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基礎研究成果の臨床応用推進研究事業

# ヒト心筋・骨格筋からの心筋幹細胞株の樹立と末期的心不全への幹細胞移植医療実現化へ向けての研究基盤形成に関する研究

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主任研究者　松原　弘明

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（総合）研究報告書

# ヒト心筋・骨格筋からの心筋幹細胞株の樹立と末期的心不全への幹細胞移植医療実現化へ向けての研究基盤形成に関する研究

主任研究者　松原　弘明　京都府立医科大学医学研究科教授

研究要旨

重症心不全への心臓移植事業はドナー不足のため充分に機能せず、これを打開するのが心筋再生医療である。ヒト心筋生検サンプルから心筋幹細胞を単離・増殖させ、移植細胞の生存・増殖を目的とした局所bFGF 徐放シートとの併用による新規の心筋再生細胞移植療法を開発し、末期的心不全の患者さんを救済する世界で初めての心筋再生医療の確立を事業目的とする。

分担研究者

王　英正　京都大学医学部　助教授

室原　豊明　名古屋大学大学院医学系研究科教授

尾池　雄一　熊本大学大学院医学薬学研究部教授

A. 研究目的

末期心不全への心臓移植事業はドナー組織不足のため十分に機能せず、実用性の高い心筋再生医療は世界中でまだ実施されていない。我々は、少量のヒト心筋材料よりヒト心筋幹細胞の単離精製・増幅法を世界に先駆けて確立した（国際特許申請済）。臨床実用化にむけて、大動物を使用した前臨床試験を実施し、安全性・有効性を検証し、臨床試験phase I/IIaのプロトコールを作製して、世界で初めての心筋再生医療の実現化を目的とする。

B. 研究方法

<u>1)ヒト心筋から心筋幹細胞の単離・増殖法の確立と大動物移植実験の実施</u>：我々はすでに、心筋幹細胞特異的マーカーとして、世界に先駆けてSca-1を発見し、その多能性分化と機能的心筋細胞分化を報告してきた。平成17年度は、より効率に幹細胞を精製するため、特定の表面抗原を用いず、幹細胞の特徴である高い増殖能によって生成される幹細胞コロニーを形成させることで、ヒト心筋幹細胞の純化に成功した。このヒト心筋幹細胞は$CD105^{+}/Nkx2.5^{+}$の間葉系幹細

胞の特徴を示し、移植実験にて心筋及び血管細胞への直接分化とparacrine効果によって虚血心機能を改善させることを確認した。初年度におけるこれらの研究成果は、特異的な体組織幹細胞とその増幅因子との併用療法へとさらに発展させる形で応用した。平成18-19年度は大型動物を用いた研究に重きを置き、ランダム割り振りによる2つの前臨床治験を行った。ヒト心臓組織は開心術中の心筋組織サンプルあるいは数mgの心筋生検サンプルを用いた。コラゲナーゼ処理による分離後、我々が独自に開発した、単一細胞無血清浮遊系システムでスフェアを形成した幹細胞群を再度酵素処理にて分離し、我々が発見した心筋幹細胞特異的増殖因子bFGF存在下にて大量に増幅させる。ブタ冠動脈を遮断・再開通させ虚血心筋モデルを作成し、1月後に心筋幹細胞を心筋内に移植し、生体吸収性bFGF徐放ゲラチンシートをその上から覆う。血管造影・エコー・MRIにて移植後の心機能・不整脈を評価する。移植後の心筋組織を免疫組織学的検索により、移植細胞の心筋分化・血管数・奇形種形成を検証した。

2)臨床試験プロトコール作成と第1相臨床試験
:大動物前臨床試験の成績をもとに第1相臨床試験(phase I/IIa)プロトコルは京大探索医療センター検証部で作成する。低心機能(EF<35%以下)で心筋壊死領域の多い、冠動脈バイパス形成術を受ける末期的心不全(虚血性心臓病)を対象に、bFGF徐放ゲラチンシート+心筋幹細胞移植(107個)を組み合わせたハイブリッド療法にて心筋再生医療の第1相臨床試験を開始する。

(倫理面への配慮)

1)前臨床試験の有効性・安全性評価、臨床試験プロトコールはTR実施に当たっての共通倫理審査指針に沿って京都大学探索医療センター検証部で作成する。患者さんに対する治療前の説明と理解を得て、またプライバシーの保護を第一とし治療経過はすべて情報公開にする。移植細胞および臨床試験は厚生労働省よりの「幹細胞の臨床応用についての治療指針」、医薬品GCP,臨床研究倫理指針(厚生労働省告示第255号)を遵守する。

2)動物操作にあたっては各施設の動物実験指針に従って行う。基礎的研究においては、遺伝子改変マウス、プラスミドDNAを用いる場合は仕様に際しては、遺伝子組み換え生物などの使用等の規則による生物の多様性の確保に関する法律に基づき研究を実施する。

C.研究結果

より効率に幹細胞を精製するため、特定の表面抗原を用いず、幹細胞の特徴である高い増殖能によって生成される幹細胞コロニーを形成させることで、ヒト心筋幹細胞の純化に成功した。この心筋幹細胞は$CD105^+/Nkx2.5^+$の間葉系幹細胞の特徴を示し、移植実験にて心筋及び血管細胞への直接分化とparacrine効果によって虚血心機能を改善させることを確認した。初年度におけるこれらの研究成果は、特異的な体組織幹細胞とその増幅因子との併用療法へとさらに発展させる形で応用した。平成18-19年度は大型動物を用いた研究に重きを置き、ランダム割り振りによる、大型動物(ブタ)を用いたヒト心臓幹細胞とbFGF徐放シートの併用療法の有効性・安全性評価を目的とする前臨床

試験を施行した。試験デザインは、ヒト臨床試験の対象となる重症虚血性心疾患患者のモデルとして陳旧性心筋梗塞による慢性虚血機能不全心を作成し、前向きランダム化試験1:bFGF徐放シート移植の有効性・安全性の評価、前向きランダム化試験2:bFGFシート単独移植に対する心臓幹細胞移植併用の有効性評価、追試1:心臓幹細胞単独移植に対するbFGFシート移植併用の有効性評価、追試2:bFGFシート＋心臓幹細胞移植術後4ヶ月の長期観察による奇形種形成の危険性評価、を行った。有効性については、試験2において用量依存性効果評価目的で低用量群($5 \times 10^5$ 個/kg)、高用量群($5 \times 10^6$ 個/kg)を設定、また心臓幹細胞の対照細胞としてヒト組織由来幹細胞の骨髄間葉系幹細胞を用い、比較検討を行った。異種間細胞移植治療に該当するため、全頭に免疫抑制剤を移植前日から治療終了まで継続して投与し、血中濃度のモニタリングを行った。

安全性に関しては、試験1及び試験2の合計、全治療ブタ70例において、全死亡は術関連死1例のみで、術後～試験終了時までの期間においての死亡例の発生は認めなかった。24時間心電図による不整脈監視においても、心室性不整脈を含む有害事象としての不整脈の発生はいずれの群においても発生は認められなかった。試験1及び試験2において、bFGFシートの心筋表面への移植に際し、術後4週後に再開胸の上確認したが、シートの脱落は1例も認めなかった。また、bFGFシート移植による術後心膜炎、心タンポナーデの発症は全70例中1例も認めず、創感染に起因する軽度の心膜外膿瘍形成を2例認めたが、全身状態及び心機能には何ら悪影響を認めなかった。その他、免疫抑制に伴う感染症、骨髄機能の低下、及び心機能低下は認めなかった。

心臓幹細胞、及び骨髄間葉系幹細胞の移植後の奇形腫形成、癌腫形成の危険性について術後4週の短期観察及び、追試2の術後4ヶ月の長期観察を行い、最終的に36頭の免疫不全ブタの細胞移植後心臓の病理学的検索を行ったが、内胚葉、中胚葉、外胚葉組織を含めいかなる奇形種、異形細胞腫の形成も確認されなかった。

有効性に関しては、試験1の無治療対象(開胸及び培養液単独投与)群とbFGFシート移植(及び培養液単独投与)群の2群比較では、bFGFシート群で明かな虚血心筋内微少循環の改善を機序とした心機能の有意な改善と梗塞部重量の減少を認めた。

心臓幹細胞単独移植に対するbFGFシート移植併用による有効性を評価した追試1では、移植したヒト心臓幹細胞のbFGFシート併用による明らかなホスト心筋内での生着率の改善が認められ、術後4週において心臓幹細胞単独移植群に対し、bFGFシート併用心臓幹細胞移植群の有意な左心機能の改善を認めた。

以上結果を踏まえ、心臓幹細胞移植の有効性評価目的の試験2を行った。心臓幹細胞移植の有効性に関して、細胞用量依存性効果の有無を評価した低用量移植及び高用量移植群の比較では、高用量群において高濃度の細胞移植による組織障害の結果、ホスト心筋への心臓幹細胞の生着能、心筋細胞への分化能は低く、移植治療の有効性は認めなかった。これに比し、低用量群では移植細胞による

組織障害は認められず、ゲラチンハイドロゲルを介したbFGFの虚血心筋組織徐放による微小心筋血流の改善と、移植したドナー幹細胞の生着性の向上により、骨髄間葉系幹細胞に比し8倍以上の実質的な心筋細胞再生を確認した。

この結果、低用量心臓幹細胞移植とbFGFシート併用移植による相乗的な心機能改善効果は治療4週後において約12%もの不全梗塞心筋の左室駆出率の改善と3%もの梗塞重量の減少を認めた。この細胞移植とbFGFシート併用移植による相乗的な心機能改善効果は骨髄由来幹細胞移植では認めず、本研究における心臓幹細胞移植の心筋再生における特異的有用性を証明した。さらに、並行して行われた追試2の長期観察試験にて、標識された心臓幹細胞は、4ヶ月後の移植後慢性期においても心臓幹細胞とbFGFシートの併用移植群では高い生着率を保ち、心機能改善効果も保持されていた。

D. 考察

1）達成度について

当初の研究目的は充分に達成されたと考えられる。大動物を用いた前臨床試験で有効性・安全性は確認された。phase I/IIa臨床試験プロトコルを作製中である。

2）学術的・国際的・社会的意義

これまで難治性重症不全心患者に対して、心臓幹細胞を用いた細胞治療を施行した報告は世界的に一例の報告もない。また急性虚血心に対する骨髄細胞移植（冠動脈注入及びカテーテルによる心筋移植）、米国で行われた小動物への心臓幹細胞単独移植と比較して下記の利点があると考えられる。

1) 体外細胞培養工程により移植細胞数を均一化することで、細胞移植の効果を正確に判定できる
2) 直視下に障害心筋に細胞移植操作を行うことで、治療有効領域への確実な移植が可能にできる
3) bFGF シート移植を併用することで、心臓幹細胞の生着率を向上させ、心筋再生を飛躍的に改善できる
4) 必要最小限の有効細胞数の移植により、移植後組織障害の軽減を図ることが可能であり、かつ体外細胞増幅にかかる期間の短縮は必要とされるヒト血清量の節減（患者侵襲の低減）および手術待機期間の短縮を図ることができる

3）今後の展望について

本研究はヒト幹細胞を用いた世界で初めての臨床研究として新規性が認められるものと考え、重症虚血性心疾患患者へのbFGFシート併用心臓幹細胞移植をphase I/IIa 臨床試験プロトコルを京大探索医療センター検証部と作成中である。

E. 結論

我々が世界に先駆けて開発したFGFシート併用心臓幹細胞移植治療は、心機能改善効果、心筋分化効率、移植後細胞生存率などから考えて世界で最も優れた心筋再生医療でると結論される。国際特許も申請しており、今後の臨床試験への展開が期待される。

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2. 実用新案登録

特になし

3. その他

上記の3つの特許に関して、米国の1社より、特許の買収及び米国内での産業展開または京都大学との共同研究を折衝中

研究成果の刊行に関する一覧

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# Skeletal muscle-derived progenitors capable of differentiating into cardiomyocytes proliferate through myostatin-independent TGF-β family signaling

Tetsuya Nomura [a,b], Tomomi Ueyama [a], Eishi Ashihara [a], Kento Tateishi [a,b], Satoshi Asada [a,b], Norio Nakajima [a,b], Koji Isodono [a,b], Tomosaburo Takahashi [b], Hiroaki Matsubara [a,b,*], Hidemasa Oh [a,*]

[a] *Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto 606-8507, Japan*
[b] *Department of Cardiovascular Medicine, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan*

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**Abstract**

The existence of skeletal muscle-derived stem cells (MDSCs) has been suggested in mammals; however, the signaling pathways controlling MDSC proliferation remain largely unknown. Here we report the isolation of myosphere-derived progenitor cells (MDPCs) that can give rise to beating cardiomyocytes from adult skeletal muscle. We identified that follistatin, an antagonist of TGF-β family members, was predominantly expressed in MDPCs, whereas myostatin was mainly expressed in myogenic cells and mature skeletal muscle. Although follistatin enhanced the replicative growth of MDPCs through Smad2/3 inactivation and cell cycle progression, disruption of myostatin did not increase the MDPC proliferation. By contrast, inhibition of activin A (ActA) or growth differentiation factor 11 (GDF11) signaling dramatically increased MDPC proliferation via down-regulation of p21 and increases in the levels of cdk2/4 and cyclin D1. Thus, follistatin may be an effective progenitor-enhancing agent neutralizing ActA and GDF11 signaling to regulate the growth of MDPCs in skeletal muscle.
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*Keywords:* Muscle progenitor cells; Follistatin; Proliferation; Myostatin; TGF-β

Cardiac myocytes undergo terminal differentiation and lose the ability to divide soon after birth, withdrawing irreversibly from the cell cycle. Thus, the loss of myocytes after myocardial infarction results in irreparable damage to the adult heart, ultimately leading to eventual heart failure. A growing number of studies focused on cell-based therapy have demonstrated the isolation of adult stem cells from a variety of tissues and shown their ability to promote cardiac repair, but the task of inducing myocyte repopulation is fraught with obstacles [1].

Adult skeletal muscle harbors a pool of satellite cells associated with single myofibers, which possess remarkable regenerative potential as endogenous muscle progenitors [2]. Although these progenitor cells preferentially generate differentiated cells of the same type as cells in their tissue of origin, several studies have indicated that at least a fraction of stem cells from skeletal muscle, termed muscle-derived stem cells (MDSCs), can generate cells of different lineages [3] and may be much more plastic than previously appreciated. Recent reports demonstrated that Pax7-expressing muscle-derived subpopulation that could be isolated from suspended clusters of cells (myosphere) may contain a heterogeneous subpopulation capable of differentiating into myogenic, osteogenic, and adipogenic lineages; however, their potential of cardiac differentiation has yet to

* Corresponding authors. Fax: +81 075 751 4741 (H. Oh).
*E-mail addresses:* matsubah@koto.kpu-m.ac.jp (H. Matsubara), hidemasa@kuhp.kyoto-u.ac.jp (H. Oh).

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be determined [4]. In addition, clonal analysis of MDSCs isolated from neonatal skeletal muscle demonstrated that they constituted a subset of a Sca-1-expressing cell population [5]. However, an additional study in the adult mice confirmed that Sca-1 negative cell population, termed skeletal-based precursors of cardiomyocytes (Spoc), also contained cells that were able to differentiate into cardiac muscle cells [6], suggesting the heterogeneity in the pool of cardiogenic progenitors within MDSCs. To study the functional properties of muscle-derived progenitor cells, we prospectively isolated progenitor clones from adult skeletal muscle, based on the characteristics of adult stem cells having a distinct proliferative potential to form floating-spheres, termed myospheres [7].

The maintenance of muscle-derived stem/progenitor cells is controlled by growth factors and cytokine signals [8,9]; however, few studies have explicitly investigated the factors contributing cardiogenic progenitor cells from adult skeletal muscle to proliferate in vitro and in vivo. In the present study, we identified follistatin, an activin-binding protein to inhibit TGF-β family members, as a direct modulator of cardiogenic progenitor cell proliferation from adult skeletal muscle. ActA and GDF11, capable of binding the activin type II receptors (ActRII) [10,11], are the potent negative regulators of MDPC activation. These results may greatly facilitate the process of cardiogenic-progenitor cell ex vivo expansion as well as the development of new transplantation technologies for regenerative medicine.

## Materials and methods

*Mice.* The *mstn−/−* mice were provided by S.-J. Lee (The Johns Hopkins University) [12]. All experimental procedures and protocols were approved by the Animal Care and Use Committee of Kyoto University.

*Progenitor cell isolataion and differentiation.* Primary hind limb muscle cells were isolated from 6- to 12-week-old C57BL/6J (Shimizu Laboratories Supplies), *mstn−/−*, or *mstn+/+* littermate mice using 15 mg collagenase type II/g tissue for digestion (Worthington). Skeletal myospheres were isolated and expanded as previously described [7]. Cells were suspended in serum-free medium consisting of Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with B27 (Invitrogen), 20 ng/ml mouse EGF (SIGMA), 40 ng/ml human recombinant bFGF (Promega), and 1% penicillin/streptomycin. Single-cell suspensions were then preplated on non-coated cell culture plates (Corning) at 20 cells/μl density for 7 days to obtain myogenic cells. Cells proliferated to form floating myospheres in cell suspension after 7 days and were selectively picked from the plates and transferred into a fresh fibronectin-coated culture plates for adherent culture. For myogenic cell isolation, the cells that adhered within first 7 days were passaged to remove fibroblasts, and then the culture was enriched with satellite cells and myoblasts. The cell growth medium was replaced with MEM, 10% FBS, insulin–transferrin–selenium supplements (Invitrogen), and $10^{-8}$ M dexamethasone (SIGMA) for cardiac differentiation. The attached cells, during preplating cell-culture, containing myogenic cells were maintained in the same growth medium for further expansion. $C_2C_{12}$ myoblasts and mouse ES cells were maintained as previously described [7,13].

*RT-PCR and Gene expression analysis.* Total RNA was extracted using TRIzol Reagent (Invitrogen) and first-strand cDNA was synthesized using a SuperScript III kit (Invitrogen). PCR reactions were performed with gene-specific primers for 30–35 cycles. The gene-specific primer sequences are shown in Supplementary Table.

*Electrophysiology.* Three days before electrophysiological experiments, the cells were transfected using polyethyleneimine with a plasmid expressing EGFP under the control of the α-MHC promoter to visualize differentiated cardiomyocytes [13]. For membrane potential measurements, current-clamp experiments were performed at 37 °C, using the whole-cell configuration of the patch-clamp technique (Axopatch 200A; Axon Instruments) [14].

*Immunofluorescence.* Cells were fixed in 4% paraformaldehyde and were stained with mouse monoclonal antibodies against cardiac troponin-T and cardiac troponin-I (Hytest). Secondary antibodies were conjugated with Alexa Fluor 488 or Alexa Fluor 555, and nuclei were visualized using DAPI (all from Molecular Probes). Images were captured with a BZ-8000 microscope (Keyence, Japan).

*Construction of knockdown vectors.* The mouse ActA and GDF11 gene sequences were analyzed for potential siRNA targets using the web-based siRNA target finder (Clontech website). The siRNA target sequences accordingly designed to suppress ActA and GDF11 were as follows: ActA siRNA-1: CTTGCTTTGGCTGAGAGGATT; ActA siRNA-2: CGAAATGAATGAACTCATGGA; ActA siRNA-3: TCTTTCCAGTGTCCAGCAGCA; GDF11 siRNA-1: TCCTCTCACAGTGGACTTTGA; GDF11 siRNA-2: GCGAATACATGTTCATGCAAA; GDF11 siRNA-3: CAATGACAAGCAGCAGATTAT. Each target sequence was subcloned into the BamHI-EcoRI site of RNAi-Ready-pSIREN-RetroQ vector (Clontech) as an inverted repeat with a hairpin loop spacer. RNAi-Ready-pSIREN-RetroQ-luciferase vector was used as a Mock vector.

*Retrovirus production and infection.* GP2-293 cells (Clontech) were cotransfected with the envelope vector pVSV-G and retrovirus vectors encoding the puromycin resistance gene using FuGENE6 (Roche). MDPCs were infected with retrovirus in the presence of 4 μg/ml polybrene for 24 h, and the infected cells were selected with 2.5 μg/ml puromycin.

*Flow cytometric analysis (FACS).* One hundred thousand cells were seeded into a 60-mm culture plate and incubated for 24 h in growth medium. After 24 h of serum and growth factor deprivation to synchronize the cell cycle, the MDPCs were re-stimulated with serum-containing medium for a further 12–16 h. Cell cycle phase was studied using a flow cytometer (FACSCalibur, BD Biosciences) for at least 30,000 individual events per reaction. Data were analyzed using Mod-fit software (Verity Software House, Inc.).

*Western blotting.* MDPCs were extracted with lysis buffer containing 50 mM Tris–HCl, (pH 7.6), 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1 mM $Na_3VO_4$, 1 mM NaF, and a protease inhibitor cocktail Kit. The transferred membranes were incubated with mouse monoclonal antibodies against p21, phospho-cdk2/4, cyclin D1/E (Santa Cruz), ActA (R&D) or GAPDH (CHEMICON); goat polyclonal anti-GDF11 (Santa Cruz); or rabbit polyclonal antibodies against phospho-Smad2/3, phospho-Smad1/5/8 (Cell Signaling), Smad2/3, or Smad1/5/8 (Upstate).

*Statistical analysis.* Data are means ± SE and were analyzed by ANOVA and Scheffe's test, using a significance level of $p < 0.05$ (StatView).

## Results

### *Myospheres contain progenitor cell able to differentiate into functional cardiomyocytes*

To determine whether myospheres could generate cardiac muscle cells, we isolated the individual spheres from floating culture as previously described [7]. For myosphere expansion, individual spheres were transferred onto fibronectin-coated 24-well plates with 2% serum-containing medium and the spheres were allowed to attach. Myospheres could be repeatedly formed, as secondary spheres, from the adherent progeny of primary spheres that had been passaged one time. Cardiac differentiation was documented at 10 days after induction with $10^{-8}$ M dexameth-

asone as verified by cardiac troponin-T (Fig. 1A; $1.4 \pm 0.3\%$, $n = 12$) and cardiac troponin-I staining (Fig. 1B; $1.7 \pm 0.3\%$, $n = 10$). To monitor differentiated cardiomyocytes more carefully, we constructed a vector in which cardiac-specific α-myosin heavy chain (α-MHC)

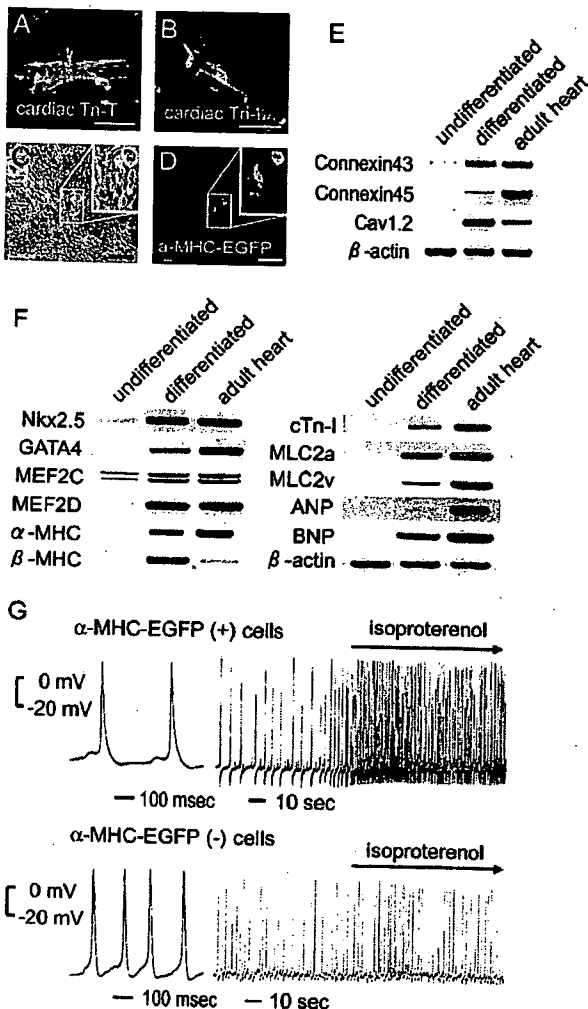

Fig. 1. Myospheres contain progenitor cells able to differentiate into functional cardiomyocytes (A and B) Myosphere-derived cells were treated with dexamethasone for 10 days and gave rise to cardiomyocytes. Cultures were stained for cardiac troponin-T (A, green) and cardiac troponin-I (B, red) to verify cardiac differentiation. DAPI, blue. (C and D) α-MHC promoter-driven EGFP plasmid was transfected into adherent progeny of MDPCs at day 7 during cardiac induction. Differentiated cardiomyocytes at day 10 are shown by phase contrast (C) and green fluorescence (D). Scale bars represent 20 μm. (E) RT-PCR showed the expression of gap junction proteins and L-type calcium channel Cav1.2 in differentiated cardiomyocytes. (F) RT-PCR for cardiac transcription factors (Nkx2.5, GATA4, MEF2C, and MEF2D) and structural genes (αβ-MHC, cTn-I, MLC2a/2v, ANP, and BNP) in differentiated and undifferentiated MDPCs. Two-month-old adult hearts were used as a positive control. (G) Action potential analysis. APs obtained from differentiated cardiomyocytes, verified as α-MHC-EGFP$^+$ cells responded to $10^{-7}$ M isoproterenol treatment (upper). APs acquired from non-cardiac muscle cells lacking EGFP fluorescence were not affected by $10^{-7}$ M isoproterenol treatment (bottom). Data shown are representative results obtained from 5 independent experiments.

promoter drives expression of an EGFP reporter cDNA. Transduction of MDPCs with the α-MHC-EGFP plasmid enabled visualization of differentiated cardiomyocytes as live cells (Figs. 1C and D). Fully differentiated cardiomyocytes expressed connexin-43/45 as well as the L-type calcium channel Cav1.2 in monolayer cell culture (Fig. 1E). The expression of cardiac differentiation markers, including cardiac transcription factors, structural proteins, and natriuretic peptides under identical differentiation conditions, was induced, as verified by RT-PCR (Fig. 1F).

To further characterize the differentiated cells from the adherent progeny of MDPCs, we then mapped the optimal action potentials (APs), focusing on EGFP$^+$ cardiomyocytes (Fig. 1G) or EGFP$^-$ non cardiac-cells under the spontaneous beating-condition after transfection of α-MHC-EGFP plasmid into undifferentiated MDPCs. Differentiated cardiac muscle cells contracted spontaneously at a lower rate than EGFP$^-$ cells ($147 \pm 14.7$ vs $309 \pm 23.3$ beats per minute; $p < 0.01$). Although the depolarization amplitude was similar between EGFP$^+$ and EGFP$^-$ cells ($66.6 \pm 5.7$ vs $68.8 \pm 4.2$ mV; n.s.), the resting membrane potential in EGFP$^+$ cardiomyocytes was higher than that in EGFP$^-$ cells ($-46.6 \pm 4.2$ vs $-54.6 \pm 3.9$ mV; $p < 0.05$). We next sought to determine whether the differentiated cardiomyocytes have β-adrenergic receptors, as assessed by their response to the β-adrenergic agonist isoproterenol. Indeed, the cardiac beating rate increased significantly in EGFP$^+$ cardiomyocytes in response to $10^{-7}$ M isoproterenol stimulation (Fig. 1G), whereas EGFP$^-$ non-cardiac cells contracted with irregular rhythm and showed a lack of increase in AP frequency.

*Follistatin is a sphere-derived intrinsic factor that increases the proliferative potential of myospheres independent of myostatin*

To investigate the molecular mechanisms of the MDPC proliferation, the expression patterns of myostatin, a secreted growth and differentiation factor (GDF8), and its antagonist follistatin were examined [15]. We found that follistatin was highly expressed in myospheres and ES cells, and was expressed to a lower extent in myogenic-lineage committed cell types (Fig. 2A). In contrast, myostatin expression was neither detectable in primary nor secondary myospheres but was abundant in myogenic cells and skeletal muscle tissue.

We examined the down-stream targets of TGF-β family signaling and cell cycle regulators in response to follistatin treatment. Follistatin inhibited Smad2/3 phosphorylation with a reciprocal increase in Smad1/5/8 phosphorylation. The effects of follistatin on MDPCs were characterized by a decrease in p21 expression and an increase in cdk2 activation (Fig. 2B). We next asked if the increased proliferation observed in follistatin-treated MDPCs is due to deregulation of the G1 to S phase transition. FACS analysis of the cell cycle distribution of a synchronous cell population revealed that follistatin significantly enhanced the