Two populations of Thy1-positive mesenchymal cells regulate in vitro maturation of hepatic progenitor cells

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Kamo N, Yasuchika K, Fujii H, Hoppo T, Machimoto T, Ishii T, Fujita N, Tsuura T, Yamashita JK, Kubo H, Ikai I. Two populations of Thy1-positive mesenchymal cells regulate in vitro maturation of hepatic progenitor cells. Am J Physiol Gastrointest Liver Physiol 292: G526–G534, 2007. First published September 21, 2006; doi:10.1152/ajpgi.00241.2006.—We previously reported that the in vitro maturation of CD49f−Thy1−CD45− (CD49f+Thy1+CD45−) fetal hepatic progenitor cells (HPCs) is supported by Thy1-positive mesenchymal cells derived from the fetal liver. These mesenchymal cell preparations contain two populations, one of a cuboidal shape and the other spindle shaped in morphology. In this study, we determined that the mucin-type transmembrane glycoprotein gp38 could distinguish cuboidal cells from spindle cells by immunocytochemistry. RT-PCR analysis revealed differences between isolated CD49f−Thy1+gp38+CD45− (gp38 positive) and CD49f+Thy1−gp38−CD45− (gp38 negative) cells, whereas both cells expressed mesenchymal cell markers. The coculture with gp38-positive cells promoted the maturation of CD49f-positive HPCs, which was estimated by positivity for periodic acid-Schiff (PAS) staining, whereas the coculture with gp38-negative cells maintained CD49f-positive HPCs negative for PAS staining. The expression of mature hepatocyte markers, such as tyrosine aminotransferase, tryptophan-2,3-dioxygenase, and glucose-6-phosphatase, were upregulated on HPCs by coculture with gp38-positive cells. Furthermore, transmission electron microscopy revealed the acquisition of mature hepatocyte features by HPCs cocultured with gp38-positive cells. This effect on maturation of HPCs was inhibited by the addition of conditioned medium derived from gp38-negative cells. By contrast, the upregulation of bromodeoxyuridine incorporation by HPCs demonstrated the proliferative effect of coculture with gp38-negative cells. In conclusion, these results suggest that in vitro maturation of HPCs promoted by gp38-positive cells may be opposed by an inhibitory effect of gp38-negative cells, which likely maintain the immature, proliferative state of HPCs.

gp38-positive cells; gp38-negative cells; coculture; immature

IN THE LIVER, several types of hepatic progenitor cells (HPCs)/hepatic stem cells (1, 4, 11, 18, 20, 23, 28–30, 33) have been isolated and examined. Due to their high proliferative potential, HPCs/hepatic stem cells are expected to be an effective cell source for transplantation therapy. The difficulty in applying these HPCs/hepatic stem cells to clinical scenarios, however, is that they exhibit lower hepatic function than terminally differentiated hepatocytes. To make cell transplantation therapy using HPCs/hepatic stem cells clinically viable, it is necessary to develop an in vitro maturation system. Many studies examining the maturation of HPCs in vitro have demonstrated the importance of several soluble factors, including hepatocyte growth factor (9, 19, 27), fibroblast growth factors (13, 34), and oncostatin M (15).

Previously, we successfully enriched and isolated HPCs [CD49f+Thy1−CD45− cells (CD49f-positive cells)] and mesenchymal cells [CD49f−Thy1+CD45− cells (Thy1-positive cells)] from embryonic day 13.5 (E13.5) fetal livers using a method to form cell aggregates similar to the neurosphere (7). Thy1-positive mesenchymal cells derived from the fetal liver promoted the functional and morphological differentiation of CD49f-positive cells into mature hepatocytes in vitro through direct cell-to-cell contacts. A number of reports have supported the requirement of cell-to-cell contacts between parenchymal cells and nonparenchymal cells in hepatocyte differentiation (2, 14, 16, 17). Therefore, it is important to elucidate the identity of the mesenchymal cells necessary for the maturation of HPCs.

In this study, to further fractionate Thy1-positive mesenchymal cells, we focused on the mucin-type transmembrane glycoprotein gp38 (also known as T1α or podoplanin). Gp38 is specifically expressed by lymphatic but not blood vascular endothelial cells and thus is well established as one of the lymphatic-specific markers (3). During embryogenesis, gp38 expression is induced in the mouse foregut endoderm prior to lung bud formation at E8.5–9.0, at which time liver specification occurs in the same region. We isolated the two cell populations as gp38-positive cells and gp38-negative cells from Thy1-positive cells by cell sorting. We then elucidated the effects of these two populations of Thy1-positive mesenchymal cells on the in vitro maturation of fetal HPCs.

MATERIALS AND METHODS

Animals. C57BL/6J Jms Slc mice were obtained from SLC (Hamamatsu, Japan). All animal experimental procedures were performed in accordance with Animal Protection Guidelines of Kyoto University.

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Isolation and culture of fetal HPCs. Fetal HPCs obtained from E13.5 fetal livers were enriched by cell aggregate formation as previously described (7, 32). A flow diagram describing the formation of cell aggregates and subsequent analysis by flow cytometry or cell sorting is shown in Fig. 1. Cell aggregates selected by gravity sedimentation were seeded on type I collagen-coated 24-well plates at a density of 2 × 10⁴ cells/well in DMEM (GIBCO-BRL, Grand Island, NY) supplemented with 10% FCS, 20 mmol/l HEPES, 25 mmol/l NaHCO₃, 0.5 mg/l insulin, 10⁻⁷ mol/l dexamethasone (Wako, Osaka, Japan), 10 mmol/l nicotinamide (Wako), 2 mmol/l L-ascorbic acid phosphate (Wako), 20 ng/ml of the deleted form of hepatocyte growth factor (kindly provided by Snow Brand Milk Products, Tokyo, Japan), and antibiotics.

**RESULTS**

Two populations of mesenchymal cells in cell aggregates. After the isolation of Thy1-positive cells by flow cytometry, it was revealed that the Thy1-positive population contained two morphologically different cell types, one cuboidal in shape and the other spindle shaped (Fig. 2A). By immunocytochemistry, gp38 expression was detected on cuboidal cells but was absent from spindle-shaped cells (Fig. 2B). We identified two sub-populations of Thy1-positive cells by flow cytometric analysis: gp38-negative cells (CD49f<sup>+</sup>Thy1<sup>+</sup>gp38<sup>−</sup>CD45<sup>+</sup>) and gp38-positive cells (CD49f<sup>+</sup>Thy1<sup>+</sup>gp38<sup>+</sup>CD45<sup>−</sup>) (Fig. 1B). Gp38-positive cells accounted for 16.31 ± 3.67% of the total Thy1-positive cells derived from the E13.5 fetal liver at the time of isolation (day 0; Fig. 1C). The proportion of gp38-positive cells increased day by day and resulted in 76.27% of the cultures 9 days after isolation (day 9; Fig. 1D).

and gp38-negative cells were separated by cell sorting with purities of 97% and 94%, respectively (Fig. 1B). Isolated cuboidal gp38-positive cells retained homogeneous staining for gp38 for at least 5 days (Fig. 2, C and D), whereas isolated spindle-shaped cells remained negative for gp38 (Fig. 2, E and F).

Table 1. Primer sequences for RT-PCR

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<th>Gene</th>
<th>Sense 1</th>
<th>Antisense 1</th>
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<td>α-Smooth muscle actin</td>
<td>5'-CTATTCAGGCTGTCGTTCCG-3'</td>
<td>5'-GGACCTCCTCTGGAAGC-3'</td>
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<td>Desmin</td>
<td>5'-GCTATCGGAAACATATTGGCG-3'</td>
<td>5'-GTGTGTTGCTGTAGCCCTG-3'</td>
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<tr>
<td>Vimentin</td>
<td>5'-TACGAGGACACTATGGGC-3'</td>
<td>5'-CTGTTGCAACAAGTTGGTCG-3'</td>
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<tr>
<td>Glial fibrillary acidic protein</td>
<td>5'-AGACAGGGAGGGAGATGAGG-3'</td>
<td>5'-AAGGCTCCCTCCGTTAACC-3'</td>
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<tr>
<td>CD31</td>
<td>5'-ACACATCATCTTCTCTCCG-3'</td>
<td>5'-CTGTCATCTGGAAGC-3'</td>
</tr>
<tr>
<td>Nestin</td>
<td>5'-CCACACTGGAGATCTCCATCC-3'</td>
<td>5'-CCACAGGAAAGAGCCAC-3'</td>
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<tr>
<td>Integrin-β1</td>
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<td>5'-TTTCAGAGTGTAGTGGTA-3'</td>
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<td>PDGF receptor-β</td>
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<tr>
<td>CFTR</td>
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<td>5'-AGGCACTCTGGAAGACTG-3'</td>
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<tr>
<td>Integrin-α5</td>
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<td>5'-GGCACTCTGGAAGACTG-3'</td>
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<td>5'-GTGCAGAGGGTCTGTGAGAC-3'</td>
</tr>
<tr>
<td>Glycogen</td>
<td>5'-AGATGATGCGTTGAGCGT-3'</td>
<td>5'-GGCACTCTGGAAGACTG-3'</td>
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<tr>
<td>Glucose-6-phosphatase</td>
<td>5'-CTGATTCCTGCTGAGTCG-3'</td>
<td>5'-GGCACTCTGGAAGACTG-3'</td>
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<tr>
<td>Albumin</td>
<td>5'-CGAGAGGGTGCGGGGATG-3'</td>
<td>5'-GTGACAGGAGGCTGCTGTG-3'</td>
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<tr>
<td>Hypoxanthine phosphoribosyltransferase</td>
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<td>5'-GGCCTATAGGCTCATG-3'</td>
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Characteristics of two mesenchymal cells. By immunostaining, both subpopulations of Thy1-positive cells were positive for α-SMA and desmin (Fig. 3, A–D). Stains for α-SMA in gp38-positive cells localized to the periphery of the cells, indicating the presence of cortical fibers (Fig. 3A). On the other hand, the distribution of α-SMA in gp38-negative cells revealed the presence of stress fibers (Fig. 3B). There were no significant differences in the staining patterns of desmin between gp38-positive cells and gp38-negative cells (Fig. 3, C and D). Next, to characterize the two populations of mesenchymal cells, we performed RT-PCR analysis for genes specifically expressed by several cells that reside in the liver. Both cell types expressed CD34 as well as mesenchymal cell markers (α-SMA, desmin, and vimentin) but did not express hepatic stellate cell marker (glial fibrillary acidic protein), endothelial cell marker (PECAM-1), and Kupffer cell marker (CD16) (Fig. 3E). Interestingly, gp38-positive cells and gp38-negative cells differentially expressed integrin-β3, CFTR, PDGF receptor-β, nestin, and integrin-α5. These data demonstrated that these two populations of Thy1-positive cells were distinct mesenchymal cells.

Coculture of HPCs and mesenchymal cells. To investigate the functional differences of the two populations of Thy1-positive cells, we tested the effect of gp38-positive or gp38-negative cells on CD49f-positive HPCs. CD49f-positive HPCs cocultured with gp38-positive cells increased intracellular granularity at day 3, proliferated until day 5, and then gradually piled up at the periphery of colonies. By day 7, the piled-up area was extensively expanded in CD49f-positive colonies, where binucleated mature cells appeared (Fig. 4A). CD49f-negative HPCs when cocultured with gp38-negative cells did not display any features of mature hepatocytes even after 14 days (Fig. 4B and data not shown). Next, to examine whether the cultured cells produced and stored glycogen, as an indicator of functional maturation, PAS staining was performed. Almost all cells in CD49f-positive HPC colonies cocultured with gp38-positive cells were strongly stained for PAS at day 7 (Fig. 4C). However, CD49f-negative HPCs cocultured with gp38-negative cells were negative for PAS staining (Fig. 4D). On the other hand, the proportion of cells incorporating BrdU among CD49f-positive HPCs cocultured with gp38-positive cells was significantly lower than that seen in CD49f-positive HPCs cultured alone (Fig. 4E). By contrast, CD49f-positive HPCs cultured with gp38-negative cells demonstrated significantly higher level of BrdU incorporation than those cells cultured alone. These results demonstrate the proliferative activities of immature CD49f-positive HPCs cultured with gp38-negative cells. Supporting these morphological and immunocytochemical findings, RT-PCR analysis revealed that the expression of mature hepatocyte markers (such as TAT, TO, and glucose-6-phosphatase) were upregulated in CD49f-positive HPCs cocultured with gp38-positive cells compared with those cells incubated with gp38-negative cells (Fig. 5A). On the other hand, CK19 and the fetal HPC marker CD49f were upregulated in CD49f-positive HPCs cocultured with gp38-negative cells. Both cell types expressed albumin strongly. Furthermore, mRNA expression of TAT and TO, as assessed by real-time RT-PCR, significantly increased in CD49f-positive HPCs cocultured with gp38-positive cells for 7 days (Fig. 5, B and C). No significant increases were observed in CD49f-positive
HPCs cocultured with gp38-negative cells. To further investigate the maintenance of the immature state of CD49f-positive HPCs cocultured with gp38-negative cells, we then carried out immunostaining for albumin and CK19. CD49f-positive HPCs cocultured with gp38-negative cells for 11 days were positive for albumin, and some were also positive for CK19, indicating the presence of bipotentiality, i.e., the immature state (Fig. 5E). Only a few of the CD49f-positive HPCs cocultured with gp38-positive cells for 11 days showed double-positive staining (Fig. 5D and F). Finally, transmission electron microscopy showed that CD49f-positive HPCs cultured with gp38-positive cells for 8 days contained multiple mitochondria, numerous peroxisomes, abundant Golgi apparatus, a large number of glycogen granules, and tight junctions with desmosomes and formed biliary canaliculi with microvilli (Fig. 6A and B). On the contrary, few of these features were observed in CD49f-positive HPCs cocultured with gp38-negative cells (Fig. 6C). These cells were thinner than those cells cocultured with gp38-positive cells.

Effect of conditioned medium of two types of mesenchymal cells on maturation of fetal HPCs. To explore the effect of soluble factors secreted from the two subpopulations of mesenchymal cells on the in vitro maturation of HPCs, CD49f-positive HPCs were incubated with conditioned medium derived from gp38-positive or gp38-negative cell cultures. CD49f-positive HPCs did not differentiate morphologically in the presence of conditioned medium from gp38-positive cells and remained negative for PAS staining, indicating that direct contact between CD49f-positive HPCs and gp38-positive cells was essential for the maturation of HPCs (Fig. 7A and B). Coculture of CD49f-positive HPCs and gp38-positive cells incubated with conditioned medium from gp38-positive cells promoted the expansion of piled-up areas in the colonies of CD49f-positive HPCs and induced many binucleated cells after

<table>
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<th>Probe and primer sequences for real-time RT-PCR</th>
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<td>Tyrosine aminotransferase</td>
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<tr>
<td>Sense 5'-TGACGAGTGGGCTGAGTCA-3'</td>
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<tr>
<td>Antisense 5'-TGACCTCAATCCCCATAGACTCA-3'</td>
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<td>Probe 5'-FAM-TGGACAGAACATCCCTCAGCCGAGG-TAMRA-3'</td>
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<tr>
<td>Tryptophan-2,3-dioxygenase</td>
</tr>
<tr>
<td>Sense 5'-GGTGAACGACGACTGTCATAACC-3'</td>
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<tr>
<td>Antisense 5'-CATGAGCCTGAATGCCATAA-3'</td>
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<tr>
<td>Probe 5'-FAM-TACAGGGAAGAGCCCTCAGGTC-TAMRA-3'</td>
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Fig. 2. A and B: phase-contrast (A) and corresponding fluorescent microscopic (B) images of isolated Thy1-positive cells derived from the fetal liver at 9 days after isolation. Note that cuboidal cells were positive for gp38 (B; red). C–F: phase-contrast (C and E) and fluorescent microscopic (D and F; red, gp38) images of isolated gp38-positive (C and D) and gp38-negative (E and F) cells. Original magnification: ×100 in A and B and ×200 in C–F.
7 days (Fig. 7C). In contrast, when CD49f-positive HPCs were cultured with gp38-positive cells in the presence of conditioned medium from gp38-negative cells, few cells showed maturate features morphologically by 7 days (Fig. 7D). PAS staining supported the morphological results (Fig. 7, E–G). The proportion of PAS-positive cells among CD49f-positive HPCs cocultured with gp38-positive cells was significantly attenuated in the presence of conditioned medium from gp38-negative cells compared with that observed in the presence of gp38-positive cell conditioned media.

**DISCUSSION**

In this study, we demonstrated that these two types of Thy1-positive cells could be separated by the expression of gp38. RT-PCR analysis indicated that both gp38-positive cells and gp38-negative cells expressed mesenchymal cell markers (α-SMA, desmin, and vimentin). Gp38 is a specific marker for lymphatic endothelium (21, 24, 25); however, both mesenchymal cells were negative for PECAM-1 and Flk-1, indicating that neither cell subtype was composed of blood or lymphatic endothelial cells. On the other hand, CD34, which is generally expressed by juvenile cells such as hematopoietic stem cells and endothelial progenitor cells, was detected on both cell subtypes. There were differences in the expression of some marker genes between gp38-positive cells and gp38-negative cells, although we cannot characterize these differences in greater detail at this point. Immunohistochemical analysis of tissue sections obtained from E13.5 fetal livers for Thy1 and gp38 could not determine the locations of Thy1-positive cells.
and gp38-positive cells (data not shown), most likely due to the very low number of mesenchymal cells.

In early liver development, fetal HPCs derived from the anterior foregut endoderm migrate into the septum transversum mesenchymal area to form the liver bud. HPCs then develop into the functionally matured hepatocytes that comprise the adult liver parenchyma. Several findings have indicated that mesenchymal cells are critical in this early stage of liver development (5, 6, 8, 12, 13, 22, 34). Colonies of CD49f-positive HPCs cocultured with gp38-positive cells developed the morphological features, marker gene expression, and glycogen storage capabilities of mature hepatocytes, whereas conditioned medium of gp38-positive cells did not promote the maturation of CD49f-positive HPCs. These results suggest that cell-cell contacts with gp38-positive cells were necessary for the maturation of HPCs into hepatocytes in vitro. This in vitro maturation effect is likely mediated by a specific surface molecule present on gp38+Thy1+ mesenchymal cells. By contrast, CD49f-positive HPCs cocultured with gp38-negative cells did not develop the feature of mature hepatocytes, and, concomitantly, those cells retained a higher proliferative activity than CD49f-positive HPCs cultured alone, as shown by BrdU incorporation. In addition, conditioned medium from gp38-negative cells suppressed the maturation of CD49f-positive HPCs that was promoted by coculture with gp38-positive cells. Taken together, gp38-negative cells maintain the proliferative and immature state of HPCs, and those cells secrete soluble factors that have an inhibitory effect on gp38-positive cell-induced HPC maturation. The effect of gp38-negative cells may resemble the effect of murine embryonic fibroblasts on embryonic stem cells; murine embryonic fibroblasts maintain the undifferentiated state of embryonic stem cells with a highly proliferative activity in vitro (31).

According to our previous study (7), Thy1-positive cells promoted the maturation of CD49f-positive HPCs during a 14-day coculture. Despite the presence of inhibitory gp38-negative cells, Thy1-positive cells mediated the maturation of CD49f-positive HPCs because the proportion of gp38-positive cells increased among the Thy1-positive cell population during the culture. By day 9, gp38-positive cells accounted for 76.27% of total Thy1-positive cells, whereas they were a minority (16.31 ± 3.67%) at isolation. Thus, the effect of gp38-positive cells would dominate during the coculture with HPCs.

Because Verfaillie and colleagues (26) reported a differentiation potential of adult bone marrow-derived mesenchymal...
stem cells toward liver cells, we investigated whether the two types of Thy1-positive fetal mesenchymal cells were able to differentiate to hepatocytes with the same medium as in previous studies. However, immunocytochemical analysis demonstrated that both gp38-positive and gp38-negative cells were negative for albumin (data not shown). As a result, we could not display the differentiation capacity of these two mesenchymal cells toward hepatocytes, although we do not exclude the possibility under other conditions. In addition, we are currently exploring the two types of mesenchymal cells in the adult liver. It would be interesting to test the two resulting cell populations in mouse models of liver disease.

In conclusion, two types of mesenchymal cells isolated from E13.5 fetal livers had profoundly different effects on the in vitro maturation of CD49f-positive HPCs. Gp38-positive cells promote HPCs differentiation, whereas gp38-negative cells promote proliferation and not differentiation. These newly identified mesenchymal cells provide an opportunity to further understand liver development and regeneration, possibly contributing to the application of HPCs/hepatic stem cells to regenerative medicine. In clinical use, isolated HPCs could be amplified by coculture with gp38-negative cells and could be differentiated into mature hepatocytes with gp38-positive cells. Such differentiated
Fig. 6. A–C: transmission electron microscopic views of CD49f-positive cells cocultured with gp38-positive cells (A and B) or gp38-negative cells (C). A and B: CD49f-positive cells cocultured with gp38-positive cells displayed many mitochondria (white arrows), possessed abundant Golgi apparatus (small solid arrow), numerous peroxisomes (long black arrow), and tight junctions with desmosomes (arrowheads) and formed biliary canaliculi with microvilli (*). Original magnification: ×7,500 in A and ×1,500 in B and C.

Fig. 7. Effect of conditioned medium (CM) from two mesenchymal cell subpopulations on in vitro maturation of CD49f-positive cells. A and B: phase-contrast (A) and PAS-stained (B) images of CD49f-positive cells cultured in the presence of CM from gp38-positive cells for 7 days. C–F: cocultures of CD49f-positive cells and gp38-positive cells in the presence of CM from gp38-positive cells (C and E) or gp38-negative cells (D and F) for 7 days. CD49f-positive cells are indicated by the dotted lines. E and F: PAS staining of CD49f-positive cells cocultured under the same conditions as C and D, respectively. G: proportion of PAS-positive cells of total CD49f-positive cells cultured in the presence of CM from gp38-positive (+) cells or gp38-negative (−) cells. Values are expressed as means ± SD; n = 3. *P < 0.05. Original magnification: ×200 in A–F.

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REFERENCES