

200806009A

# 厚生労働科学研究費補助金

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

(H20-再生-一般-004)

平成 20 年度 総括研究報告書

研究代表者 小室 一成

平成 21 (2009) 年 3 月

## 目 次

I. 総括研究報告書	
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.....	1
小室 一成	
II. 分担研究報告書	
1. 末梢性血管疾患に対する治療・基礎研究.....	5
南野 徹	
2. 虚血性心疾患に対する治療.....	8
宮内 秀行	
III. 研究成果の刊行に関する一覧表.....	10
IV. 研究成果の刊行物・別刷.....	11

## 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* が起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、十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70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を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、本年度、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。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）

南野 徹 千葉大学医学部附属病院  
循環器内科 助教

宮内秀行 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

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70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を進めることに

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) 次世代の血管再生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。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・利益を十分に説明し、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。

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。

### D. 考察

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ド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。

### F. 健康危険情報

該当なし

### G. 研究発表

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## 2. 学会発表

### 国内

(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-Lessons from PERISCOPE」

・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)小室一成. 「心肥大はなぜ恐いか!? 癌より怖い心不全」

・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」

(南野徹)

・Minamino T. 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 第24回日本医工学治療学会 シンポジウム 2008/4/18-20 千葉

・Minamino T. Therapeutic neovascularization using peripheral mononuclear cells. 第40回日本動脈硬化学会総会シンポジウム 2008/07/10-11 つくば市 プログラム・抄録集 page139.

### 海外

(小室一成)

・The 2<sup>nd</sup> 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.

### 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

## 研究要旨

末梢血中に骨髄由来の血管内皮前駆細胞が存在することが報告されて以来、骨髄単核球細胞を用いた虚血性心血管疾患の治療は著しい早さでヒトへと臨床応用された。しかし未だにその明確な作用メカニズムは不明である。これに対して我々は、独自に末梢血単核球細胞の有用性について基礎的な検討を行った。その結果、末梢血単核球細胞は骨髄由来の単核球と比較して血管新生効果が勝るとも劣らないことなどを明らかにし、末梢血単核球細胞を用いたヒト重症末梢性動脈疾患に対する治療について臨床研究を開始した。これまでに 70 数例に対して本治療を行い、70%以上の症例に有用性を認めた。レスポonderとノンレスポonderとの臨床データの比較から、その治療効果は単核球移植により惹起される虚血組織における血管増殖因子の産生によることが明らかとなりつつある。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を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。本年度は、重症虚血下肢症例に対する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。末梢性血管疾患による重症間欠性跛行（Fontaine 2B 相当）を有する症例に対する臨床試験については、その登録を開始した。

### 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 70 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を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、下肢シリンチ、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) 次世代の血管再生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。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・利益を十分に説明し、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。それらに対して、非透析症例においては、閉塞性動脈硬化症とパージャーマ病ともに治療効果を認めたが、治療後の生存率や心血管イベントフリー率については、パージャーマ病の症例で有意に高かった。再発率に関しては、透析症例と非透析症例、閉塞性動脈硬化症とパージャーマ病の間にそれぞれ有意差はなかった。レスポンドとノンレスポンドの解析では、レスポンドの生存率が高い傾向にあった。

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の治療にて症状が改善せず、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。

## D. 考察

重症下肢虚血患者を対象にした臨床研究における治療効果や長期予後について検討では、透析患者に対する本治療の妥当性を考慮していく必要があると考えられた。レスポンドの生存率が高い傾向にあったという結果は、本治療の安全性を示唆している。

## E. 結論

透析症例に対しては、血管再生治療の効果や治療後の予後について十分に検討した上で、本治療の適応を決定する必要がある。

## 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

## 研究要旨

再生医療は多くの臓器・病態の分野において目覚ましい発展を遂げているが、虚血性疾患も例外ではない。虚血性心疾患、重症下肢虚血に対する血管再生治療が世界規模で行われているが、その安全性・有用性についてのエビデンスは不十分である。これに対して我々は、独自に末梢血単核球細胞の有用性について基礎的な検討を行った。その結果、末梢血単核球細胞は骨髓由来の単核球と比較して血管新生効果が勝るとも劣らないことなどを明らかにし、末梢血単核球細胞を用いたヒト重症末梢性動脈疾患に対する治療について臨床研究を開始した。これまでに 70 数例に対して本治療を行い、70%以上の症例に有用性を認めた。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、重症虚血性心疾患症例に対して用量エスカレーション試験を行う。当初は、プラセボをコントロールとした 2 重盲験試験を計画していたが、プロトコールを一部変更し、登録を開始した。

## 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、その効果は骨髓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 70 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、重症虚血性心疾患症例に対して用量エスカレーション試験とプラセボをコントロールとした 2 重盲験試験を行う。

## B. 研究方法

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。初期の 15 症例に対しては低用量、後期の 15 症例に対しては高用量の治療を行い、その治療効果と安全性についてバイパス術 6 ヶ月後に評価する。

（倫理面への配慮）

### (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) 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・利益を十分に説明し、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。

## (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。

## D. 考察

プラセボをコントロールとした2重盲験試験に先駆けて、用量エスカレーション試験を行うことにより、適切な用量を再確認するとともに、評価項目について妥当性についても検証することができると考えられた。

## E. 結論

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。

## F. 健康危険情報

該当なし

## G. 研究発表

なし

## 2. 学会発表

なし

## H. 知的財産権の出願・登録状況

1. 特許取得 該当なし
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研究成果の刊行に関する一覧表

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

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

Insulin-like growth-factor-binding proteins (IGFBPs) bind to and modulate the actions of insulin-like growth factors (IGFs)<sup>1</sup>. 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<sup>1,2</sup>. 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<sup>3</sup>. Molecules that mediate cardiogenesis are of particular interest because of their potential use for cardiac regeneration<sup>4,5</sup>. 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<sup>3,4</sup>. 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)<sup>6</sup>. 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,

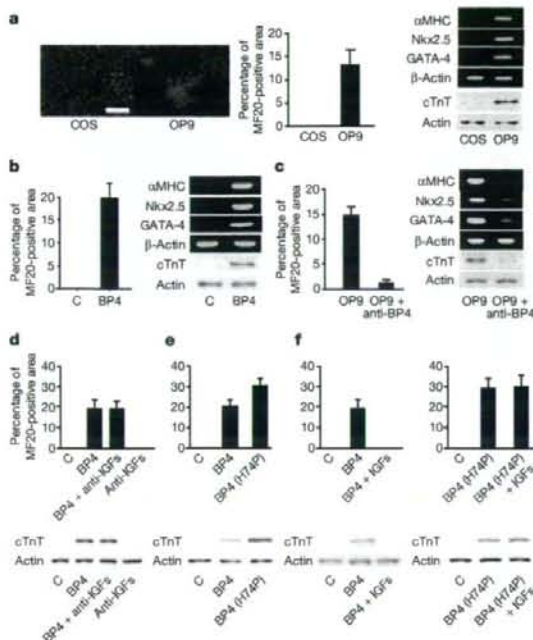
right panel). In contrast, culture media conditioned by COS7 cells, mouse embryonic fibroblasts, NIH3T3 cells, HeLa cells, END2 cells (visceral endoderm-like cells), neonatal rat cardiomyocytes and neonatal rat cardiac fibroblasts did not induce cardiomyocyte differentiation of P19CL6 cells in the absence 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<sup>7</sup> 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; His74 replaced by Pro)<sup>8</sup> 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

<sup>1</sup>Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan. <sup>2</sup>ICORP Organ Regeneration Project, Japan Science and Technology Agency (JST), Tokyo 153-8902, Japan. <sup>3</sup>Institute of Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, California 94305, USA. <sup>4</sup>Department of Reproductive Biology, National Institute for Child Health and Development, Tokyo 157-8535, Japan. <sup>5</sup>Department of Biochemistry, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima 734-8551, Japan. <sup>6</sup>Department of Life Sciences (Biology), Graduate School of Arts and Science, The University of Tokyo, Tokyo 153-8902, Japan. <sup>7</sup>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  $\mu$ m. Error bars show s.d. **b**, Treatment with IGFBP-4 (1  $\mu$ g ml<sup>-1</sup>) 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  $\mu$ g ml<sup>-1</sup>) 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  $\mu$ g ml<sup>-1</sup> 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<sup>-1</sup> 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<sup>3,4</sup>. In P19CL6 cells, Wnt3A treatment activated  $\beta$ -catenin-dependent transcription of the TOPFLASH reporter gene, and this activation was attenuated by IGFBP-4 (Fig. 2a). Wnt/ $\beta$ -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)<sup>9</sup> 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

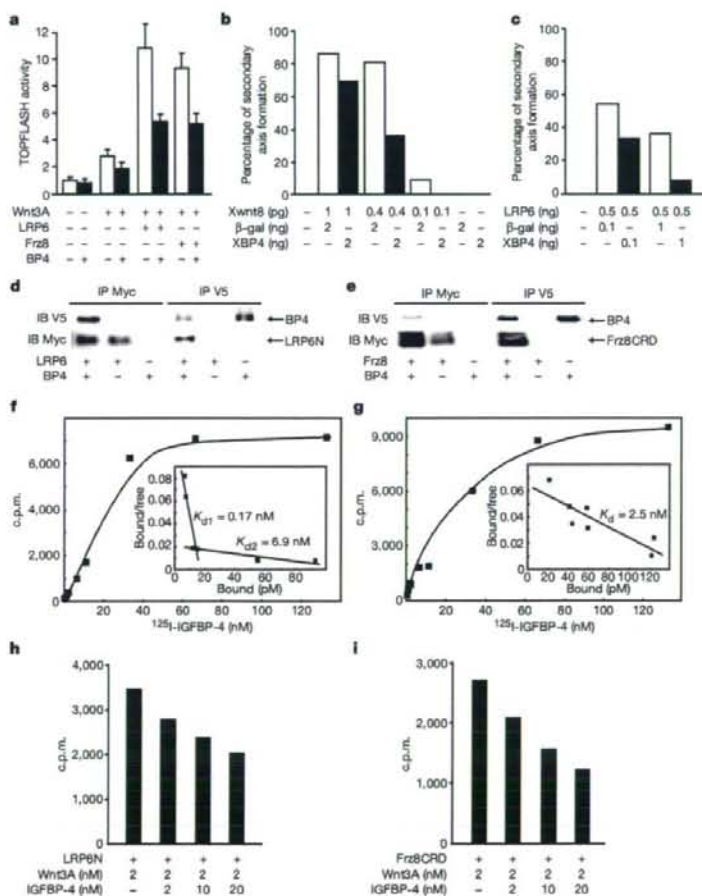
formation. However, *Xwnt8*-induced or LRP6-induced secondary axis formation was efficiently blocked by coexpression of *XIGFBP-4* (Fig. 2b, c), indicating that IGFBP-4 inhibits canonical Wnt signalling *in vivo*. To explore the mechanisms of Wnt inhibition by IGFBP-4, *Xenopus* animal cap assays and TOPFLASH reporter gene assays were performed. In animal cap assays, IGFBP-4 inhibited LRP6-induced but not  $\beta$ -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  $\beta$ -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 <sup>125</sup>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 <sup>125</sup>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 Frz8CRD (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<sup>4,10,11</sup>. These results therefore collectively suggest that IGFBP-4 promotes cardiogenesis by antagonizing the Wnt/ $\beta$ -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/ $\beta$ -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/ $\beta$ -catenin pathway. The temporal profile of cardiac defects induced by *XIGFBP-4* knockdown was also examined by *in situ* hybridization with *cardiac troponin 1* (*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

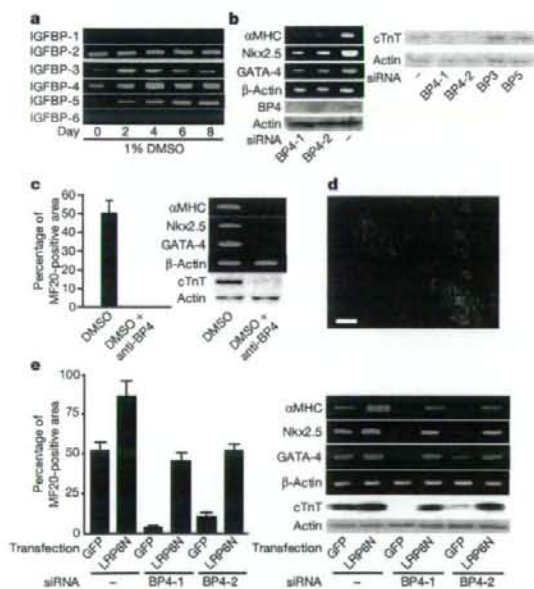


**Figure 2** | IGFBP-4 inhibits Wnt/ $\beta$ -catenin signaling through direct interactions with Wnt receptors. **a**, IGFBP-4 attenuated  $\beta$ -catenin-dependent transcription in P19CL6 cells. P19CL6 cells were transfected with TOPFLASH reporter gene and expression vectors for LRP6 or Frz8, and then 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**). **f**, 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.

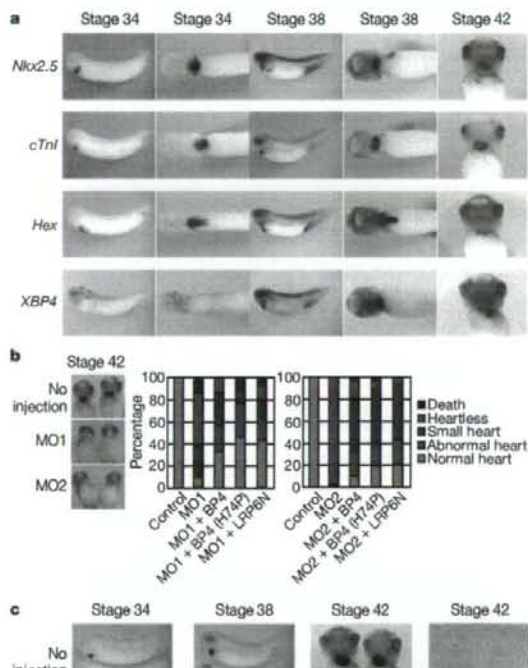
promotes cardiogenesis by maintaining the proliferation and/or survival of embryonic cardiomyocytes.

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<sup>4</sup>. 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/ $\beta$ -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<sup>10–12</sup>. 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



**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  $\mu$ g ml<sup>-1</sup>) 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  $\alpha$ MHC-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  $\mu$ m. **e**, Attenuated cardiomyocyte differentiation of P19CL6 cells by *Igfbp4* knockdown was rescued by inhibiting Wnt/ $\beta$ -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.

time-dependent effects of Wnt/ $\beta$ -catenin signalling on cardiogenesis has been shown in zebrafish embryos<sup>11</sup>. Moreover, several recent reports suggest that Wnt/ $\beta$ -catenin signalling is a positive regulator of cardiac progenitor-cell proliferation in the secondary heart field<sup>13</sup>. 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 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<sup>14</sup>, 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  $\beta$ -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<sup>15</sup> 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/ $\beta$ -catenin signalling may also have some implications for cancer biology<sup>16</sup>. 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<sup>17,18</sup>, 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<sup>19</sup>. 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. <sup>125</sup>I-labelled IGFBP-4 and Wnt3A was performed with IODO-BEADS Iodination Reagent (Pierce). A liquid-phase binding assay was performed essentially as described<sup>19</sup>.

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

**Full Methods** and any associated references are available in the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

Received 22 August 2007; accepted 24 April 2008.

Published online 4 June 2008.

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

**Acknowledgements** We thank E. Fujita, R. Kobayashi and Y. Ishiyama for technical support; T. Yamauchi and K. Ueki for advice on binding assays; and Y. Onuma and S. Takahashi for advice on *Xenopus* electroporation. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), the Ministry of Health, Labour, and Welfare, and the New Energy and Industrial Technology Development Organization (NEDO).

**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.J., 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.

**Author Information** Reprints and permissions information is available at [www.nature.com/reprints](http://www.nature.com/reprints). Correspondence and requests for materials should be addressed to I.K. ([kumuro-ky@umin.ac.jp](mailto:kumuro-ky@umin.ac.jp)).

## 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). Histagged human wild-type IGFBP-4 and mutant IGFBP-4-H74P (vectors provided by X. Qin)<sup>9</sup> were produced and purified with HisTrap HP Kit (Amersham). Full-length Frz8, Frz8CRD and LRP6N were provided by X. He<sup>22,23</sup>. Full-length LRP6, membrane-bound forms of LRP6 deletion mutants, and Dkk1 were from C. Niehrs<sup>24</sup>. pXwnt8 and pCSKA-Xwnt8 were from J. Christian<sup>25</sup>. pCS2- $\beta$ -catenin was from D. Kimelman<sup>26</sup>.  $\alpha$ MHC-GFP was from B. Fleischmann<sup>27</sup>. BRE-luc was from P. ten Dijke<sup>28</sup>. pCGN-Dvl-1 was described previously<sup>29</sup>. 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- $\beta$ -actin, anti- $\beta$ -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<sup>30</sup>. 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  $\alpha$ MHC promoter driven-GFP were generated by transfection of  $\alpha$ MHC-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<sup>10</sup>. 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  $\beta$ -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). <sup>125</sup>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<sup>19</sup>. In brief, conditioned media containing LRP6N-Myc or Frz8CRD-Myc were mixed with various concentrations of <sup>125</sup>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 <sup>125</sup>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<sup>30</sup>. 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<sup>31</sup>. Injection of mRNA (5 ng in 5  $\mu$ l 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<sup>30</sup>.

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# Vascular aging: insights from studies on cellular senescence, stem cell aging, and progeroid syndromes

Tohru Minamino and Issei Komuro\*

## SUMMARY

Epidemiological studies have shown that age is the chief risk factor for atherosclerotic cardiovascular diseases, but the molecular mechanisms that underlie the increase in risk conferred by aging remain unclear. Evidence suggests that the cardiovascular repair system is impaired with advancing age, thereby inducing age-associated cardiovascular dysfunction. Such impairment could be attributable to senescence of cardiovascular tissues at the cellular level as a result of telomere shortening, DNA damage, and genomic instability. In fact, the replicative ability of cardiovascular cells, particularly stem cells and/or progenitor cells, has been shown to decline with age. Recently, considerable progress has been made in understanding the pathogenesis of human progeroid syndromes that feature cardiovascular aging. Most of the genes responsible have a role in DNA metabolism, and mutated forms of these genes result in alterations of the response to DNA damage and in decreased cell proliferation, which might be common features of a phenotype of aging. Here we review the cardiovascular research on cellular senescence, stem cell aging, and progeroid syndromes and discuss the potential role of cellular senescence in the mechanisms underlying both normal aging and premature aging syndromes.

**KEYWORDS** DNA damage, progenitor cell, progeria, senescence, telomere

## REVIEW CRITERIA

We searched PubMed for papers published between 1980 and 2008 by using combinations of the following search terms: "aging", "senescence", "telomere", "stem cell", "progenitor cell", "EPC", "progeroid", "Hutchinson-Gilford progeria syndrome" and "Werner syndrome". We also searched the reference lists of papers identified for other relevant manuscripts.

T Minamino is Assistant Professor at the Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, and a Research Scientist at Japan Science and Technology Agency, Saitama, and I Komuro is Professor and Chairman of the Department of Cardiovascular Science and Medicine at Chiba University Graduate School of Medicine and Director of the Center for Cardiovascular Interventions at Chiba University Hospital, Chiba, Japan.

## Correspondence

\*Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan  
komuro-iky@umin.ac.jp

Received 4 March 2008 Accepted 1 July 2008 Published online 2 September 2008

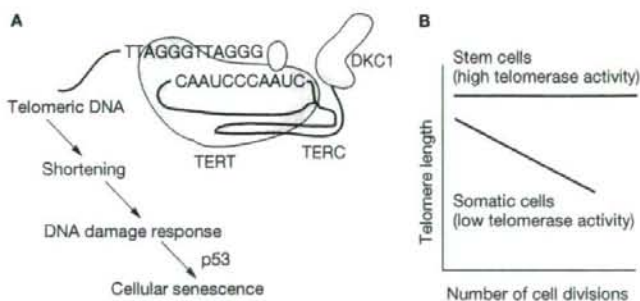
www.nature.com/clinicalpractice  
doi:10.1038/npcardio1324

## INTRODUCTION

Aging is a known major risk factor for cardiovascular disease.<sup>1</sup> The aging process is also associated with adverse hemodynamic and metabolic changes that accelerate the development of cardiovascular disease. It is now accepted that changes in cardiovascular structure and function occur in healthy individuals as they age. These alterations precede the onset of clinical disease and predict the future risk of developing atherosclerosis, hypertension and heart failure. Such age-related changes could, therefore, be potential targets for new treatments.

The age-associated changes in blood vessels that occur in healthy individuals include increased arterial wall thickness, luminal dilatation and reduced compliance.<sup>2</sup> In addition to these structural changes, endothelial function becomes impaired with increasing age, thereby increasing arterial stiffness.<sup>2</sup> This endothelial dysfunction is partly caused by decreased production of vasodilators, such as nitric oxide and prostacyclin, and a reduction in the responsiveness of vascular smooth muscle cells (VSMCs) to these vasodilators. Moreover, evidence indicates that cardiovascular repair systems become progressively impaired with aging. For example, neovascularization of ischemic tissue and re-endothelialization after vascular injury are impaired in aged animals.<sup>3,4</sup> These impairments are attributed to the reduced production of proangiogenic factors, impaired cell replication, and a decrease in the number and function of stem and/or progenitor cells. Even if these age-associated changes do not result in overt cardiovascular disease *per se*, they impair the capacity of the cardiovascular system and influence the severity and prognosis of subsequent disease.

Cellular and molecular mechanisms underlying age-associated changes of the cardiovascular system have been studied, but it remains unclear exactly how these alterations occur with advancing age. Evidence suggests that cardiovascular cells, including stem and/or progenitor cells, undergo senescence, and that this process



**Figure 1** Telomeres and telomerase. (A) Telomerase consists of a RNA component, a catalytic component and cofactors including DKC1. The RNA moiety serves as a template for the synthesis of new telomeric repeats by the catalytic component. Critically short telomeres resemble damaged DNA and thus trigger cellular senescence via a p53-dependent pathway. (B) Stem cells have high telomerase activity and maintain telomere length, whereas most somatic cells including vascular cells show progressive telomere shortening due to low telomerase activity. Abbreviations: DKC1, dyskeratosis congenita 1, dyskerin; TERC, telomerase RNA component; TERT, telomerase reverse transcriptase.

contributes to age-associated alterations of cardiovascular structure and function.<sup>5,6</sup> In this Review we discuss the potential impact of cellular senescence of vascular and stem cells on age-related cardiovascular disease and examine experimental changes in relation to premature aging syndromes.

#### CELLULAR SENESCENCE AND VASCULAR AGING

Replicative senescence was originally defined by the finite replicative life span of human somatic cells in culture. Senescent cells enter irreversible growth arrest, exhibit a flattened and enlarged shape, and express a set of genes—including negative regulators of the cell cycle such as p53 and p16—that differs from those normally expressed. The growth potential of cultured cells correlates well with the mean maximum life span of the species from which the cells are derived; therefore, phenotypic changes associated with senescence have been suggested to be involved in human aging. This hypothesis of cellular aging is supported by the finding that primary cells from patients with progeroid syndromes have a shorter life span than cultured cells from age-matched healthy persons.<sup>7</sup> A number of studies have shown that many of the changes seen in senescent vascular cells, such as decreased production of nitric oxide, are consistent with the aforementioned cellular changes seen in

patients with age-related cardiovascular disease, suggesting that cellular senescence has a role in the aging of the cardiovascular system.

#### *In vivo* evidence of vascular cell senescence

Human atherosclerotic lesions have been studied extensively, and within the plaques studied both endothelial cells and VSMCs have been found that exhibit the morphological features of cellular senescence.<sup>8,9</sup> Vascular cells that are positive for senescence-associated  $\beta$ -galactosidase (SA $\beta$ -gal) activity, a biomarker of senescence, have been found in atherosclerotic plaques obtained from the coronary arteries of patients with ischemic heart disease.<sup>10</sup> These SA $\beta$ -gal-positive cells are predominately localized on the luminal surface of the plaque and have been identified as endothelial cells. Interestingly SA $\beta$ -gal-positive cells have not been observed in the internal mammary arteries of the same patients, where atherosclerotic changes are minimal.<sup>10</sup> In advanced plaques, however, SA $\beta$ -gal-positive VSMCs are only detected in the intima and not in the media,<sup>11</sup> possibly the result of extensive replication in these lesions. SA $\beta$ -gal-positive VSMCs cells from human atheromas show increased expression of p53 and p16—both markers of cellular senescence.<sup>11</sup> These cells also exhibit various functional abnormalities, including decreased expression of endothelial nitric oxide synthase and increased expression of proinflammatory molecules.<sup>11</sup> Such findings support the theory that cellular senescence occurs *in vivo* and contributes to the pathogenesis of human atherosclerosis.

#### Telomere shortening in aged arteries

Telomeres are non-nucleosomal DNA-protein complexes located at the ends of chromosomes and serve as protective caps and act as the substrate for specialized replication mechanisms. As a consequence of semiconservative DNA replication, the extreme terminals of chromosomes are not duplicated completely, causing successive shortening of the telomeres with each round of cell division. Telomerase is an enzyme that adds telomeres to the ends of chromosomes.<sup>12</sup> This enzyme consists of a RNA component, a catalytic component, and various cofactors including dyskeratosis congenita 1, dyskerin. The RNA moiety serves as a template for new telomeric repeats synthesized by the catalytic component (Figure 1A). In contrast with