition

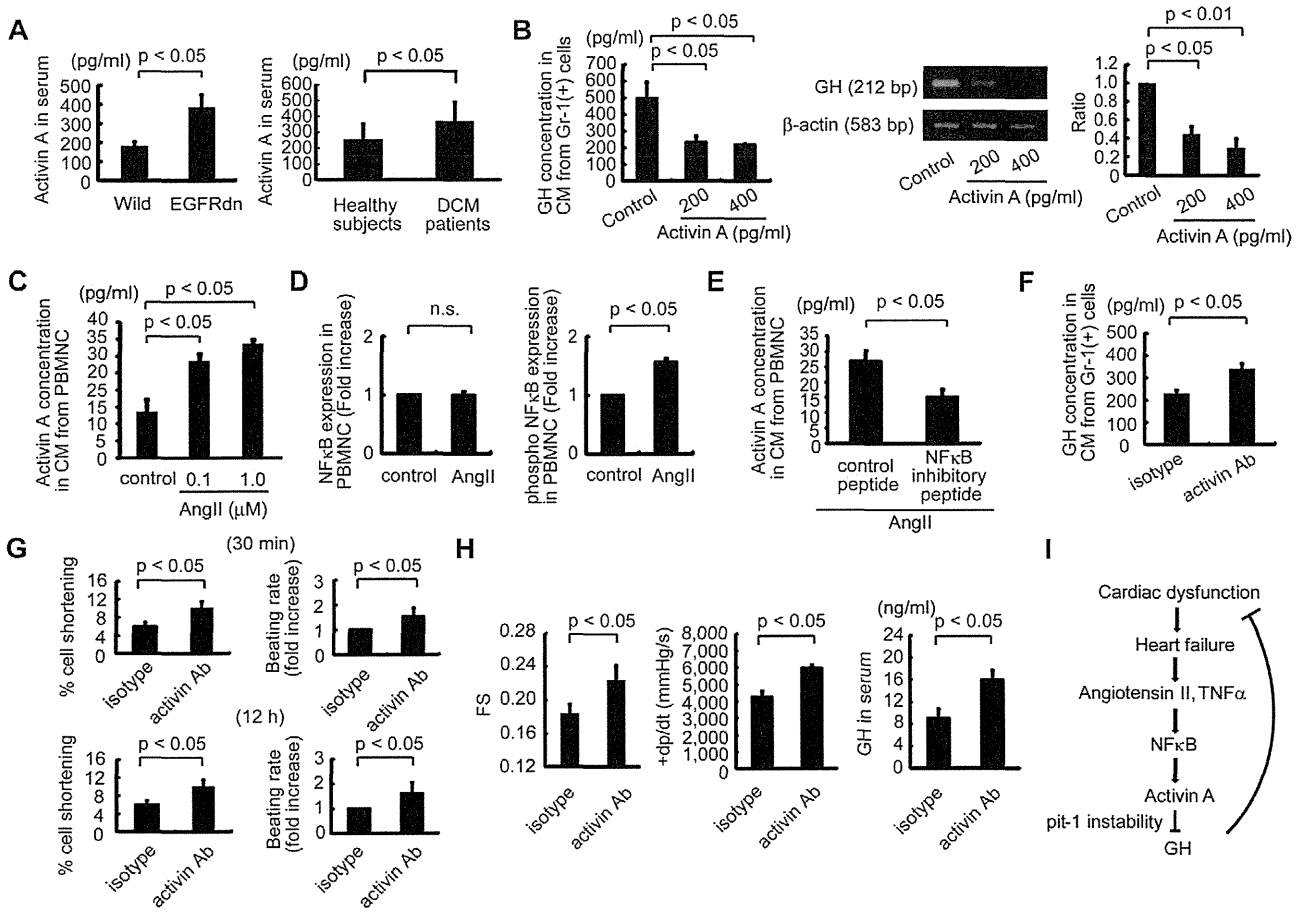

Figure 6. Regulatory mechanisms of GH in heart failure. (A) The serum activin A concentration was higher in EGFRdn mice (left, $n=5$) and in DCM patients (right, $n=10$) than in wild-type mice ($n=5$) and healthy subjects ($n=11$). (B) Activin A downregulated GH mRNA expression in Gr-1(+) cells and GH protein levels in Gr-1(+) cell CM. Left graph, GH protein concentration; middle photographs, representative semi-quantitative RT-PCR images; right graph, GH mRNA expression ($n=3$). (C, D) AngII upregulated activin A secretion (C, $n=4$) and phosphorylated NFκB expression (D, $n=5$) in wild-type PBMNC. (D) Left graph, total NFκB; right graph, phosphorylated NFκB. (E) Inhibition of NFκB [50 μM; NFκB p65 (Ser276) inhibitory peptide] suppressed AngII (10 μM)-mediated upregulation of activin A in CM derived from wild-type PBMNC ($n=5$). Isotype peptide was used as control. (F) The GH concentration in CM from EGFRdn Gr-1(+) cells ($n=5$) was significantly increased by treatment with an anti-activin A antibody ($n=5$). (G) Effects of anti-activin A antibody treatment on cell shortening and the beating rate of cardiomyocytes induced by CM from Gr-1(+) cells isolated from EGFRdn mice ($n=18$ cells per group). (H) Treatment with the anti-activin A antibody improved the cardiac function of EGFRdn mice. Left graph, echocardiography ($n=7$). Middle graph, miller catheter results ($n=7$). Right graph, serum GH concentration in EGFRdn mice after antibody treatment ($n=7$). Data are means ± s.e.m. (I) Proposed mechanism underlying impaired GH expression by activin A in heart failure.
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of activin A and enhancement of GH levels might offer novel therapeutic strategies for heart failure.

We used EGFRdn for DCM model mice in this study. It has been reported that cardiac-specific mutant of *ErbB2*, a member of the EGFR/erbB family, shows a severe dilated cardiomyopathy in mice [39]. In the clinical setting, trastuzumab, an anti-cancer agent, is humanized monoclonal antibody that targets the extracellular domain of the human epidermal growth factor receptor 2 and the use of trastuzumab demonstrated an unexpectedly high incidence of both asymptomatic and symptomatic cardiomyopathy. EGFRdn is a compatible DCM model mouse, resembling the cardiotoxic effects observed in patients treated with trastuzumab [40], [41].

There is a limitation in this study. We examined the surface area of neonatal rat cardiomyocytes after the treatment with CM from Gr-1(+) cells or BMMNC as an index for cardiac hypertrophy. However, the surface area not only depends on cell volume, but also on the degree of adhesion and spreading on the culture dishes.

Materials and Methods

Ethics Statement. The ethical committee of Tokyo Women's Medical University reviewed and approved the study protocol (approval ID: 1795). The study was conducted in accordance with the Declaration of Helsinki. We obtained informed consent from the all patients and the all healthy subjects by written before inclusion in this study.

Animals. Wild-type mice (C57BL/6) were purchased from Japan SLC. Adult GFP transgenic mice (C57BL/6) were a kind gift from Dr. M. Okabe (Osaka University). Cardiac-specific dominant-negative STAT3 mice were a kind gift from Dr. K. Yamauchi-Takihara (Osaka University). Neonatal Wistar rats (0–1 d old) were purchased from Saitama Experimental Animals Supply. All protocols were approved by the Institutional Animal Care and Use Committee of Tokyo Women's Medical University and Chiba University. The approval IDs for the animal experiments were 11–34 in Tokyo Women's Medical University