Scaffolding for human pluripotent stem cell lineage specification

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Scaffolding for Human Pluripotent Stem Cell Lineage Specification

An Undergraduate Honors College Thesis

in the

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College of Engineering
University of Arkansas
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by

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1-Introduction

1-1 Stem Cell Background

Stem cell research has been a booming topic in many laboratories. This is due to the unique properties that these stem cells possess (“Stem Cell Basics”). To begin, what are stem cells? These cells have the potential to develop into any kind of cell in the human body. The more specialized cells such as heart muscle cells, liver cells, pancreatic cells, and even nerve cells all begin as stem cells during the initial stages of growth. Stem cells also act as a repair mechanism to replace non-functioning cells in the body. For example, they are able to continually renew themselves during the later stages of growth to repair exhausted or damaged tissue such as red blood cells. Other stem cells are forever locked as the specialized cell they form into and can only divide under certain conditions (i.e. heart or pancreas cells) or not at all (i.e. nerve cells).

There are two types of stem cells: embryonic and adult stem cells. Embryonic stem cells (ES cells) originate from the inner cell mass of the blastocyst of early embryos. This inner mass consists of three body lineages: ectoderm, mesoderm, and endoderm. This is the stage where all the specialized cells originate from that give rise to an entire organism’s body as the stem cells transition or differentiate through each stage of growth. Adult stem cells or somatic stem cells are stem cells not originating from the germ layer, but comes from body. Although it is an undifferentiated cell, its primary role is to maintain and repair tissue where they are found. They still retain extensive regeneration potential throughout adult life (Czyz et al., 2003). For the majority of this paper, the main focus will be on embryonic stem cells.

1-2 Stem Cells’ in Pancreatic beta cell research
Before research was experimented with human stem cells due to moral complications, there was already extensive research done with mouse stem cells. It was not until 1981 that scientists found a way to derive mouse embryonic stem cells from early mouse embryos (“Stem cell basics”). Then in 1998, following this discovery in mouse stem cells, the same could be done with human embryonic stem cells when the National Institute of Health approved of Wicell Research Institute’s use and distribution of hESC (human embryonic stem cells). These hESCs are derived from the H9 stem cell line which is mainly used for research and clinical purposes (“Stem cell basics”). Many researchers are using the concept of induced differentiation of stem cells to create certain cells to replace the ones that are damaged in certain diseases. The main challenge is, however, controlling and guiding the pathways to induce differentiation of stem cells to the cell of choice.

A disease that is targeted by most stem cell researchers is diabetes mellitus. Diabetes patients have impaired insulin metabolism, lack the ability for its production in sufficient concentrations, have cells that do not respond to its signaling, or have its catabolism through the immune system. Impaired insulin metabolism prevents the body to regulate the levels of blood glucose. The insulin-producing cells in the pancreas are the pancreatic beta-cells found in the Islets of Langerhans. The production of pancreatic insulin-producing cells from hESCs has emerged as one of the most attractive cell therapy strategies for insulin deficient diabetes treatment (Tateishi et.al., 2008).

1.3 The Extracellular Matrix

Previous studies in mentor’s cell culture laboratory demonstrated that pancreatic differentiation of mouse and human embryonic stem cells could benefit from three dimensional (3D) cultures. These 3D cultures mimic the cellular environment in which hESCs grow and
function (Weber et al., 2008). These 3D cultures are also known as the extracellular matrix (ECM), providing support for cellular tissues as well as physical sites of cellular attachment. They not only provide structural support, but also provide signals capable of supporting appropriate cell differentiation and tissue development (Yang et al., 2010). The extracellular matrix is partly responsible for directing the stem cell to grow into whichever specialized cell the signal pathways lead. This extracellular matrix is what is experimented on as researchers develop protocols aimed at replacing damaged pancreatic beta cells with the new ones grown from stem cells.

There are still many unanswered questions about the complexity of the ECM-cell interactions. Some of the ECM proteins that are known specifically for pancreatic beta-cells are the following: collagen I, laminin, fibronectin, and vitronectin. Collagen I is mainly found in connective tissue and it functions as structural support for the cells. It is also the most abundant protein in the human body. Laminin is the basic component of basal membranes that provide a protein network foundation of many cells and organs. Fibronectin major functions are cell adhesion, growth, migration, and differentiation which are critical for embryonic development. Vitronectin promotes cell adhesion during the early stages of development and then it becomes absent in adult, mature tissues. It is known that in mature islet cells, these interactions have shown to regulate survival, secrete insulin, proliferate, and preserve the cell’s morphology (cell shape) in pancreatic beta cells. These functions are the target goal after successful guidance of embryonic stem cells to pancreatic beta cells.

1-4 Purpose

Previous experimental results in our lab have shown that the efficiency of induced hESCs differentiation to pancreatic lineage can be significantly enhanced when cells are cultured in a
collagen scaffold environment. Addition of multiple ECM protein, such as laminin, vitronectin, and fibronectin, to scaffolds lead to considerable promotion of the lineage-specific differentiation. Thus, it is critical to investigate the composition of ECM protein in the 3D scaffold. On the other hand, the definitive endoderm (DE) differentiation is the first step and also the most critical step of hESC pancreatic differentiation. Previous laboratory studies indicate that the ECM protein combination of collagen I (1.5 mg/mL), fibronectin (25 µg/mL), laminin (25 µg/mL), and vitronectin (5 µg/mL) can significantly enhance hESC differentiation into pancreatic lineages. Thus, the goal of this project is to not only investigate the effect of scaffold composition on DE tissue production but to also see if there is any deviation of the concentrations used from the previous set of ECM proteins. The project will provide knowledge on how soluble signals and physiochemical signals synergistically control the specification of hESCs. Investigation along this direction will lead to a robust and efficient hESC differentiation protocol for producing mature beta cells for cell-based diabetes therapy.

2-Materials and Methods:

2-1. Cell Culture and Passage

This experiment followed the protocol provided by Stemcell Technologies. All materials and reagents used were called by the protocol. The hESC line H9 (Wicell Research Institute) was maintained on Matrigel-coated plates in mTeSR1 medium and passaged every 3-5 days at a split ratio of 1:5. Medium changes were performed daily. Once the colonies were judged sufficiently large and beginning to merge, the cells were deemed ready for passage within a 24 hour time frame. Regions of differentiated cells were marked and then removed before the next passage. Undifferentiated H9 cells were seeded into the scaffolds and cultured in mTeSR1 medium at 37°C and 5% CO₂ incubator. This is prepared (after pre-coating the new plates with Matrigel) by
first detaching the undifferentiated cells using the enzyme dispase. DMEM/F-12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) solution was used as a wash to ensure the collection of all cells. The collection tube with the cell aggregate solution was then centrifuged at 300 x g for 5 minutes at room temperature (15-25°C). Cell aggregates were clumped at the bottom of the tube. The supernatant was then aspirated. mTeSR1 medium was added to the cell aggregates and is then transferred to the new Matrigel-coated plate.

2-2 Scaffold Preparation

The protocol used for collagen gel scaffold preparation was established by the laboratory. Rat tail Collagen Type I (Col I) hydrogels (1.5 mg/mL) were prepared by diluting Col I Stock Solution (3.54 mg/mL, company purchased from, where the company is located) with 10x phosphate buffered saline (PBS). The pH was adjusted using sterile NaOH to 7.4. Varied amounts of extracellular matrix proteins were interspersed in the Col I hydrogels. Fibronectin (FN), Laminin (LM), and Vitronectin (VN) were used. FN stock solution was added to the Col I hydrogel solution for a final concentration of 10, 25, or 50 µg/mL. LM stock solution was added to the Col I and FN hydrogel solution for a LM final concentration of 10, 25, or 50 (µg/mL). VN stock solution was added to the Col I, FN, and VN hydrogel solution for a VN final concentration of 2, 5, and 8 (µg/mL). A 5X supplement was also added to the hydrogel solutions for added cellular nutrition. Nuclease-free water was added to the hydrogels to bring the final volume to whichever cell plate was used. These solutions were also neutralized to pH of 7.4 and then kept on ice before proceeding to prepare the undifferentiated and passaged hESCs for the hydrogels.

After the H9 cells had been passaged to a workable volume, the Accutase enzyme was added to detach the cells from the cell plate. DMEM/F-12 was used as a wash to collect all of the
cells. The wash was added to a collection tube which was then centrifuged for 5 min. The supernatant was aspirated and mTeSR1 solution was added to the remaining cells in the tube. An amount of the mTeSR1 and H9 cell solution was added into each of the hydrogel solutions before adding the hydrogels to a welled plate. The plates were incubated for 1 h at 37°C for gelation. All experiments were done in duplicates. Once the hydrogels had become gelatinous, mTeSR1 solution (prepared with 5X supplement) was added to each well.

2-3 Cell Differentiation on Scaffolds

Once undifferentiated H9 cells had been seeded onto optimized scaffolds and cultured in mTeSR1 medium for 24 h, the cells were induced to DE differentiation by passaging with DE differentiation medium, which was developed in our laboratory. The system was cultured at 37°C in a 5% CO₂ supplemented incubator for 4 days. Medium changes were performed daily. Cells cultured in tissue culture plate (2D) were induced to differentiation as well for comparison. The differentiation medium consisted of DMEM/F-12 solution, 1% BSA (Bovine Serum Albumin), supplements B27 and N2, Activin A, P/S (penicillin/streptomycin) solution, and Wortmannin.

2-4 Calcein-AM/propidium iodide (PI) staining

After 4 days of differentiation, the cells were ready for experimental analysis. The Live/Dead Cell Double Staining Kit (Sigma-Aldrich) was used to visualize the ratio of live cells to dead cells. Two colored dyes were used. Calcein-AM dyed the viable cells green. Propidium Iodide (PI) solution dyed the dead cells red. Pictures were taken under an inverted microscope.

2-5 Immunoflourescent Staining

After 4 days of differentiation, the cells were prepared for immunoflourescent staining with the protocol developed in the laboratory. The cells in the hydrogels were fixed in position and stage of differentiation was observed using 4% paraformaldehyde. A Blocking Buffer was
used to pre-treat the hydrogel samples before introducing the antibodies. This step reduces background staining from reactive sites that the antibodies may unnecessarily bind to. The Blocking Buffer solution consisted of 1% Bovine Serum Albumin, 1X PBS, 0.3% Triton X-100, Donkey Serum, and Goat Serum. The indirect method of immunostaining was used with a primary and secondary antibody solution. These antibodies bind to the target antigen with a fluorescent dye that can be detected. The primary antibody used contained the DE marker proteins Sox17 (1:200) and Foxa2 (1:1000) which were mixed in 1% Bovine Serum Albumin, 1X PBS, and 0.3% Triton X-100. Cold 1X PBS was used as a wash between the addition of 4% paraformaldehyde, Blocking Buffer, and primary antibody. Once the primary antibody was added, the cells were kept at 4˚C for 24 hours. After 24 hours, 0.1% BSA/PBS was used as a wash (and is continued to being used as a wash for the rest of the procedure). The secondary antibody used contained TRITC (1:100) and anti-mouse IgG (1:500) diluted in a solution of 0.1% BSA/PBS. The final dye DAPI (1:100) was added to dye the nuclei of the cells in the hydrogels. The samples were then examined under a fluorescent microscope. Images were taken to visually observe the presence of DAPI, Sox17, and Foxa2 proteins. Hydrogel samples with no antibodies stains was used as a control.

2-6 qRT-PCR

Gene expression levels were quantified using qRT-PCR. The genes examined were Sox17, Foxa2, CXCR4, and Oct4. Before beginning the qRT-PCR analysis, the cells were prepped as cDNA. First, the RNA from the hESCs grown in the hydrogels was extracted. The protocol from the RNeasy Mini Kit (Qiagen) was used. RNA concentrations for each set of hydrogels were determined using a microplate reader. Based on these concentrations, reverse transcription into cDNA was performed. The cDNA protocol used was from the High Capacity
cDNA Reverse Transcription Kits (Applied Biosystems). Once the RNA was converted to cDNA, the cDNA was then used to perform the qRT-PCR analysis. The Power SYBR Green PCR Master Mix and RT-PCR protocol (Applied Biosystems) was used to prepare the cDNA with SYBR green detecting agents. A 2D sample of cells was used as a control for the qRT-PCR analysis. The gene β-actin was used as the housekeeping gene.

3-Experimental Results and Discussion

Collagen I was the ECM protein chosen to be the main structural component to be used in the 3D scaffolds. It was set at a fixed concentration throughout all the combinations. Collagen I was then intermixed with other ECM proteins such as fibronectin, laminin, and vitronectin which were readily available in the laboratory.

The experiments used to test the proteins were split into three phases: one for each ECM protein. After each phase, once the best ECM protein concentration was determined, this value was used for the next phase. Phase 1 tested solely fibronectin. Phase 2 tested the established fibronectin concentration with variations in laminin concentration. Phase 3 tested the established fibronectin and laminin concentration with variations in vitronectin concentration. Table 1 below also shows the concentrations that were used.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 mg/mL (fixed)</td>
<td>FN10 = 10 µg/mL</td>
<td>LM10 = 10 µg/mL</td>
<td>VN2 = 2 µg/mL</td>
</tr>
<tr>
<td></td>
<td>FN25 = 25 µg/mL</td>
<td>LM25 = 25 µg/mL</td>
<td>VN5 = 5 µg/mL</td>
</tr>
<tr>
<td></td>
<td>FN 50 = 50 µg/mL</td>
<td>LM50 = 50 µg/mL</td>
<td>VN8 = 8 µg/mL</td>
</tr>
</tbody>
</table>

Table 1: ECM concentrations used for each phase

Note: [X] denotes the best concentration found for FN through experimentation. [Y] denotes the best concentration for LM through experimentation.
The morphology of undifferentiated cells versus differentiated cells is shown in Figures 1 and 2, respectively. Human ESCs look small and circular in size as seen in Figure 1. The tendril-like arms emanating from the cell is what separates the differentiated cell from the hESCs as shown in Figure 2.

**Figure 1:** H9 hESCs before differentiation

**Figure 2:** H9 hESCs after differentiation
3-1 Phase I: Fibronectin

The batch of concentrations that were used in Phase 1 is located in Table 2. This set of criteria was used to make the 3D scaffolds/hydrogels for further testing.

<table>
<thead>
<tr>
<th>Col I Alone</th>
<th>Col I + FN(10 µg/mL)</th>
<th>Col I + FN(25 µg/mL)</th>
<th>Col I + FN(50 µg/mL)</th>
</tr>
</thead>
</table>

**Table 2**: Combinations used for Phase 1

Three tests were performed: live/dead cell staining, Immunostaining, and quantitative Real Time Polymerase Chain Reaction (qRT-PCR). Each protocol was modified to fit experimental components and quantity amounts that were used throughout this phase. This was done to complete the procedure more effectively, to ensure complete use of all materials, as well as to avoid the risk of contamination to the samples.

3-1.1 qRT-PCR

After running each sample in duplicate through the RealPlex Eppendorf qRT-PCR program, the data exported Ct values. The Ct value represented the number of cycles after which fluorescence was detectable above the background during the exponential phase of the reaction. SYBR green is the fluorescence dye that was detected once added to the cDNA samples. As the sample continued to amplify and make more strands of the sample product, there was an increase in fluorescence to a point where it was detected, resulting in the Ct value.

Ct analysis involved computing the relative amount of gene expression by comparing the target gene to the housekeeping or reference gene. The housekeeping gene used was β-actin. A change in a single Ct value corresponded to a two-fold difference from the starting material. This was mathematically determined from the mathematical expression: $2^n$ where $n$ equals the change in Ct value ($\Delta$Ct). For example, a change of 3 represented an 8-fold difference in starting
material. Several mathematical steps were utilized until the relative gene expression level was determined (Denman & McSweeney, 2005). Calculations were as follows:

1. Average of the Ct values for all gene replicates was calculated.

2. $\Delta C_{t}$ was calculated.

$$\Delta C_{t} = C_{t}(\text{target gene}) - C_{t}(\text{housekeeping gene})$$

3. $\Delta \Delta C_{t}$ was calculated.

$$\Delta \Delta C_{t} = \Delta C_{t}(\text{target gene}) - \Delta C_{t}(\text{control gene})$$

4. Fold Change $= 2^{-\Delta \Delta C_{t}}$

The initial qRT-PCR results for expression of the genes of focus (Sox17, Foxa2, and Oct4) are shown in Figures 3, 4, and 5 using cells differentiated in a 2D culture environment as a negative control. The lines above each column are the error bars that represent the standard deviation of the duplicates tested for each sample.

![Figure 3: Sox17 Expression for Phase 1 - FN](image)
Sox17 is a gene located in hESCs that regulates cells differentiation. Foxa2 is a gene that regulates metabolism and more specifically, the differentiation of pancreas and liver cells. Higher levels of these two genes compared to the negative control (2D) are considered favorable. From this initial set of data, it was concluded that the concentrations for FN10 and FN25 needed to be re-tested due to the higher levels of gene expression when testing for the expression of the
Oct4 gene compared to the 2D control. The Oct4 gene signifies the presence of undifferentiated hESCs. If the Oct4 gene is expressed, undifferentiated cells possibly present are in the sample. This gene will disappear as the hESCs move into the next cell stage through differentiation.

The second trial of qRT-PCR focused on the Sox17 and Foxa2 gene for the concentrations for FN10 and FN25.

![Sox17](image1)

**Figure 6**: Secondary Test for Sox17 Expression for Phase 1 –FN

![Foxa2](image2)

**Figure 7**: Secondary Test for Foxa2 Expression for Phase 1 –FN
From the second trial, it was concluded that in the first phase, FN25 proved to have the highest level of both Sox17 and Foxa2 gene expression. Thus, fibronectin concentration at 25 µg/mL was the best concentration for the stem cells to differentiate into definitive endoderm.

3.1.2 Calcein-AM/propidium iodide (PI) staining

The 04511 Sigma-Aldrich Cell Staining protocol was modified then applied to the combination of stem cells and ECM proteins used in this sample. On Day 4 of differentiation, each combination of cells were stained to visualize the ratio of live:dead cells.

From these pictures taken with an inverted fluorescent microscope, it can be seen visually that most of the cells were alive in the 3D culture environment.
3.1.3 Immunofluorescent Staining

On Day 4 of differentiation, each combination of cells were prepared and stained, according to the protocol established in the laboratory, to visualize the expression of the definitive endoderm (DE) protein markers Sox17 and Foxa2, with a fluorescent microscope. DAPI is a fluorescent stain that can pass through the cell membrane of the hESCs to bind strongly to the DNA. Thus, it is used to stain the nucleus. Blue represents the DAPI dye, green represents Sox17, and red represents the Foxa2.

**Figure 12:** Col I 20x  
DAPI/SOX17/FOXA2

**Figure 13:** FN10 20x  
DAPI/SOX17/FOXA2

**Figure 14:** FN25 20x  
DAPI/SOX17/FOXA2

**Figure 15:** FN50 20x  
DAPI/SOX17/FOXA2
It was clear from these microscopic images that Sox17 (green) and Foxa2 (red) could be expressed. However, it was not possible to quantify the exact level of protein expression just from the images alone. Therefore, qRT-PCR was implemented to quantify the gene expression level as detailed above. From the images though, it can be inferred that Foxa2 expressed the highest at the FN concentration of 50 µg/mL. This may imply that the mRNA level may not always reflect the protein expression level.

3.2 Phase 2: Laminin

The batch of concentrations that were used in Phase 2 is the following:

<table>
<thead>
<tr>
<th>Col I +FN (25 µg/mL)</th>
<th>Col I + FN(25 µg/mL) + LM (10 µg/mL)</th>
<th>Col I + FN(25 µg/mL) + LM (25 µg/mL)</th>
<th>Col I + FN(25 µg/mL) + LM (50 µg/mL)</th>
</tr>
</thead>
</table>

**Table 3:** Combinations used for Phase 2

This set of criteria was used to make the 3D scaffolds/hydrogels for further testing. Phase I’s concentration, Col I +FN (25 µg/mL), was tested again to compare FN alone with a combination of FN and LM.

3.2.1 qRT-PCR

The qRT-PCR results for expression of the genes of focus (Sox17, Foxa2) are as follows (using a 2D culture of cells to use as a control):
From the data that qRT-PCR gave, it is LM50 that proved to have the highest level of gene expression. So far, a combination of Col I + FN25 + LM50 proved to be the best composition for stem cells to differentiate into definitive endoderm.

Calcein-AM/propidium iodide (PI) staining and immunostaining were not performed for Phase 2 due to the lack of evidence supported by these two procedures in the middle of the
phases. It was decided that once the specific concentrations were found for each ECM protein, then calcein-AM/propidium iodide (PI) staining and immunostaining was to be implemented for additional evidential support to the qRT-PCR results.

**3-3 Phase 3: Vitronectin**

The batch of concentrations that were used in Phase 3 is the following:

<table>
<thead>
<tr>
<th>Col I + FN(25 µg/mL) + LM (50 µg/mL)</th>
<th>Col I + FN(25 µg/mL) + LM (50 µg/mL) + VN(2 µg/mL)</th>
<th>Col I + FN(25 µg/mL) + LM (50 µg/mL) + VN(5 µg/mL)</th>
<th>Col I + FN(25 µg/mL) + LM (50 µg/mL) + VN(8 µg/mL)</th>
</tr>
</thead>
</table>

*Table 4: Combinations used for Phase 3*

This set of criteria was used to make the 3D scaffolds/hydrogels for further testing. Phase 2’s established concentration, Col I + FN (25 µg/mL) + LM (50 µg/mL), was tested again to compare FN and LM with a combination of FN, LM, and VN.

**3-3.1 qRT-PCR**

The qRT-PCR results for expression of the genes of focus (Sox17, Foxa2, Oct4) are as follows (using a 2D culture of cells to use as a control as well as the previous Phase’s concentration: FN25/LM50):
**Figure 20**: Sox17 Expression for Phase 3 - VN

**Figure 21**: Foxa2 Expression for Phase 3 - VN
Instead of using the 2D sample for a control in the Oct4 gene comparison, undifferentiated H9 cells were used instead. Since Oct4 is present in undifferentiated cells in a 3D scaffold, H9 was chosen as a better comparison when testing the other ECM combinations.

From the data that qRT-PCR gave, it is VN5 that proved to have the highest level of gene expression when all three ECM proteins are mixed together. In conclusion, a combination of Col I + FN25 + LM50 + VN5 proves to be the best composition for stem cells to differentiate in.

### 3.3.2 Calcein-AM/propidium iodide (PI) staining

The 04511 Sigma-Aldrich Cell Staining protocol was modified and applied to this combination of stem cells and ECM proteins. On Day 4 of differentiation, each combination of cells were stained to visualize the ratio of live:dead cells.
From these pictures taken with an inverted fluorescent microscope, it can be seen visually that there was a bigger percentage of live cells compared to dead cells.

3-3.3 Immunoflourescent Staining

On Day 4 of differentiation, each combination of cells were prepared and stained, according to the protocol establish in the laboratory, to visualize the expression of the DE markers Sox17 and Foxa2, with a fluorescent microscope.
Figure 27: FN25/LM50 40X
DAPI/SOX17/FOXA2

Figure 28: VN2 40x
DAPI/SOX17/FOXA2

Figure 29: VN5 40x
DAPI/SOX17/FOXA2

Figure 30: VN8 40x
DAPI/SOX17/FOXA2

Figure 31: Negative Control (Sox17) (Wang)

Figure 32: Negative Control (Foxa2) (Wang)
4-Conclusion

In conclusion, it was demonstrated that various combinations of extracellular matrix proteins affected hESC growth and differentiation in 3D hydrogels. Experimental techniques like the calcein-AM/propidium iodide (PI) staining, immunoflourescent staining, and qRT-PCR provided the evidence to reach a final set of concentrations: Col I (1.5 mg/mL) + FN(25 µg/mL) + LM(50 µg/mL) + VN(5 µg/mL). Both staining techniques provided the visual evidence; however, it was shown that it was difficult to see the differences between the tested ECM protein combinations. Real-time PCR provided the quantitative evidence to determine which concentration was best as well as support the visual evidence. The final concentrations for fibronectin, laminin, and vitronectin did deviate from previous lab studies. This project was performed to create a more robust protocol in the future for stem cell studies in the laboratory.

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6-References


