特別掲載

ヒト幹細胞を用いた細胞・組織加工医薬品等の品質・安全性確保に関する研究（その８）

ヒト幹細胞加工医薬品等の品質及び安全性の確保に関する指針（案）

－ヒト体性幹細胞，iPS（様）細胞又はES細胞を加工して製造される医薬品等（ヒト幹細胞加工医薬品等）の非臨床試験及び臨床試験について－

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研究の経緯と視点

本研究の経緯については，本シリーズ第１報[1]において詳細に述べた。平成20年度から22年度に至る間，ヒト幹細胞由来製品の実用化の推進を図ることを目的として厚生労働科学研究事業「ヒト幹細胞を用いた細胞・組織加工医薬品等の品質・安全性確保に関する研究（研究代表者：早川堯夫）」が遂行された。その結果，体性幹細胞，iPS細胞，ES細胞などに由来する製品の薬事法下での臨床応用に向けて，研究・開発，確認申請（治験開始〔First-in-Man〕），評価等を効率的，効果的，合理的に行う上で，必要と思われる技術，製造方法，特性解析方法，品質管理方法及び安定性評価に関する具体的留意事項，安全性及び有効性に関する各種データとしてどのようなものがあるかに関しては，これらの３種類の原料細胞それぞれに特化した形でまとめる方向性が打ち出された。この方向性と科学的原則の一貫性という観点から，平成20年２月及び９月に通知された自己細胞・組織加工医薬品等全般に関する指針「ヒト（自己）由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針（薬食発第0208003号）（ヒト自己親指針）」[2]をベースとして，ヒト（自己）体性幹細胞及びヒト（自己）iPS（様）細胞加工医薬品等に関するそれぞれの指針案（中間報告）[3,4]を作成した。また，平成20年９月に通知された同種細胞・組織加工医薬品等全般に関する指針「ヒト（同種）由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針（薬食発第0912006号）（ヒト同種親指針）」[5]をベースとして，ヒト（同種）体性幹細胞，ヒト（同種）iPS細胞及びES細胞加工医薬品等に関する指針案（中間報告）を作成し，公表した[6-8]。平成22年度，これをベースにさらに諸外国での状況，その後の当該分野の進歩，さまざまな観点からの論議を踏まえて最終案を作成した。

この中で，「製造方法のうち原材料及び製造関連物質，製造工程」に関しては，体性幹細胞，iPS細胞，ES細胞のいずれを原材料にするか，あるいは自己由来か，同種由来か，などにより区別して留意事項を明確にすることが望ましいと考え，その内容を本シリーズの第２報から第６報までに報告してきた。

一方，「最終製品の品質管理や安定性評価のあり方」については，由来する細胞に特化した留意事項に重きを置くと云うよりもむしろ，最終製品そのものに焦点をあてた留意事項として捉えることがより重要であると考えて第７報で一括して報告した[9]。非臨床試験及び臨床試験についても製品レベルで考慮することであるので，本報で一括して報告する。ここで「ヒト体性幹細胞，iPS（様）細胞又はES細胞を加工して製造される医薬品等」はとくに断らない限り一括して「ヒト幹細胞加工医薬品等」と総称する。

医薬品の研究開発段階で動物などを用いて実施される非臨床安全性試験の主な目的としては，一般に以下のようなものが挙げられている。

①当該医薬品をヒトに適用する際の用量および用法を設定するための安全性情報を可能なかぎり得ておくこと
②医薬品として期待される「目的の作用」以外の望ましくない作用（毒性）が発現するおそれのある臓器・組織を可能なかぎり特定し，かつその毒性の種類・程度・可逆性や発現機序を検討しておくこと
③臨床試験を含めた臨床使用時にモニタリングするべき具体的な安全性評価項目を見いだしておくこと

④承認・上市前にヒトでの知見を十分に得ることが事実上困難なケースが多い安全性（例えば，がん原性，生殖・発生毒性，遺伝毒性）に関する情報を得ておくこと

すなわち，新医薬品の研究開発の全段階を通じて，*in vitro*および*in vivo*での非臨床安全性試験の実施は，安全性薬理試験も含めて一般的に必要不可欠なものであるということである。これはタンパク質性医薬品においても例外ではないが，タンパク質性医薬品においては，目的タンパク質の構造の多様性や不均一性，作用発現の動物種特異性，抗原性・免疫原性，予期しない部位での作用発現の可能性などの物性面や作用面での特徴・特殊性から，従来の医薬品（特に化学合成医薬品）における非臨床安全性試験の種類・項目および試験方法をそのまま機械的に適用することは必ずしも妥当ではなく，従来とは異なる観点や方法で試験を実施するべき場合が多いとされている。そして全製品いずれにも画一的に適用可能な非臨床安全性試験のプロトコールなるものは存在せず，対象とする製品の特性や臨床上の適用法などを考慮しながら製品ごとにケースバイケースで合理的かつ柔軟に対応することが重要であるとされている。

一方，「ヒト幹細胞加工医薬品等」の場合，上記①～④いずれも，一部を除いて目的に沿って非臨床安全性試験を実施することは容易ではなく，また適用することの意義を明確に示すことも容易ではない。これは，製品である細胞・組織医薬品等の特性が化学薬品はもとより，タンパク質性医薬品とも著しく異なっているからである。

ヒト型タンパク質性医薬品の場合，最も重要な留意事項として「適切な動物種」を使用することが推奨されている。「適切な動物種」とは，標的組織に当該医薬品の受容体が存在し，目的とする薬理学的活性を示す動物種のことである。そして適切でない動物種を用いた毒性試験については，誤った結論に導かれる可能性があるので勧められないとされている。

「ヒト幹細胞加工医薬品等」では，単一のタンパク質に適用されるような「標的組織に受容体が存在し，目的とする薬理学的活性を示す動物種」という基準で「適切な動物種」を選ぶことは，その特性上，必ずしも容易ではない。また皮膚，角膜，軟骨等，細胞・組織として機能不全のみならず物理的不全や欠損を補充するための治療では，その目的とするところをふまえて，薬理学的反応性などとは別の基準で選ぶことが必要な場合もある。

ある細胞・組織医薬品等の効能・効果のメカニズムが生理活性タンパク質の産生にあった場合で，製品から産生されるさまざまな生理活性タンパク質群のうち，どのような活性タンパク質群が治療効果と結びつき，逆に安全性上問題となるのか，あらかじめある程度明らかになっていれば，「適切な動物種」を選択することができるかも知れない。また，産生する生理活性タンパク質の種類やその量は置かれた細胞環境における他の細胞等とのクロストーク等によっても変わることが予測されるが，これに対する知見の多寡は「適切な動物種」の選定の妥当性に影響する。分泌生理活性タンパク質とは別の機能が移植された製品の効能・効果のもとであるような場合には，当然その機能発揮を評価できる試験動物が「適切な動物」といえる。結局，対象としている「ヒト幹細胞加工医薬品等」の特性についていかに多くのことを知り，正しく把握しているか，使用目的に応じた試験・評価計画の適切性が肝要である。遺伝子導入をして，その発現産物に効能・効果を期待するような場合は，よりわかりやすく「適切な動物を」選ぶことができる。しかし，一般には「適切な動物」を選択することが困難な場合が多いと考えられる。

特定できない多数の生理活性物質を産生している可能性がある「ヒト幹細胞加工医薬品等」のような場合，安全性薬理試験のようなものが包括的試験として意味あるかも知れない。例えば循環器系，呼吸器系，腎臓系，中枢神経系などの主要な生理的機能を営む系に及ぼす影響を明らかにできる可能性が考えられるからである。またこれは，薬理試験あるいは動物モデルを用いての薬効薬理試験の一部ともなるかも知れない。さらに，こうした試験は，特定の臓器における安全性上の問題発現に関する知見をもたす可能性がある。これは，ひいてはヒトでの臨床使用・適応に関して十分に考慮すべき情報となるかも知れない。

タンパク質性医薬品の場合のもう一つの動物選択基準に免疫応答に関しての留意がある。当該医薬品の薬理作

用や毒性作用が免疫応答によって中和されるような場合，試験そのものが意味をなさなくなるからである。

「ヒト幹細胞加工医薬品等」では実験動物に免疫応答を引き起こす可能性がきわめて高いが，それがどのような影響を及ぼすか，単一タンパク質の場合とは異なり，関係する抗原及び抗体を特定することが困難を極めるところから，アレルギー等観察できる現象を除いて，個々の抗体等を解析し，その位置づけを評価することはほとんどできない。対策としては免疫不全動物を使用することが多い。これはある種の「適切な動物」と言えるかも知れない。なお，「ヒト幹細胞加工医薬品等」のヒトでの抗原性に対する評価を実験動物で試験しようとするのは常識的に考えて意味に乏しいと思われる。

「ヒト幹細胞加工医薬品等」の適用法としてタンパク質性医薬品等と異なるところは，ほとんどの場合，局所に移植されることである。皮膚，心筋，角膜など特定の部位に，例えば細胞シートとして移植される状態を考えると，局所での機能や有害作用は試験や評価の対象となるにしても，局所を大きく離れた部位，特に全身的に安全性上の問題を引き起こすことはほとんど考えられない。

「ヒト幹細胞加工医薬品等」は総体としてきわめて複雑な構造物で多様な特性を有する点やヒトへの適用法でタンパク質性医薬品とは著しく異なる。またタンパク質性医薬品は，その登場以来蓄積されてきた多くの経験が精査・評価され，非臨床安全性試験のあり方がICHガイドラインとして作成されているが，ヒト幹細胞加工医薬品等についての蓄積や体系的な考え方の整備・構築は充分なされていない。今後，さまざまな知見や経験を積み重ねて行くことでより適切な非臨床試験に関する基本的考え方やあり方を構築していく他ないであろうと考えられる。試行錯誤を重ねながら，臨床使用という出口にでたものから振り返って，個別事例における非臨床試験のあり方を検証することで，試験の種類や試験内容の妥当性を論ずることができる日が遠からず来ることを期待したい。

今回，通知案として提示する下記の内容は，いわば途半ばにおける検討，考慮事項を記述したものである。要求事項として示したものではない。文中に登場する，「技術的に可能であれば，科学的に合理性のある範囲で」，「試験の採用，実施及び評価にあたっては，慎重な事前検討や対応が必要である」，「これらは例示であって，合理性のない試験の実施を求める趣旨ではなく，製品の由来，製品の特性及び臨床適用法等を考慮して，必要かつ適切な試験を実施し，その結果について総合的な観点から評価，考察する」，「必要に応じて」，「検討，考察すること」，「実施を考慮すること」などの表記は，ケースバイケースで，試験の実施が科学的に合理性のある意義あるもので必要なことであるかをまず考慮し，また技術的に可能で結果を評価できるものであるかを問いながらの対応が肝要であることを示している。

繰り返し述べてきたように，本指針を解釈し，運用していくにあたって，前提と考えるべきことがある。本来の目的は再生医療という新たな医療によって病に苦しむ患者さんが救われる機会を提供することである。指針の役割は，最も効率的，効果的に所定の目標に達するための要素と方策の提示である。指針にはさまざまな事態，状況を想定して，網羅的に留意事項が記述されているが，これらは，細胞の特性や臨床目的，適用法等によって取捨選択されるべきものであり，また適用項目についても適切，柔軟に解釈・運用すべきものである。新たな治療法への可能性が期待できること(Proof of Concept：POC)，ヒトに初めて適用しても差し支えない程度に既存の知見の中で想定し得る安全性上の問題がクリアされていること，倫理的妥当性の確保・堅持(ヘルシンキ宣言遵守，ドナー/患者に対する徹底的な説明と同意や自己決定権が前提)は当然であるが，手段である指針への遵守が主となり，他に代え難い患者さんへの医療機会の提供という目標が従になるような解釈や運用は本末転倒であり，避けなければならない。試験動物を用いた非臨床安全性試験等は，上記の目標に向かうための欠かせぬ要素であるが，「ヒト幹細胞加工医薬品等」の特性や適用法，対象疾患の特殊性と，試験動物で得られる情報の意義，限界を考慮して過度な不合理が生じないような適切なアプローチが望まれる。

ヒト幹細胞加工医薬品等の品質及び安全性の確保に関する指針(案)

ーヒト体性幹細胞，iPS(様)細胞又はES細胞を加工して製造される医薬品等(ヒト幹細胞加工医薬品等)の非臨床試験及び臨床試験についてー

1　ヒト体性幹細胞，iPS(様)細胞，又はES細胞を加工して製造される医薬品等(ヒト幹細胞加工医薬品等)の非臨床安全性試験

製品の特性及び適用法から評価が必要と考えられる安全性関連事項について，技術的に可能であれば，科学的合理性のある範囲で，適切な動物を用いた試験又は*in vitro*での試験を実施すること。なお，非細胞成分及び製造工程由来の不純物等については，可能な限り，動物を用いた試験ではなく理化学的分析法により評価すること。また，特にiPS(様)細胞，又はES細胞由来の最終製品においては，未分化細胞の存在が異所性組織形成や腫瘍形成・がん化の可能性など安全性上の重要な関心事であるが，可能な限り，セル・バンクや中間製品段階等での徹底的な解析により，混在の可能性を否定するか，あるいは，目的細胞から未分化細胞の効果的分離・除去法や不活化法を開発し，活用することにより，混在の可能性を最小限にする努力が求められる。さらに，投与経路等の選択も安全性上の懸念を最小限にするための有用な方策であるかも知れない。

ヒト由来の試験用検体は貴重であり，また，ヒト由来の製品を実験動物等で試験して必ずしも意義ある結果が得られるとは限らない。このため，動物由来の製品モデルを作成し適切な実験動物に適用する試験系により試験を行うことで，より有用な知見が得られると考えられる場合には，むしろ，このような試験系を用いることに科学的合理性がある場合があるかも知れない。その際は，対象疾患ごとに適切な中・大動物を用いた試験の実施を考慮する(注：例えば神経疾患ならばサル等，循環器疾患ならばブタ・イヌ等が適している場合がある)。ただし，ヒト体性幹細胞，ヒトiPS(様)細胞，ヒトES細胞加工医薬品等(ヒト幹細胞加工医薬品等)を構成する細胞と同一の特徴を有する細胞集団が同一の手法にてヒト以外の動物種からも得られるとは限らず，また同様の培養条件等で同等/同質な製品が製造できるとも限らないことから，このような試験の採用，実施及び評価にあたっては，慎重な事前検討や対応が必要である。ヒト以外の動物種から得た幹細胞加工製品を用いて動物実験を行った場合，その外挿可能性を説明すること。場合によっては細胞を用いる試験系も考慮し，このようなアプローチにより試験を行なった際には，その試験系の妥当性について明らかにすること。

以下に，必要に応じて非臨床的に安全性を確認する際の参考にすべき事項及び留意点の例を示す。これらは例示であって，合理性のない試験の実施を求める趣旨ではなく，製品の由来が自己細胞か同種細胞か，体性幹細胞，iPS(様)細胞由来，あるいはES細胞由来かなどの点や，製品の特性及び臨床適用法等を考慮して，必要かつ適切な試験を実施し，その結果について総合的な観点から評価，考察すること。

1　培養期間を超えて培養した細胞について，目的外の形質転換を起こしていないことを明らかにすること。ヒトiPS(様)細胞又はES細胞に由来する製品の場合には，目的細胞以外の細胞が異常増殖していないことを明らかにすること。

2　必要に応じて細胞・組織が産生する各種サイトカイン，成長因子等の生理活性物質の定量を行い，生体内へ適用したときの影響に関して考察を行うこと。

3　製品の適用が患者の正常な細胞又は組織に影響を与える可能性，及びその安全性について検討，考察すること。

4　製品の種類に応じて，患者への適用により，製品中の細胞や混入する未分化細胞が，異所性組織を形成する可能性，及びその安全性について検討，考察すること。その際，製品の種類や特性，投与経路，対象疾患及び試験系の妥当性等を総合的に勘案すること。

5　製品及び導入遺伝子の発現産物等による望ましくない免疫反応が生じる可能性，及びその安全性について検討，考察すること。

6　良性腫瘍を含む腫瘍形成及びがん化の可能性については，製品の種類や特性，投与量・投与経路，対象疾患，及び試験系の妥当性等を総合的に勘案して考察すること。体性幹細胞加工製品の場合には必要に応じて，iPS(様)細胞やES細胞加工製品の場合には原則として適切な動物モデル等を利用した検討を行うこと。また，腫瘍形成またはがん化の可能性がある場合には，期待される有効性との関係等を勘案して，使用することの妥当性及び合理性について明らかにすること(注：造腫瘍性試験において最も重要なのは，最終製品が患者に適用された場合の製品の造腫瘍性を的確に評価することである。しかし，十分な細胞数が得られない等の理由により最終製品を構成する細胞を用いることができず，中間製品の細胞を用いて最終製品の造腫瘍性を評価しなければならない場合も想定される。また，動物モデルを使用した造腫瘍性試験においては，細胞の分散や足場への接着，細胞密度，投与部位等の条件が最終製品と必ずしも一致するものではない。さらに，動物の種・系統・免疫状態による感度差もある。これらの事情を総合的に勘案して，最終製品の造腫瘍性を評価する必要がある。また，最終製品の造腫瘍性に起因する患者へのリスクについては，対象疾患を治療することによる患者へのベネフィット等とのバランスを踏まえて合理的に評価すること。)。

7　製造工程で外来遺伝子の導入が行われ，最終製品中で機能している場合や残存している場合には，遺伝子治療用医薬品指針に定めるところに準じて試験を行うこと。特に，ウイルスベクターを使用した場合には増殖性ウイルスがどの程度存在するかを検査するとともに，検査方法が適切であることについても明らかにすること。

また，導入遺伝子及びその産物の性状について調査し，安全性について明らかにすること。細胞については，増殖性の変化，良性腫瘍を含む腫瘍形成及びがん化の可能性について考察し，明らかにすること。

8　動物由来のモデル製品を含めて製品の入手が容易であり，かつ臨床上の適用に関連する有用な安全性情報が得られる可能性がある場合には，合理的に設計された一般毒性試験の実施を考慮すること。

なお，一般毒性試験の実施に当たっては，平成元年9月11日付け薬審1第24号厚生省薬務局新医薬品課長・審査課長連名通知「医薬品の製造(輸入)承認申請に必要な毒性試験のガイドラインについて」の別添「医薬品毒性試験法ガイドライン」等を参照すること。

2　ヒト幹細胞加工医薬品等の効力又は性能を裏付ける試験

1　技術的に可能かつ科学的に合理性のある範囲で，実験動物又は細胞等を用い，適切に設計された試験により，ヒト幹体性幹細胞，ヒトiPS(様)細胞，又はヒトES細胞加工医薬品等の機能発現，作用持続性及び医薬品・医療機器として期待される臨床効果の実現可能性(Proof-of-Concept)を示すこと。

2　遺伝子導入細胞にあっては，導入遺伝子からの目的産物の発現効率及び発現の持続性，導入遺伝子の発現産物の生物活性並びに医薬品等として期待される臨床効果の実現可能性(Proof-of-Concept)を示すこと。

3　適当な動物由来細胞・組織製品モデル又は疾患モデル動物がある場合には，それを用いて治療効果を検討すること。

4　確認申請(治験開始〔First-in-Man〕)段階では，当該製品の効力又は性能による治療が他の治療法と比較したときはるかに優れて期待できることが国内外の文献又は知見等により合理的に明らかにされている場合には，必ずしも詳細な実験的検討は必要とされない。

3　ヒト幹細胞加工医薬品等の体内動態

1　製品を構成する細胞・組織及び導入遺伝子の発現産物について，技術的に可能で，かつ，科学的合理性がある範囲で，実験動物での吸収及び分布等の体内動態に関する試験等により，患者等に適用された製品中の細胞・組織の生存期間，効果持続期間を推測し，目的とする効果が十分得られることを明らかにすること。(注：体内動態に関する試験等には，例えば組織学的検討，AluPCR法，磁気共鳴画像診断法(MRI)，陽電子放射断層撮影法(PET)，単一光子放射断層撮影法(SPECT)，バイオイメージングなどがある)。

2　ヒト幹細胞加工医薬品等の用法(投与方法)について，動物実験を通してその合理性を明らかとすること。特に，全身投与にあっては投与後の細胞の全身分布を動物実験などから外挿し，有用性の観点から議論すること。(注：投与経路ごとにどこに生着するかは不明であるが，全身投与よりも局所投与が望ましいと想定される。しかし，全身投与であってもその有用性において被投与患者に有益であると合理的に説明が可能である場合には用法として設定可能である。例えば，あるヒト幹細胞加工医薬品等を肝疾患治療剤として肝臓への生着を期待する場合，肝臓へ効率よく到達させかつその他の臓器への分布を最低限に抑えることが合理的な投与方法であると想定されるが，経末梢静脈投与により当該細胞が肝臓に集積し，他臓器に生着しないことが証明できれば良い。しかし，異所性生着しても，被投与患者にとって不利益(生体機能への悪影響)が生じない場合は用法として肯定できるかも知れない。異所性分化による不利益とは，例えば間葉系幹細胞が心臓に異所性生着して骨形成する場合が想定され，それが不整脈を惹起したような場合である。)

3　当該細胞・組織が特定の部位(組織等)に直接適用又は到達して作用する場合には，その局在性を明らかにし，局在性が製品の有効性・安全性に及ぼす影響を考察すること。

4　臨床試験

ヒト幹細胞加工医薬品等の治験を開始する(First-in-Man)に当たって支障となる品質及び安全性上の問題が存在するか否かの確認申請等の段階における安全性については，臨床上の有用性を勘案して評価されるものであり，ヒト幹細胞加工医薬品等について予定されている国内の治験計画について以下の項目を踏まえて評価すること。その際，明らかに想定される製品のリスクを現在の学問・技術を駆使して排除し，その科学的妥当性を明らかにした上で，なお残る未知のリスクと，重篤で生命を脅かす疾患，身体の機能を著しく損なう疾患，身体の機能や形態を一定程度損なうことによりQOLを著しく損なう疾患などに罹患し，従来の治療法では限界があり，克服できない患者に対する不作為のリスクとのリスクの大小を勘案し，かつ，これらすべての情報を開示した上で患者の自己決定権に委ねるという視点を導入することが望まれる。

1　対象疾患

2　対象とする被験者及び被験者から除外すべき患者の考え方

3　ヒト幹細胞加工医薬品等及び併用薬の適用を含め，被験者に対して行われる治療内容(注：投与・移植した細胞の機能を維持・向上・発揮させるために併用する薬剤が想定される場合，当該薬剤の作用を*in vitro*あるいは*in vivo*で検証すること)。

4　既存の治療法との比較を踏まえた臨床試験実施の妥当性

5　現在得られている情報から想定される製品及び患者のリスク及びベネフィットを含め，被験者への説明事項の案

なお，臨床試験は，適切な試験デザイン及びエンドポイントを設定して実施する必要があり，目的とする細胞・組織の由来，対象疾患及び適用方法等を踏まえて適切に計画すること。

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AW551984: a novel regulator of cardiomyogenesis in pluripotent embryonic cells

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An understanding of the mechanism that regulates the cardiac differentiation of pluripotent stem cells is necessary for the effective generation and expansion of cardiomyocytes as cell therapy products. In the present study, we have identified genes that modulate the cardiac differentiation of pluripotent embryonic cells. We isolated P19CL6 cell sublines that possess distinct properties in cardiomyogenesis and extracted 24 CMR (cardiomyogenesis-related candidate) genes correlated with cardiomyogenesis using a transcriptome analysis. Knockdown of the CMR genes by RNAi (RNA interference) revealed that 18 genes influence spontaneous contraction or transcript levels of cardiac marker genes in EC (embryonal carcinoma) cells. We also performed knockdown of the CMR genes in mouse ES (embryonic stem) cells and induced *in vitro* cardiac differentiation. Three CMR genes, *AW551984*, *2810405K02Rik* (RIKEN cDNA 2810405K02 gene) and *Cd302* (CD302 antigen), modulated the cardiac differentiation of both EC cells and ES cells. Depletion of *AW551984* attenuated the expression of the early cardiac transcription factor *Nkx2.5* (NK2 transcription factor related locus 5) without affecting transcript levels of pluripotency and early mesoderm marker genes during ES cell differentiation. Activation of Wnt/β-catenin signalling enhanced the expression of both *AW551984* and *Nkx2.5* in ES cells during embryoid body formation. Our findings indicate that AW551984 is a novel regulator of cardiomyogenesis from pluripotent embryonic cells, which links Wnt/β-catenin signalling to Nkx2.5 expression.

Key words: cardiac differentiation, cardiomyogenesis, embryonal carcinoma cell, embryonic stem cell, Wnt signalling.

INTRODUCTION

The heart is the first organ to form in the vertebrate embryo. Cardiac progenitor cells are derived from the mesoderm, which emerges from the primitive streak during gastrulation. Cardiac progenitor cells migrate into the anterolateral regions of the embryo to form the cardiac crescent and subsequently contribute to the myocardium and endocardium of the heart [1,2]. The *in vitro* differentiation of ES (embryonic stem) cells assembling into aggregates, which are called EBs (embryoid bodies), mimics early embryonic development and is commonly conducted for cardiomyogenesis [3]. Wnt/β-catenin signalling is crucial for the differentiation of ES cells into cardiomyocytes as well as for heart development [4]. Recent studies have reported that Wnt/β-catenin signalling plays biphasic roles in cardiomyogenesis. Activation of Wnt/β-catenin signalling in the early phase of EB cultures promotes cardiac differentiation, whereas late activation of Wnt/β-catenin signalling inhibits cardiac differentiation [5,6]. Extracellularly secreted Dkk-1 (Dickkopf-1) interacts with a Wnt co-receptor LRP6 (low-density lipoprotein-receptor-related protein 6) [7] and modulates cardiac differentiation owing to its antagonistic inhibition of Wnt/β-catenin signalling [4–6].

Heart diseases such as myocardial infarction damage cardiomyocytes and consequently lead to a significant loss of the contractile capacity of the heart [8]. To repair functions of the injured heart, a great deal of research has attempted to develop regenerative medicine using ES cell- or iPS (induced pluripotent stem)-cell-based cardiomyocytes as cell therapy products [9]. ES cells and iPS cells are pluripotent and able to differentiate spontaneously into a variety of cell types, including cardiomyocytes [3,10,11]. However, the efficiency of the current methods available for the cardiac differentiation of pluripotent stem cells is insufficient for clinical settings [3,12]. A comprehensive understanding of the mechanism involved in the cardiac differentiation of pluripotent stem cells is necessary to improve the differentiation efficiency.

In the present study, we provide a list of genes whose expression is significantly correlated with efficiency in mouse EC (embryonal carcinoma) cell cardiomyogenesis induced by DMSO. RNAi (RNA interference) experiments for these genes revealed that *AW551984*, *2810405K02Rik* (RIKEN cDNA 2810405K02 gene) and *Cd302* (CD302 antigen) effectively modulate the cardiac differentiation of both EC cells and ES cells. *AW551984* transcriptionally increased in response

Abbreviations used: *2810405K02Rik*, RIKEN cDNA 2810405K02 gene, *Adm*, adrenomedullin; BMP, bone morphogenetic protein; *Cd302*, CD302 antigen; CMR, cardiomyogenesis-related candidate; Dkk-1, Dickkopf-1; EB, embryoid body; EC, embryonal carcinoma; ES, embryonic stem; *F2r*, coagulation factor II (thrombin) receptor; FBS, fetal bovine serum; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Gata4*, GATA binding protein 4; iPS, induced pluripotent stem; LIF, leukaemia inhibitory factor; *Mef2c*, myocyte enhancer factor 2C; MEF, mouse embryonic fibroblast; *Mesp1*, mesoderm posterior 1; MHC, mysoin heavy chain; *Mlc2a*, myosin light chain 2a; *Mlc2v*, myosin light chain 2v; *Myh6*, α-MHC; *Myh7*, β-MHC; *Nkx2.5*, NK2 transcription factor related locus 5; *Oct3/4*, octamer-binding protein 3/4; PCA, principal component analysis; *Ptprb*, protein tyrosine phosphatase receptor type B; qRT-PCR, quantitative real-time PCR; RNAi, RNA interference; siRNA, small interfering RNA; *Tbx5*, T-box 5; *VWA5A*, von Willebrand factor A domain containing 5A.

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to Wnt/β-catenin signalling stimulation during ES cell differentiation and regulated the expression of *Nkx2.5* (NK2 transcription factor related locus 5), an early cardiac transcriptional factor. Our findings demonstrate that AW551984 is a novel regulator of cardiomyogenesis that links Wnt/β-catenin signalling to Nkx2.5 expression.

MATERIALS AND METHODS

EC cell culture and differentiation

P19 cells and P19CL6 cells were obtained from the A.T.C.C. and RIKEN Cell Bank respectively. Mouse EC cells were maintained in α-minimum essential medium (Invitrogen) supplemented with 10% FBS (fetal bovine serum; Cell Culture Technologies), 2 mM L-glutamine (Sigma–Aldrich), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 37 °C in an atmosphere containing 5% CO_2. To prepare P19CL6 cell sublines, P19CL6 cells were co-transfected with a DNA plasmid that encoded the EGFP (enhanced green fluorescent protein) gene (Clontech) driven by mouse *Myh6* [α-MHC (myosin heavy chain)] promoter (a gift from Professor Jeffrey Robbins, Children's Hospital Medical Center, Cincinnati, OH, U.S.A.) and pcDNA3.1 (Invitrogen) using Lipofectamine™ 2000 (Invitrogen), followed by selection with 1 mg/ml G418 (Sigma–Aldrich). A total of 72 G418-resistant clones were isolated, out of which four clones (CL6G26, CL6G36, CL6G45 and CL6G52) with a wide range of cardiac differentiation efficiencies were selected for further analysis. For differentiation, cells were trypsinized and suspended in EC cell growth medium containing 1% DMSO [13]. The cells were seeded at a density of 4×10^4 cells/well on six-well plates (BD Biosciences) or Lab-Tek Chamber Slide two-well Permanox Slides (Nunc). To evaluate the differentiation efficiency, the number of nodules/cm^2 that were beating spontaneously was counted under a microscope. In the GeneChip experiments, the differentiation efficiency was assessed non-parametrically by the number of beating nodules/cm^2 (grade 1, <0.098/cm^2 corresponding to one beating nodule/six-well plate; grade 2, 0.098 to 157/cm^2 corresponding to one beating nodule/well on six-well plates; and grade 3, >157/cm^2 corresponding to one beating nodule/microscopic image magnified ×200). EC cell differentiation medium was replaced every other day. The day of beginning the differentiation was set as day 0.

qRT-PCR (quantitative real-time PCR)

Total RNA was isolated from cells with an RNeasy Mini kit (Qiagen) or a BioRobot M48 Workstation (Qiagen), according to the manufacturer's instructions. One-step qRT-PCR was performed with a QuantiTect Probe RT-PCR kit (Qiagen) on an ABI Prism 7000 Sequence Detection System or a 7300 real-time PCR System (Applied Biosystems). The expression levels of target genes were normalized to those of 18S rRNA or the *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) transcript, which were quantified using TaqMan rRNA control reagents or TaqMan rodent *Gapdh* control reagents (Applied Biosystems) respectively. Probes and primers were obtained from Applied Biosystems and Sigma–Aldrich. The sequences of probes and primers used in the present study are shown in Supplementary Table S1 (at http://www.BiochemJ.org/bj/437/bj4370345add.htm.

GeneChip and biostatistical analysis

Total RNA isolated from undifferentiated EC cell strains (P19 cells, P19CL6 cells and four P19CL6 cell sublines) was converted into biotinylated cRNA using Two-Cycle Target Labelling and Control Reagents (Affymetrix). Labelled RNA was processed for microarray hybridization to MOE430A and MOE430B GeneChips (Affymetrix), which contain 22626 and 22511 probe sets (13410 and 9249 mouse RefSeq transcripts) respectively. An Affymetrix GeneChip Fluidics Station was used to perform streptavidin/phycoerythrin staining. The hybridization signals on the microarray were scanned using a GeneChip Scanner 3000 (Affymetrix) and analysed using GCOS software (Affymetrix). Normalization was performed by global scaling with the arrays scaled to a trimmed average intensity of 500 after excluding the 2% of probe sets with the highest and the lowest values. The hybridization experiments were performed in five samples of each EC cell strain. The NCBI GEO (National Center for Biotechnology Information Gene Expression Omnibus) accession number for the microarray data is GSE26875. To extract the informationally significant probe sets from the data set, we filtered probe sets using the following three steps. First, probe sets were regarded as 'absent' when indicated as 'present' by 'absolute analysis' using GCOS software in less than three samples from one strain. Probe sets regarded as 'absent' in all strains were eliminated from the data set. Secondly, when no significant difference was observed among strains using ANOVA ($P \geqslant 0.05$), probe sets were also eliminated from the data set. Thirdly, if the difference between the maximum and minimum mean values of probe sets in the strains was equal to or more than 2.5-fold, probe sets were used for further analysis.

After data standardization (*z*-scoring) of the cardiac marker genes [*Nkx2.5* (NK2 transcription factor related locus 5), *Gata4* (GATA binding protein 4), *Mef2c* (myocyte enhancer factor 2C), *Myh6* (α-MHC), *Myh7* (β-MHC), *Mlc2a* (myosin light chain 2a) and *Mlc2v* (myosin light chain 2v)] expressed in the differentiated EC cell strains (P19 cells, P19CL6 cells and four P19CL6 cell sublines), PCA (principal component analysis) was performed to project the data into a lower-dimensional space using SYSTAT 10.2 software. Eigenvalues of the first and second factors equal 4.43 and 1.20 and represent 63.3 and 17.1% of the total variability. The factor loading plot indicates the coefficients of all variables on the first and second principal components (Supplementary Figure S1A at http://www.BiochemJ.org/bj/437/bj4370345add.htm). The first and second principal component scores were calculated and averaged at days 8, 12, 16 and 20 for each strain (Supplementary Figures S1B and S1C). Cardiac differentiation evaluated by lag time to the onset of beating and by the number of beating nodules was shown among EC cell strains (Figure S1D). To identify probe sets related to the cardiac differentiation of EC cells, the correlation between the intensity values of the filtered probe sets and three variables (the maximum of the first principal component scores in each strain, lag time to the onset of beating and beating nodule numbers assessed non-parametrically at day 20) was determined by calculating Spearman's rank correlation coefficients [14] and their *P* values. Probe sets exhibiting statistically significant correlations with all three variables ($P < 0.05$) were selected.

ES cell culture and differentiation

Mouse ES cells (R1 [15]; A.T.C.C.) were maintained with mitomycin C-inactivated MEFs (mouse embryonic fibroblasts; Millipore) on gelatin-coated dishes in DMEM (Dulbecco's modified Eagle's medium; Sigma–Aldrich) supplemented with 20% (v/v) FBS (ESGRO), 1000 units/ml LIF (leukaemia inhibitory factor; ESGRO), 1 mM sodium pyruvate (Invitrogen),

0.1 mM 2-mercaptoethanol (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 37 °C in an atmosphere containing 5% CO_2. Before differentiation, R1 cells were dissociated by trypsinization and separated from MEFs by pre-plating on non-coated dishes for 30 min at 37 °C. To initiate differentiation, 750 ES cells in 20 μl of ES cell growth medium without LIF were cultured in a hanging drop for 3 days. The EBs formed were transferred on to non-adherent 96-well round-bottom plates (PrimeSurface; Sumitomo Bakelite) and cultured for an additional 2 days. At day 5 of differentiation, each EB was transferred to a well on a gelatin-coated 48-well plate (Iwaki). EBs exhibiting spontaneous contraction were counted daily to calculate the percentage of beating EBs. The differentiation medium was replaced every other day. The day of starting the hanging-drop formation was set as day 0. A total of 48 EBs were observed in one group for each experiment.

siRNA (small interfering RNA) transfection

CL6G52 cells were plated at 1×10^4 cells/well in 400 μl of EC cell growth medium on 24-well plates. The next day, cells were transfected with 100 nM Stealth RNAi™ siRNA (Invitrogen) using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's instructions. After 48 h of transfection, cells were used for further analysis. R1 cells seeded at a density of 5×10^5 in 5 ml of ES cell growth medium on a gelatin-coated 60-mm-diameter dish were transfected with 50 nM Stealth RNAi™ siRNA using Lipofectamine™ RNAiMAX (Invitrogen), according to the manufacturer's instructions. After 24 h of transfection, cells were used for further analysis. Stealth RNAi™ siRNA negative control duplex (Invitrogen) was used as a control. The sequences of siRNAs used in the present study are shown in Supplementary Table S2 (at http://www.BiochemJ.org/bj/437/bj4370345add.htm).

Western blot analysis

Undifferentiated R1 cells and EBs were lysed in RIPA buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA) containing protease inhibitor mixture (Roche). Cell lysates were centrifuged at 12000 *g* for 10 min to prepare the supernatant. Proteins were separated using SDS/PAGE, transferred on to PVDF membranes and probed with primary antibodies. The immunoreactive bands were visualized with HRP (horseradish peroxidase)-conjugated anti-(mouse IgG) or anti-(rabbit IgG) antibodies (Jackson ImmunoResearch) and an ECL (enhanced chemiluminescence) Plus Western Blotting Detecting System (GE Healthcare), and were detected using a LAS 4000 mini (Fujifilm).

Antibodies and reagents

To raise the anti-AW551984 antibody, a synthetic peptide LMPNGTPQQRQNSQKKK(C) (amino acids 654–671 of mouse AW551984) was coupled to KLH (keyhole limpet haemocyanin) and used as an antigen. Rabbits were immunized by multiple injection of the cross-linked peptide with adjuvant. The antibody was affinity-purified with antigen peptide coupled to gel (Medical and Biological Laboratories). The anti-β-actin antibody (AC15) was obtained from Sigma–Aldrich. Recombinant mouse Wnt3a and Dkk-1 was purchased from R&D Systems.

RESULTS

Identification of CMR (cardiomyogenesis-related candidate) genes

We attempted to isolate genes related to the DMSO-induced cardiomyogenesis of mouse EC cells. Our approach to identify genes for cardiac differentiation is essentially based on statistical comparison of GeneChip signal intensities of undifferentiated EC cell strains with their cardiac differentiation. The scheme highlighting the gene identification process is shown in Figure 1. To search for genes that correlated with the cardiac differentiation of EC cells, GeneChip data were obtained from undifferentiated EC cell strains and filtered by the three steps described in the Materials and methods section. The correlations of signal intensities of the filtered probe sets with three variables, i.e. (i) first principal component scores of cardiac marker gene expression, (ii) lag time to the onset of beating, and (iii) number of beating nodules at day 20, were determined by calculating Spearman's rank correlation coefficients. Finally, probe sets derived from 24 genes {*Prdm5* (PR domain containing 5), *AW551984*, *D430028G21Rik* [*Mavs* (mitochondrial antiviral signalling protein)], *5330410G16Rik* [*Tmem59l* (transmembrane protein 59-like)], *Tmem98* (transmembrane protein 98), *Ctsc* (cathepsin C), *F2r* [coagulation factor II (thrombin) receptor], *Sema3e* (sema domain Ig domain short basic domain secreted 3E), *Maged2* (melanoma antigen family D2), *2810405K02Rik*, *Rhox4b* (reproductive homeobox 4B), *Cd302*, *Fzd1* (Frizzled homologue 1), *Adarb1* (adenosine deaminase RNA-specific B1), *Gpaa1* (GPI anchor attachment protein 1), *Chst2* (carbohydrate sulfotransferase 2), *9830115L13Rik* [*Zc3hav1* (zinc finger CCCH type, antiviral 1)], *9630055N22Rik* [*Mfsd7b* (major facilitator superfamily domain containing 7B)], *Ptprb* (protein tyrosine phosphatase receptor type B), *Gstz1* (glutathione transferase ζ1), *1110021L09Rik* (RIKEN cDNA 1110021L09 gene), *Tsga14* (testis-specific gene A14), *Hnrnpa1* (heterogeneous nuclear ribonucleoprotein A1) and *Adm* (adrenomedullin)} exhibited statistically significant correlations with all of these variables, and we named these 24 genes CMR genes for convenience (Table 1 and Supplementary Table S3 at http://www.BiochemJ.org/bj/437/bj4370345add.htm). Nine of the 24 genes also had significant correlations between the intensity values of probe sets and the second principal component scores of cardiac marker gene expression (Supplementary Table S3).

Validation of CMR genes as regulators of cardiac differentiation in EC cells

To examine whether or not the selected 24 CMR genes function as regulators of cardiac differentiation, we first attempted to knockdown CMR gene expression in CL6G52 cells by siRNA transfection. qRT-PCR analysis verified that the siRNAs used in the present study successfully suppressed the expression of 21 CMR genes (excluding CMR11, CMR19 and CMR22) in the EC cells at 48 h after transfection (Supplementary Figure S2 at http://www.BiochemJ.org/bj/437/bj4370345add.htm). The mRNAs for CMR11, CMR19 and CMR22 were not significantly reduced by the siRNAs that we used in the present study. To determine the differentiation states of CL6G52 cells transfected with the validated siRNAs, the number of nodules beating spontaneously was counted in cells, the differentiation of which was initiated by DMSO. The number of beating nodules decreased significantly in cells transfected with siRNAs targeting CMR2–CMR5, CMR9, CMR10, CMR13, CMR18 and CMR24 (Table 2). In contrast, RNAi against CMR7, CMR12, CMR16,

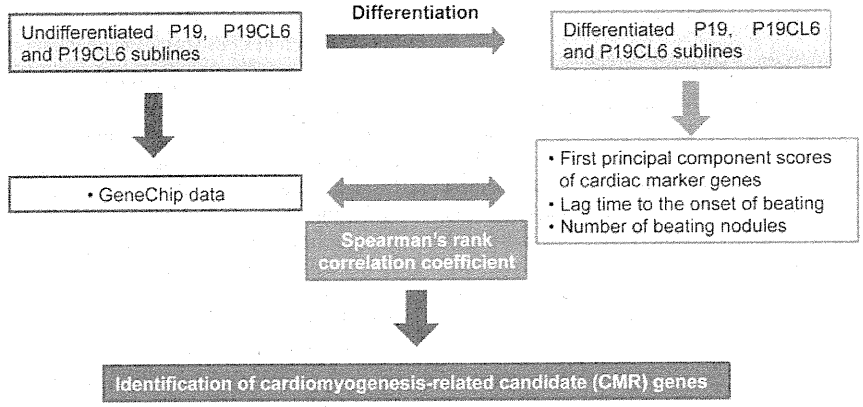

Figure 1 Biostatistical-based approach for identification of CMR genes

Genome-wide expression analysis on GeneChips of undifferentiated P19 cells, P19CL6 cells and P19CL6 cell sublines (CL6G26, CL6G36, CL6G45 and CL6G52) was performed as described in the Materials and methods section. The differentiation of EC cells into cardiomyocytes was induced by stimulation with DMSO. PCA was conducted using standardized data of cardiac marker gene expression in differentiated cells, and the first principal component scores were calculated. Statistical correlation of the probe-set intensities with the first principal component scores, lag time to the onset of beating and number of nodules was determined using Spearman's rank correlation coefficient. Genes of probe sets exhibiting significant correlation with the three variables ($P < 0.05$) were identified as CMR genes.

Table 1 Identified CMR genes

CMR gene number	Gene name	Gene symbol	GenBank® accession number
CMR1	PR domain containing 5	*Prdm5*	NM_027547
CMR2	Expressed sequence AW551984	*AW551984*	NM_178737
CMR3	RIKEN cDNA D430028G21 gene	*D430028G21Rik*	NM_144888
CMR4	RIKEN cDNA 5330410G16 gene	*5330410G16Rik*	NM_182991
CMR5	Transmembrane protein 98	*Tmem98*	NM_029537
CMR6	Cathepsin C	*Ctsc*	NM_009982
CMR7	Coagulation factor II (thrombin) receptor	*F2r*	NM_010169
CMR8	Semaphorin 3E	*Sema3e*	NM_011348
CMR9	Melanoma antigen family D 2	*Maged2*	NM_030700
CMR10	RIKEN cDNA 2810405K02 gene	*2810405K02Rik*	NM_025582
CMR11	Reproductive homeobox 4B	*Rhox4b*	NM_133362
CMR12	CD302 antigen	*Cd302*	NM_025422
CMR13	Frizzled homologue 1	*Fzd1*	NM_021457
CMR14	Adenosine deaminase RNA-specific B1	*Adarb1*	NM_130895
CMR15	GPI anchor attachment protein 1	*Gpaa1*	NM_010331
CMR16	Carbohydrate sulfotransferase 2	*Chst2*	NM_018763
CMR17	RIKEN cDNA 9830115L13 gene	*9830115L13Rik*	AK036481
CMR18	RIKEN cDNA 9630055N22 gene	*9630055N22Rik*	AK044206
CMR19	Protein tyrosine phosphatase receptor type B	*Ptprb*	BB119527
CMR20	Glutathione transferase ζ1	*Gstz1*	NM_010363
CMR21	RIKEN cDNA 1110021L09 gene	*1110021L09Rik*	AK003902
CMR22	Testis-specific gene A14	*Tsga14*	NM_031998
CMR23	Heterogeneous nuclear ribonucleoprotein A1	*Hnrnpa1*	NM_010447
CMR24	Adrenomedullin	*Adm*	NM_009627

CMR17, CMR21 and CMR23 significantly facilitated the beating nodule development. No significant effect of CMR1, CMR6, CMR8, CMR14, CMR15 or CMR20 on beating nodule number was observed.

To evaluate precisely the degree of cardiac differentiation of CL6G52 cells transfected with siRNA, we measured the mRNA levels of cardiac markers after the addition of DMSO by qRT-PCR. mRNA levels of cardiac markers [*Nkx2.5*, *Gata4*, *Mef2c*, *Myh6* (α-MHC), *Myh7* (β-MHC), *Mlc2a* and *Mlc2v*] in non-transfected CL6G52 cells changed in a time-dependent manner (Supplementary Figure S3 at http://www.BiochemJ.org/bj/437/bj4370345add.htm). Transcript expression of *Myh6* (α-MHC) at day 14 was significantly decreased by the knockdown of CMR2–CMR5, CMR10, CMR13 and CMR18, whereas it was increased by knockdown of CMR12 and CMR23 (Figure 2A). Knockdown of CMR2 and CMR13 significantly inhibited *Myh7* (β-MHC) expression (Figure 2B). In contrast, knockdown of CMR16, CMR17 and CMR23 markedly elevated *Myh7* (β-MHC) expression. The expression of *Mlc2a* was significantly inhibited by knockdown of CMR2, CMR4, CMR8, CMR10, CMR20 and CMR23, and enhanced by CMR16 knockdown (Figure 2C). Knockdown of CMR2–CMR6, CMR10 and CMR13 significantly decreased MLC2v expression, whereas knockdown of CMR7, CMR12, CMR16 and CMR23 increased its expression (Figure 2D). Collectively, RNAi against 17 CMR genes exhibited significant effects on marker genes in the cardiac differentiation of EC cells.

Table 2 Effects of CMR gene knockdown on the number of beating nodules in EC cells

CL6G52 cells were transfected with 100 nM siRNA targeting the CMR genes or the negative control siRNA. After 48 h of transfection, differentiation of CL6G52 cells was initiated with 1% DMSO. Nodules beating spontaneously were counted per well with a microscope at day 14. Results are means ± S.E.M. ($n = 4$). Statistical significance was determined using a Student's t test (*$P < 0.05$ compared with control).

	Number of beating nodules (% of the control)	
CMR gene number	Negative control siRNA	CMR gene siRNA
CMR1	100.0 ± 3.0	93.1 ± 2.7
CMR2	100.0 ± 7.8	3.4 ± 1.3*
CMR3	100.0 ± 1.5	52.7 ± 4.9*
CMR4	100.0 ± 2.7	22.9 ± 4.2*
CMR5	100.0 ± 2.7	47.9 ± 5.3*
CMR6	100.0 ± 1.5	85.0 ± 6.6
CMR7	100.0 ± 1.1	115.0 ± 2.2*
CMR8	100.0 ± 4.9	95.7 ± 4.7
CMR9	100.0 ± 3.8	60.5 ± 3.8*
CMR10	100.0 ± 14.5	13.7 ± 0.7*
CMR12	100.0 ± 7.1	179.6 ± 9.1*
CMR13	100.0 ± 9.2	55.3 ± 2.6*
CMR14	100.0 ± 4.8	106.4 ± 5.2
CMR15	100.0 ± 9.9	91.5 ± 6.3
CMR16	100.0 ± 6.0	151.7 ± 9.7*
CMR17	100.0 ± 4.8	172.2 ± 2.7*
CMR18	100.0 ± 3.5	67.0 ± 2.4*
CMR20	100.0 ± 6.3	102.1 ± 4.6
CMR21	100.0 ± 5.9	132.2 ± 6.5*
CMR23	100.0 ± 6.4	151.3 ± 8.0*
CMR24	100.0 ± 3.8	35.7 ± 6.0*

Validation of CMR genes as regulators of cardiac differentiation in ES cells

To initiate ES cell differentiation into cardiomyocytes, EBs were formed by hanging-drop culture and cultured further on non-adherent round-bottom plates. The EBs transferred to adherence culture began to exhibit spontaneous beating at day 8 and an elevated rate of beating by day 11 (Figure 3A). To examine whether or not the expression of CMR genes changed in a time-dependent manner, transcript levels of CMR genes were measured at days 0, 3, 5 and 11 by qRT-PCR. A statistically significant correlation ($P < 0.05$) between gene expression and days was observed in CMR2, CMR4–CMR9, CMR12–CMR18 and CMR20 (Figure 3B). To verify the roles of CMR genes in the cardiac differentiation of ES cells, R1 cells were transfected with siRNAs targeting the CMR genes, the knockdown efficiency of which was confirmed (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/437/bj4370345add.htm), and were subjected to EB formation 24 h after transfection. We analysed the expression of cardiac marker genes at day 8 of differentiation. RNAi against CMR2, CMR8, CMR10, CMR14, CMR15, CMR20 and CMR23 significantly inhibited the expression of both *Myh6* (α-MHC) and *Mlc2a*, almost consistent with the case of *Myh7* (β-MHC) and *Mlc2v* (Figure 4). In contrast, knockdown of CMR7, CMR9, CMR12, CMR21 and CMR24 significantly enhanced the expression of both *Myh7* (β-MHC) and *Mlc2a*. Moreover, knockdown of CMR9 and CMR12 also increased levels of *Mlc2v* and *Myh6* (α-MHC) respectively. The effects of CMR genes on the cardiac differentiation of EC cells and ES cells are summarized in Supplementary Table S4 (at http://www.BiochemJ.org/bj/437/bj4370345add.htm).

Depletion of the CMR10 gene, *2810405K02Rik*, exhibited significant inhibitory effects on cardiac differentiation in both EC cells and ES cells. Depletion of the CMR12 gene, *Cd302*, however, increased most of the cardiac marker genes and promoted cardiomyogenesis in both EC cells and ES cells. It is of note that depletion of the CMR2 gene, *AW551984*, whose function is almost unknown, markedly blocked the expression of all cardiac gene markers that we measured in both EC cells and ES cells when these cells were differentiated. These results indicate that, in our identified CMR genes, CMR2 is the most potent regulator of cardiac differentiation in EC cells and ES cells.

AW551984 regulates *Nkx2.5* gene expression during cardiac differentiation of ES cells in response to Wnt3a

We attempted to analyse the function of CMR2, *AW551984*, in ES cell differentiation into cardiomyocytes. As shown in Figure 3(B), the mRNA level of the *AW551984* gene was drastically elevated during the differentiation of ES cells. We confirmed the protein levels of AW551984 during the EB differentiation of R1 cells by immunoblotting. The band corresponding to AW551984 was almost undetectable in undifferentiated cells but clearly appeared at day 8 (Figure 5A), supporting the increase in transcript levels of *AW551984* during differentiation. In addition, we counted the number of EBs exhibiting spontaneous beating on a daily basis after transfection with siRNA targeting *AW551984*. Consistent with the inhibited expression of cardiomyocyte markers (Figure 4), knockdown of *AW551984* markedly decreased the number of EBs with beating activities (Figure 5B). These results strongly support the notion that AW551984 that is elevated during differentiation regulates the cardiac differentiation of ES cells. To identify the developmental stage regulated by AW551984, we examined whether or not AW551984 affects the expression of embryonic markers in R1 cells. No significant effect of *AW551984* knockdown on *Nanog* and *Oct3/4* (octamer-binding protein 3/4) levels was observed at day 0, indicating that AW551984 does not modulate the stemness of ES cells (Figure 5C). Moreover, neither early mesoderm marker *T/brachyury* nor the cardiac mesoderm marker *Mesp1* (mesoderm posterior 1) were significantly affected by knockdown of *AW551984* (Figure 5D). This indicated that AW551984 is not involved in the development of ES cells into cardiac mesoderm. We next examined the expression of early cardiac transcription factors *Gata4*, *Mef2c*, *Nkx2.5* and *Tbx5* (T-box 5) during the differentiation of ES cells. Interestingly, the RNAi against *AW551984* significantly reduced the expression of *Nkx2.5* at days 6 and 7, but did not affect those of *Gata4*, *Tbx5* or *Mef2c* (Figure 5E). These results strongly suggest that AW551984 mainly regulates cardiac differentiation via the expression of Nkx2.5.

Wnt/β-catenin signalling has recently been reported to regulate the cardiac differentiation of mouse ES cells biphasically. Stimulation of ES cells with Wnt3a in the early phase for EB formation enhanced the cardiac differentiation associated with the development of cardiac markers such as Nkx2.5 [5,6]. Therefore we next examined whether or not Wnt3a in the early phase for EB formation influenced AW551984 expression. The addition of Wnt3a (100 ng/ml) into differentiation medium from day 2 to day 5 markedly increased the transcript of *AW551984* as well as that of *Nkx2.5* in differentiated ES cells at day 5 (Figure 5F). Reciprocally, the addition of Dkk-1 (500 ng/ml), an extracellular inhibitor of Wnt/β-catenin signalling from days 2–5 significantly inhibited the expression of both *AW551984* and *Nkx2.5* at day 5 (Figure 5G) and at day 7 (results not shown). Thus Wnt/β-catenin signalling enhanced the expression of AW551984 during EB formation, leading to the commitment of ES cells into a cardiac lineage through Nkx2.5 expression.

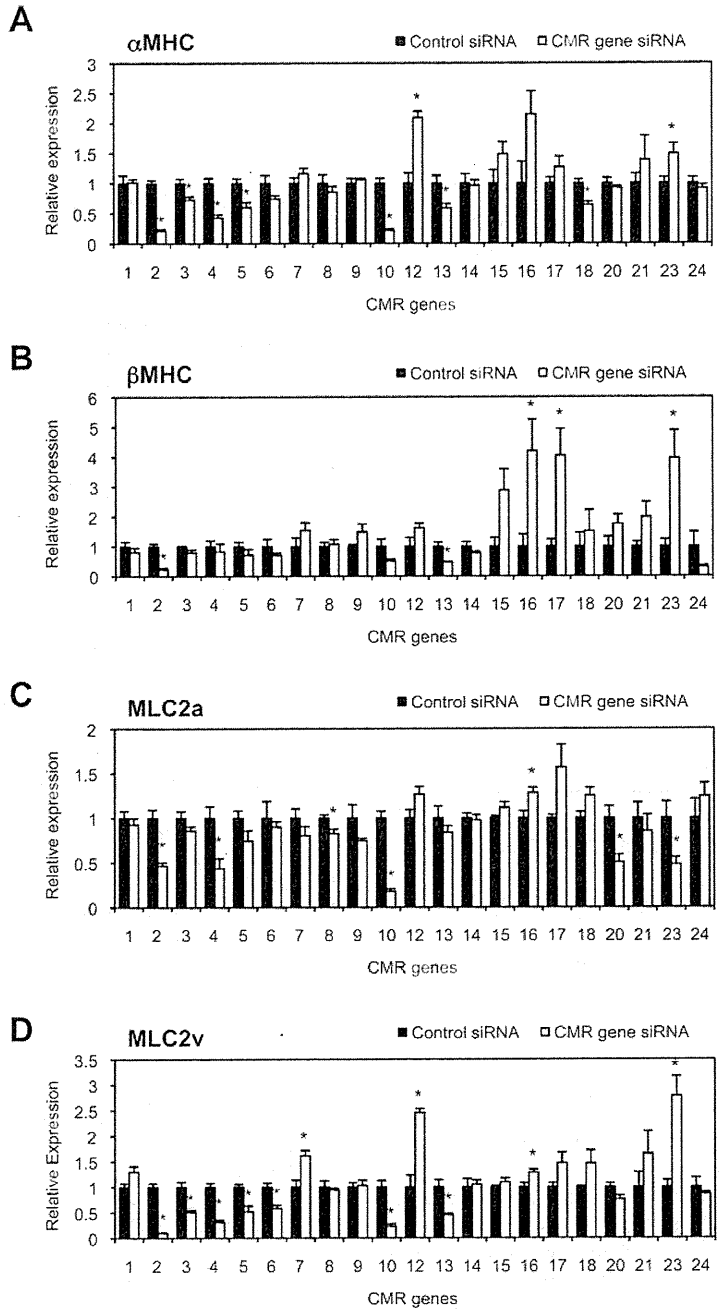

Figure 2 Effects of CMR gene knockdown on mRNA levels of *Myh6* (α-MHC), *Myh7* (β-MHC), *Mlc2a* and *Mlc2v* in differentiated EC cells

CL6G52 cells were transfected with 100 nM siRNA targeting CMR genes or the negative control siRNA. After 48 h of transfection, differentiation of CL6G52 cells was initiated with 1% DMSO. At day 14, total RNA was isolated from differentiated CL6G52 cells and subjected to one-step qRT-PCR. mRNA levels were normalized to 18S ribosomal RNA levels. Expression levels of *Myh6* (α-MHC) (**A**), *Myh7* (β-MHC) (**B**), *Mlc2a* (**C**) and *Mlc2v* (**D**) in cells transfected with the negative control were set to 1. Results are means ± S.E.M. ($n = 4$). Statistical significance was determined using a Student's *t* test (*$P < 0.05$ compared with control).

DISCUSSION

EC cells and ES cells are used as *in vitro* models of early embryonic development, and they are useful for studying the mechanism underlying the cardiac differentiation of stem cells [3]. In the present study, we have identified cardiomyogenesis-related candidate genes expressed in EC cells through comprehensive analysis. A knockdown study using siRNA revealed that the candidate genes actually influence the cardiac differentiation of EC cells and ES cells. Furthermore, *AW551984*, one of the candidate genes, potently regulated the cardiac differentiation via the expression of the *Nkx2.5* transcript and is suggested to act downstream of Wnt/β-catenin signalling.

Several studies have attempted to identify the factors involved in the cardiac differentiation of EC cells and ES cells [12,13].

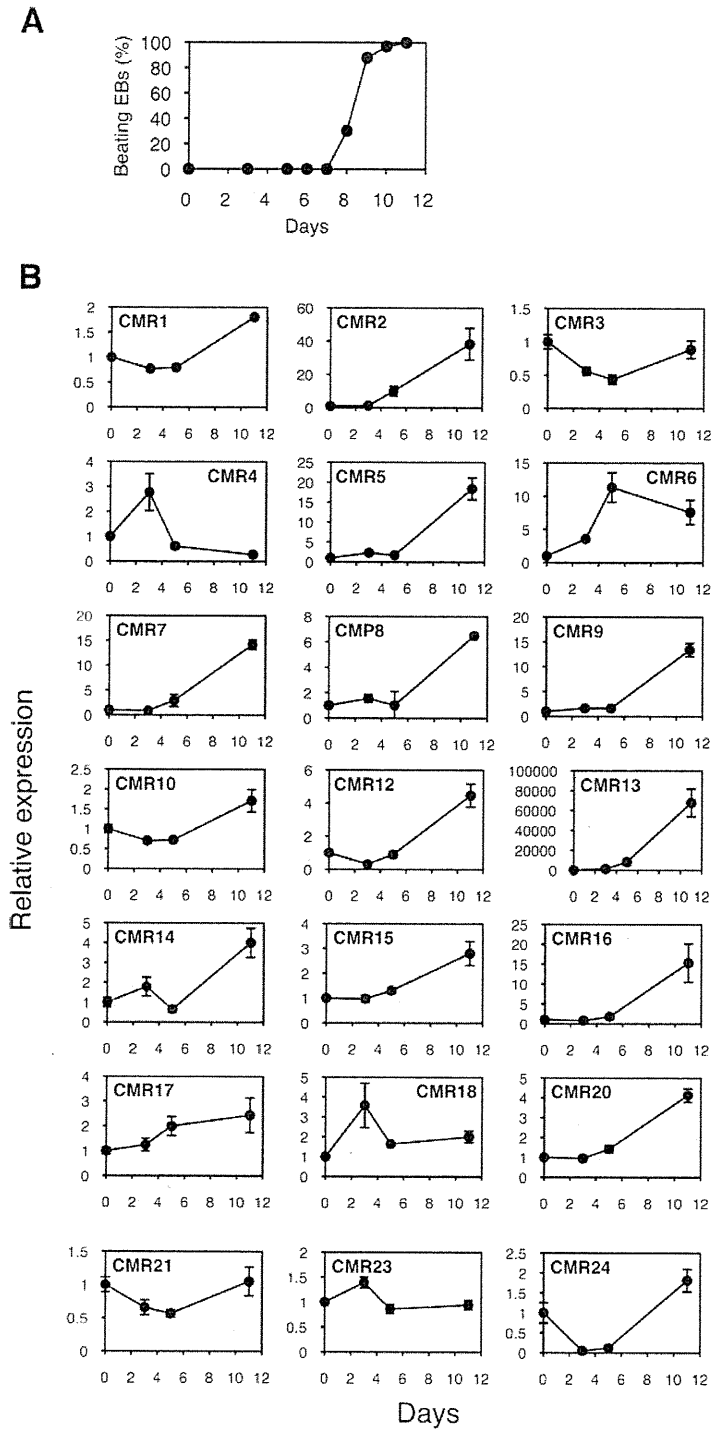

Figure 3 Time courses of beating development and CMR gene expression in ES cells during differentiation

Differentiation of R1 cells was initiated by forming EBs without LIF. (**A**) EBs exhibiting spontaneous beating were counted and the percentages were calculated. (**B**) Total RNA of R1 cells at days 0, 3, 5 and 11 was isolated and subjected to one-step qRT-PCR. mRNA levels of the CMR genes were normalized to those of *Gapdh*. Expression levels of cells at day 0 were set to 1. Results are means ± S.E.M. ($n = 5$). Statistical significance between gene expression and days was determined using a Spearman's rank correlation coefficient test ($P < 0.05$) and were observed for CMR2, CMR4–CMR9, CMR12–18 and CMR20.

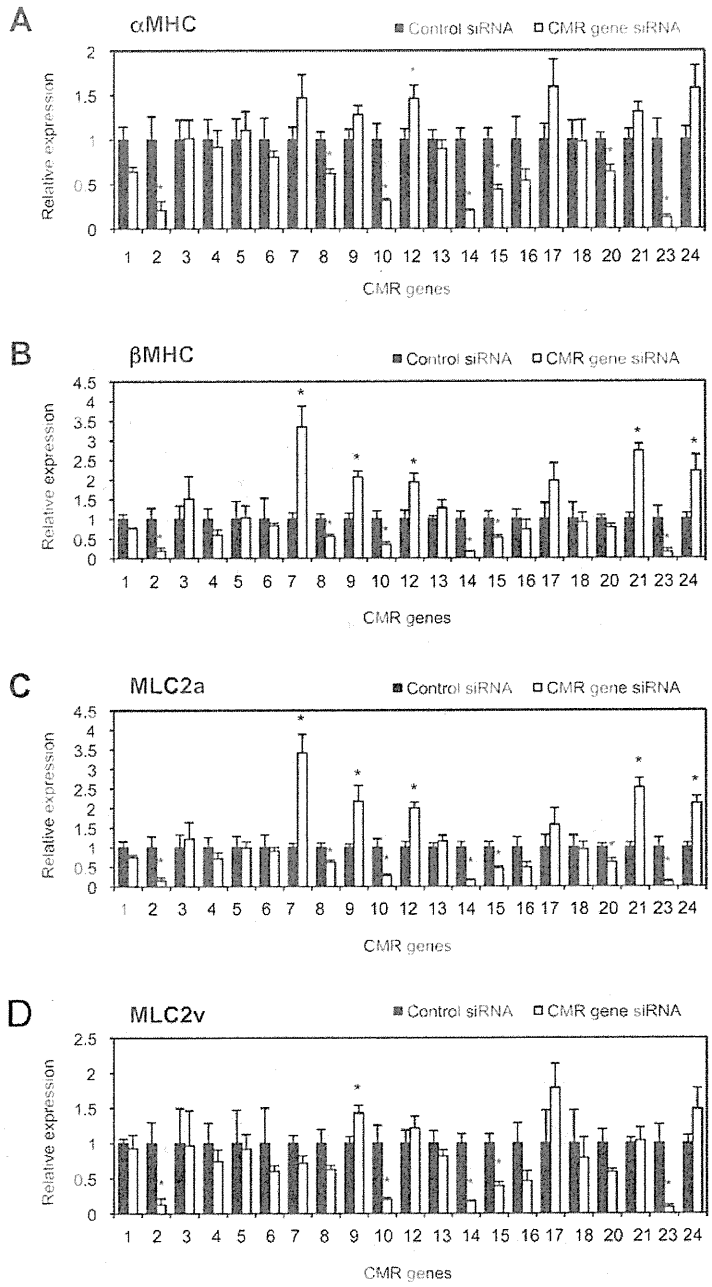

Figure 4 Effects of CMR gene knockdown on mRNA levels of *Myh6* (α-MHC), *Myh7* (β-MHC), *Mlc2a* and *Mlc2v* in differentiated ES cells

R1 cells were transfected with 50 nM siRNA targeting the CMR genes or the negative control siRNA in the presence of 1000 units/ml LIF. After 24 h of transfection, differentiation of R1 cells was initiated by forming EBs without LIF. At day 8, total RNA was isolated from differentiated R1 cells and subjected to one-step qRT-PCR. mRNA levels were normalized to those of *Gapdh*. Expression levels of *Myh6* (α-MHC) (**A**), *Myh7* (β-MHC) (**B**), *Mlc2a* (**C**) and *Mlc2v* (**D**) in cells transfected with the negative control were set to 1. Results are means ± S.E.M. ($n = 5$). Statistical significance was determined using a Student's *t* test (*$P < 0.05$ compared with control).

We sought to determine whether or not any additional factors existed to achieve efficient differentiation into cardiomyocytes. To address this question, we statistically compared gene expression patterns in an undifferentiated state with cardiomyogenic potential of the EC cell strains and successfully identified 24 CMR genes (Table 1). The CMR genes contained *F2r*, *Ptprb* and *Adm* genes, which are associated with cardiac development *in vivo*. Knockout mouse studies have demonstrated that the ablation of *F2r* [16], *Ptprb* [17] and *Adm* [18] leads to fetal bleeding, defects of angiogenesis and the heart, and cardiovascular defects respectively, indicating the high reliability of our approach in the identification of cardiomyogenesis candidate genes. It is worth noting that our screen did not isolate any cardiac transcription factors reported previously. Expression of cardiac transcription factors increases approximately in a time-dependent manner 'after' induction of differentiation. However, it is kept low

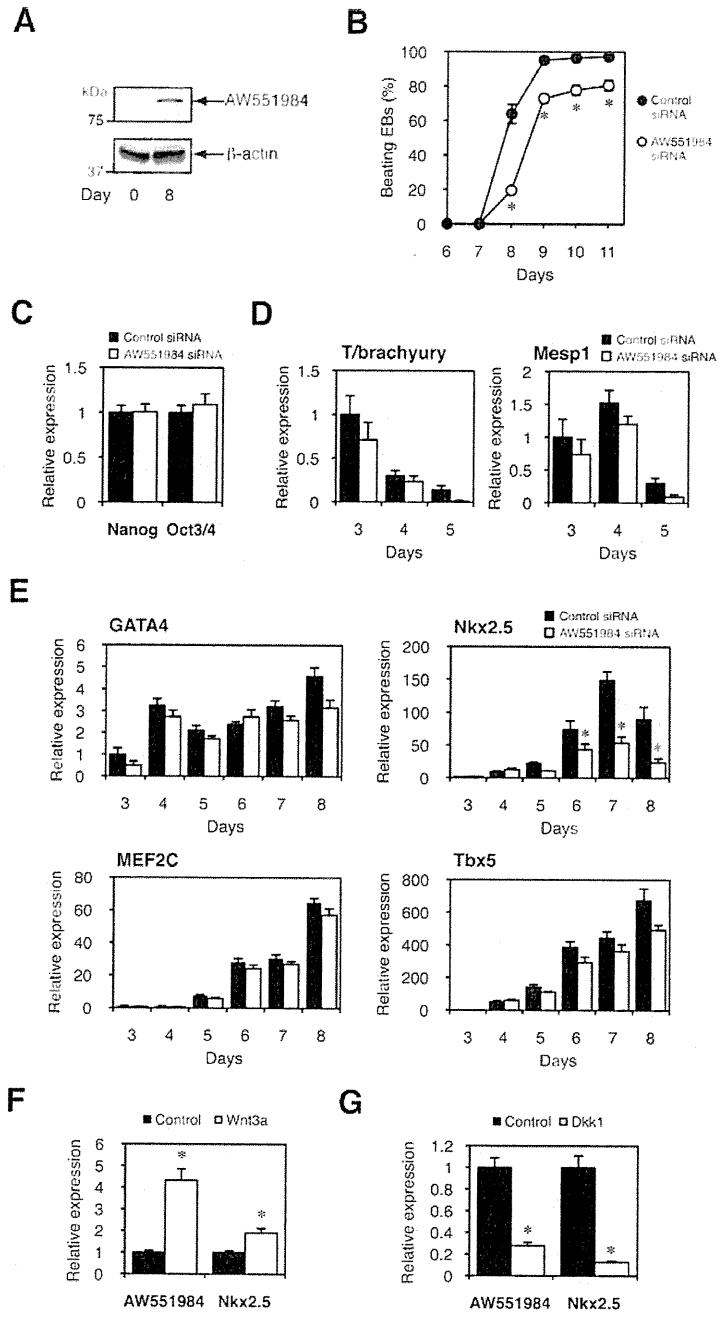

Figure 5 AW551984 is required for *Nkx2.5* gene expression during cardiac differentiation of ES cells and is regulated by Wnt/β-catenin signalling

(**A**) Cell lysates from R1 cells at days 0 and 8 were subjected to Western blot analysis. AW551984 in the cell lysates was detected using the anti-AW551984 antibody. (**B–E**) R1 cells were transfected with 50 nM siRNA targeting *AW551984* or the negative control siRNA in the presence of 1000 units/ml LIF. After 24 h of transfection, differentiation of R1 cells was initiated by forming EBs. (**B**) EBs exhibiting spontaneous beating were counted and the percentages were calculated. Results are means ± S.E.M. from three independent experiments. Statistical significance was determined using two-way repeated measures ANOVA and Bonferroni's post-hoc test (*P<0.01 compared with control). (**C**) After 24 h of transfection, total RNA was isolated from the cells. mRNA levels of *Nanog* and *Oct3/4* were measured by one-step qRT-PCR and normalized to those of *Gapdh*. Expression levels in cells transfected with the negative control were set to 1. Results are means ± S.E.M. ($n = 5$). No statistical significance was observed using a Student's t test ($P \geqslant 0.05$ compared with control). (**D**) Total RNA was isolated from the cells at days 3, 4 and 5 and subjected to one-step qRT-PCR. mRNA levels of *T/brachyury* and *Mesp1* were normalized to those of *Gapdh*. Expression levels in cells that were transfected with the negative control and differentiated at day 3 were set to 1. Results are means ± S.E.M. ($n = 5$). No statistical significance on interaction between days and siRNAs was observed using two-way ANOVA ($P \geqslant 0.05$). (**E**) Total RNA of R1 cells at days 3–8 was isolated and subjected to one-step qRT-PCR. mRNA levels of *Gata4*, *Nkx2.5*, *Mef2c* and *Tbx5* were normalized to those of *Gapdh*. These expression levels in cells that were transfected with the negative control and differentiated at day 3 were set to 1. Results are means ± S.E.M. ($n = 5$). Statistical significance was determined using two-way ANOVA and Bonferroni's post-hoc test (*P < 0.01 compared with control). (**F** and **G**) EBs were cultured in the absence or presence of 100 ng/ml Wnt3a (**F**) or 500 ng/ml Dkk-1 (**G**) from days 2–5. Total RNA was isolated at day 5 and subjected to one-step qRT-PCR. Results are means ± S.E.M. ($n = 5$). Statistical significance was determined using a Student's t test (*P < 0.01).

in the undifferentiated state. We isolated genes whose expression 'before' induction was associated with cardiomyogenesis in EC cells. Therefore some of the CMR genes are presumed to play more significant roles at initiation or a very early stage of cardiac differentiation in EC cells, compared with cardiac transcription factors. In addition, we confirmed the effects of the CMR genes on cardiac differentiation of EC cells and ES cells through RNAi experiments. *AW551984*, *2810405K02Rik* and *Cd302* were particularly effective at modulating the cardiac differentiation of both EC cells and ES cells (Figures 2 and 4, Table 2 and Supplementary Table S4).

RNAi against *AW551984* notably inhibited the cardiac differentiation of mouse EC cells and ES cells (Figures 2 and 4, and Table 2). To the best of our knowledge, we are the first to demonstrate that AW551984 is involved in the cardiac differentiation of stem cells. Furthermore, we revealed that AW551984 selectively regulates the expression of a cardiac transcription factor *Nkx2.5*, but not *Gata4*, *Mef2c* or *Tbx5* (Figure 5E). Because Nkx2.5 is a homeobox transcription factor that is essential for the development of ventricular cardiomyocytes [19], AW551984 regulates the cardiac differentiation of ES cells mainly through Nkx2.5 activities. Ritner et al. [20] have recently reported that the expression of Nkx2.5 is shown to spike and then decreases during EB differentiation in human ES cells [20]. Expression of *Nkx2.5* peaked at day 7 of differentiation in our experimental conditions, and knockdown of *AW551984* inhibited *Nkx2.5* expression at days 6–8 (Figure 5E). Therefore AW551984 exerts its effects on cardiomyogenesis due to modulating levels but not the peaking time of *Nkx2.5* transcripts. Wnt/β-catenin signalling is known to regulate the biphasic cardiac differentiation of ES cells. Treatment of EBs with Wnt3a in the early stages of ES cell differentiation is known to facilitate cardiac differentiation following an increase in Nkx2.5 expression [5,6]. However, factors elevating early cardiac transcription factors are poorly known downstream of Wnt/β-catenin signalling. In the present study, we have demonstrated that Wnt/β-catenin signalling in the early stages of ES cell differentiation enhances the expression of *AW551984* as well as that of *Nkx2.5* (Figures 5F and 5G). Collectively, our results suggest that AW551984 plays a significant and critical role in the cardiac differentiation of ES cells as a novel intermediate molecule linking Nkx2.5 expression to Wnt/β-catenin signalling. The transcription-factor-binding site prediction web server (http://www.sabiosciences.com/chipqpcrsearch.php?app=TFBS) predicted that p300 acetyltransferase could regulate the gene expression of both *AW551984* and the human orthologue of AW551984 *VWA5A* (von Willebrand factor A domain containing 5A). p300 binds to β-catenin and serves as a co-activator of β-catenin, regulating the β-catenin–Tcf4 (T-cell factor) interaction [21]. β-Catenin stabilized by Wnt signalling may inversely influence the function of p300, leading to up-regulation of AW551984 expression. Identifying the mechanism by which AW551984 regulates Nkx2.5 expression should be carried out to enhance our understanding of cardiac differentiation in the future.

AW551984 has been reported by several groups to be associated with cancer. The human orthologue *VWA5A* is located at chromosome 11q23-q24, which corresponds to regions of frequent loss of heterozygosity in solid tumours [22]. *AW551984* was also identified as a metastasis-related gene, and knockdown of this gene by shRNA (small hairpin RNA) accelerated cell migration in a melanoma cell line [23]. Thus AW551984 had been recognized as a tumour suppressor. Interestingly, mRNA expression of *AW551984* decreased in NIH 3T3 cells responding to Wnt3a [24], in direct contrast with our results with EBs treated with Wnt3a (Figure 5F). This inverse effect of Wnt3a may be explained by the difference in the cell types. Alternatively, non-canonical Wnt signalling may contribute to the decrease in *AW551984* expression in NIH 3T3 cells.

2810405K02Rik has been identified as a novel type of prostamide/prostaglandin F synthase belonging to the thioredoxin-like superfamily [25]. Prostamide/prostaglandin F synthase has been reported to be abundantly expressed in the spinal cord and is thought to play an important role in the central nervous system [26]. However, its function in terms of cardiac development remains unknown.

Cd302 is a C-type lectin receptor and has been reported to be involved in cell adhesion and migration as well as endocytosis and phagocytosis [27]. Knockdown of *Cd302* significantly facilitated cardiac differentiation in both EC and ES cells, although expression of the *Cd302* gene in undifferentiated EC cells was positively correlated with the first principal component score and number of nodules, and was negatively correlated with lag time to the onset of beating (Table S3). This may be due to the effective timing of *Cd302* suppression by RNAi on cardiomyogenesis. The direction of regulating ES cell cardiomyogenesis by Wnt/β-catenin and BMP (bone morphogenetic protein) signalling can actually be determined by the timing of the addition of ligands and their antagonists [5,28,29]. Cd302 may possibly act downstream of the Wnt/β-catenin and/or BMP signalling.

Recently, differentiated ES cells and iPS cells have been extensively explored as cell therapy products for regenerative medicine because of their pluripotency and unlimited growth. To yield the expected differentiated cells, it is necessary to increase the differentiation efficiency of ES and iPS cells. The CMR genes identified in the present study will enable the more efficient differentiation of ES cells and iPS cells into cardiomyocytes *in vitro*, thereby adding to the known factors that facilitate cardiac differentiation. The functional roles of the CMR gene homologues need to be clarified in human ES cells and iPS cells, the properties of which are similar to those of mouse epiblast stem cells [30]. In addition, the CMR genes may contribute not only to cardiomyogenesis, but also to cardiac organogenesis and may assist in our understanding of the mechanism of cardiac development *in vivo* in the future.

AUTHOR CONTRIBUTION

Satoshi Yasuda contributed to the conception and design of the study, and performed the ES cell experiments. Tetsuya Hasegawa conducted the microarray and RNAi experiments in the EC cell lines with Tetsuji Hosono. Kei Watanabe analysed the phenotypes of the EC cell lines with Kageyoshi Ono. Mitsutoshi Satoh, Shunichi Shimizu, Takao Hayakawa, Teruhide Yamaguchi and Kazuhiro Suzuki provided theoretical input and critical advice for the stem cell phenotyping. Yoji Sato led the conception and design of the study, established the CL6 cell sublines and directed the work. Satoshi Yasuda, Mitsutoshi Satoh, Takao Hayakawa, Teruhide Yamaguchi, Shunichi Suzuki and Yoji Sato also contributed to securing funding. Satoshi Yasuda wrote the manuscript together with Tetsuya Hasegawa, Kei Watanabe, Mitsutoshi Satoh, Shunichi Shimizu and Yoji Sato. The remaining authors commented on the final text.

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online data

SUPPLEMENTARY ONLINE DATA

AW551984: a novel regulator of cardiomyogenesis in pluripotent embryonic cells

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See the following pages for Supplementary Figures S1–S4 and Supplementary Tables S1–S4.

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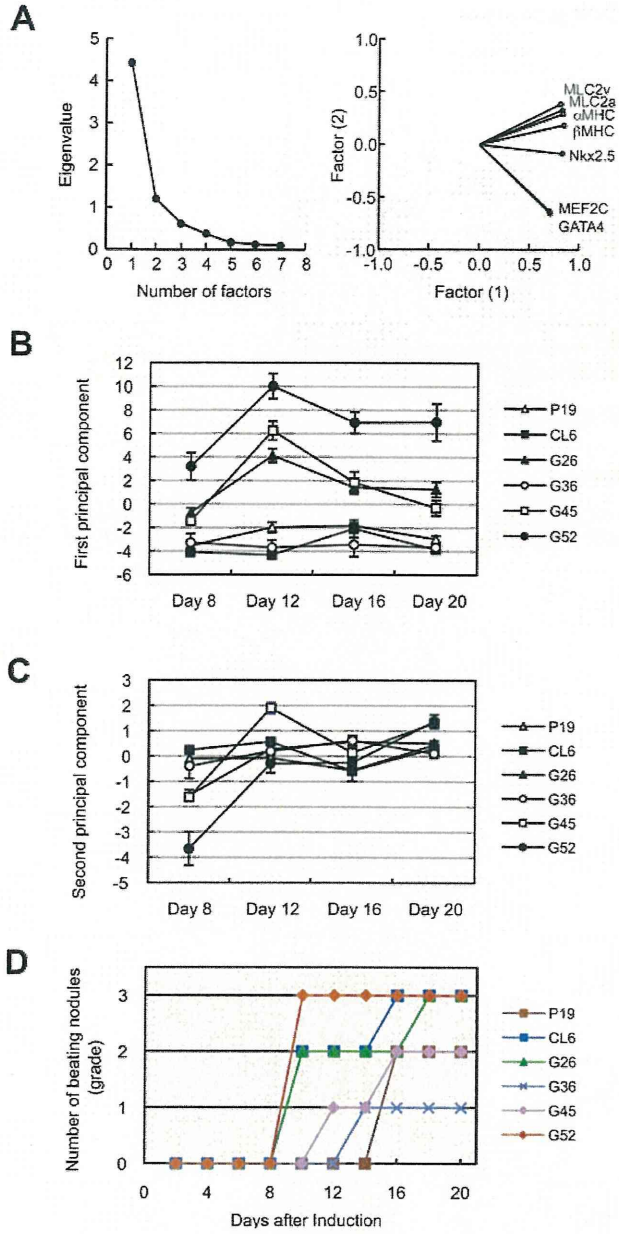

Figure S1 Cardiomyogenesis of P19 cells, P19CL6 cells and P19CL6 cell sublines

(**A–C**) PCA was performed using expression data of cardiac marker genes [*Nkx2.5*, *Gata4*, *Mef2c*, *Myh6* (α-MHC), *Myh7* (β-MHC), *Mlc2a* and *Mlc2v*] in differentiated P19 cells, P19CL6 cells and P19CL6 cell sublines (CL6G26, CL6G36, CL6G45 and CL6G52). (**A**) Scree plot indicates eigenvalues plotted against the factor numbers (left-hand panel). Factor loadings plot compares the two factor analyses (right-hand panel). (**B**) First component scores of P19 cells, P19CL6 cells and P19CL6 cell sublines at days 8, 12, 16 and 20. (**C**) Second component scores of P19 cells, P19CL6 cells and P19CL6 cell sublines at days 8, 12, 16 and 20. (**D**) P19 cells, P19CL6 cells and P19CL6 cell sublines (CL6G26, CL6G36, CL6G45 and CL6G52) were differentiated in the presence of 1 % DMSO. Nodules exhibiting spontaneous beating were counted with a microscope every other day and assessed non-parametrically, as described in the Materials and methods section of the main paper.

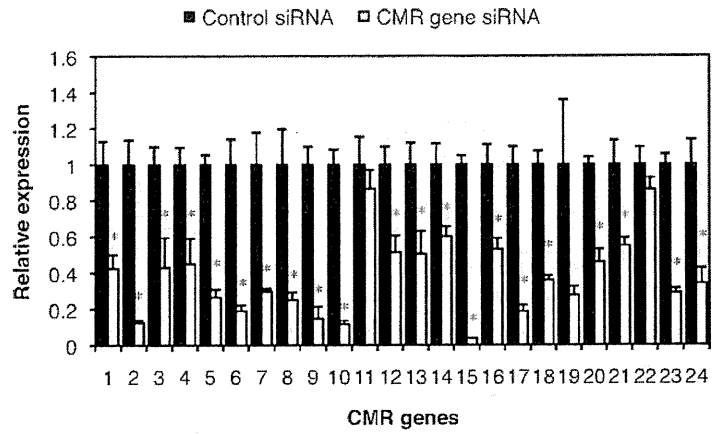

Figure S2 Efficiency of CMR gene knockdown by siRNA targeting the CMR genes in EC cells

CL6G52 cells were transfected with 100 nM siRNA targeting the CMR genes or the negative control siRNA. After 48 h, total RNA was isolated from the transfected cells. mRNA levels of the CMR genes were measured by qRT-PCR and normalized to 18S rRNA levels. Expression levels in cells transfected with the negative control were set to 1. Results are means $\pm$ S.E.M. ($n = 6$). Statistical significance was determined using a Student's t test (*$P < 0.05$ compared with control).