厚生労働科学研究費補助金

難治性疾患克服研究事業

新規拡張型心筋症モデルマウスを用いた拡張型心筋症発症機序の解明

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研究代表者　小室　一成

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総括研究報告書

新規拡張型心筋症モデルマウスを用いた拡張型心筋症発症機序の解明

研究代表者　小室一成　千葉大学大学院医学研究院循環病態医科学　教授

研究要旨　拡張型心筋症の原因の約30%は遺伝子変異であるが、それらの遺伝子変異により拡張型心筋症を発症する機序に関しては不明である。最近我々が確立した拡張型心筋症モデルマウスを用い、その機序の解明を目的に実験を行った。この拡張型心筋症モデルマウスでは、カルシウム依存性脱リン酸化酵素 calcineurin とリン酸化酵素 CaMKIIδ の活性が亢進し、癌抑制遺伝子 p53 の発現量が増加していた。calcineurin の阻害薬では心機能の改善は認めず、CaMKIIδ の阻害薬で心機能の改善を認めた。また p53 遺伝子欠損マウスとの交配により、拡張型心筋症モデルマウスの心機能は改善した。以上の結果より、CaMKIIδ と p53 が遺伝子変異による拡張型心筋症の発症に重要であることが示唆された。

（研究代表者）

小室一成　千葉大学大学院医学研究院
循環病態医科学 教授

（研究分担者）

塩島一郎　大阪大学大学院医学系研究科
先進心血管治療学寄附講座
准教授

赤澤宏　千葉大学大学院医学研究院
循環病態医科学 講師

東口治弘　千葉大学大学院医学研究院
循環病態医科学 助教

A. 研究目的

拡張型心筋症の予後は、非常に不良であり、現在拡張型心筋症の最終的な治療法は心臓移植しかなく、新たな治療法の確立が切望されている。拡張型心筋症の原因の約30%が遺伝子変異であることが明らかとなってきたが、それらの遺伝子変異により収縮機能不全をきたす機序は未だ不明である。この発症機序の解明が新規の治療法の開発に必要であると考え、拡張型心筋症の発症機序を明らかにすることを本研究の目的とする。

B. 研究方法

これまで拡張型心筋症の発症機序が明らかにならなかった理由の一つに、拡張型心筋症を解析するための適切なモデル動物が存在しなかったことが挙げられる。ヒト拡張型心筋症の原因遺伝子のひとつに、心筋αアクチン遺伝子がある。我々は、ヒトで報告されている変異型心筋αアクチン遺伝子を心臓特異的に過剰発現させた遺伝子改変マウスを作成し、拡張型心筋症モデルの確立に成功した。初年度（20年度）までの予備的な研究で，拡張型心筋症モデルマウスでカルシウム依存性脱リン酸化酵素 calcineurin とリン酸化酵素 CaMKIIδ の活性が亢進し、癌抑制遺伝子 p53 の発現量が増加していることを見いだしていた。これらの活性化および蛋白量の増加の重要性を検討するため、この拡張型心筋症モデルマウスに calcineurin と CaMKIIδ のそれぞれの阻害薬を投与した。また p53

ヘテロノックアウトマウスと交配させた。

拡張型心筋症モデルで認めたcalcineurin、CaMKIIδの活性化やp53の発現増加が変異型心筋αアクチンの直接的な影響によるのか、もしくは心機能低下により誘導される神経液性因子による二次的なものか検討するため、拡張型心筋症モデルマウス作成に用いた変異型心筋αアクチン遺伝子を培養心筋細胞に導入した。

（倫理面への配慮）

実験動物を用いる研究については、千葉大学動物実験指針に準拠して研究を実施する。特に、動物愛護と動物福祉の観点から実験動物使用は、目的に合致した最小限にとどめる。またその際、麻酔等手段により苦痛を与えない等の倫理的配慮をおこない、実験者は、管理者と相互協力のもと適切な環境のもと飼育管理を行った。

C. 研究結果

1） カルシウム依存性脱リン酸化酵素calcineurinの役割

拡張型心筋症モデルマウスではcalcineurinの活性化が野生型と比較し増加していた。これまでcalcineurinは心不全発症に重要であることが報告されている。この拡張型心筋症モデルマウスでもcalcineurinが拡張型心筋症の発症に重要であると予想し、その阻害剤であるFK506を投与した。予想と反し、FK506投与による心拡大縮小効果や心機能改善効果は認めなかった。

2） カルシウム依存性リン酸化酵素CaMKIIδの役割

拡張型心筋症モデルマウスではCaMKIIδの活性化が野生型と比較し増加していた。CaMKIIδの過剰発現マウスは、心機能が低下することが報告されている。この拡張型心筋症モデルマウスでもCaMKIIδが拡張型心筋症の発症に重要であると予想し、その阻害剤であるKN-93を投与した。結果、KN-93投与により拡張型心筋症モデルマウスの心拡大を抑制し、心収縮力を改善した。

3） 癌抑制遺伝子p53の役割

拡張型心筋症モデルマウスではp53の蛋白量が野生型と比較し増加していた。癌抑制遺伝子であるp53が、心不全発症に重要であることを我々は最近報告している。この拡張型心筋症モデルマウスにおいてもp53の発現増加が拡張型心筋症の発症に関与しているのと予想し、p53遺伝子欠損マウスを購入し拡張型心筋症モデルマウスと交配した。結果、p53遺伝子欠損により拡張型心筋症モデルマウスの心臓サイズは減少し、心機能は改善した。

4） 培養心筋細胞への変異型心筋αアクチン遺伝子の導入

変異型心筋αアクチンと野生型心筋αアクチンの発現プラスミドを作成した。両者をそれぞれ培養心筋細胞に遺伝子導入し、mRNAおよび蛋白量の変化を検討した。結果、野生型心筋αアクチン遺伝子を導入した培養心筋細胞と比較し、変異型心筋αアクチン遺伝子を導入した培養心筋細胞では、CaMKIIδとp53の蛋白量の増加を認めた。

D. 考察

遺伝子変異による拡張型心筋症の発症にCaMKIIδの活性化とp53の蛋白量の増加が重要であることが明らかとなった。しかし、CaMKIIδを活性する機序やp53を増加させる機序に関しては不明であり、またそれぞれの標的因子に関しても不明である。さらに、CaMKIIδの抑制には薬理学

的な手法を用いたが、その効果が十分であるかははっきりしない。今後、CaMKIIδ と p53 のそれぞれの上流、下流の因子の検索および CaMKIIδ の遺伝子学的な抑制、具体的には CaMKIIδ 特異的阻害ペプチドの過剰発現マウスとの交配を行う予定である。

E. 結論

遺伝子変異による拡張型心筋症の発症に CaMKIIδ の活性化と p53 の蛋白量の増加が重要であることが明らかとなった。今後、両因子を標的とした治療が拡張型心筋症の治療となり得る可能性が示唆された。

F. 健康危険情報

該当なし

G. 研究発表

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（小室一成）

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・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」

・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」

・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」

・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」

・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「心肥大はなぜ恐いか！？癌より恐い心不全」

・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」

（赤澤 宏）

・第5回GPCR研究会（東京：平成20年5月9日）「インバースアゴニストによるアンジオテンシンII受容体活性の抑制機構」

・第12回日本心不全学会学術集会（東京：平成20年10月16-18日）シンポジウム「遺伝子改変マウスを用いた心不全・心肥大の病態解明」

Homeostatic role of PDK-1 in the regulation of beta-adrenergic response and cell survival in the hearts

・日本循環器学会学術集会（大阪：平成21年3月20-22日）Symposium 9. Cardiomyocyte Death and Cardiac Remodeling. Homeostatic role of Atg7 in normal and stressed hearts

（東口 治弘）

・成人病の病因病態に関する研究会（平成20年7月5-6日、軽井沢）「拡張型心筋症発症におけるCaMKIIδの役割」

海外

（小室 一成）

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

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

（赤澤 宏）

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H. 知的財産権の出願・登録状況

1. 特許取得　　該当なし
2. 実用新案登録　該当なし
3. その他　　該当なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
アデノウィルスコンストラクトの作成、in vitro の実験
分担研究報告書

研究分担者　塩島一朗　大阪大学大学院医学系研究科先進心血管治療学寄附講座 准教授

研究要旨　拡張型心筋症の原因の約 30%は遺伝子変異であるが、それらの遺伝子変異より拡張型心筋症を発症する機序に関しては不明である。この機序を明らかにするために、既にヒトの拡張型心筋症の原因として報告されている変異型心筋αアクチン遺伝子を培養心筋細胞に導入した。変異型心筋αアクチンにより、カルシウム依存性リン酸化酵素 CaMKIIδ と癌抑制遺伝子 p53 の発現量が増加した。

A. 研究目的

拡張型心筋症の予後は、非常に不良であり、現在拡張型心筋症の最終的な治療法は心臓移植しかなく、新たな治療法の確立が切望されている。拡張型心筋症の原因の約 30%が遺伝子変異であることが明らかとなってきたが、それらの遺伝子変異により収縮機能不全をきたす機序は未だ不明である。この発症機序の解明が新規の治療法の開発に必要であると考える。研究分担者は、新規に確立した拡張型心筋症モデルマウスを用い得られた結果に関して、培養心筋細胞を用いさらに詳細な機序の解明を行うことを目的とする。

B. 研究方法

1．野生型心筋αアクチンと変異型心筋αアクチンの発現プラスミドを作成した。両プラスミドには HA タグをつけた。

2．培養心筋細胞に野生型並びに変異型心筋αアクチンを遺伝子導入し、mRNA や蛋白レベルの変化を検討した。

C. 研究結果

1．作成した野生型心筋αアクチンと変異型心筋αアクチンの発現プラスミドを COS7 細胞に遺伝子導入し、発現を確認した。Western blot にて両プラスミドともに HA が発現することを確認した。

2．野生型心筋αアクチンと変異型心筋αアクチンの発現プラスミドを心筋細胞に導入した。結果、野生型心筋αアクチンと比較し変異型心筋αアクチンを導入した心筋細胞は CaMKIIδ および p53 の発現が増加した。

D. 考察

変異型心筋αアクチンの培養心筋細胞への遺伝子導入により、CaMKIIδ および p53 の発現が増加した。CaMKIIδ と p53 の発現増加の機序は現在不明であり、今後さらなる実験が必要と考えている。

E. 結論

拡張型心筋細胞の原因遺伝子変異の導入により、培養心筋細胞の CaMKIIδ と p53 の発現が増加した。

F. 健康危険情報

該当なし

G. 研究発表

1．論文発表

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H. 知的財産権の出願・登録状況

1. 特許取得 該当なし
2. 実用新案登録 該当なし
3. その他

厚生労働科学研究費補助金（難治性疾患克服研究事業）
遺伝子改変マウスの作成
分担研究報告書

研究分担者　赤澤宏　千葉大学大学院医学研究院循環病態医科学 講師

研究要旨　拡張型心筋症の原因の約30%は遺伝子変異であるが、それらの遺伝子変異より拡張型心筋症を発症する機序に関しては不明である。最近我々が確立した拡張型心筋症モデルマウスを用い、その機序の解明を目的に実験を行った。この拡張型心筋症モデルマウスでは、癌抑制遺伝子p53の発現量が増加していた。p53遺伝子欠損マウスとの交配により、拡張型心筋症モデルマウスの心機能は改善した。p53が遺伝子変異による拡張型心筋症の発症に重要であることが示唆された。

A. 研究目的

拡張型心筋症の予後は、非常に不良であり、現在拡張型心筋症の最終的な治療法は心臓移植しかなく、新たな治療法の確立が切望されている。拡張型心筋症の原因の約30%が遺伝子変異であることが明らかとなってきたが、それらの遺伝子変異により収縮機能不全をきたす機序は未だ不明である。この発症機序の解明が研究分担者の目的であり、そのために最近我々が確立した拡張型心筋症モデルマウスを用い実験を行った。

B. 研究方法

我々は最近、既にヒト拡張型心筋症の原因遺伝子として報告されている変異型心筋αアクチン遺伝子を心臓特異的に過剰発現させることにより、ヒトの拡張型心筋症の表現系と非常に類似した拡張型心筋症モデルマウスの確立に成功した。この拡張型心筋症モデルマウスでは、p53の蛋白量が野生型と比較し増加していた。癌抑制遺伝子であるp53が、心不全発症に重要であることを我々は最近報告している。この拡張型心筋症モデルマウスにおいてもp53の発現増加が拡張型心筋症の発症に関与しているのと考え、p53遺伝子欠損マウスを購入し拡張型心筋症モデルマウスと交配させた。

（倫理面への配慮）

実験動物を用いる研究については、千葉大学動物実験指針に準拠して研究を実施する。特に、動物愛護と動物福祉の観点から実験動物使用は、目的に合致した最小限にとどめる。またその際、麻酔等手段により苦痛を与えない等の倫理的配慮をおこない、実験者は、管理者と相互協力のもと適切な環境のもと飼育管理を行った。

C. 研究結果

本拡張型心筋症モデルマウスでは、野生型マウスと比較しp53およびその標的因子であるBaxの増加を認めた。

p53ヘテロノックアウトマウスと拡張型心筋症モデルマウスと交配した。p53ヘテロノックアウトマウスとの交配により、拡張型心筋症モデルマウスで認める左室内腔の拡大は軽減し、左室収縮力の低下は改善した。また、拡張型心筋症モデルマウスで認めたp53とBaxの増加は、p53ヘテロノックアウトマウスとの交配により減少した。

D. 考察

遺伝子変異による拡張型心筋症の発症にp53の増加が重要であることが明らかとなった。しかし、

p53 を増加させる機序や p53 増加による心機能低下の機序に関しては不明である。今後、さらなる詳細な解明が必要と考える。

E. 結論

遺伝子変異による拡張型心筋症の発症に p53 の増加が重要であることが示唆された。

F. 健康危険情報

該当なし

G. 研究発表

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・第12回日本心不全学会学術集会（東京：2008年10月16-18日）シンポジウム「遺伝子改変マウスを用いた心不全・心肥大の病態解明」
Homeostatic role of PDK-1 in the regulation of beta-adrenergic response and cell survival in the hearts

・日本循環器学会学術集会(大阪:2009年３月20-22日) Symposium 9. Cardiomyocyte Death and Cardiac Remodeling. Homeostatic role of Atg7 in normal and stressed hearts

H. 知的財産権の出願・登録状況

1. 特許取得　該当なし
2. 実用新案登録　該当なし
3. その他　該当なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
遺伝子改変マウスの解析
分担研究報告書
研究分担者　東口治弘　千葉大学大学院医学研究院循環病態医科学 助教

研究要旨　拡張型心筋症の原因の約30%は遺伝子変異であるが、それらの遺伝子変異より拡張型心筋症を発症する機序に関しては不明である。最近我々が確立した拡張型心筋症モデルマウスを用い、その機序の解明を目的に実験を行った。この拡張型心筋症モデルマウスでは、カルシウム依存性脱リン酸化酵素 calcineurin とリン酸化酵素 CaMKIIδ の活性が亢進していた。calcineurin の阻害薬では心機能の改善は認めず、CaMKIIδ の阻害薬で心機能の改善を認めた。CaMKIIδ が遺伝子変異による拡張型心筋症の発症に重要であることが示唆された。

A. 研究目的

拡張型心筋症の予後は、非常に不良であり、現在拡張型心筋症の最終的な治療法は心臓移植しかなく、新たな治療法の確立が切望されている。拡張型心筋症の原因の約30%が遺伝子変異であることが明らかとなってきたが、それらの遺伝子変異により収縮機能不全をきたす機序は未だ不明である。この発症機序の解明が新規の治療法の開発に必要であると考える。最近我々が確立した拡張型心筋症モデルマウスを用い、拡張型心筋症の発症機序を明らかにすることが研究分担者の目的である。

B. 研究方法

これまで拡張型心筋症の発症機序が明らかにならなかった理由の一つに、拡張型心筋症を解析するための適切なモデル動物が存在しなかったことが挙げられる。ヒト拡張型心筋症の原因遺伝子のひとつに、心筋αアクチン遺伝子がある。我々は、ヒトで報告されている変異型心筋αアクチン遺伝子を心臓特異的に過剰発現させた遺伝子改変マウスを作成し、拡張型心筋症モデルの確立に成功した。この拡張型心筋症モデルマウスを用いて、以下の実験を行った。

（1）FK506 投与

これまでの実験によりこの拡張型心筋症モデルマウスでは calcineurin の活性化が野生型と比較し増加していることを確認している。calcineurin の活性化が心不全発症に重要であることがこれまで報告されている。この拡張型心筋症モデルマウスでも calcineurin が拡張型心筋症の発症に重要であると考え、calcineurin 阻害剤である FK506 を2ヶ月齢より投与し、心エコー検査で心機能を評価した。

（2）KN-93 投与

この拡張型心筋症モデルマウスでは CaMKIIδ の活性化が野生型と比較し増加していた。CaMKIIδ の過剰発現マウスは、心機能が低下することが報告されている。この拡張型心筋症モデルマウスでも CaMKIIδ が拡張型心筋症の発症に重要であると考え、CaMKIIδ の阻害剤である KN-93 を2ヶ月齢より3ヶ月間投与し、心機能を評価した。

（倫理面への配慮）

実験動物を用いる研究については、千葉大学動物実験指針に準拠して研究を実施する。特に、動

物愛護と動物福祉の観点から実験動物使用は、目的に合致した最小限にとどめる。またその際、麻酔等手段により苦痛を与えない等の倫理的配慮をおこない、実験者は、管理者と相互協力のもと適切な環境のもと飼育管理を行った。

C. 研究結果

（1）拡張型心筋症モデルマウスに calcineurin の阻害剤である FK506 を投与した結果、拡張型心筋症モデルマウスで認める左室内腔の拡大や心収縮力の低下に対する改善効果は認めなかった。

（2）拡張型心筋症モデルマウスに CaMKIIδ の阻害剤である KN-93 を投与した。拡張型心筋症モデルマウスでは野生型マウスと比較し CaMKIIδ の標的蛋白であるホスホランバンのリン酸化が亢進していたが、そのリン酸化の増加は KN-93 投与により抑制された。さらに、KN-93 投与により拡張型心筋症モデルマウスで認める左室内腔拡大は抑制され、心機能は改善した。

D. 考察

遺伝子変異による拡張型心筋症の発症に CaMKIIδ の活性化が重要であることが明らかとなった。しかし、CaMKIIδ を活性する機序や標的因子に関しては不明である。さらに、CaMKIIδ の抑制には薬理学的な手法を用いたが、その効果が十分であるかははっきりしない。今後、CaMKIIδ の上流、下流の因子の検索および CaMKIIδ の遺伝子学的な抑制、具体的には CaMKII 特異的阻害ペプチドの過剰発現マウスとの交配を行う予定である。

E. 結論

遺伝子変異による拡張型心筋症の発症に CaMKIIδ の活性化が重要であることが示唆された。

F. 健康危険情報

該当なし

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得　　該当なし
2. 実用新案登録　該当なし
3. その他　　該当なし

研究成果の刊行に関する一覧表

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LETTERS

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

Weidong Zhu[1]*, Ichiro Shiojima[1]*, Yuzuru Ito[2]*, Zhi Li[1], Hiroyuki Ikeda[1], Masashi Yoshida[1], Atsuhiko T. Naito[1], Jun-ichiro Nishi[1], Hiroo Ueno[3], Akihiro Umezawa[4], Tohru Minamino[1], Toshio Nagai[1], Akira Kikuchi[5], Makoto Asashima[2,6,7] & Issei Komuro[1]

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

[1]Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan. [2]ICORP Organ Regeneration Project, Japan Science and Technology Agency (JST), Tokyo 153-8902, Japan. [3]Institute of Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, California 94305, USA. [4]Department of Reproductive Biology, National Institute for Child Health and Development, Tokyo 157-8535, Japan. [5]Department of Biochemistry, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima 734-8551, Japan. [6]Department of Life Sciences (Biology), Graduate School of Arts and Science, The University of Tokyo, Tokyo 153-8902, Japan. [7]National Institute of Advanced Industrial Sciences and Technology (AIST), Ibaraki 305-8562, Japan.
*These authors contributed equally to this work.

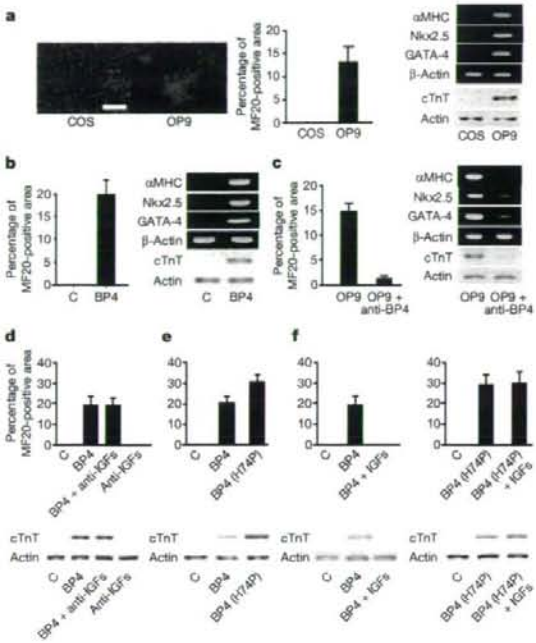

Figure 1 | IGFBP-4 promotes cardiomyocyte differentiation in an IGF-independent manner. a, Culture media conditioned by OP9 cells but not by COS7 cells induced cardiomyocyte differentiation of P19CL6 cells as assessed by MF20-positive area, cardiac marker-gene expression and cTnT protein expression. 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

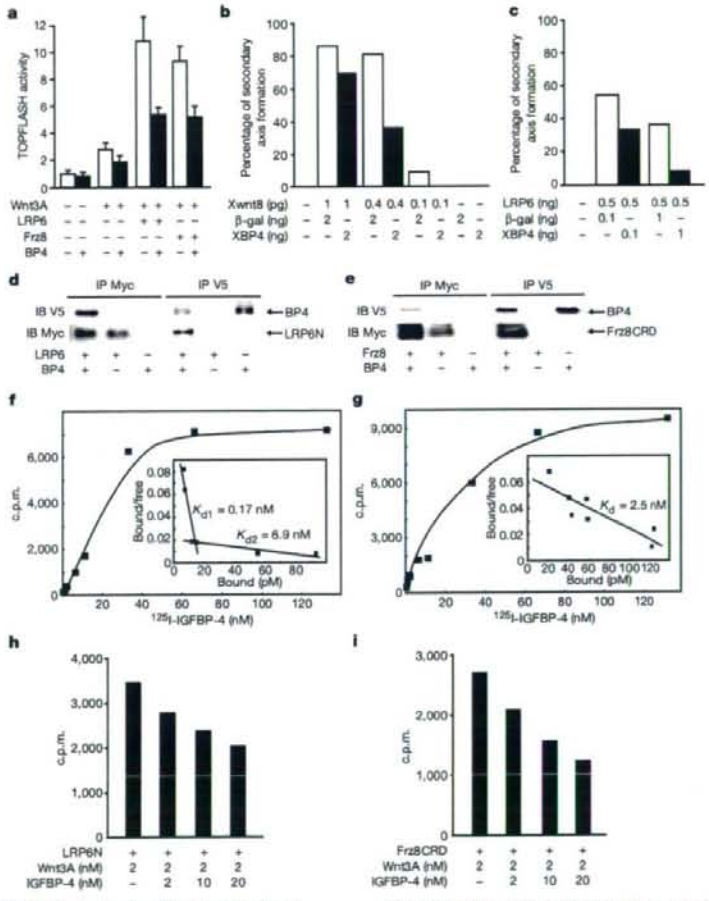

Figure 2 | IGFBP-4 inhibits Wnt/β-catenin signalling through direct interactions with Wnt receptors. **a**, IGFBP-4 attenuated β-catenin-dependent transcription in P19CL6 cells. P19CL6 cells were transfected with TOPFLASH reporter gene and expression vectors for LRP6 or Frz8, and then treated with Wnt3A 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

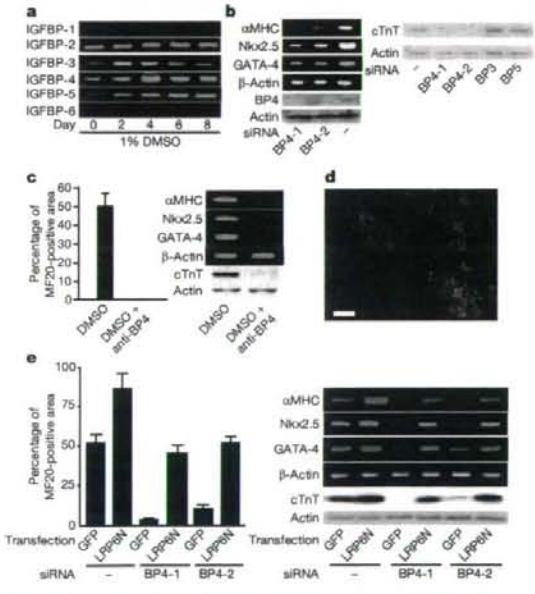

Figure 3 | IGFBP-4 is required for the differentiation of P19CL6 cells into cardiomyocytes. **a**, Expression analysis of IGFBP family members by RT–PCR during DMSO-induced cardiomyocyte differentiation of P19CL6 cells (from day 0 to day 8). **b**, Left: knockdown of *Igfbp4* in P19CL6 cells attenuated 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.

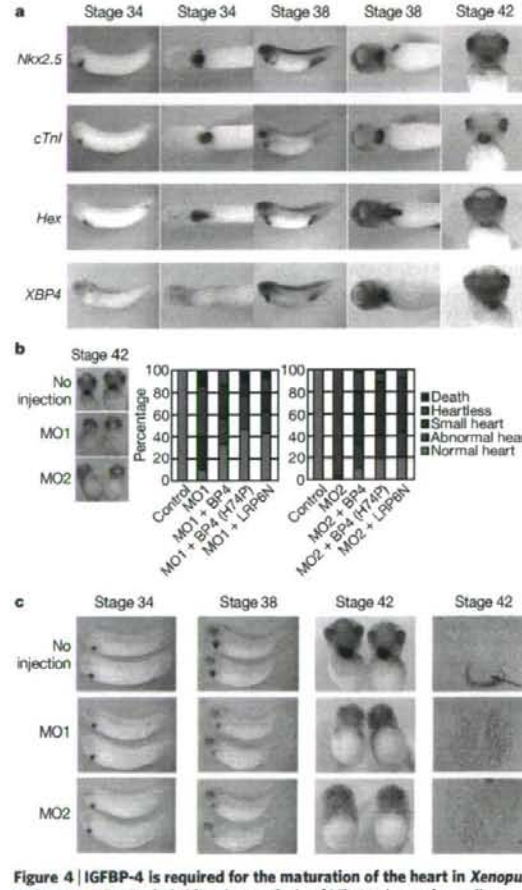

Figure 4 | IGFBP-4 is required for the maturation of the heart in *Xenopus* embryos. **a**, *In situ* hybridization analysis of *Nkx2.5* (an early cardiac marker), *cTnI* (a mature cardiac marker), *Hex* (a liver marker), and *XIGFBP-4* (*XBP4*) mRNA expression at stages 34, 38 and 42. **b**, Knockdown 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[8,10]. 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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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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METHODS

Plasmids and reagents. cDNA clones encoding mouse IGFBPs and *Xenopus* IGFBP-4 were purchased from Open Biosystems. XIGFBP-4–H74P mutant was generated with a QuickChange Site-Directed Mutagenesis kit (Stratagene). His-tagged human wild-type IGFBP-4 and mutant IGFBP-4-H74P (vectors provided by X. Qin)[8] were produced and purified with HisTrap HP Kit (Amersham). Full-length Frz8, Frz8CRD and LRP6N were provided by X. He[22,23]. Full-length LRP6, membrane-bound forms of LRP6 deletion mutants, and Dkk1 were from C. Niehrs[24]. pXwnt8 and pCSKA-Xwnt8 were from J. Christian[25]. pCS2–β-catenin was from D. Kimelman[26]. αMHC–GFP was from B. Fleischmann[27]. BRE–luc was from P. ten Dijke[28]. pCGN-Dvl-1 was described previously[29]. Soluble forms of LRP6 deletion mutants and probes for *in situ* hybridization analysis (Nkx2.5, cTnI and Hex) were generated by PCR. IGFBP-4, Wnt3A, IGF-I, IGF-II and BMP2 were from R&D. Neutralizing antibodies were from R&D (anti-IGFBP-4), Sigma (anti-IGF-I and anti-IGF-II), and Oncogene (anti-type-I IGF receptor). The antibodies used for immunoprecipitation, western blotting and immunostaining were from Invitrogen (anti-Myc, anti-V5), Santa Cruz (anti-cTnT, anti-IGFBP-4, anti-topoisomerase I (TOPO-I)), Sigma (anti-β-actin, anti-β-catenin, anti-FLAG (M2)) and Developmental Studies Hybridoma Bank (anti-sarcomeric myosin heavy chain (MF20)).

Cell culture experiments. P19CL6 cells and ES cells were cultured and induced to differentiate into cardiomyocytes essentially as described[6,30]. P19CL6 cells (2,000 cells per 35-mm dish) were treated with various conditioned media for screening of their cardiogenic activities. P19CL6 cells or ES cells stably transfected with αMHC promoter driven–GFP were generated by transfection of αMHC–GFP plasmid into P19CL6 cells or ht7 ES cells followed by G418 selection. Luciferase reporter gene assays, western blot analyses, immunostaining and RT–PCR were performed as described[10]. Reporter gene assays were repeated at least three times. PCR primers and PCR conditions are listed in Supplementary Table 1. For siRNA-mediated knockdown, siRNAs were expressed with pSIREN-RetroQ vector (Clontech). Oligonucleotide sequences used are listed in Supplementary Table 2. pSIREN-RetroQ vectors ligated with double-stranded oligonucleotides were transfected into P19CL6 cells or ES cells, and puromycin-resistant clones were isolated and expanded. For β-catenin stabilization assays, nuclear extracts of L cells were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Data are shown as means and s.d.

IP/western blot analyses and binding assays. Conditioned media for IP/western blot analyses containing full-length or various deletion mutants of IGFBPs, LRP6, Frz8CRD and Dkk1 were produced with 293 cells. Binding reactions were performed overnight at 4 °C. Immunoprecipitation was performed with Protein G–Sepharose 4 Fast Flow (Amersham). ^{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]. In brief, conditioned media containing LRP6N–Myc or Frz8CRD–Myc were mixed with various concentrations of ^{125}I-labelled IGFBP-4 and incubated overnight at 4 °C. LRP6N–Myc or Frz8CRD–Myc was immunoprecipitated and the radioactivity of bound IGFBP-4 was measured after extensive washing of the Protein G–Sepharose beads. For a competitive binding assay, conditioned media containing LRP6N–Myc or Frz8CRD–Myc were mixed with ^{125}I-labelled Wnt3A and unlabelled IGFBP-4, and incubated overnight at 4 °C. LRP6N–Myc or Frz8CRD–Myc was then immunoprecipitated and the radioactivity of bound Wnt3A was measured.

***Xenopus* experiments and mouse *in situ* hybridization analysis.** Axis duplication assays, animal cap assays and *in situ* hybridization analyses in *Xenopus* were performed essentially as described[20]. Two independent cDNAs for *XIGFBP-4*, presumably resulting from pseudotetraploid genomes, were identified by 5′ rapid amplification of cDNA ends (Supplementary Fig. 4a). Two different MOs targeting both of these two IGFBP-4 transcripts were designed (Gene Tools) (Supplementary Fig. 4a and Supplementary Table 2). MO-sensitive *XIGFBP-4* cDNA including a 41-base-pair 5′-untranslated region (UTR) was generated by PCR. MO-resistant *XIGFBP-4* cDNA (wild-type and H74P mutant) was generated by introducing five silent mutations in the MO1 target sequence and excluding the 5′-UTR (Supplementary Fig. 4a). To determine the specificity of MOs, MO-sensitive or MO-resistant *XIGFBP-4–myc* mRNA was injected into *Xenopus* embryos with or without MOs, and protein/mRNA expression was analysed. PCR primers and PCR conditions are listed in Supplementary Table 1. MOs and plasmid DNAs were injected at the eight-cell stage into the dorsal region of two dorsal–vegetal blastomeres fated to be heart and liver anlage. Electroporation of mRNA was performed essentially as described[21]. Injection of mRNA (5 ng in 5 nl of solution) into the vicinity of heart anlage and application of electric pulses were performed at stage 28. Whole-mount *in situ* hybridization analysis of murine IGFBP-4 was performed as described[30].

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