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Novel Technology for Liver Regeneration and Replacement

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Organ transplantation has been grounded in the development of new technologies, both scientific and technical, over the past 50 years that have escalated organ and patient survival rates and have led organ transplantation to become the “gold standard” treatment for almost any type of organ failure.⁽¹⁾ This success has correspondingly led to an increase in the number of patients waiting for an organ for transplant and highlights the grave disparity between those patients requiring tissue replacement and available tissues and organs for transplantation. New discoveries in molecular and stem cell biology over the past 10 years have created opportunities to once again develop game-changing therapeutic technologies to repair damaged liver tissue or develop bioartificial replacement tissue when injury is too far advanced.^(2,3) This summary of the field in experimental liver regeneration and tissue replacement will describe the following:

1. Novel methods to reset the transcription factor network to repair damaged liver tissue.
2. New advances to bring nontransplantable organs into acceptable criteria for transplantation.
3. Strategies now on the horizon to develop humanized tissues within either chimeric animals or through new research in ex vivo tissue biomanufacturing.

Given the time and space limitations, an exhaustive discussion of all new technologies currently being investigated is not practical and so additional material will be referenced to provide additional sources for expanded review.

Reprogramming Transcription Networks

Transcription factors are regulatory networks of small molecules that activate or repress combinatorial clusters of downstream genes. This process in turn regulates cell function, phenotype as well as the degree of cellular maturation or pluripotency.⁽⁴⁾ Nishikawa et al., using a chronic liver injury model, showed that a cluster of transcription factors, specifically

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hepatocyte nuclear factor (HNF) 4 α , forkhead box A2, peroxisome proliferator-activated receptor (PPAR) α , HNF1 α , and CCAAT/enhancer binding protein α , are collectively down-regulated in the setting of hepatic failure.⁽⁵⁾ To establish their model, the group induced liver injury in Lewis rats by administering phenobarbital in the drinking water and carbon tetrachloride by gavage. Importantly, the investigators found significant down-regulation of the transcription factor HNF4 α , which controls the expression of multiple downstream genes within the liver. Further investigation with microarray analysis revealed the coordinated repression of the above-mentioned transcription factor groups in liver dysfunction. This finding raises the possibility that deficiencies in key transcription factors may enable, or worsen, liver dysfunction. In addition to new advances in strategies to reprogram cells to manipulate the degree of pluripotency/maturity or to induce repair, this work suggests a means by which HNF4 α could be replenished within injured cells to enhance organ recovery. The investigators developed an adeno-associated virus carrying HNF4 α linked to green fluorescent protein that was used to inoculate rodents with hepatic failure. Treated animals showed increased activity, improved neurologic function, and liver tests that returned toward normal levels. The authors concluded that reconstitution of the HNF4 α pool is due to correcting phenotypically abnormal hepatocytes and not by augmenting replacement through differentiation of progenitor cells. Other recent studies evaluating cellular renewal and repair within the liver are those by Yanger et al.⁽⁶⁾ and Schaub et al.⁽⁷⁾ and suggest similar results using other strategies, such as lineage tracing. These noteworthy studies by Fox et al. highlight the development of both new scientific data that potentially uncovers governing pathways leading toward liver injury and also describes novel treatment strategies that may be effective at reversing parenchyma disease or minimizing the rate of functional decompensation that may have the longterm effect of reducing the number of patients who require liver transplantation.

REPAIRING MARGINAL DONORS BY MACHINE PERFUSION

Elsewhere in this transplantation course, we will learn the pros and cons of machine perfusion for liver preservation and the degree to which this new technology will be applied; however, these devices not only maintain organ viability, they also provide a means to deliver novel restorative treatments to a damaged organ parenchyma. Organ perfusion has been used in lung transplantation for quite some time and has been effective at bringing those organs that may not meet standard criteria into the acceptable range for transplantation.⁽⁸⁾ Perfusion circuits not only have the potential to provide a biochemical readout of an organ's function and measure hydrodynamic profiles that may suggest chronic injury, but they also provide a conduit to administer agents that improve cell phenotype and potentially alter organ function.^(9–11) One common reason for rejecting livers for transplantation is increased steatosis, or fat content of the liver.⁽¹²⁾ As more than two-thirds of Americans are considered overweight and greater than one-third are obese (body mass index >30 kg/m²) macrosteatosis and microsteatosis in the liver will continue to be commonly encountered during liver recovery.^(13,14) Although liver perfusion has been well characterized in the literature and evaluated in limited clinical trials,^(15,16) Nagrath et al. published early work on “defatting” livers with steatosis using ex vivo organ perfusion along with infusion of specific agents that increase lipid export or its oxidation.⁽¹⁷⁾ Lipophilic ligand-activated transcription factors (eg, PPARs) are potent regulators of lipid oxidation and

transport. In their study, livers removed from obese Zucker rats supplied hepatocytes and whole livers to evaluate for “defatting” using combinations of agents. Isolated hepatocytes were cultured in fatty acid-supplemented media. Those hepatocytes that were subsequently cultured in a cocktail of defatting agents led to a 24% decrease in lipid content at 24 hours compared with untreated controls. Additionally, ketone body production (a surrogate marker for β -oxidation) was decreased in treated cells. Whole livers taken from Zucker rats were subsequently perfused with the defatting cocktail. Organs that were perfused without defatting treatment resulted in a 30% decrease in triglyceride content with the perfusate alone, which further decreased to 65% by the addition of defatting agents. Moreover, the defatting cocktail led to increased β -oxidation as measured by ketone body production that also required higher oxygenation to support lipid oxidation. A particular advantage of organ perfusion systems, such as the normothermic liver perfusion system described in this work, is the ability to deliver nutrients and oxygen to growing cells and may be responsible for the accelerated lipid oxidation seen in whole livers, as commented by Yarmush et al. in this study. Such a rapid reduction in steatosis within these fatty rodent livers suggest a timeframe that may be acceptable for eventual translation to human transplantation, though work in large animals and scaled-up perfusion systems are required to establish adequate oxygen delivery to these correspondingly larger liver grafts.

Induced Pluripotent Stem Cells

Induced pluripotent stem cells (IPSCs) were first developed in 2006 using mouse and later in 2007 using human cells.^(18–20) IPSCs are multipotential cells derived from somatic populations. Mature cells, such as fibroblasts or peripheral blood mononuclear cells, were initially transformed with retroviruses encoding for defined genes, octamer-binding transcription factor 4 3/4, SRY sex determining region Y-box 2, Kruppel-like factor 4, and c-myc,^(18,19) and later the latter 2 genes were substituted by NANOG and LIN28.⁽²⁰⁾ These defined factors “turn back the biological clock” of somatic cells to produce pluripotent stem cells that may then be coaxed to develop into all 3 germ layers. The potential benefits of IPSCs, beyond embryonic stem cells, are the possibility to derive a pluripotent stem cell population directly from mature cells from the cell donor. This relatively new technology, just 10 years old, opens the door for more accurate disease modeling with improved ability to reflect the condition within the donor patient.⁽²¹⁾ Moreover, IPSCs would potentially allow the development of cells and tissues with the same genetic makeup as the donor, providing an opportunity for “autologous” cell or tissue transplantation. In theory, these derived cell populations should elicit a minimal or no immune response as cells would be considered “self” by the immune system. Over the last several years, data pointing to this possibility have yielded conflicting results, as pluripotent IPSCs have been rejected upon implantation into syngeneic rodent recipients though as these cells progress toward maturity the corresponding immune response is progressively decreased.^(22–26) Furthermore, the process by which IPSCs are developed may also either prompt an immune response or predispose toward malignant potential as retroviruses may integrate into genomic DNA and thus risk activating oncogenes. Recent research using nonintegrative viruses, such as adenoviral and Sendai vectors, or episomal DNA or small molecules indicate that these strategies may reduce the potential for malignant transformation.

Presently, iPSCs have effectively been used to model patient disease within an in vitro setting. The next step as it applies to organ transplantation, is to develop a pathway to advance iPSCs toward cell and tissue therapy.⁽²⁷⁾ To realize this potential, large-scale and high throughput systems must be developed to mass-produce iPSCs and their mature progeny such that the final therapeutic product has adequate function and adheres to standardized quality measures. Presently, iPSC-derived hepatocytes still do not contain comparable potency to endogenous cells and express alpha fetal protein, indicating a degree of immature phenotype. Various strategies have been employed to date to enhance maturation of iPSC-derived hepatocytes including 3-dimensional culture conditions and growth in coculture environments with supportive fibroblasts or stromal cells.^(28,29) iPSCs have been used to develop human “liver buds” upon progressive differentiation within in vitro culture.^(30,31) After differentiating toward a hepatocyte lineage, Takebe et al.^(30,31) added endothelial and mesenchymal cells that resulted in self-assembly into organoid-like clusters. Upon transplantation into recipient rodents, these clusters ramified with the vasculature of the host and some human cells within these clusters produced albumin.

Blastocyst Complementation

A subsequent extension of cell reprogramming technology seen in iPSC development has been used to create chimeric animals in which pluripotent donor cells complement a functional defect in organogenesis within the recipient to develop an organ derived from the donor cells used to restore the defect. The potential end result is the development of an xenogeneic organ within an animal host. An early example of this technology was studied by Hiromitsu Nakauchi's both in rodents⁽³²⁾ and subsequently in pigs.⁽³³⁾ By altering the dosage of an exogenously administered *Hes1* gene through development of a genetic construct encoding the *Pdx1* promoter upstream of *Hes1*, the group introduced the transgene in vitro into matured pig oocytes via intra cytoplasmic sperm injection, resulting in the development of an atretic pancreas within 2 of 5 progeny animals. Fibroblasts recovered from a male offspring were used as nucleus donor cells for somatic cell cloning and resulted in cloned pigs that lacked a pancreas. Embryos harboring the transgene leading to defective pancreas formation taken at the morula stage were injected with blastomeres of donor embryos to add back the functional capacity to develop a pancreas by forming chimeric blastocysts that were transferred to recipient pigs (Fig. 1). These donor embryos were from female pigs and expressed orange fluorescent protein humanized Kusabira-Orange, allowing chimeric offspring to be identified. The authors found that chimeric animals were uniformly male and systemically expressed orange fluorescent protein. Chimeric offspring developed normal appearing pancreata that were densely cellular with islets of Langerhans dispersed throughout the organ parenchyma that were stained with the antibody to humanized Kusabira-Orange. Pigs with ineffective complementation were stillborn, whereas others developed hypoglycemia commonly seen in the newborn state while a few that survived up to 3 days displayed marked hyper-glycemia with glucose levels that were >600 mg/dL. Pigs with effective establishment of cellular chimerism developed normally with a complete pancreas and had normal serum glucose concentrations at 4, 6, and 12 months.⁽³³⁾ Interestingly, all organs, including testes, contained cells of donor origin; however, progeny from these chimeric animals failed to develop a pancreas indicating that the donor cells,

which effectively complemented the defect in organogenesis, were not carried within the germline. Though this study used pigs as the donor-recipient pair, it suggests a model in which pluripotent and xenogeneic cells (such as human iPSCs) may complement a genetic defect in organogenesis to produce an xenogeneic organ, such as a human organ within a porcine recipient. Next steps for this type of research, from both a scientific and ethical viewpoint, are to understand trafficking of the donor cells within the developing organism to evaluate target microenvironments that may become chimeric or develop largely from the donor cell populations. Furthermore, from a technical point of view, it will be important to delineate the origin of the vasculature that supports the parenchyma, which may still retain recipient cells. The US National Institutes of Health sought public comment in the summer of 2016 regarding the scope of certain human-animal chimera research that may eventually allow consideration for federal funding of certain types of research involving human tissue chimerism in animals under defined situations.⁽³⁴⁾

Ex Vivo Tissue Biomanufacturing

Ex vivo biomanufacturing of tissues is an alternative strategy to develop human-scale organs and cells for therapeutic purposes. Using technology to strip endogenous cells from donor organs via a process called “decellularization” results in the retention of the 3-dimensional scaffold, or otherwise called the extracellular matrix, that holds cells in place, facilitates cell-cell crosstalk, and gives organs and cells structure.⁽³⁵⁾ Such a process can be used to produce extracellular matrix scaffolds from nontransplantable human livers or organs from pigs.⁽³⁶⁾ Over the last few years, specific technologies such as bioreactors have been developed to add back donor cells to the scaffolds to reconstitute the parenchyma and recreate a functional organ.⁽³⁷⁾ The ability to reconstitute the scaffold of nontransplantable organs with iPSC-derived hepatocytes of recipient origin would potentially produce genetically identical and functional organs from grafts deemed nontransplantable due to high steatotic content. Alternately, the addition of human iPSC-derived cells to xeno-derived scaffolds may lead to the development of “humanized” organs that are likewise immunologically similar to the intended recipient. Specific protocols have been developed to isolate extracellular matrix scaffolds from livers as well as strategies to add back the cells.^(38–40) A specific obstacle still needed to be overcome is the development of large-scale quantities of functional cells that may be developed by iPSC technology. Alternatively, the development of rodents and pigs with a defect in the fumarylacetoacetate hydrolase gene, which causes hereditary tyrosinemia type 1, allows for the ability of donor human hepatocytes to proliferate and may be a source of functional human cells.⁽⁴¹⁾

Final Thoughts

In summary, new inroads in regenerative medicine have defined the tools that may one day change the way physicians treat organ failure. This overview provides a description of a subset of these technologies currently under development that still require refinement, an improved understanding of the longterm implications of these potential treatments, and development of ethical guidelines where appropriate. Academic, industry, and government partnerships will need to work in collaboration to advance these technologies and formulate functional standards to ensure potency, quality control, safety, and efficacy. Current

initiatives are doing just that, but it will be a gradual process to advance the technology to eventually develop tissues for those in need or the ability to repair injury and prevent development of end-stage disease.

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Abbreviations

HNF	hepatocyte nuclear factor
IPSC	induced pluripotent stem cell
PPAR	peroxisome proliferators-activated receptor

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Key Points

- 1.** Transcription factors may be harnessed to turn-back liver damage.
- 2.** Organ perfusion provides new routes to administer agents that improve graft function.
- 3.** New cell technologies provide for the development of new cells of recipient origin for disease modeling and transplantation.

FIG. 1.

The figure represents the process by which the defective phenotype in pancreatic organogenesis in Pdx1-Hes1 cloned pig embryos (A, lineage animas) are restored by infusion of donor blastomeres (B, lineage cells) encoding orange fluorescent protein humanized Kusabira-Orange. Figure reproduced with permission from Ref. 33.