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学 位 論 文 題 目	Characterization and evolutionary inference of cold-shock domain proteins enhancing the reprogramming of differentiated gametophore leaf cells to chloronema apical stem cells in the moss *Physcomitrella patens*
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(別紙様式 2)
(Separate Form 2)

論文の要旨
Summary (Abstract) of doctoral thesis contents

Both plants and animals have the capacity to reprogram differentiated cells to stem cells. In mammals, the induction of four factors is sufficient to reprogram somatic cells to pluripotent stem cells. Oct4, Sox2, cMyc, and Klf4 were first reported as induced pluripotent stem cell (iPSC) factors able to reprogram mouse fibroblast cells into pluripotent stem cells. Later, the application of the same factors to human fibroblast cells also induced pluripotency. Meanwhile, another set of iPSC factors Oct4, Sox2, Nanog, and Lin28 was identified. Although several factors involved in reprogramming have also been reported in land plant lineages, no common factor has been identified between the land plant and metazoan lineages.

To analyze the molecular mechanisms of reprogramming in land plants, the moss *Physcomitrella patens* (Physcomitrella) was chosen as a useful model for its remarkable reprogramming ability. When a differentiated leaf is excised from the gametophore and cultivated on a medium without phytohormones, leaf cells facing the cut change to chloronema apical stem cells with tip growth and divide approximately 30 hours after excision. The transition of differentiated leaf cells to chloronema apical stem cells enables researchers to investigate reprogramming processes at the cellular level. In addition, the sequenced genome and the high efficiency for gene targeting enable reverse-genetics approaches to investigate gene functions.

Since multicellularity with stem-cell systems has evolved independently in land plant and metazoan lineages, molecular mechanisms underlying reprogramming were thought to be different between these lineages. Unexpectedly, it was found that *Physcomitrella patens* Cold-Shock Domain Protein 1 (PpCSP1), which shares highest sequence similarity and domain structure with the iPSC factor Lin28 in mammals, regulates reprogramming of differentiated leaf cells to chloronema apical stem cells. CSPs were first identified in bacteria as proteins expressed under cold shock conditions, and were later implicated in the process of cold acclimation in flowering plants as CSP transcripts accumulate after cold treatment in *Arabidopsis thaliana* and wheat. PpCSP1 contains a Cold Shock Domain (CSD) and two CCHC zinc-finger domains, which are capable of binding to single-stranded DNA and RNA as well as to double-stranded DNA.

Firstly, Lin28 was shown to be an evolutionarily related protein of PpCSP1 in metazoa based on the phylogenetic analysis. Then, a dual reporter line to detect *PpCSP1* promoter activity and PpCSP1 protein amounts was established to examine the spatiotemporal expression pattern of PpCSP1 in the cut leaves at a single-cell level. The coding sequence of *Citrine*, a modified yellow fluorescent protein, was inserted at the C terminal of native *PpCSP1* gene to produce a fluorescent fusion protein. Subsequently, the coding sequence of *luciferase* was driven by a 1.8-kb *PpCSP1* promoter and integrated into a

neutral site in PpCSP1-Citirne protein fusion background to detect transcription activity as well as the fusion protein level. PpCSP1 transcripts and proteins accumulated in the reprogramming cells and were maintained throughout the reprogramming process and in the resultant stem cells. However, the inconsistency between the promoter activity and protein levels was detected, suggesting the potential involvement of post-transcriptional regulation of *PpCSP1*. Next, the expression of *PpCSP1* was found to be negatively regulated by its 3' untranslated region (UTR). Removal of the 3' UTR stabilized *PpCSP1* transcripts, resulting in elevated levels of *PpCSP1* transcripts and proteins, as well as enhanced reprogramming. A quadruple deletion mutant of *PpCSP1* and three closely related *PpCSP* genes exhibited attenuated reprogramming, indicating that *PpCSP* genes function redundantly in cellular reprogramming. Taken together, these data demonstrate a positive role of PpCSP1 in reprogramming, which is similar to the function of mammalian Lin28.

PpCSP1 functions in Physcomitrella in a similar manner as its mammalian closely related protein Lin28, suggesting that PpCSP1/Lin28 is a common factor controlling stem cell formation in land plants and metazoa. Both *PpCSP1* and *Lin28* are dispensable for reprogramming, rather function in the enhancement of reprogramming. Lin28 is dispensable for iPSC formation and promotes the maturation of iPSCs, although Lin28 participates in the effective iPSC reprogramming from human fibroblast cell. In the *ppcsp* quadruple deletion line of Physcomitrella, reprogramming was attenuated in edge cells but was not completely arrested, and non-edge cells were reprogrammed in the PpCSP1 transcript-increased lines. However, the molecular mechanisms underlying *PpCSP1* and *Lin28* regulation appear to be different. Lin28 binds to precursors of microRNA *let-7* and inhibits its processing, while *let-7* leads to the degradation of *Lin28* transcripts. Therefore, this negative feedback loop functions as a bistable switch to regulate cell fate. Although *PpCSP1* transcripts were regulated by its 3' UTR, microRNA binding sites were not found in this region and *let-7* homologs were not found in the Physcomitrella genome. Furthermore, the degradation of *PpCSP1* transcripts was not specific to the differentiated cells. The activation of the *PpCSP1* promoter in the reprogramming cells resulted in the increase of *PpCSP1* transcripts. Even though the different regulatory mechanisms at the 3' UTR, future studies are warranted to investigate both the PpCSP1 and Lin28 regulatory networks in order to find molecular mechanisms underlying the common positive reprogramming functions between PpCSP1 and Lin28.

(別紙様式 3)
(Separate Form 3)

博士論文審査結果の要旨

Summary of the results of the doctoral thesis screening

幹細胞は細胞分裂によって、自分自身と同じ細胞と、自分自身とは異なった性質を持つ細胞を作り出す能力を持った細胞である。幹細胞から生じた細胞は、特有の性質を持った細胞へと分化する。植物の茎や根の先端には幹細胞があり、自分自身を維持しながら、茎や葉、根になる分化細胞を作り出す。一方、分化した細胞をもう一度幹細胞に戻すことが可能である。哺乳類では、近年可能になった誘導多能性幹細胞（iPS 細胞）が一例である。他方、植物は容易に葉挿しや挿し木ができ、動物よりも分化細胞が幹細胞化する能力が高いことが知られている。これまで動物と植物の幹細胞形成研究は独立に進んできた。その理由は共通の遺伝子や分子機構が見つからなかったからである。しかし、生物全般における幹細胞形成の共通原理が存在するのか、するならどのようなものかを明らかにするためには、動物と植物の共通点を探すことが求められていた。

Li, Chen 氏は植物の低温ショックドメインタンパク質遺伝子機能解析に興味を持ち、陸上植物の中でもとりわけ幹細胞化能が高いコケ植物ヒメツリガネゴケ *Physcomitrella patens*を用い、相同組換え実験系により *Physcomitrella patens Cold Shock Domain Protein 1 (PpCSP1)*遺伝子末端に *Citrine* 遺伝子を導入することで、*PpCSP1* 局在を調べた。その結果、発生過程で生じる原糸体幹細胞や原糸体側枝始原幹細胞、茎葉体の葉細胞が原糸体幹細胞へ変化する過程で、幹細胞化する細胞特異的に PpCSP1 タンパク質が蓄積することを明らかにした。また、mRNA の 3’非翻訳領域によって *PpCSP1* 伝令 RNA 量は負に制御されており、3’領域を取り除くと PpCSP1 タンパク質量が増加するとともに幹細胞化が促進されることを解明した。そして、*PpCSP1* とそれに近縁な３つの遺伝子の合計４つの遺伝子を欠失させると、幹細胞化が遅延することを明らかにした。

さらに、遺伝子系統解析の結果、*PpCSP1* 遺伝子は哺乳類の幹細胞化を誘導する iPS 因子の一つである Lin28 に最も近縁であることを明らかにした。*Lin28* は *let-7* などのマイクロ RNA によって制御されていることが知られているが、これらのマイクロ RNA はヒメツリガネゴケゲノムには見つからなかった。また、3’末端非翻訳領域の段階的欠失や *in silico* 解析から、特定のマイクロ RNA ターゲットサイトは見つからなかった。このことから、Li 氏は *PpCSP1/Lin28* は動植物共通の幹細胞誘導因子であるが、制御機構は異なっていると考察した。*PpCSP1* の 3’末端非翻訳領域がどのような分子機構で *PpCSP1* を分解するのかはわかっておらず、今後、*PpCSP1* がどのような遺伝子を制御するかを調べることによって、*PpCSP1* と *Lin28* に幹細胞化の制御機構について共通性があるのかどうかが明らかとなり、生物に共通の幹細胞化機構の解明への糸口が見いだせることが期待できる。以上より、本研究は学位授与に相応しいと審査委員全員が一致して判断した。