厚生労働科学研究費補助金
（医療技術実用化総合研究事業：基礎研究成果の臨床応用推進研究）
分担研究報告書

臨床治験の準備に関する研究

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研究要旨
エクソン・スキップ治療を臨床応用するための基盤整備として、筋ジス犬と同じ方法であるエクソン6と8のスキップが有効なエクソン7単独欠失を有するDMD患者を見いだし、治験の可能性を検討するために臨床評価と皮膚由来の線維芽細胞の初代培養の確立を行った。またエクソン・スキップ治療の対象患者のリストアップを念頭においた患者データベースの作成を行った。

A．研究目的

Duchenne 型筋ジストロフィー（DMD）に対する、エクソン・スキップ治療を臨床応用するための臨床的な基盤を整備する。

B．研究方法

1，エクソン7単独欠失患者の臨床評価ならびにin vitroエクソン・スキップのための試料採取

国内において、筋ジス犬と同じ方法が適用できる、エクソン6と8のスキップが有効なエクソン7単独欠失を有する22歳のDMD患者を1名見いだした。患者及び家族との良好な関係を築いたうえで、インフォームド・コンセントを得て、平成２０年９月国立精神・神経センター倫理委員会に申請書を提出し、審議、承認を受けた。書面による承諾書を得たうえで臨床評価と皮膚生検を行って、皮膚線維芽細胞の初代培養の確立を試みた。臨床評価は呼吸機能、心機能、側わん症、栄養評価、運動機能評価、骨格筋画像評価などを標準的な方法を用いて行った。

2，エクソン・スキップ治療の治験の基盤となる患者データベースの作成

エクソン・スキップ治療は患者それぞれの遺伝子変異に基づく、テーラーメイド医療の典型ともいえる方法であり、臨床情報と遺伝子変異情報を併せた患者データベースの構築はエクソン・スキップ治療の対象となる患者のリストアップにおいて非常に重要な役割を担うものである。そこで当センター受診歴のある患者に関する患者データベースの作成を行うこととした。平成２０年９月国立精神・神経センター倫理委員会に申請書を提出し、審議、承認を受けたうえで研究を開始した。対象は当院小児神経科通院中のDMD患者130名、登録項目として、身体計測値、関節可動域、歩行の有

無、歩行不能年齢、定量的筋力評価、10 m 歩行時間、起立時間、階段登り時間、ADL 評価、筋生検所見、遺伝子診断の詳細な結果、認知機能、呼吸機能、人工呼吸器使用など、心機能、骨折の既往、消化器症状の有無、ステロイド使用、心筋症治療の有無、側わん、骨密度、骨格筋画像診断を設定した。

C．研究結果

1，エクソン 7 単独欠失を有する DMD 患者の臨床評価ならびに in vitro エクソン・スキッピングのための試料採取

局所麻酔のもと左上腕より皮膚を採取、直ちに培養を開始し、約 1 ヶ月で皮膚由来の線維芽細胞初代培養を確立することができた。

呼吸機能では肺活量は 2180 ml と年齢標準と比較するとやや低下していたものの、夜間の低酸素血症、高炭酸ガス血症は認めなかった。側わんもごく軽度認めるのみであった。心機能は心臓超音波検査にて左室駆出率は 18%と著明に低下しており、び慢性の壁運動低下を認めた。血漿脳性ナトリウム利尿ペプチド（BNP）は 148 pg/ml（正常 18.4 以下）と高値を示していた。骨格筋 MRI ではほとんどの筋がほとんど脂肪置換していたが、後頚骨筋は比較的保たれていた。

2，エクソン・スキップ治験にむけた、患者データベースの作成

今年度は倫理審査、承認の手続き、エクソン・スキップ治療を念頭においた遺伝子変異情報の解析を主に行った。遺伝子変異の解析では 130 名中、エクソン単位の欠失を有する患者は 72 名、エクソン単位の重複を有する患者は 15 名、点変異などの微小変異を有する患者は 14 名、残り 29 名は未確定ないし未検査という結果であった。

エクソン・スキップ治療を対象者の分析を行ったところ、エクソ 51 番スキップ対象患者（エクソン 50 ないし 52 の単独欠失、エクソン 48-50、エクソン 49-50、エクソン 45-50 欠失例が 14 名（15 歳未満 14 名）、エクソン 53 番スキップ対象患者が 10 名（15 歳未満 8 名）、エクソン 45 番スキップ対象患者が 4 名（15 歳未満 3 名）、エクソン 44 番スキップ対象患者が 4 名（15 歳未満 2 名）であった。

D．考察

1，エクソン 7 単独欠失を有する DMD 患者の臨床評価

患者は側わん、呼吸機能、栄養状態、運動機能に関しては同年齢の DMD 患者と比較すると良好な状態を維持していた。しかしながら心筋症の程度は重篤であり著明な心機能の低下を認めていた。モルフォリノは心筋でのスキッピング効率が比較的悪いとされているので、今後この患者のエクソン・スキップ治療を考えるうえでは問題となる所見であり、治験を行うにあたって今後慎重な検討を要する。

2，エクソン・スキップ治験にむけた、患者データベースの作成現在までの知見で推定されているほぼ同じ頻度でエクソン・スキップ治療の対象となり得る患者が存在することが判明した。ホットスポットとされるエクソン 50 付近の欠失を有する患者は当センターの患者でも多数存在することが判明した。

E．結論

エクソン・スキップ治療の実現には基礎研究と臨床研究の両面からのアプローチが必

要であり、今後もエクソン・スキップ治療の基盤となる臨床面からのアプローチを進めていく。

F．健康危険情報
なし

G．研究発表

I．論文発表
なし

II．学会発表
なし

H．知的財産権の出願・登録状況
なし

厚生労働科学研究費補助金
（ 医療技術実用化総合研究事業：基礎研究成果の臨床応用推進研究 ）
分担研究報告書

アンチセンス・モルフォリノによるDuchenne型筋ジストロフィーのエクソン・スキップ治療に向けた臨床応用研究

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研究要旨
筋ジストロフィーにおけるエクソンスキッピング治療を臨床試験の段階に移して行くには、一定の研究時間を確保された研究者、プロジェクトマネジャーをはじめとして医療、科学のみならず、財務、法律など他職種に渡る支援人材、多大な資金と時間を要することが明確になった。

A. 研究目的

基礎研究から見いだされた新しい医療技術をヒトに対して有効性・安全性を検証しながら開発していくことは非常に困難な事業である。筋ジストロフィーに対するエクソン・スキッピング治療を日本において臨床開発に移していく準備をすることが分担研究の目的である。

B. 研究方法

2002年の薬事法改正に伴って製薬企業にしか許されていなかった承認申請のための臨床試験（治験）が2003年7月から医師が主導して実施することが可能になり、実際に小児科領域ではクエン酸フェンタニルの適応拡大が2007年9月に医師主導治験で初の承認を受けた。

本研究においても基礎研究で見いだされた医療技術を高い水準の臨床研究に移行していくため、日本及び各国の状況を調査することとした。また、上記は日本においては医師主導治験にて実施されることが想定され、関係各機関への調査、調整が必要となり、これも実施することとした。

C. 研究成果

1. 医師主導治験に関する調査

2002年の薬事法改正に伴って製薬企業にしか許されていなかった承認申請のための臨床試験（治験）が2003年7月から医師が主導して実施することが可能になった。

臨床研究（人を対象とした研究）には様々なタイプのものがあるが、大きく分けると観察研究と介入研究とに分かれ、臨床研究は人を対象とするものだけに、日本においても様々な倫理指針、法律などで規制されている。このうち介入研究は治療などの介入を含むため2008年7月末に改正のあった「臨床研究に関する倫理指針」で示されたように事前の登録、補償、などが規定され、さらに介入試験のうちでも医師主導治験ではGCP (Good Clinical Practice) の遵守が科せられる。（現在実施されている治験はすべてGCPの遵守が求められる。）

欧米ではSponsor-investigator trialと呼ばれるこの医師主導治験は、医師が治験の企画立案・実施、研究費の調達、厚生労働省への治験届も自ら行うことになる。GCPでは、このような医師は製薬企業のように治

験を推進する役割と、製薬企業主導の治験と同じような治験責任医師の両方の役割を果たすことが求められている。また、厚生労働省からの承認を得ても、製造販売は医師自らが行うことはできず、製薬企業との連携は欠かすことができない。

医師主導治験は、医療上の必要性は高いにもかかわらず承認されていない効能や、市場性の問題などから製薬企業が開発を行わない新規の薬剤などを医師自身が開発することが想定される。医師主導治験によって承認を得た事例も出てきたが、日本医師会治験促進センター（http://www.jmacct.med.or.jp/）や、各大学の支援部門、ナショナルセンターなどの治験中核病院など充分な知識・経験を持った組織に支援を受けることは実施における欠かせない条件であろう。

わが国では医師の教育課程において臨床研究に関する教育がほとんど行われず、生物統計の考え方、データマネジメントなどの品質管理が充分導入されなかったこともあり、臨床試験の基本的考え方や方法論を十分に理解せずに臨床試験が実施されることも多いため、実施者にとっては大きなチャレンジである。

2. 海外の現状に関する調査

米国 4 施設を訪問し、多くの臨床研究者、臨床研究支援組織の方にインタビューをおこなった。精神科研究者として、NIMH (National Institute of Mental Health: Intramural) の Donald Rosenstein, M.D.、Carlos Zarate, M.D.、Jose Antonio Apud M.D., Ph.D.、Marlyland Pao, M.D.、Johns Hopkins Hospital の Akira Sawa, M.D.、コロンビア大学の J. John Mann, M.D.、Michael F. Grunebaum, M.D.、MGH (Massachusetts General Hospitao)の Maurizio Fava, M.D.、David C. Henderson, M.D.、神経内科研究者として、Johns Hopkins Hospital の Paul S. Lietman M.D., Ph.D.、John W. McDonald, M.D., Ph.D.、Justin McArthur, M.D., M.P.H. 放射線科研究者として、Johns Hopkins Hospital の Dean F. Wong, M.D., Ph.D.、研究支援組織の現状を聞くために、NIMH (Extramural) の Richard Nakamura Ph.D.、Philip Wang MD, Dr.P.H.、Benedetto Vitiello, M.D.、Jena Baum、Adam Heier、Farris Tuma, Sc.D, MHS、Johns Hopkins (CTSA)の Daniel Ford, M.D., M.P.H.、Johns Hopkins (JHMI)の Mohan Chellappa, M.D.、Sanford Wu, M.B.A.、コロンビア大学 GCRC の Janelle R., M.D.、David A. Dierkens, M.P.H.、Henry N. Ginsberg Kahn, M.D.であり、約 25 名の研究者および、研究支援者にインタビューを行った。

米国では、国が資金を提供し、臨床研究の専門家を保有する臨床研究専門センター General Clinical Research Center (GCRC)を各施設に設置してきており、1960 年以降、NIH の補助金によって主要 78 施設に設置されてきたが、その特徴としては、研究被験者専用のリラクゼーションルームなどを含めた臨床研究専用施設、臨床研究の患者の優先的診察(外来)および入院、小規模な病床数（10～20 床）、看護師・CRC・ソーシャルワーカー・事務職員といった臨床研究に特化した専門スタッフの配置、基本検査機能の一括配置によるコストの削減が挙げられる。

また、CTSA (Clinical and Translational Science Award) は、2006 年に主要研究機関における臨床研究支援組織設置のための NIH (National Institute Health) 補助金制度として発足した。その支給対象研究機関は 2012 年に 60 施設、年間 500 億円の支給を目標として設定され、2006～07 年にそれぞれ 12 施設、合計 24 施設が現在参加している。補助金の対象となる支援内容は施設によって様々で、今回視察およびインタビューを行ったうちで、CTSA による支援を受けていたのは、Johns Hopkins Hospital、コロンビア大学であった。Johns Hopkins Hospital では、プロジェクトマネジャー、CRC

(Clinical Research Coordinator)、などの研究実施支援、補助金申請書類作成などの事務支援機能、統計家などと時間を予約をした上で実施されるプロトコル作成支援、統計家、プログラマー、データマネジャーなどによるデータベース設計・管理、小規模ではあるが設備の整った研究実施用病棟、論文の作成支援、研究被験者に対して窓口を一元化して行われる患者アドボカシー、研究ネットワーク構築、次世代主任研究者育成などが行われ、コロンビア大学では、臨床研究 PhD コースの設立、奨学金の増設、ユニークな研究への補助金付与、GCRC の拡充が行われていた。しかし、CTSA には課題もあり、予算配分・支援内容のベストプラクティスが確立されているわけではなく現在各施設で試行錯誤されていること、支援対象の拡大（小規模研究以外の研究者）も必要なことである。これらの課題の解決は今後の日本にも適用できる可能性が高く、その動向を注視していく必要がある。

3．実施に対する準備

医師主導治験を実施すべく、日本医師会治験推進室に相談の上、標準手順書の整備などを行った。また、筋ジストロフィー治療の第一相国際共同医師主導治験を実施するため、医薬品医療機器総合機構との事前面談を行った。国内での開発・輸入・販売を前提にパートナーとなる企業と守秘義務契約を結び役割分担など協議を進め、プロトコールの日本語版の作成も行った。平成20年度になって生じた経済情勢の混乱から薬剤供給予定であった米国企業の問題もあり臨床試験の実施に若干の困難を生じているが、平成21年度に米国、英国との共同で臨床試験を実施することを念頭に準備を進めている。

D．考察

臨床研究において支援システムが重要であることは繰り返し述べられていることである。米国視察で明確になったことは、臨床研究実施支援のみならず、生物統計、財務・経営的観点を持った人材からなる分厚いインフラが米国の臨床研究を支えていることである。したがって、医学、研究を中心に携わる人材のみならず、それぞれの専門領域で修士から博士レベルの人材を集め、医師主導治験を実施していく仕組みを作ることが重要であることを確認した。

筋ジストロフィーに対するエクソン・スキップ治療をすすめていく上で、医薬品機構、日本医師会治験推進室、米国・英国の共同研究者及び化合物提供企業、日本における提携企業との協議を行い、開発の道筋を明確にすることができた。一方で、対外的な交渉や開発戦略の策定などプロジェクトマネジャーを置く必要があり、なおかつそのプロジェクトマネジャーに高い専門性とコーディネート能力、実務負担が重くかかることがわかった。このような人材の獲得は今後の非常に大きな課題である。

E．結論

筋ジストロフィーにおけるエクソンスキッピング治療を臨床試験の段階に移して行くには、一定の研究時間を確保された研究者、プロジェクトマネジャーをはじめとして医療、科学のみならず、財務、法律など他職種に渡る支援人材、多大な資金と時間を要することが明確になった。

F．健康危険情報

特記すべきことなし

G．研究発表

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H．知的財産権の出願・登録状況

1. 特許取得
なし
2. 実用新案登録
なし
3. その他
なし

別紙4

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Recent Advances in Skeletal Muscle Differentiation, 2008: 61-78 ISBN: 978-81-308-0232-9
Editors: Kunihiro Tsuchida and Shin'ichi Takeda

4 Side population (SP) cells and skeletal muscle differentiation

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Abstract

Side population (SP) cells are isolated from various tissues by their ability to efficiently exclude the vital DNA dye Hoechst 33342. The clearance of the dye from the cells is thought to be mediated by ABC transporters. Bone marrow SP cells are rich in hematopoietic stem cells and have been demonstrated to participate in muscle fiber repair. Similarly, SP cells from skeletal muscle were shown to reconstitute the bone marrow of lethally irradiated mice and, at the same time, participate in muscle fiber regeneration.

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Several reports, however, suggest that muscle-derived SP cells are heterogeneous in origin, gene expression, and function. To further elucidate their functions and relationships with the other myogenic cells identified to date and their potential as a tool for cell-based therapy of muscular dystrophies, it might be necessary to refine the protocol for SP cell preparation and combine Hoechst staining with identification of several molecular markers.

Introduction

Duchenne muscular dystrophy (DMD) is a progressive, ultimately lethal X-linked muscle disorder, caused by mutations of the DMD gene [1], which encodes a large cytoskeletal protein, named dystrophin. Dystrophin forms the large dystrophin/dystrophin-associated protein complex at the sarcolemma of myofibers, linking the basal lamina and cytoskeleton. Dystrophin deficiency causes structural weakness of the sarcolemma. The defective sarcolemma easily ruptures under mechanical stress, leading to muscle fiber necrosis, and finally results in loss of myofibers and reduced contractile power.

Skeletal muscle regenerates when injured. Muscle satellite cells, which are muscle progenitor cells located between the muscle basal lamina and myofibers, are largely responsible for this activity [2], and were expected to be a cell source for cell-based therapy of DMD. However, transplantation of satellite cells or their progeny (myoblasts) into skeletal muscle showed insufficient regenerative efficiency, and failed to ameliorate the dystrophic phenotypes of animal models and DMD patients (reviewed in [3, 4]).

On the other hand, several reports have suggested that stem cell-like activities are found in non-satellite cell fractions derived from adult skeletal muscle or in non-muscle tissues and participate in muscle fiber regeneration [5-8]. Therefore, stem cells other than satellite cells could be an alternative cell source for cell-based therapy of muscle diseases such as DMD.

Among the myogenic stem cells reported to date are side population (SP) cells. Originally, SP cells were isolated from bone marrow as highly purified hematopoietic stem cells on the basis of their ability to efflux Hoechst 33342 dye [9]. Since then, cells with the SP phenotype have been found in a wide variety of mammalian tissues, cell lines, and tumor cells, some of which have shown to possess stem cell-like properties (reviewed in [10, 11]).

In this chapter, we review papers characterizing the properties of bone marrow SP cells and muscle SP cells. Importantly, many reports show that SP cells are highly heterogeneous. To correctly understand the therapeutic potential of SP cells, it might be necessary to combine Hoechst staining with identification of several cell surface markers and perform functional analysis using a limited number of SP cells.

I. Bone marrow side population cells

1. Discovery of SP cells as hematopoietic stem cells

Side population (SP) cells were discovered as highly purified hematopoietic stem cells [9]. While using Hoechst 33342 vital dye staining to study the cell cycle of bone marrow (BM) cells, Goodell et al. found that simultaneously displaying Hoechst fluorescence at two emission wavelengths (red 675 nm and blue 450 nm) localizes a distinct, small, non-stained cell population (0.1% of the total BM cells) that expresses markers of multipotent hematopoietic stem cells (HSC) ($Sca1+lin^{neg/low}$). *In vivo* competitive repopulation experiments revealed that HSC activities were enriched at least 1,000-fold in the SP fraction. The majority of BM SP cells were not cycling: only 1-3% of bone marrow SP cells were in S-G_2M stages of the cell cycle, whereas 20% of main population (MP) cells were [9]. Because the SP fraction disappears when staining is performed in the presence of verapamil, Goodell et al. speculated that the exclusion of Hoechst 33342 by SP cells is an active process involving multidrug resistance protein (mdr) or mdr-like transporters [9]. Later, Zhou et al. demonstrated that breast cancer resistance protein (BCRP), also known ABCG2, is the molecular determinant of the SP phenotype [12, 13]. Interestingly, more detailed fractionation studies indicated that the SP tail can be further divided into subregions according to their dye efflux abilities, and that the tip of the SP cells (which have the highest Hoechst efflux activity) shows higher progenitor activity than the distal portion [14-16].

Although BM SP cells are widely accepted as highly enriched hematopoietic stem cells, it seems that not all SP cells possess HSC activities [14]. Further, a recent study showed that hematopoietic stem cells are present in both SP and non-SP fractions [17]. Therefore, the properties of BM SP and HSC cells are not completely identical.

2. Role of bone marrow SP cells in myogenesis

Ferrari et al. reported that BM-derived cells participated in repair of muscle fibers [8], suggesting that at least a fraction of myogenic precursor cells originate in the bone marrow, circulate throughout the body, and are mobilized to damaged muscle to regenerate muscle fibers. Later, Gussoni et al. injected BM SP cells from wild-type male mice intravenously into lethally irradiated *mdx* female mice, and demonstrated that bone marrow SP cells contain both myogenic and hematopoietic precursors, i.e., they are multipotent stem cells with great plasticity [6]. These results gave us hope of recovering dystrophin expression in the whole musculature of patients with DMD by systemic delivery of BM-derived wild-type stem cells. Stimulated by these reports, researchers intensively investigated the properties of side population cells in bone marrow, especially the contribution of BM cells [18, 19] or BM SP cells [20] to muscle

regeneration. BM cells and BM SP cells prepared from GFP-transgenic or LacZ-expressing mice were indeed found to differentiate into muscle fibers *in vivo* after transplantation. Disappointingly, however, the percentage of myofibers formed by donor-derived cells delivered via the circulation was very low (1-2 %) and therapeutically not significant in most skeletal muscles.

Fusion or stepwise myogenic differentiation?

LaBarge and Blau reported that BM cells differentiate stepwise into myogenic precursor cells (e.g. satellite cells) and then, response to muscle injury, proliferate, fuse, and finally develop into mature myofibers [18]. Similarly, several reports suggested that BM-derived cells can differentiate into satellite cells [6, 19]. On the other hand, Sherwood et al. demonstrated that cells of bone marrow or hematopoietic origin did not give rise to functional adult myogenic progenitors [21]. Several reports provided evidence that the plasticity of hematopoietic stem cells shown in BM transplantation experiments can be explained simply as fusion events [22, 23]. Further, additional concerns have arisen from studies demonstrating that while BM cells or BM SP cells are able to fuse with myofibers, a large proportion of incorporated cells do not actually enter the myogenic program [24, 25].

II. Muscle SP cells

1. Protocol for isolation of muscle SP cells

Although SP-like cells are found in mononuclear cells prepared from skeletal muscle (Figures 1 and 2), there are often discrepancies among reports in abundance, cell surface markers, and differential potentials of muscle SP cells (Table 1). This may be due to the many variables involved in the preparation and staining for isolation of SP cells by FACS. Montanaro et al. investigated the effects of isolation parameters on viability, yield, and phenotype of SP cells [26], and found that 1) the enzymatic dissociation procedure, 2) cell-counting method, 3) Hoechst concentration, and 4) SP gating are important parameters to minimize the heterogeneity of SP cells prepared from bone marrow, skeletal muscle, or skin. They showed that when isolated using stringent criteria, muscle SP cells are CD45-negative and Sca1-positive, and show very low Hoechst uptake. The Hoechst concentration seems to be the most critical. For example, Hoechst 33342 staining at a concentration of 5 μg/ml allows contamination by CD45-positive and Sca-1-negative cells. In contrast, 12.5 μg/ml Hoechst reduces the yield of SP cells and increases the percentage of CD45-negative Sca-1-positive cells. Because the percentage of CD45-positive SP cells tends to decrease at higher concentrations of Hoechst 33342 in both BM and non-hematopoietic tissues, muscle- SP cells seem to

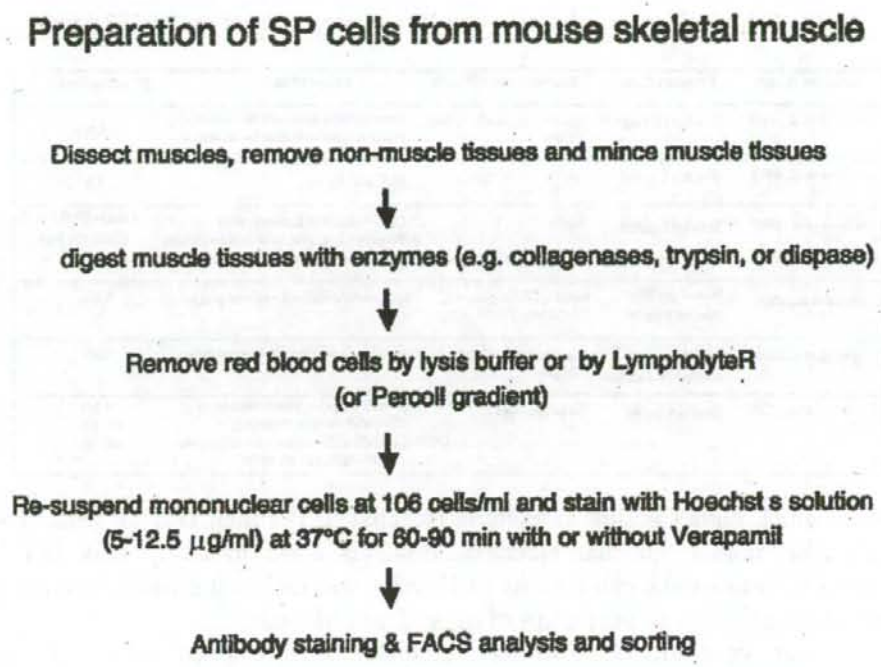

Figure 1. Preparation of SP cells from mouse skeletal muscle.

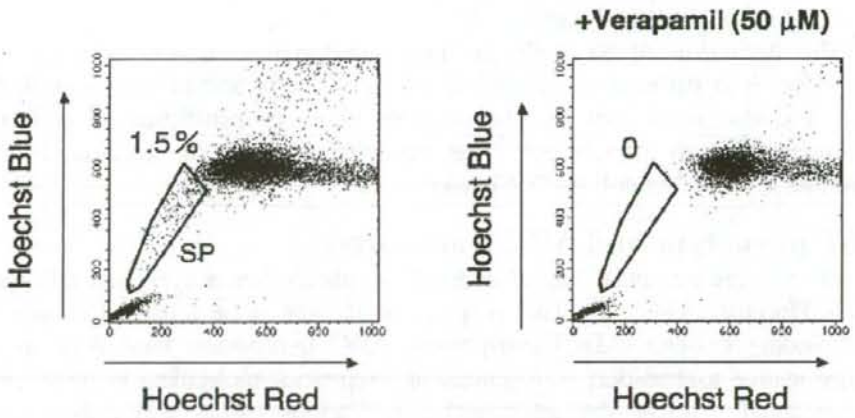

Figure 2. SP cells in adult skeletal muscle. Left panel: Muscle mononuclear cells were isolated from C57BL/6 mice, stained with 5 μg/ml Hoechst 33342 dye, and analyzed on a dual laser FACS Vantage SE (Becton-Dickinson). Right panel: A calcium channel blocker verapamil was added to Hoechst solution to confirm the SP fraction.

Table 1. Variations in isolation protocols and properties of muscle SP cells.

Authors (year)	Preparation	Markers on SP cells	Properties	SP abundance
Gussoni et al., 1999	Hoechst 12.5 μg/ml	Sca-1+, lin-, c-kit-, CD45-, CD43-	hematopoietic stem cell-like activity produce myofibers after i.v. injection	1-1.5%
Jackson et al., 1999	Hoechst 5 μg/ml	Sca1+, c-kit+, CD45-	HSC activity (+)	1%
Asakura et al., 2002	Hoechst 5 μg/ml	Sca1+	CD45+ fraction: hematopoietic differentiate into muscle cells in co-culture differentiate into satellite cells and muscle fibers *in vivo*	CD45+SP: 0.3-0.5% CD45-SP: 2-3%
Majka et al., 2003	Percoll gradient Hoechst 5 μg/ml	c-met+, CD45+, Sca-1+, PE-CAM+, Tie-2+	bone marrow-derived vascular progenitor	0.2%
Meeson et al., 2004	Percoll gradient Hoechst 12.5 μg/ml	Sca-1+, c-kit+, CD31-, Abcg2+	increase during muscle regeneration	0.2%
Uezumi et al., 2006	Hoechst 5 μg/ml	heterogeneous	CD31+SP: Bcrp-1+, vessel-associated CD45+SP: bone marrow origin CD31-CD45-SP:differentiate into adipocyte, osteocyte, and myocyte	1-3% < 0.1% < 0.1%

have a much higher ability to exclude Hoechst 33342 than BM SP cells. The study also points out that Hoechst 33342 is toxic to cells. This fact is important, because the cell toxicity of Hoechst dye makes it difficult to directly compare the biological properties of SP and non-SP cells.

In our opinion, one further parameter that has an effect on the heterogeneity of muscle SP cells is pre-fractionation by a Percoll gradient before Hoechst staining [27] (Table 1). In our experience, this procedure eliminates CD45-negative SP cells, resulting in enrichment of CD45-positive SP cells (our unpublished data).

The definition of SP cells for non-hematopoietic tissues is not clear. Therefore, it is difficult to determine which SP cells are the true muscle SP cells. We also think that Hoechst staining alone is insufficient to collect a homogeneous cell population. This approach should be combined with identification of other cell surface markers.

2. SP phenotype and ABC transporters

SP cells are generally defined as a cell population that actively and efficiently expels Hoechst 33342 dye. This property is thought to be mediated mainly by ATP-binding cassette (ABC) transporters. ABC transporters bind ATP as an energy source to transport endogenous or exogenous molecules, in most cases unidirectionally, across the cell membrane. Various tissues and cells express different combinations of ABC transporters, therefore, it is no wonder that SP cells from different tissues are heterogeneous in phenotypes and functions [10].

Muscle SP phenotype is not simply determined by Bcrp-1 expression

The ABCG2/Bcrp1 transporter is most often related to the SP phenotype. Bcrp-1-null mice show reduced numbers of SP cells in bone marrow and

skeletal muscle [13], but expression of this transporter is often found in non-SP cells [12]. Furthermore, isolation of human hematopoietic stem cells using an anti-ABCG2 antibody does not work [28]. These observations suggest that Bcrp-1 expression is not sufficient to endow the cell with the SP phenotype. In addition, it seems that a certain percentage of muscle SP cells are not Bcrp-1-dependent. When stained with an anti-Bcrp-1 antibody, the CD31-positive SP fraction is found to strongly express Bcrp-1, whereas two fractions, CD45-negative CD31-negative SP cells and CD45-positive SP cells reacted weakly with the anti-Bcrp-1 antibody [29]. Thus, transporters other than Bcrp-1 likely efflux Hoechst dye in a subset of muscle SP cells.

On the other hand, Meeson et al. examined the muscle SP fraction on a fluorescence-activated cell sorter (FACS) using both verapamil and fumitremorgin C (FTC), and confirmed that muscle-SP cells are highly sensitive to both of them [30]. Verapamil is a calcium channel blocker widely used to confirm the SP fraction on FACS profile, but shows no specificity toward a single transporter. FTC was shown to be a specific inhibitor of Abcg2, where it functions to inhibit Abcg2-associated ATPase activity [31]. Thus, the study showed that muscle SP cells are dependent on Abcg2/Bcrp-1 for the SP phenotype. SP preparation by Litman et al. employed a pre-fractionation of the muscle mononuclear cells using a Percoll gradient and a high concentration of Hoechst dye (12.5 μg/ml) for 90 min. The percentage of SP cells (0.19 %) was low, compared with other reports (see Table 1). Therefore, this result does not exclude the contribution of ABC transporters other than ABCG2/Bcrp-1 to the SP phenotype.

3. Other properties of muscle-derived SP cells

Most muscle SP cells are in quiescent stage

Freshly isolated SP cells from muscle are often reported to be non-adherent in culture, and are characterized by small cell size, stages G0/G1 of the cell cycle, and low metabolic activity, elements common to stem cells. Indeed, small size (6.6 ± 0.1 μm) and a high nucleus-to-cytoplasm ratio were reported for muscle-derived SP cells [30]. However, when we analyzed muscle SP cells during muscle regeneration, they discovered CD31-negative CD45-negative SP cells, which are large in size and Ki-67-positive. Thus, at least one subset of SP cells is not small and is cycling.

Cell surface markers on muscle SP cells

Reflecting the variety of experimental protocols for SP preparation, there are discrepancies in reported cell surface markers of SP cells (Table 1). In 1999, Gussoni et al. described the isolation of SP cells from skeletal muscle for the first time [6]. They used a higher concentration of Hoechst dye (12.5 μg/ml) than the original protocol (5 μg/ml) described by Goodell et al. [9], and

reported that SP cells in muscle are CD45-negative, c-kit-negative, and Sca-1-positive [6]. On the other hand, Jackson et al. reported that muscle-derived SP cells are Sca-1-positive (79 %), c-kit-positive (75 %), and CD45-negative [32]. Using a 5 μg/ml solution of Hoechst 33342, Asakura et al. reported that most muscle-derived SP cells are CD45-negative Sca-1-positive, but more than 10% of SP cells are CD45-positive [33]. Using an almost identical isolation procedure and Hoechst staining conditions, we reported that muscle-derived SP cells isolated from uninjured adult skeletal muscle have three phenotypically distinct SP subpopulations [29]. The report shows that approximately 90 % of muscle SP cells are CD31-positive CD45-negative. This fraction strongly expresses Bcrp-1 on the cell surface. About 5 % of muscle SP cells are CD45-negative CD31-negative, and approximately 3-5 % of muscle SP cells are CD45-positive.

Cell surface markers and origin of muscle SP cells

Interestingly, the difference in cell surface markers on SP subfractions is clearly related to their developmental origins. CD45-positive SP cells are thought to originate from BM and home into skeletal muscle via the circulation [29, 34]. Further, the HSC activity found in skeletal muscle was proven to be almost completely limited to the CD45-positive SP fraction [34]. On the other hand, the majority, but not all, of the CD45-negative SP cells in limb muscles were shown to be derived from the hypaxial somite and to have higher myogenic potentials than CD45-positive SP cells [35]. Interestingly, the study also revealed that a certain percentage of CD45-negative SP cells are not likely derived from the somite and are less myogenic than SP cells of somitic origin.

4. Gene expression in skeletal muscle SP cells

No expression of myogenic regulators in SP cells

Asakura et al. showed that the muscle SP fraction expresses no desmin or Pax7. Furthermore, muscle SP cells prepared from Myf-5-nLacZ mice do not express β-galactosidase [33]. Using sensitive RT-PCR analysis, we also showed that muscle SP cells prepared from non-injured or regenerating muscles do not express Pax7, Pax3, Myf-5, or MyoD [29]. These observations suggest that muscle SP cells are not committed to a myogenic lineage.

Molecular signatures of SP cells revealed by microarray analysis

Genome-wide gene expression analyses of SP fractions have been performed to elucidate the molecular regulation of SP cells. Meeson et al. examined the gene expression profiles of muscle SP cells isolated from uninjured and regenerating muscles, bone marrow-SP cells, and embryonic stem (ES) cells [30]. The results showed that skeletal muscle SP cells express Abcg2 (Bcrp-1) and endothelial and hematopoietic transcripts. They concluded

that muscle SP and BM SP cells have distinct molecular programs. Liadaki et al. reported the gene expression of muscle SP and BM SP cells. The analysis revealed that BM SP and muscle SP cells share a transcriptome signature but at the same time express tissue-specific markers [36]. When compared with MP cells within the same tissues, SP cells were found to underexpress genes reflecting tissue-specific functions [36]. Rochon et al. reported gene expression analysis of SP cells isolated from adult mouse bone marrow, adult male germinal cells, muscle primary culture, and mesenchymal cells [37]. These four types of SP cells are proposed to be a "stem cell-like" population. Transcriptional profiles for SP and the more differentiated non-SP cells isolated from these four tissues were compared by microarray analysis. The authors reported that the genes commonly upregulated in SP cells are implicated in the quiescent status of cells, maintenance of their pluripotency, and capacity to undergo asymmetric division, and that the repression of lineage-affiliated genes in SP cells is responsible for their undifferentiated state.

5. Location of SP cells in skeletal muscle

SP cells are defined by FACS analysis, but their location in muscle is ill-defined. Because Bcrp-1 is largely responsible for the SP phenotype [12,13], it is informative to stain muscle tissues sections with an anti-Bcrp-1 antibody. Using a polyclonal antibody against Bcrp-1, we showed that CD31-positive SP cells on glass slides are strongly stained with the antibody after sorting. Interestingly, CD31-positive Bcrp-1-positive cells are found in capillaries and venous endothelium, suggesting that the majority of muscle SP cells are associated with blood vessels [29]. Meeson et al. also showed that Abcg2 (Bcrp-1)-expressing cells are closely associated with the vasculature [30]. However, Bcrp-1 positive cells are also found in the MP fraction [29]. Therefore, not all Bcrp-1-positive cells found on muscle cross sections have the SP phenotype [29]. On the other hand, CD45-positive SP cells express quite low levels of Bcrp-1 [29]. Currently CD45-positive SP cells lack a distinctive marker available for use on tissue sections. Therefore, it is difficult to see BM-derived CD45-posotive SP cells on muscle cross sections. Likewise, CD45-negative CD31-negative SP cells on glass slides are hardly stained with anti-Bcrp-1 antibody after cell sorting, so it is also difficult to find CD45-negative CD31-negative SP cells in muscle sections [29].

6. Origin of muscle SP cells

Satellite cells originate in somite

Repair of mature skeletal muscle is largely mediated by the muscle progenitor cells referred to as satellite cells [2]. Satellite cells reside beneath the basal lamina of adult skeletal muscle juxtaposed against skeletal muscle

fibers and account for 2-5 % of sublaminal nuclei in adult muscle. The developmental origin of satellite cells is reported to be the somite [35, 38, 39].

Satellite cells and muscle SP cells are distinct populations

In the beginning, the relationship between muscle SP cells and satellite cells was controversial. Seale et al. reported that Pax7-null mice have severely reduced numbers of satellite cells, but show normal levels of muscle SP cells [40]. Asakura et al. showed that satellite cells and muscle SP cells have distinct differentiation potentials both *in vitro* and *in vivo* [33]. Fukada et al. established an antibody, named SM/C-2.6, that can purify quiescent satellite cells from muscle efficiently [41], and directly showed that almost all satellite cells are found in the MP fraction [41]. Furthermore, it was shown that muscle SP cells are negative for Pax7 or Pax3 and hardly differentiate into myotubes *in vitro* without co-culturing with myoblasts [29, 33]. Taken together, satellite cells and muscle SP cells seem to be distinct populations. Some reports, however, suggest that when transplanted into skeletal muscle, a portion of muscle SP cells differentiate into Pax7-positive cells [6, 33] and express Pax7. Although a recent study demonstrated that satellite cells vigorously self-renew, denying the existence of muscle stem cells that replenish the satellite cell pool [42], muscle SP cells might slowly supply severely damaged muscle with myogenic precursor cells.

Majority of limb muscle SP cells are derived from hypaxial somite

The source of muscle SP cells has long been debated. Schienda et al. explicitly tested and quantified the contribution of embryonic somitic cells to side populations [35]. Chick somitic cells were labeled by using replication-defective retroviruses or quail/chick chimeras, and mouse cells were labeled by crossing somite-specific, Pax3-derived Cre driver lines with a Cre-dependent reporter line. The results showed that a significant number, but not all, of limb muscle SP cells are derived from the hypaxial somite. Notably, the developmental origin of SP cells is related to their potentials; somitically derived CD45-negative SP cells are intrinsically more myogenic than CD45-negative SP cells from other sources. As mentioned above, CD45-positive SP cells are thought to be derived from bone marrow [29, 34].

7. Differentiation potential of muscle SP cells

The differential potentials of muscle SP cells have long been controversial. However, their cell surface markers reveal their developmental origin and their differential potentials.

Hematopoietic activities of muscle SP cells

Skeletal muscle-derived cells have the potential to repopulate the major peripheral blood lineages of lethally irradiated mice and thus behave like HSC

[6, 32]. To further clarify the properties of muscle-derived HSC, skeletal muscle-derived cells were fractionated based on the expression of CD45 and c-kit and Hoechst 33342 efflux, and examined for HSC activity in vivo [34]. The results revealed that muscle-derived HSC activities fall exclusively in the c-kit (dim) CD45 (pos) compartment of the muscle side population (msSP). Furthermore, it was shown that the CD45-positive msSP compartment of skeletal muscle is derived from whole bone marrow HSC. CD45-positive muscle SP cells are, however, shown to be much less potent in HSC activity than bone marrow HSC cells in competitive repopulation assays [34].

Muscle SP cells contain vascular progenitors

Muscle SP cells have also been shown to contribute to vascular regeneration after local injection into chemically damaged regenerating muscle [27]. Majka et al. showed that more than 70 % of muscle SP cells are CD45-positive and derived from bone marrow. The higher percentage of CD45-positive cells in the muscle-derived SP fraction, compared with those reported by other laboratories, may be due to pre-fractionation by a Percoll gradient of crude muscle-derived mononuclear cells prior to Hoechst staining (Table 1).

Myogenic potential of muscle SP cells

Gussoni et al. reported that muscle SP cells fail to settle on the plate during the first two weeks after cell sorting and that they then differentiate as a mixture of myoblasts and fibroblasts [6]. In contrast, Asakura et al. showed that skeletal muscle SP cells cultured in myoblast growth medium do not give rise to myogenic progenitors unless cultured with primary myoblasts [33]. We also observed that muscle SP cells alone hardly form myotubes *in vitro* [29]. After transplantation into muscle, however, muscle-SP cells generate both satellite cells [6, 33] and mature myofibers [6, 29, 33].

Mesenchymal potential of muscle SP cells

We showed that a minor subset of muscle SP cells (the CD31-negative CD45-negative fraction) differentiate into adipocytes or osteocytes *in vitro* upon induction. Furthermore, when these cells are transplanted into irradiated muscle, muscle fiber regeneration is severely impaired, and transplanted CD31-negative CD45-negative SP cells differentiate into many adipocytes and fibrotic cells (our published data). This observation suggests that they might be a source of the adipogenesis seen in advanced muscular dystrophy. CD31-positive CD45-negative SP cells uptake 1,1'-dioctadecyl- 3,3,3',3'-tetramethylindo-carbocyanine perchlorate-acetylated-low density lipoprotein (Dil Ac-LDL), suggesting that they possesses endothelial cell-like properties [29], but their functions *in vivo* remain to be determined.