EXPLORING CURRENTLY INVESTIGATED METHODS OF REGENERATIVE MEDICINE BASED THERAPIES FOR THE TREATMENT OF ACUTE KIDNEY INJURY

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LIST OF ABBREVIATIONS

2D	two-dimensional
AKI	Acute kidney injury
ANG1	angiopoietin-like 1
ANGPTL-4	angiopoietin-like 4
ATN	Acute tubular necrosis
ATP	adenosine triphosphate
BMA	bone marrow ablation
BMP	bone morphogenetic protein
BUN	blood urea nitrogen
CKD	Chronic kidney disease
ECM	Extra-cellular matrix

ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
ESRD	end-stage renal disease
EV	Extra-cellular vesicle
FBS	fetal bovine serum
FGF2	fibroblast growth factor 2
GFR	glomerular filtration rate
HBSP	helix-β surface peptide
HGF	hepatocyte growth factor
HPSC	human placental stem cell
HSC	hematopoietic stem cell
HUVECs	human umbilical vein endothelial cell
I/R	ischemia-reperfusion
ICP	inductive conformal prediction
IGF	insulin-like growth factor
iPSC	induced pluripotent stem cell
KDIGO	Kidney Disease Improving Outcomes
LC-MS	liquid chromatography mass spectrometry
LLM	large language models
miRNA	micro-RNA
ML	machine learning
MRPC	mouse renal progenitor cell

MSC	mesenchymal stem cell
MSH	Melanocyte stimulating hormone
NADPH	Nicotinamide adenine dinucleotide phosphate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCNA	Proliferating cell nuclear antigen
PRP	platelet rich plasma
RNA	ribonucleic acid
ROS	reactive oxidase species
scRNA-seq	single-cell RNA sequencing
SS-13	Szetso-Schiller 13
uPAR	urokinase receptor
VEGF	vascular endothelial growth factor

ABSTRACT

Acute kidney injury (AKI), most commonly a result of prolonged blood flow reduction, grows increasingly prevalent in hospitalized patient populations both within the United States and globally. Patients who have previously resolved AKI or who have sustained a severe AKI are at a greater risk for developing chronic kidney conditions. Currently available therapies for chronic conditions, such as dialysis and transplantation, are not options for acute injury, and current pharmacological approaches include reno-protective anesthetic alternatives such as propofol, as well as small molecule therapeutics with anti-inflammatory or selective vasoconstrictive effects to treat AKI after the fact, but with no documented functional restorative effects. For this reason, regenerative medicine-based therapies are a necessary approach in order to both mitigate damage and return normal tissue function. Here, we review the currently investigated regenerative medicine therapies to provide a comprehensive understanding of the state of latest progress within the field to better inform future research directions.

Stem cell implantation therapies are effective but have a primarily paracrine effect on regenerating injured tissue, and thus secretome- and extracellular vesicle-derived therapies become another viable avenue of treatment. To lower cost, minimize the need for cultures, and maximize shelf-stability, pre-mixed cocktails of identified relevant proteins and peptides are explored both independently and in conjunction. Implementation of computational analysis shows promise to target genetic up- and down-regulation surrounding the injury timeline, as well as deep-learning models to simulate designer

proteins' conformational design and unexpected adverse or secondary effects in digital injury models.

Future directions in regenerative medicine for treating acute kidney injury will mostly lean towards controlled trophic factor administration, as this allows the maximum effect with the minimum number of variables. The usage of machine learning models developed on aggregate proteomic data will allow for novel design and applications of multiple permutations of proteins in conjunction with one another at scale.

INTRODUCTION

Within the renal organ system, the kidneys are responsible for filtration of the blood and excretion of waste produced by normal bodily functions[1]. The minimum functional unit of the kidney is the nephron, which has its structures spread across both regions of the kidney: the cortex and the medulla. The glomerulus, the initial section of the nephron, is responsible for directly interfacing with capillary vasculature and allowing certain filtrates to pass through the membranes and foot processes of the podocytes. Capillary hydrostatic pressure becomes a regulating factor in determining which filtrates pass through which barriers, in turn affecting glomerular filtration rate (GFR), the net volume of fluid filtered per minute[1–3]. After this, the filtered fluid gets passed through the proximal renal tubules, where the high surface area and abundance of membrane transporter proteins like aquaporins along the brush border allows for appropriate water and nutrient reabsorption. From here, the nephron passes this filtered fluid through the tubules and collecting ducts and stores the resulting excrement as urine to be disposed of.

Acute kidney injury grows increasingly prevalent in hospitalized patient populations both within the United States and globally. Within hospitalized populations, incidence rates have gone up from 15.5% to 26.8% from 2011 to 2021[4]. Acute kidney injury is defined as an increase in serum creatinine by over 0.3 mg/dl within 48 hours, or over 1.5 times baseline within the previous 7 days, or a total urine output of less than 0.5 mL/kg/h for at least six hours, according to the Kidney Disease Improving Global Outcomes (KDIGO) Clinical Practice Guidelines for Acute Kidney Injury[5]. AKI injury

classification can be further organized into stages 1 to 3 (where 3 is the most severe with the least optimal patient outlook) and duration ("transient" injury that will resolve itself within 48 hours and "persistent" injury that does not resolve within 48 hours) - these classifications tend to be correlated with one another, as higher grades of injury tend to require more recovery time to return to baseline.

Most patients who develop AKI suffer from acute tubular necrosis (ATN). Due to the high metabolic activity of the proximal renal tubule cells, when the kidneys sustain any injury that affect local blood flow or oxygen availability, these are the first cells to go, and also typically where the worst of the damage can be seen. AKI also affects the GFR, worsened by the inactivity of the proximal tubule. This tends to manifest across multiple different causes of AKI whether it be induced perioperatively, as a result of preexisting health conditions that cause hypotension such as diabetes, due to drugs with side effects that lower renal perfusion, sepsis, or even from post-transplant ischemia-reperfusion injury[6–9].

AKI can resolve on its own, especially if it is a lesser injury. However, in the case that the injury is severe, the risk of developing long-term complications or chronic condition increases, and options for treatment become significantly more limited. Conditions like chronic kidney disease (CKD) and end-stage renal disease (ESRD), where functional capability of the kidney is severely or totally compromised and can be fatal[10,11], are not reversible, and the currently available therapies for such later-stage kidney diseases tend to centralize around 2 methods of treatment: dialysis to replace kidney function, and transplantation once an organ becomes available. These methods are

not without their negatives - both avenues of treatment require incredible lifestyle adjustments for the patient and can result in a weakened or otherwise compromised immune system; this is nothing to say of pressing issues such as a lack of donor availability compared to transplantation needs, or for dialysis, the incomplete replacement it offers not fully encompassing all kidney functions[12–15]. Furthermore, due to the nature of organ transplantation, there is a non-negligible chance of developing AKI even after transplant.

To mitigate the risk of kidney injury progressing to an irreversibly damaged state, treatments designed to protect renal cell populations and encourage proliferation at an earlier stage are ideal. Various approaches in the field of regenerative medicine have been investigated as viable alternatives for treatment: autologous and allogenic progenitor and stem cell therapies provide necessary cellular functions once differentiated into local cell populations that pharmacological approaches lack. Simplifying it further to simply the excreted factors allows for maximum control when administering, testing, and scaling the treatment for practical use at the bedside. To understand the breadth of what is available, both clinically and bench side, and aid future directions in AKI therapy research, a comprehensive review of current clinical approaches and actively investigated regenerative medicine-based approaches applicable to emergent AKI is paramount.

CHAPTER I

CAUSES OF ACUTE KIDNEY INJURY AND CURRENT CLINICAL APPROACHES

While the majority of AKI patients are the result of complications of post-surgical procedures, there are a variety of ways in which AKI can develop. Generally, all causes are categorized as pre-renal, intrarenal, and post-renal[16]. Prerenal AKI is a result of a significantly reduced rate of blood flow into the kidney, and can be caused by simple blood loss, septic shock, heart medication, or as a not-uncommon postoperative complication, typically of cardiac surgery. This disturbance in total fluid volume affects the pressure differential found in the Bowman's capsule and thus alters the GFR. This disrupts the process and restricts both nutrient and oxygen flow through the nephron, which can lead to intra-renal injury.

Intra-renal injury is not necessarily preceded by pre-renal injury. Prescription medication can cause tubular injury within the kidney without damaging the body outside of the kidney. Analgesics, for example, can reduce overall GFR by inhibiting prostaglandins responsible for vasodilation; some antibiotics can, in high enough concentrations, bind to the tubular basement membrane and be recognized by dendritic cells, kickstarting an immune response[17]. This may lead to acute interstitial nephritis, which can further lead to AKI should the damage be sustained long enough. However, the single most common intrarenal cause of AKI is ischemia-reperfusion (I/R) injury. During the initial ischemia, the dearth of available oxygen begins anaerobic glycolysis,

which breaks down adenosine triphosphate (ATP) into small molecules that do not necessarily provide the energy needed to allow the cells to continue functioning. Specifically, ATP-dependent transmembrane pumps cease function, and the failure to maintain ionic gradients allows for oxidase formation, phospholipase activation, and cell membrane degradation[18]. Reperfusion, paradoxically, does not ameliorate the situation when reintroducing oxygen into this anaerobic environment. Rather, it becomes a source for reactive oxidase species (ROS) to form, which disrupt cell membrane lipid structures and ultimately lead to cell death[7,18]. In the nephron, proximal tubular epithelial cells are the most vulnerable due to their proximity and high metabolic activity (and therefore increased dependency on oxygen and ATP) and thus suffer first – acute tubular necrosis sets in, leading to tubule dilation and brush border loss, further exacerbating the level of injury[18,19]. Finally, post-renal causes of AKI typically include blockages or obstructions, such as tumors, calculus, blood clots, or pregnancy[6].

Dialysis is the artificial or manual removal of waste and water from the blood, which can be done either by hemodialysis, which involves continuous blood flow through a dialyzer multiple times a week, or peritoneal dialysis, which is the process of adding dialysate into the lining of the abdomen, then draining about three to five times per day. Dialysis in treating AKI has been hotly contested as AKI can be a transient injury, healing on its own with minimal or manageable lasting damage. Therefore, introducing renal replacement therapy is not always seen as a particularly efficient approach to treatment, especially when considering the fact that the exact timing of when to introduce a patient to dialysis is not widely agreed upon due to inconclusive and contradictory

information. Some studies indicate no meaningful effect, some instead say the earlier the better to attenuate organs from damage, others say that it only would show any effect in AKI severe enough to stop kidney function outright, others still say that due to dialysis' single function as filtration replacement, it does not have enough power as an independent variable to sway mortality rates[20–24]. Other functions of the kidney, such as blood pressure modulation and hormone production, can hardly be addressed with dialysis alone.

Transplantation is the surgical removal and replacement of diseased or injured tissue with healthy donor tissue. Generally partial transplantation is preferred over radical or whole transplantation, especially considering that estimated GFR in patients with radical nephrectomy decline more than with partial nephrectomy. Overall, transplantation is generally a last resort when all other avenues of treatment have been exhausted. In fact, it is not very popular for acute kidney injury for a number of reasons: firstly, as mentioned previously, AKI can be transient and resolve on its own. A kidney transplant is typically saved for CKD or ESRD that cannot be reversed or managed without intervention. Moreso, kidney transplantation itself is considered a risk factor in developing AKI[25–27]. The case for transplantation for AKI is not helped by the fact that due to a shortage of available organs suitable for transplant, the demand already greatly surpasses the supply, even without AKI being a qualifying condition to receive a transplant[12]. For these reasons, alternative approaches to treating AKI must be investigated.

CHAPTER II

PHARMACOLOGICAL AND MOLECULAR APPROACHES TO AKI THERAPY

Prescription drug and pharmacological compound approaches encompass a broad field of study, as it refers to any study of the therapeutic effects of drugs or chemicals on living organisms. Specific interventions to address AKI in earlier stages have been investigated, but none have been approved by the FDA so far due to either still being in clinical trial phases or targeting too late past onset[28]. Most of these products have been shown to have some sort of ameliorative effect on injured cells in other medical applications and are then indicated as potential interventions to prevent or slow AKI. Often times, these products are used to treat some of the underlying causes of AKI, as opposed to reversing damage after the fact. Multiple ongoing clinical trials are observing the effect that certain drugs have on AKI, each one addressing a different method by which AKI develops.

Due to post-operative development of renal damage after transplant, specifically that caused by significantly decreased blood flow during surgery due to anesthetic, different types of anesthetics are also investigated to absolutely minimize the amount of damage that occurs during surgery. Propofol is an anesthetic with widespread use as a sedative among its alternatives. It has demonstrated free radical scavenging capabilities[29], as well as bone morphogenetic protein 2 (BMP2) and transforming growth factor β1 (TGF-β1) upregulation effects, which has been implicated in neo-angiogenesis[29–31]. Clinical trials investigating the difference between desflurane as an anesthetic and propofol as an

anesthetic, to determine if propofol has more renal-protective effects both in brain-dead donor kidney transplantation[32] and valvular heart surgery[33] were conducted at Yonsei University, although results were inconclusive[34]. RBT-1, by Renibus Therapeutics, Inc., is a combination drug of stannic protoporfin and iron sucrose that is investigated for its anti-oxidant and anti-inflammatory effects demonstrated in human patients post-cardiac surgery[35], reducing I/R AKI as a result of post-operative complications[36,37].

Certain proteins have been indicated as potential avenues of treatment. Hepatocyte growth factor (HGF) is known to be a potent hepatotropic factor with anti-apoptotic effects on renal cells, as well as agnogenic effects on endothelial cells[38,39]. However, elevation of HGF has been paradoxically indicated to worsen patient outcomes in the case of AKI[40], likely due to overexpression inhibiting pathways that produce HGF as a byproduct of their cellular protective effects[28,36,41]. Insulin-like growth factor (IGF) is another popular target protein indicated in AKI, naturally secreted by the collecting duct and a key regulator of cell proliferation and apoptosis. IGF infusions in fasted rats have previously been shown to have net positive effects on GFR in both healthy and AKI-model rats[42,43], however it failed to show functional efficacy in I/R injured rat models, resulting in an exacerbated inflammatory response[44]. In addition, human trials for treating delayed graft function also were futile, as symptoms such as recurrent renal injury and multi-organ failure preclude direct IGF administration as a therapy for AKI[42,45].

Small molecule therapies include AptaBio Therapeutics' APX-115 aim to address the ROS that generate during in contrast-induced AKI. As an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase enzymes, the drug aims to reduce

oxidative stress patients experience when receiving contrast media[46]. The Phase 2 clinical trial is ongoing and actively recruiting participants. OCE-205, a novel vasopressin mixed agonist/antagonist peptide therapy by Ocelot Bio, Inc. has also undergone clinical trials to determine the safety and efficacy of the treatment for AKI developed from cirrhosis[47]. A single intravenous infusion of OCE-205 was determined to be safe and well tolerated in 64 healthy human subjects 18 to 45 years of age, with an overall increase in mean arterial pressure, mild adverse events such as abdominal pain and diarrhea, and only one recorded severe adverse event of bradycardia[48].

Peptides have also been investigated for the treatment of AKI. Szeto-Schiller-13 (SS-13) has been shown to protect mitochondria during oxidative stress and improve inflammation in I/R mice[49,50]. In addition, tubular injury scores were noticeably reduced in histological analysis of I/R mouse kidney tissue treated with SS-13 as opposed to I/R tissue without treatment, as well as an increase in proliferating cell nuclear antigen (PCNA), decrease in oxidative stress, and limited tubular detachment. Erythropoietin, a growth factor secreted and used by the kidney, has a peptide analogue called helix-β surface peptide (HBSP) that has led to a decrease in apoptosis and upregulation of erythropoietin production, signaling repair after I/R injury[49,51,52]. In human trials as well, human brain natriuretic peptide injected intravenously into patients with ESRD as a result of cardiorenal syndrome showed potential for reducing emergency dialysis need and overall renal protective effects, indicating potential for AKI therapy as well[49,53].

The common drawback between all drug- and bioactive molecule-based approaches to treating AKI, however, unfortunately lies in the nature of the treatment. Each

previously listed therapy addresses the underlying cause, but they do not necessarily help restore tissue or organ function – at most, it will stall further damage, which is helpful in cases of mild AKI or when AKI is predicted or spotted early. However, if the damage is severe or noticed after some time from initial onset, this is not enough. For this reason, methods in regenerative medicine that allow for both cell protection *and* encouragement of cell proliferation simultaneously is paramount.

CHAPTER III

CELL THERAPIES

Cell therapies involve the direct transfer of a specific autologous or allogeneic cell population into a patient for therapeutic purposes[54]. The design may include biocompatible scaffolds, hydrogels, manipulation of genetic material, multi-cell populations, and so forth. This method has been widely investigated as an option to mitigate damage sustained by injury and encourage injured cell populations to self-regenerate[55,56], broadly across multiple injury types. Due to this demonstrated effectiveness, this is considered a promising viable therapy for treating AKI.

The potential for mesenchymal stem cells (MSCs) to proliferate rapidly with little encouragement identify the cell population as a potential source for regenerative therapies[57]. In theory, the implanted stem cells would proliferate and, using the host's environmental biomarkers from the site of injury, self-differentiate into the necessary cells required for healing, including but not limited to tubule cells, podocytes, and vasculature. In a previous study[58], an MSC-based cell therapy aimed at creating scaffold-free two-dimensional (2D) sheets of transplantable cells that secreted select factors known for their therapeutic effects, then tested in mice with ischemia-reperfusion (I/R) induced AKI. The MSCs were harvested from human umbilical cord, then genetically engineered using zinc finger nuclease-assisted transfection to integrate puromycin resistance at targeted genes responsible for producing certain growth factors: VEGF,

angiopoietin-like 1 (ANG1), erythropoietin (EPO), and alpha-melanocyte stimulating hormone (MSH). Testing *in vitro* was conducted using a cell proliferation assay on human umbilical vein endothelial cells (HUVECs) to ensure the angiogenic effects of the engineered populations of MSCs. The sheets themselves were generated by seeding the cells onto a N-isopropylacrylamide coated dish, then exposing the dish to varying temperatures to shrink and detach the sheet from the plate. This sheet was then applied to the surface of the kidney in I/R injured mice (model created via bilateral clamping of the renal pedicle for 30 minutes). The engineered cells were also injected intravenously, but serum BUN and creatinine levels were overall higher than observed in the cell sheet treatment. This approach is beneficial overall, but in practice, the time required to culture cells into a two-dimensional monolayer may past the threshold for irreversible damage. Other studies conducted using MSCs have administered the cells intravenously, intra-arterially, and topically, but recent research also investigates the direct administration to the site of injury[59].

In clinical trials[60] conducted in post-cardiac surgical patients where MSCs were directly injected into the femoral artery of patients via intra-aortal catheter, few benefits were noticed. Multiple plausible explanations as to why this therapy essentially failed to yield results that were significantly better than the placebo group are numerous: the time of diagnosis, as the administration of the stem cell implantation would be applied too late, and ideal administration time is quite early; the very ischemia causing the injury in the first place also made it difficult for the cells to travel into the injured tissue effectively and thus hinder their performance[61]. In high enough doses, MSCs have been found in the

lungs of I/R injured rats designed to better understand the mechanism of the ameliorative effect MSCs have on AKI[62]. The implanted MSCs may have differentiated into some type of immunogenic cell population(s) and induced more harm in the tissue, a behavior observed in other studies conducted in other tissues using MSC implantation therapies[63].

Human placental derived stem cells (HPSCs) are a population of stem cells that share many characteristics with MSCs like the ability to be cultured in standard cell medium and a high differentiation capability, while having low to no carcinogenic risk[71] and higher plasticity and proliferation potential than adult tissue-sourced MSCs[72,73]. This population of cells, typically derived from umbilical cord blood, tends to have similar immunomodulatory effects within injured tissue as MSCs derived across multiple sources[72,74], indicating it as a potential source population for cell therapies. For example, in a study conducted by Gryguc, et al., HPSCs were suspended in phosphate-buffered saline (PBS) and injected into the corticomedullary region of I/R injured kidneys of rats. These rats then had blood and tissue samples assessed for biomarkers of injury, and had a marked improvement in kidney outcomes compared to untreated rats[75]. In humans, although no clinical trials currently exist to treat specifically AKI with HPSCs, a dose escalation study was conducted in human patients with critical limb ischemia to determine the safety of PLX-PAD, an injectable placental stem cell therapy, as a treatment due to known cytokine secretions such as VEGF, angiopoietins, hepatocyte growth factor, and more, being linked to angiogenesis and cell proliferation while downregulating inflammation after injury[76]. Aside from injection site reactions, transient allergic reactions, bruising, and other mild adverse events, the therapy was determined to

be acceptably safe, and even had some positive clinical effect documented. Pain scores decreased, amputation-free survival rate was higher than comparable groups, and tissue perfusion increased over time after PLX-PAD injection, all positive indications of therapy efficacy.

Autologous renal stem cells and progenitor cells have been previously shown to ameliorate injury when directly engrafted or injected into the site of injury[64]. When sourced from the host itself, there is also the decreased risk of graft rejection. In a study conducted in mice, progenitor cells were harvested from mice and characterized to determine expression of renal progenitor cell markers, then subcultured to quantify differentiation potential[65–67]. These mouse renal progenitor cells (MRPCs) were then injected, suspended in 50 μL of PBS, erythropoietin, or suramin, and the mice were observed for seven days. In all groups treated with MRPCs, with or without erythropoietin or suramin, serum creatinine levels, blood urea nitrogen levels, and histological evaluation of acute tubular necrosis all indicated a strong therapeutic effect when administered to I/R injured mice. In a similar fashion, a study conducted 25 human kidney transplant patients who were suffering from acute tubular necrosis (a marker for AKI) due to delayed graft function aimed to determine if the regenerative effects seen as they recover come from renal progenitor cells positive for CD133, Pax-2, and CD24[64]. As histological analysis showed, the populations of renal cells taken from biopsies positive for these markers were markedly increased post-transplantation as compared to pre-transplantation, implying these renal progenitor cells heavily proliferate in response to injury and therefore likely play a major role in human renal tissue regeneration.

Another popular and widely studied source of cells for is hematopoietic stem cells (HSCs) found in bone marrow, as these stem cells have previously demonstrated a capability of being recruited to ischemic tissue and differentiating into vascular structures and promoting neo-angiogenesis[68]. In a study conducted in mice[69], bone marrow was harvested from donor mice, depleted of lineage positive cells to ensure a population of undifferentiated cells, then injected intravenously via the retroorbital sinus into I/R injured mice that also received bone marrow ablation (BMA), I/R injured mice without BMA, and control (sham operation) mice that received BMA. These mice were then sacrificed 24 hours afterwards, with samples taken from both kidneys to quantify healing from injury and blood to determine mobility of the injected stem cells. In control mice, circulating implanted lineage-negative cells were not detectable above background, whereas in I/R injured mice, anywhere from 20-24% of all non-red blood cells in the sample were lineage-negative HSCs. Kidney tissue histological analysis also showed that compared to the control, I/R mice with BMA had better tubular necrosis recovery and overall lower levels of blood urea nitrogen (BUN).

A different study also used mice to harvest lineage-negative and $Rh^{lo}Lin^{-}Sca\text{-}1^{+}ckit^{+}$ cells, then administered to I/R injured mice (15-minute clamp time + reperfusion) intravenously via the tail vein[70]. X-Gal staining showed donor-derived cells found throughout the kidney in the injured models that received HSCs, but not in the control or in injured samples without HSCs. The tissue samples were also stained with beta-galactosidase and renal proximal tubular marker to determine if the identity of the HSCs that already express beta-galactosidase may be aligning more with renal cell markers, and

positive staining on the I/R injured samples that received HSC transplant therapy indicate that is the case.

Induced pluripotent stem cells (iPSCs) are yet another popular source of progenitor cells due to their high versatility, differentiable ability, and ability to be reverse engineered via gene modification from various populations of somatic cells. One method is encouraging induced iPSCs to self-organize into necessary structures that may be lost with severe AKI, such as nephrons, in theory to eventually be able to have progenitors self-organize *ex-vivo* into enough tissue to graft or even transplant [77,78]. Hydrogels like alginate, Type 1 collagen, and decellularized renal extracellular matrix are used as vehicles for the delivery of engineered kidney tissue, partially due to the convenience of being able to inject gels into the body, and partially due to the fact that these hydrogels can be fabricated with total control, such as optimizing stiffness or including various growth factors such as VEGF, HGF, and IGF-1 to help stimulate differentiation and integration into the host environment[79–81]. Unfortunately, the accurate and consistent self-development for more delicate and minuscule structures such as renal tubules, collecting ducts, and glomeruli "remain challenging," as the tissues struggle to generate these structures in studies conducted so far *in vitro*[4,77,82]. iPSCs have been successfully assembled into renal proximal tubule-like structures[83], but these cells are assembled using stepwise growth factor exposure methods that produce an organoid monoculture. This makes tissue constructs ideal for developing injury models for the purpose of experimental study, but it is not preferred for direct tissue implantation. In addition, much like the MSC sheet approach, the time required to culture and

differentiate these cells becomes prohibitive in urgent cases. Recurrent issues with these methods of engineering tissue to simulate the function of the kidney also include cost, host microenvironment integration and rejection, time spent harvesting tissue or cells for and preparing necessary cultures, and post-transplant maintenance of structural integrity[4,84]. In addition, most of the approaches require quite invasive surgical interventions; although it may be necessary in certain cases, limiting the invasiveness of a procedure is often the more greatly beneficial option, and should be explored if possible.

As *ex-vivo* differentiation tends to lack the structural complexity found naturally in a nephron, allowing the stem cells to differentiate after engraftment in vivo becomes an option instead, as Lee, et. al discuss[85]. In that study, iPSC cells were administered intra-arterially in I/R injured mice which displayed renal cell markers after implantation, improved overall renal function after injury, and are implicated in reducing oxidative stress. However, differentiating the iPSCs prior to transplantation, so as not to rely on inconsistent differentiation after implantation, has also been studied. Xia, et al., discusses a method of differentiation of human iPSCs into mesodermal cells that present with gene signatures like that of renal progenitor cells[86]. Utilizing growth factors associated with renal lineage commitment, such as retinoic acid, activin A, fibroblast growth factor 2 (FGF2), and bone morphogenetic protein (BMP) 2 and 4, the cells began to specify themselves towards "renal-like lineages," with a significant diminishing effect on pluripotent markers. Polymerase chain reaction (PCR) analysis also suggested, thanks to a weak displayed induction of genes related to the metanephric mesenchyme, that these iPSCs would differentiate into multiple types of renal-cell-like populations.

The specific mechanism by which all implanted cell therapies greatly increased regeneration of injured tissue and protection from further injury are not fully understood, but data from previously established studies show that the paracrine effects of the secreted factors produced by the implanted stem cell populations are the drivers of cell proliferation and regeneration. Paired with issues of implanted cell death[87] and a near total lack of observed differentiation into renal cells once implanted *in vivo*[84], the potential for utilizing the conditioned medium stem cells are grown in is another consideration for treating AKI is substantial[57]; in fact, in studies across various conditions, stem cell secretome therapy showed great promise[88] as a form of preventing further injury or cell death and encouraging healing.

CHAPTER IV

STEM CELL SECRETOME-BASED THERAPIES

The secretome of a cell is collection of all of the proteins, extracellular vesicles, cytokines, lipids, and other signaling molecules secreted by a cell or tissue into the extracellular space[89]. The phrase "stem cell secretome" specifically refers to the cell supernatant collected over the culture of a given population of stem cells. Typically, this supernatant is collected over cells incubated for one to three days in medium without fetal bovine serum (FBS) to minimize any interference provided by serum proteins[90]. However, the proteins found in this collected secretome tend to be in low concentration and thus susceptible to noise interference during analysis, so techniques such as centrifugation and evaporation to increase the concentration of the secretome are employed (Figure 1) [91,92]. Secretome analysis can be conducted with liquid-chromatography mass spectrometry (LC-MS), enzyme-linked immunosorbent assay (ELISA), 2-dimensional gel electrophoresis, and ribonucleic acid (RNA) extraction and sequencing to determine the identities of as many unknown components within the secretome as possible[90–93]. Previous direct stem cell implantation therapies indicate a paracrine effect[94], as not all implanted progenitor or stem cells will differentiate into local cell populations and instead signal the cells in their immediate environment to regenerate. For this reason, stem-cell conditioned medium has been indicated as a potential avenue for treating AKI[88].

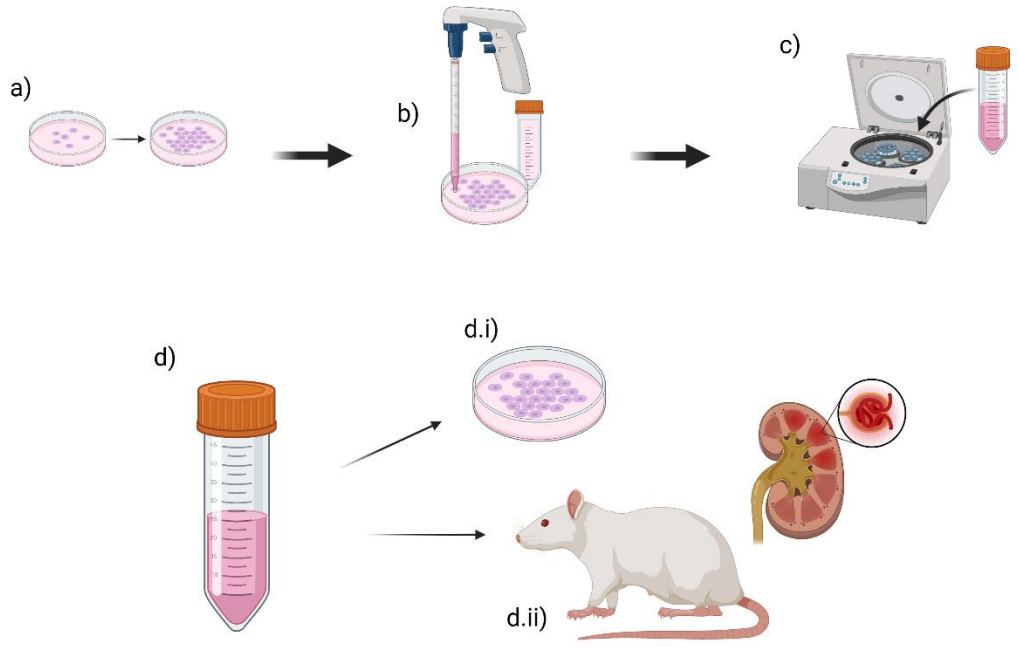

Figure 1: Graphical depiction of collection and concentration of stem cell secretome via centrifugation. a) Cell culture in serum-free media. b) Collection of conditioned medium. c) Centrifugation of medium until desired concentration. d) Application of conditioned medium in in-vtro experiments (d.i) or in-vivo (d.ii).

As discussed earlier, HPSCs secrete a wide variety of cytokines and growth factors indicated in angiogenesis and anti-inflammatory response. Proteins with known immunomodulatory and cell protective effects, such as HGF, VEGF, IGF-1, TGF-β, and more have been identified in secreted factor analysis of HPSC culture supernatant[76,95]. Given placental stem cells' protective and anti-fibrotic effects in acute kidney injury models[96], the HPSC secretome, cultured and concentrated by 4 times, is used to study the effects of direct administration to the site of injury[97]. Using platelet-rich plasma gel as a vehicle to sustain the delivery of concentrated HPSC medium over time, the administered therapy provided noticeably improved results in rodent AKI models within 7 days after injury. This effect was noticed both as tubular reconstruction (histological analysis) and kidney function retention (serum analysis).

Other methods of delivering the excreted factors of stem cells for the purpose of tissue regeneration have been studied: the exosomes and microRNA (miRNA) of MSCs have been used to observe potential regenerative effects in various types of tissue, including but not limited to skin, nerve, and bone tissue[18]. For exosomes specifically, research on micro-vesicles derived from MSCs has been shown to reduce ROS in I/R injured rats, with lowered tubular lesion scores, lower TUNEL-positive and higher KI67-positive histological analysis, and even lowered fibrosis in vesicle treated groups compared to control groups[98,99]. In real-time, MSC-derived extracellular vesicles have also been shown to be accumulated in renal tubules, then up-taken by the tubular epithelium in I/R injured mice, inducing mitochondrial protective mechanisms by limiting fragmentation and increasing miRNA-200a-3p expression, activating Keap1-Nrf2 signaling pathway and ultimately having renal protective effects[99,100].

Bone marrow stem-cell (BMSC) derived extracellular vesicles (EVs) have also been tested in vivo as a source of secreted factors with potential therapeutic effects. In rats with bilateral fibrotic renal injury[101], increased markers of fibrotic injury expression, such as tubular atrophy, glomerular enlargement, and increased collagen deposition, were seen at higher rates than inured rats that also received BMSC-EVs collected from the centrifuged media of BMSCs from 4-week old donor rats. In a similar study specifically investigating BMSC-EVs on AKI, rats with gentamicin-induced injury that received the conditioned medium from BMSCs had lowered expressions of pro-inflammatory cytokines such as interleukin 6, and increased expressions of anti-inflammatory cytokines interleukin-10[102]. The levels of expression also were found to be similar to the group of

gentamicin-injured rats that were treated with an injected BMSC transplant, supporting the idea of the cell therapies' paracrine effect rather than differentiable and establishing secretome injection therapy as a comparably effective option.

Unfortunately, the methods of isolating exosomes are difficult, as the cells themselves can be quite heterogenous and thus their excreted factors change with minute alterations to their microenvironment, the ability to appropriately dose amounts of extracellular vesicles is inconsistent, and miRNA, even delivered via EVs, tends to have low cell membrane permeability[88,99,103,104]. Furthermore, the inherent heterogeneity of cell culture that originates from a donor can result in variations across batches of both cell therapies and secretome therapies, which is a problem when considering scaling for mass production. Even if the cell line is controlled and immortalized, minuscule and undetectable variations in their microenvironment due to handling can also introduce variation in concentration and identity of factors introduced. The precise identity and concentrations of the growth factors, signaling molecules, and extracellular vesicles found in the secretome have not been completely discovered. Therefore, to some degree, there is an unknown factor in what is being administered when using the secretome directly. To combat this issue, distilling a secretome into the fundamental components that encourage all the associated positive effects and mitigate the negative can be developed to better control outcomes. Therefore, secretome-informed bioactive- and small molecule-based therapies might provide maximal control in effect with minimal or predictable side effects.

Factors such as HGF, IGF-1, and VEGF have already been associated with pro-renal and pro-angiogenic effects[6,38,39,105]. Isolation of individual growth factors secreted by stem cells, such as in one study involving genetically modified MSCs with silenced IGF-1, allow us to identify the specific effects of individual factors – in this instance, IGF-1 was found to limit apoptosis in cisplatin-induced AKI[106]. Transplantation of IGF-1 blocked MSCs showed a significant decrease in cell proliferation in comparison. Clinical analysis of hospital patients with AKI also demonstrated higher mortality rates associated with lower serum IGF-1[107]. In this way, independently administering such growth factors has been indicated

Determination of dosage and joint usage with multiple factors at a time are difficult to ascertain in a vacuum; for this reason, using the stem cell secretome as a model provides adequate base information for *in vivo* experimentation. In one such study, a recombinant protein cocktail containing only the proteins specifically identified for their angiogenic, anti-apoptotic, and proliferative effects is studied as a potential therapy for AKI[108]. Secretome analysis conducted on HPSC cell culture medium via multiplex ELISA identified the most abundantly present proteins associated with general healing. Five that were associated with cell proliferation, anti-apoptosis, and angiogenesis were identified in the analysis: follistatin, angiopoietin-like 4 (ANGPTL-4), urokinase receptor (uPAR), VEGF, and HGF[105,109–114]. Reconstituting all these proteins at concentrations akin to that found in the 4X concentrated HPSC conditioned medium, in platelet-rich plasma (PRP) hydrogel, allowed for easy injection into the site of injury in a rodent AKI model. This showed significant anti-apoptotic, proliferative, and angiogenic effects in

ischemia/reperfusion (I/R) injury rat models. Provided that the PRP method of creating a hydrogel to sustain the delivery of the cocktail over the course of seven days has in fact documented adverse side effects in the form of thrombus formation[115], other hydrogels such as collagen-based hydrogels are being investigated as potential alternatives with low- to no-immunogenicity[116]. As commercially synthesized proteins have long shelf stability times when stored correctly at low temperatures[117] once produced (compared to culture medium), this can provide a viable scalable approach to treating AKI.

CHAPTER V

COMPUTATIONAL METHODS OF INNOVATION IN DEVELOPMENT AND TESTING OF THERAPIES

With the advent of large language models (LLMs) such as OpenAI's ChatGPT, Anthropic's Claude, and Google's Gemini, it would be remiss to overlook the new space that seems to have been carved out for the usage of artificial intelligence, machine learning algorithms, and deep learning models specifically for biomedical research, and how it may pertain to the future stem-cell- and secretome-based regenerative medicine therapies. Genetic sequencing data, such as that gained from single-cell RNA sequencing (scRNA-seq), have been previously used to establish the genetic mechanisms involved in controlling kidney recovery after injury[118]. By analyzing large datasets of genomic and transcriptomic data, the exact gene expressions responsible for regulating the body's response to renal injury can be uncovered and utilized to guide further research optimizing how therapies are developed. The issue, however, is that the large data sets are unconscionably large, and sorting through it all is a laborious and time-intensive task riddled with redundancy and noise - which is where deep learning models come in.

Using machine learning algorithms attuned to clearing noise from large datasets such as those acquired via RNA sequencing, identifying redundant information, and isolating key features of interest for future analysis is a textbook application of deep learning algorithms. Inductive conformal prediction (ICP) has been studied as a method

of using such algorithms to clean and identify mislabeled biomedical data[119]. Simulated noise and necessary reclassification were successfully conducted by the model, even with incompletely processed datasets, indicating a possible future application in larger sequencing data sets (Figure 2).

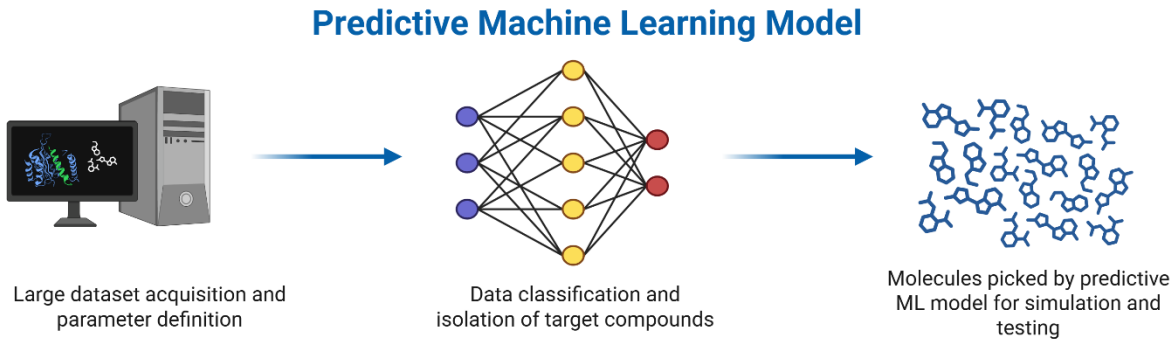

Figure 2: Predictive machine learning model for identification and simulation of compounds

Beyond analyzing raw data collected from cell cultures that exist in vitro, machine learning can also be used to simulate protein-ligand interactions in silico. In protein–ligand modeling, supervised machine learning (ML) models trained on large structural and biochemical datasets can learn complex, non-linear relationships between molecular features and binding affinity, kinetics, or specificity, allowing rapid virtual screening of vast chemical libraries that would be infeasible with docking or molecular dynamics alone. An open-access project "Target 2035" is an initiative in the infancy stages of creating a large, annotated database for the binding information of over 2,000 proteins and small molecules. This database is then intended to be used to train a deep learning algorithm to be able to successfully predict interactions between lesser-studied proteins and the potential hit that could be used to identify the presence of said protein

using a chemical probe. This is beneficial for the purposes of drug discovery, as interactions between biologically available small molecules and proteins, especially lesser-studied or novel proteins, can be simulated and predicted in a controlled virtual environment without worrying about the heterogeneity of cell culture or potential adverse effects in live subjects - animal or otherwise[120]. This would limit the amount of time spent in trial-and-error developing small molecules or designer growth factors and allow for more efficient development and testing. It also may be able to reveal certain protein-protein interactions within the human biological environment and reveal relationships that may be beneficial or detrimental to AKI regenerative and therapeutic purposes.

Deep learning algorithms can also be used to design proteins that specifically respond to certain small molecules and produce an intended effect. Diffusion models and deep-learning-guided models have been shown to be capable of simulating dynamic proteins that can then be used to model biological interactions[121]. In fact, these models can conduct physics-based simulations that mimic the biological environment of these novel proteins. These generative models can be conditioned on functional constraints—such as binding pocket geometry, catalytic residue placement, or stability metrics—to design proteins that adopt specific conformational states or undergo controlled conformational transitions upon ligand binding. Whole safety experiments can be pre-simulated before real clinical trials, potentially allowing them to predict adverse events, unforeseen side effects, and more well in advance.

CONCLUSION

The development of regenerative medicine therapies for the treatment of kidney diseases is an ever-evolving field. Pharmacological intervention as it currently stands has a fairly well-established ability to ameliorate blood flow and reduce further injury once administered early or pre-emptively. In the event of a later diagnosis or emergent situation, however, stem cell-derived therapies are a promising way to address kidney injury in both early and late stages. Allowing progenitor or stem cells to be injected intravenously or implanted, with or without guiding hormones and biomarkers, directly into the site of injury has shown great ameliorative effects. To limit the variability of direct application of active cells, secretome and extracellular vesicle administration allows for a concentrated approach to treating AKI. For even further control, specific growth factor and protein administration, with dosage and joint usage informed by analysis of presence and abundance found in the effective stem cell secretome, can further simplify and increase the efficacy of the therapy, providing a shelf-stable and more readily scalable therapy with fewer costs. Future directions of regenerative medicine therapies for AKI may be pioneered by machine learning algorithms in designing new pharmaceutical protein designs, modifying them as indicated by simulations, and predicting side effects prior to clinical trials to maximize patient safety and trial efficacy.

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CURRICULUM VITAE

Anisha Krishnan

EDUCATION

M.S., Translational Biotechnology, Wake Forest, Winston-Salem, NC (May 2026)

B.S., Neuroscience, George Mason University, Fairfax, VA (May 2023)

AWARDS

2026 Best Poster, WFIRM All-Hands Retreat (February 2026)

ACTIVITIES

2026 Medical Students for a Sustainable Future

2026 Biomedical Graduate Student Association Liaison

2023 Learning Assistant

Poster Presentations

Anisha Krishnan, Olivia Clemensen, Sunil George, Hemanth Thangappazham, Young Min Ju, James Yoo, Anthony Atala, John Jackson, Ji Hyun Kim

Exploring Methods of Regenerative Medicine Based Therapies for the Treatment of Kidney Injuries: A Systematic Review

2026 WFIRM All-Hands Retreat