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再生医療実用化研究事業

末梢血単核球移植による血管再生治療と次世代の再生治療を目指した基盤研究

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総合研究報告書

末梢血単核球移植による血管再生治療と次世代の再生治療を目指した基盤研究

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研究要旨

末梢血中に骨髄由来の血管内皮前駆細胞が存在し、成体においても胎児期同様に血管の分化形成、すなわちvasculogenesisがおこるということが報告されて以来、ヒト虚血性心血管疾患に対して骨髄細胞を用いた細胞治療が、主にアジアとヨーロッパで盛んに行われ、その有効性を検証する研究が始まっているがまだ不確定な点が多い。このような中、我々は独自に末梢血単核球を用いた血管新生治療について基礎研究を重ねてきた。その結果、末梢血単核球は高度な血管新生効果を持つこと、その効果は骨髄由来の単核球と比較して、勝るとも劣らないことを見出した。そこで我々は、充分な血管新生効果がより安全に期待できる、自家末梢血単核球移植を臨床応用する方針とし、本学倫理委員会承認のもと、重症末梢性動脈疾患（安静時疼痛や虚血性潰瘍あり）を対象とした臨床研究を2002年7月より開始し、以後これまでに90例以上に対して本治療を行っている。その結果、約60-70％の症例において治療効果を認めた。さらに臨床データや基礎的検討の結果、末梢血単核球移植によって虚血肢の筋組織の再生がおこり、その再生過程において筋組織が分泌する血管増殖因子が持続的に虚血肢に作用し、血管再生を誘導することによって筋組織の再構築を促進しているという新しい作用メカニズムを明らかにした。そこで本研究では、末梢血単核球を用いた血管新生治療の有効性をさらに確認するため、重症間欠性跛行症例、重症虚血性心疾患症例に対してプラセボをコントロールとした2重盲験試験を行う。さらに動物モデルを用いた基礎的な研究も進めることによって、次世代の血管再生治療の開発を行う。重症虚血肢に対する末梢血単核球移植による血管再生治療に関しては、安静時疼痛や虚血性潰瘍のある重症下肢虚血患者を対象にした臨床研究において、治療効果や長期予後について検討したところ、透析症例においては、その治療効果が低いこと、予後も悪いことが明らかとなった。また、重症虚血肢に対する末梢血単核球移植による血管再生治療と重症虚血性心疾患に対する末梢血単核球細胞移植による血管再生治療の臨床研究登録を開始し、初期症例の結果ではそれぞれ治療の有効性と安全性が確認された。また、遺伝子改変マウスモデルを用いて、Notchシグナルの重要性について検証したところ、本治療におけるNotchシグナルの活性化の重要性が示唆された。

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A. 研究目的

我々はこれまでに末梢血単核球を用いた血管新生治療について基礎研究を重ねてきた。そ

の結果、末梢血単核球は高度な血管新生効果を持つこと、その効果は骨髄由来の単核球と比較して、勝るとも劣らないことを見出した。重症末梢性動脈疾患を対象とした臨床研究を2002年7月より開始し、以後これまでに90例以上に対して本治療を行い、その安全性と有効性を確認した。そこで本研究では、末梢血単核球を用いた血管新生治療の安全性や有効性をさらに確認するため、重症間欠性跛行症例、重症虚血性心疾患症例に対してプラセボをコントロールとした2重盲験試験を行う。さらに動物モデルを用いた基礎的な研究も進めることによって、次世代の血管再生治療の開発、治療効果の予想指標の解明を行う。

B. 研究方法

(1) 重症虚血肢に対する末梢血単核球移植による血管再生治療

これまでは、安静時疼痛や虚血性潰瘍のある重症下肢虚血患者を対象にパイロット研究を行ってきた。これらの対象者においては、倫理的に二重盲験とし難いため、本研究における対象は、末梢性血管疾患による重症間欠性跛行（Fontaine 2B相当）を有するが、従来の内科および外科的治療にても症状が改善せず、将来的に虚血症状の悪化が見込まれる患者とした。インフォームドコンセントを得た後、症例は細胞移植群とプラセボコントロール群の2群に無作為に分類する。治療前と移植治療6ヶ月後に、トレッドミル検査による最大歩行距離（一次評価項目）、跛行出現距離、下肢血圧回復時間、ABPI、レーザードップラー、下肢シンチ、MRI、下肢動脈造影、QOLによる判定（二次評価項目）を行い、治療効果を詳細に評価する。

(2) 重症虚血性心疾患に対する末梢血単核球細胞移植による血管再生治療

我々はウサギなどを用いた動物実験を重ね、慢性心筋虚血において、末梢血単核球を開胸下に心筋内に注射すると筋注部位の血管数の増加及び著明な血流改善を認め、その結果心機能の改善が得られることを確認した。また、同時に行ったテレメトリーを用いた心電図解析においても、単核球の心筋内注射に伴う有害不整脈の出現を認めなかった。以上の結果より、虚血性心疾患における末梢血単核球細胞移植は安全かつ有益であると考えた。特に冠動脈バイパス術との併用療法は従来の外科的治療では治療し得なかった心筋虚血を有する症例に対して効果的である可能性がある。このような背景から今回我々は従来の治療法にても虚血症状が改善しない重症虚血性心疾患患者を対象として、自家末梢血単核球細胞移植による血管新生治療を実施することを計画した。対象は、重症虚血性心疾患を有し、冠動脈バイパス術を施行予定であるが、バイパス術施行後も残存する心筋虚血領域があることが予測される患者とした。

(3) 次世代の血管生再治療を目指した基礎研究

我々は既に本治療の作用メカニズムの一部を解明した。予備実験では、その作用機序にNotchシグナルが重要であることも確認している。本研究では、次世代の再生治療を目指すため、そのシグナルの強弱と臨床効果との相関を検証するとともに、そのシグナルを増強することによって治療効果を増幅する方法を開発していく。

（倫理面への配慮）

(1) 動物を用いた研究は、千葉大学動物実験指針に基づき行う。遺伝子改変マウスの取り扱いや、拡散防止などについては、その指針に従って、十分注意を払いながら実験を行う。

(2)-1 研究対象個人の人権の庇護

患者自らの意志にて本申請医療を希望する場合のみ施行する。患者本人の意志を尊重すると

ともに、マスメディアからは可能な限り隔離し臨床成績発表の際にも患者のプライバシーに関わる情報の公表は避けるなど、最大限人権保護を優先するように努める。

(2)-2 同意を求める方法

添付説明文書にて末梢血単核球移植で発生する医学的合併症・効能・不利益・利益を充分に説明し、患者自らの意志にて移植医療を希望する場合のみ施行する。また細胞移植の実施中に患者さんより医療中断の意志が表明された際には即座に中止する。

(2)-3 個人への不利益ならびに危険性への配慮

本治療に同意を得られた患者については治療に不利益となる他疾患の有無をスクリーニングするための検査を治療前に全例について行う。治療後は入院中・外来通院を通じて合併症の早期発見とその早期対応に努める。

C. 研究結果

重症虚血肢に対する末梢血単核球移植による血管再生治療に関しては、安静時疼痛や虚血性潰瘍のある重症下肢虚血患者を対象にした臨床研究において、治療効果や長期予後について検討した。その結果、透析症例においては、その治療効果が低いこと、予後も悪いことが明らかとなった。

末梢性血管疾患による重症間欠性跛行（Fontaine 2B相当）を有するが、従来の内科および外科的治療にても症状が改善せず、将来的に虚血症状の悪化が見込まれる症例に対する臨床試験については、その登録を開始した。初期の12症例の検討では、本治療の有効性が示唆された。

次世代の血管生再治療を目指した基礎研究については、遺伝子改変マウスモデルを用いて、Notchシグナルの重要性について検証した。その結果、本治療により虚血組織に置けるNotchシグナルは活性化したが、その活性阻害によって本治療の効果は著しく低下したこと。さらに、Notch活性化抗体の投与によって、本治療の効果が増強されたことから、本治療におけるNotchシグナルの活性化の重要性が示唆された。

重症虚血性心疾患に対する末梢血単核球細胞移植による血管再生治療に関しては、プラセボコントロール試験に先駆けて、まず用量エスカレーション試験を施行することが、臨床研究の評価委員会で決定されたため、プロトコールの一部を変更した。全例において、細胞移植を行う方針とし、登録を開始した。初期の18症例の検討では、本治療と因果関係の疑われる有害事象の報告はなかった。

D. 考察

初期の症例の検討では、本治療の安全性と有効性が示唆された。本研究により重症虚血性心血管疾患に対する有効性が確定すれば、エビデンスのある血管再生治療として多施設において臨床応用を進めることができる。また、末梢血単核球移植による筋組織再生メカニズムが解明されれば、その鍵分子を用いた新たな血管再生医療やレスポンダーの選別方法の開発にもつながる。

E. 結論

重症虚血肢に対する末梢血単核球移植による血管再生治療と重症虚血性心疾患に対する末梢血単核球細胞移植による血管再生治療の臨床研究登録が開始された。初期の症例の検討では、本治療の安全性と有効性が示唆された。

F. 健康危険情報

該当なし

G. 研究発表

（平成20年度）

A. 研究発表

1. 論文発表

・Zhu, W., Shiojima, I., Ito, Y., Li, Z., Ikeda, H., Yoshida, M., Naito, A.T., Nishi, J.I., Ueno, H., Umezawa, A., Minamino, T., Nagai, T., Kikuchi, A., Asashima, M., Komuro, I. IGFBP-4 is an inhibitor of canonical Wnt signalling required for cardiogenesis. Nature 454:345-349, 2008.

・Minamino, T., and Komuro, I. Vascular aging: insights from studies on cellular senescence, stem cell aging, and progeroid syndromes. Nat Clin Pract Cardiovasc Med 5: 637-648, 2008.

・Toko, H., Minamino, T., and Komuro, I. Role of heart shock transcriptional factor 1 and heart shock proteins in cardiac hypertrophy. Trends Cardiovasc Med 18:88-93, 2008.

・Monzen, K., Ito, Y., Naito, A.T., Kasai, H., Hiroi, Y., Hayashi, D., Shiojima, I., Yamazaki, T., Miyazono, K., Asashima, M., Nagai, R., Komuro, I. A crucial role of a high mobility group protein HMGA2 in cardiogenesis. Nat Cell Biol 10:567-574, 2008.

・Nishi, J., Minamino, T., Miyauchi, H., Nojima, A., Tateno, K., Okada, S., Orimo, M., Moriya, J., Fong, G.H., Sunagawa, K., Shibuya, M., Komuro, I. Vascular endothelial growth factor receptor-1 regulates postnatal angiogenesis through inhibition of the excessive activation of Akt. Circ Res 103:261-268, 2008.

・Takano, H., Komuro, I. Peroxisome proliferators activated receptor gamma and cardiovascular diseases. Circ J 73:214-220, 2009.

・南野徹. 末梢血を用いた血管新生療法. 循環器科 66:359-366, 2009.

2. 学会発表

・小室一成. 第51回日本糖尿病学会年次学術集会イブニングセミナー 7（平成20年5月23日、東京）「心血管イベント抑制から考えた糖尿病治療ーLessons from PERISCOPE」

・Komuro I. The 2nd Oriental Congress of Cardiology(May 30-June1, 2008, Shanghai, China) "Molecular Mechanisms of Heart Failure"

・小室一成. 第50回日本老年医学会学術集会（平成20年6月19日、千葉）「高血圧治療における血管保護の重要性」

・小室一成. 第21回東海心筋代謝研究会（平成20年7月4日、名古屋）「心不全モデルマウスから考える分子メカニズム」

・小室一成. 第27回高血圧シンポジウム-基礎から臨床まで-（平成20年7月12日、大阪）「心肥大から心不全発症の分子機序」

・小室一成. 第45回日本臨床分子医学会学術集会（平成20年7月24日、大阪）「心不全発症の分子機序」

・Stems of the Heart: Myocardial and Vascular Rebirth(Sep 11-13, 2008, Boston, USA) A Novel Cardiomyocyte Differentiation Factor.

・小室一成. 日本血管生物医学会 プレスセミナー（平成20年9月24日、東京）小室一成.「心肥大はなぜ恐いか！？ 癌より恐い心不全」

・小室一成. 第8回日本再生医療学会総会（平成21年3月6日、東京）「老化という視点から考える血管疾患と血管新生」

・Minamino T. 第24回日本医工学治療学会 シンポジウム（平成20年4月18日ー20日、千葉）「血管再生療法の臨床研究と次世代の再生治療を目指した基礎研究」

・Minamino T. 第40回日本動脈硬化学会総会 シンポジウム（平成20年7月10日ー11日、茨城）"Therapeutic neovascularization using peripheral mononuclear cells"

（平成21年度）

1. 論文発表

・Ito, K., Akazawa, H., Tamagawa, M., Furukawa, K., Ogawa, W., Yasuda, N., Kudo, Y., Liao, C.H.,

Yamamoto, R., Sato, T., Molkentin, J.D., Kasuga, M., Noda, T., Nakaya, H., Komuro, I. PDK1 coordinates survival pathways and beta-adrenergic response in the heart. Proc Natl Acad Sci USA 106:8689-8694, 2009.

・Qin, Y., Yasuda, N., Akazawa, H., Ito, K., Kudo, Y., Liao, C.H., Yamamoto, R., Miura, S.I., Saku, K., Komuro, I. Multivalent ligand-receptor interactions elicit inverse agonist activity of AT(1) receptor blockers against stretch-induced AT(1) receptor activation. Hypertens Res 32:875-883, 2009.

・Yoshida, M., Shiojima, I., Ikeda, H., Komuro, I. Chronic doxorubicin cardiotoxicity is mediated by oxidative DNA damage-ATM-p53-apoptosis pathway and attenuated by pitavastatin through the inhibition of Rac1 activity. J Mol Cell Cardiol 47:698-705, 2009.

・Matsuura, K., Honda, A., Nagai, T., Fukushima, N., Iwanaga, K., Tokunaga, M., Shimizu, T., Okano, T., Kasanuki, H., Hagiwara, N., Komuro, I. Transplantation of cardiac progenitor cells ameliorates cardiac dysfunction after myocardial infarction in mice. J Clin Invest119:2204-2217, 2009.

・Kayama, Y., Minamino, T., Toko, H., Sakamoto, M., Shimizu, I., Takahashi, H., Okada, S., Tateno, K., Moriya, J., Yokoyama, M., Nojima, A., Yoshimura, M., Egashira, K., Aburatani, H., Komuro, I. Cardiac 12/15 lipoxygenase-induced inflammation is involved in heart failure. J Exp Med 206:1565-1574, 2009.

・Minamino, T., Orimo, M., Shimizu, I., Kunieda, T., Yokoyama, M., Ito, T., Nojima, A., Nabetani, A., Oike, Y., Matsubara, H., Ishikawa, F., Komuro, I. A crucial role for adipose tissue p53 in the regulation of insulin resistance. Nat Med 15:1082-1087, 2009.

・Ikeda, H., Shiojima, I., Ozasa, Y., Yoshida, M., Holzenberger, M., Kahn, C.R., Walsh, K. Igarashi,T., Abel, E.D., Komuro, I. Interaction of Myocardial insulin receptor and IGF receptor signaling in exercise-induced cardiac hypertrophy. J Mol Cell Cardiol 47:664-675, 2009.

・Akazawa, H., Komuro I. "Change Can Happen" by PKA: Proteasomes in in vivo Hearts. J Mol Cell Cardiol 46:445-447, 2009.

・Akazawa H, Yasuda N, Komuro I. Mechanisms and functions of agonist-independent activation in the angiotensin II type 1 receptor. Mol Cell Endocrinol 302:140-147, 2009.

・Liao, C.H., Akazawa, H., Tamagawa, M., Ito, K., Yasuda, N., Kudo, Y., Yamamoto, R., Ozasa, Y., Fujimoto, M., Wang, P., Nakauchi, H., Nakaya, H., Komuro, I. Cardiac mast cells cause atrial fibrillation through PDGF-A-mediated fibrosis in pressure-overloaded mouse hearts. J Clin Invest 120:242-253, 2010.

・Moriya, J., Minamino, T., Tateno, K., Okada, S., Uemura, A., Shimizu, I., Yokoyama, M., Nojima, A., Okada, M., Koga, H., Komuro, I. Inhibition of semaphorin as a novel strategy for therapeutic angiogenesis. Circ Res 106:391-398, 2010.

・森谷純治，南野徹. 次世代の血管新生治療の開発. 脈管学 50: 351-355, 2010.

・南野徹. 次世代の血管新生治療. 循環器専門医 18:251-257, 2010.

・南野徹：循環器疾患に対する再生医療 心臓 42:1563, 2010.

・森谷純治，舘野馨，南野徹. 末梢血管疾患に対する再生医療. 心臓 42:1582-1586, 2010.

2. 学会発表

・小室一成. 第46回日本臨床分子医学会（平成21年4月12日、東京）「心不全の新しい発症機序と治療」

・小室一成. 第17回アジア太平洋心臓病学会イブニングセミナー(平成21年5月21日、京都)
"Molecular mechanism and new theraphy for heart failure"
・小室一成. ISHNE 2009 ファイアサイドセミナー(平成21年6月4日, 神奈川)"Regeneration Theraphy for Heart Failure"
・小室一成. 日本循環器学会北海道地方会教育セッション（平成21年6月13日, 北海道）「心不全の新しい発症機序と治療」
・小室一成. 第19回日本心臓核医学会総会・学術大会（平成21年6月26日, 東京）「糖尿病における心臓の異常について」
・小室一成. The 9th World Congress on Inflammation（July 9, 2009, Tokyo)
"Regeneration Theraphy For Heart Failure"
・小室一成. 第15回日本遺伝子治療学会（平成21年7月10日, 大阪）"Molecular Mechanisms and Novel Treatments For Heart Failure"
・小室一成. 第15回日本心臓リハビリテーション学会（平成21年7月18日, 東京）「骨格筋の再生を介した単核球によるPAD治療」
・Komuro I. Basic Cardiovascular Sciences Conference 2009-Molecular Mechanisms of Cardiovascular Diseases（July 20-23, 2009, Las Vegas, USA） "Wnt-IGFBP4 Signaling in the Heart"
・小室一成. 第57回日本心臓病学会学術集会ランチョンセミナー（平成21年9月19日, 北海道）「心不全の新しい発症機序と再生治療」
・Komuro I. Advances in heart development:from molecules to cures（Sep 28-Oct 3, 2009, Nice, France） "Wnt signaling regulates cardiomyocyte differentiation and cardiac remodeling"
・小室一成. 第23回日本臨床内科医学会（平成21年10月11日、埼玉）「心不全の新しい発症機序と再生治療」
・小室一成. 第40回日本内科学会九州支部生涯教育講演会（平成21年11月1日、福岡）「心不全の新しい発症機序と再生治療」
・Komuro I. The Third International Conference on Cell Therapy(IRICT) (Nov 12, 2009, Seoul, Korea) "Long-Term Outcome of Therapeutic Neovascularization Using Peripheral Blood Mononuclear Cells for Limb Ischemia"
・Komuro I. Zhongshan Lecture（Dec 7, 2009, Shanghai, China）"The Role of Statin in the Treatment of Heart Failure"
・Komuro I. SIRIC International Forum 2009: Theme: Imaging for Shedding light to Atherosclerosis（Dec 11, 2009, Seoul,Korea）
"Senescenec as a cause of atherosclerosis and therapeutic angiogenesis using peripheral blood mononuclear cells"
・小室一成. 第23回日本冠疾患学会ランチョンセミナー（平成21年12月19日、大阪）「心血管発症抑制を目指した糖尿病治療戦略」
・Komuro I. 2010Angiotensin Gordon Conference（Feb 21-26, 2010 California, USA）"Molecular & Therapeutic implications of stretch-sensing by the AT1R"
・Minamino T. 第54回透析医学会学術集会・総会 ランチョンセミナー（平成21年6月5日ー7日、神奈川）血管再生治療のUpdateと次世代治療法の開発
・Minamino T. 第41回日本動脈硬化学会総会シンポジウム(平成21年7月17日ー18日、山口）Long-term outcome of therapeutic neovascularization using peripheral blood mononuclear cells for limb ischemia
・Minamino T, Komuro I. 第50回日本脈管学会総会 シンポジウム(平成21年10月29日ー31日、東京）「バージャー病に対する血管新生療法」
・Minamino T, Komuro I. 第74回日本循環器学会総会・学術集会 Plenary Session（平成22

年3月5日ー7日、京都）“Another phase of therapeutic angiogenesis for cardiovascular disease”

（平成22年度）

1．論文発表

・Minamino T. Role of Cellular Senescence in Lifestyle-Related Disease. Circ J 74:2527-2533, 2010.

・Shimizu I, Minamino T, Toko H, Okada S, Ikeda H, Yasuda N, Tateno K, Moriya J, Yokoyama M, Nojima A, Koh GY, Akazawa H, Shiojima I, Kahn CR, Abel ED, Komuro I. Excessive cardiac insulin signaling exacerbates systolic dysfunction induced by pressure overload in rodents. J Clin Invest 120:1506-1514, 2010.

・Naito AT, Okada S, Minamino T, Iwanaga K, Liu ML, Sumida T, Nomura S, Sahara N, Mizoroki T, Takashima A, Akazawa H, Nagai T, Shiojima I, Komuro I. Promotion of CHIP-mediated p53 degradation protects the heart from ischemic injury. Circ Res 106:1692-1702, 2010.

・Toko H, Takahashi H, Kayama Y, Okada S, Minamino T, Terasaki F, Kitaura Y, Komuro I. ATF6 is important under both pathological and physiological states in the heart. J Mol Cell Cardiol 49:113-120, 2010.

・Ueda K, Takano H, Niitsuma Y, Hasegawa H, Uchiyama R, Oka T, Miyazaki M, Nakaya H, Komuro I. Sonic hedgehog is a critical mediator of erythropoietin-induced cardiac protection in mice. J Clin Invest 120:2016-2029, 2010.

・Toko H, Takahashi H, Kayama Y, Oka T, Minamino T, Okada S, Morimoto S, Zhan DY, Terasaki F,Anderson ME, Inoue M, Yao A, Nagai R, Kitaura Y, Sasaguri T, Komuro I. Ca^{2+}/calmodulin-dependent kinase IIdelta causes heart failure by accumulation of p53 in dilated cardiomyopathy. Circulation 122:891-899, 2010.

・Ikeda H, Shiojima I, Oka T, Yoshida M, Maemura K, Walsh K, Igarashi T, Komuro I. Increased Akt-mTOR signaling in lung epithelium is associated with respiratory distress syndrome in mice. Mol Cell Biol 31:1054-1065, 2011.

・Aoki A, Ozaki K, Sato H, Takahashi A, Kubo M, Sakata Y, Onouchi Y, Kawaguchi T, Lin TH, Takano H, Yasutake M, Hsu PC, Ikegawa S, Kamatani N, Tsunoda T, Juo SH, Hori M, Komuro I, Mizuno K, Nakamura Y, Tanaka T. SNPs on chromosome 5p15.3 associated with myocardial infarction in Japanese population. J Hum Genet 56:47-51, 2011.

・Shioyama W, Nakaoka Y, Higuchi K, Minami T, Taniyama Y, Nishida K, Kidoya H, Sonobe T, Naito H, Arita Y, Hashimoto T, Kuroda T, Fujio Y, Shirai M, Takakura N, Morishita R,Yamauchi-Takihara K, Kodama T, Hirano T, Mochizuki N, Komuro I. Docking Protein Gab1 Is an Essential Component of Postnatal Angiogenesis After Ischemia via HGF/c-Met Signaling. Circ Res 108:664-75. 2011.

・南野徹. バージャー病に対する血管新生療法 J Jpn Coll Angiol 51:67-72, 2011.

2. 学会発表

・小室一成. 第53回日本腎臓学会学術講演会（平成22年6月16日、兵庫）「心腎連関を考えたARBによる降圧療法の重要性」

・Komuro I. Basic Cardiovascular Sciences Scientific Sessions 2010（Jul 19-22, 2010, Rancho Mirage, USA）“Wnt Signaling and Age-Associated Diseases”

・小室一成. 第58回日本心臓病学会学術集会（平成22年9月17日～9月19日、東京）「心房細動発生の新しい機序をエビデンス」

・小室一成. 第33回日本高血圧学会総会（平成

22年10月15日～10月17日、福岡）「高血圧治療の新たな治療戦略—ARB/CCB配合剤への期待-心肥大の形成を抑制するにはどうしたらよいか」

・小室一成．第18回日本血管生物医学会（平成22年12月3日、大阪）「降圧は心血管病の最高の予防である～併用療法を必要とする高血圧患者の治療戦略～」

・小室一成．第18回日本血管生物医学会（平成22年12月3日、大阪）「老化から診た心血管病」

・小室一成．第18回日本血管生物医学会（平成22年12月3日、大阪）“Angiogenic Therapy for heart failure”

・・Komuro I. Keystone Symposia (Feb 22 – 27, 2011, Keystone, USA) p53 as a Mediator of Cardiovascular Diseases.

・Minamino T. 第16回日本遺伝子治療学会学術集会（平成22年7月1日ー3日、栃木）シンポジウム“Another phase of therapeutic angiogenesis for cardiovascular disease”

・Minamino T. 第51回日本脈管学会総会シンポジウム（平成22年10月14日ー16日、北海道）「分子標的医薬による血管新生治療の開発」

H. 知的財産権の出願・登録状況

該当なし

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Zhu, W., Shiojima, I., Ito, Y., Li, Z., Ikeda, H., Yoshida, M., Naito, A.T., Nishi, J.I., Ueno, H., Umezawa, A., Minamino, T., Nagai, T., Kikuchi, A., Asashima, M., Komuro, I	IGFBP-4 is an inhibitor of canonical Wnt signalling required for cardiogenesis	Nature	454	345-349	2008
Minamino, T., and Komuro, I	Vascular aging: insights from studies on cellular senescence, stem cell aging, and progeroid syndromes	Nat Clin Pract Cardiovasc Med	5	637-648	2008
Toko, H., Minamino, T., and Komuro, I	Role of heart shock transcriptional factor 1 and heart shock proteins in cardiac hypertrophy	Trends Cardiovasc Med	18	88-93	2008
Monzen, K., Ito, Y., Naito, A.T., Kasai, H., Hiroi, Y., Hayashi, D., Shiojima, I., Yamazaki, T., Miyazono, K., Asashima, M., Nagai, R., Komuro, I	A crucial role of a high mobility group protein HMGA2 in cardiogenesis	Nat Cell Biol	10	567-574	2008
Nishi, J., Minamino, T., Miyauchi, H., Nojima, A., Tateno, K., Okada, S., Orimo, M., Moriya, J., Fong, G.H., Sunagawa, K., Shibuya, M., Komuro, I	Vascular endothelial growth factor receptor-1 regulates postnatal angiogenesis through inhibition of the excessive activation of Akt	Circ Res	103	261-268	2008
Takano, H., Komuro, I	Peroxisome proliferator activated receptor gamma and cardiovascular diseases	Circ J	73	214-220	2008

研究成果の刊行に関する一覧

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ito, K., Akazawa, H., Tamagawa, M., Furukawa, K., Ogawa, W., Yasuda, N., Kudo, Y., Liao, C.H., Yamamoto, R., Sato, T., Molkentin, J.D., Kasuga, M., Noda, T., Nakaya, H., Komuro, I	PDK1 coordinates survival pathways and beta-adrenergic response in the heart	Proc Natl Acad Sci USA	106	8689-8694	2009
Akazawa, H., Komuro I	"Change Can Happen" by PKA: Proteasomes in in vivo Hearts	J Mol Cell Cardiol	46	445-447	2009
Akazawa, H., Yasuda, N., Komuro I	Mechanisms and functions of agonist-independent activation in the angiotensin II type 1 receptor. Mol Cell Endocrinol	Mol Cell Endocrinol	302	140-147	2009
Minamino, T., Orimo, M., Shimizu, I., Kunieda, T., Yokoyama, M., Ito, T., Nojima, A., Nabetani, A., Oike, Y., Matsubara, H., Ishikawa, F., Komuro, I	A crucial role for adipose tissue p53 in the regulation of insulin resistance	Nat Med	15	1082-1087	2009
Qin, Y., Yasuda, N., Akazawa, H., Ito, K., Kudo, Y., Liao, C.H., Yamamoto, R., Miura, S.I., Saku, K., Komuro, I	Multivalent ligand-receptor interactions elicit inverse agonist activity of AT(1) receptor blockers against stretch-induced AT(1) receptor activation	Hypertens Res	82	875-883	2009
Yoshida, M., Shiojima, I., Ikeda, H., Komuro, I	Chronic doxorubicin cardiotoxicity is mediated by oxidative DNA damage-ATM-p53 -apoptosis pathway and attenuated by pitavastatin through the inhibition of Rac1 activity	J Mol Cell Cardiol	47	698-705	2009
Matsuura, K., Honda, A., Nagai, T., Fukushima, N., Iwanaga, K., Tokunaga, M., Shimizu, T.,Okano, T., Kasanuki, H., Hagiwara, N., Komuro, I	Transplantation of cardiac progenitor cells ameliorates cardiac dysfunction after myocardial infarction in mice	J Clin Invest	119	2204-2217	2009

研究成果の刊行に関する一覧

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kayama, Y., Minamino, T., Toko, H., Sakamoto, M., Shimizu, I., Takahashi, H., Okada, S., Tateno, K., Moriya, J., Yokoyama, M., Nojima, A., Yoshimura, M., Egashira, K., Aburatani, H., Komuro, I	Cardiac 12/15 lipoxygenase-induced inflammation is involved in heart failure	J Exp Med	206	1565-1574	2009
Ikeda, H., Shiojima, I., Ozasa, Y., Yoshida, M., Holzenberger, M., Kahn, C.R., Walsh, K., Igarashi, T., Abel, E.D., Komuro, I	Interaction of myocardial insulin receptor and IGF receptor signaling in exercise-induced cardiac hypertrophy	J Mol Cell Cardiol	47	664-675	2009
Liao, C.H., Akazawa, H., Tamagawa, M., Ito, K., Yasuda, N., Kudo, Y., Yamamoto, R., Ozasa, Y., Fujimoto, M., Wang, P., Nakauchi, H., Nakaya, H., Komuro, I	Cardiac mast cells cause atrial fibrillation through PDGF-A-mediated fibrosis in pressure-overloaded mouse hearts	J Clin Invest	120	242-253	2010
Moriya, J., Minamino, T., Tateno, K., Okada, S., Uemura, A., Shimizu, I., Yokoyama, M., Nojima, A., Okada, M., Koga, H., Komuro, I	Inhibition of semaphorin as a novel strategy for therapeutic angiogenesis	Circ Res	106	391-398	2010

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Minamino T	Role of Cellular Senescence in Lifestyle-Related Disease	Circ J	74	2527-2533	2010
Shimizu I, Minamino T, Toko H, Okada S, Ikeda H, Yasuda N, Tateno K, Moriya J, Yokoyama M, Nojima A, Koh GY, Akazawa H, Shiojima I, Kahn CR, Abel ED, Komuro I	Excessive cardiac insulin signaling exacerbates systolic dysfunction induced by pressure overload in rodents	J Clin Invest	120	1506-1514	2010
Naito AT, Okada S, Minamino T, Iwanaga K, Liu ML, Sumida T, Nomura S, Sahara N, Mizoroki T,Takashima A, Akazawa H, Nagai T, Shiojima I, Komuro I	Promotion of CHIP-mediated p53 degradation protects the heart from ischemic injury	Circ Res	106	1692-1702	2010
Toko H, Takahashi H, Kayama Y, Oka T, Minamino T, Okada S, Morimoto S, Zhan DY, Terasaki F,Anderson ME, Inoue M, Yao A, Nagai R, Kitaura Y, Sasaguri T, Komuro I	Ca^{2+}/calmodulin-dependent kinase IIdelta causes heart failure by accumulation of p53 in dilated cardiomyopathy	Circulation	122	891-899	2010
Ueda K, Takano H, Niitsuma Y, Hasegawa H, Uchiyama R, Oka T, Miyazaki M, Nakaya H, Komuro I	Sonic hedgehog is a critical mediator of erythropoietin-induced cardiac protection in mice	J Clin Invest	120	2016-2029	2010
Ikeda H, Shiojima I, Oka T, Yoshida M, Maemura K, Walsh K, Igarashi T, Komuro I	Akt-mTOR signaling in lung epithelium is associated with respiratory distress syndrome in mice	Mol Cell Biol	31	1054-1065	2011

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Toko H, Takahashi H, Kayama Y, Okada S, Minamino T, Terasaki F, Kitaura Y, Komuro I	ATF6 is important under both pathological and physiological states in the heart	J Mol Cell Cardiol	49	113-120	2010
Aoki A, Ozaki K, Sato H, Takahashi A, Kubo M, Sakata Y, Onouchi Y, Kawaguchi T, Lin TH, Takano H, Yasutake M, Hsu PC, Ikegawa S, Kamatani N, Tsunoda T, Juo SH, Hori M, Komuro I, Mizuno K, Nakamura Y, Tanaka T	SNPs on chromosome 5p15.3 associated with myocardial infarction in Japanese population	J Hum Gene	56	47-51	2011
Shioyama W, Nakaoka Y, Higuchi K, Minami T, Taniyama Y, Nishida K, Kidoya H, Sonobe T, Naito H, Arita Y, Hashimoto T, Kuroda T, Fujio Y, Shirai M, Takakura N, Morishita R, Yamauchi-Takihara K, Kodama T, Hirano T, Mochizuki N, Komuro I	Docking Protein Gab1 Is an Essential Component of Postnatal Angiogenesis After Ischemia via HGF/c-Met Signaling	Circ Res	108	664-675	2011

LETTERS

IGFBP-4 is an inhibitor of canonical Wnt signalling required for cardiogenesis

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Insulin-like growth-factor-binding proteins (IGFBPs) bind to and modulate the actions of insulin-like growth factors (IGFs)[1]. Although some of the actions of IGFBPs have been reported to be independent of IGFs, the precise mechanisms of IGF-independent actions of IGFBPs are largely unknown[1,2]. Here we report a previously unknown function for IGFBP-4 as a cardiogenic growth factor. IGFBP-4 enhanced cardiomyocyte differentiation *in vitro*, and knockdown of *Igfbp4* attenuated cardiomyogenesis both *in vitro* and *in vivo*. The cardiogenic effect of IGFBP-4 was independent of its IGF-binding activity but was mediated by the inhibitory effect on canonical Wnt signalling. IGFBP-4 physically interacted with a Wnt receptor, Frizzled 8 (Frz8), and a Wnt co-receptor, low-density lipoprotein receptor-related protein 6 (LRP6), and inhibited the binding of Wnt3A to Frz8 and LRP6. Although IGF-independent, the cardiogenic effect of IGFBP-4 was attenuated by IGFs through IGFBP-4 sequestration. IGFBP-4 is therefore an inhibitor of the canonical Wnt signalling required for cardiogenesis and provides a molecular link between IGF signalling and Wnt signalling.

The heart is the first organ to form during embryogenesis, and abnormalities in this process result in congenital heart diseases, the most common cause of birth defects in humans[3]. Molecules that mediate cardiogenesis are of particular interest because of their potential use for cardiac regeneration[4,5]. Previous studies have shown that soluble growth factors such as bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), Wnts and Wnt inhibitors mediate the tissue interactions that are crucial for cardiomyocyte specification[3,4]. We proposed that there might be additional soluble factors that modulate cardiac development and/or cardiomyocyte differentiation.

P19CL6 cells differentiate into cardiomyocytes with high efficiency in the presence of 1% dimethylsulphoxide (DMSO)[6]. We cultured P19CL6 cells with culture media conditioned by various cell types in the absence of DMSO, and screened the cardiogenic activity of the conditioned media. The extent of cardiomyocyte differentiation was assessed by the immunostaining with MF20 monoclonal antibody that recognizes sarcomeric myosin heavy chain (MHC). Among the several cell types tested, culture media conditioned by a murine stromal cell line OP9 induced cardiomyocyte differentiation of P19CL6 cells without DMSO treatment (Fig. 1a, left and middle panels). Increased MF20-positive area was accompanied by the induction of cardiac marker genes such as *αMHC*, *Nkx2.5* and *GATA-4*, and by the increased protein levels of cardiac troponin T (cTnT) (Fig. 1a, right panel). In contrast, culture media conditioned by COS7 cells, mouse embryonic fibroblasts, NIH3T3 cells, HeLa cells, END2 cells (visceral endoderm-like cells), neonatal rat cardiomyocytes and neonatal rat cardiac fibroblasts did not induce cardiomyocyte differentiation of P19CL6 cells in the absence of DMSO (Fig. 1a and data not shown). From these observations, we postulated that OP9 cells secrete one or more cardiogenic growth factors.

To identify an OP9-derived cardiogenic factor, complementary DNA clones isolated by a signal sequence trap method from an OP9 cell cDNA library[7] were tested for their cardiogenic activities by transient transfection. When available, recombinant proteins were also used to confirm the results. Among candidate factors tested, IGFBP-4 induced cardiomyocyte differentiation of P19CL6 cells, as demonstrated by the increase in MF20-positive area and the induction of cardiac markers (Fig. 1b). We also cultured P19CL6 cells with OP9-conditioned media pretreated with an anti-IGFBP-4 neutralizing antibody. The application of an anti-IGFBP-4 neutralizing antibody attenuated the efficiency of cardiomyocyte differentiation induced by OP9-conditioned media (Fig. 1c). These findings strongly suggest that IGFBP-4 is a cardiogenic factor secreted from OP9 cells.

Because IGFBPs have been characterized as molecules that bind to and modulate the actions of IGFs, we tested whether IGFBP-4 promotes cardiogenesis by either enhancing or inhibiting the actions of IGFs. We first treated P19CL6 cells with a combination of anti-IGF-I and IGF-II-neutralizing antibodies or a neutralizing antibody against type-I IGF receptor. If IGFBP-4 induces cardiomyocyte differentiation by inhibiting IGF signalling, treatment with these antibodies should induce cardiomyocyte differentiation and/or enhance the cardiogenic effects of IGFBP-4. In contrast, if IGFBP-4 promotes cardiogenesis by enhancing IGF signalling, treatment with these antibodies should attenuate IGFBP-4-mediated cardiogenesis. However, treatment with these antibodies did not affect the efficiency of IGFBP-4-induced cardiomyocyte differentiation (Fig. 1d and data not shown). Treatment of P19CL6 cells with IGF-I and IGF-II also did not induce cardiomyocyte differentiation (data not shown). Furthermore, treatment with an IGFBP-4 mutant (IGFBP-4-H74P; His 74 replaced by Pro)[8] that is unable to bind IGFs induced cardiomyocyte differentiation of P19CL6 cells even more efficiently than wild-type IGFBP-4 (Fig. 1e). This is presumably due to the sequestration of wild-type IGFBP-4 but not mutant IGFBP-4-H74P by endogenous IGFs. In agreement with this idea, exogenous IGFs attenuated wild-type IGFBP-4-induced but not IGFBP-4-H74P-induced cardiogenesis (Fig. 1f). Taken together, these observations indicate

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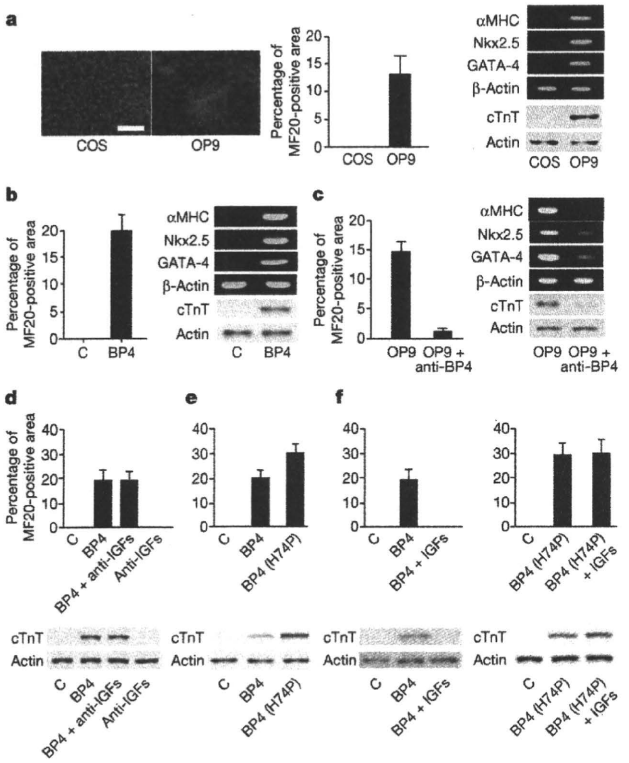

Figure 1 | IGFBP-4 promotes cardiomyocyte differentiation in an IGF-independent manner. a, Culture media conditioned by OP9 cells but not by COS7 cells induced cardiomyocyte differentiation of P19CL6 cells as assessed by MF20-positive area, cardiac marker-gene expression and cTnT protein expression. Scale bar, 100 μm. Error bars show s.d. **b,** Treatment with IGFBP-4 (1 μg ml^{-1}) induced cardiomyocyte differentiation of P19CL6 cells in the absence of DMSO. Error bars show s.d. **c,** Treatment with a neutralizing antibody against IGFBP-4 (anti-BP4; 40 μg ml^{-1}) attenuated cardiomyocyte differentiation of P19CL6 cells induced by OP9-conditioned media. Error bars show s.d. **d,** Treatment with neutralizing antibodies against IGF-I and IGF-II (anti-IGFs; 5 μg ml^{-1} each) had no effect on IGFBP-4-induced cardiomyocyte differentiation of P19CL6 cells. Error bars show s.d. **e,** Mutant IGFBP-4 (BP4(H74P)) that is incapable of binding to IGFs retained cardiomyogenic activity. Error bars show s.d. **f,** IGFs (100 ng ml^{-1} each) attenuated wild-type IGFBP-4-induced but not mutant IGFBP-4-H74P-induced cardiomyocyte differentiation of P19CL6 cells. Error bars show s.d.

that IGFBP-4 induces cardiomyocyte differentiation in an IGF-independent fashion.

To explore further the mechanisms by which IGFBP-4 induces cardiomyogenesis, we tested the hypothesis that IGFBP-4 might modulate the signals activated by other secreted factors implicated in cardiogenesis. It has been shown that canonical Wnt signalling is crucial in cardiomyocyte differentiation[3,4]. In P19CL6 cells, Wnt3A treatment activated β-catenin-dependent transcription of the TOPFLASH reporter gene, and this activation was attenuated by IGFBP-4 (Fig. 2a). Wnt/β-catenin signalling is transduced by the cell-surface receptor complex consisting of Frizzled and low-density-lipoprotein receptor (LDLR)-related protein 5/6 (LRP5/6)[9] and IGFBP-4 attenuated TOPFLASH activity enhanced by the expression of LRP6 or Frizzled 8 (Frz8) (Fig. 2a). As a control, IGFBP-4 did not alter BMP-mediated activation of a BMP-responsive reporter BRE–luc (Supplementary Fig. 1b). These findings suggest that IGFBP-4 is a specific inhibitor of the canonical Wnt pathway. To examine this possibility *in vivo*, we performed axis duplication assays in *Xenopus* embryos. Injection of *Xwnt8* or *Lrp6* mRNA caused secondary axis formation, and injection of *Xenopus IGFBP-4* (*XIGFBP-4*) mRNA alone had minimal effects on axis formation. However, Xwnt8-induced or LRP6-induced secondary axis formation was efficiently blocked by coexpression of XIGFBP-4 (Fig. 2b, c), indicating that IGFBP-4 inhibits canonical Wnt signalling *in vivo*. To explore the mechanisms of Wnt inhibition by IGFBP-4, *Xenopus* animal cap assays and TOPFLASH reporter gene assays were performed. In animal cap assays, IGFBP-4 inhibited LRP6-induced but not β-catenin-induced Wnt-target gene expression (Supplementary Fig. 1c). Similarly, IGFBP-4 attenuated Wnt3A-induced or LRP6-induced TOPFLASH activity but did not alter Dishevelled-1 (Dvl-1)-induced, LiCl-induced or β-catenin-induced TOPFLASH activity (Supplementary Fig. 1d, e). These findings suggest that IGFBP-4 inhibits canonical Wnt signalling at the level of cell-surface receptors. To examine whether IGFBP-4 antagonizes Wnt signalling via direct physical interaction with LRP5/6 or Frizzled, we produced conditioned media containing the Myc-tagged extracellular portion of LRP6 (LRP6N–Myc), the Myc-tagged cysteine-rich domain (CRD) of Frz8 (Frz8CRD–Myc), and V5-tagged IGFBP-4 (IGFBP-4–V5). Immunoprecipitation (IP)/western blot experiments revealed that IGFBP-4 interacted with LRP6N (Fig. 2d) and Frz8CRD (Fig. 2e). A liquid-phase binding assay with ^{125}I-labelled IGFBP-4 and conditioned media containing LRP6N–Myc or Frz8CRD–Myc demonstrated that the interaction between IGFBP-4 and LRP6N or Frz8CRD was specific and saturable (Fig. 2f, g). A Scatchard plot analysis revealed two binding sites with different binding affinities for LRP6N (Fig. 2f, inset) and a single binding site for Frz8CRD (Fig. 2g, inset). A similar binding assay with ^{125}I-labelled Wnt3A demonstrated that IGFBP-4 inhibited Wnt3A binding to LRP6N (Fig. 2h) and Frz8CRD (Fig. 2i), and a Lineweaver–Burk plot revealed that IGFBP-4 was a competitive inhibitor of the binding of Wnt3A to Frz8CDR (Supplementary Fig. 2a). IP/western blot analyses with various deletion mutants of LRP6 and IGFBP-4 revealed that IGFBP-4 interacted with multiple domains of LRP6 and that the carboxy-terminal thyroglobulin domain of IGFBP-4 was required for IGFBP-4 binding to LRP6 or Frz8CRD (Supplementary Fig. 2b–f). It has been shown that inhibition of canonical Wnt signalling promotes cardiomyocyte differentiation in embryonic stem (ES) cells and in chick, *Xenopus* and zebrafish embryos[4,10,11]. These results therefore collectively suggest that IGFBP-4 promotes cardiogenesis by antagonizing the Wnt/β-catenin pathway through direct interactions with Frizzled and LRP5/6.

Next we investigated the role of endogenous IGFBP-4 in P19CL6 cell differentiation into cardiomyocytes. Reverse transcriptase-mediated polymerase chain reaction (RT–PCR) analysis revealed that the expression of *Igfbp4* was upregulated during DMSO-induced P19CL6 cell differentiation (Fig. 3a). Expression of *Igfbp3* and *Igfbp5* was also upregulated in the early and the late phases of differentiation, respectively. Expression of *Igfbp2* was not altered, and that of *Igfbp1* or *Igfbp6* was not detected. When IGFBP-4 was knocked down by two different small interfering RNA (siRNA) constructs, DMSO-induced cardiomyocyte differentiation was inhibited in both cases (Fig. 3b). In contrast, knockdown of *Igfbp3* or *Igfbp5* did not inhibit DMSO-induced cardiomyocyte differentiation (Fig. 3b, right panel). Treatment with an anti-IGFBP-4 neutralizing antibody also blocked DMSO-induced cardiomyocyte differentiation (Fig. 3c). Secretion of endogenous IGFBP-4 is therefore required for the differentiation of P19CL6 cells into cardiomyocytes. Immunostaining for IGFBP-4 revealed that cardiac myocytes were surrounded by the IGFBP-4-positive cells, suggesting that a paracrine effect of IGFBP-4 on cardiomyocyte differentiation is predominant (Fig. 3d). Essentially the same results were obtained in ES cells (Supplementary Fig. 3d–g). To investigate whether IGFBP-4 promotes the differentiation of P19CL6 cells into cardiomyocytes by the inhibition of the canonical Wnt pathway, we expressed dominant-negative LRP6 (LRP6N) in P19CL6 cells. Expression of LRP6N enhanced cardiomyocyte differentiation of P19CL6 cells and reversed the inhibitory effect of *Igfbp4*

knockdown on cardiomyogenesis (Fig. 3e). These observations suggest that endogenous IGFBP-4 is required for cardiomyocyte differentiation of P19CL6 cells and ES cells, and that the cardiogenic effect of IGFBP-4 is mediated by its inhibitory effect on Wnt/β-catenin signalling.

The role of endogenous IGFBP-4 in cardiac development *in vivo* was also examined with *Xenopus* embryos. Whole-mount *in situ* hybridization analysis revealed that strong expression of *XIGFBP-4* was detected at stage 38 in the anterior part of the liver adjacent to the heart (Fig. 4a). Knockdown of *XIGFBP-4* by two different morpholino (MO) constructs resulted in cardiac defects, with more than 70% of the embryos having a small heart or no heart (Fig. 4b). The specificity of MO was confirmed by the observation that simultaneous injection of MO-resistant *XIGFBP-4* cDNA rescued the MO-induced cardiac defects (Fig. 4b, Supplementary Fig. 4c). Coexpression of IGF-binding-defective XIGFBP-4 mutant (XIGFBP-4-H74P) or dominant-negative LRP6 (LRP6N) also rescued the cardiac defects induced by *XIGFBP-4* knockdown (Fig. 4b), whereas overexpression of Xwnt8 in the heart-forming region resulted in cardiac defects similar to those induced by *XIGFBP-4* knockdown (Supplementary Fig. 4d–f), supporting the notion that the cardiogenic effect of IGFBP-4 is independent of IGFs but is mediated by inhibition of the Wnt/β-catenin pathway. The temporal profile of cardiac defects induced by *XIGFBP-4* knockdown was also examined by *in situ* hybridization with *cardiac troponin I* (*cTnI*) (Fig. 4c). At stage 34, morphology of the heart was comparable between control embryos and MO-injected embryos. However, at stage 38, when *XIGFBP-4* starts to be expressed in the anterior part of the liver, the expression of *cTnI* was markedly attenuated in MO-injected embryos; expression of *cTnI* was diminished and no heart-like structure was observed at stage 42. Thus, the heart is initially formed but its subsequent growth is perturbed in the absence of XIGFBP-4, suggesting that IGFBP-4

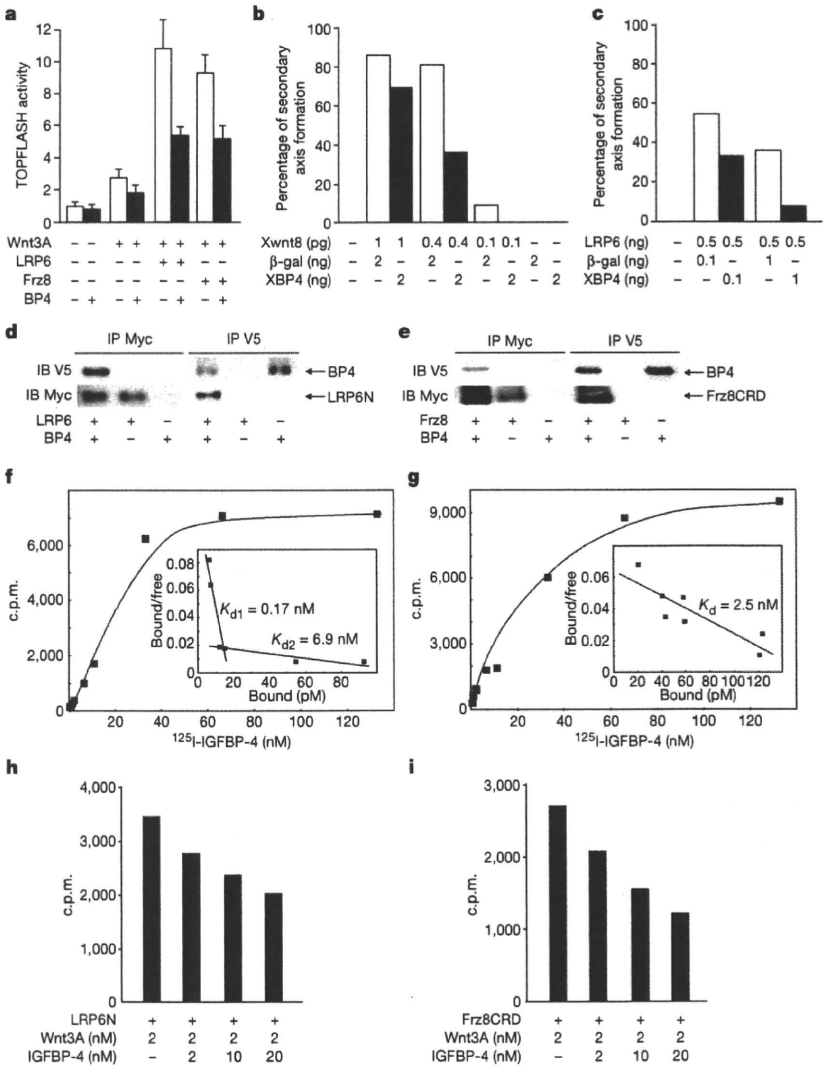

Figure 2 | IGFBP-4 inhibits Wnt/β-catenin signalling through direct interactions with Wnt receptors. **a**, IGFBP-4 attenuated β-catenin-dependent transcription in P19CL6 cells. P19CL6 cells were transfected with TOPFLASH reporter gene and expression vectors for LRP6 or Frz8, and then treated with Wnt3A or Wnt3A plus IGFBP-4; luciferase activities were then measured. Error bars show s.d. **b**, XIGFBP-4 (XBP4) inhibited Xwnt8-induced secondary-axis formation in *Xenopus* embryos ($n = 20$ for each group). **c**, IGFBP-4 inhibited LRP6-induced secondary-axis formation in *Xenopus* embryos ($n = 30$ for each group). **d**, **e**, IGFBP-4 interacted directly with LRP6N (**d**) and Frz8CRD (**e**). IB, immunoblotting; IP, immunoprecipitation. **f**, A binding assay between ^{125}I-labelled IGFBP-4 and LRP6N. The inset is a Scatchard plot showing two binding sites with different binding affinities. **g**, A binding assay between ^{125}I-labelled IGFBP-4 and Frz8CRD. The inset is a Scatchard plot showing a single binding site. **h**, **i**, IGFBP-4 inhibited Wnt3A binding to LRP6N (**h**) or Frz8CRD (**i**). ^{125}I-labelled Wnt3A binding to LRP6N or Frz8CRD was assessed in the presence of increasing amounts of IGFBP-4.

promotes cardiogenesis by maintaining the proliferation and/or survival of embryonic cardiomyocytes.

It has been shown that canonical Wnt signals inhibit cardiogenesis in chick and frog embryos, and that Wnt antagonists such as Dkk1 and Crescent secreted from the anterior endoderm or the organizer region counteract the Wnt-mediated inhibitory signals and induce cardiogenesis in the anterior lateral mesoderm[4]. However, IGFBP-4-mediated Wnt inhibition is required at later stages of development, when the heart is already formed at the ventral portion and starts to grow and remodel to maintain embryonic circulation. It has been shown that Wnt/β-catenin signalling has time-dependent effects on cardiogenesis in ES cells: canonical Wnt signalling in the early phase of ES-cell differentiation promotes cardiomyogenesis, whereas it inhibits cardiomyocyte differentiation in the late phase[10–12]. In agreement with this notion, IGFBP-4 promoted cardiomyocyte differentiation of ES cells only when IGFBP-4 was applied in the late phase after embryoid body formation (Supplementary Fig. 3a–c). Similar time-dependent effects of Wnt/β-catenin signalling on cardiogenesis has been shown in zebrafish embryos[11]. Moreover, several recent reports suggest that Wnt/β-catenin signalling is a positive regulator of cardiac progenitor-cell proliferation in the secondary heart field[13]. It therefore seems that canonical Wnt signalling has divergent effects on cardiogenesis at multiple stages of development: first, canonical Wnt signalling promotes cardiogenesis at the time of gastrulation or mesoderm specification; second, it inhibits cardiogenesis at the time when cardiac mesoderm is specified in the anterior lateral mesoderm; third, it promotes the expansion of cardiac progenitors in the secondary heart field; and fourth, it inhibits cardiogenesis at later stages when the embryonic heart is growing. It is interesting to note that IGFBP-4 is expressed predominantly in the liver. Mouse IGFBP-4 is

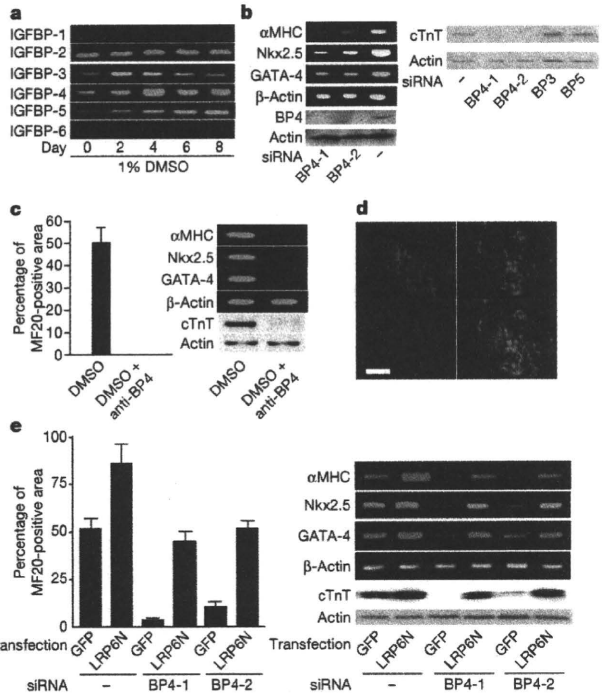

Figure 3 | IGFBP-4 is required for the differentiation of P19CL6 cells into cardiomyocytes. **a**, Expression analysis of IGFBP family members by RT–PCR during DMSO-induced cardiomyocyte differentiation of P19CL6 cells (from day 0 to day 8). **b**, Left: knockdown of *Igfbp4* in P19CL6 cells attenuated cardiac marker expression in response to treatment with DMSO. BP4-1 and BP4-2 represent two different siRNAs for IGFBP-4. Right: knockdown of *Igfbp3* or *Igfbp5* had no effect on cTnT expression in response to DMSO treatment. **c**, Treatment with a neutralizing antibody against IGFBP-4 (anti-BP4; 40 μg ml^{-1}) attenuated DMSO-induced cardiomyocyte differentiation of P19CL6 cells. Error bars show s.d. **d**, IGFBP-4 immunostaining during DMSO-induced differentiation of P19CL6 cells stably transfected with αMHC–green fluorescent protein (GFP) reporter gene. Top left, IGFBP-4 staining (red); top right, GFP expression representing differentiated cardiomyocytes; bottom left, nuclear staining with DAPI (4′,6-diamidino-2-phenylindole); bottom right, a merged picture. Scale bar, 100 μm. **e**, Attenuated cardiomyocyte differentiation of P19CL6 cells by *Igfbp4* knockdown was rescued by inhibiting Wnt/β-catenin signalling. Control and *Igfbp4*-knocked-down P19CL6 cells were transfected with an expression vector for GFP or LRP6N (a dominant-negative form of LRP6) and induced to differentiate into cardiomyocytes by treatment with DMSO. LRP6N overexpression rescued the attenuated cardiomyocyte differentiation induced by *Igfbp4* knockdown as assessed by MF20-positive area (left panel), cardiac marker-gene expression and cTnT protein expression (right panel). Error bars show s.d.

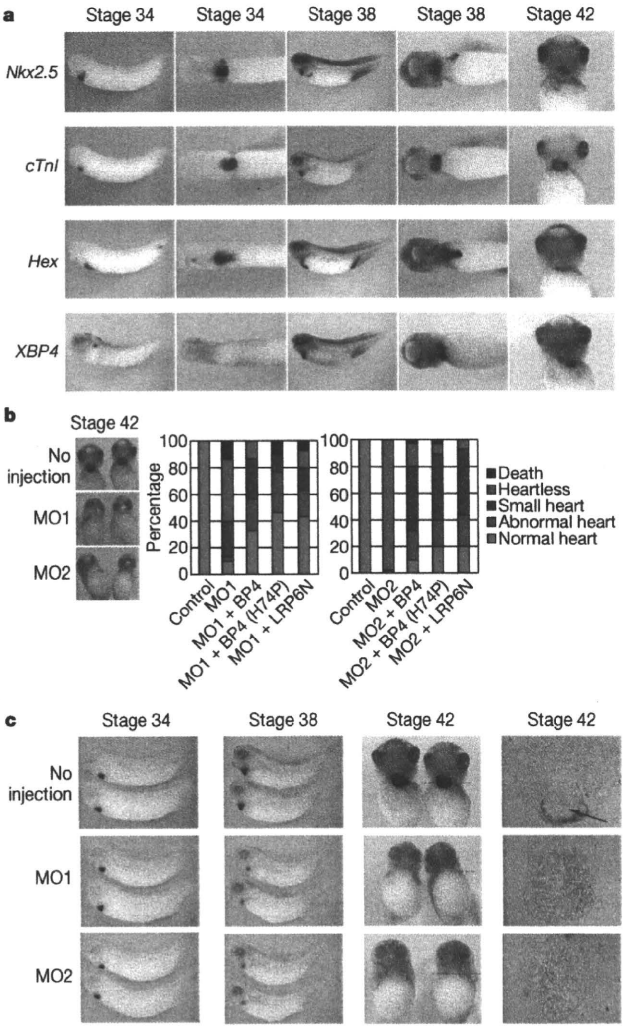

Figure 4 | IGFBP-4 is required for the maturation of the heart in *Xenopus* embryos. **a**, *In situ* hybridization analysis of *Nkx2.5* (an early cardiac marker), *cTnI* (a mature cardiac marker), *Hex* (a liver marker), and *XIGFBP-4* (*XBP4*) mRNA expression at stages 34, 38 and 42. **b**, Knockdown of *XIGFBP-4* by two different morpholinos (MO1 and MO2) resulted in severe cardiac defects as assessed by *cTnI in situ* hybridization at stage 42 (left). These cardiac defects were rescued by simultaneous injection of MO-resistant wild-type XIGFBP-4, mutant XIGFBP-4-H74P (BP4(H74P) and LRP6N ($n = 30$ for each group). **c**, Temporal profile of cardiac defects induced by *XIGFBP-4* knockdown. Morphology of the heart as assessed by cTnI *in situ* hybridization was almost normal at stage 34 but was severely perturbed at stages 38 and 42. The right column shows sections of control and MO-injected embryos. The arrow indicates the heart in control embryos. No heart-like structure was observed in MO-injected embryos.

also strongly expressed in the tissues adjacent to the heart such as pharyngeal arches and liver bud at embryonic day (E)9.5 (Supplementary Fig. 3h). These observations and the results of IGFBP-4 immunostaining in P19CL6 cells and ES cells suggest that IGFBP-4 promotes cardiogenesis in a paracrine fashion. Together with a previous report showing that cardiac mesoderm secretes FGFs and induces liver progenitors in the ventral endoderm[14], these observations suggest that there exist reciprocal paracrine signals between the heart and the liver that coordinately promote the development of each other.

IGFBPs are composed of six members, IGFBP-1 to IGFBP-6. Reporter gene assays and β-catenin stabilization assays revealed that IGFBP-4 was the most potent canonical Wnt inhibitor and that IGFBP-1, IGFBP-2 and IGFBP-6 also showed modest activity in Wnt inhibition, whereas IGFBP-3 and IGFBP-5 had no such activity (Supplementary Fig. 5a–c). In agreement with this, IP/western blot analyses demonstrated that IGFBP-1, IGFBP-2, IGFBP-4 and IGFBP-6 but not IGFBP-3 or IGFBP-5 interacted with LRP6 or Frz8CRD (Supplementary Fig. 5d, e). Thus, the lack of cardiac phenotypes in IGFBP-4-null mice or IGFBP-3/IGFBP-4/IGFBP-5 triple knockout mice[15] may be due to genetic redundancies between IGFBP-4 and other IGFBPs such as IGFBP-1, IGFBP-2 and/or IGFBP-6.

The identification of IGFBP-4 as an inhibitor of Wnt/β-catenin signalling may also have some implications for cancer biology[16]. It was shown that treatment with IGFBP-4 reduces cell proliferation in some cancer cell lines *in vitro*, and that overexpression of IGFBP-4 attenuates the growth of prostate cancer *in vivo*. Decreased serum levels of IGFBP-4 are associated with the risk of breast cancer. Because the activation of Wnt signalling is implicated in several forms of malignant tumours[17,18], it is possible that the inhibitory effect of IGFBP-4 on cell proliferation is mediated in part by the inhibition of canonical Wnt signalling.

METHODS SUMMARY

Cell culture. P19CL6 cells and ES cells were cultured and induced to differentiate into cardiomyocytes essentially as described[6,10]. P19CL6 cells (2,000 cells per 35-mm dish) were treated with various conditioned media for screening of their cardiogenic activities. For siRNA-mediated knockdown, pSIREN-RetroQ vectors (Clontech) ligated with double-stranded oligonucleotides were transfected into P19CL6 cells or ES cells, and puromycin-resistant clones were selected.

IP/western blot analyses and binding assays. Conditioned media for IP/western blot analyses were produced by using 293 cells. Binding reactions were performed overnight at 4 °C. ^{125}I-labelling of IGFBP-4 and Wnt3A was performed with IODO-BEADS Iodination Reagent (Pierce). A liquid-phase binding assay was performed essentially as described[19].

***Xenopus* experiments.** Axis duplication assays, animal cap assays, and *in situ* hybridization analyses in *Xenopus* were performed essentially as described[20]. Electroporation of mRNA was performed at stage 28 essentially as described[21].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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1. Firth, S. M. & Baxter, R. C. Cellular actions of the insulin-like growth factor binding proteins. *Endocr. Rev.* **23,** 824–854 (2002).
2. Mohan, S. & Baylink, D. J. IGF-binding proteins are multifunctional and act via IGF-dependent and -independent mechanisms. *J. Endocrinol.* **175,** 19–31 (2002).
3. Olson, E. N. & Schneider, M. D. Sizing up the heart: development redux in disease. *Genes Dev.* **17,** 1937–1956 (2003).
4. Foley, A. & Mercola, M. Heart induction: embryology to cardiomyocyte regeneration. *Trends Cardiovasc. Med.* **14,** 121–125 (2004).
5. Leri, A., Kajstura, J. & Anversa, P. Cardiac stem cells and mechanisms of myocardial regeneration. *Physiol. Rev.* **85,** 1373–1416 (2005).
6. Monzen, K. *et al.* Bone morphogenetic proteins induce cardiomyocyte differentiation through the mitogen-activated protein kinase kinase kinase TAK1 and cardiac transcription factors Csx/Nkx-2.5 and GATA-4. *Mol. Cell. Biol.* **19,** 7096–7105 (1999).
7. Ueno, H. *et al.* A stromal cell-derived membrane protein that supports hematopoietic stem cells. *Nature Immunol.* **4,** 457–463 (2003).
8. Qin, X., Strong, D. D., Baylink, D. J. & Mohan, S. Structure–function analysis of the human insulin-like growth factor binding protein-4. *J. Biol. Chem.* **273,** 23509–23516 (1998).
9. Moon, R. T., Kohn, A. D., De Ferrari, G. V. & Kaykas, A. WNT and β-catenin signalling: diseases and therapies. *Nature Rev. Genet.* **5,** 691–701 (2004).
10. Naito, A. T. *et al.* Developmental stage-specific biphasic roles of Wnt/β-catenin signaling in cardiomyogenesis and hematopoiesis. *Proc. Natl Acad. Sci. USA* **103,** 19812–19817 (2006).
11. Ueno, S. *et al.* Biphasic role for Wnt/β-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proc. Natl Acad. Sci. USA* **104,** 9685–9690 (2007).
12. Liu, Y. *et al.* Sox17 is essential for the specification of cardiac mesoderm in embryonic stem cells. *Proc. Natl Acad. Sci. USA* **104,** 3859–3864 (2007).
13. Cohen, E. D., Tian, Y. & Morrisey, E. E. Wnt signaling: an essential regulator of cardiovascular differentiation, morphogenesis and progenitor self-renewal. *Development* **135,** 789–798 (2008).
14. Jung, J., Zheng, M., Goldfarb, M. & Zaret, K. S. Initiation of mammalian liver development from endoderm by fibroblast growth factors. *Science* **284,** 1998–2003 (1999).
15. Ning, Y. *et al.* Diminished growth and enhanced glucose metabolism in triple knockout mice containing mutations of insulin-like growth factor binding protein-3, -4, and -5. *Mol. Endocrinol.* **20,** 2173–2186 (2006).
16. Durai, R. *et al.* Biology of insulin-like growth factor binding protein-4 and its role in cancer. *Int. J. Oncol.* **28,** 1317–1325 (2006).
17. Logan, C. Y. & Nusse, R. The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20,** 781–810 (2004).
18. Clevers, H. Wnt/β-catenin signaling in development and disease. *Cell* **127,** 469–480 (2006).
19. Semenov, M. V. *et al.* Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. *Curr. Biol.* **11,** 951–961 (2001).
20. Kobayashi, H. *et al.* Novel Daple-like protein positively regulates both the Wnt/β-catenin pathway and the Wnt/JNK pathway in *Xenopus*. *Mech. Dev.* **122,** 1138–1153 (2005).
21. Sasagawa, S., Takabatake, T., Takabatake, Y., Muramatsu, T. & Takeshima, K. Improved mRNA electroporation method for *Xenopus* neurula embryos. *Genesis* **33,** 81–85 (2002).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions W.Z., I.S. and Y.I. contributed equally to this work. I.K. designed and supervised the research. W.Z., I.S., Y.I., Z.L., H.I., M.Y. and A.T.N. performed experiments, J.N., H.U., A.U., T.M., T.N., A.K. and M.A. contributed new reagents and/or analytical tools. W.Z., I.S., Y.I., A.K. and I.K. analysed data. W.Z., I.S., Y.I. and I.K. prepared the manuscript.

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