Induction of mESCs into Hepatic Stem Cells by using Embryonic Chicken Hearts

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Background: *Many researchers have been trying different methods for obtaining stem cells. Some studies have failed due to the growth of a tumor after stem cells transplantation. Several successful tries for getting stem cells or stem cell like cells: direct isolation from tissue, direct isolation from blood or fluids, iPS cells, small molecules induced stem cells. However, none have used real organ stimulation in the induction of a specific stem cell lineage.*
Objective: *To induce a lineage specific hepatic stem cell using isolated embryonic organs.*
Material and Method: *The embryonic stem cells were cultured through confluence. After observing several colonies formations, we put freshly isolated chicken embryonic hearts onto the colonies. After, at least, four days, we started looking for hepatic plate-like formations.*
Results: *After several trials, we found that the chicken embryonic hearts, on day 4, could actually induce a hepatic cell fate for the mouse embryonic stem cells. We were able to show specific marker for early hepatic lineage such as the production of Albumin, AFP. When these cells were tested for a hepatocyte function, we found glycogen formation inside the cells.*
Conclusion: *Isolated early embryonic chicken hearts are acceptable for inducing embryonic stem cells into the hepatic stem cell lineage.*

Keywords: *Mouse embryonic stem cells, Hepatic stem cells, Early embryonic chicken heart*

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Until early September 2014, the first human stem cell-based therapy had been tried with the so-called induced pluripotent stem (iPS) cells, which were prepared from the patient's own cell[1,2]. Due to ethical issues, the field of stem cell applications has been halted for quite some time by regulations, religious beliefs, personal commitments, and so forth[3,4]. The only stem cells that can be used for medical applications are the inner cells derived from discarded embryos resulting from in vitro fertilization (IVF)[5-8]. With such restrictions, the limitations on stem cells necessitated more and better ways of preparing stem cells. At an earlier time, many scientists had tried to isolate directly from the bone marrow and some had tried to isolate stem cells from the blood[9-11].

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However, several studies revealed the difficulties as an extremely low number of stem cells had been isolated[10,11]. Furthermore, there is a controversy about whether those cells are really "true stem cells", will these cells actually work[12], and how they incorporate into the right microenvironment (Homing). Later, many markers had been introduced into the field such as Oct4, NANOG, SOX2, KLF4, CD34, LIF1, TRA-1 60, TRA-1-81, SSEA-1, and others[13-15]. Then, the field was stirred up by the unexpected success of iPS cells developed by Takahashi et al[16-18]. Still, the important question remained, how to solve the tumor development after stem cell transplantation[19-23]. The unknown mechanism underneath this phenomenon needs more time for further investigation. In the meantime, it is worth working toward getting proper stem cells, particularly lineage specific stem cells that may give us some clues to unveil the answers.

Researchers have been trying many paths in an attempt to get cells that have stem cell like properties; such as the self-renewal capability and unlimited cell

fate differentiation[24-27]. Many studies had successfully recapitulated the body generating of several stem cell lineages, fat cells, neuronal cells, pancreatic cells, hepatic cells, and others[28-32]. However, these studies could not answer if these inductions were truly the same as in vivo. We wanted to try inducing a specific stem cell lineage that possesses property as close as possible to the physiological condition of in vivo stem cells. We believe that the closer the induced stem cells get close to physiological condition, the better the likelihood of having no tumor formation. Therefore, we want to try a partial in vivo experiment for inducing hepatic stem cells. In early studies, it was found that during hepatogenesis the important piece of information regarding the earliest hepatic induction was a specific signal from the lower part of the early embryonic heart, the so-called septum transversum[33-35]. We may receive a similar induction if we mimic the process by using an isolated embryonic heart to stimulate pluripotent stem cells in vitro yielding a specific hepatic stem cell lineage.

Material and Method

Isolation of embryonic chicken hearts

The committee for animal use in research has approved this study before the beginning of the processes. Dr. Suksaweang is an expert who has had good experience in using chicken embryos in his earlier studies. Chicken eggs were incubated at 37.5 degree Celsius for four days in the humidified incubator with rocking every 2 to 4 hours. The eggs were removed from the incubator and placed into a clean laminar flow hood before spraying 70% alcohol onto the shells and left for about five minutes to kill any possible contamination microorganisms. The eggshells were opened from the top where the embryos had developed (See A). The embryos day 4 (E4) were removed and the hearts were isolated before putting them onto the mouse embryonic stem cells (mES cells) (See B and C). By soaking the isolated hearts in an MEM medium (Sigma) for 5 to 10 minutes, the remaining blood cells were washed away. Then, the isolated chicken hearts (H) were kept in cold MEM until use. The above steps were all executed in the clean laminar flow hood. See Fig. 1.

Culture of mouse embryonic stem cell

About two weeks before the start of the experiment, mESC cells stored in liquid nitrogen were thawed and the viable cells were counted. Then, the $10x10^{12}$ cells were placed into the dish containing mouse embryonic stem cells (mESCs) and a culture medium, as previously described (Ye, 2011). Briefly, the culture medium included mESCs (mESCs-SUT-1, strain C57BL/6) (Tanthanuch et al., 2010), DMEM (Invitrogen) supplemented with 10% FBS (Hyclone, Logan, UT, USA), 200mM l-glutamine (In vitrogen), 0.1mM mercaptoethanol (Sigma), 1x minimum essential amino acid (Invitrogen), 1x penicillin/streptomycin (Invitrogen) and 1000 IU/ml hLIF (Chemicon), on feeder free gelatin coated plates maintaining their pluripotency. Subsequently, mESCs colonies with ES cell morphology were selected for subculture and maintained by standard methodology. After one passage, the chicken embryonic hearts were isolated freshly and put onto the mESCs, with at least 3 or 4 hearts per dish and incubation was continued for 3, 4, 5, 6, 7, and 11 days (see Fig. 2).

Characterization of the induced hepatic stem cells

A. Morphology: Cell morphology was observed under a light microscope at low magnification (4x) and high magnification (20x) looking for hepatic plate like cells. Some cells with big two nuclei in one cell may be present, which is one specific characteristic of hepatocyte.

B. Immuno-fluorescent staining: The immunocytochemistry was done with specific antibodies for Albumin (1:500; sigma goat anti rabbit), Oct-4 (1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Nanog (1:50; Santa Cruz Biotechnology), and SSEA-1 (1:50; Chemicon) and a commercially available kit for detecting alkaline phosphatase (Vector Laboratories, Burlingame, CA, USA), following the method of Lorthongpanich et al (2008).

C. PAS staining: The staining for glycogen storage properties of hepatocytes were done as previously described (Suksaweang et al, 2004). Briefly, the cell culture was removed and washed three times with PBS before fixing with cold 4% paraformaldehyde for 5 minutes. Rinse with tap water for 1 minute and continue with recommended protocol from Sigma as described in details at this website, http://www.sigmaaldrich.com/catalog/product/sigma/395b?lang=en&region=US

Results

The embryonic chicken hearts of day 4 were successfully isolated (Fig. 2) as described in material and method section. The embryonic chicken hearts were dissected and transferred into the clean dish until use.

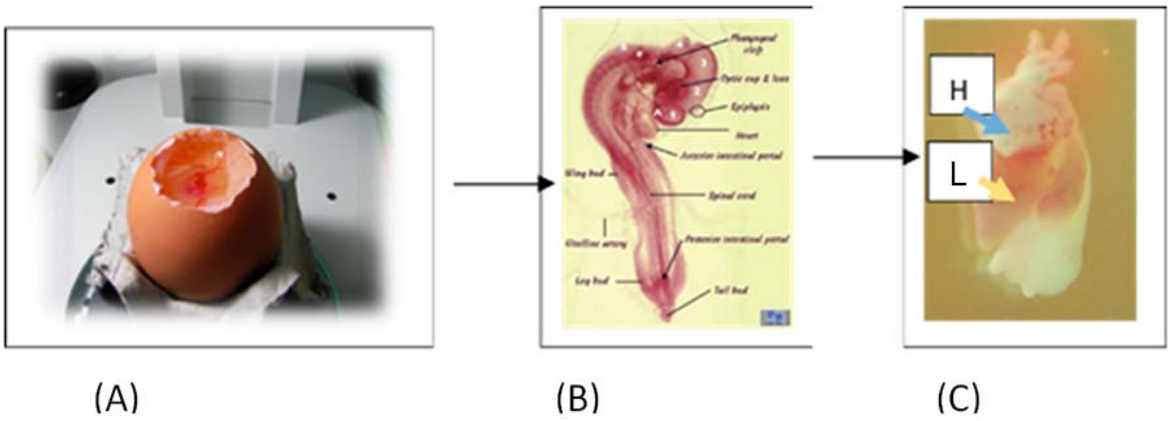

Fig. 1 The chicken embryo model.
We were using HH staging as the standard method for choosing embryo appropriate embryo. (A) Chicken embryonic day 4, (B) An embryo day 3 at a closer look, (C) Isolated heart [H].

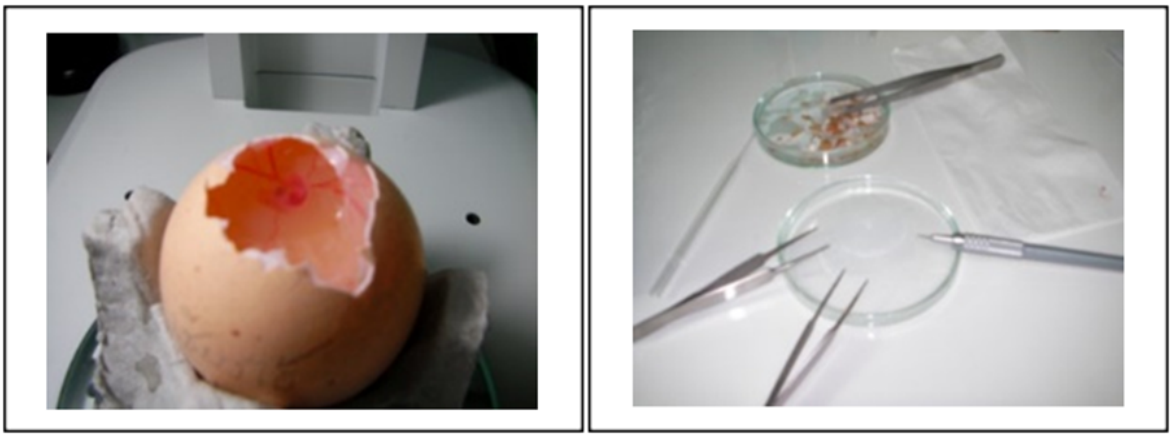

Fig. 2 The isolation of embryonic chicken heart.
The eggshell was opened and the embryo was scooped out by a spatula before put into the PBS. The amniotic sac was cut and the embryonic heart was dissected with sharp forceps. The whitish muscle beating heart was submerged into cold DMEM until use.

The fertilized chicken eggs from the Suranaree University of Technology Farm were purchased and incubated at 37.5 degree Celsius in the humidified incubator with rocking every four hours. The eggs were removed and sprayed with 70% alcohol before being put under the hood. The eggshells of the desired eggs were opened and the embryos were removed into a petri dish filled with PBS. The embryonic chicken hearts were then dissected and transferred into the clean dish until use as seen in Fig. 2.

Embryonic chicken hearts (Dark shadow in Fig. 3B and 3D) induced differentiation of mouse embryonic stem cells (mESC) to hepatic cell lineage.

As presented in Fig. 3A, the embryonic stem cells will form colony after 14 days of induction. These cells differentiated quickly to become hepatic cord like cells in the absence of LIF (Fig. 3D; see also hepatic cord in chicken liver, Fig. 3G). The monitoring of cells proliferation and migration were observed by using Q-track fluorescent dye staining (Fig. 3E and 3F). The intensity of the fluorescent dye decreased as the cell number increased.

The omission of LIF has been repeated to assure the consistency. As shown in Fig. 4, the omission of the LIF resulted in cell differentiation greatly (Fig. 4A and 4D) while keeping LIF could prolong stem cell colony a little longer (Fig. 4B and 4E). The isolated hearts could, some degree, help stem cells stay intact a little further as well (Fig. 4C and 4F).

The embryonic stem cells could be kept for

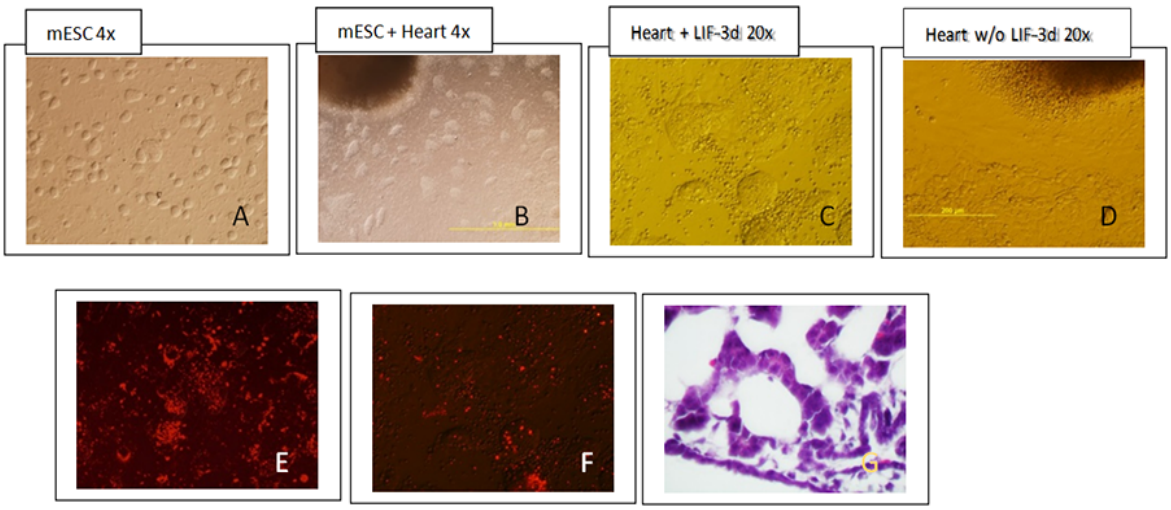

Fig. 3 Embryonic chicken hearts induced differentiation of mESC.
The hepatic stem cells were induced by the specific factors released from the chicken embryonic heart. The round stem cell colony was exposed to those factors similar in the in vivo condition.

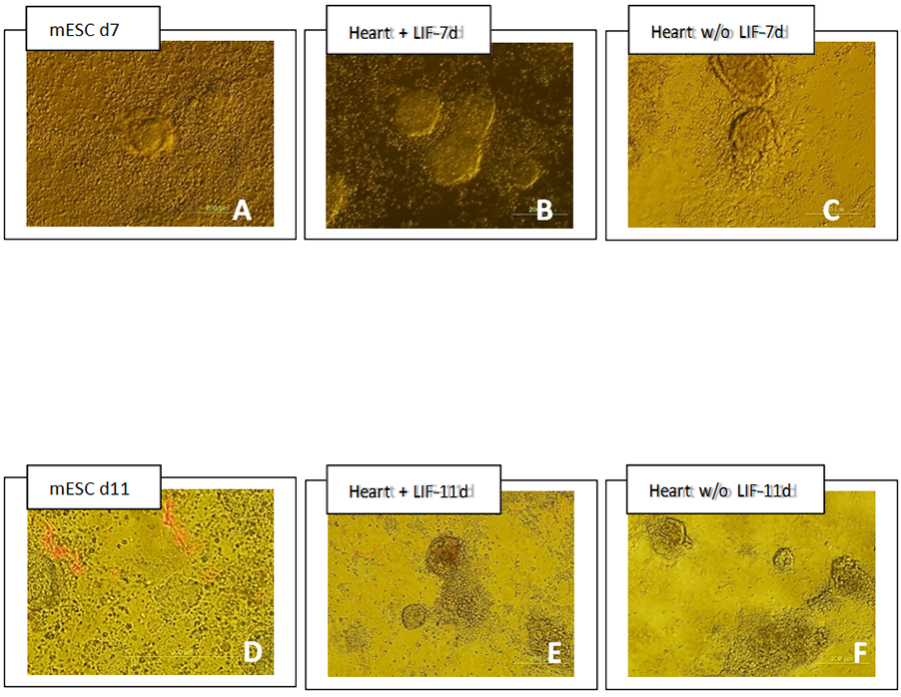

Fig. 4 The differentiation of mESC at 1 and 2 weeks.

several days before the colony differentiated into embryoid body, which some parts of this body developed into cardiac tissue with beating cells (Fig. 6A). After adding embryonic heart into this culture, the embryoid body attached to the surface and showed somewhat hepatic likes structure (Fig. 6C) compared to stem cell colony in (Fig. 6B). However, these cells failed to form three-dimensional structure of hepatic cords (Fig. 6E), which is possible only when the endothelial cells are present. Several possible signals may play a crucial role during such sophisticated histological structure (Fig. 6D).

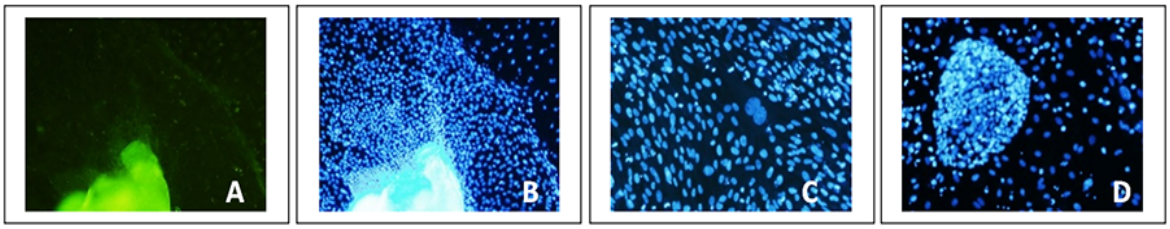

Fig. 5 Some features of cardiac cells and hepatic cells.
Some cardiac cells attached to the surface and slowly spreaded outward (Fig. 5A and 5B). The hepatic cell lineage displayed two nuclei (Fig. 5C) or just big single nucleus (Fig. 5D) in some cells.

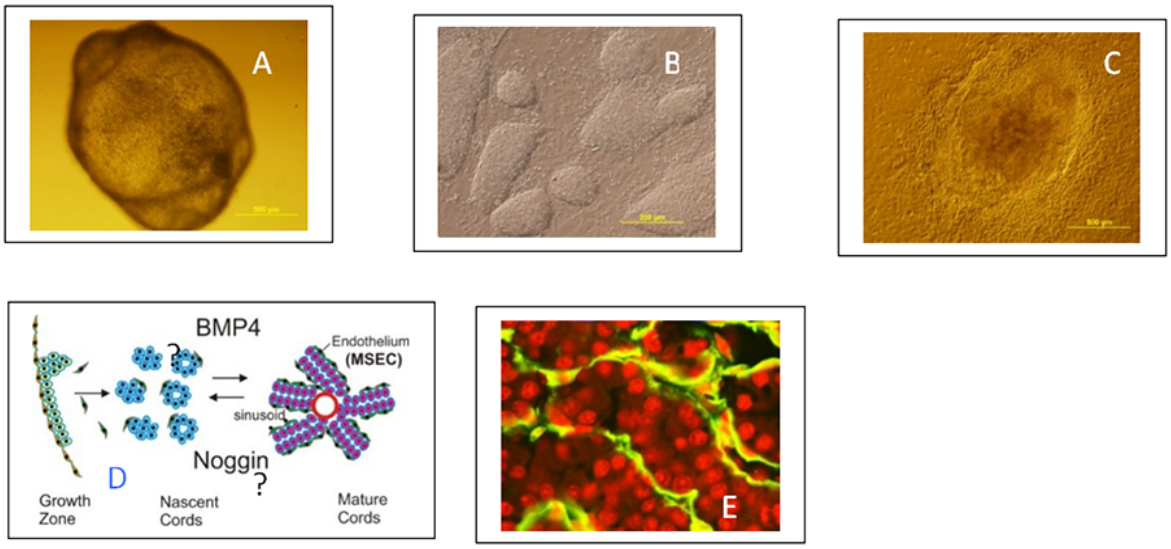

Fig. 6 The embroid body and three dimentional structure of the liver cells.

Table 1. Differentiation study

Experiment	Morphology	Anti-albumin	Glycogen storage (PAS)
mES + LIF	Undifferentiated	Negative	Negative
mES + CH	Differentiated	Positive	Positive
mES + FBS	Differentiated	Negative	Negative

In this study, hepatic stem cell lineage was identified using PAS and immunofluorescent stainings. Table 1 shows the results of mES differentiation to become a hepatic stem cell lineage (Anti-albumin positive), capable of function as liver cells (Glycogen positive) once the cells were exposed to the embryonic chicken hearts.

Discussion

As mentioned earlier that many ways had been applied in order to induce embryonic stem cells into several different cell types. Few have been used for specific hepatocytes induction such as Shiraki et al has successfully used specific number of factors that can induce embryonic stem cells into endodermal cell fate including pancreatic cells[38]. In another study, Pei et al had used embryoid body (EB) as the starting point before stimulating with sequential factors and coculture with liver stromal cells, in which the endothelial cells were also included in the system. This system was able to show the characteristic of hepatic cord in zone 1 of the liver[39]. However, there is no proving whether those

cells were from the embryonic stem cells or from the stromal cells. Later, Shiraki's group has further found a component of basement membrane, laminin 5, that is important for embryonic stem cells lineage specification[40]. Other had used growth factor, meaning that containing surface could also induce cell lineage[41] and alike. Mouse iPS cells were recently on the try as well[42]. In our study, which very much mimic that study, in vivo induction was done by using the entire isolated early embryonic chicken heart to give the right combination of physical inducers and inhibitors to stimulate mouse embryonic stem cells into hepatic cell lineage successfully. If this were the case, then patients who are waiting for liver transplantation would benefit tremendously from liver stem cells therapy soon.

Conclusion

Taken together, we found that the isolated early chicken embryonic hearts can induce mouse embryonic stem cells into a hepatic stem cells lineage in vitro. Therefore, we would like to claim that this is the first time we can mimic phenomenon in vivo in term of specific induction during hepatogenesis. In addition, this is one of the methods that one can use to prepare hepatic stem cells lineage in the future.

What is already known on this topic?

There are several ways of inducing stem cells successfully. However, no one ever used a real organ to induce specific stem cell lineage.

What this study adds?

This study is the first to use real organ induction property in inducing a hepatic stem cell lineage.

Acknowledgements

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Potential conflicts of interest

None.

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การเหนี่ยวนำเซลล์ต้นกำเนิดสายพันธุ์ตับด้วยหัวใจของเอมบริโอไก่

สนอง สุขแสวง, แดนนา เย, รังสรรค์ พาลพ่าย

ภูมิหลัง: หลังจากที่เราสามารถจำลองสิ่งมีชีวิตขึ้นมาใหม่ได้ความเจริญก้าวหน้าทางการแพทย์อย่างหนึ่งที่กำลังได้รับความสนใจอย่างมากคือเทคโนโลยีด้านเซลล์ต้นกำเนิด เมื่อประมาณสามปีที่ผ่านมานักวิทยาศาสตร์ชาวญี่ปุ่นสามารถทำการใช้เซลล์ต้นกำเนิดของตนเองเพื่อรักษาโรคในมนุษย์เป็นครั้งแรกแต่เพียงอยู่ระหว่างการรอผล ซึ่งมีนักวิทยาศาสตร์ได้พยายามหลากหลายวิธีในการเตรียมเซลล์ต้นกำเนิดหนึ่งในนั้นที่ได้รับรางวัลโนเบลก็คือการใช้เซลล์ที่เกิดจากการกระตุ้นด้วยสารจำนวนหนึ่งเรียกว่า ไอพีเอสเซลล์ (iPS) แต่ก็ยังมีอีกหลายคำถามที่เซลล์ชนิดนี้ไม่สามารถให้คำตอบได้ และบางการทดสอบได้เซลล์ต้นกำเนิดเซลล์มะเร็งแทน ดังนั้นจึงจำเป็นต้องคิดใหม่ว่ามีวิธีที่ดีกว่านี้หรือไม่ สิ่งที่เคยๆ ทำมาแล้ว อาทิ การแยกโดยตรงจากชิ้นส่วนเนื้อเยื่อ จากเลือด ไอพีเอส หรือการใช้สาร โมเลกุลเล็ก แต่ยังไม่เคยมีใครคิดใช้อวัยวะจริงทำการเหนี่ยวนำ

วัตถุประสงค์: การวิจัยนี้จึงอยากทำการเลียนแบบธรรมชาติโดยการใช้หัวใจระยะเอมบริโอในการเหนี่ยวนำ เซลล์ต้นกำเนิดสายพันธุ์ตับ

วัสดุและวิธีการ: จากความรู้ในการศึกษาก่อนหน้านี้ที่ตับจะเกิดขึ้นได้ต้องได้รับการกระตุ้นจากหัวใจในระยะตัวอ่อนเท่านั้น ซึ่งมีส่วนที่เรียกว่าเซปตั้มทรานซเวอซั่ม เป็นที่ปล่อยปัจจัยกระตุ้น เราจึงทำการแยกหัวใจไก่ในระยะเอมบริโอนี้มากระตุ้นเซลล์ต้นกำเนิดของหนูให้กลายเป็นเซลล์ต้นกำเนิดสายพันธุ์ตับ

ผลการศึกษา: พบว่าหัวใจของเอมบริโอไก่อายุ สี่ วันสามารถเหนี่ยวนำเซลล์ต้นกำเนิดหนูให้เป็นเซลล์ต้นกำเนิด สายพันธุ์ตับได้ โดยดูสารบ่งชี้การทำงานจำเพาะของเซลล์ตับได้แก่ อัลบูมิน และไกลโคเจน เป็นต้น

สรุป: เราพบว่าหัวใจไก่ระยะเอมบริโอสามารถกระตุ้นและเหนี่ยวนำเซลล์ต้นกำเนิดให้เป็นเซลล์ต้นกำเนิดตับได้