ORIGINAL ARTICLE

Generation of Hepatocyte-like Cells from Human Induced Pluripotent Stem (iPS) Cells By Co-culturing Embryoid Body Cells with Liver Non-parenchymal Cell Line TWNT-1

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ABSTRACT

Objective: To generate a homogeneous population of patient-specific hepatocyte-like cells (HLCs) from human iPS cells those show the morphologic and phenotypic properties of primary human hepatocytes.

Study Design: An experimental study.

Place and Duration of Study: Department of Surgery, Okayama University, Graduate School of Medicine, Japan, from April to December 2011.

Methodology: Human iPS cells were generated and maintained on ES qualified matrigel coated plates supplemented with mTeSR medium or alternatively on mitotically inactivated MEF feeder layer in DMEM/F12 medium containing 20% KOSR, 4ng/ml bFGF-2, 1 x 10^{-4} M 2-mercaptoethanol, 1 mmol/L NEAA, 2mM L-glutamine and 1% penicillin-streptomycin. iPS cells were differentiated to HLCs by sequential culture using a four step differentiation protocol: (I) Generation of embryoid bodies (EBs) in suspension culture; (II) Induction of definitive endoderm (DE) from 2 days old EBs by growth in human activin-A (100 ng/ml) and basic fibroblasts growth factor (bFGF2) (100 ng/ml) on matrigel coated plates; (III) Induction of hepatic progenitors by co-culture with non-parenchymal human hepatic stellate cell line (TWNT-1); and (IV) Maturation by culture in dexamethasone. Characterization was performed by RT-PCR and functional assays.

Results: The generated HLCs showed microscopically morphological phenotype of human hepatocytes, expressed liver-specific genes (ASGPR, Albumin, AFP, Sox17, Fox A2), secreted human liver-specific proteins such as albumin, synthesized urea and metabolized ammonia.

Conclusion: Functional HLCs were generated from human iPS cells, which could be used for autologus hepatocyte transplantation for liver failure and as *in vitro* model for determining the metabolic and toxicological properties of drug compounds.

Key Words: *Hepatocyte-like cells. Liver failure. iPS cells. Embryoid body cells.*

INTRODUCTION

Liver failure is a devastating condition which if not treated leads to significant morbidity and mortality. At present, whole-organ liver transplantation is the only curative treatment at this time for patients with liver failure,[1] but this procedure is costly, limited by scarcity of donor livers and associated with high rate of complications.[2] Furthermore, significant numbers of patients with acute liver failure (ALF) and a majority of patients with acute-on-chronic liver disease are not even placed on the transplant waiting list due to multi-organ failure, sepsis, psychosocial reasons or the lack of adequate health care facility. Therefore, current interest has been focused on the possibility of utilizing cellular resources to sustain patients until liver transplantation or to renovate liver function.

The use of *ex-vivo* adult human hepatocytes is a desirable option for cellular therapies. However, these cells are scarce, have limited proliferation potential, and lose function and viability upon isolation. Therefore, in last decade, the scientists have been able to derive human liver cells from other sources, in particular human embryonic stem cells (hESCs). However, hESCs have certain ethical issues and rejection problems.

The generation of human induced pluripotent stem cells (iPSCs) from human somatic cells has revolutionized the stem cell field.[3] The iPS cells are similar to embryonic stem cells in morphology, proliferation, gene expression, epigenetic status and *in vitro* differentiation. Reprogramming of somatic cells is achieved with the introduction of a defined set of transcription factors. This was first demonstrated by the Yamanaka's group in both mouse and human fibroblasts using retroviral insertion of four transcription factors, Oct 3/4, Sox2, cMyc and Klf4.[4,5]

The advent of iPS technology has given rise to the possibility of patient specific cell therapy, where the use of genetically identical cells would prevent immune

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Received: October 10, 2012; Accepted: August 31, 2013.

rejection and the ethical issues associated with human ES cells. A variety of functional cell types have been derived from iPS cells including neurons, hematopoietic cells, cardiomyocytes and hepatocytes.[6-10] Together, it is speculated that human iPS cells could be used in future regenerative medicine for a variety of patient specific therapies.

Heterotypic cell interactions between parenchymal cells and non-parenchymal neighbours have been recognized to be central to the function of many organ systems. Cells constituting various organs develop into either epithelial cells or mesenchymal cells, depending on their developmental process. In the liver, the hepatocytes and cholangiocytes that conduct liver functions are epithelial parenchymal cells, whereas liver macrophages, hepatic stellate cells and liver endothelial cells are mesenchymal cells. These mesenchymal cells produce and secrete hepatocyte growth factor (HGF) and other cytokines in the process of hepatic regeneration from liver damage.

Among the known functional linkages between hepatocytes and non-parenchymal cells, hepatic stellate cells are believed to play an essential role. Therefore, when liver endothelial cells and hepatic stellate cells are incorporated as components of cell-based liver therapies, it is possible to promote and maintain the function of liver parenchymal cells. In such case, stellate cells produce collagen as a scaffold for hepatocyte proliferation and secrete important regulating factors for hepatocyte function. Therefore, the authors established a protocol for hepatic differentiation of human iPS cells by co-culturing them with immortalized human hepatic stellate cell line, as a reproducible, readily available and cost effective approach.

The aim of this study was to describe the results of production of HLCs from human iPS cells by co-culture with immortalized human hepatic stellate cell line.

METHODOLOGY

This study was conducted at the Department of Surgery, Okayama University, Graduate School of Medicine, Japan, from April to December 2011.

Mouse embryonic fibroblasts (MEFs) were prepared in the study centre laboratory by a method modified from "Teratocarcinoma and Embryonic stem cells: a practical approach"[11] and were maintained in DMEM medium containing 10% defined fetal bovine serum (dFBS) (Hyclone, New Zealand) and 1% non-essential amino-acids (NEAA) (Invitrogen, CA). MEFS were passaged after 2 - 3 days using trypsin-EDTA (Invitrogen, CA), keeping the cells sub-confluent.

Human iPS cell were generated from human lung fibroblasts cell line, IMR-90, by optimizing the retroviral system published by Dr. Yamanaka from Kyoto University, Japan and were maintained on ES qualified matrigel (B.D, Bedford, MA) coated plates (non-feeder layer) supplemented with mTeSR medium (Wicell, Madison, Wis) or alternatively on mitotically inactivated MEF feeder layer in DMEM/F12 medium containing 20% Knockout Serum Replacement (KOSR) (Invitrogen), 4 ng/ml fibroblast growth factor (bFGF-2), 1 x 10^{-4} M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 1 mmol/L NEAA, 2 mM L- glutamine (MP Biomedicals, OH) and 1% penicillin-streptomycin (P/S) (Sigma, Irvine, UK). For passaging, iPS cells were washed once with PBS (Sigma) and then incubated with DMEM/F12 containing 1 mg/ml dispase or collgenase IV (Invitrogen) for 5 minutes. When colonies at the edge of dish started dissociating from the bottom, DMEM/F12/collagenase was removed and washed with ES media, and the cells were transferred to a new dish on MEF-feeder cells or matrigel coated plates. The split ratio was routinely 1:3. For feeder-free culture, the plate was coated with 0.3 mg/ml matrigel at 4°C overnight. The plate was warmed to room temperature before use. Unbound matrigel was aspirated off and washed out with DMEM/F12.

iPS cells were differentiated by sequential culture in bFGF-2 and human activin-A through a step involving generating EBs, definitive endoderm (DE), co-culture with human immortalized non-parenchymal hepatic stellate cell line, TWNT-1 (generated in our laboratory) and dexamethasone. Strategy of protocol has been depicted in Figure 1.

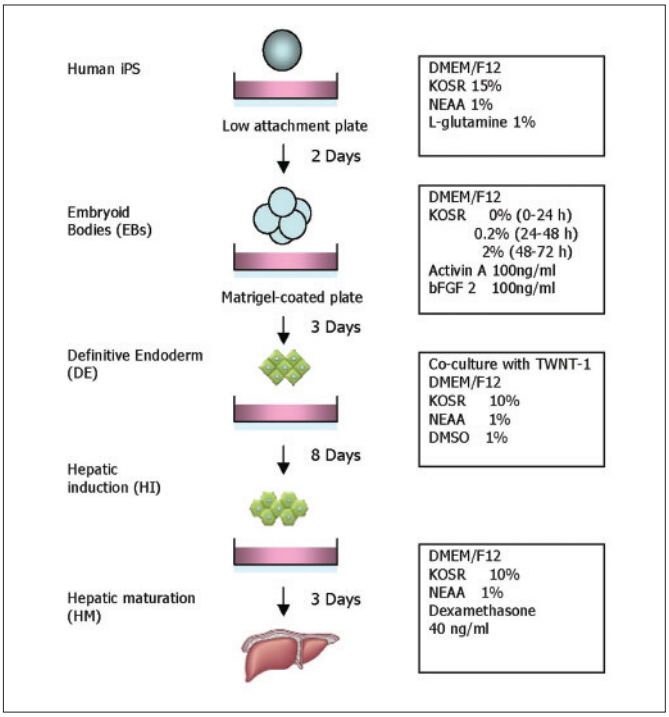

Figure 1: Schematic representation of differentiation induction protocol. Schematic representation of the strategy for differentiation of human iPS cells to hepatocyte-like cells. iPS cells were cultured in suspension method on ultra low attachment plate for 2 days to form embryoid bodied (EBs). The resulting EBs were transferred to matrigel-coated plates and treated with basic fibroblast growth factor (FGF-2) (100 ng/ml) and activin-A (100 ng/ml) for three days to form definitive endoderm (DE). The cells then were subjected to hepatic induction by co-culture with hepatic stellate cell line TWNT-1 and 1% dimethyl sulfoxide (DMSO) for 8 days, followed by culture in dexamethasone (40 ng/ml) for additional three days for hepatic maturation.

Embryonic bodies (EBs) were generated by plating dispase/collagenase passaged cells at a density of 1-5 x 10^4 cells per cm^2 on ultra low attachment plates (Corning, NY) for 48 hours in DMEM/F12 supplemented with 15% KSR, 1 Mm NEAA, L-glutamine and 1% 2 -mercapto-ethanol. For differentiation, EBs were plated on 5% Matrigel-Growth Factor Reduced (R&D Systems Inc. MN), and maintained for 3 days in DMEM/F12 media supplemented with 100 ng/ml Recombinant human activin-A (R&D Systems Inc., MN), 100 ng/ml FGF-2 (Millipore, Temecula, CA). The concentration of serum (fetal bovine serum-FBS or KSR) was 0% for the first 24 hours, 0.2% for the second 24 hours, and 2.0% for the last 24 hours. Cells were then grown for 8 days in DMEM-F12 containing 10% FBS or KSR, 1 mM NEAA, L-glutamine, 1% dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) and 100 ng/ml by co-culture with TWNT-1 using cell inserts, followed by culture for three additional days in DMEM-F12 containing 10% FBS or KSR, 1 mM non-essential amino-acids, L-glutamine, 10^{-7} M dexamethasone (Sigma-Aldrich). Hepatocyte-like cells were examined for morphology and growth, using an inverted phase contrast microscope Olympus I x 51 (Olympus, Tokyo, Japan).

Total RNA was purified from generated iPS derived hepatocyte-like cells using the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer`s instructions. Chromosomal DNA was removed using RNase-free DNase (Qiagen). Complementary DNA was prepared using the superscript first-strand synthesis system (Invitrogen, CA) and RNase inhibitor (Applied Biosystems, CA) from 2 μg of total RNA. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with AmpliTaq gold DNA polymerase, GeneAmp PCR gold buffer and $MgCl_2$ (Applied Biosystems, CA). PCR products were resolved on 2.5% agarose gels and visualized by ethidium bromide staining. Total RNA from human ES cells (HH1796) was used as positive control and from human fetal lung fibroblasts (IMR-90) cells as a negative control. GAPDH was used as an internal control. Primers used for RT-PCR in this study are listed below.

Oct 3/4 (78 base pair (bp)): sense, 5'-TGGGCTCGAGAAGCATGTG-3', anti-sense, 5'-GCATA GTCGCTGCTTGATCG- 3'.

Sox17 (186 bp): sense, 5'-CGCTTTCATGGTGTGGG CTAAGGACG-3', anti-sense, 5'-TAGTTGGGGTGGTCC TGCATGTGCTG- 3'.

ASGPR (230 bp): sense, 5'-CCACACTCCCCTAAGTT CCA-3', anti-sense, 5'-CTGTGCAGATGCCGAACTAA- 3'.

Albumin (598 bp): sense, 5'-ACTTTTATGCCCCGGAA TC-3', anti-sense, 5'-AGCAGCAGCACGACAGAGTA- 3'.

AFP (174 bp): sense, 5'-ACTGAATCCAGAACACTGCA-3', anti-sense, 5'-TGCAGTCAATGCATCTTTCA- 3'.

GAPDH (700 bp): sense, 5'-GTTCCATCAATGACCCC TTCATTG-3', anti-sense, 5'-GCTTCACCTTCTTCTTGA TGTCATC- 3'.

To assess the synthetic function of generated hepatocyte-like cells, we measured 24-hour secreted albumin secretion into culture medium using human albumin enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, TX) and metabolic rate was calculated as previously reported.[12] At the end of differentiation, cells were also assayed for ammonia-metabolizing capacity and urea production. Ammonium sulphate (Sigma-Aldrich, Irvine, UK) was added into the culture medium at the final concentration of 0.56 mM. The concentration of each reagent was measured 24 hours later to estimate the metabolic capacities. The ammonia concentration was determined using a Fuji Dri-Chem slide (Fuji, Japan), as per manufacturer's instructions. iPS cells were used as controls.

RESULTS

Directed differentiation of hiPS cells showed morphological characteristics of hepatocytes. Morphologically, the generated HLCs revealed polygonal shape with prominent nuclei, scant cytoplasm and enriched cytoplasmic granules under phase contrast microscope, which are comparable with the characteristics of normal human hepatocytes in culture (Figure 2 E).

iPS induced hepatocytes expressed human hepatocyte-specific gene markers. RT-PCR showed that the expression of albumin (Alb.), a marker for matured hepatocytes, progressively increased over the course of differentiation (Figure 3). Gene expression levels of hepatocyte-enriched markers,[13] such as Alb., ASGPR and cytosolic key control enzymes of gluconeogenesis phosphoenolpyruvate carboxykinase 1 (data not shown) also intensified in a time dependant manner. After 3 days of activin-A treatment, iPS cell-derived cells expressed endodermal markers Sox 17 and Fox A2. Pluripotency markers such as Oct 3/4 are gradually reduced and virtually absent in DE. Alpha fetoprotein (AFP) expression, which is present in endoderm and early hepatocytes development but is not expressed by mature hepatocytes, was still detected 16 days after culture, indicating that differentiation towards mature hepatocytes was not uniform.

Human iPS cell-induced HLCs secreted albumin into the culture medium, synthesized urea and metabolized loaded ammonia (Figure 4). Urea synthesis of the differentiated cells was tested with a urea nitrogen determination system. At the end of hepatic maturation stage, urea could be detected in the culture medium supernatant of the day 16 differentiated human iPS cells. The above data is indicative of three separate experiments. The result showed that hepatocyte-like cells hold a synthetic and metabolic activity as functional hepatocytes.

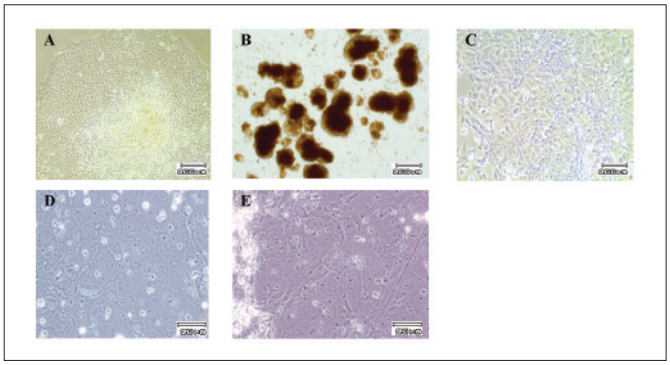

Figure 2: Morphology and growth of hepatocyte-like cells
Morphology of human iPS cells at various levels of differentiation toward hepatocyte-like cells. Round shaped colonies of iPS cells (A), suspended clustered cells called EBs (B), triangular spiky shaped cells referred as DE (C) and characteristic hepatocytic polygonal shape with prominent nuclei and scant cytoplasm (D&E). (Magnification: A, B & C (10x), D & E (20x)).

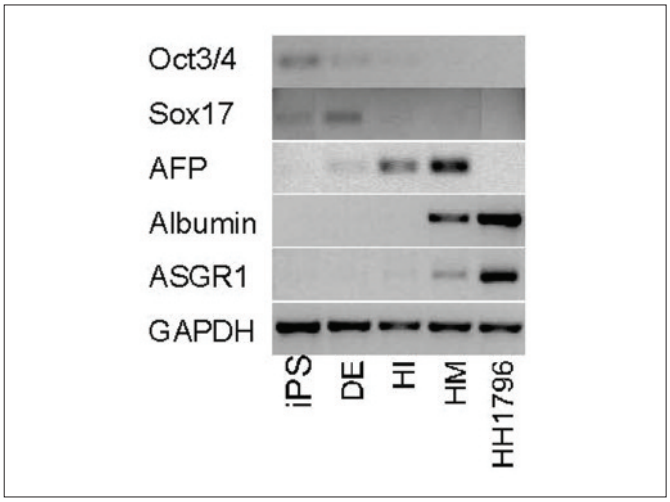

Figure 3: Human hepatocyte-specific gene markers expression.
RT-PCR gene expression of various hepatocyte-specific markers in hiPS-derived HLCs. Endodermal markers (Sox17), early hepatic development marker (AFP) and hepatocyte enriched markers (Albumin and ASGPR) are strongly expressed at various levels of differentiation.

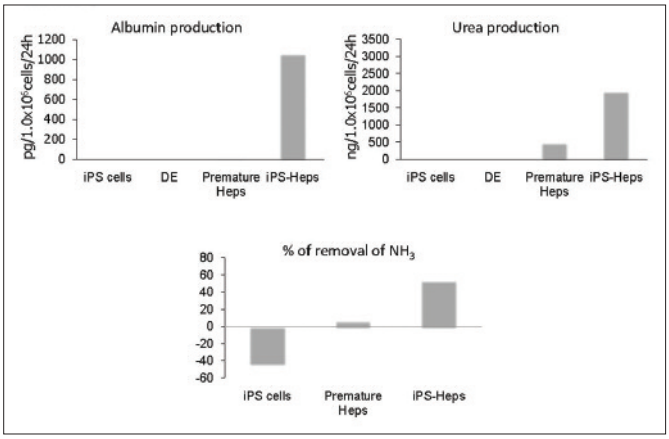

Figure 4: Functional assay of generated hepatocyte-like cells.
Metabolic activities of human iPS derived hepatocytes. (A) albumin secretion and (B) urea synthesis was determined during differentiation. Removal of loaded ammonia (C) was confirmed by iPS-derived hepatocytes, whereas ammonium concentration was increased in the culture of undifferentiated iPS cells.

DISCUSSION

Stem cell research offers great promise for understanding the basic mechanisms of human development and differentiation, as well as the hope for new treatments for diseases such as diabetes, Parkinson's disease, myocardial infarction, spinal cord lesions and hepatic failure. Because of their pluripotency and therapeutic potential in cell replacement therapy, iPS cells have become the most active field of biomedical research. Without the ethical and rejection concerns associated with hES cells, iPS cell technology has emerged as the most promising method for cell-based therapies of regenerative medicine.[14]

After establishment of iPS cells, the ultimate goal of stem cell researchers and clinicians is to treat patients with patient-specific iPS derivative cells. Hepatocytes, the primary cells of the liver, have attracted particular attention, as liver plays a central role in multiple functions of human body and liver failure is often only treatable by liver transplantation. However, the number of donors is insufficient to cope with growing demand for transplantation. While little can be done to increase the numbers of donor livers, it is reasonable to search for artificial means of liver replacement and/or assistance with the aim of either supporting patients with borderline functional liver cell mass until an appropriate organ become available for transplantation or until their livers recover from injury (regenerate). Hepatocyte transplantation, to increase the number of functional hepatocytes, could be employed as an alternative therapeutic approach to whole organ transplantation. Stem cells are the prime source to obtain the unlimited number of functional hepatocytes. The authors have previously shown that mouse ES cells can be differentiated to hepatocytes by sequential culture in activin-A, basic FGF, HGF and dexamethasone, and can be isolated by albumin promoter-based cell sort to generate functional hepatocytes.[15] They also successfully applied the differentiation protocol to human ES cells with minor modifications.[16] Therefore, in the present study, the authors applied the strategy to obtain relatively homogeneous population of hepatocyte-like cells from human iPS cells. To differentiate iPS cells to functional hepatocytes, floating cultivation was used to form EBs.[17] The iPS cells (Figure 2A) formed EBs after 2 days in suspension cultures (Figure 2B). EBs were maintained in adherent culture for 3 days in activin A and basic FGF and converted to the cells resembling definitive endoderm (Figure 2C).

Although NODAL, a member of transforming growth factor (TGFß) super family, is an attractive candidate for inducing DE differentiation,[18] another TGFß family member activin binds the same receptors as NODAL, triggering similar intracellular events,[19] and, therefore, can be used to mimic NODAL activity *in vitro*. This was characterized by gradual down regulation of undifferen-

tiated gene expression, such as Oct 3/4 and Nanog and up regulation of Sox 17 and Fox A2; the genes continuously expressed in definitive endoderm progenitors (Figure 3).[20] The cells were further differentiated in co-culture system for 8 days in the presence of human hepatic stellate cells and 3 days with dexamethasone. Co-culture of iPS cells maintained the favourable morphology of iPS-derived hepatocyte-like cells (Figure 2E) and synthetic functions as shown by the gradual sequential up regulation of AFP, ASGPR and albumin (Figure 3). The cell morphology changed from a triangular spiky shape to the characteristics polygonal shape with prominent nuclei and scant cytoplasm (Figure 2C-E). The cells exhibited the characteristic morphology of hepatocytes. The functional assay showed that these HLCs produced albumin, synthesized urea and metabolized ammonia (Figure 4). This data shows that human iPS cells could be differentiated to functional hepatocyte-like cells.

The precise mechanism of co-culture is not clearly known and is now under investigation. One explanation could be that enhanced hepatic differentiation of iPS cells was due to the presence of HGF and other important regulatory cytokines produced by stellate cells.[21] HGF is a mesenchymally derived potent mitogen for hepatocytes and acts as a growth factor for a broad spectrum of tissues and cell types.[22] It is speculated that lipid droplets in stellate cells may contribute to hepatic differentiation of iPS cells. Lipid droplets in hepatic stellate cells contain not only vitamin A, but they also contain triglycerides, cholesterol, and phospholipids.[23] The differentiation-inducing action of vitamin A has been well investigated.[24] More recently, unsaturated fatty acids have been recognized as important signals in diverse processes such as differentiation, development and proliferation.[25]

From the gene expression level (Figure 3), human iPS-derived hepatocytes can be used for transplantation, however, further improvement may be required particularly on maturation. The current protocol has significantly improved differentiation efficiency but iPS-derived HLCs are probably still at fetal liver developmental stage as indicated by high gene expression level of AFP. Further studies are needed to purify the protocol in order to determine whether differentiation protocol and enrichment strategy can be scaled up for use in humans and be modified to eliminate the risk of contamination of undifferentiated cells. In addition, there are concerns shown about the potential activation of viruses and genome mutations during the process of iPS induction that involves virus-based delivery of transcription factors. To overcome these issues, non-retroviral methods such as adenoviruses or cell-permeable recombinant proteins are already under investigation. Identification of small molecules to induce iPS cells without gene transfer may be another possibility.

CONCLUSION

The authors presented an efficient, cost-effective and reproducible instant hepatic differentiation of human iPS cells using co-culture system. It is hoped that this system will be useful in further development of using human iPS cell-derived hepatocytes for liver targeted cell therapies and for biomedical and clinical research and applications. Moreover, iPS-derived hepatocytes could be utilized to understand disease mechanisms and for assessing the toxicity of new drugs, liver being a major site for metabolism of many drugs.

Acknowledgement: This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Technology (to N.K), Japan. The authors are thankful to Professor Dr. Ira J Fox, Dr. Soto-Gutiérrez Aljandro and Dr. Nagaya Masaki, Assistant Professor, Department of Surgery, University of Pittsburgh of UPMC, U.S.A, for their technical and moral assistance and advice in carrying out this work.

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