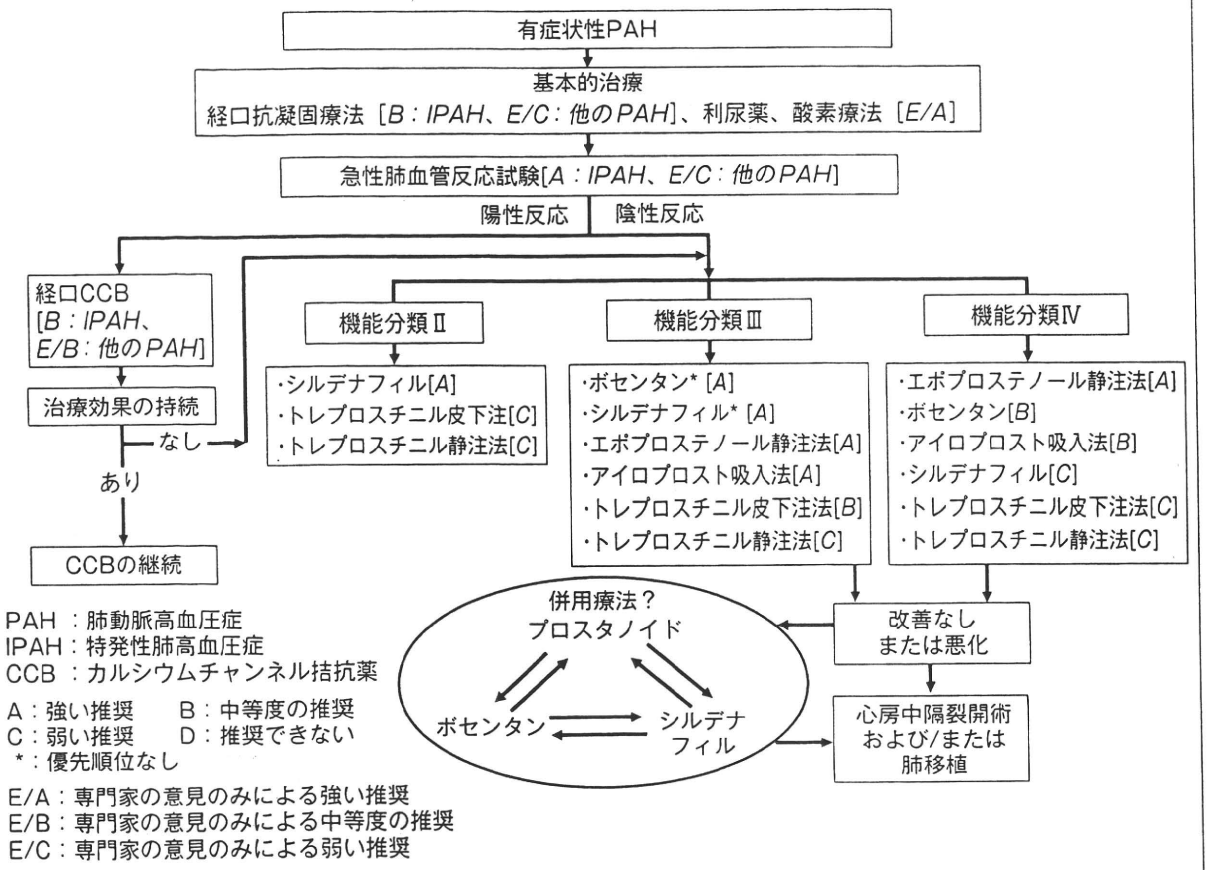

図 14-1　肺高血圧症治療ガイドライン

2007 年 ACCP ガイドラインより一部改変

が異なるため、正確な原因疾患の診断が重要といえる。**図 14-1** に 2007 年に Chest 誌に発表された ACCP（American College of Chest Physician）のガイドラインを示す。現在日本で使用可能な治療薬を順に概説したい。なお、以下の治療薬の多くは、投与初期（エポプロステノールは増量期間中）には血圧が低下することがあり患者に血圧測定を義務づけた方がよい。

内科的治療法；

酸素療法

内服薬：利尿薬、抗凝固療法（ワルファリン）、カルシウム拮抗薬、ベラプロスト（プロサイリン®、ベラサス LA®）、ボセンタン（トラクリア®）、シルデナフィル（レバチオ®）

持続静注薬：エポプロステノール（フローラン®）

外科的治療法；

肺移植

以下にそれぞれの治療法について解説する。

酸素療法

肺高血圧症が進行すると、血中酸素濃度が低下する。血中酸素濃度の低下は、息切れや呼吸困難の原因となる。また、血中酸素濃度が低下した状態では、肺血管が収縮して肺高血圧症がさらに悪化する。そのため、一部の患者では酸素の継続投与が必要で、在宅酸素療法を行う場合がある。

利尿薬

右心不全が進行してくると、心不全コントロールのために利尿薬が必要となる。

抗凝固療法：ワルファリン

ワルファリンは肺高血圧症の予後を改善するとも報告されている。そのため、出血のリスクがない場合にはワルファリンが適応となる場合がある。

カルシウム拮抗薬

一部の肺高血圧症の患者でカルシウム拮抗薬に反応して肺血管が拡張し、肺動脈圧が低下することが報告されている。しかし、カルシウム拮抗薬に反応性がある患者は少なく、反応性がある場合にのみ適応となる。カルシウム拮抗薬が有効であるのは、カテーテル検査中に急性血管反応性検査（肺血管拡張作用のあるどのような血管拡張薬でも構わないので血圧低下が限界に達するまで投与量を増やして肺動脈圧の低下度をみる）を行い急性血管拡張反応が陽性（肺動脈圧が前値の20%以上低下しかつ平均肺動脈圧が40 mmHg以下となる）となる患者に限られる。**図14-1**に示すガイドラインで、一般的ケアのあと急性血管反応性試験に進むフローチャートが示されている。急性血管反応性検査が陽性だとカルシウム拮抗薬により特発性肺動脈性肺高血圧症の5年予後は95%と報告されている[4]。欧米では全患者の10-20%に急性血管反応陽性者が存在するとされるが本邦では5%以下といわれている。

ベラプロスト（プロサイリン®、ベラサスLA®）

ベラプロストは、肺血管を拡張させる作用があるプロスタグランジンI2製剤のうち、内服で投与できる製剤である。また、ベラサスLA®は、成分はプロサイリン®と同じベラプロストだが、徐放性製剤であり、血中濃度をより安定して持続させる効果がある。ベラサスLA®は前向き研究で6分間歩行距離等が改善しているが、開発会社の行った治験以外にエビデンスがない。従来のベラプロストは、6カ月までは有意に6分間歩行距離の改善効果があるが、9カ月で投与前に戻り長期効果がないことからアメリカでは認可とならなかった。軽症例には有効であることは示されており、今後は徐放性製剤についても効果を確認していく必要がある。

エポプロステノール（フローラン®）；プロスタグランジンI2製剤）

肺動脈に対する強い血管拡張作用と抗血小板凝集作用を有する薬剤である。1999年より、日本で認可された。重度の肺高血圧症に対して使用され、現在使用できる治療薬の中で最もエビデンスが蓄積されており、治療効果があるとされる。エポプロステノールが使用できるようになってから、肺高血圧症患者の予後は飛躍的に改善した。欧米では特発性肺動脈性肺高血圧症の5年生存率を30%から70%前後にまで改善した[5]。

図14-1のガイドラインでWHO　Ⅳ度（WHOの分類はNYHA心機能分類とほぼ同様の基準で、症状は呼吸困難を対象とした臨床症状からの重症度分類、ちなみにNYHA心機能分類は胸痛や動悸など他の心臓病の症状も対象としている）においてはエポプロステノールのみが「強く推奨」とされている。すなわち重症例ではエポプロステ

ノールが適応となる。肺高血圧症の予後には右心不全の程度が強く関連するが、以前の我々の検討では肺血管抵抗値 20 Wood unit が右心不全出現の目安であった。また実際に WHO Ⅳ度となるとⅢ度に比べて予後が不良であるため、真に重症化する少し手前と考えて肺血管抵抗値 15 Wood unit あたりからエポプロステノールの適応を考え始める必要があると思われる。原因疾患も適応決定の考慮要素となるが、膠原病を原因とする患者ではエポプロステノールに対する反応がより良好で、逆に他の内服の治療薬は膠原病に対して効果がやや弱い傾向を示す。

エポプロステノールは、在宅持続点滴静注療法となるため清潔操作などの自己管理が十分にできることが必要となる。また、薬剤の半減期が約 6 分と非常に短いため、液体で中心静脈カテーテルから体内へ持続静注投与が必要となる。中心静脈カテーテルはヒックマンカテーテルといわれるもので、皮下に埋め込む処置が必要である。ヒックマンカテーテルの植え込みとエポプロステノールを含有した携帯ポンプを常時携帯する必要があり、患者の quality of life を低下させるという欠点がある。また、ヒックマンカテーテルの皮下留置部からの感染の危険性があり、感染によりヒックマンカテーテルが抜けて再挿入が必要となる場合や、感染により心不全が増悪する場合もありえる。

薬剤自身の副作用は頭痛、顔のほてり、皮膚の発赤、下痢、骨痛などで頻度は低くないが対照的に対処していける。投与前から右心不全がある症例ではエポプロステノールによる心拍出量増加の結果右心不全が悪化することもあることも心得ておく。

投与法は、ヒックマンカテーテル挿入後、1 ng/mL/min より開始し 4 日毎程度で 1 ng/mL/min ずつ増量し、2 - 3 カ月経過したら 7 日毎の増量とする。半年で増量を中止するか 1 年間増量するかはカテーテル検査を施行して判断する。初回治療で血圧低下を生じない範囲でスピーディに増量していくことが治療成功の鍵になる。

海外では、他のプロスタグランジン I 2 製剤で、吸入ができるものや皮下注射ができるものがあり、本邦でも将来的に認可される可能性もある。

ボセンタン（トラクリア®）；エンドセリン受容体拮抗薬

肺高血圧症患者では、血液中および肺組織中のエンドセリン - 1 という強力な血管収縮物質が多く作られているといわれている。このため、エンドセリン - 1 の作用を阻害すれば、肺高血圧症が改善できることが報告されている。エンドセリン受容体拮抗薬であるボセンタンは、肺血管収縮と肺血管平滑筋増殖を阻害することにより効力を発揮する。

欧米の前向き研究では WHO Ⅱ度およびⅢ度に対して投与され、2 年生存率 89% とコントロールの 57% を大幅に上回った。日本でも WHO Ⅱ度およびⅢ度の 21 例に対し、3 カ月後に 6 分間歩行距離 86 m の延長等の改善効果が認められた。

投与法は、1 日量 2 錠（1 錠 62.5 mg）を分 2 で投与開始し、1 カ月後副作用の出現がなければ 1 日量 4 錠を 1 日 2 回投与する。

自覚的な副作用は頭痛、顔面紅潮、筋痛などで多くは自制できる。他覚的な副作用が問題で、肝機能検査を 1 カ月に一度施行し、AST・ALT 値が正常値の 3 倍までは経過観察し、5 倍まではボセンタン減量で対処する。汎血球減少の副作用もあり、1 カ月に一度は末梢血検査を行う。妊産婦・授乳婦への投与は禁止となる。シクロスポリン、タクロリムスはボセンタンの血中濃度を上昇させ、グリベンクラミドは肝障害を増悪させるなど薬剤相互作用に注意する。

静脈圧，循環血液量，循環時間

1 静脈圧 venous pressure

一部の施設（手術後，集中治療室，小児科など）では中心静脈圧の代替として末梢静脈にカニュレーションして測定している．

1940年代に，侵襲を伴う中心静脈圧測定の代替として容易で安全に測定できる末梢静脈圧測定が行われるようになったが，その後両者の関連性に疑問がもたれ廃れていた．1970年代に一部で見直され現在では少数の施設で中心静脈圧の推定のために使用されている．末梢静脈の還流障害（四肢などの抑制，腫瘍，血栓など）があると中心静脈圧より異常に高値となる．

①方法：末梢静脈にカニュレーション後マノメーターへ接続しトランスデューサーを経て通常のカテーテル心内圧測定装置で計測される．

②正常値：中心静脈圧は右房圧（＜7～8 mmHgあるいは＜10 cm水柱）にほぼ等しいが，静脈圧はより末梢側で測定されるほど軽度高値となる（肘静脈で3 mmHg以内）．

③疾患との関連：右房圧が上昇する病態として，循環血液量の増加，右心不全，胸腔内圧上昇で高値となるほか，末梢静脈を収縮させるカテコールアミン，末梢静脈還流障害でも高値となる．

2 循環血液量 circulating blood volume

各種重症疾患や術後などにおいて，輸液量の決定などの目的でまれに測定される．

体内総血液量や循環血液量（体内総血液量の60％前後とされる）を厳密に知りたいときに測定されるが，

動脈にセンサー付きカテーテルを留置すると連続的に測定できる．

①方法：無害で血管外に漏出されず排泄・代謝もされにくい試薬を静注し，動脈でサンプリングして希釈度から計算する．

②試薬：インドサイアニングリーン（ICG），エバンスブルー，バイタルレッドなどがあるがICGが主に使用される．^{131}I，^{125}Iヒト血清アルブミンなどの放射性標識物質を使用することもある．ICGの希釈度は吸光計で計測され，動脈採血してサンプリングする原法から，非侵襲的に指の皮動脈からプローベで計測するもの，計測器が内蔵され循環血液量が自動計算されるカテーテル型のものもある．

③正常値：体内総血液量は60～70 mL/kgとなる．

④疾患との関連：心不全，腎不全などでは増加し，脱水，出血などでは減少する．

3 循環時間 circulation time

心機能をみる手段として以前は使用されたが現在では使用されることは非常に少ない．中枢性睡眠時無呼吸症の機序として心機能障害による循環時間の延長が注目されており，この分野の研究でしばしば計測されている．

心拍出量と反比例することから古くは心機能の指標として使用された．試薬を静注して臭気を感ずるまでの時間を腕肺時間と呼び，右心系の循環時間に相当し，試薬を静注して舌に苦みを感じるまでの時間を腕舌時間と呼び，両心の循環時間となる．

①方法：腕肺時間測定にはアリナミン10～20 mg，エーテル0.1 mLを，腕舌時間には20％コレレチン3～5 mL，10～20％塩化カルシウム1～2 mLを静注する．

②正常値：腕肺時間は4～9秒，腕舌時間は10～16秒．

③疾患との関連：心不全では腕舌時間は延長するが，右心不全があれば腕肺時間も延長する．高心拍出状態では短縮する．

容積脈波，サーモグラフィ

1 容積脈波 plethysmogram

末梢動脈の硬化の度合いが推定されるほか，末梢動脈は自律神経分布が豊富なため自律神経の状態を判定できる．

日本独自に発展した検査法で，特定の波長の近赤外光を末梢血管に当てヘモグロビンで反射する光量を連続的に測定して得られる．

①方法：脈波を検出するプローベを指に装着し数分で記録される．二次微分は加速度波と呼ばれ，記録が安定しており解析にはこちらが使用されることのほうが多い．図101に示すように加速度はa波～e波に分けられ各波高をa波の波高で除した値を評価する．

②正常値：図101は50歳の正常パターンを示し，動脈硬化が進行するとb波が浅くなり，d波が深くなる．

③疾患との関連：動脈硬化の程度を推定できるほか，連続測定したものをフーリエ解析して自律神経機能の評価に使われる．

2 サーモグラフィ thermography

血管狭窄・閉塞による血流障害，脊椎・神経障害，皮膚疾患，炎症，癌など，皮膚温度に変化をきたす疾

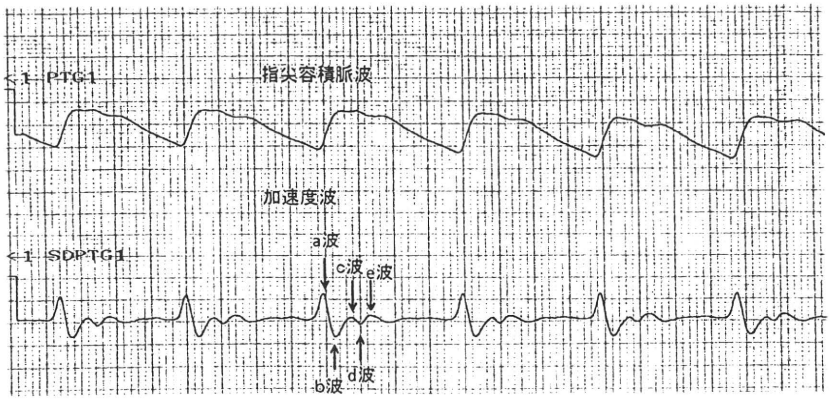

図101 容積脈波

患の診断に使用される.

体表の温度を赤外線センサーで感知して温度分布を表示する装置で，各種疾患による皮膚温度変化を無侵襲で表示して，診断や治療効果判定に使用される.

①方法：赤外線を検出するカメラを使用して目的部を撮影し，これをコンピュータ解析して温度に応じて着色して画像として表示する.

②正常値：正常の皮膚温分布と対照させるか左右差をみることにより異常を検出する.

③疾患との関連：(1)閉塞性動脈硬化症（ASO），Buerger病，糖尿病性末梢血管障害などの血流障害をきたす血管疾患により皮膚温が低下する.(2)脊椎疾患，片麻痺などの神経疾患により皮膚温が低下する.(3)種々の皮膚疾患で皮膚温が変化する.(4)関節などの炎症をきたす疾患で皮膚温が上昇する.

〔佐藤　徹〕

シルデナフィル（レバチオ®）；ホスホジエステラーゼ-5阻害薬

元々、勃起障害の治療薬として開発された薬剤である。ホスホジエステラーゼ-5という受容体を阻害する働きがあるが、陰茎の血管と同様に、肺血管にもホスホジエステラーゼ-5が多く存在することがわかり、肺高血圧症に使用されるようになった。本邦でも2008年に保険適応として認可されている。WHO Ⅱ度からⅣ度で適応となる。臨床的改善作用はエポプロステノールよりは劣るがボセンタンにほぼ匹敵するとされる。

シルデナフィルはホスホジエステラーゼ-5を阻害することにより血管内皮でのサイクリックGMPを増量して効果を発揮するが、サイクリックGMPには血管拡張作用と独立して後負荷増を原因とする心機能障害の強力な改善作用がある。肺高血圧症に伴う右心不全がまさにこれに相当し、右心不全を伴う症例では第一適応となる。

投与量は、1日量3錠（1錠20 mg）の分3が標準量とされる。血圧が低い患者では1/2量、1/4量から開始して可能なら増量する。シルデナフィルは効果に用量依存性がなく少量でも有効との意見もあり、これに関する前向き試験が予定されている。

副作用は、頭痛、顔面紅潮、下痢などでボセンタン同様自制可能な患者が多いが、中にどうしても継続が難しい患者もいる。ボセンタンと併用するとボセンタンの血中濃度を上げてボセンタンの副作用が発現しやすくなる。50歳以上の男性で糖尿病や高血圧を有する患者に勃起障害治療のためにシルデナフィルを使用すると失明が多かったとの報告があるが、肺高血圧症では視力障害等も報告されていない。視力障害を認めたら報告するよう念のため患者に伝えておく。

肺移植

一部の患者では上記までの現在使用可能な治療法での治療を継続しても治療抵抗性で右心不全が進行する場合がある。そのような患者では肺移植を検討する場合がある。

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Methods for Differentiation of Bone-Marrow-Derived Stem Cells into Myocytes

Shinji Makino and Keiichi Fukuda

Abstract Although heart transplantation is the ultimate therapy for severe heart failure, it is not widely used owing to the inadequate supply of donor hearts. Therefore, cell-based therapies for the prevention or treatment of cardiac dysfunction have attracted significant interest. Since we first reported (in 1999) that bone marrow (BM) mesenchymal stem cells (MSCs) could differentiate into cardiomyocytes in vitro [1], research on regenerative medicine has advanced dramatically [2, 3]. In addition to BM MSCs, embryonic stem cells, cardiac tissue stem cells, adipose tissue stem cells, and induced pluripotent stem cells undergo myocardial differentiation; additional cell types may also prove to have cardiac cell differentiation abilities. An early-phase clinical trial involving the direct infusion of BM mononuclear cells and peripheral blood mononuclear cells into coronary arteries and the myocardium has been undertaken. However, there is a vast gap between demonstrating that a cell type can differentiate into myocardium and translating this result into clinical practice. The major challenges for the therapeutic use of stem cells include the effective harvesting and in vitro expansion of cells to ensure sufficient numbers and purity of the cells. This chapter focuses on methods for the differentiation of BM-derived stem cells into myocytes.

Keywords Bone marrow stem cells • Mesenchymal stem cells • Cardiac stem cells • Hematopoietic stem cells • Endothelial progenitor cells • Cell transplant • Myocardial infarction • Myocytes

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1 Bone-Marrow-Derived Stem Cells

Stem cells are clonogenic cells that are capable of both self-renewal and differentiation into more specialized progeny. Traditionally, stem cells have been divided into two broad categories: adult stem cells and embryonic stem cells. Adult stem cells are derived from postnatal somatic tissues and are considered to be multipotent, meaning they can give rise to multiple differentiated cell types. Embryonic stem cells, which are derived from the inner cell mass of blastocyst-stage embryos, are pluripotent, meaning they can give rise to all the differentiated cell types of the postnatal organism. Differentiated somatic cell types can also be reprogrammed into a pluripotent state similar to that of embryonic stem cells via the forced expression of stem-cell-related genes, which represents the basis for a recent report on induced pluripotent stem cells [4].

Approximately one decade ago, several studies challenged the long-held view that adult stem cells give rise to only a restricted set of differentiated cell types. These reports described "transdifferentiation" events, whereby adult stem cells differentiated into unexpected cell types, and even across embryonic germ layer boundaries. Cardiac differentiation has been reported for a variety of expected and unexpected stem cell types. These manifestations of transdifferentiation continue to be sources of controversy.

The bone marrow (BM) is a very heterogeneous compartment that contains multiple stem cell populations with putative cardiac potential, e.g., hematopoietic stem cells (HSCs) [5], mesenchymal stem cells (MSCs) [1, 6–11], very small embryonic-like stem cells [12], and multipotent adult progenitor cells (MAPCs) [13]. In this chapter, we focus on these BM-derived progenitors, which have attracted considerable attention.

1.1 Mesenchymal Stem Cells

Friedenstein et al. first reported the existence of MSCs in the BM in 1966, terming them "bone formation progenitors" [14]. Subsequently, MSCs were reported to constitute 0.001–0.01% of the total nucleated cell population in the BM, which is far lower than the content of HSCs in the BM [15, 16]. BM-MSCs were initially believed to be the stem cells that gave rise to osteoblasts, chondroblasts, adipocytes, and connective tissues [17, 18]. Recent studies have demonstrated that BM-MSCs can also differentiate into neurons [19], skeletal muscle cells [20], and cardiomyocytes [1, 21, 22], both in vitro and in vivo. BM-MSCs are found in the stromal cell fraction, which can be easily separated from hematocytes in culture. These stem cells were initially isolated from the BM stromal cells on the basis of their characteristic proliferative activities and multipotencies. Cell-surface markers that can be used to isolate MSCs have yet to be determined. CD29, CD44, CD105, and Sca-1 (only in the mouse) are widely accepted cell-surface markers for MSCs, whereas the value of other markers is debated among researchers. In 1999, we [1, 22]

observed that the exposure of immortalized murine MSCs to 5-azacytidine (5-AzaC), which demethylates methylcytosine and induces the transcription of critical transcription factors by demethylating the CpG islands in the promoter regions, resulted in the appearance of spontaneously beating foci. We have termed these cell lines "CMG" (cardio*myo*genic), as they are from adult BM stromal cells. Through repeated limiting dilutions, we isolated hundreds of clones, and we identified several clones that could differentiate into cardiomyocytes that exhibited spontaneous beating. These experiments were repeatable and reproducible, although the percentages of cardiomyocyte differentiation varied among these clones. Phase-contrast photography revealed that the CMG cells had a fibroblast-like morphology before 5-AzaC treatment (week 0), and this phenotype was retained through repeated subcultures under nonstimulating conditions. After 5-AzaC treatment, the morphology of the cells gradually changed. Approximately 30% of the CMG cells increased gradually in size, attaining a ball-like appearance or lengthening in one direction, and showed a sticklike morphology after 1 week. These cells connected with adjoining cells after 2 weeks, and formed myotube-like structures at 3 weeks (Fig. 1). The differentiated CMG myotubes retained the cardiomyocyte phenotype and beat vigorously for at least 8 weeks after the final 5-AzaC treatment.

The cardiac phenotype of the treated cells was confirmed by a variety of techniques, including reverse transcription PCR (for the markers of atrial natriuretic peptide, myosin light chain 2a and myosin light chain 2v, GATA4, and Nkx2.5), immunocytochemistry (for the markers of sarcomeric myosin heavy chain (MHC) and α-actinin), and electron microscopy. An electrophysiology study was performed on the differentiated CMG cells 2–5 weeks after 5-AzaC treatment. Two types of morphologic action potentials were distinguishable: sinus-node-like potentials (Fig. 2a); and ventricular-myocyte-like potentials (Fig. 2b). All the action potentials recorded for the CMG cells until 3 weeks of 5-AzaC treatment were sinus-node-like action potentials. Ventricular-myocyte-like action potentials were recorded after 4 weeks, and the percentage of these action potentials gradually increased thereafter.

This outcome was surprising because at the time BM cells were thought to form only blood cell lineages or bone cells. This finding was followed up using a variety of approaches, revealing the potential of BM cells to differentiate into a variety of tissues, including cardiomyocytes. Although similar findings with 5-AzaC have been reported by others [6], some investigators have suggested that this type of cardiac induction requires "immortalized" MSCs [23]. Currently, less is known about methods for the specific induction of differentiation than is known about embryonic stem cells.

Shim et al. [7] isolated MSCs from the BM of human patients who were undergoing coronary artery bypass surgery, and treated the cells with insulin, dexamethasone, and ascorbic acid. The authors reported that the treated cells immunostained positively for α-MHC, β-MHC, and GATA4, but not for skeletal muscle markers, such as skeletal MHC and MyoD. However, the efficiency of cardiogenesis achieved using this approach appeared to be poor. The resultant "cardiomyocyte-like" cell cultures lacked appreciable spontaneous contractile activity, and only a

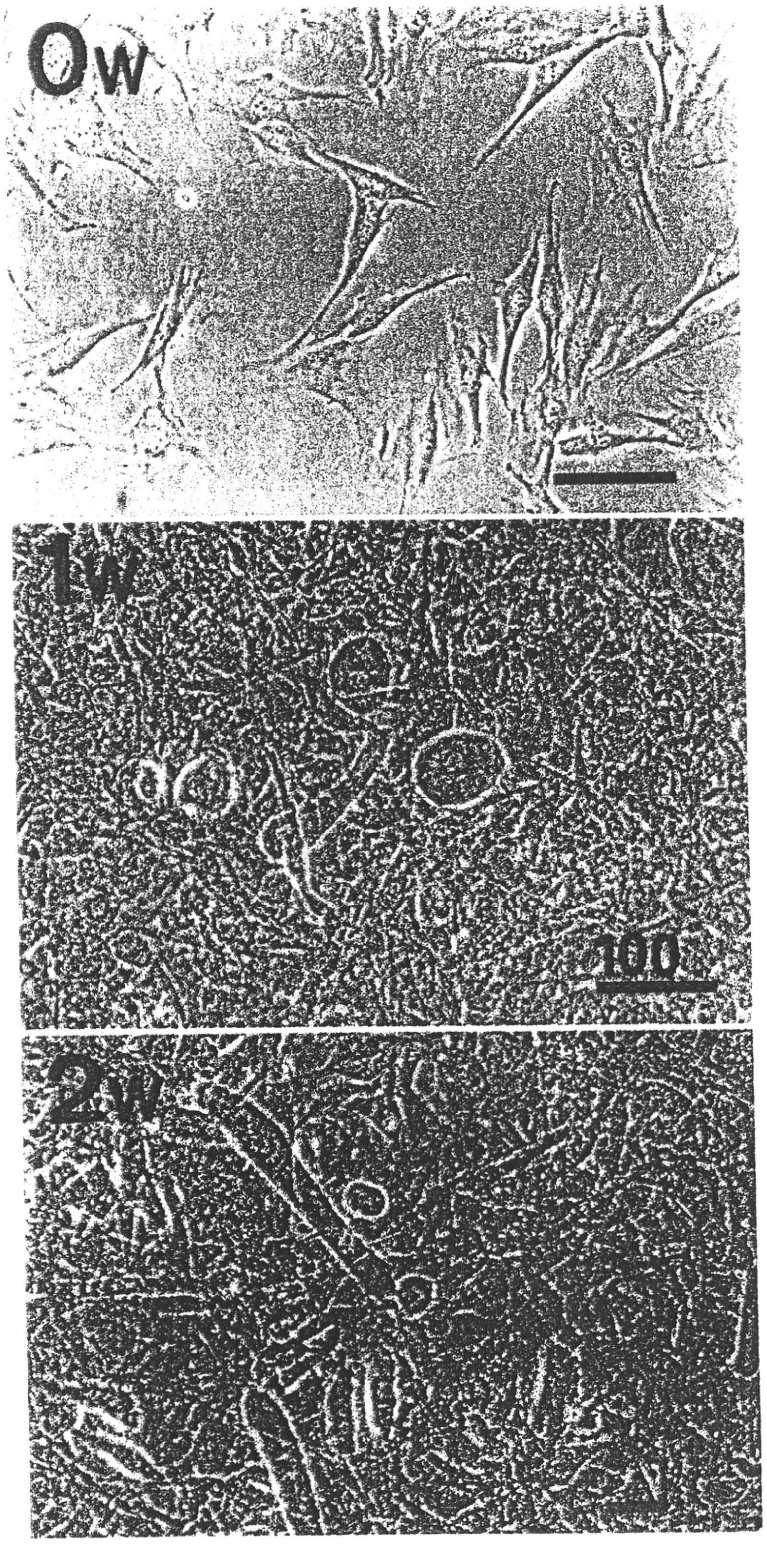

Fig. 1 Phase-contrast micrographs of CMG cells before and after 5-azacytidine (5-AzaC) treatment *Top*: CMG cells show fibroblast-like morphology before 5-AzaC treatment (week 0). *Middle*: CMG cells 1 week after treatment. Some of the cells have increased in size, assuming a ball-like or sticklike appearance. These cells began beating spontaneously thereafter. *Bottom*: CMG cells 2 weeks after treatment with 5-AzaC. Ball-like or sticklike cells are connected to adjoining cells, and are beginning to form myotube-like structures. *Bars* 100 μm

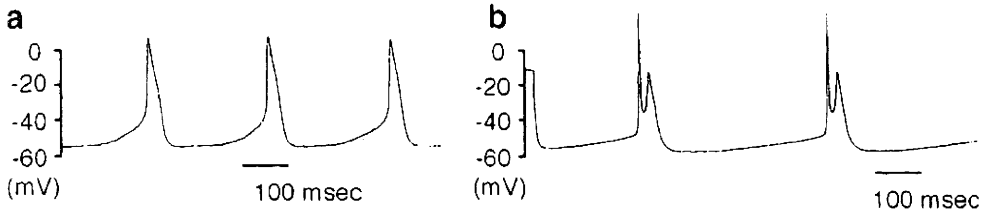

Fig. 2 Representative tracing of the action potential of CMG myotubes Action potential recordings were obtained for the spontaneously beating cells on day 28 after 5-AzaC treatment using a conventional microelectrode. These action potentials are categorized as a sinus-node-like action potential (**a**) and a ventricular-cardiomyocyte-like action potential (**b**)

small subset of the cells exhibited α-actinin-positive cross-striations. More recently, Shiota et al. have reported the cardiac induction of MSC-like progenitors derived using a complex culturing protocol that involves the formation of spheres by BM-derived adherent cells [24]. After treatment with 5-AzaC, the spheres showed spontaneous beating activity, as well as immunoreactivity for cardiac markers, including Nkx2.5 and myosin light chain 2v. The authors tested the capacity of these preparations to mediate cardiac repair in a murine infarct model. They reported functional improvements following the transplantation of green fluorescent protein (GFP)-tagged, sphere-derived cells, although the degree of remuscularization was extremely low. The latter study is one of many preclinical studies that assert beneficial effects for contractile function following the transplantation of MSCs in models of cardiac injury. Some [6, 8, 11], but not all [25], of these studies conclude that MSCs transdifferentiate into cardiomyocytes in vivo. In general, reports favoring myocardial repopulation by MSCs have shown only rare clusters of cells that lack the typical cardiomyocyte morphology but that immunostain positively for one or more cardiac markers.

In 2001, Beltrami et al. observed cardiomyocyte mitotic figures in human hearts after myocardial infarction (MI) [26]. In 2009, Bergmann et al. reported that cardiomyocytes undergo renewal, with a gradual decrease in annual turnover from 1% at 25 years of age to 0.45% at 75 years of age, according to carbon-14 measurements. Fewer than 50% of cardiomyocytes are exchanged during a normal life span [27]. Their report, which sparked controversy regarding cardiomyocyte induction, investigated the following possibilities: (1) whether the cells, which were thought to be terminally differentiated, had acquired the ability to proliferate; (2) whether immature cardiomyocytes differentiated from stem cells into cardiomyocytes and then began to proliferate; or (3) whether mature cardiomyocytes acquired the ability to proliferate by fusing with cells that had retained the proliferative capabilities of stem cells.

1.2 Hematopoietic Stem Cells

Recent advances in fluorescently activated cell sorting (FACS) techniques have enabled the prospective isolation of HSCs on the basis of their cell-surface antigen expression patterns and fluorescent dye efflux characteristics [28–30].

The FACS-derived $CD34^-$ $c\text{-}kit^+$ $Sca\text{-}1^+$ Lin^- tip side population (SP) cell fractions contained the HSC population in mice; [30] c-kit is a stem cell factor receptor, Sca-1 is a stem cell antigen that is specifically expressed in various stem cells (only in the mouse), and Lin is a mixture of antibodies against lineage markers for hematocytes (in mouse, Gra-1, Mac-1, B220, CD3, and Ter119; in human, CD3, CD4, CD8, CD19, CD33, and glycophylin A). In 2001, Orlic et al. reported cardiomyocyte differentiation following the transplantation of $c\text{-}kit^+$ Lin^- BM cells into peri-infarct tissue after MI [26]. They demonstrated directly that BM cells become cardiomyocytes in vivo. However, $c\text{-}kit^+$ Lin^- BM cells are predominantly HSCs, and even if BM cells differentiate into a variety of cells, including cardiomyocytes, controversy persists regarding, for example, whether HSCs transdifferentiate or MSCs differentiate. Moreover, in 2002, fluorescent in situ hybridization analysis revealed the presence of numerous cardiomyocytes that seemed to be recipient-derived after human heart transplantation [31]. In contrast, in 2003, numerous BM-derived cardiomyocytes were shown to be present in the recipient heart after BM transplantation [32]. In an experiment that gave very different results, Wagers et al. examined a variety of organs after transplanting GFP-labeled single HSCs ($c\text{-}kit^+$, Lin^-, $Sca\text{-}1^+$) into irradiated mice, and they concluded that if HSC transdifferentiation does occur, it is extremely rare, and that cardiomyocyte differentiation does not occur as a result of MI or induced injury [33]. Goodell et al. transplanted highly enriched HSCs into lethally irradiated mice, which were subsequently rendered ischemic by coronary artery occlusion for 60 min, followed by reperfusion; they reported that the transplanted BM cells differentiated into cardiomyocytes in the peri-infarct region at a prevalence of 0.02% [34]. In 2004, Balsam et al. investigated whether the $c\text{-}kit^+$ HSCs in BM are capable of differentiating into cardiomyocytes [35], by directly injecting BM cells into myocardial tissue instead of transplanting BM cells after irradiation as other groups had done. Importantly, they conducted their study to exclude irradiation, given the possibility that invasive treatment, including irradiation, contributes to a fusion phenomenon. They concluded that $c\text{-}kit^+$ HSCs do not include cells that are capable of differentiating into cardiomyocytes. Murry et al. investigated this differentiation ability in a similar manner, by directly infusing $c\text{-}kit^+$ Lin^- HSCs into the heart [36] and, as expected, they found that the HSCs were unable to differentiate into cardiomyocytes. In the same year, we examined the differentiation capabilities of HSCs using a $c\text{-}kit^+$ $Sca\text{-}1^+$ $CD34^-$ Lin^- SP ($CD34^-$KSL-SP) of HSCs [37]. When we transplanted whole BM cell populations, which included both HSCs and MSCs, from GFP-transgenic mice into lethally irradiated mice and subsequently induced MI, we found very few GFP^+ (BM-derived) cardiomyocytes. Interestingly, granulocyte colony stimulating factor (G-CSF) increased the number of GFP^+ cardiomyocytes and nonmyocytes in the infarcted or border zone area. In contrast, when we performed HSC transplantation followed by induction of MI and administration of G-CSF, cardiomyocytes were rarely found in the group that was transplanted with HSCs alone, although fibroblast-like cells were observed, and G-CSF increased their number. Moreover, we confirmed the predominance of MSC-derived GFP^+ cardiomyocytes in the group that was transplanted with cardiomyogenic cells, i.e., purified MSCs. It should be emphasized that in this type of BM

transplantation experiment the dosage of radiation must be carefully determined, as the sensitivity to radiation of MSCs is much higher than that of HSCs. We propose that the differentiation by whole BM cells into organs (cells) other than hematopoietic populations is attributable to MSCs rather than HSCs, and that MSCs are mobilized from the BM into the bloodstream, in similarity to HSCs.

Nonetheless, the cardiac potential of HSCs remains controversial. The authors of the original study by Orlic et al. recently revisited this issue, and they concluded once again that in a mouse infarct model, the c-Kit$^+$ BM cells transdifferentiated following transplantation and formed extensive replacement myocardium [38].

1.3 BM-Derived Endothelial Progenitor Cells

Endothelial progenitor cells (EPCs) should be viewed as both circulating and BM stem cell types, since they are known to reside in both compartments. In 1997, Asahara and colleagues described the phenotype of EPCs, which proliferate in response to tissue ischemia, home to areas of injury, and either incorporate within or otherwise promote neovascularization [39, 40]. EPCs express the markers of Flk-1, CD34, and CD133, and can differentiate into definitive endothelial cells [39, 41–43]. Initial interest in the application of EPCs to cardiac repair was naturally focused on their angiogenic properties. The capacity of EPCs to transdifferentiate into cardiomyocytes was first reported by Dimmeler and colleagues in 2003 [44]. In that study, CD34$^+$ human EPCs were obtained from peripheral blood mononuclear cells of healthy adults or from patients with coronary artery disease. After coculture with neonatal rat cardiomyocytes, EPCs were reported to transdifferentiate into cardiomyocytes on the basis of morphology, α-sarcomeric actinin immunoreactivity (as assessed by flow cytometry), and the expression of other cardiac markers (as assessed by immunostaining or reverse transcription PCR with species-specific probes). Furthermore, the EPCs showed calcium transients that synchronized with adjacent rat cardiomyocytes, suggesting communication with the host myocardium through gap junctions. Coculturing experiments with paraformaldehyde-fixed cardiomyocytes revealed that cell fusion was not required for EPCs to acquire the cardiac phenotype [44–47]. However, the efficiency of cardiac induction by EPCs was very low; even after enhancement through inhibition of Notch signaling, less than 1% of the EPCs expressed α-sarcomeric actinin [47]. Asahara and colleagues reported even lower rates of cardiac transdifferentiation in vitro following coculturing of EPCs with the rat heart-derived H9C2 cell line [48]. The latter authors also reported the in vivo cardiac differentiation of a related preparation of human circulating cells following transplantation into a rodent infarct model. However, this conclusion is complicated by the definitive demonstration of cell fusion between host myocytes and graft cells, using species-specific fluorescent in situ hybridization probes [49]. Moreover, Gruh et al. were unable to confirm the in vitro cardiac differentiation of EPCs following coculturing with primary myocytes [50]. These authors found no expression of human cardiac transcripts,

and they concluded that the rare, ostensibly transdifferentiated EPCs observed by FACS or epifluorescence microscopy were artifacts that resulted from overlying cells and/or autofluorescence. Thus, although the cardiac potential of EPCs remains a source of controversy, the report of Gruh et al. underscores the challenges inherent to interpreting coculture experiments.

1.4 Very Small Embryonic-Like Stem Cells

In 2006, employing multiparameter sorting, Kucia and colleagues identified in murine BM populations a homogenous population of rare (approximately 0.02% of BM mononuclear cells) Sca-1$^+$ Lin$^-$ CD45$^-$ cells that express SSEA-1, Oct-4, Nanog, and Rex-1 [51]. These cells are very small and display several features that are typical of primary embryonic stem cells. In vitro cultures of these cells are able to differentiate into all three germ layer lineages, including cardiomyocytes. For cardiac differentiation, GFP$^+$ Sca-1$^+$ Lin$^-$ CD45$^-$ or Sca-1$^+$ Lin$^-$ CD45$^+$ cells together with unpurified GFP$^-$ BM cells were plated in Dulbecco's modified Eagle's medium that was supplemented with 10% fetal bovine serum, 10 ng/ml basic fibroblast growth factor, 10 ng/ml vascular endothelial growth factor, and 10 ng/ml transforming growth factor β_1. Growth factors were added every 24 h, and the medium was replaced every 2–3 days.

Dawn et al. have reported that the transplantation of a relatively low number of very small embryonic-like stem cells is sufficient to improve left ventricular function and to alleviate myocyte hypertrophy after MI [12]. In that report, 10,000 very small embryonic-like stem cells in a 50-μl volume were injected intramyocardially using a 30-gauge needle.

1.5 Multipotent Adult Progenitor Cells

In 2002, Jiang et al. reported on pluripotent BM-derived cells, which they referred to as multipotent adult progenitor cells (MAPCs) [52]. When transplanted into blastocysts, MAPCs had the potential to differentiate into the three germ layers both in vitro and in vivo. These MAPCs were maintained using a low-density culture method, making independent corroboration of the findings by other laboratories rather difficult. In 2006, Zeng et al. showed that MAPCs could be derived from both postnatal and fetal swine BM. Swine MAPCs are negative for CD44, CD45, and major histocompatibility complex classes I and II, express octamer-binding transcription factor 3a messenger RNA and protein at levels close to those seen in human embryonic stem cells, and have telomerase activity, which prevents telomere shortening.

Transplantation of MAPCs (injected directly into heart at ten million cells per location diluted in 400 μl of saline) at the time of coronary artery ligation resulted in improved infarct zone contractile function and prevented peri-infarct border zone bioenergetic deterioration [13]. The left ventricular chamber response to cell transplantation resulted from the beneficial effects of sparing myocytes and

increasing revascularization in both the infarct zone and the peri-infarct border zone. A direct structural contribution of the engrafted cells to cardiomyocyte regeneration appears to be unlikely.

2 Other BM-Derived Cells and Cell Fusion

In 2005, Yoon et al. identified a subpopulation of human BM stem cells (hBMSCs) that did not belong to the previously described class of BM-derived stem cells [53]. These cells were $CD29^-$, $CD44^-$, $CD73^-$, demonstrating minimal expression of CD90, CD105, and CD117, and could differentiate into the three germ layers. Intramyocardial transplantation of hBMSCs after MI resulted in robust engraftment of transplanted cells, which exhibited smooth muscle cell identity and colocalization with markers of cardiomyocytes and endothelial cells, which is consistent with the differentiation of hBMSCs into multiple lineages in vivo. Coculturing of hBMSCs with cardiomyocytes revealed that phenotypic changes in the hBMSCs result from both differentiation and fusion. Other laboratories have identified additional multipotent, $CD45^-$, nonhematopoietic BM-derived cells [40, 54, 55]. In some cases, it is likely that similar or overlapping populations of primitive stem cells in the BM detected using various experimental strategies have been assigned different names. The relationships among the BM-derived stem cells reported from different laboratories need to be clarified.

In 2002, Terada et al. suggested that a cell fusion phenomenon had to be considered with regard to the plasticity of the BM cells reported thus far [56]. Their coculture of adult animal BM cells with embryonic stem cells induced cell fusion naturally in the presence of interleukin-3, and although the karyotype was tetraploid, the cells acquired pluripotency and proliferative ability. More recently, the transplantation of whole BM cells into lethally irradiated mice resulted in fused cardiomyocytes but no transdifferentiation [57]. In addition, the same study aimed to identify the cell lineages in whole BM populations that are responsible for cell fusion, by transplanting CD45-Cre mouse BM into R26R mice. Fused cardiomyocytes were observed in this experimental system, and BM-derived leukocyte lineage cells were found to be responsible for the fusion. The lack of a clear definition for cell plasticity has led to confusion, with several reports failing to demonstrate that a single cell can indeed differentiate into multiple lineages at significant levels.

Studies using the Cre-lox recombination system revealed only rare MSC-derived cardiomyocytes, nearly all of which resulted from cell fusion [58].

3 Specific Culture Method for Cardiac Differentiation and Cell Fusion

Another obstacle to cell therapy is that specific culture methods for differentiating BM cells are only available for some target cells. Specific differentiation is achievable for osteoblasts, chondroblasts, and adipocytes. The use of 5-AzaC is effective for cardiomyocyte differentiation but it is clinically toxic. For cardiomyocytes,

no methods have been established that use physiologic growth factors, cytokines, or nontoxic chemical compounds. Perhaps the most studied strategy to date with adult stem cells is the effect of 5-AzaC, a DNA demethylation reagent, on cardiac protein expression in MSCs [1, 59]. Several studies have demonstrated an increase in cardiac protein expression after treatment of MSCs with 5-AzaC [1]. Importantly, studies have consistently demonstrated improvement in cardiac function after the transplantation of 5-AzaC-treated MSCs, as compared with the transplantation of control MSCs [59–61]. As we begin to define the pathways, we can attempt to optimize further cardiac differentiation and functional effects [61]. For the further development of this field, it is necessary to find the small molecule and to elucidate the epigenetic status that can enhance cardiac differentiation from these stem cells [62].

Recently, Ge et al. reported the cardiomyocyte differentiation of rat BM-MSCs by treating the stem cells under conditions similar to those seen during MI [63]. The extract from the infarcted rat myocardium contained the same biochemical factors that arise after MI. Ge et al. found that the extract of infarcted myocardium could induce cardiomyocyte differentiation of BM-MSCs, as shown by the expression of cardiomyocyte-specific genes, including those for α-actin, connexin 43, Nkx2.5, MEF2c, GATA4, α-MHC, and troponin I. This approach could represent an alternative means of inducing cardiomyogenic differentiation in that it does not rely on gene demethylation or the use of viral vectors. The findings of that study appear to support the use of autologous extracts for the induction of stem cell differentiation and may have clinical implications for cardiac cell therapy.

Significant work has been performed to further understand the regulatory pathways involved in embryonic stem cell differentiation to cardiac myocytes [64–66]. These studies have suggested potential pathways that could be activated in adult stem cells so as to induce them to take on a cardiac phenotype [64, 66, 67].

Another approach that is being developed to direct the cardiac differentiation of adult stem cells is the delivery of chimeric proteins that encode cell-penetrating peptides (CPPs) and cardiac-specific transcription factors [68, 69] CPPs cause non-secreted proteins to be secreted and to be internalized by surrounding cells. Bian et al. have demonstrated that the transplantation into the myocardium of cells that are genetically enhanced to express a CPP-GFP protein results in GFP expression in native cardiac myocytes [69]. To deliver functional transcription factors to the myocardium, Bian et al. developed a CPP-GATA4 construct and transplanted cardiac fibroblasts that were stably transfected with the CPP-GATA4 construct, 1 month after MI in the Lewis rat. The infarct border zones of the animals that received CPP-GATA4 demonstrated increased expression of cardiac myosin and Bcl-2 [69]. The modulation of GATA4-responsive gene expression led to hypertrophy of the cardiac myocytes at the infarct border zone and a global improvement in cardiac function [69]. These findings suggest that combining genetic enhancement of stem cells to deliver CPP–transcription factor chimeric proteins together with either stem cell homing agents or additional stem cells could lead to an increase in cardiac protein expression in the stem cells, cardiac myocyte regeneration, and further improvements in cardiac function.

4 Cardiospheres and Cardiac Extracts for Cardiomyogenesis

In 2004, Messina et al. described a novel technique for isolating resident cardiac progenitors from murine hearts, as well as subcultures of human atrial or ventricular specimens [70]. Mild enzymatic digestion of the tissue specimens yielded small, round, phase-bright cells that clustered together in suspension. These sphere-generating cells were allowed to adhere to poly(L-lysine)-coated plates, and were cultured in a medium that was supplemented with cytokines (epidermal growth factor, basic fibroblast growth factor, cardiotrophin-1, and thrombin). These "cardiosphere"-derived cells were self-renewing, clonogenic, and expressed both endothelial markers (KDR in human, flk-1 in mouse cells, and CD31) and stem cell markers (CD34, c-Kit, and Sca-1). Murine cardiosphere-derived cells showed spontaneous contractile activity, whereas human cardiosphere cells did so only after 24 h of coculturing with postnatal rat cardiomyocytes. The cardiosphere-derived cells from both human and mouse demonstrated trilineage differentiation into cardiomyocytes and endothelial and smooth muscle cells. However, quantitative data on the frequencies of these events were not reported. Cardiosphere-derived cardiomyocytes express cardiac markers, including cardiac troponin I, atrial natriuretic peptide, and cardiac MHC. In vivo, cardiosphere-derived cells have been reported to regenerate the infarcted mouse heart [70]. Subsequently, Smith et al. expanded on these findings by isolating cardiosphere-forming cells from human biopsy specimens [71]. These human cardiospheres, which were successfully isolated from 69 of the 70 biopsies tested, consistently expressed c-Kit but not the multidrug resistance gene MDR1, indicating that these cells were phenotypically distinct from the resident cardiac progenitors previously identified in situ (c-Kit$^+$, MDR1$^+$) [31, 72]. Consistent with the findings of Messina et al. [70] human cardiosphere-derived cells did not spontaneously contract, whereas coculturing with neonatal rat cardiomyocytes evoked calcium transients in synchrony with neighboring cardiomyocytes, action potentials, and fast inward sodium currents. Smith et al. also injected lentivirally transduced LacZ$^+$ human cardiosphere-derived cells into the border zones of infarcted SCID beige mice [71]. Twenty days later, the cardiosphere-derived cells were detected throughout the border regions of the mouse hearts, and occasional donor cells were immunostained for α-sarcomeric actin and von Willebrand factor. Echocardiography showed improvements in global left ventricular function, although given the apparently limited cardiomyocyte repopulation by LacZ$^+$ cells, these functional effects were attributed to a combination of regeneration and paracrine effects. On the basis of these studies, explant-derived cardiospheres appear to have cardiomyogenic potential and considerable promise for cardiac repair.

The differentiation of human adipose tissue stem cells to take on cardiomyocyte properties occurs following transient exposure to a rat cardiomyocyte extract [73–75]. Adult cardiomyocytes retain the capacity to induce cardiomyogenic differentiation of adult human MSCs. This approach could represent an alternative strategy to induce cardiomyogenic differentiation that does not rely on gene demethylation or the use of viral vectors.

5 Conclusions

Advances in stem cell and developmental biology have resulted in the identification of numerous candidate stem cell types with putative cardiogenic potential. The ideal cell type remains to be confirmed, despite all claims to the contrary. The cardiogenic potentials of BM-derived and circulating stem cells appear limited, whereas other candidates, including pluripotent stem cells, are clearly capable of more efficient cardiogenesis. We are optimistic that research into cell-based cardiac repair will eventually yield effective myogenic therapies, although success in this area will require rigorous cardiac phenotyping, cell fate mapping, and preclinical and clinical testing.

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