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| 氏　　名 | Chanthra Nawin（じゃんたらー　なーうぃん） |
| 学位の種類 | 博士（医学） |
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| 学位論文名 | **心筋細胞成熟における細胞外マトリックスの機能解明** |
| 論文審査委員 | (委員長)　教授　古川雄祐 |
| | (委　員)　教授　川人宏次　　准教授　金田るり |

# 論文内容の要旨

## 1　研究目的

**AIM 1: Development of assessment tools for cardiomyocyte (CM) maturation.**

A lack of assessment tools for CM maturation is a key road brick to obtain fully mature pluripotent stem cell-derived cardiomyocytes (PSC-CMs). Thus, I have developed qualitative and quantitative methods to assess CM maturation. For qualitative method, I generated a novel fluorescence reporter line, Myom2-RFP. For quantitative method, I updated RNA sequencing method using Quant-seq and evaluated the maturation degree of PSC-CMs compared to *in vivo* data.

**AIM 2: Evaluation of the effects of extracellular matrices (ECMs) on CM maturation.**

Previous study has shown that ECMs such as laminin, collagen, and fibronectin, could enhance CM maturation. However, how those ECMs promote CM maturation are largely unknown, and what the maturity of PSC-CMs with those ECMs. Thus, I aimed to examine if the ECMs could promote CM maturation. If so, which ECM is superior to others and what maturity of PSC-CMs.

## 2　研究方法

**1) Development of assessment tools for CM maturation.**

1.1) *Generation of a qualitative method for CM maturation*

As a lack of assessment tools for CM maturation is a major obstacle to obtain mature PSC-CMs effectively, I first generated a fluorescent CM maturation reporter line. Our transcriptome data indicated that Myom2, encoding to M-protein, one of sarcomere protein, starts to express around late-embryonic stage and increases subsequently. Therefore, I decided to knock-in RFP to 3' end of Myom2 genomic locus, called Myom2-RFP. To achieve knock-in efficiency, I used CRISPR/Cas9 system, a genome-editing tool, to generate double strand break at the target region. Myom2 is localized to M-line of the sarcomeres, thus I expected that RFP would be observed in the same region as Myom2 protein *in vivo*.

1.2) *Development of a quantitative method for CM maturation*

Previously, our group has developed a microarray-based quantitative assay for CM

maturation. However, this method is an expensive method. Thus, I recently updated the method with Quant-seq, using poly T primer to synthesize cDNA and sequence predominantly 3' end of mRNA. This approach requires less read depth and allows more multiplexing samples per run with affordable cost. With the update method, I have set a weight for each gene to calculate the maturation score. The maturation score is sum of the expression levels of each gene multiplied by the weight. With this method, I could determine maturation status of PSC-CMs and also examine gene expressions which related to CMs.

**2) Evaluation of the effects of ECMs on CM maturation.**

Mouse PSC-CMs exhibited more mature properties on native cardiac ECMs such as laminin, collagen, and fibronectin, which are secreted from cardiac fibroblasts. Here, I examined if ECMs promote CM maturation using qualitative and quantitative methods. And if so, what the maturation degree of PSC-CMs achieved by the ECM treatments. To this end, PSC-CMs generated from the reporter line were plated on different types of ECMs at day 10 of cardiac differentiation. Then, morphological, physiological, and functional analysis, were conducted at day 38, to evaluate the effects of those ECMs on CM maturation. Moreover, I also corrected RNA from all of the conditions and performed RNA-sequencing to assess the maturation degree of the PSC-CMs with ECM treatments. With these methods, I could identify ECMs which are able to enhance the maturation of PSC-CMs.

## 3 研究成果

I identified that ECMs including laminin, collagen, and fibronectin, showed temporal upregulation during heart development. To test if the ECMs have impacts on CM maturation, I examined the dose-dependencies of the ECMs using Myom2-RFP reporter line. To this end, I plated Myom2-RFP PSC-CMs at day 10 of differentiation on different concentrations of ECMs ranging from 0.125 μg/cm$^2$ - 1 μg/cm$^2$, and cultured up to day 38 of cell culture. I found that high concentrations of the ECMs, especially laminin-511/521, significantly increased Myom2-RFP expression. In addition, morphological, physiological, and functional analysis demonstrated that laminin-511/521 treatments promoted PSC-CMs towards adult-like CMs such as long sarcomere length, increase cell size, increase percent of binuclear cell, inducing connexin 43 to lateral cell-axis, improving mitochondrial function, as well as improving calcium handling and cell shortening properties.

Next, I performed an RNA sequencing to assess maturation statuses of the PSC-CMs treated with laminin-511/521. The transcriptome of the treated PSC-CMs were compared to mouse heart counterparts. The result revealed that PSC-CMs plated on laminin-511 at day 38 of cell culture had the highest maturation score compared to gelatin and laminin-521. Moreover, specific genes related to CMs were slightly upregulated in laminin-511/521 treatments such as cardiac marker (*Tnnt2*), sarcomere genes (*Actc1*, *Mybpc2*, *Mybpc3*, *Myh7*, *Myl2*), transcriptional regulator (*Ankrd23*), and calcium handling (*Casq2*).

**4　考察**

In this study, I successfully developed assessment methods for CM maturation. Using these methods, I could identify laminin-511/521 as enhancers for CM maturation *in vitro*. Although PSC-CMs displayed more mature phenotypes on laminin-511/521 treatments, these cells were still immature compared to adult CMs. Combinations of ECMs and other maturation strategies are required to enhance the maturity of PSC-CMs similar to adult CMs.

For molecular mechanism, integrin-binding ECMs are able to activate several intracellular signaling cascades, which relate to cell survival, proliferation, motility, and differentiation. Previous study has shown that the interactions between laminin-511/521 E8 fragments are primarily α6β1 integrin-dependent. Thus, I believe that the interactions of laminin-511/521 with α6β1 integrin are the important route for cell-ECM binding and enhancing PSC-CMs maturation.

**5　結論**

Laminin-511/521 promoted morphological, physiological, functional, and transcriptional changes of PSC-CMs, and also enhanced Myom2-RFP expression. Therefore, this study highlight laminin-511/521 as potent enhancers for CM maturation.

## 論文審査の結果の要旨

多能性幹細胞から心筋細胞を作製することは循環器領域の再生医療の進歩に必須である。しかしながら現在のところ、成熟心筋段階まで分化を誘導することができず、臨床応用に当たって大きな課題となっている。

本研究において申請者らは、心筋細胞の分化段階を簡便かつ定量的にモニターできる方法を確立し、心筋細胞を成熟させる因子をスクリーニングした。さらにスクリーニングで同定された因子がマウス ES 細胞由来の心筋細胞において分化を促進させることを確認した。

１）心筋細胞の分化段階のモニター法の確立：胎仔型から成体型心筋細胞に分化する際に発現が特異的に上昇するサルコメア分子 Myom2 に着目し、Myom2 遺伝子の 3’末端に RFP を knock-in した reporter line をマウス ES 細胞（mESC）より樹立した。また正常心筋細胞の RNA-seq データを元に心筋細胞の分化段階を遺伝子発現プロファイルによってスコアリングする系を確立した。この系を用いると、Myom2-RFP reporter mESC line において RFP が陽性となるタイミングは、正常心筋における P10 stage に相当することが示された。

２）心筋細胞成熟因子のスクリーニング：Myom2-RFP reporter line を心形成に伴って発現が上昇する ECM タンパク質 15 種類の存在下に培養し、心筋分化の促進を RFP 発現にてモニタリングした。その結果、Laminin-511/521 が最も RFP 発現を増強することが明らかになった。

３）Laminin-511/521 によるマウス ES 細胞由来心筋細胞の分化促進：マウス ES 細胞由来心筋細胞を Laminin-511/521 の存在下に培養し、分化スコアが対照と比べて有意に上昇することを確認した。この際、実際に発現が上昇する因子として、心筋特異的遺伝子・サルコメア遺伝子・心筋細胞においてカルシウムのバッファーとして働く因子等が認められた。

本研究は循環器領域の再生医療の進歩に貢献する可能性の大きい有意義なものである。英文論

文はすでに査読付きの英文国際誌 Scientific Reports に投稿されており、revision 中である。学位論文も非常に丁寧に作成されており、現状のまま合格とした。

## 最終試験の結果の要旨

最終審査会に際しては、論文の内容について明快にプレゼンテーションがなされた。

審査委員からの質問として、１）今後の方向性とくに臨床応用を最終目標としたロードマップの提示、２）Laminin がどのようなシグナル伝達を介して心筋細胞の分化を促進するのか、３）心筋分化における細胞接着の意義についてなどが挙げられた。

申請者は以上の質問に適切に返答した。関連する過去の知見にも精通し、研究者として充分な資質・能力を有していると考えられた。

以上の観点から、申請者は学位に値する学識が備わっていると判断し、審査委員全員の合意をもって最終試験に合格とした。