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学位（専攻分野）	博士(理学)
学位記番号	総研大甲第682号
学位授与の日付	平成１５年３月２４日
学位授与の要件	生命科学研究科　遺伝学専攻 学位規則第４条第１項該当
学位論文題目	Studies on molecular mechanisms underlying early mesoderm and endoderm specification in zebrafish.
論文審査委員	主　査　教授　相賀　裕美子 教授　桂　勲 助教授　平田　たつみ 助教授　川上　浩一（国立遺伝学研究所） 助教授　今井　義幸（東京工業大学）

The formation of the vertebrate body plan begins with the segregation of undifferentiated mass of cells into three germ layers: ectoderm, mesoderm and endoderm. The understanding of molecular mechanisms underlying germ layer formation is important not only for developmental biology, but also for regenerative tissue engineering in the future.

In zebrafish, mesoderm and endoderm form from the margin of the blastoderm as the mesendoderm, a mixture of cells that fated to mesoderm and/or endoderm. Previous experiments demonstrated that the extra-embryonic yolk cell, especially associating yolk syncytial layer (YSL), is necessary and sufficient for zebrafish mesendoderm formation.
In addition, the YSL is known to be a signaling center that required for many developmental events such as induction of dorsal organizer and epiboly cell movement. However, molecular processes underlying these inducing activities remained unclear. To understand them, I have isolated novel genes expressed in the YSL and identified the genes responsible for mesendoderm formation.

To efficiently isolate genes, I have developed an original *in situ* hybridization screening method in which all the procedures were done on 96-well plates. Then I constructed a subtracted cDNA library in which yolk-cell specific genes were enriched. Evaluation of the subtraction step revealed that the subtraction worked as expected. Therefore, I picked up about 600 clones from this library and screened them by their expression using the *in situ* hybridization screening method. I successfully obtained nearly 80 clones showing the YSL specific expression at the blastula stage. Clustering analysis based on their sequences demonstrated that these clones were classified into 33 independent clusters. Blast similarity analysis revealed that there are 6 previously reported YSL genes in the obtained clusters, indicating that my screening strategy worked well. Since I used 4-base-recognition restriction enzyme to make a subtraction cDNA library, these obtained clusters are mainly cDNA fragments that do not contain full-length cDNAs. Therefore, it is not clear how many genes are included in the obtained clusters. To verify this, I obtained their full-length cDNAs or 5'-terminal regions. Thus far, I have identified · 21 clusters that consist of at least 17 genes (Chapter 1).

Among isolated genes expressed in the YSL, I initially focused on a clone, 226D7 that encodes a novel sox transcriptional factor, because the first round of anti-sense experiments provided a drastic phenotype, a lack of endoderm-derived tissues. Predicted 226D7 protein shows high similarity to other sox proteins such as sox17. However, the HMG domain of 226D7 has three amino acids change in sox-signature region that is conserved in nearly all sox proteins reported. The result strongly supports that 226D7 is a novel sox transcription factor, related to sox17. 226D7 gene starts to express in the YSL at the blastula stage, and the positive region extends from the YSL to presumptive endoderm cells located near the blastoderm margin at the gastrula stage. Since Nodal signaling is required for endoderm formation, I examined the relationship between Nodal signal and 226D7 expression by examining

226D7 expression in both Nodal signal up-regulated and down-regulated embryos. Then, I found that 226D7 expression in endoderm cells is positively regulated by Nodal signaling. Next I performed loss-of-function experiment by injecting morpholino antisense oligonucleotids, and found that loss of 226D7 function resulted in a lack of the endoderm regions. The earliest endoderm molecular marker, sox17, was completely lost in injected embryos, indicating that 226D7 is required for the initial step of entire endoderm development. I expected from these results that augmentation of 226D7 function could also affect endoderm development. I found that early endoderm region drastically expanded after226D7 RNAs injection. Furthermore, 226D7 was able to induce endoderm marker in the absence of Nodal signal, indicating that 226D7 acts downstream of Nodal signal and is sufficient for endoderm formation. In brief, I revealed that the novel sox gene, 226D7, is a key player in zebrafish endoderm formation, acting downstream of Nodal signal (Chapter 2).

During my *in situ* hybridization screening, I happened to isolate 5 clones showing region-specific expression but not in the YSL. 3 clones out of 5 were expressed in the shield, equivalent to Spemann's organizer in zebrafish, at the early gastrula stage. Among them, I focused on a 109G3 clone encoding a novel BTG/Tob family gene. 109G3 gene, designated as zebrafish *btg-b*, is expressed in the shield at gastrula stage, and in the polster, hindbrain region and somites during segmentation stages. BTG family is thought to be a tumor suppressor and involved in regulation of cell proliferation. Murine BTG1 and BTG2 were recently shown to be cofactors for HoxB9. In this study, I demonstrated that zebrafish *btg-b* gene were co-expressed with zebrafish *hoxb9a* in the posterior trunk region at somite forming stages by a precise expression analysis. These results indicated the possibility that btg/hox complex works in this region (Chaptor3).

論文の審査結果の要旨

脊椎動物の発生過程において、中胚葉、内胚葉の形成機構は重要な問題である。ゼブラフィッシュでは、これらの組織は内中胚葉と呼ばれる共通の前駆細胞が卵黄細胞からの誘導を受けて分化すると考えられているが、その誘導現象に関わる分子機構、また内胚葉と中胚葉の分化の機構に関してはほとんど理解されていない。そこで、坂口君はこれらの現象に関わる重要な遺伝子が卵黄細胞に発現していると考え、卵黄細胞特異的な遺伝子の同定を試みた。まず cDNA ライブラリーを作成し、サブトラクション法により、特異的遺伝子を濃縮した後、whole mount in situ hybridization を用いた独自のスクリーニング法を開発し、遺伝子の発現スクリーニングを行った。その結果、スクリーニングした 576 個の遺伝子のうち、34 個の特異的発現を示す独立クローンが得られ、このなかには少なくとも 16 個の独立した遺伝子を含むことを示した。その中から、特に興味深い発現パターンを示した遺伝子 226D7 に関して詳細な機能解析を行った。226D7 は Sox ファミリーに属する新規の遺伝子で、卵黄多核層及び内胚葉前駆細胞に発現しており、この遺伝子の発現は Nodal シグナルにより、正の制御をうけていた。坂口君は Morpholino アンチセンスオリゴを用いたノックダウン法を用いてこの遺伝子の機能解析を行った。その結果、この遺伝子の機能阻害が内胚葉の分化をほぼ完全に抑制し、その結果、発生した個体は腸管を欠損しており、この遺伝子が内胚葉細胞の分化に重要な働きを担うことを示した。また、この遺伝子の過剰発現は、Nodal シグナルを誘導せずに予定内胚葉を増加させることから、より直接的に内胚葉の分化に関与することを示した。以上のように、この論文の内容は、内胚葉の初期分化に非常に重要な機能をもつ遺伝子の同定につながったものであり、学位に充分ふさわしい内容と判断できる。

博士論文審査に関わる公開発表会の後に、審査委員と坂口君の間で質疑応答がなされた。坂口君は博士論文に関わる研究分野に関して充分な知識をもっており、その知識に基づいて考察する能力を持つことが示された。またこの学位論文は英語で記載されており、さらにこの内容がすでに国際誌に掲載されていることから英語の能力にも問題なく、審査員全員一致で学位にふさわしいと判断した。