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
諫田泰成	再生心筋細胞を用いた安全性薬理評価系の開発	エイブル株式会社和田昌憲	再生医療における臨床研究と製品開発	株式会社技術情報協会	東京都品川区	2013	572-576

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nakamura Y., Matsuo J., Miyamoto N., Ojima A., Ando K., Kanda Y., Sawada K., Sugiyama A., Sekino Y.	Assessment of Testing Methods for Drug-Induced Repolarization Delay and Arrhythmias in an iPS-Derived Cardiomyocyte Sheet: Multi-site Validation Study	Journal of Pharmacological Sciences			印刷中

IV.　研究成果の刊行物・別刷

再生医療における臨床研究と製品開発

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(株)技術情報協会

「再生心筋細胞を用いた安全性薬理評価系の開発」　抜刷

2013年9月発刊

第3節　再生心筋細胞を用いた安全性薬理評価系の開発

はじめに

ヒトiPS細胞は，今まで入手が困難であったヒト細胞の作製が可能となるため，「再生医療」と「創薬」の実用化が期待されている（図1）。再生医療への注目度は非常に高く，ヒトiPS細胞から作成した網膜色素上皮細胞を移植する臨床研究が2012年度に申請され，正式に承認された。創薬応用としては，医薬品の安全性や有効性の評価に対する利用が考えられ，創薬プロセスの早い段階で医薬品候補化合物の副作用などを予測できれば，臨床試験における予測性の向上や安全性確保，開発コスト削減などが期待される（図1）。

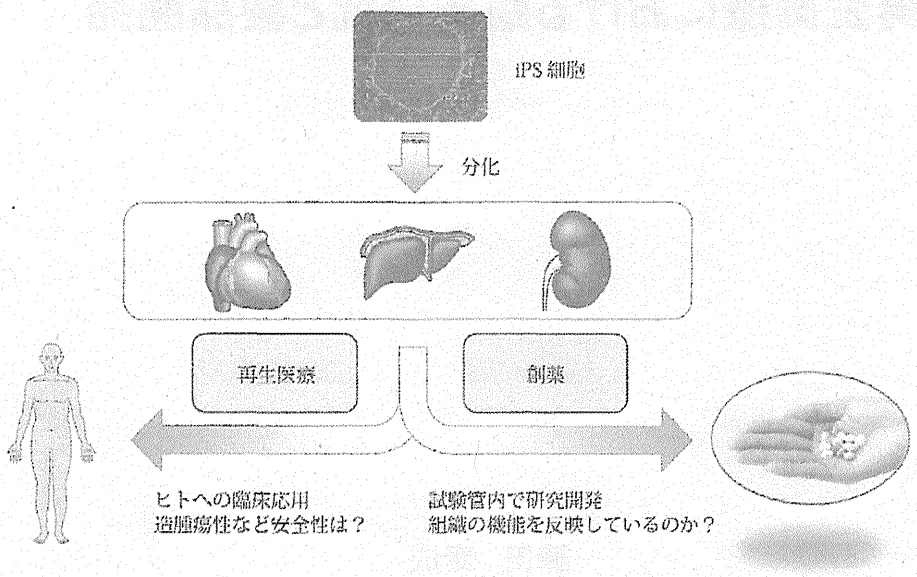

図1　ヒトiPS細胞の医療応用への可能性

これらのヒトiPS細胞の実用化に向けた課題として，ヒト未分化iPS細胞の品質，すなわち株間の差，継代数や研究室間の差などが明らかになり，国内外のプロジェクトにより標準化の作業が進められている[1]。しかし，ヒトiPS細胞由来の分化細胞に関してはほとんど標準化が手付かずであり，分化誘導の標準プロトコールや分化細胞の品質評価，分化指向性などあまり明らかになっていない。

また，再生医療と創薬応用でそれぞれ克服すべき課題も明らかになってきている。再生医療においては，分化細胞の安全性の確保が必須であり，残存している未分化iPS細胞の検出など造腫瘍性の評価法の開発が進められている[2]。分化心筋細胞に関しては，移植によって不整脈が誘発されないかなどの検証も必要である。創薬応用は，in vitroのアッセイ系で分化細胞を使用するため再生医療とは異なり安全面のハードルが低く分化製品に対するウイルス導入などの加工も可能であるが，元の臓器の性質を反映した成熟した細胞が必要と考えられる。

そこで本稿では，ヒトiPS細胞から心筋細胞への分化誘導技術ならびに分化心筋細胞の電気生理学的特性を概説し，将来的にヒトiPS細胞の創薬応用実用化に向けて整備すべき課題について考察したい。

1.　ヒトiPS細胞の心筋分化誘導法

1.1　EB形成法

ヒトiPS細胞から分化心筋細胞の一般的な作製法として，図2Aに示すような胚様体（Embryoid body, EB）を形成させる方法が知られている。胚の発生過程をin vitroで模倣するためにマウスES細胞から擬似的な胚であるEBを形成さ

せる方法が開発され，ヒト iPS 細胞にも応用されている。

マウス ES 細胞の場合には，未分化 ES 細胞をトリプシン処理により single cell にして，非接着コート処理済の 96 ウエルプレートなどを用いて EB を形成する。一方，ヒト iPS 細胞の場合には single cell にすると細胞死が起きるため，最初は小さな細胞塊を用いて EB を作成する必要がある。小さな細胞塊はヒト iPS 細胞のコロニーを CTK 溶液（トリプシン，コラーゲナーゼを含む分散液）などで処理した後ピペッティングすることによって作製し，低接着ディッシュに移す。すぐに 10% 血清を含む DMEM などの分化培地に変更すると細胞死が誘導されることがあるため，我々は basic fibroblast growth factor（bFGF）を含む未分化維持培地で数日間培養して EB を形成させてから，3 日ごとに半量を分化培地（10% 血清，0.1mM 2- メルカプトエタノール，非必須アミノ酸を添加した DMEM）に切り替えている。未分化維持に重要な bFGF の除去により分化スイッチが ON になり，外・中・内胚葉の三方向へ分化が誘導される。中胚葉を経由して心筋にも分化が誘導され，分化培地で EB の培養を続けると 2 ～ 3 週間後に拍動する EB が観察される。

EB 法の欠点は，三方向へ分化が誘導されるため，特定の細胞への分化効率が低いことである。EB の中でも心筋細胞は 10% 程度しか含まれていない。そこで，心筋分化効率を高くするために分化を亢進する液性因子が探索され，BMP-4，Wnt3a，G-CSF など様々な因子が報告されている[3-5]。もう一つの欠点は，EB のサイズが不均一で形状もばらばらであるため，EB 間の差が大きく拍動数などもバラつくことである。我々は EZ passage（Invitrogen #23181-010）を使用してコロニーを処理し，できる限り均一なサイズとなるように心掛けている。最近，V 字型ウエルの中で強制的に EB を形成させるプレートがいくつか報告されており，形やサイズのそろった EB 作成法の開発が期待される。

このように EB 形成を介する分化プロトコールは比較的簡便であり，特別な試薬を用いることなく血清を含む培地で分化誘導も可能であるが，問題点として，ヒト iPS 細胞には分化指向性が存在し，株間で心筋分化能に差が認められることがあげられる。公的な細胞バンクから入手可能な株の中で，201B7 株，253G1 株は心筋分化能が高いが，Tic 株（JCRB1331）はほとんど拍動が認められないことから，ヒト iPS 細胞には分化の方向性を規定する特性（分化指向性）があると考えられる。Tic 株は肝臓への分化効率が高いと報告されており[6]，心筋に対しては分化抵抗性を有する可能性がある。分化指向性に関するメカニズムや選別のマーカーなどは明らかになっていないので，今後の研究の進展が待たれる。目的に応じて最適なヒト iPS 細胞株を入手できるように、分化特性も含めた細胞バンクの整備が望まれる。また、そのような情報を共有するシステムも必要である。

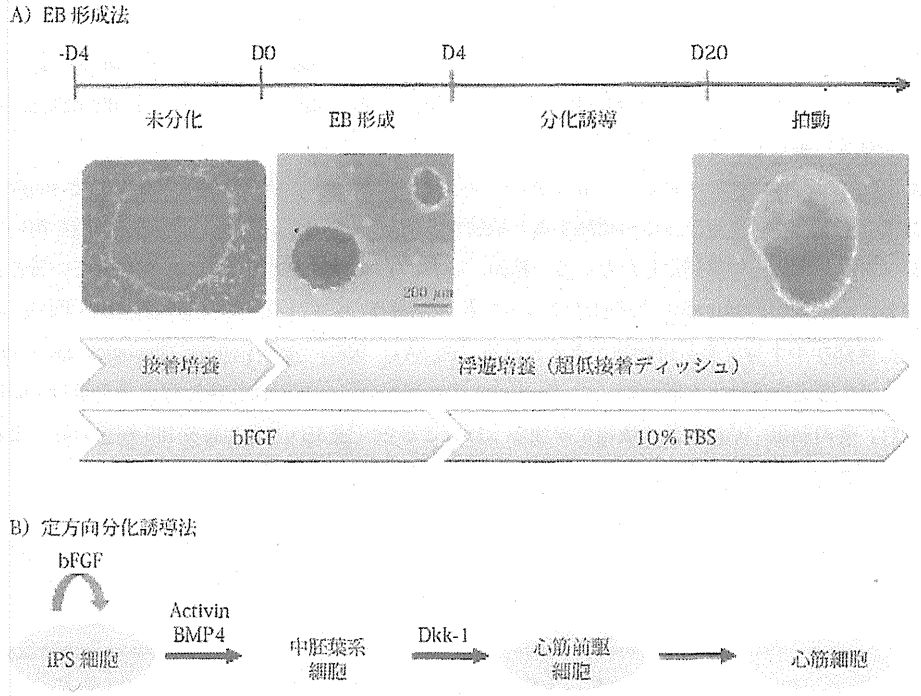

図 2　ヒト iPS 細胞の心筋分化誘導法

1.2 定方向分化誘導法

定方向分化誘導法とは，液性因子などを用いて特定の方向へ分化を特徴付ける方法であり，心筋細胞の場合は，中胚葉⇒心筋前駆細胞⇒心筋細胞，と段階的に分化誘導を行うことを指す（図2B）。この際，各ステップで添加する液性因子は，心筋細胞の発生過程やES細胞由来分化細胞に発現している受容体などを元にして，液性因子のスクリーニングや最適化が行われている[7]。

まず，ヒトiPS細胞をマトリゲルでコートしたディッシュに高密度で播種して，数回継代を行うことによりフィーダーをできる限り除去した後，Activinにより中胚葉や内胚葉の元となる原条へ分化誘導する。BMP-4の添加により中胚葉へ分化誘導がかかる。次に，Wnt antagonistであるDickkopf-1（Dkk-1）などにより心筋前駆細胞の方向へ分化を行い，最終的には心筋細胞の拍動が観察される。

EB形成法と比較すると，定方向分化誘導法は拍動までの期間が約1週間と顕著に短縮できる特徴がある。実際，酵素処理により分散しトロポニンで染色すると40%以上の細胞が心筋細胞であることから，分化効率も非常に高いことを確認している。また，接着したまま分化誘導を行うのでシートのような状態となり，ダイナミックな拍動が観察できる。欠点は，分化誘導のステップが増えてEB形成法よりも手間とコストがかかること，ヒトiPS細胞のコロニーの密度により分化効率が大きく異なることなどがあげられる。また，添加する因子は濃度に加えてタイミングも重要である。Wntシグナルは心筋分化の初期には促進的に作用し，後期では抑制的に作用することが知られており，タイミングが合わない場合には全く逆の作用をもたらすことが起こり得る。従って，液性因子を添加する時期・濃度・順序などに関して慎重に最適化する必要がある。

このように心筋細胞への分化プロトコールはEB法，定方向分化法ともに改良が加えられてきているが，分化心筋細胞における遺伝子発現やイオンチャネルの発現は幼若タイプである[8,9]。後述するように、分化心筋細胞は電気生理学的にも未成熟であるため，現在，我々は成熟させる分化誘導法を構築中である（投稿準備中）。再生医療においては細胞移植により心機能が回復するのかが焦点となるので分化細胞の成熟度はあまり問題にならず，むしろ液性因子の産生が重要と言われているが，創薬応用の場合は成熟度が薬剤のスクリーニング効率に影響をあたえる可能性があり，今後の研究に進展が期待される。

2. 分化心筋細胞の電気生理学的な特性

一般的に細胞の特性の指標として遺伝子発現やマーカー分子の発現などがあげられるが，心筋細胞で最も重要なことは電気生理学的な特性である。筆者らは創薬応用に向けて，国内の公的な細胞バンクよりヒトiPS細胞株を入手して，分化心筋細胞の品質を検証している。

201B7株由来の拍動EBをピンセットで引っ張るかカッターで刻んだ後にトリプシン処理をするとsingle cellを単離でき，ラミニンでコートしたディッシュに再播種すると拍動する細胞が得られる。この単離拍動細胞を用いてマニュアルパッチクランプにより活動電位を測定したところ，結節，心房，心室型の各サブタイプが存在していることが明らかになった。しかし，心筋細胞の静止膜電位が通常は-90mV程度であるのに対して分化心筋細胞はいずれも-40mV程度と浅いことから，成熟が不十分である可能性が考えられる（図3A）。次に，米国Cellular Dynamics International社で販売されているヒトiPS細胞由来分化心筋細胞（iCell）をiPSアカデミアジャパン株式会社経由で入手し，同様に活動電位の測定を行った。その結果，やはり静止膜電位が浅かったことから（図3B），元となるiPS細胞の株によるのではなく分化心筋細胞の共通の課題であることが示唆された。

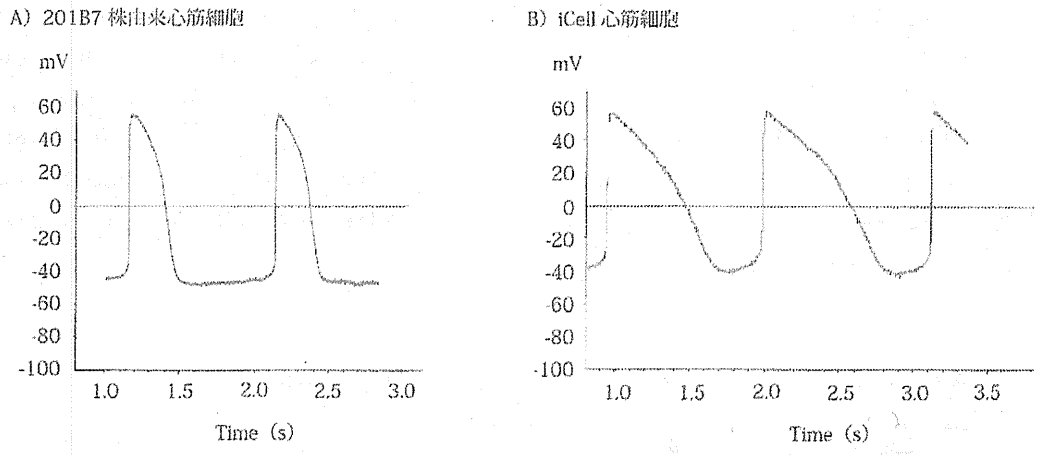

図 3　分化心筋細胞の活動電位

このような分化心筋細胞を用いた薬理作用の評価においては，様々なヒト iPS 細胞株から種々の手法を用いて心筋へ分化誘導すると，心筋細胞のサブタイプの割合や成熟度が不均一となり，薬剤に対する反応性についてもばらつくことが予想される。同一の化合物に対して同様の薬理作用が観察できない限り、催不整脈の予測が困難であり、バラつきを抑える必要がある。そこで，我々は次に述べるように，心筋シートを用いた実験プロトコールを整備している。

3.　分化心筋細胞を用いた安全性薬理試験

分化心筋細胞を用いた創薬応用の例として，薬剤性不整脈のリスク評価が考えられる。医薬品によって発生する副作用の中でもトルサード・ド・ポアント（TdP）とよばれる重篤な不整脈は重要で，発生頻度は極めて少ないもののまれに心室細動に移行し突然死に至る[10]。TdP は QT 間隔（心室の興奮から再分極までの時間）の延長を伴うことから，現在 TdP のリスクは，非臨床試験としてカリウムチャネル（IKr）を発現させた HEK293 細胞を用いてカリウム電流阻害作用を検討し（hERG 試験法），次いで in vivo で動物の QT 延長作用を評価し，その後臨床において Thorough QT/QTc 試験により厳密にヒトの QT 間隔に対する作用を調べることにより，一定の評価が可能である（図 5）。しかしながら，hERG 試験法は疑陽性が多く，有用な化合物を化合物のスクリーニングのプロセスで除外してしまう可能性がある。ヒト iPS 細胞由来の分化心筋細胞は，カリウム（IKs/IKr）に加えて，カルシウム，ナトリウムなど複数のチャネルが発現しているため再分極電流への影響を総合的に評価できる利点があり，疑陽性が減少して予測性が向上することが期待される。今までは，心筋への分化誘導条件（細胞株、分化誘導分化誘導法、日数，培養細胞密度など）が異なるばかりではなく，心筋細胞の電気生理学的機能の測定方法も異なっていたために，実験データを研究間で比較検討ができず，実験結果の再現性を確かめることが困難であった。そこで，我々は電気生理学的特性の解析法を比較検討し、不整脈検出プロトコルの標準化作業を行っている。

個々の心筋細胞の場合は，パッチクランプで解析が可能である。一つ一つの細胞の活動電位の波形をもとに QT 間隔を評価するため，心筋細胞のサブタイプの情報も同時に得られる利点がある。さらに，QT 延長などに起こることが多い早期後脱分極も直接検出することができる。しかし，前述したように個々の波形にバラつきが認められること，スループット性が低く大規模なスクリーニングには向かないこと，侵襲があるので短時間の薬理作用に限定されてしまうことなどを考慮すると，現実的には薬理作用の検出には向かないと考えられる。

細胞塊の場合は，図 4A に示すような多点電極システムを用いて心電図の解析が可能である。電極を埋め込んだディッシュに細胞塊を接着させると，細胞外電位（Field Potential: FP）が測定できる。FP は細胞内で記録される活動電位の微分波形に一致し，心電図によって得られる信号と同様の変化を記録する事ができる[11]。従って，ナトリウムによるピークから活動電位再分極時に観察されるカリウムのピークまでの時間である FPD は，心電図における QT 間隔に相当する。

FPDを用いる利点は，侵襲がないため医薬品候補化合物の長時間曝露による薬理作用が調べられることである。しかし，細胞塊ごとのFPの波形が一定の範囲内になるよう均一な塊の作製技術が必要不可欠である。さらに，EBが電極に接していなければシグナルが取得できない。もともと心筋細胞はガラスに張り付きにくい性質がある上に，拍動によってディッシュから剥がれたり接着場所が変わったりするので，ディッシュをコートする基材やデバイスの改善が求められる。我々が作製したヒトiPS細胞由来の拍動EBも市販のヒトES細胞由来細胞塊（スウェーデンCellectis社）も細胞外電位の測定に手間と時間がかかり，スループット性も低く，現時点では薬理作用の検出にはまだまだ遠いと言わざるを得ない。

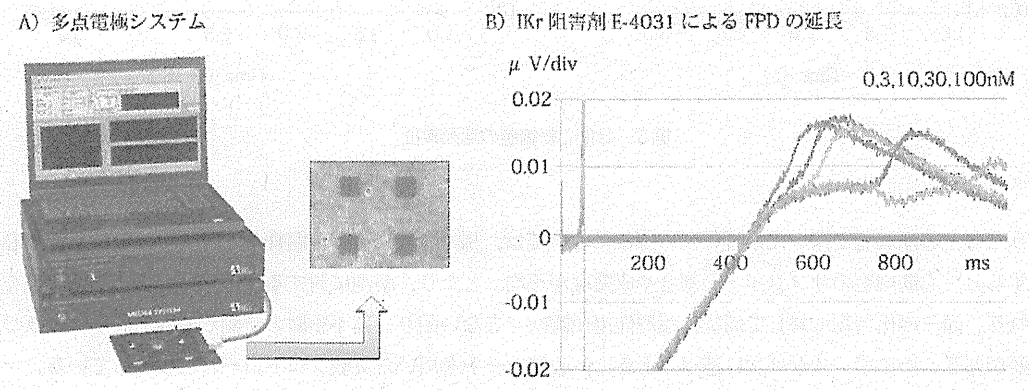

図4　心筋シートにおけるFPの解析

そこで，我々は心筋シートを用いて多点電極システムによるQT延長評価系の開発に着手した。心筋シートは分化心筋細胞を密に播種することで容易に作成することができ同期した拍動が観察される。シート形成によりEB間や個々の心筋細胞などのバラつきが平均化されるため安定したデータが得られると期待される。また，シートは多点電極全体を覆うことになるので，FPのデータ取得も大幅に改善される。実際例として，陽性対照物質であるIKr阻害剤E-4031によって心筋シートのFPD延長が検出できる（図4B）。現在、産官学の枠組みで市販の分化心筋細胞を用いて、薬理作用の再現性，メーカの相違，ロット間差など比較を行っており、不整脈検出プロトコールの標準化を行っている。

最終的に、分化心筋細胞を用いた試験法として確立するためには，検出感度，再現性，信頼性などを検証する必要がある。検出感度に関しては，今のところ，分化心筋細胞を用いた評価系は感受性が高いような結果が得られており，hERG試験で落とされた医薬品候補化合物を拾えるようなフォローアップに使用できるのかは不明である。再現性や信頼性を明らかにするためには，特定のラボ内のデータのみでは評価できないので，施設内及び多施設間で多くの化合物を用いたバリデーションを行う必要がある。本当に分化心筋細胞を用いる試験系がhERG試験よりも優位性があるのか？分化心筋細胞の試験法が手間やコストをかけるだけの価値があるのか？ヒトにおける予測性が向上するのか？臨床試験を代替しうるのか？などを明らかにしなければならない。

おわりに

ヒトiPS細胞の分化誘導技術などの研究の進展により，ヒトiPS細胞の実用化に向けた動きが盛んである。創薬応用の実用化に向けては，ヒトiPS細胞の株間の差，分化細胞の規格，安全性薬理試験のプロトコール整備など再生医療とは異なる多くの課題が残されている。薬剤性不整脈のリスク評価に関しては，現行のhERG試験法に対する優位性も重要なポイントである。これらの課題を克服することにより、創薬プロセスの効率が向上し，将来的には承認審査の迅速化が実現することが期待される。

謝辞

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Full Paper

Assessment of Testing Methods for Drug-Induced Repolarization Delay and Arrhythmias in an iPS-Derived Cardiomyocyte Sheet: Multi-site Validation Study

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Abstract. A prospective comparison study across 3 independent research laboratories of a pure I_{Kr} blocker E-4031 was conducted by using the same batch of human iPS cell–derived cardiomyocytes in order to verify the utility and reliability of our original standard protocol. Field potential waveforms were recorded with a multi-electrode array system to measure the inter-spike interval and field potential duration. The effects of E-4031 at concentrations of 1 to 100 nM were sequentially examined every 10 min. In each facility, E-4031 significantly prolonged the field potential duration corrected by Fridericia's formula and caused early after-depolarizations occasionally resulting in triggered activities, whereas it tended to decrease the rate of spontaneous contraction. These results were qualitatively and quantitatively consistent with previous non-clinical in vitro and in vivo studies as well as clinical reports. There were inter-facility differences in some absolute values of the results, which were not observed when the values were normalized as percentage change. Information described in this paper may serve as a guide when predicting the drug-induced repolarization delay and arrhythmias with this new technology of stem cells.

Keywords: E-4031, iPS cell–derived cardiomyocyte, multi-site validation, field potential, TdP

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Introduction

Drug-induced proarrhythmia has been a major safety concern about the development of new drugs, leading to issuing of ICH E14 and S7B guidelines in May 2005 (1, 2). The guidelines have effectively reduced risks of a new compound causing torsades de pointes, whereas non-clinical and clinical studies in the current approach still remain imperfect because they identify many drugs as being "positive" despite a lack of demonstrable pro-arrhythmic risk (3 – 6). In a recent workshop held in July 2013 by the US Food and Drug Administration (FDA), the Cardiac Safety Research Consortium and the non-profit Health and Environmental Sciences Institute (HESI), a new paradigm was proposed and discussed, focusing on a comprehensive assessment of multi ion channel effects to determine actual proarrhythmic risk of drugs (7). This new approach will include a stem-cell technology that has the potential to improve the currently used assessment of cardiotoxicity; however, more work is required prior to the use of stem cell–derived cardiomyocyte models to accurately predict proarrhythmias in humans (7).

There have been a large number of various studies with stem cell–derived cardiomyocytes examining electrophysiological effects of drugs (8 – 13). In an effort to further improve upon the assay system, this report describes a more simple and reliable protocol of an induced pluripotent stem (iPS) cell–derived, cardio-

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myocyte-sheet model. Extensive preliminary studies have confirmed that the protocol proposed in this paper could be optimal for assessing E-4031-induced repolarization delay and arrhythmias and would qualitatively and quantitatively reflects its electropharmacological profile in humans. This is a critically new finding and a significant improvement over the previous in vitro I_{Kr} assay systems including the hERG potassium channels and the papillary muscle of guinea pigs. In this study, in order to start verifying the reproducibility of our protocol, a prospective comparison study of E-4031 was conducted across 3 independent research laboratories with the same batch of iPS cell–derived cardiomyocytes.

Materials and Methods

Cell culture and plating

Each facility (E, N, T) obtained the same batch (#1089404) of cryopreserved human iPS cell–derived cardiomyocytes [iCells; Cellular Dynamics International (CDI), Madison, WI, USA]. The cells were thawed in specially prepared medium (Plating Media, CDI), which were plated onto 0.1% gelatin-coated, 6-well tissue-culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of $1.3 - 2.6 \times 10^6$ (E: 1.3×10^6, N: 2.0×10^6, T: $2.4 - 2.6 \times 10^6$) of cells per well. Two days after plating, Plating Media was replaced to specially prepared culture medium (Maintenance Media, CDI). Then, the culture medium was changed with fresh one every 2 days. The cells were cultured for 3.7 ± 1.4 days (2 – 7 days) after thawing at 37°C with 5% CO_2 prior to re-plating.

The electrical activity of cardiomyocytes was measured by using our original protocol. Briefly, the recording area of probes with 64 of the recording electrodes (MED probe; MED-P515A, Alpha Med Scientific, Osaka) of the MED64 System (Alpha Med Scientific) was coated with 2 μL of fibronectin (50 μg in 1 mL of distilled water), which was incubated at 37°C for $\geq$ 1 h. The cells cultured in the 6-well tissue-culture plates were dispersed with 0.25% trypsin-EDTA or TrypLE Select, which were re-plated onto the MED probes at a density of 3×10^4 cells in a 2 μL of the culture medium. The cells were incubated at 37°C with 5% CO_2 for 2 – 18 h (E: 4 – 12 h, N: 2 – 18 h, T: 12 – 18 h) in moisture condition prior to filling each probe with 1 mL of the culture medium. The half volume or all of the culture medium of the probes was changed with the culture medium, which had been warmed to 37°C, every 2 days thereafter. The cells were cultured for 5.2 ± 1.6 days (3 – 7 days) to obtain a sheet of cardiomyocytes with spontaneous and synchronous electrical automaticity.

Field potentials (FPs) assay

Maintenance Media was used as a culture medium throughout the experiment. Prior to the measurement of FPs, cardiomyocyte sheets were equilibrated for $\geq$ 30 min in the CO_2 incubator in 1 or 2 mL of fresh culture media. After equilibration, the probes were kept at 36°C – 37°C with thermo-control systems and covered with a lid, through which aeration of 95% O_2 / 5% CO_2 gas was provided. FPs from spontaneously beating cardiomyocyte sheets were recorded and digitized at 20 kHz by using the MED64 System. The stability and constancy of the waveforms, inter-spike interval, and field potential duration (FPD) were confirmed for $\geq$ 20 min. FPD was defined as an interval from the initial sharp deflection to the peak of the dome (8). Using the information obtained in this observation period, we selected 3 – 6 electrodes, which would be suitable for continuous monitoring of the FP configuration consisting of spike and dome. After recording the basal control state, the effects of 1, 3, 10, 30, and 100 nM of E-4031 were assessed by adding stock solution cumulatively to the culture medium to obtain target concentrations. The final concentration of DMSO was limited to be < 0.6%, since DMSO at a concentration of < 0.6% has been reported to hardly affect any of the variables assessed in this study (8). At each concentration, the FP was recorded for $\geq$ 10 min and the last 30 beats were extracted as a dataset to analyze waveforms, inter-spike interval, and FPDs according to the previous report (8). The datasets of concentrations were excluded from the statistical analysis, when early after-depolarization and/or triggered activity were observed. Early after-depolarization was defined as deflection occurring at the plateau of the dome, and sharp deflection originating from early after-depolarization was judged as a triggered activity. FPD was corrected with Fridericia's formula, which was defined as the primary method of correction in this study [$FPDcF = FPD / (\text{inter-spike interval} / 1000)^{1/3}$] (14). The values of inter-spike interval and FPDcF from the last 30 waves at each concentration were averaged.

Drugs and chemicals

E-4031 was obtained from WAKO (Osaka) or synthetized at Eisai Co., Ltd. (Tsukuba). Gelatin was obtained from Sigma (St. Louis, MO, USA). Fibronectin was obtained from Becton Dickinson or Invitrogen (Carlsbad, CA, USA). Trypsin-EDTA and TrypLE Select were obtained from Invitrogen.

Data analyses and statistical assessment

In each experiment, one electrode that satisfied the following two conditions was chosen: 1) FP was recorded whole through the experiment, and 2) The amplitude of

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the dome was the largest. The data were expressed as the mean ± S.E.M. The effects of the drug on inter-spike interval and FPDcF obtained in each facility were evaluated with the paired *t*-test or one-way repeated-measures analysis of variance (ANOVA) followed by Contrasts for mean values comparison between the baseline value (0 nM) and others. Meanwhile, inter-facility variability was assessed with one-way factorial ANOVA followed by Fisher's test or unpaired *t*-test. A P value < 0.05 was considered statistically significant.

Results

The effects of E-4031 in concentrations of 0, 1, 3, 10, 30, and 100 nM were examined in each facility, except that 1 nM was not performed in facility N. The number of preparations that can be used for the assessment of inter-spike interval, field-potential duration, and categorical analysis decreased due to the onset of early after-depolarization and/or triggered activity as the concentration of drug increased.

Inter-spike interval

The effects of the drug on the inter-spike interval (ms) are summarized in Fig. 1 (upper panel). The baseline values (0 nM) were 926 ± 44 ms in facility E, 1,216 ± 56 ms in facility N, and 956 ± 22 ms in facility T. Inter-facility difference was observed between N and E besides between N and T, which was not detected between E and T. No significant change from the respective baseline values was detected at 1, 3, or 10 nM in E and T and at 3 nM in N. Inter-facility difference was observed at 3 nM between N and E besides between N and T, which was not detected at any concentration between E and T. Meanwhile, the effects of the drug on the inter-spike interval (%) are summarized in Fig. 1 (lower panel). The significant increase was observed at 3 nM in N, which was not detected at 1, 3, or 10 nM in E or T, although the similar trend was observed. Inter-facility difference was not detected at any concentration.

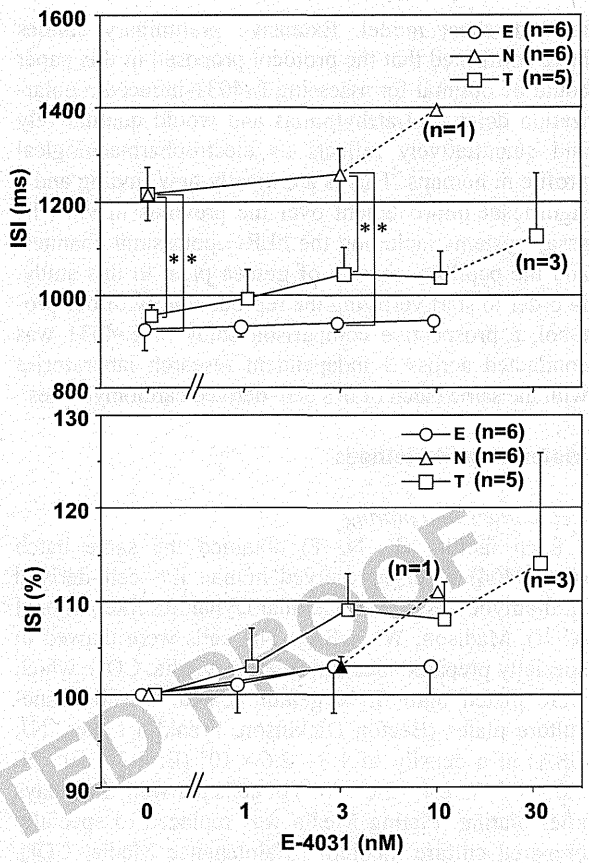

Fig. 1. Summary of the results showing the actual measurement values (upper) and their percentage changes (lower) in inter-spike interval (ISI) of E-4031 in human iPS cell–derived cardiomyocytes in each facility. Each value represents the mean ± S.E.M. of 6 preparations for facility E and facility N and 5 preparations for facility T. Values in parentheses represent the number of the preparations. An asterisk indicates significant difference between the facilities, whereas a closed symbol represents significant change from the respective baseline value.

Prolongation of field-potential duration

The effects of the drug on the FPDcF (ms) are summarized in Fig. 2 (upper panel) and typical tracings of field potential before and after the drug treatment are depicted in Fig. 3. The baseline values (0 nM) were 430 ± 12 ms in E, 443 ± 5 ms in N, and 320 ± 15 ms in T. Inter-facility difference was detected between T and E besides between T and N, which was not detected between E and N. FPDcF was prolonged at 3 and 10 nM in E and at 10 nM in T, which tended to be prolonged at 3 nM in N without statistical significance. Inter-facility difference was detected at 1, 3, and 10 nM between E and T and at 3 nM between N and T. Inter-facility difference was not detected at any concentration between E and N. Meanwhile, the effects of the drug on the FPDcF (%) are summarized in Fig. 2 (lower panel). FPDcF was prolonged at 3 and 10 nM in E and at 10 nM in T, whereas it tended to be prolonged at 3 nM in N without statistical significance. Inter-facility difference was not detected at any concentration.

Incidence of early after-depolarization or triggered activity

The incidence of early after-depolarization or triggered activity is summarized in Table 1 and typical tracing of field potential with triggered activity is depicted in

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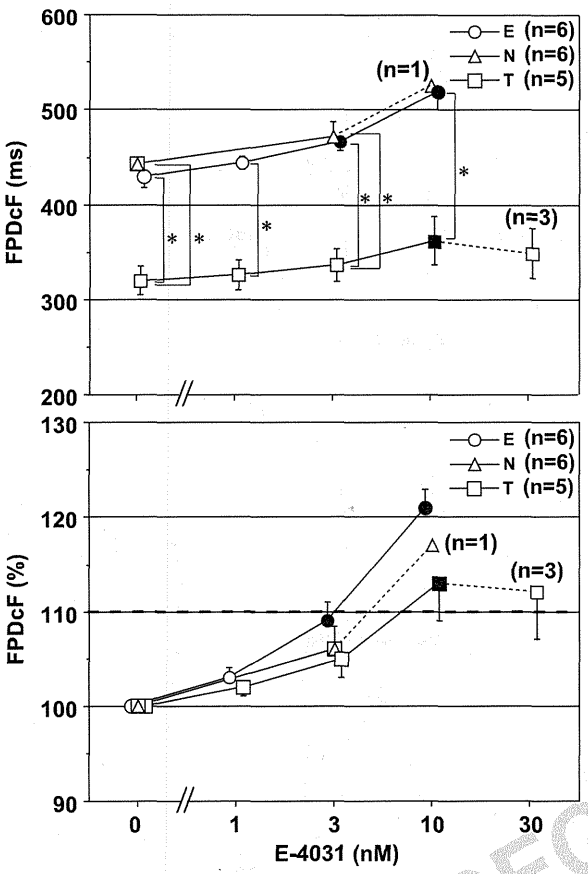

Fig. 2. Summary of the results showing the actual measurement values (upper) and their percentage changes (lower) in FPDcF of E-4031 in human iPS cell–derived cardiomyocytes in each facility. Each value represents the mean ± S.E.M. of 6 preparations for facility E and facility N and 5 preparations for facility T. Values in parentheses represent the number of the preparations. An asterisk indicates significant difference between facilities, whereas a closed symbol represents significant change from the respective baseline value.

Fig. 3. Early after-depolarization or triggered activity was induced at ≥ 10 nM in N and at ≥ 30 nM in E and T.

Categorical analysis of FPDcF

The results of categorical analysis of absolute FPDcF (ms) are summarized in Table 2. At the baseline and 1 nM, all FPDcF in each facility were categorized in ≤ 480 ms, whereas FPDcF of ≥ 500 ms was observed at ≥ 3 nM in N and at ≥ 10 nM in E, which was not observed in T.

The results of categorical analysis of ⊿FPDcF are summarized in Table 3. At 1 nM, all ⊿FPDcF in E and T were categorized in ≤ 60 ms. ⊿FPDcF of > 60 ms was observed at ≥ 3 nM in E and N and at ≥ 10 nM in T.

Discussion

In this study, a prospective comparison study of E-4031 was conducted with the same batch of human iPS cell–derived cardiomyocytes in order to start verifying the reproducibility of our original standard protocol across 3 independent research laboratories. We demonstrated that the protocol can be reliable in detecting the drug-induced repolarization delay and arrhythmias with high reproducibility.

E-4031 tended to show a negative chronotropic effect at concentrations of ≥ 3 nM; however, a significant change was detected only at 3 nM in facility N when assessed by percentage change (Fig. 1). A more potent negative chronotropic effect was observed by higher concentrations of E-4031 in each facility, although we did not perform the statistical analyses on inter-spike interval at concentrations of ≥ 10 nM in facility N and ≥ 30 nM in facilities E and T because of the limited number of experiments (n = 0 – 3). These results are in good accordance with a previous observation in patients with supraventricular tachyarrhythmias (15), in which E-4031 at a plasma concentration of 4.85 ± 1.35 ng/ml (11 nM) modestly prolonged RR interval, but it did not achieve statistical significance. Meanwhile in the single sinoatrial nodal cells of rabbits, E-4031 at a concentration of 100 nM suppressed or blocked the spontaneous activity (16), and moreover in the Langendorff-perfused whole hearts of guinea pig, E-4031 at concentrations of 30 – 300 nM or 5 μM significantly reduced the heart rate (17, 18). Thus, our testing method using the human iPS cell–derived cardiomyocytes can be considered to be more sensitive than currently available in vitro non-clinical models in detecting the E-4031-induced negative chronotropic effect.

E-4031 caused early after-depolarization and/or triggered activity in a concentration-related manner as shown in Fig. 3 and Table 1. In previous studies using the Langendorff-perfused rabbit heart, 0.5 μM of E-4031 induced early after-depolarization and triggered activity (19, 20). Also, in human embryonic stem cell–derived cardiomyocyte clusters, 1 μM of E-4031 induced early after-depolarization in half of the clusters (11). Meanwhile, in the human engineered heart tissue sheet made of the human embryonic stem cells, 10 nM of E-4031 was reported to induce arrhythmias (12). Thus, our testing method as well as the previous human engineered heart tissue sheet is considered to have higher sensitivities than the human cardiomyocyte clusters or the Langendorff-perfused rabbit heart in detecting E-4031-induced early after-depolarization and/or triggered activity.

E-4031 prolonged the FPDcF in a concentration-related manner as shown in Figs. 2 and 3. A wide variety

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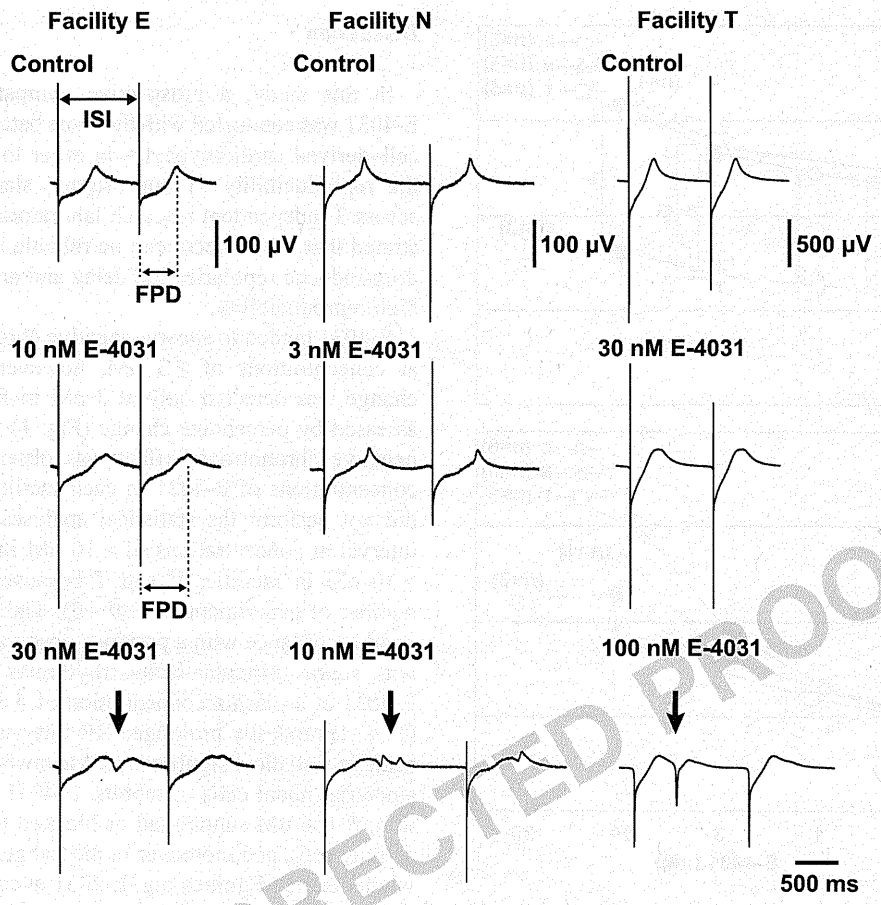

Fig. 3. Typical tracings of the field potential from each facility. Upper traces show the field potential at control (Control); middle traces indicate the prolongation of field potential duration (FPD) with 3 – 30 nM of E-4031; and lower traces represent the onset of early after-depolarization or triggered activity with 10 – 100 nM E-4031 (arrow). ISI: inter-spike interval.

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Table 1. Incidence of E-4031-induced early afterdepolarization or triggered activity

Concentration (nM)	Facility			All
	E	N	T	
0	0% (0/6)	0% (0/6)	0% (0/5)	0% (0/17)
1	0% (0/6)	NT	0% (0/5)	0% (0/11)
3	0% (0/6)	0% (0/6)	0% (0/5)	0% (0/17)
10	0% (0/6)	83% (5/6)	0% (0/5)	29% (5/17)
30	100% (6/6)	100% (6/6)	40% (2/5)	82% (14/17)
100	100% (6/6)	100% (6/6)	100% (5/5)	100% (17/17)

Note: The numerator in parentheses shows the number of preparations exerting early after-depolarization or triggered activity, whereas the denominator indicates the total number of preparations assessed. NT: Not tested.

of analyses have been performed to clarify the effects of E-4031 on the repolarization process as summarized in Table 4 (12, 15, 17 – 26). The effects of E-4031 in these previous reports are directionally the same as the currently observed results, although their potency varied greatly. Thus, the present findings suggest that the sensitivity of current testing method to detect drug-induced repolarization delay can be considered to be comparable to hERG assay and human subjects, but it may be higher than those of in vivo and in vitro animal models.

Table 2. Summary of categorical analysis of absolute FPDcF values

Concentration of E-4031 (nM)	FPDcF (ms)	Facility			All
		E	N	T	
0	≤ 450	67% (4/6)	83% (5/6)	100% (5/5)	82% (14/17)
	> 450	33% (2/6)	17% (1/6)	0% (0/5)	18% (3/17)
	> 480	0% (0/6)	0% (0/6)	0% (0/5)	0% (0/17)
	> 500	0% (0/6)	0% (0/6)	0% (0/5)	0% (0/17)
1	≤ 450	50% (3/6)	NT	100% (5/5)	73% (8/11)
	> 450	50% (3/6)		0% (0/5)	27% (3/11)
	> 480	0% (0/6)		0% (0/5)	0% (0/11)
	> 500	0% (0/6)		0% (0/5)	0% (0/11)
3	≤ 450	17% (1/6)	17% (1/6)	100% (5/5)	41% (7/17)
	> 450	50% (3/6)	50% (3/6)	0% (0/5)	35% (6/17)
	> 480	33% (2/6)	17% (1/6)	0% (0/5)	18% (3/17)
	> 500	0% (0/6)	17% (1/6)	0% (0/5)	6% (1/17)
10	≤ 450	17% (1/6)	0% (0/1)	80% (4/5)	42% (5/12)
	> 450	0% (0/6)	0% (0/1)	20% (1/5)	8% (1/12)
	> 480	17% (1/6)	0% (0/1)	0% (0/5)	8% (1/12)
	> 500	67% (4/6)	100% (1/1)	0% (0/5)	42% (5/12)
30	≤ 450	— (0/0)	— (0/0)	100% (3/3)	100% (3/3)
	> 450	— (0/0)	— (0/0)	0% (0/3)	0% (0/3)
	> 480	— (0/0)	— (0/0)	0% (0/3)	0% (0/3)
	> 500	— (0/0)	— (0/0)	0% (0/3)	0% (0/3)

Note: The numerator in parentheses shows the number of preparations exerting respective FPDc values in each category, whereas the denominator indicates the total number of preparations assessed. FPDcF = FPD / (inter-spike interval / 1000)$^{1/3}$. NT: Not tested.

Table 3. Summary of categorical analysis of ΔFPDcF values

Concentration of E-4031 (nM)	ΔFPDcF (ms)	Facility			All
		E	N	T	
1	≤ 30	83% (5/6)	NT	100% (5/5)	91% (10/11)
	> 30	17% (1/6)		0% (0/5)	9% (1/11)
	> 60	0% (0/6)		0% (0/5)	0% (0/11)
3	≤ 30	67% (4/6)	67% (4/6)	100% (5/5)	76% (13/17)
	> 30	0% (0/6)	17% (1/6)	0% (0/5)	6% (1/17)
	> 60	33% (2/6)	17% (1/6)	0% (0/5)	18% (3/17)
10	≤ 30	0% (0/6)	0% (0/1)	60% (3/5)	25% (3/12)
	> 30	17% (1/6)	0% (0/1)	20% (1/5)	17% (2/12)
	> 60	83% (5/6)	100% (1/1)	20% (1/5)	58% (7/12)
30	≤ 30	— (0/0)	— (0/0)	33% (1/3)	33% (1/3)
	> 30	— (0/0)	— (0/0)	33% (1/3)	33% (1/3)
	> 60	— (0/0)	— (0/0)	33% (1/3)	33% (1/3)

Note: The numerator in parentheses shows the number of preparations exerting respective ΔFPDc values in each category, whereas the denominator indicates the total number of preparations assessed. ΔFPDcF: Increase from baseline in FPDcF. NT: Not tested.

Since 82% of the basal FPDcF was ≤ 450 ms, which is the upper limit of the normal range of QTc in human subjects, we examined the repolarization delays with the categorical analysis described in the ICH E14 guideline. FPDcF > 500 ms and/or ΔFPDcF > 60 ms were detected at concentrations of ≥ 3 nM in some preparations (Tables 2 and 3), indicating that 3 nM of E-4031 will be a critical concentration for inducing the excessive QT-interval prolongation by this testing method.

The major purpose of this study was to clarify the

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Table 4. Summary of nonclinical and clinical reports regarding the effects of E-4031 on the repolarization markers

Model	Method	Marker	Change (%)	Concentration (nM)	References
hERG		IC_{50}		7.7	21
Guinea pig	Ventricular myocytes	APD_{90}	26	5,000	22
		APD_{90}	71	100	23
	Papillary muscles	APD_{70}	9 – 68	30 – 300	23
		APD_{90}	10	20	24
		$APD_{30\text{-}90}$	10	7	24
	Langendorff heart	QTc	5 – 27	3 – 300	18
		MAP_{90}	3 – 18	3 – 300	18
		QTc	26	5,000	17
Rabbit	Langendorff heart	QT	51	500	19
		MAP_{90}	50	500	19
Dog	In vivo (Anesthetized)	QT	57	6.2	25
		QTc	10	5.1	24
	In vivo (Conscious)	QTc	10	19.2	24
Monkey	In vivo (Conscious)	QTc	10	3.1	24
Human		QTc	14	12.1	15
		QTc	4.7 – 15	5.1 – 27	26
iPS cell	Single cell	APD_{90}	40 – 70	30 – 100	20
		APD_{90}	5 – 11	10 – 1,000	12
		$APD_{30\text{-}90}$	15 – 29	10 – 1,000	12

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extent of the inter-facility difference in sensitivity and reliability of this new testing method. The concentrations of E-4031 that caused early after-depolarization and/or triggered activity were close to each other among the 3 facilities; however, there were some variations in the basal absolute values of inter-spike interval and FPDcF. Since we used the same batch of the cardiomyocytes, these differences might be induced by small inter-facility variability in the net culture period, the cell density on recording electrodes and/or experimental temperature. It should be noted that there was no difference in inter-spike interval or FPDcF among the 3 facilities when compared using percentage change.

In conclusion, we demonstrated that the use of the standardized protocol for the iPS cell–derived cardiomyocyte sheets can minimize inter-facility difference in detecting the drug-induced repolarization delay and arrhythmia. While further studies are needed to establish the currently proposed protocol including the assessment of variability across batches, more reference compounds and clinical predictability, information described in this paper may help predict the potential of the drug-induced repolarization delay and arrhythmias with this new technology. Also, methodological work for high-throughput evaluation is now ongoing.

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Conflicts of Interest

The authors declare no conflicts of interest.

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V.　ヒューマンサイエンス財団・規制基準委員会
勉強会　講演資料
平成 26 年 2 月 21 日開催
「ヒト iPS 細胞を用いた心毒性評価の現状と課題」

2014-2-21 HS財団・規制基準委員会勉強会

ヒトiPS細胞を用いた心毒性評価の現状と課題

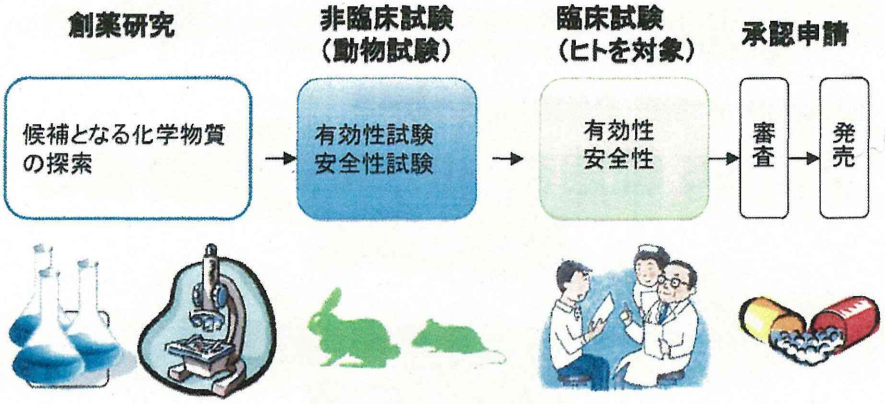
国立医薬品食品衛生研究所
薬理部第二室
諫田 泰成

本発表の見解は発表者の私見であって、国立医薬品食品衛生研究所
および厚生労働省の公式な見解ではありません。

本日のお話

- 創薬プロセスにおけるヒトiPS細胞の応用
  - 国内外の動向
- ヒトiPS細胞由来心筋細胞を用いた安全性薬理試験法の開発
  - 標準化の意義
  - 試験法としての科学的根拠（バリデーション）
- 今後の展望
  - ガイドラインに向けて

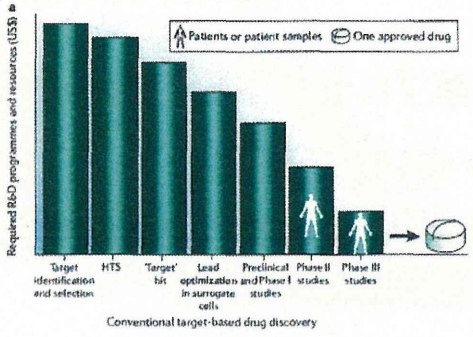
創薬プロセス



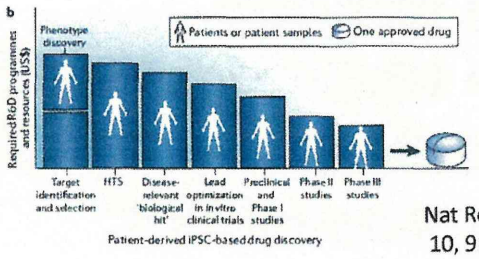
- 動物とヒトの種差の問題
- 現在の安全性の評価手法の限界
- 個人差

創薬プロセスにおけるヒトiPS細胞の応用

Conventional drug development



Perspective – iPS cell based drug development



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