UNIVERSITA' DEGLI STUDI DI PADOVA

DIPARTIMENTO DI SCIENZE FARMACEUTICHE

SCUOLA DI DOTTORATO DI RICERCA
BIOLOGIA E MEDICINA DELLA RIGENERAZIONE
INDIRIZZO IN INGEGNERIA DEI TESSUTI E DEI TRAPIANTI
XXI CICLO

TESI DI DOTTORATO

AMNIOTIC FLUID: STEM CELLS AND PROGENITOR CELLS FOR KIDNEY REGENERATION

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To my Family

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ABSTRACT

End Stage Renal Disease (ESRD) is a condition of kidney failure with loss of functionality in all the primary renal functions. The major cause of ESRD is Chronic Kidney Disease (CKD) characterized by a slow progression of kidney injury fibrosis and glomerular sclerosis. Many are the causes for Chronic Kidney Disease and treatments are directed to slow down the progress of the renal functional deterioration before the necessity for dialysis and/or transplant.

Alport Syndrome (AS) is a well known model for CKD. AS is a hereditary disease caused by mutations in the genes that codify for collagen IV α chains. Collagen IV is the main constituent of the glomerular basal membrane (GBM), essential for the proper filtration activity of the nephron. In the fetal stage Collagen IV network is constituted by α 1-2 chains that are normally replaced in the adult with α 3-4-5 chains networks. In AS, the absence of Collagen IV α 3-4-5 leads to the deposition of the α 1-2 chains, unable to sustain the ultrafiltration and the blood pressure. Therefore the collagen scaffold of the GBM is defective and, over time, the GBM splits and thins. A cascade of events leads to GBM destruction and eventually ESRD. No definitive treatments are available for Alport Syndrome and CKD. The use of antihypertensive slows the injury progression but eventually dialysis or transplants are required. Nonetheless, the budget cost and the side effects of dialysis and the shortage of organ donors are major limits to these therapies and scientists and clinicians are investigating new tools for kidney regeneration.

The use of stem cells has been a breakthrough for Regenerative Medicine in the last years. In this study we are investigating potential of stem cells and progenitor cells from Amniotic Fluid (AF) for renal regeneration, in particular for CKD.

Amniotic fluid fills the amniotic cavity, providing an environment in which floats the developing embryo and late the fetus. The volume and composition of the amniotic fluid changes during pregnancy and reflects the physiology of the fetus. Amniotic Fluid is principally composed by fetal urine and fetal lung liquid, with minor contribution from the amnion. In 2007, the presence of a pluripotent population of Amniotic Fluid Stem Cells (AFSC) was reported.

Beside AFSC that constitutes about the 0.8-1% of the total population, mesenchymal and hematopoietic cells have been shown to be present within Amniotic Fluid but its composition is still poorly understood.

Two different approaches were investigated in this study for possible use of stem cells in renal regeneration. Since Amniotic Fluid by 8 weeks of gestation is mainly derived by fetal urination, it could represent a source for kidney derived cells. In order to better understand Amniotic Fluid cell composition a wide characterization has been performed for cells derived from the three germ layers (ectoderm, mesoderm, endoderm), pluripotent cells and organ-specific progenitor cells, focusing mainly on renal precursors.

The expression of markers from endoderm and mesoderm was seen to be higher in samples of earlier gestational age, while ectodermal markers showed constant rates of expression across gestational ages. A population of cells positive for CD24 and OB-Cadherin was isolated as uninduced Metanephric Mesenchyme derived population from human Amniotic Fluid. From this cell line, immunoseparation was used to characterize four distinct subpopulations specific for expression of E-Cadherin, Nephrin, NGF high affinity receptor, and PDGFR α. These subpopulations are possibly representative of different cells committed to four of the renal cell types: mesangial cells, expressing PDGFR α, podocytes, expressing nephrin, stromal cells, positive for TrKA and Mesenchymal to Epithelial Transition cells, expressing E-Cadherin.

In our second approach, we injected AFSC into Alport Syndrome mice. We investigated life span, levels of haematic creatinine and nitrogens,

urinary albumin and collagen IV α 1 deposition in the glomerular compartment.

AFSC injected AS mice where compared with non treated AS mice and Wild Type mice.

We have shown a decrease in haematic creatinine and nitrogens, decrease of urinary albumin, decrease in deposition of Collagen IV α 1 and prolongation of life in injected Alport Syndrome mice. Evaluation of the results obtained, allows us to speculate that AFSC can be useful for CKD treatment.

In summary, Amniotic Fluid is a repository for many cells that could be used for kidney regeneration. Specific progenitor cells are residing within Amniotic Fluid and may be tested for kidney differentiation in *in vitro* and *in vivo* models.

In addition, AFSC are able to integrate within the glomerular structure and participate to the restoration of the renal homeostasis. Investigation about AFSC *in vivo* mechanism of action and evaluation with multiple injections and different time points could give us better information about their potential to cure AS and CKD.

ABSTRACT IN LINGUA ITALIANA

Negli ultimi anni l'incidenza di malattie renali croniche ha visto un netto incremento. L'allungamento dell'aspettativa di vita e il numero crescente di pazienti con malattie quali diabete e ipertensione, che provocano danno renale e possono portare allo sviluppo di malattia renale cronica, sono considerate causa principale di questo aumento.

I trattamenti farmacologici disponibili sono scarsi e si limitano a rallentare la progressione della malattia e la dialisi presenta importanti effetti collaterali. La mancanza di organi rende il trapianto d'organo scarsamente accessibile all'elevato numero di pazienti nelle liste d'attesa. La ricerca di terapie alternative ci ha spinto allo studio di nuovi approcci per la rigenerazione renale mediante l'utilizzo di cellule staminali e progenitori renali.

La Sindrome di Alport (AS) rappresenta un ben noto modello per l'insufficienza renale di tipo cronico. AS e' una malattia ereditaria provocata da una mutazione nel gene codificante il Collagene IV, costituente principale della membrana basale glomerulare (GBM), responsabile principale dell'attivita' filtrante del rene. La mancata produzione di catene α 3-4-5, sostituite con le catene fetali α 1-2, porta ad un progressivo deterioramento della funzione renale che si conclude con il collasso dei reni e la morte del paziente.

In particolare, le cellule staminali da liquido amniotico (AFSC), si sono dimostrate, in studi precedenti, capaci di integrarsi e differenziare in reni embrionici, provando la loro potenzialita' per la rigenerazione renale. L'assenza di teratomi, la facilita' di coltura e l'accessibilita' alla fonte rendono le AFSC candidati ideali per la loro applicazione clinica. Le AFSC sono solamente circa 0.8-1% della totale popolazione amniotica. Il liquido amniotico contiene diverse popolazioni cellulari tra cui cellule pluripotenti,

progenitori di diversi organi e cellule differenziate, e poco si conosce della sua specifica composizione cellulare.

Nel presente studio abbiamo scelto due diversi approcci per la cura della'insufficienza renale cronica: 1. Identificazione di specifici progenitori renali all'interno del liquido amniotico per un eventuale utilizzo in Medicina Rigenerativa. 2. Valutazione *in vivo* della capacita' di AFSC nel supportare la rigenerazione renale in un modello murino di AS.

Nel primo esperimento sono state studiate ed identificate le varie popolazioni cellulari presenti nel liquido amniotico a diverse settimane di gestazione. I campioni, di eta' compresa tra le 15 e le 20 settimane di gestazione, sono stati ottenuti tramite amniocentesi, tecnica usata per studiare il cariotipo del feto durante lo sviluppo.
Sono stati valutati differenti terreni di coltura, indagando proliferazione e conservazione della morfologia nei campioni ottenuti.
L'analisi e la caratterizzazione della popolazione totale presente nel liquido amniotico e' stata effettuata utilizzando RT-PCR, Real Time PCR e Western Blotting, analizzando l'espressione specifica di geni che sono coinvolti nel mantenimento della pluripotenzialita', geni che identificano specificatamente i tre foglietti embrionali (endoderma, mesoderma ed ectoderma) ed infine geni che identificano progenitori organo-specifici. Sono state inoltre identificate popolazioni specifiche renali, tramite immunoseparazione con biglie magnetiche (MASC). L'espressione di marcatori per i foglietti embrionali endoderma e mesoderma e' piu' alta in campioni piu' giovani rispetto a campioni con tempo di gestazione maggiore mentre, per l'ectoderma, rimane pressoche' invariata nel tempo.
La presenza di cellule pluripotenti e' costante cosi' come le cellule staminali mesenchimali mentre le cellule progenitrici ematopoietiche, investigate tramite CD34, fanno la loro comparsa successivamente alle 17 settimane di gestazione.

La presenza di progenitori tessuto specifici già “committed” e’ evidente nei campioni di gestazione più avanzata sia per quantitita’ che per specificità dell’organo preso in esame.

E’ stata approfondita l’analisi di cellule progenitrici renali, utilizzando un ampio pannello di marcatori che identificano sia la componente tubulare che quella glomerulare del nefrone, struttura fondamentale per la filtrazione renale. I risultati ottenuti confermano la presenza di cellule progenitrici renali dopo le 17 settimane di gestazione.

E’ stata identifica e studiata una popolazione esprimente CD24 e OB-Cadherin isolata da campioni di liquido amniotico di 17 o piu’ settimane.

CD24 e OB-Cadehrin sono stati identificati nel topo come co-espressi *in vivo* nel mesenchima metanefrico. Dal mesenchima metanefrico ha origine il nefrone ed e’ una delle due strutture embrionali fondamentali per lo sviluppo del rene. Da questa popolazione principale sono state ottenute 4 nuove sottopopolazioni che identificano sottocompartimenti del glomerulo, come per esempio le cellule corticali stromogeniche (tramite selezione per la Tyrosin Kinase, TrKA), i podociti (selezionati per la Nefrina), le cellule del mesangio (con selezione positiva per Platelet Derived Growth Factor Receptor α, PDGFRα) e le cellule in transizione mesenchima-epitelio (con selezione per la E-Cadherin). Tramite PCR e Real Time PCR e’ stata dimostrata la forte specificita’ di ogni singola linea cellulare.

Parallelamente uno studio e’ stato effettuato *in vivo*, con l’utilizzo di un modello animale affetto da Sindrome di Alport. AFSC sono state iniettate intracardialmente e i progressi sono stati seguiti comparando il progresso della malattia con quelli in topi AS non iniettati e topi Wild Type.

I risultati preliminari ottenuti hanno evidenziato un allungamento dell’aspettativa di vita nei topi AS trattati, con diminuzione dei livelli di proteinuria, livelli ematici di nitrogeni e creatinina e di albumina nelle urine.

Analisi istologiche hanno evidenziato una diminuzione di produzione di collagene fetale IV α 1 nella capsula di Bowman e nella membrana basale glomerulare degli animali trattati.

E’ necessario uno studio approfondito che preveda per le AKPC differenziazioni *in vitro*, utilizzando diversi fattori di crescita nefro-specifici

in modo tale da confermare la loro completa differenziazione in cellule renali mature.

Un approfondimento sul meccanismo d'azione e sulle migliori tempisitiche di somministrazione, sono i punti fondamentali da chiarire per comprendere il meccanismo d'azione delle AFSC *in vivo*.

Questa ricerca puo' essere una base fondamentale per future applicazioni cliniche in pazienti che soffrono di nefropatie.

INTRODUCTION

1. End Stage Renal Disease, causes and outcomes

End Stage Renal Disease (ESRD) is a condition of chronic and progressive injury of the kidney, leading to a complete failure of the renal system. ESRD usually occurs when renal functionality is less than 10% of normal activity[1].

According to the 2007 United States Renal Data System, the number of United States patients in treatment for ESRD was 400,000, with more than 20,000 waiting for organ transplantation. Predictions for the year 2020 are showing an increase in patients undergoing dialysis and in need of kidney replacement.

Progression to ESRD can be simplified within two major processes known as Acute Kidney Failure and Chronic Kidney Disease.

Acute Kidney Failure (AKF) is characterized by sudden and fast kidney function deterioration. Pathological kidney functionality is characterized by a decrease in filtration rate, starting from previously called pre-renal acute kidney injury and up to unresponsiveness. In the acute setting, the two most significant threats to renal perfusion pressure are systemic arterial hypotension and increased intra-abdominal pressure[2] and about 4% of all critically ill patients with AKF will require dialysis. Kidney stones, infections, cancer or drugs intoxication can be causes for AKF[3]. Common treatments to increase renal perfusion are increasing cardiac output, replenishing the circulating volume, enhancing cardiac inotropy, and inducing vasoconstriction.

Chronic Kidney Disease (CKD) is recognized as a major health problem affecting approximately 13% of the United States population. Numbers of

prevalent CKD patients will continue to rise, reflecting the growing elderly population[4].

CKD is defined as the presence of kidney damage, manifested by abnormal albumin excretion or decreased kidney function that persists for more than 3 months.

Typically, kidney function is quantified by glomerular filtration rate (GFR), the rate at which an ultrafiltrate of plasma is produced by glomeruli per unit of time and is the best estimate of the number of functioning nephrons or functional renal mass. To obtain the GFR it's required to measure the renal clearance of molecules with a steady blood concentration, in order to minimize errors in the evaluation. In the current clinical practice GFR value is established considering many factors such as age, weight, race and most frequently estimated using equations that incorporate serum creatinine along with demographic data[5].

To facilitate assessment of CKD severity, the National Kidney Foundation developed criteria as part of its Kidney Disease Outcomes Quality Initiative (NKF K/DOQI) to stratify CKD patients:

- Stage 1: normal eGFR ≥ 90 mL/min per 1.73 m2 and persistent albuminuria
- Stage 2: eGFR between 60 to 89 mL/min per 1.73 m2
- Stage 3: eGFR between 30 to 59 mL/min per 1.73 m2
- Stage 4: eGFR between 15 to 29 mL/min per 1.73 m2
- Stage 5: eGFR < 15 mL/min per 1.73 m2 or End-Stage Renal Disease

The prevalence of these stages of CKD in the United States population is as follows: 1.8% for stage 1, 3.2% for stage 2, 7.7% for stage 3, and 0.35% for stages 4 and 5. Patients with stage 3 or 4 disease progress to End-Stage Renal Disease or stage 5 at a rate of 1.5% per year. Stage 1 or 2 CKD patients progress to more advanced stages at approximately 0.5% per year[6].

The early stages of CKD (stages 1 and 2) are manifested by kidney damage and are generally asymptomatic: the kidney functions normally but the risk for progressive disease is significant.

As kidney disease worsens, renal function begins to deteriorate (stages 3 and 4 CKD). Eventually, kidney failure (stage 5) occurs and kidney replacement therapy is required[7].

Common origins for CKD are pathologies affecting the kidney compartment like analgesic nephropathy, glomerulonephritis, kidney stones, obstructive uropathy and reflux nephropathy, lupus, and polycystic kidney disease, genetic malformations or diseases affecting other organs like diabetes and hypertension[8].

Nearly 45% of incident kidney failure is attributed to diabetes and another 20% is attributed to chronic hypertension. More than 10 million Americans are diabetic and 40 to 50 million American adults have hypertension, constituting an enormous at-risk population for kidney disease[9].

Complications derived from CKD are various. In chronic renal failure the loss of function is usually coupled with an increase of fibrosis, amyloid deposition and glomeruli destruction. Major sequelae of CKD include continued progression of CKD and development of kidney failure requiring kidney replacement therapy, development and/or progression of cardiovascular disease, anemia, and bone disease.

1.1 Chronic Kidney Disease Therapy

Many are the therapeutic and pharmacological tools used by clinicians to slow the progression and symptoms of ESRD but the only effective treatments up to now are dialysis and transplantation.

In the treatment of patients with impaired kidney functionality, it is important to recognize that the major risk for patients with CKD is death from cardiovascular disease or diabetic complications.

Currently CKD drug therapy in the early stages is limited to administration of antihypertensive medication to decrease blood pressure, and

consequentially decrease the risk of injury provoked by high blood pressure, and limit proteinuria[4]. The use of a particular class of antihypertensives, called Angiotensin-Converting Enzyme (ACE) inhibitors, was reported to give additional renoprotection[10].

Generally, two major mechanisms have been implicated to explain the additional effects of ACE inhibitors: (i) suppression of angiotensin II formation (ii) increase of kinin concentration via inhibition of bradykinin degradation. Both pathways can cause a decrease in glomerular capillary pressure, proteinuria, or growth of renal cells, accompanied by increased degradation of extracellular matrix[11-14].

Treatments with pharmaceuticals are a good start point to treat kidney disease but at long term are not effective because they interfere with very specific pathways. Mechanisms that lead to ESRD are multiple and very complex and the administration of one or more medicines is not enough to treat and cure the CKD.

Even if on pharmacological therapy many patients eventually require renal replacement therapy, or dialysis. Dialysis is a clinical procedure that substitutes the loss function of the kidney for what concerns the blood purification. The use of external hemodialyzer or “inside the body” dialysate administration is the current therapy for dialysis. Although either intermittent or continuous current artificial renal replacement therapies can administer substantive small- and middle-molecule solute and fluid clearance, dialysis is not a complete replacement therapy. In addition to its major role in maintaining the constant extracellular environment, the kidney has many other roles. It is regarded as an endocrine organ, responsible for the secretion of hormones that are critical in maintaining hemodynamics (renin, angiotension II, prostaglandins, nitric oxide, endothelin, and bradykinin), red blood cell production (erythropoietin), bone metabolism (1,25-dihydroxyvitamin D 3 or calcitriol). The traditional renal replacement therapies, based on diffusion, convection, or absorption, provide only filtration; they do not replace these lost homeostatic, regulatory, metabolic, and endocrine functions of the kidney[15].

In addition, although life-sustaining, dialysis does not provide a high quality

of life to most patients and several side effects may occur during dialysis such as hypotension, arrhythmia, and complications of vascular access placement. Plus, some studies highlighted concerns regarding an increased risk of slowing the recovery of renal function and developing ESRD by dialysis treatment. [16-20]

Several studies reported that a kidney transplant from a live donor should be promoted as the first choice for eligible patients who require renal replacement therapy. Outcomes with deceased donor kidneys are also significantly better than with dialysis[21]. Since the first kidney transplants were performed in the 1950s[22], there have been major advances in both transplantation and dialysis and the risks and benefits for both options have changed. Kidney transplantation has lifestyle advantages and is cheaper than dialysis and this makes transplantation the first choice treatment for ESRD patients. Nonetheless, availability of donor kidneys is very limited. Many adults on the deceased donor waiting list will die on dialysis before they receive an organ. Unrelated kidney transplantation is increasing due to organ shortages. [23]

Increase of risk factors such as age, obesity and hypertension is raising the demand for organs and by now is possible to transplant not well matched organs, thanks to the use of immunosuppressive drugs. At first, kidney transplant regimens relied on steroids and azathioprine to prevent rejection with some success, but the real success was the introduction of cyclosporin over 30 years ago[24]. More recently, new and potent immunosuppressive drugs have been introduced including tacrolimus, mycophenolate and sirolimus. As might be expected with better immunosuppressive regimens, acute rejection rates fell over the decades. However, immunosuppression takes its toll in both the short and long term. In the short term, infection is a particular concern, especially with viruses such as cytomegalovirus. However, in the long term, the incidence of most cancers is increased in patients who are immunosuppressed[25]. The risk of cancer incidence in patients now under the novel, more aggressive immunosuppressive therapies, may be higher than expected.

1.2 Alport Syndrome

Alport Syndrome (AS), identified for the first time in the early 1900', is a well known model for CKD leading to ESRD. AS is a hereditary disease characterized by hematuria and proteinuria caused by structural defects of the Glomerular Basement Membrane (GBM), the main responsible for the filtration process. This rare disease, affecting 1 out of 20,000 people, is leading to a progressive loss of renal function ending in 10-12 years to ESRD. Etiology and clinical outcome are heterogeneous and often kidney defects are associated with deafness and ocular lesions[26]. Mutations in the gene codifying for the major α chains (3, 4 and 5) of type IV collagen are preventing the correct assembly of the extracellular membrane network in the GBM. Any of the genes encoding these three chains can be involved in the pathogenesis of this syndrome[27].

Mutation in COL4A5, encoding for the collagen IV α 5 chain causes about 85% of all the AS cases (X-linked AS). The other 15% (14% recessive and 1% dominant) presents a mutation affecting the expression of COL4A3 or COL4A4. The first subtle manifestations of classical AS (X-Linked) usually appear early in life. Hematuria may be detectable by one year of age in about 15% of cases, and by six years of age in about 70% of cases. Occasionally, there may be brief episodes lasting for days during which the blood is visible to the eye; Proteinuria is not usually evident in infancy, but increases during the first two decades.

If a renal biopsy is performed in the first few years of life, segments of the renal basement membranes may be somewhat thin, but the characteristic unraveled appearance of basement membrane collagen may not be evident until several years later. Overtime, the basement membranes become progressively thickened or fibrosed in parallel with decreasing overall function of the kidney[28].

Progressive loss of kidney functions in AS is variable. In some families, affected males may require dialysis as early as 20 years and invariably by 31 years of age; in others, dialysis is not needed until about 40 years. Deafness is bilateral, but may be subtle in childhood. Formal audiometric

studies detect some level of hearing abnormality in about 85% of affected boys by age 15 and many require hearing aids by the age of 25. About 25-30% of patients have a characteristic abnormality of the shape of the lens, as well as changes in the retina at the back of the eye. Although useful in diagnosis, these problems are not usually associated with severe loss of vision[29].

Currently, there is no definitive therapy to delay progression to ESRD for patients with AS and the only treatment currently in use is administration of Angiotensin – Converting Enzyme Inhibitors (ACE Inhibitors) to slow the damage progress[30].

2. Regenerative Medicine and Kidney regeneration

In order to overcome the limits of current therapies, scientists and clinicians have looked for alternative approaches for CKD management.

In the last years Regenerative Medicine has grown as an alternative for many diseases.

Regenerative Medicine defines a wide field of both research and clinical therapies involving the improvement of healing and reconstruction of tissues and/or organs. The main goal of Regenerative Medicine is the complete replacement of a damaged organ through the regeneration *in situ* or the transplantation of a bio-engineered and functional organ reconstructed *in vitro*[31]. In the last two decades Regenerative Medicine has been increasing the efforts of the scientific community toward the discovery of new clinical tools for treatment of acute, chronic and genetic kidney disorders.

2.1 Tissue Engineering for Kidney regeneration

Tissue Engineering, that combines natural or biodegradable polymers with cells and growth factors, has contributed in the recent years to the field of kidney regeneration. The perfect implantable device needs to mimic main

physiological function of the native kidney and it needs to operate incessantly to remove solutes. It will be optimum to design perfect membrane that has the same filtration capability as the nephron.

Humes *et al*[32] showed the creation of membrane that has both pore selectivity and at the same time hydraulic permeability as the native kidney. The creation of better bioartificial hemofilters is important to overcome the problem of loss of filtration due to thrombotic occlusion and protein deposition and that exclude the use of anticoagulant in the extracorporeal units that very often results in bleeding for the patient[32].

Few experiments have been conducted where renal cells were cultured *in vitro*, seeded into a polyglycolic acid polymer scaffold and subsequently implanted into athimic mice[33]. Over time, formation of nephron-like structures was observed within the polymer. These preliminary results when improved could easily be used to produce three-dimensional functional renal structures that can be used in *ex-vivo* or *in vivo* filtering units. This approach is called cell-based tissue-engineering as it refers to the use of scaffolds (natural or synthetic) and cells mixed together to recreate a tissue that mimics the physiological one by size and functionality. An important study by Lanza *et a*l[34], used therapeutic cloning to produce genetically identical renal tissue in a bovine model. Nonetheless since adult cells are completely differentiated and their response to growth factors can be absent or different from cells in the developing kidney, the seeding, the integration and the interaction *in vitro* or *in vivo* systems can fail or be partial. Therefore a great improvement came from the discovery of stem cells and their pluripotential capability.

2.2 Stem Cells

In recent past, the potential use of stem cells and the advancement in stem cell research for Regenerative Medicine and in particular for repairing kidney injury is considered as an alternative therapeutic strategy.

The interest about stem cells has been increasing over the past years, since their discovery in the early ’90s. Stem cells might be a promising tool for regenerative purposes because of their capability to become almost any cell of an adult organism. The definition of stem cell is not yet clear, but it is universally accepted that a stem cell possesses two fundamental characteristics: long term self-renewal and pluripotentiality.

Self renewal describes the unique capability of these cells to reproduce itself indefinitely while producing also cell progeny that mature into more specialized, organ specific cells. In this process, called asymmetric division, a stem cell divides into another stem cell and a cell that is going to differentiate and divide symmetrically. Pluripotentiality of stem cells is defined as the ability of a stem cell to give rise to different tissues. The fertilized oocyte is totipotent, able to differentiate into all the embryonic and extraembryonic tissues. Pluripotent stem cells are defined by the ability to differentiate, under certain stimuli from the surrounding environment, into many different mature cells of all the three germ layers and germ cells. A cell is defined as multipotent if can give rise to more than one cell type and unipotent if it can differentiate into one cell type. Based on their capability to differentiate into different cell types, stem cells are divided into different categories.

Embryonic Stem Cells

Embryonic Stem Cells (ESC) are collected from the Inner Cell Mass (ICM) of the blastocyst at five days from the fertilization of the egg. The blastocyst includes three structures: the trophoblast, which is the layer of cells that surrounds the blastocyst; the blastocoel, which is the hollow cavity inside the blastocyst; and the ICM, which is a group of approximately 30 cells at one end of the blastocoel. ESCs are defined as pluripotent, meaning they have the capability to give rise to cells derived from all the three germ layers (endoderm, ectoderm and mesoderm). For this reason, ESCs have been widely investigated for their wide capability

to differentiate into any cell line of the body, being a reliable tool for cell development and differentiation pathways studies.

Primordial Germ Cells

Primordial Germ Cells (PGC) are retrieved from the embryo at 5-9 weeks of gestation. The germ cell lineage is discriminated from somatic cell lineage during development and repression of the somatic cell fate is therefore a key event during germ cell specification. The specification is initiated by signals provided by the Extraembryonic Ectoderm (ExE), and the Visceral Endoderm (VE) that surrounds the epiblast cells and instructs a small number of epiblast cells to become PGCs. Since PGCs are retrieved at a later time when the original ESCs have started to commit to a specific pathway of differentiation, they present limited plastic properties if compared with ESCs.

Adult Stem Cells

Adult Stem Cells (ASC) are located within the tissues of the adult body. Their function is, under specific stimulation, to undergo differentiation and replace the loss of cells in an injured compartment. A specific organ localization called niche is thought to harbor the stem cells in an environment that protect cells from differentiation. One example of ASC are hematopoietic stem cells (HSCs). HSCs, localized within the bone marrow, are the most important adult stem cell line, discovered over 30 years ago. HSCs are commonly used for transplant for the treatment of leukemia diseases. In bone marrow another population called bone marrow stromal stem cells (MSC) shows to differentiate into mesenchymal lineages. *In vivo* they support hematopoietic events establishing a microenvironment with function of stem cell niche. MSC were shown capable to differentiate *in vitro* into adipocytes, chondrocytes, muscular cells, tendons, osteoblasts and endothelial cells. *In vivo* experiment

reported successful differentiation of mouse Bone Marrow MSC cells into brain astrocytes, glial cells and CNS cells, hepatocytes, endothelial and myocardial cells in adult mice. HSC cells were shown to differentiate into myocites, endothelial cells, hepatocytes and epithelial cells of liver, gut, lung and skin[35].

Somatic Cell Nuclear Transfer and Induced Pluripotent Stem Cells

In the most recent years many studies were published regarding the retrieval or the discovery of stem cells populations. Scientific knowledge allows us to modify cell genetic background and gene expression and to create, in the laboratory, different types of exogenous stem cells. Somatic Cell Nuclear Transfer (SCNT) cells were derived from the injection of an endogenous somatic cell nucleus within an oocyte. The result was the creation of a pluripotent cell, capable of being implanted in utero or used to retrieve new stem cell lineages. Induced Pluripotent Stem Cells (iPS) were obtained with insertion of pluripotent genes within the DNA of a somatic cell. Retroviral introduction of transcription factors OCT-4, SOX-2, KLF4 and MYC induced pluripotency within somatic cells. Recent studies have shown that OCT-4 and SOX-2 could be combined with other genes to produce iPS cells. IPS cells were able to participate to the embryonic development when injected in a blastocyst.

2.3 Stem Cells and Kidney regeneration

In the last few years some important scientific publications have shown evidence that stem cells, mainly mesenchymal stem cells derived from bone marrow, or of kidney-specific progenitors[36], have the capability to ameliorate renal injury. Transplanted bone marrow stem cells were found integrated into damaged kidney[37-38]. Morigi *et al*[39-40] and Herrara *et al*.[41] demonstrated that MSC are capable of integrating into damaged tubules and speculated that exogenous MSC from bone marrow have the ability to

differentiate into renal epithelial cells. Yokoo *et al.*[42] injected MSC from bone marrow into developing kidneys and confirmed their integration into various renal compartments, suggesting engraftment of these cells within nephron structures. However, whether there is any physiologic benefit of incorporation of these cells within damaged tubules of the kidney is still unclear. In contrast, there have been other groups, who have shown that MSC have a role in restoring function to damaged kidneys through some other mechanism other than incorporation and replication[43-45]. Bonventre *et al*.[46] underscored the importance of MSC in renal repair and raised the possibility that MSC may mediate their reparative effect by affecting the inflammatory response following acute renal injury.

With the possibility to choose from so many different pluripotential cell lineages, each one with different characteristics such as differentiation potential, committed status that could increase tissue specificity, autologos source and easiness of collection, stem cells have been the center of interest for scientists, clinicians and patients.

Despite the discoveries and the promising results, many are the controversies raised by stem cells. ESCs are strongly opposed by many for ethical reasons regarding their source and because of their ability to form teratomas *in vivo*. PGCs present a more limited pluripotentiality and share the same ethical issues as ESCs, making them even less attractive for clinical purposes. Free from moral argumentations are ASCs but their yet partially committed state makes them less reliable for a wide broad use for many applications. In fact, HSCs are broadly used in leukemia and some types of anemia therapy with discrete success.

A different problem is presented by SCNT where the incomplete technical knowledge and the know-how are still the major opposition for their therapeutic use as well as for iPS, where safety concerns for the use of retroviral infection are debated in the scientific community.

Feasibility of stem cells for human therapeutic use is regulated by many requirements such as safety, accessibility to a source that can provide an

adequate amount of cells for *in vitro* expansion, absence of ethical issues and repeatability of the results.

3. Amniotic Fluid as an alternative source of Stem Cells and Progenitors

To overcome all the above described concerns we and others are investigating new sources of pluripotent cells with low risk for their use, easy access to the source and capacity of giving rise to many mature cells through a safe and specific pathway.

Amniotic Fluid (AF) derives mostly from fetal urine and lung secretions, with minor contribution from the amnion[47]. Due to its contact with the developing fetus during the gestation, many cells are present within AF. In the last years many are the studies performed about the cells populating AF. Pluripotent cells were found within AF based on the expression of pluripotency marker OCT-4[48] and is demonstrated the presence of mesenchymal and hematopoietic cells[49] with pluripotent or multipotent characteristics. But mostly the AF cell population is still poorly known.

In 2007 Atala[49] widely described a pluripotent population characterized by the expression of c-Kit, a surface marker expressed by stem cells of mesenchymal origin. The c-Kit receptor is a protein-tyrosine kinase that is specific for stem cell factor. This complex has been suggested to be involved in embryogenesis as well as carcinogenesis[50]. The stem cells population is about 0.8%-1% of the entire AF population and presents a fibroblast-like morphology.

AFSC express some surface markers and transcription factors distinctive of ESC such us OCT-4 and SSEA-4 indicating they can actually posses some important characteristics that also ESC have, showing their pluripotential capability. In addition, they stained positively for a number of surface markers characteristic of mesenchymal and/or neural stem cells, including CD29, CD44 (hyaluronan receptor), CD73, CD90 and CD105[49].

The cells were positive for Class I major histocompatibility (MHC) antigens (HLA-ABC), and some were weakly positive for MHC Class II (HLA-DR). The AFSC cells were negative for markers of the hematopoietic lineage (CD45) and of hematopoietic stem cells (CD34, CD133).

AFSC have a very high proliferative capacity exceeding Hayflick's limit and the doubling time is around 36 hours with some variation between samples. Over the population doublings the cells maintain a normal karyotype, and also they present normal regulation of the control checkpoints of the cell cycle, in particular the G1 and G2, in preparation for chromosomes replication and entrance into mitosis. When injected into a blastocyst AFSC were able to integrate and participate to the development of several embryonic organs.

In vitro c-Kit+ cells were able to differentiate into adipocytes, myocytes, neurons, and bone[49] as well as *in vivo*.

Recently, Perin *et al.*[10] showed the capability of AFSC to participate *in vitro* to the development of embryonic kidneys. AFSC labeled with the surface marker CM-DiI were integrating within the developing structures of the kidney. Integration of AFSC into the metanephric structures was additionally confirmed by the migration of the injected cells from the site of injection, the center of the embryonic kidney, to the periphery, strongly correlates to the centrifugal pattern of induction, morphogenesis and differentiation of the metanephros, proceeding from the center to the periphery of the embryonic organ[49].

In 2008 Carraro *et al*[51] demonstrated *in vivo* integration and differentiation of AFSC into murine injured lungs. Since safety of stem cells is essential for a human therapeutic application tumorogenicity was tested and AFSC were not showing any carcinogenic potentiality when injected *in vivo*.

This preliminary work sustains the possible capability of using AFSC in therapeutic applications, especially for Kidney regeneration.

OBJECTIVES

Chronic Kidney Disease (CKD) is a major public health problem that often goes unrecognized until late-stage disease. Many are the causes for Chronic Kidney Disease and treatments are directed to slow down the progress of the renal functional deterioration before the necessity for dialysis and/or transplant.

None of the existing therapies are exempt from side effects and kidney physiological functionality is never completely restored. Transplantation has been reported as the preferred cure for CKD management but organ shortage and risks due to the immunosuppressive therapy make it far from being the perfect treatment for ESRD.

In this study we have focused our attention on finding novel cellular therapy candidates for kidney regeneration.

1. It is known that by 8 weeks of gestation Amniotic Fluid derives for the most part by fetal urination, and it could represent a repository of cells with kidney commitment and these progenitors (for example podocytes-like cells) may be a potential source of cells for kidney regeneration.

Beside the well known population of stem cells (AFSC), corresponding to 1% of the total cell population, the most part of the cells within Amniotic Fluid are poorly characterized. To better identify and select a renal population from Amniotic Fluid we performed a wide characterization of the cells present in the liquid, ranging from cells derived from all the three germ layers and organ specific progenitors, from multipotent to unipotent cells. In addition, we focused our attention in the isolation of specific kidney progenitor cells.

2. In the recent past, research groups have used stem cells derived from bone marrow (MSC) in order to treat acute and Chronic Kidney Diseases in experimental animals. In our laboratory we have well

characterized the c-Kit+ stem cells derived from Amniotic Fluid. In a parallel study conducted in the lab for the last 4 years it was shown that c-Kit AFSC, when injected into a mouse model of Acute Tubular Necrosis (ATN), are able to restore the normal physiological parameters and integrate into damaged tubules increasing the functionality of the organ. We decided to investigate the role of AFSC in Chronic Kidney Disease model, prevailing ailment among the population.

We focus our attention in a model used for CKD affected by Alport Syndrome. AFSC have been delivered through cardiac injection in order to verify their capability of integration in the glomeruli and the ability to slow down the fibrotic processes that can lead to ESRD.

MATERIALS AND METHODS

1. Expansion of human Amniotic Fluid Total Cell Population

Under Institutional Review Board approval of Children's Hospital Los Angeles, 28 human amniotic samples were obtained from discarded amniocentesis fluid between 15 and 20 weeks of gestation. Samples with normal male karyotype and normal fetal ultrasound were collected from discarded cultures (Genzyme Pasadena, CA). Cells were expanded in Tissue Culture Dishes (BD Falcon, Franklin Lakes, NJ) with three different culture media.

1. Chang's media (α MEM, 20% Chang B and 2% Chang C) (Irvine Scientific, Santa Ana, CA), L-Glutamine 20% of ES-FBS (Gibco/Invitrogen, Carlsbad, CA) and 1% of antibiotic (Pen/Strep, Gibco/Invitrogen, Carlsbad, CA).

2. Amniomax II was added as supplied (Gibco).

3. DMEM was supplemented with 10% FBS (Gibco/Invitrogen, Carlsbad, CA) and 1% antibiotic.(Gibco/Invitrogen, Carlsbad, CA)

Cells were trypsinized using trypsin 0.25% EDTA (Gibco/Invitrogen, Carlsbad, CA).

Cells were cultured at 37° Celsius and 5% CO2 for 50 passages.

2. Characterization of Amniotic Fluid Cells

16 samples of AF cells were analyzed by RT-PCR for a wide panel of markers for all the three germ layers, early progenitor cells from different organs and pluripotent cells (Table 1 A-B-C).

12 samples were analyzed by real-time PCR to determine the quantitative variation in the expression of the different markers at different gestational ages.

2.1 Analysis and characterization by RT-PCR

Between passages 4 and 5 and after trypsinization, cells were collected for RT-PCR.

Total RNA was isolated using the RNeasy Mini Kit (Invitrogen, Carlsbad, CA) as described on the data sheet. Briefly, with the use of silica-gel columns RNA is separated from DNA through centrifugation after lysis and homogenization of the samples. Ethanol addition allows RNA to bind the silica-gel before the centrifugation step. The RNA solution obtained was then processed with DNAse treatment (DNAse I, Invitrogen, Carlsbad, CA) to avoid any possible genomic contamination. 1μg of total RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The cDNA was amplified with Taq Polymerase (Invitrogen, Carlsbad, CA) in the presence of gene specific primers (Operon, Huntsville, AL). Amplification conditions were as follows: 94°C, 3 minutes; 94° C, 45 seconds; annealing temperature specific for each primer between 55° and 62° C, 30 seconds; 72°C, 1:30 minutes in a total of 36 cycles followed by 10 minutes at 72°C. RT-PCR products were separated in a 1.0% agarose/ethidium bromide gel and visualized using Blue/Orange Loading Dye (Promega, San Luis Obispo, CA).

Gene	Primer Sequenze (5'→3')	Size (bp)	Annealing Temperature	Function
AFP	AGCTTGGTGGTGGATGAAAC CCCTCTTCAGCAAAGCAGAC	248	56.2	Early expressed by the visceral yolk sac and, later, by the visceral endoderm
GOOSECOID	**AGGAGAAAGTGGAGGTCTGGTT TACAGCTCCTCGTTCCTCTTTC**	**299**	**58.6**	**Transcription factor expressed in the definitive endoderm. Eessential for normal embryo development.**
SOX-17	AGCGCCCTTCACGTGTACTA CTTGCACACGAAGTGCAGAT	216	56.2	Extraembryonic visceral and definitive endoderm. Leading cells toward a pathway of endodermal differentiation
CXCR-4	**TGAGAAGCATGACGGACAAG GACGCCAACATAGACCACCT**	**275**	**56.2**	**Chemokine Receptor 4, expressed by pre lymphocytes B cells. Involved in haematopoiesis**
E-Cadherin	TGCCCAGAAAATGAAAAAGG GTGTATGTGGCAATGCGTTC	200	58.8	Cell adhesion molecule expressed in the primitive ectoderm and down regulated in cells of primordial germ layer origin. Is also expressed by cells going through Mesenchymal to Epithelial Transition (MET)
FGF-5	**AAAGAGGAAAAGCCAAACGAG CCAAAGCGAAACTTGAGTCTG**	**223**	**54.7**	**Structurally related mitogen that promotes neural development**
NCAM	GGAGGACTTCTACCCGGAAC CTTTGGGGCATATTGCACTT	200	58.8	In nerves, regulates interactions between neurons and muscle; stimulates tyrosine kinase activity of receptor to induce neurite outgrowth. Contributes to cell-cell or cell-matrix adhesion during development.
TAL-1	**CTTCCCTATGTTCACCACCAAC CTCATTCTTGCTGAGCTTCTTGT**	**208**	**56.9**	**Transcription factor that plays many important roles in embryo growth ,including the development of the putative hemangioblast**
BRACHYURY	ACCCAGTTCATAGCGGTGAC ATGAGGATTTGCAGGTGGAC	216	56.2	Required for the development of the posterior mesoderm in mice. The absence of this molecule leads to death of the embryo
FLK-1	**GTGACCAACATGGAGTCGTG TGCTTCACAGAAGACCATGC**	**218**	**56.2**	**Migration of haematopoietic and endothelial cell progenitors to the yolk sac and also for generation of definitive haematopoietic precursors. Its absence is followed by death of the embryo because of defects in vascular development**
PDX-1	GATGAAGTCTACCAAAGCTCACG CTTGACCGAGAGACACATCAAGAT	211	57.6	Homeodomain transcription factor essential for pancreas development, insulin production, and glucose homeostasis
TTF-1	**ACAAGAAAGTGGGCATGGAG GCTGTTCCTCATGGTGTCCT**	**251**	**56.2**	**Transcription factor whose expression has been showed in thyroid, lung and some specific regions of the forebrain**
CEBPG	CATGGATCGAAACAGTGACG ACGTTGTCTGCAAGGTTGTG	228	58.8	Transcription factor of the family of the CCAAT/enhancer-binding protein and is expressed in the fetal liver at the onset of definitive haematopoiesis
NKX2.5	**ACATCCTAAACCTGGAGCAGCAG CCAAGTGTGCGTCTGCCTTTC**	**300**	**58.8**	**Encodes a homeodomain-containing transcription factor with major role in heart development. It's the earliest marker for pre cardiac differentiation**
GDNF	TATGGGATGTCGTGGCTGT ACACCTTTTAGCGGAATGCTT	624	58.2	Essential for nephrogenesis. Involved in stimulating UB branching. The highest expression of GDNF is found in the developing kidney while is not present in the adult organ
β-actin	**AGAAAATCTGGCACCACACC CTCCTTAATGTCACGCACGA**		**55.4**	**Housekeeping Gene**

Table 1A - *Panel of markers used for the characterization of human AF populations for the three germ layers and organ progenitor cells by RT-PCR*

Gene	Primer Sequenze (5'→3')	Size (bp)	Annealing Temperature	Function
OCT-4	**TATGAATTCCCAGCCGCAGCTTAGCTTCAAGAACATGTGT TATGGATCCGGCAGGCACCTCAGTTTGAATGC**	**620**	**57.6**	**Transcription factor involved in the conservation of pluripotent character of stem cells. It is involved in the self renewal of undifferentiated state of stem cells**
c-KIT	GGATTTTCTCTGCGTTCTGC TCCGTGATCCATTCATTCTG	250	59.6	Receptor for Stem Cells Factor and its expression has been shown in a pluripotent population residing into Amniotic Fluid as described by Atala *et al*.
CD90	**AGCATCGCTCTCCTGCTAAC ACGTGCTTCTTTGTCTCACG**	**380**	**56.4**	**Mesenchymal progenitor marker. It is a GPI linked glycoprotein member of the Immunoglobulin superfamily**
CD34	AATGAGGCCACAACAAACATCACA CTGTCCTTCTTAACCTCCGCACAGC	400	57.6	Called Hematopoietic Stem Cells marker. This protein is present on endothelial cells and hematopoietic progenitor cells

Table 1B *- Panel of markers used for the characterization of human AF populations for pluripotent cells by RT-PCR.*

Gene	Primer Sequenze (5'→3')	Size (bp)	Annealing Temperature	Function
CD24	**ACCCAGCATCCTGCTAGAC CTTAAGAGTAGAGATGCAGAA**	**286**	**59**	**Sialoglycoprotein anchored to the cell external surface and is usually expressed by hematopoietic cells**
OB CADHERIN	CACTGTCTTTGCAGCAGAAATC TACAATGACCAAGGAGAATGACG	437	55.6	Cadherin 11 is a cell surface glycoprotein and seems to be involved in dendritic spine morphogenic
LIM1	**TCAGGAGFCGAAGTAGGAAC AAGAGCGAGGATGAAGATGG**	**627**	**59.3**	**Transcription factor widely expressed in the renal compartment during development and evidences of its presence are shown in developing tubules, UB branches, S and comma shaped bodies**
PAX-2	AGGATGAGGGACCAACTGC AACGACAGAACCCGACTATGTT	738	59.5	Transcription factor known to be a WT1 inductor and a key ruler of mesenchymal to epithelial transiction
OCCL	**GCCCTCGCAACCCAAATTTTA TCATTCACTTTGCCATTGGA**	**425**	**58.2**	**Protein present on tight junctions with the role of sealing tight junctions between cells in a cell layer**
AQP1	CACCTCCTCCCTGACTGG GGTTGCTGAAGTTGTGTGTGA	290	58.8	Water channel protein, expressed by red blood cells, renal proximal tubules and descending limbs of loop of Henle
TRKA	**CCATCGTGAAGAGTGGTCTC GGTGACATTGGCCAGGGTCA**	**476**	**61.4**	**NGF High Affinity Receptor (chained with Tyrosin Kynase, TrKA) is expressed in the stromogenic compartment**
PGFR α	TGGAAGAAATCAAAGTCCCATCC GGATCAGCATTAATTTGCAACG	720	63	α isoform of the Plateled Derived Growth Factor, shown to be expressed in cells of the nephrogenic mesenchyme
Nephrin	**ACACGGAGCACACATACCAC GGATTGGAGAGGAGCAGAAG**	**568**	**59.8**	**Essential transmembrane protein expressed by podocytes at the slit between foot processes**
ZO-1	GCTGGTTTTGCTGTTGTTGA AGGAGAGGTGTTCCGTGTTG	773	59.9	Involved in the assembly and function of various tight junctions. Expressed by podocytes with the slit diaphragm

Table 1C *- Panel of markers used for the characterization of human AF populations and derived subpopulations for kidney commitment by RT-PCR.*

2.2 Analysis and characterization by Real Time PCR

Total RNA was isolated from cell cultured for 4 to 5 passages and retrotranscribed as previously stated starting from an RNA concentration equal to 800ng/μL.
Quantitative real-time PCR was carried out using the Roche Light Cycler 480 and the Light Cycler TaqMan Master Mix.
Real Time PCR conditions were as follows: 90°C for 10 minutes, 60°C for 10 seconds, 72°C 1 second with the analysis of the fluorescent emission at 72°C.
35 cycles were performed for each experiment. All primers and probes were designed by Roche.

2.3 Analysis and characterization by Western Blotting

Total cell lysates were prepared using the Nuclear Extract Kit (Active Motif). After washing the plate with a phospatase inhibitor, solution cells were scraped from the plate, collected, and centrifuged at 500 rpm for 5 minutes at 4° C. After incubation on lysis buffer at 4° C for 20 minutes cells were vortexed and successively centrifuged at 14,000 rpm, for 20 minutes at 4° C. Supernatant was collected and concentration measured with UV-VIS Spectroscopy. Each sample was prepared with Loading Buffer containing 250 mM Tris HCl (Sigma-Aldrich, St. Louis, MO) pH 6.8, 10% SDS (USBio, Cleveland, OH), 30% Glycerol (Sigma-Aldrich, St. Louis, MO), 5% B-Mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 0,02% Bromophenol blue (Sigma-Aldrich, St. Louis, MO-Aldrich). Equal amounts (20 ml, 1ng/ul) of individual protein solution were separated from each sample, after 1 minute boiling step, by SDS-PAGE with 4%-20% Glycin gels (Individual solutions were then transferred to PVDF 0.45 um membrane (Millipore, Billerica, MA) and probed with a various range of antibodies at 1:1,000 concentration (Table 2). Peroxide conjugation of secondary antibodies was performed (Sigma-Aldrich, St. Louis, MO) with

concentrations as follows: 1:10,000 for Anti-Mouse, 1:15,000 for Anti-Rabbit, 1:8,000 anti-Chicken and 1:120,000 for anti-Goat secondary antibodies. The blocking steps were performed with 10% Dry Fat Milk (Santa Cruz Biotech., Santa Cruz, CA) in TBS. TBS-T (1% Triton) was used as washing solution. Detection of antigens was performed using the ECL Western Blotting detection Reagents (Amersham Biosciences/GE Healthcare, Buckinghamshire, UK), impressed on Biomax Light Film (GE Healthcare, Buckinghamshire, UK) with a 1 minute exposure.

Antigen	Company	Host	Antigen	Company	Host
AFP	Santa Cruz	Goat	GDNF	Santa Cruz	Rabbit
GOOSECOID	Santa Cruz	Goat	OCT-4	Abcam	Mouse
SOX-17	Santa Cruz	Goat	CKIT	Santa Cruz	Rabbit
CXCR-4	Abcam	Rabbit	CD24	Abcam	Mouse
E-Cadherin	US Biological	Chicken	OB CADHERIN	Abcam	Mouse
FGF-5	Santa Cruz	Goat	LIM1	Abcam	Rabbit
NCAM	Abcam	Mouse	PAX-2	Abcam	Rabbit
TAL-1	Santa Cruz	Goat	OCCL	Santa CruzG	Goat
BRACHYURY	Santa Cruz	Goat	AQP1	Santa Cruz	Mouse
FLK-1	Abcam	Rabbit	Nephrin	Abcam	Rabbit
PDX-1	Abcam	Rabbit	ZO-1	US Biological	Rabbit
TTF-1	Abcam	Mouse	β-actin	Santa Cruz	Mouse
CEBPG	Abcam	Mouse	CD34	Santa Cruz	Rabbit
NKX2.5	Santa Cruz	Goat	CD90	Abcam	Mouse

Table 2 *- Panel of markers used for the characterization of human AF populations and derived subpopulations by Western Blotting assay.*

3. Selection and characterization of Metanephric Mesenchyme derived cells (MMDC) and kidney progenitor cells (AKPC) from the whole Amniotic Fluid

3.1 Immunoseparation of MMDC and AKPC from whole Amniotic Fluid

A positive population from AF for both CD24 and OB-Cadherin (MMDC) was selected incubating the total cell population with these two specific antibodies for 30 minutes at 4° C on a rocking platform, followed by second incubation with immunomagnetic microbeads for 5 minutes at 25° C and then 15 minutes at 4° C followed by immunoseparation by MS columns (Miltenyi Biotech, Germany).

Positive and negative (used as a negative control) selected populations were replated on Tissue Culture dishes with Chang's Media for subsequent expansion

A further immunoselection from the MMDC to identify 4 subpopulations of renal progenitors (AKPC) was performed as above described, using anti-Human antibodies for Nephrin, TrKA, PDGFR α and E-Cadherin following the previously described immunoseparation technique.

Final subpopulations were obtained after a total of 18 passages from the original samples. Cells were reseeded under the same conditions used for the total AF cell population and the main selection for MMDC cells.

3.2 Characterization of MMDC and AKPC by RT-PCR

The CD24+OB-Cadherin+ population (MMDC) was investigated by RT-PCR for early and mature kidney markers.

After the immunoselection, the four subpopulations of AKPC were analyzed according to the same panel of markers in order to investigate differences and common traits between the Nephrin+, TrKA+, PDGFR α+ and E-Cadherin+ AKPC derived populations following the protocol previously described.

3.3 Analysis and characterization of MMDC and AKPC by Real Time PCR

MMDC and AKPC were analyzed by Real Time PCR as previously described. Markers analyzed were: GDNF, LIM-1, PAX-2, Nephrin, OCT-4, TrKA, PDGFRα, E-Cadherin, ZO-1 and Occludin. Analysis was performed following the previously described protocol.

4. Selection of Amniotic Fluid Stem Cells (AFSC)

Stem cell population was separated from the general cellular milieu using standard Magnetic Sorting (MACS) techniques (Miltenyi Biotech) against cell surface marker c-Kit as described by Atala *et al*[49]. Pluripotential characteristics of the clonal and subclonal groups were tested according to protocols also outlined in Atala *et al*. Clones were then cultured in petri dishes in medium containing α-MEM supplemented with 20% Chang B and 2% Chang C solutions, 20% Fetal Bovine Serum, 1% L-Glutamine, and 1% antibiotics (pen-strep, Gibco).

5. Mouse model of Chronic Kidney Disease: Alport Syndrome

5.1 Animal models

Mouse AFSCs are derived from C57BL/C6 background. The Alport mice (B6.Cg-*Col4a5*^{tm1Yseg}/J, from Jackson labs) are derived from a C57Bl/6J background; they begin to die at 8 weeks of age with a median survival of 39 weeks[26]. Cells injected presented the same background for the infused cells and the Alport mice to avoid confounding background effects (Figure 1).

5.2 Injection of mAFSC

Before injections, a clonal AFSC population was trypsinized and centrifuged at 1500rpm for 5 min. and then labelled with a cell surface marker CM-DiI (Molecular Probe) following the manufacturer's instructions in order to track the cells during and after injection. Briefly, the cells were incubated with a working solution of CM-DiI of 1mg/ml for 5 minutes at 37^0C followed by an incubation of 15 minutes at 4^0C and 3 washes with PBS.

In addition, cells were investigated by RT-PCR for the expression of markers of the early and mature kidney with the protocol previously described in order to exclude previous commitment to renal differentiation.

We administered labeled AFSC (~ 800,000 AFSC) by intra-arterial (intracardiac) injection through the chest wall into the left ventricle using a 29 gauge needle with careful monitoring under isofluorane inhalation anesthesia. Weight-matched littermates were used to minimize inter-individual variation.

These procedures and protocols are approved by the IACUC at Children's Hospital Los Angeles. IACUC is the Institutional Animal Care and Use Committee, on charge of oversees CHLA's animal programs, animal facilities, and policies ensuring appropriate care, ethical use and humane treatment of animals.

Strain Information

Type Congenic; Mutant Strain; Targeted Mutation;
Mating System Homozygote x Hemizygote (Female x Male)
Generation N1S+F8 (08-JAN-09)

Donating Investigator Yoav Segal, University of Minnesota

Description
Heterozygous females and hemizygous males for the targeted mutation are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities. Immunofluorescence analysis of kidney tissue from mutant male mice does not detect gene product (protein), but in kidney from mutant female mice a mosaic pattern of gene product (protein) is detected (due to X-inactivation). RNase protection assay analysis of kidney tissue from mutant male mice detects a significantly lower level than wildtype. Hemizygous male and heterozygous female mice develop progressive kidney disease. By 7 weeks of age, 97% of mutant male mice and 78% of heterozygous female mice exhibit proteinuria. Histological analysis of kidneys from mutant male mice at 4 weeks of age reveals thickened capillary walls and mesangial hypercellularity. The pathology progresses to interstitial inflammation, glomerular abnormalities, and focal sclerosis. Electron microscopic examination of glomerular basement membrane ultrastructure shows lamellation and splitting abnormalities. Female mice heterozygous for the mutation exhibit similar progressive kidney disease but at later onset. These mice do not contain the neo selection cassette. This mutant mouse strain may be useful in studies of X-linked Alport syndrome (XLAS).

Development
A targeting vector containing a *loxP* site flanked neomycin resistance gene and a point mutation was used to insert a G213T transversion into exon 1. The construct was electroporated into 129SvJ derived ESVJ-1182 embryonic stem (ES) cells. Correctly targeted ES cells were injected into C57BL/6 blastocysts. The resulting chimeric animals were crossed to FVB/N-Tg(ACTB-cre) 2Mrt/J (JAX STOCK#3376) mice to remove the neo selection cassette and then backcrossed to C57BL/6 for 1S generations.

Control Information

Control
000664 C57BL/6J

Gene & Allele Information

Allele Symbol *Col4a5*^{tm1Yseg}
Allele Name targeted mutation 1, Yoav Segal
Allele Type Targeted (knock-in)
Common Name(s) COL4A5';
Mutation Made By Yoav Segal, University of Minnesota
Strain of Origin 129
ES Cell Line Name ESVJ-1182
ES Cell Line Strain 129
Gene Symbol and Name *Col4a5*, collagen, type IV, alpha 5
Chromosome X
Gene Common Name(s) ASLN; ATS; CA54; MGC167109; MGC42377; RGD1565499;
Molecular Note A targeting construct was designed to insert a G213T transversion into exon 1. This mutation was predicted to convert codon 5 from a glycine to a stop codon. Expression of the alpha5(IV) chain was lost in mutant kidneys from males. Basement membrane expression of the alpha 3(IV) chain was lost as well as that of the alpha6(IV) chain. Expression of alpha1(IV) and alpha2(IV) was conserved. Expression of the alpha5(IV) and alpha3(IV) chains in kidneys from females was mosaic, reflecting X-inactivation. [MGI Ref ID J:102306]

Phenotype Information

Related Disease (OMIM) Terms

Alport Syndrome, X-Linked; ATS - Models with phenotypic similarity to human disease where etiologies involve orthologs.[1]

[1] Human genes are associated with this disease. Orthologs of those genes appear in the mouse genotype(s).

Mammalian Phenotype Terms

assigned by genotype

Col4a5^{tm1Yseg}/Col4a5^{+}

B6.Cg-Col4a5^{tm1Yseg}

life span-post-weaning/aging
premature death (MGI Ref ID J:102306)
- females become ill and begin to die at 8 weeks of age with a median survival age of 39 weeks; however, some survive to 50 weeks of age

renal/urinary system phenotype
abnormal kidney morphology (MGI Ref ID J:102306)
- in older, visibly ill mice, kidneys appear pale and dull and are pockmarked
- the appearance of kidney abnormalities is delayed relative to hemizygous mutant males

abnormal renal glomerulus morphology (MGI Ref ID J:102306)
- abnormalities similar to those in males are seen but at later times
- at 17 weeks of age some glomeruli develop focal areas of lamellation and other diffuse changes with widespread abnormalities seen at 30 weeks of age

abnormal renal tubule morphology (MGI Ref ID J:102306)
- abnormalities similar to those in males are seen but at later times

proteinuria (MGI Ref ID J:102306)
- urine concentrations of protein greater than 10 mg/mg creatinine are seen in 78% of females after 9 weeks of age

homeostasis/metabolism phenotype
increased blood urea nitrogen level (MGI Ref ID J:102306)

proteinuria (MGI Ref ID J:102306)
- urine concentrations of protein greater than 10 mg/mg creatinine are seen in 78% of females after 9 weeks of age

Col4a5^{tm1Yseg}/Y

B6.Cg-Col4a5^{tm1Yseg}

life span-post-weaning/aging
premature death (MGI Ref ID J:102306)
- males become ill and begin to die at 6 weeks of age with none surviving beyond 34 weeks of age
- median survival is 23 weeks of age

renal/urinary system phenotype
abnormal kidney morphology (MGI Ref ID J:102306)
- in older, visibly ill mice, kidneys appear pale and dull and are pockmarked

abnormal kidney vasculature (MGI Ref ID J:102306)
- at 4 weeks of age capillary wall thickening is seen

abnormal renal glomerular capsule (MGI Ref ID J:102306)
- at 17 weeks of age capsular collapse may be seen

abnormal renal glomerulus morphology (MGI Ref ID J:102306)
- at 4 weeks of age lamellation of glomerular basement membranes is present and by 17 weeks of age diffuse basement membrane abnormalities including lamellation and splitting are seen
- at 17 weeks of age glomeruli display many, variable abnormalities including capillary loop dilation and simplification, capillary tuft collapse, and focal sclerosis

abnormal mesangial cell (MGI Ref ID J:102306)
- at 4 weeks of age mesangial hypercellularity is seen; however, the tubulointerstitium is similar to wild-type

abnormal podocyte (MGI Ref ID J:102306)
- at 17 weeks of age signs of podocyte injury, including foot process effacement, vesiculation, and denudation, are present

podocyte foot process effacement (MGI Ref ID J:102306)
- at 17 weeks, foot process effacement is seen

dilated renal tubules (MGI Ref ID J:102306)
- at 17 weeks of age tubular atrophy and dilation are seen

kidney inflammation (MGI Ref ID J:102306)
- at 17 weeks of age widespread interstitial inflammation is seen

proteinuria (MGI Ref ID J:102306)
- urine concentrations of protein greater than 10 mg/mg creatinine are seen in 97% of males after 7 weeks of age

homeostasis/metabolism phenotype
increased blood urea nitrogen level (MGI Ref ID J:102306)

proteinuria (MGI Ref ID J:102306)
- urine concentrations of protein greater than 10 mg/mg creatinine are seen in 97% of males after 7 weeks of age

immune system phenotype
kidney inflammation (MGI Ref ID J:102306)
- at 17 weeks of age widespread interstitial inflammation is seen

cardiovascular system phenotype
abnormal kidney vasculature (MGI Ref ID J:102306)
- at 4 weeks of age capillary wall thickening is seen

Figure 1 – Datasheet for the mouse model B6.Cg-Col4a5^{tm1Yseg}/J. *Phenotype information about the mouse model used for our study from the datasheet of Jackson Laboratories.*

5.3 Blood Collection, Creatinine and Statistical Analysis

Blood and urine were collected in AS injected mice, AS mice not injected and WT mice.

For blood collection, the facial vein was lanced with 5mm point length animal lancet using standard protocols approved by Animal Core Facility at Children's Hospital. The blood samples (30µL) were collected into plasma separation tubes with lithium heparin. They were centrifuged at 13,000-RPM for 3 min and the plasma (upper layer) was removed and stored at –80^{O}C until processed. A maximum of 15% of circulating blood was sampled in a given 14 day period (total blood volume ~0.6% of total body weight). The sampled blood was used to monitor the renal function, by analyzing for creatinine levels, both during renal function loss and after the injection of AFSC into the kidney. 30µL serum samples were loaded into 96-well microplates including the standards. Working Solution prepared by mixing 100µl reagent A and 100µl reagent B per well was quickly added to all wells, and optical density was read at 1 min (OD_1) and 5 min (OD_5) at 490nm absorbance. (BioAssay Systems, Cat # DUCT-500). The amount of creatinine expressed in mg/dl was calculated as suggest from the kit. Comparison between groups was made using an unpaired *t* test. A value of $p< 0.05$ was considered statistically significant. Data are shown as mean, SD.

Renal function was evaluated using urinary albumin to creatinine ratios, serum creatinine and urinary BUN concentrations, determined using routine colorimetric assays according to the manufacturer's instructions. Animals were monitored weekly (from the age of 2 weeks) by measuring the level of albuminuria (albumin/creatinine ratio) to determine the earliest manifestations of, and the progression of, the disease. The control groups for the experiments included α5(IV) knockout mice with no injection and normal mice C57BL/C.

5.4 Tissue Processing

At different time points, the injected and the control mice were scarified, the kidneys were removed and washed in PBS buffer for 5-10 min x 2. The kidneys were processed by one of two methods depending on the analysis performed.

For histological analysis, kidneys were fixed in 4% buffered paraformaldehyde [Sigma-Aldrich] for 8 hr at 4^0C, routinely processed, embedded in paraffin, and sectioned at 5 μm. Briefly, the kidneys were washed in alcohol 70% for 2 hour, followed by two washes in alcohol for 2 hour and placed in toluene, twice for 40 minutes, then one hour in a solution of toluene/paraffin and paraffin overnight. The following morning the kidney were entirely embedded in paraffin and prepared for sectioning. The sections were stained with hematoxylin and eosin (H&E) [Sigma-Aldrich] or PAS (Periodic Acid Schiff. Sigma-Aldrich) using standard histological protocols to study the morphology of the damaged organs over time and the expression of collagen type IV α1 the main types of collagen affected in AS and collagens type 1 as marker of interstitial fibrosis, by immunohistochemistry. In addition, some kidneys were frozen with liquid nitrogen, were stored at -20°C and when necessary they were cryosectioned at 5 μm and then used for immuno-histochemistry.

For immunostaining, frozen and paraffin slides were stained with florescence. Paraffin slides were deparaffinized, placed in 1% Triton for 5 min and briefly washed in PBS. . The frozen slides were fixed for 5 min. in 80% methanol. After the block of Avidin/Biotin (Vector Laboratories) a second block was carried for 30 min. using the appropriate 5% normal serum in PBS. The slides were incubated in primary antibody (table) solution for 1 hr at room temperature or overnight at 4°C. Afterwards, the slides were washed in PBS for 5 min. x 3. Secondary antibody (Vector Laboratories) concentration was 1:200 in 5% normal serum – slides were incubated in this solution for one ½ hr at room temperature, followed by 5 min. x 3 PBS. The appropriate fluorescent marker (Texas Red or Fluorescein Avidin DCS from Vector Laboratories) was then applied in a

concentration of 1:500 in PBS buffer for 5-10 min., followed by a final 5 min. x 3 PBS wash. Slides were mounted with Vector DAPI mounting medium (Vector Laboratories). A Leica DM RA fluorescent microscope was used in conjunction with Open Lab 3.1.5 software to image the staining.

RESULTS

1. Characterization of Amniotic Fluid cells by expression of markers for the three germ layers and progenitor cells

1.1 Amniotic Fluid Total Cell Population Culture

The morphology of the total cell population is very heterogeneous with a preponderance of fibroblastoid shapes (Figure2A-B). The expansion of the total population of Amniotic Fluid (AF) was possible for up to 10 passages using DMEM. Therefore, after these few passages, the cells stopped growing and started dying. Nevertheless when the cells were cultured in Amniomax II and Chang media they could be expanded for more then 50 passages in culture. The cells cultured with Amniomax II acquired a defined fibroblastoid shape. We therefore chose to culture cells in Chang media for all the experiments, since cell morphology did not change significantly over subsequent population doublings.

1.2 Analysis and characterization of human Amniotic Fluid cells by RT-PCR

AF Total Cell Population was stratified by week of gestation (from 15 to 20 weeks) and analyzed using RT-PCR. As shown in Figure 3 A-B-C, expression of markers for the three germ layers, for pluripotent cells as well as for mesenchymal, hematopoietic and early progenitor cells of several organs were analyzed.

Expression of genes characteristics of the Endodermal and Mesodermal germ layers was found to decrease over time, while ectodermal markers remained constantly expressed (Figure 3A).

Pluripotent markers were expressed in all samples younger than 19 weeks.
While mesenchymal marker CD90 is expressed at all the time points analyzed, CD34 (Marker for mature haematopoietic lineages) was absent in early gestation samples, but appeared in samples of 18 weeks of gestation or older (Figure 3A).
Early progenitor markers were expressed in 18 weeks and older samples (Figure 3B) as well as specific kidney markers (Figure 3C).

1.3 Analysis and characterization of human Amniotic Fluid cells by Western Blotting

The cells protein expression showed a decrease on endodermal and mesodermal layers over the course of gestation while, as seen with the mRNA expression, ectodermal proteins are constantly present along the gestational age investigated in this study. Pluripotent, hematopoietic and mesenchymal markers follow the same trend seen in the RT-PCR analysis (Figure 4A).
The early proteins of progenitor cells from different organs, as shown in Figure 4B, show increased expression into AF cells over the course of gestation. Kidney specific proteins were shown to increase around 17-18 weeks of gestation as shown in Figure 4C.

1.4 Analysis and characterization of total Amniotic Fluid cell population by Real Time PCR

4 samples for each chosen time point (15-16, 17-18 and 19-20 gestational age weeks) for a total of 12 samples were analyzed. The investigated samples for all the markers analyzed confirmed the same overall trends showed by RT-PCR analysis. However, some of the markers such as Brachyury, Tal-1, Nephrin, GDNF, TrKA were not expressed in one or

more samples. Goosecoid and PDX-1 were not found in any of the twelve samples analyzed (Figure 5).

The ectodermal marker E-Cadherin increased 15-fold between 15-16 and 17-18 weeks of gestation. In contrary NCAM and FGF5, while confirmed to be present, did not change significantly between 15 and 20 weeks (Figure 5A).

The Endodermal marker CXCR-4 increased 3.5-fold between 15-16 and 19-20 weeks while the other Endodermal markers Sox-17 and AFP tended to decrease (Figure 5A).

The mesodermal marker Brachyury was expressed at 15-16 weeks in one sample but not later on. TAL-1 appeared to decrease over time, while FLK1 increased 4-fold (Figure 5A) .

Pluripotency marker OCT-4 didn't change over the investigated weeks of gestation while c-Kit marker increased by 3-fold between 15-16 and 17-18 weeks and disappeared in the older samples (Figure 5B).

Hematopoietic marker CD34 decreased between the 17-18 and the 19-20 weeks in contrast with mesenchymal marker CD90 that increased 2 fold by 17-18 weeks old samples (Figure 5B).

Progenitor markers, excluded PDX-1 that showed no expression, generally increased with the progression of the gestation; NKX2.5, early cardiac marker showed a 6-fold increase between 17-18 and 19-20 weeks while lung/Thyroid marker TTF-1 increased 1 fold between 15-16 weeks and 17-18 weeks and an additional 2.5 fold to 19-20 weeks (Figure 5C).

CEBPG showed a 5 fold increased expression at 17-18 weeks if compared with the 15-16 and 19-20 weeks of gestation (Figure 5C).

Renal marker GDNF was expressed only in two of the samples, increasing 5 fold between the 15-16 sample and the 19-20 weeks sample (Figure 5D).

Undifferentiated Metanephric Mesenchyme markers CD24 and OB Cadherin increased 1 fold between 15-16 and 17-18 weeks. While CD24 expression remained unchanged between 17-18 weeks and 19-20 weeks, OB-Cadherin decreased to the previous expression (Figure 5D).

PAX-2 increased slightly between 15-16 and 19-20 weeks while LIM1 didn't change its expression (Figure 5D).

One sample for each time period was positive for Nephrin, showing a constant increase over the time with a 1-fold increase between 15-16 weeks and 19-20 weeks of gestation (Figure 5D).

Zo-1 and Aquaporin-1 marker didn't change significantly overtime while Occludin increased 8-fold between 17-18 weeks and 19-20 weeks (Figure 5E).

PDGFR α expression did not change significantly over the progression of the gestation while TrKA expression of the only three positive samples decreased 4 fold between 17-18 and 19-20 weeks (Figure 5E).

2. Selection and characterization of Metanephric Mesenchyme derived cells (MMDC) and Kidney Progenitor cells (AKPC) from the whole Amniotic Fluid

2.1 Immunoseparation of MMDC and AKPC from whole Amniotic Fluid

A specific cell population positive for CD24 and OB-Cadherin (MMDC) was successfully isolated from whole AF and cultured under the conditions described above.

MMDC selected cells present a different morphology with typical fibroblast shapes (Figure 6A). From the CD24+OB-Cadherin+ population, AKPC subpopulations expressing TrKA, Nephrin, PDGFR-α and E-Cadherin were successfully obtained by immunoseparation.

2.2 Characterization of MMDC and AKPC by RT-PCR

The MMDC population (CD24+ Ob Cadherin+) (Figure 6B) and the four derived AKPC subpopulations were characterized by RT-PCR for early and mature kidney markers, as well as pluripotency markers (Figure 7). Expression of renal markers differed in the investigated populations as shown in the figures. The AKPC E-Cadherin+ population expressed E-Cadherin, GDNF and was slightly positive for Nephrin.

AKPC Nephrin+ cells were positive for Nephrin, Aquaporin 1 and Zona-Occludens 1. The population immunoselected for AKPC PDGFR α+ was positive for ZO-1 and PDGFR α while the AKPC TrkA+ population expressed TrkA, ZO-1 and, slightly, PDGFR α (Figure 7).

2.3 Analysis and characterization of MMDC and AKPC by Real Time PCR

The main population MMDC and the four AKPC subpopulations were analyzed by Real Time PCR for the expression of specific kidney markers. GDNF, Metanephric Mesenchyme specific marker, was expressed by the main population and was almost 12 fold greater in the Nephrin+ subpopulation, but absent in the other subpopulations.

LIM-1 was 11 folds greater in the Nephrin population and increased 9 folds in E-Cadherin population. It was increased in the TRKA+ population.

PAX-2 increased 4 fold in Nephrin. Nephrin expression was 2 fold greater in Nephrin population. (Figure 8A)

Pluripotent marker OCT-4 was increased 10 fold in Nephrin+ AKPC, 15 fold in E-Cadherin+ population and 2 fold in TRKA. (Figure 8A) E-Cadherin+ cells showed a 3 fold increase in E-Cadherin expression, decreased in PDGFRα+ and slightly in TRKA. (Figure 8B) TRKA showed no increase in TrKA expression if compared with the main population but the other population were negative for TrKA. PDGFRα increased 2 fold in PDGFRα+ APKC, decreased in Nephrin+ and was practically absent in E-Cadherin+ APKC. ZO-1 was increased 7 fold in the Nephrin population but

absent or slightly expressed in the other subpopulations. Occludin was 3.5 fold greater in Nephrin+ APKC, 7 fold greater in E-Cadherin and 1.5 fold greater in PDGFRα+ cells (Figure 8B).

3. Selection of Amniotic Fluid Stem Cells

AF Stem Cells were successfully isolated by immunoseparation with magnetic beads from C57BL/6J Amniotic Fluid sample. Cells were then expanded with a maximum confluence of about 60%.

3.1 mAFSC before injection

mAFSC present a fibroblastoid shape as shown in Figure 9A. mAFSC were negative for most important kidney markers, ranging from transcription factors expressed during early kidney development to late differentiation markers. This allowed us to confirm that the mAFSC are not specifically committed to be kidney progenitor cell when cultured *in vitro* (Figure 9B)

3.2 Analysis of kidneys of mAFSC injected Alport Mice

Periodic Acid Staining of paraffin embedded kidneys section for WT and AS mice was used to compare the morphological changes in the renal compartment (Figure 10).

Collagen VI α1 immunofluorescence analysis in WT mice was positive in the Bowman Capsule's and in the tubular basal membrane (Figure 11A). In AS mice staining for α1 was showing an increase in thickness in the Bowman's Capsule as shown in Figure 11B and was positive in the GBM. AS mice Injected with AFSC presented less deposition of α 1 in the

Bowman's capsule and absence of collagen IV α1 in the GBM. The data presented allows us to confirm a positive effect of AFSC in the AS mice (Figure 11C).

3.3 Analysis of creatinine, albumin, BUN and Life span.

At 5 months after birth, corresponding to 12 weeks after the injection of mAFSC, from WT, AS and AS-AFSC injected mice were collected blood and urine samples for albumin, creatinine and BUN analysis. Levels of albuminuria, creatinine and BUN decreased in the injected mice compared with their siblings that were not injected. The life–span of the injected mice also increased significantly (Figure 12 A-B-C-D).

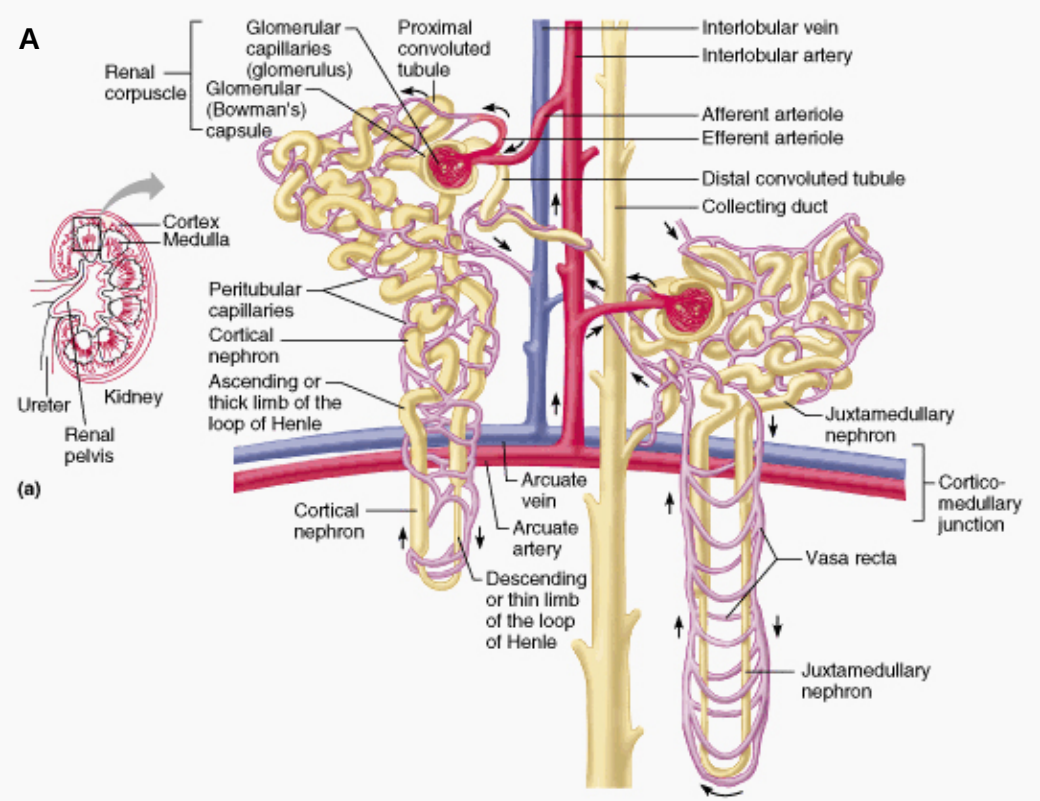
DISCUSSION

The adult kidney is a highly vascularized organ, receiving about 20% of the blood supply of the body. The kidneys filter about 180 liters of fluid per day with an electrolyte composition similar to that of plasma and the nephrons have the responsibility of handling this large volume of filtrate and separating that which must be conserved and that which needs to be excreted. The basic functional unit of the kidney is the nephron, composed of a glomerulus and a tubular system that includes a proximal tubule, a loop of Henle and a distal tubule that is connected with the collecting tubule, where the urine is collected and delivered to the collecting duct (Figure 12A).

In the kidneys, the role of tubules is the re-absorption of water and electrolytes in order to maintain the body homeostasis and each segment is specialized in a different process of water and electrolytes intake.

In the proximal tubule, water, electrolytes and glucose are reabsorbed from the filtrate. The connection between the proximal tubule and the loop of Henle is called descending loop. In the descending loop, the concentrations inside and outside the tubule are increasing with the current, with the maximum concentration being reached at the bottom of the loop. The increased concentration is the result of the passive diffusion of Na+ into the tubule and water out of the tubule. When the filtrate reaches the distal tubule, a net loss of Na+ and water has occurred through the loops of Henle.

In the ascending loop, Na+ (or any solute) is actively pumped out of the tubule. As flow continues up the loop, the tubular concentration decreases as does the interstitial concentration. Because water is impermeable in the ascending loop, the volume at the bottom of the loop is the same as the



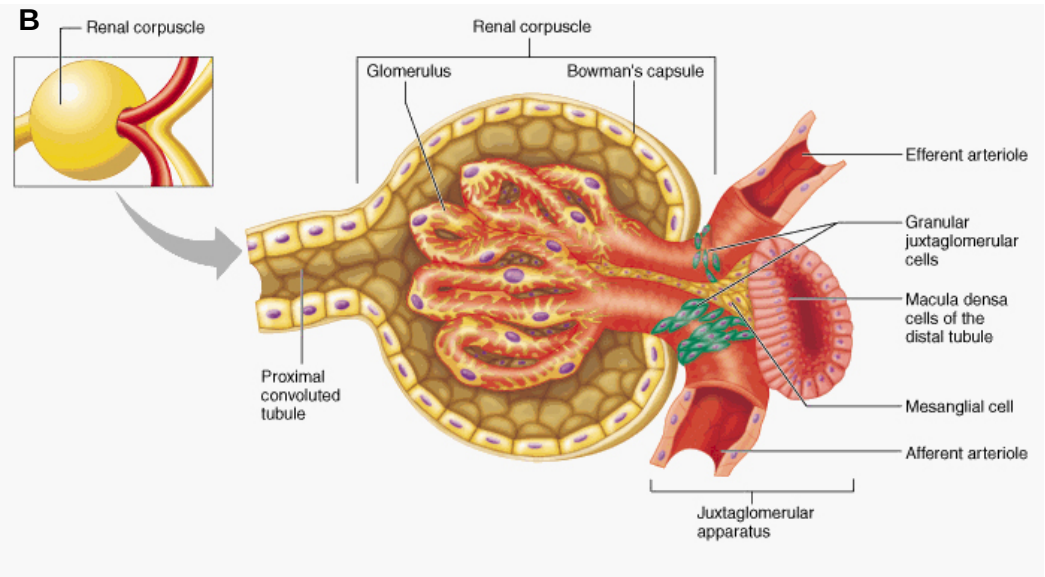


Figure 12A-B Schematic representation of kidney structure. ***A*** *General representation of the kidney showing the tubular, glomerular and vascular organization in the renal compartment.* ***B.*** *Organization of the glomerulus with the capillary network surrounded by the Bowman's Capsule*.

volume entering the distal tubule. At the bottom of the loop, the tubular and interstitial concentrations are equal. Inside the distal and collecting tubules, the filtrate is either diluted or concentrated to form urine.

But the kidney function is not only to maintain the balance of water and electrolytes. A key role for the kidney is to avoid the loss of any essential molecule from the blood. The glomerulus is the compartment in charge of discriminate what may be discarded and what needs to be preserved.

The glomerulus is a specialized capillaries network of interconnected loops surrounded by Bowman's capsule (Figure 12B). The glomerular capillaries have unique characteristics that contribute to its filtering capabilities. The porosity of the endothelial layer increases capillary permeability, the mesh-like structure of the basement membrane provides a barrier to large molecules, and the portal structure allows maintenance of an intracapillary pressure that favors filtration.

The Bowman's capsule is made up of two cell layers: the visceral layer that forms the epithelial layer (podocytes) of the filtration barrier and the parietal cell layer that forms the outer layer of the capsule. The space between the two cell layers, referred to as Bowman's space, collects the filtered fluid and solutes and directs this filtrate toward the proximal tubule. The glomerular membrane has three layers: (a) endothelial, (b) basement membrane, and (c) epithelial. The endothelium lines the capillary lumen and contains many pores, or fenestrae, that favor the filtration of fluid and small solutes. The Glomerular Basement Membrane (GBM), mainly produced by podocytes, is a matrix of collagen and similar proteins as well as glycosaminoglycans that provides a size and charge barrier to the movement of large particles out of the capillary lumen. The visceral epithelial cells of Bowman's capsule, or the podocytes, have cytoplasmic foot processes that extend over the basement membrane. Spaces between these foot processes are called slit-pores and allow the filtrate into Bowman's space. Mesangial cells are located between the capillary loops of the glomerulus and form a support network within the tuft. The glomerular membrane allows filtration of fluid and small molecules. Large molecules are prevented from entering the filtrate in two ways. First the

size of spaces in the glomerular epithelium and basement membrane limits the passage of these larger molecules and cells such as the white and red blood cells and albumin. Second, the podocytes and, to some extent, the GBM have a net negative charge that repels large negatively charged molecules, particularly the plasma proteins. Small anions that easily filter through the pores are not influenced by the negative electrical charge.

Since kidney is in charge of blood purification (ultrafiltration) with maintenance of acid/basic balance, metabolites elimination and blood pressure control, when damage occurs, the effects are notable in many other organs and tissues of the body.

In this work we focus mainly our attention on Chronic Kidney Disease (CKD). CKD is often referred as a “silent” disease because no evident symptoms or pain are caused. Nonetheless, many are the chemical changes that origin from CKD injury.

Neuropathies and bone disorders are common in patients with CKD and more than 70% of patients with CKD were reported to undergo hypertension. High blood pressure is well known as involved in cardiac and vascular diseases such as heart enlargement, congestive heart failure, heart attacks and strokes. In addition, hypertension is causing even a faster loss of kidney function.

Since kidney is involved in the production and release of hormones like erythropoietin, that stimulates proliferation and differentiation of erytrhoid cells, a loss in renal endocrine function can lower levels of red blood cells with subsequent anemia. The loss of proteins (proteinuria), due to the destruction of the tubular system, can worse the malnutrition caused by absence of appetite, low protein intake and loss of weight that is typical in patients with CKD.

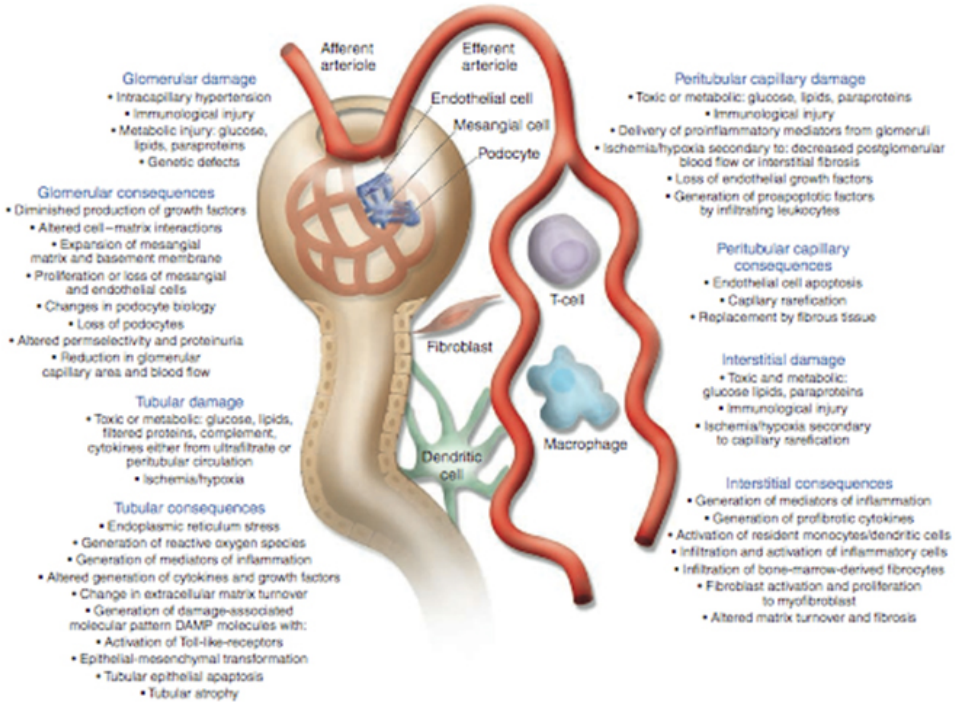


Figure 13 – Specific kidney damages and their effects *Graphic representation of the most frequent kidney damages and their effect at a cellular and molecular level.*

At a cellular and molecular level CKD causes a progressive scarring that ultimately affects all kidney structures leading to fibrosis, activated after the initial injury[52]. Tubular damage can lead to apoptosis of tubular cells, release of toxic substances, excretion of cytokines and other inflammatory molecules and eventually tubular atrophy. Decrease in peritubular capillary density has been proposed as a mechanism for tubulointerstitial fibrosis characterized by an abnormal production of extracellular matrix and maybe infiltration of macrophages[52].

One important aspect of CKD is the subsequent glomerular damage leading to decrease of growth factors, alterations in the cell-matrix interactions, altered selectivity and permeability of the GBM and a reduction of the blood flow (Figure 13). All these consequences can damage the podocytes leading to loss of function and apoptosis. Since podocytes are thought to be the key cell of the glomerular compartment,

their malfunctionality induces a cascade of other effects that can lead to ESRD like alterated proliferation of mesangial cells and changes in microvascular permeability[53] (Figure 13).
As described in the introduction one of the main challenges in therapeutic treatment of CKD using stem cells and regenerative medicine principles, is to be able to identify a podocyte precursor cell that can rescue the functionality of the glomerulus. In addition, it is fundamental to understand the processes involved in the progression of fibrosis in order to slow down the progression of the disease.

In this work we have decided to use two different strategies as experimental approaches to cure CKD in the future as explained in the objectives with isolation of kidney progenitor cells and the use of c-Kit AFSC for treatment of CKD in Alport mice.

1. Amniotic Fluid Cells characterization and isolation of Metanephric Mesenchyme Derived cells (MMDC) and Amniotic Fluid Kidney Progenitor Cells (AKPC)

AF is known source of stem cells, but almost 99% of cell population is still poorly investigated. It seems reasonable to speculate that within AF there is a repository of different progenitor cells included, due to its origin, progenitors derived from the renal compartment.
AF fills the amniotic cavity, providing an environment in which floats the developing embryo and later on the fetus. The volume and the composition of the AF change during pregnancy following the physiological variations of the developing fetus. During embryogenesis, maternal plasma is the main protagonist of AF volume increase and water flows osmotically, though fetal membranes, and, later on, through the placental membrane. The composition of AF during the first weeks of gestation is similar to the fetal plasma with a volume of 25 ml at 10 weeks to about 400

ml at 20 weeks[47]. By 8 weeks of gestation the fetal kidney begins fluid production that rapidly increases in volume during the second trimester. The exchange of fluids through the skin is present until keratinisation that occurs between 20 and 24 weeks of gestation. The molecular composition of AF and the presence of nutritive substances have been shown to play a key role, in animals, in the proliferation and differentiation of various intestinal cell types such as epithelial and mucosa cells. AF has been used as a safe and reliable screening tool for genetic and congenital diseases in the fetus for many years and, by now, is being deeply investigated trying to match its molecular composition (or its variation) with preterm delivery, infective processes and embryo diseases.

Contact between AF and compartments of the developing fetus, such as lung and gastrointestinal tract could explain the presence of different cell types in the milieu of the AF as reported in literature.

The first approach was to characterise more specifically the presence of cells derived from all the three germ layers. The development of the embryo goes through a defined pathway of differentiation that follows specific steps of maturation. From the small cell agglomerate call blastula, derived from the fertilized oocyte, a subsequent separation of the cells in agglomerates gives rise to three layers called germ layers. From this three germ layers (Endoderm, Mesoderm and Ectoderm) are deriving the cells that originate all the tissues and organs. The cells within each germ layer are multipotent, able to give rise only to some cell lineages. Each germ layer develops along the first phases of the gestation to form different compartments of the fetal body[54].

In addition to the multipotent cells belonging to the three germ layers were investigated the presence of more committed cells and in particular organ specific progenitor cells focusing on kidney precursors.

Since the composition of AF changes over time as the fetus develops during pregnancy, we divided samples according to gestational age. Since the identification of a single marker to identify a specific

embryological tissue is difficult, do to the frequent co-expression of a gene in many cells at the same or different time, we found the necessity to analyze several markers for each germ layer in order to obtain more precise results. The choice of the markers was made according to gene expression in the early passages of germ layer specification, in order to avoid the chance of false positives. Endodermal marker Sox-17, for example, is expressed in the early stages of endoderm specification but, in the mouse, expression was confirmed in mature lung cells. But since Sox-17 was not revealed before E18 and our human amniotic fluid samples are far from the end of gestation, we can speculate that positive results obtained were not from mature cells derived from lung[55]. On the other hand, AFP was shown to be expressed in endodermal tissues and only at very low levels in the adult[56] as well as Goosecoid[57]. Cells from mesodermal lineages were detected through expression of FLK-1, Brachyury and SCL/TAL-1. FLK-1 is expressed broadly and transiently in the mesodermal cells in the embryo but is known to be expressed in adults too[58]. TAL-1 has been shown to be essential for hematopoiesis in the early stages of life, and was shown to be expressed in the adult. But its expression is limited to a short term repopulating cell population, with characteristics of pluripotentiality, confirming that the marker was specific enough for our purposes[59]. Brachyury was shown to be present in adult almost only in pathological situation, becoming a reliable marker for endodermal specification[60]. The expression of markers from endoderm and mesoderm was higher in samples of AF of earlier gestational age versus those samples taken from later gestational age. In contrast, ectodermal markers NCAM, FGF-5 and E-Cadherin were equally expressed in early and later samples of human AF maybe due to the turnover of the fetal skin cells during the development (Figure 3A-4A).

The presence of pluripotent cells within AF is well known since the studies of Prusa[49] but possible information about pluripotent cells variation over the gestational time could give us better data for the best time for their collection. Therefore OCT-4, pluripotent marker essential for the

development of the embryo, and c-Kit, characterizing a population of mesenchymal stem cells, were analyzed.

Expression of OCT-4 and c-Kit was constant over time, indicating the presence of pluripotent cells in AF in a large number of the samples investigated, with no significant difference in presence between the earlier and the later samples. These data prove that the pluripotent population within AF is maintaining a strong presence over the time, with no overall difference in the choice of the time for the collection, at least for the time range considered in this study.

We investigated also the presence and variation of markers for mesenchymal and hematopoietic cells through expression of CD90 and CD34.

Results revealed a constant and strong expression of mesenchymal-derived cells, while hematopoietic cells were absent from the most part of AF samples at 15-16 weeks (Figure 3A-4A) and only increased slightly in older samples. Real Time PCR showed an increase number of samples positive for CD34 in older weeks but with a decreased expression of the marker (Figure 5B). Previous studies about AF MSC[61] confirmed the expression of CD90 in this population. CD34 is a well known marker for hematopoietic lineage[62], even if recent publications are debating the necessity of CD34 expression for cells to undergo hematopoietic differentiation[63].

Interestingly previous studies showed that hematopoietic cells were present before the 12 weeks of gestation but no further characterizations were performed on older samples.

Secretions from the lung, gastrointestinal tract, skin and the urinary system are the origin of a repository of a heterogeneous population of cells within AF that has been demonstrated in our study. The development of the human embryo follows a precise pathway and the cells committed to various organs are differentiating at different time points.

Any adult cell follows a specific pathway of gene expression that leads to maturation with specificity.

We investigated, for this analysis, early markers of differentiation to the specific organ cell lineage in order to identify cells committed, but not yet mature, through a precise pathway. The choice of the markers was done looking for an early expression but with a certain specificity that could grant the definitive commitment to a cell lineage or group of cell lineages. Since Nkx2.1 expression is limited to lung, thyroid and certain regions of the forebrain[64], was used for the identification of lung progenitors as well as Nkx2.5, essential for cardiac differentiation, is present at low levels in other organs of the murine embryo[65]

In our study we found the presence of early transcription factors that regulate differentiation processes of pluripotent cells into mature cells including Nkx2.1 (TTF-1, Thyroid Transcription Factor-1) for lung, Nkx2.5 (CSX, Cardiac Specific Homeobox) for cardiomyocytes, CEBPG (CCAAT/enhancer binding protein, gamma) for liver, PDX-1 (pancreatic and duodenal homeobox 1) for pancreas, and GDNF (Glial Cell Line-Derived Neurotrophic Factor) for kidney. As expected, the expression of markers for committed cell populations is increased in the older samples but is weak or absent in samples of earlier weeks of gestation. (Figure 3B-4B) As the fetus develops, pluripotential cells subsequently may then give way to multipotent cells, which in turn become committed to tissue-specific lineages. These data suggest the presence of cells undergoing specific differentiation. A deeper analysis should be performed within these populations to evaluate cells expression paired with other markers that could confirm the cells commitment toward a definitive differentiation. On the other hand, PDX-1 and GDNF are pretty specific and are identifying populations committed to pancreas[66] and kidney[51], respectively.

Since the major part of the liquid in AF derives from fetal urine, progenitors for the kidney could be floating in the liquid and, therefore, we focused our attention to the isolation and characterization of renal precursor cells from Amniotic Fluid since the overall aim of our research is to identify lineages that may be useful for kidney therapeutic purposes.

Kidney complex development required to select AF cells derived in order to obtain a specific progenitor cell population.

In fact, kidney, unlike other organs, has two different embryological origins. Two different structures derive from the intermediate mesoderm: the Ureteric Bud (UB), an extroflection of the Wolffian duct and the Metanephric Mesenchyme (MM). The two structures show a common expression of LIM-1, essential for the development from the intermediate mesoderm.

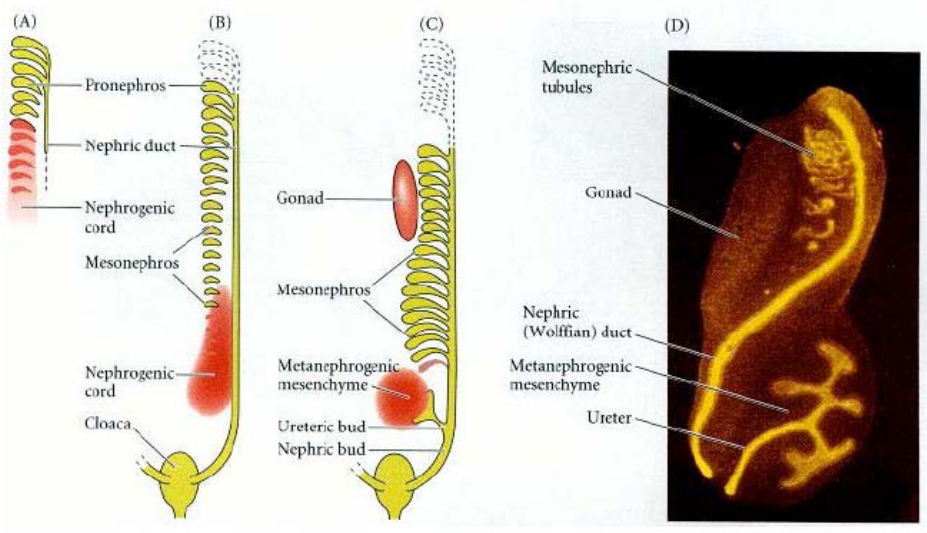


Figure 14 – Early kidney development *The picture shows kidney development from the pronephros to the formation of nephric duct and the nephrogenic cord leading to the formation of the metanephros.*

The evolution of the kidney reflects the need of land-adaptive vertebrates to conserve water, excrete waste and maintain electrolyte homeostasis within a variety of challenging environments[68].

Human kidney development starts around E18 and is ending between gestational weeks 32-36 and also after birth; in the mouse is starting around E7.5 and is ending 2 weeks after birth. Kidney develops from the intermediate mesoderm located between the axial, or somitic, mesoderm and the lateral plate mesoderm[67].

The UB gives rise to two transient structures called the pronephros and the mesonephros and extend caudally along the axis with a structure called Nephric Duct (Figure 14). At the caudal end of the UB is present an agglomerate of cells, called Metanephric blastema, that forms the MM. While the pronephros and the mesanephros degenerate, the metanephros, a structure present only in mammals, goes through an intensification of signals between two structures: the MM and the UB, an outgrowth of the nephric duct[67].

The invasion of the UB into the MM represents a crucial point for the kidney development. This process is probably guided by signals secreted from the MM, although this has not yet been demonstrated. Inductive signals exchanged between the MM and the UB are pushing the bud to grow and branch while the MM cells replicate themselves prior to condensate around the UB tips[67].

GDNF is secreted by the MM and addressed to the RET receptor expressed on the UB cells surface starting the UB branching. This process is essential to induce the UB branching that stimulates MM condensation through expression of factors such as molecules of the FGF's family. The induced MM at this point starts to express PAX-2, first indicator of the nephrogenic cell lineage (Podocytes, mesangial cells, stromal cells and MET cells) at this stage[68].

The condensed mesenchymal cells induce the branching of UB giving rise to two new ureteric tips that are going to form pretubular aggregates. These pretubular aggregates undergo a mesenchyme to epithelial transition and form an epithelial tubule. Kidney tubules develop into nephrons, through several stages of development. First, a tubule develops into a comma shaped body and then into an S-shaped body. In the S-Shaped stage is possible to retrieve mesangial cell precursors expressing PDGF receptor and podocyte cells precursors beginning to express Nephrin at the cleft of the S-shaped body[69-70]. The most proximal end of the S-Shaped body undergoes angiogenesis[71]. These processes are started by repression of PAX-2 expression, and consequentially the S-Shaped body forms the glomerulus and proximal and distal tubules, which

fuse to the UB. The middle part of the tubule develops into the loop of Henle. The proximal tubule and the loop of Henle are the kidney regions that are responsible for most of the reabsorption of essential molecules and salts from urine, before they pass to the collecting duct system. During the branching morphogenesis of the epithelial UB, each tip acts as an inductive center to initiate nephrogenesis, after it has elongated and branched to generate new epithelial tips. The branches of the UB eventually form the collecting-duct system, which collects urine into the renal pelvis and urinary bladder. These stages are repeated to generate ~12,000 nephrons in the mouse kidney and almost 1,000,000 nephrons in the human kidney. In humans, nephrogenesis is complete around 35-37 weeks while in rats and mice it continues postnatally for about 2 weeks.

The understanding of the kidney development helped us to determine which are the more specific genes involved in particular pathways that identify the different cells present in the nephron.
We focus our attention in defining and characterizing a more specific cell population isolated from AF for kidney regenerative purposes, capable of more precise homing, integration and differentiation in the injured glomerular structures.

In order to increase the specificity of a stem cell lineage for the glomerulus compartment, and in particular for the podocytes cell type, we investigated reliable markers that could be used to retrieve a progenitor cell population from the AF.
Since many are the cells constituting the glomeruli, the first necessary step was the identification of markers that allow us to separate the different progenitor cells.
Based on literature review we identified Nephrin as a reliable marker for immature and mature podocytes, PDGFR α for mesangial cell progenitors, TrKA for stromogenic cortical mesenchymal cells and E-Cadherin for epithelial cells of the Bowman's Capsule[68]. It is important to mention that the identification of these particular markers was not granting us the

selection of a reliable renal population since their expression is not exclusive of the kidney compartment, but they are expressed also in other organs pathways development. Nephrin, for example, expressed in the differentiating and mature podocytes, has been found in other extrarenal tissues such as brain and pancreas[71] and E-Cadherin, epithelial marker, has been known to be present, beside in the first steps of gastrulation, in many epithelial tissues[72-73].

One important *in vivo* study[74] for our purpose reported that CD24 and OB Cadherin are co-expressed in cells of the undifferentiated MM in a mouse developing kidney.
Since Metanephric Mesenchyme is the fetal structure that gives rise only to the nephron, it was a reliable starting point for our purposes, giving us the capability to select a more specific MM population from which retrieves the more specific progenitor populations. In this way we could exclude the possibility of isolating other progenitors that would eventually give rise to cells with no specific renal fate.

We showed that in the total population of AF is evident the expression of renal markers (early and mature) such as PAX-2, LIM-1, Nephrin, PDGFRα, NGF High Affinity Receptor (chained with Tyrosin Kynase, TrKA) , E- Cadherin, CD24, and OB-Cadherin (Cadherin 11) with a strong increased expression by the end of the 17th week of gestation, when kidney C-Shaped bodies are formed and are turning into S-Shaped bodies (Figure 3C-4C). Real time PCR revealed an increase expression for Nephrin and GDNF, markers of the MM derived lineages (Figure 5D-5E).

After having confirmed that within AF we can identify the expression of renal markers, we focused our attention on the isolation of the MM lineage using immunoseparation techniques for the CD24 and OB Cadherin. Since the origin of cells within AF is from different organ, as described previously, the first necessary step was to confirm that the MMDC

population was sharing, with the *in vivo* renal population, specific traits of expression patterns. We confirmed that CD24+ OB-Cadherin+ population (MMDC) expresses several kidney markers including AQP1, LIM-1, PAX-2, Nephrin, GDNF, Occludin and ZO-1 by RT-PCR.

Subsequently we performed specific selection from MMDC population in order to obtain four different subpopulations, called AKPC, expressing for ***1.*** E-Cadherin (MET cells), ***2.*** Nephrin (podocytes), ***3.*** TrkA (for stromogenic mesenchymal cells) and ***4.*** PDGFR α (mesangial cells) respectively. In doing this we could be confident that the expression of these markers was not involved in other organs pathways, but very specific for the kidney since they were immunosepareted from an already renal induced population, the MM.

These four subpopulations were then analyzed by the more sensitive Real Time PCR. The expression pattern for the four population confirmed that the four progenitor cell lineages are very distinct populations, with specific and particular roles into the glomerular structure of the nephron.

Among others, expression of LIM-1 and PAX-2 was evaluated in order to confirm AKPC non complete maturation, since the two transcription factors are not present in the renal adult cells (Figure 7).
TrKA+ APKC cells showed expression of TrKA, LIM-1, PAX-2 and OCT-4 at important levels and expressed E-Cadherin, Occludin and PDGFRα at low levels (Figure 8A-B). No expression of Nephrin or GDNF was shown. TrKA has been shown not to be expressed in other than Stromogenic Cortical Mesenchymal cells[69] and since the expression of this marker has been found only within this selection, we can with high confidence speculate that this population represents the progenitors of the renal stromal cells (PDGFRαAKPC).
Mesangial precursor cells, selected for PDGFRα were positive at different levels for PDGFRα and E-Cadherin (Figure 8A-8B). Expression of E-Cadherin is normally not reported in cells of the mesangium but since is

evident the slightly expression of uninduced mesenchyme markers (LIM-1, PAX-2 and OCT-4) and E-Cadherin is expressed in the uninduced mesenchyme, it is not unreasonable to think that the differentiation process is still in progress.

E-Cadherin+ APKC expressed high levels of OCT-4 and Occludin and high levels of E-Cadherin and LIM-1, both expressed in the uninduced mesenchyme. E-Cadherin+ cells expressed low levels of PAX-2 and showed no expression of TrKA, PDGFRα and ZO-1 (Figure 8A-8B). These data may indicate that, since E-Cadherin is expressed in the uninduced mesenchyme, the selection performed was not precise enough to retrieve cells undergoing Mesenchymal to Epithelial Transition but more analysis have to be performed.

As reported by Pavenstadt *et al*.[75] mature podocytes are expressing several markers such as Nephrin and ZO-1 but data are lacking regarding eventual precursors of this visceral epithelial cells. Our Nephrin+ APKC population showed the expression of pluripotent marker OCT-4 and of LIM-1 and PAX-2, that may let us confirm the traits as a renal precursor that haven't yet committed to glomerulus or proximal/distal tubules, due to the presence of E-Cadherin (Figure 8A-8B). At the same time, the expression of Nephrin, localized in the kidney only in the differentiating and mature podocytes, may indicate these cells are undergoing through visceral epithelial cell maturation. An additional data in support of this thesis is the expression of Zo-1 that usually co-localizes within the podocytes, coupled with Nephrin.

The knowledge about AF composition and its variations may provide useful information for clinical diagnosis or the collection of specific cell populations. In addition, presence of progenitor cells within AF and in particular the existence of kidney progenitor cells (AKPC) within AF may provide new tools for therapeutic applications.

2. *In vivo* Amniotic Fluid Stem Cells application for Chronic Kidney Disease treatment; Injection in Alport mice

In this study we have chosen a mouse model of CKD, the AS due its peculiar proprieties related to the GBM and the activity of the podocytes, the key cell in the functional kidney. In addition, this animal model represents very well the progression of the renal disease that involves fibrosis as it happens in human patients.

GBM during glomerulogenesis is generated by glomerular and endothelial cells as two distinct layers that fuse together to form the mature GBM consisting of laminin, and integrins in a network mainly composed by collagen IV[75].

Six individual chains (α 1-6) have been identified and their assembly in different protomers gives rise to collagen IV with distinct characteristics and localizations.

In the fetal kidney the collagen IV is mostly composed of α1/α2 dimers that are replaced in many locations in the adult GBM.

In the adult, α3-4-5 trimer substitutes the α1-2 chains network in the glomerular basement membrane and some tubules whereas the α 5-5-6 becomes predominant in the Bowman's capsule[76] (Figure 15).

In mice affected by AS, the fetal α 1 and α 2 chains persist in adult GBM and are not replaced with the α 3-4-5 network. While the α1/2 network is essential for the normal glomerular development, it lacks of the long term stability of the α3/4/5 collagen IV and this cause the malfunction of the entire glomerulus.

After birth a progressive thickening and splitting of the GBM, a subsequent postnatal proteolysis and a greater susceptibility to mechanical strain caused by the ultrafiltration are presumed to cause progressive deterioration of the GBM due to the absence of the required adult collagen IV[77].

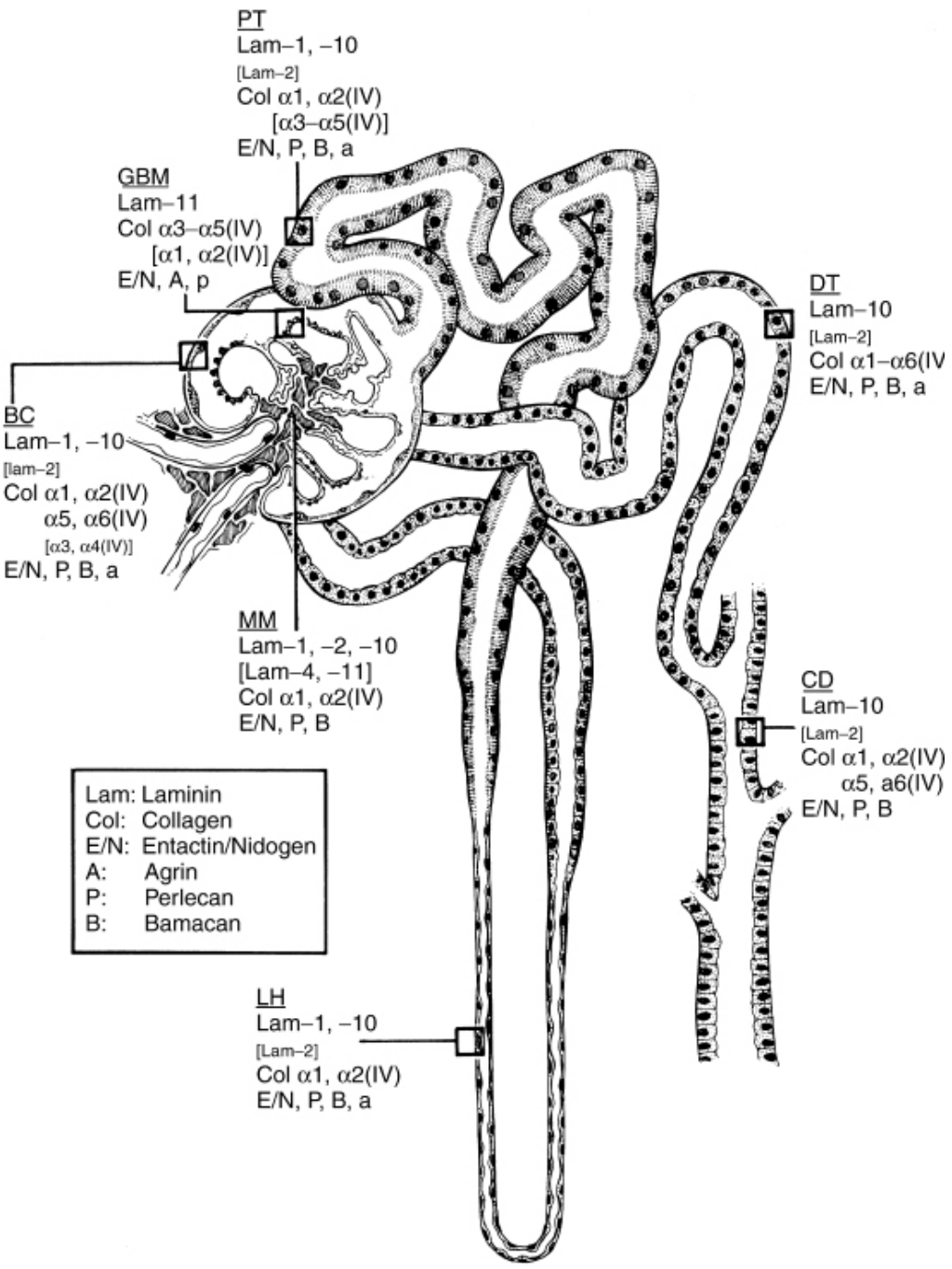


Figure 15 –Composition of the basement membrane in the different compartments of the kidney. *Deposition of Collagen IV α 3-4-5 is limited to the adult glomerular structure and some tubules. Presence of Collagen IV α 1-2 in the normal glomerulus is limited to the fetal age.*

Damages in the GBM are inducing apoptosis in podocytes which present a limited ability regeneration and repair and have been considered critical and vulnerable targets in glomerular injury[78].

The disruption of both GBM and podocytes layer leads to hematuria and proteinuria and the progression into CKD is associated with glomerular sclerosis and tubulo-interstitial fibrosis, fibroblast activation, inflammation and reorganization of extracellular matrix[79]. The effects of AS on the renal compartment is evident as shown in the Figure 10 were we compared a WT kidney with a kidney from an AS mice stained with PAS solution.

The destruction of all the organized structures of the kidney is complete and both the glomeruli and the tubules are deeply affected by the progression of the disease.

In the C57BL/6J murine model of AS (B6.Cg-*Col4a5*^{tm1Yseg}/J), presenting the most common form of the syndrome (X-Linked), the first clinical outcomes are evident at 2 weeks and the injury slowly progresses until around 8 weeks after birth, ending with the dead of the animals due to a massive disruption of the kidney structures.

We performed some preliminary experiments and Alport mice were injected with about 800,000 mouse AFSC at 8 weeks after birth, when typically happens the outcome of CKD with increasing proteinuria. In the first set of injection we follow the mice until the time of death due to ESRD. When compared with the control (not injected mice) the cells-injected Alport mice were able to survive longer with an increase of life- span of the 25% (Figure 12). From these data we can speculate that injected cells where able to slow down the progression of the disease.

In addition, AS mice, either injected or not, and Wild Type mice, were checked for blood levels of creatinine and nitrogens and for urine levels of albumin.

Since the use of only one parameter to evaluate kidney functionality is not completely reliable, we chose to perform three distinct tests to assess kidney functionality (Figure 12).

In the Wild Type mice the level of creatinine is usually around 0.6-0.7 mg/DL but in the AS mice the level was increased to 1.4 mg/DL showing a dramatic loss of renal functionality. In the AFSC injected mice the average of blood creatinine was 0.978 mg/DL showing a clear improvement in the Glomerular Filtration Rate. The same trend was found in the BUN measurement were the nitrogen level was decreasing from 53.13 mg/DL of AS mice to a 46.08 mg/DL in the injected murine model with no significant difference from the WT value of 45.30 mg/DL.
In addition, albumin urine levels felt from the 600 mg/DL of the AS mice to a 273 mg/DL of the injected AS mice, not far from the WT average of 255 mg/DL. The results, retrieved around 5 weeks after the cell injection, are indicating that the physiological parameters were restored to normal compare with their siblings not injected (Figure 12).

Histological analysis of the kidneys showed that AFSC, labeled with red fluorescent dye CM-DiI, were integrated within the glomeruli with only few cells homed in tubular structures.
The specificity of cell homing may be explained with the chronic inflammation of the kidney and in particular the degeneration of glomeruli that provides a molecular recognition sink, with excretion of chemokynes and other molecules able to lead stem cells to the injured site.
This hypothesis is confirmed by previous works done with BM-MSC. In particular, Sugimoto H. *et al*.[80], Prodromidi EI. *et al*.,[81] and Ninichuk V. *et al*.,[82] have shown that stem cells derived from bone marrow can be used to treat animal models of AS. However, the beneficial effects of BM transplantation from WT mice, although statistically significant, have been modest in scope and Kalluri[80] in 2006 reported similar results.
In AS the absence of the three adult chains (α3-4-5) of collagen IV are replaced with the fetal chains α 1 and α 2, usually not or poorly expressed in the normal renal background. When kidneys from AS mice are stained for Collagen IV α 1, the presence of the α1 chain is detected in thick agglomerates around the Bowman's capsule and within the GBM, while in

the Wild Type the Bowman's capsule is slightly positive and the GBM is negative for α1.

Immunofluorescence analysis of the injected murine kidneys showed that deposition of α1 was lower in the Bowman's capsule if compared with the AS not injected mice as shown in FIG 11. And most importantly, a lot less COL4α1 was found in the GBM where AFSC were integrated. This is an indication that AFSC may be able to slow down the progression of the deposition of the wrong form of col4α1.

The decrease of protein loss with amelioration of the ultrafiltration process in the injected AS mice, the decreased thickening of the GBM due to α1-2 chains and the increased life span of the mice versus the controls are promising results that need further analysis about homing, mechanism and capacity of AFSC to slow and control proteinuria, podocytes loss and fibrosis.

CONCLUSIONS

In summary, in this study we assessed the potential of AFSC for Chronic Kidney Disease therapy through the evaluation of survival, amelioration of the physiological parameters and morphological investigation of the injured organ.

Nonetheless, beside the presence of a small number (1%) of cells with pluripotent characteristics, the composition of the other 99% of AF cells is diverse, with a great amount of cells exhibiting commitment to a defined germ line or cellular endpoint.

The evidence for the existence of progenitor cells in Amniotic Fluid, which can generate different cell types of mature organs, seems to be clear.

By 17 weeks of gestation is notable an increase tissue specific cellular presence and this data may indicate that the choice of the time point for cell selection is fundamental. In addition we demonstrated the presence of a renal population with specific traits of commitment. In particular, the presence of podocytes at both undifferentiated and almost mature stage could prove their use for kidney regeneration *in vitro* and *in vivo* animal models. The presence and successful identification of specific renal progenitors in AF, committed to different compartments of the kidney environment, could represent a valuable new tool for regenerative purposes with regards to the treatment of a broad range of renal diseases.

In conclusion, the discovery of renal specific progenitor cells within AF could bring a breakthrough in the study for novel, more specific approaches in the renal therapy. However, the real pluripotential capability of these progenitors cells, in particular the kidneys progenitors to present mature renal cells characteristics, has to be established. Moreover, their potential for survival, integration, proliferation and differentiation needs to be assessed in *in vivo* models involving different types of renal damage.

In addition, this study has shown AFSC as a reliable tool for regenerative medicine. The ability of stem cells to home, integrate and ameliorate AS damage through diminished proteinuria, decrease of fetal collagen IV α 1 and prolongation of life time has been proved. The investigation of the therapeutic effects of AFSC should be now performed through a study of mechanism of action, the evaluation of multiple cell injections administration and the assessment of the optimal doses.

Future aims of this project are:

1. assessment of differentiation potential for AKPC cells, performing *in vitro* and *in vivo* studies, in order to evaluate their specificity for the glomerular structures. *In vitro* differentiation and functional investigation will be used as a preliminary evaluation of their regenerative capability. *In vivo* studies on AKF, ATN or CKD will be performed to understand their integration, differentiation and immunogenicity. The understanding of AKPC maturation pathways can reveal new tools for kidney development and pathology formation comprehension;

2. Investigation of AFSC mechanisms in the healing process for Alport Syndrome and Chronic Kidney Disease. In addition, the establishment of a more effective administration of the cells, like injection at different time points along the progression of the disease or multiple injections, are a priority in order to understand the mechanism of action and maximize their beneficial effect.

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TABLES AND FIGURES

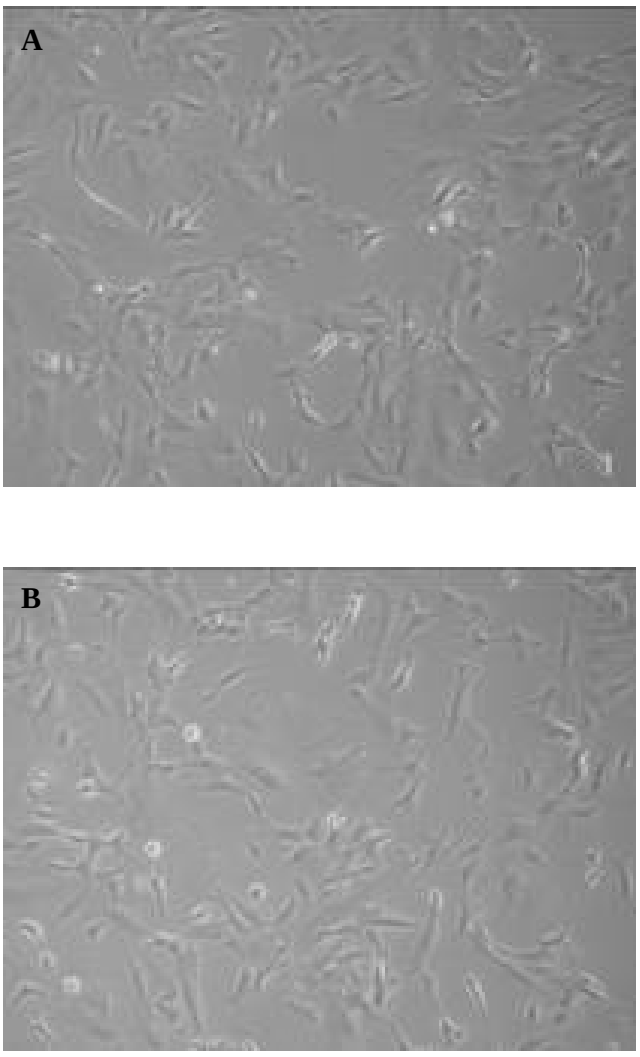


Figure 2 A - B – Morphology of Total amniotic fluid cell population. *The morphology of total amniotic fluid populations is various with a prevalence of fibroblastoid shapes. In Chang's medium the morphology is unchanged for up to 50 passages in culture (A, B x20).*

A

	Weeks of Gestation	15	16	16	17	17	17	17	17	18	18	18	18	18	19	20	20
	Sample	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Pluripotency	CD34										▪		▪	▪	▪	▪	▪
	CD90	▪	▪	▪	▪	▪	▪	▪		▪	▪	▪		▪	▪	▪	▪
	Ckit	▪	▪	▪		▪		▪		▪	▪	▪		▪			
	OCT-4	▪	▪			▪	▪			▪	▪	▪	▪	▪			
Ectoderm	NCAM	▪	▪	▪	▪	▪			▪	▪	▪	▪		▪	▪	▪	
	FGF-5	▪	▪	▪	▪	▪	▪		▪	▪	▪	▪		▪	▪	▪	▪
	E-Cadherin	▪		▪	▪					▪	▪	▪	▪	▪		▪	
Mesoderm	Flk-1	▪	▪			▪	▪			▪		▪		▪		▪	▪
	Brachyury		▪			▪											
	Tal-1	▪		▪												▪	
Endoderm	Cxcr-4	▪	▪	▪		▪	▪				▪						
	Sox-17	▪	▪														
	Goosecoid	▪		▪													
	AFP		▪	▪	▪	▪			▪	▪		▪		▪	▪		▪

B

Weeks of Gestation	15	16	16	17	17	17	17	17	18	18	18	18	18	19	20	20
Sample	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
CEBPG	▪	▪	▪	▪		▪		▪	▪	▪	▪		▪	▪	▪	▪
GDNF	▪						▪		▪	▪			▪		▪	▪
TTF-1						▪			▪		▪	▪			▪	
NKX2.5			▪						▪	▪	▪		▪		▪	▪
PDX-1					▪					▪						

C

Weeks of Gestation	15	16	16	17	17	17	17	17	18	18	18	18	18	19	20	20
Sample	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
OB-Cad									▪	▪		▪	▪	▪	▪	▪
CD24									▪		▪	▪	▪	▪	▪	▪
GDNF	▪						▪		▪	▪			▪		▪	▪
PAX-2		▪			▪					▪		▪			▪	
NEPHRIN											▪		▪	▪		▪
LIM1	▪	▪	▪		▪		▪		▪	▪	▪		▪	▪		
OCCLUDIN		▪			▪				▪	▪	▪		▪		▪	▪
AQP-1	▪	▪	▪		▪		▪		▪	▪	▪		▪	▪	▪	▪
ZO-1	▪	▪	▪		▪		▪		▪	▪	▪		▪	▪	▪	▪

Figure 3 A-B-C – Expression of markers for the three germ layers, for progenitor cells and for kidney specific progenitor cells over the time by RT-PCR. *Colored areas are including the time range in which the expression is significant for different groups of correlated markers.*
Expression of Endodermal and Mesodermal markers decreases in older samples while Ectodermal and pluripotency markers expression is constant over the time (Table 3A). By 17 weeks of gestation it's evident an increase in expression of several progenitor markers (Table 3B) including renal specific proteins (Table 3C).

A

	Weeks of Gestation	15	16	16	17	17	17	17	17	18	18	18	18	18	19	20	20
	Sample	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Pluripotency	CD34										▪		▪	▪	▪	▪	▪
	CD90	▪	▪	▪	▪	▪	▪	▪		▪	▪	▪		▪	▪	▪	▪
	Cktt	▪	▪	▪				▪	▪	▪	▪	▪		▪		▪	▪
	OCT-4	▪	▪			▪	▪	▪		▪		▪	▪	▪		▪	▪
Ectoderm	NCAM	▪	▪	▪	▪		▪		▪	▪	▪	▪		▪	▪	▪	
	FGF-5		▪	▪		▪			▪		▪		▪	▪	▪		
	E-Cadherin		▪	▪		▪						▪	▪		▪	▪	▪
Mesoderm	Flk-1	▪	▪			▪	▪			▪		▪		▪		▪	▪
	Brachyury		▪			▪											
	Tal-1	▪		▪												▪	
Endoderm	Cxcr-4		▪	▪		▪											
	Sox-17	▪	▪		▪	▪				▪							
	Goosecoid			▪		▪	▪			▪							
	AFP	▪	▪	▪		▪	▪	▪		▪	▪	▪	▪	▪	▪	▪	

B

	Weeks of Gestation	15	16	16	17	17	17	17	17	18	18	18	18	18	19	20	20
	Sample	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
	CEBPG	▪	▪	▪	▪		▪		▪	▪	▪	▪		▪	▪	▪	▪
	GDNF	▪						▪		▪	▪			▪		▪	▪
	TTF-1						▪	▪		▪		▪	▪			▪	▪
	NKX2.5			▪						▪	▪	▪	▪	▪		▪	▪
	PDX-1					▪				▪	▪						

C

	Weeks of Gestation	15	16	16	17	17	17	17	17	18	18	18	18	18	19	20	20
	Sample	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
	OB-Cad								▪	▪	▪	▪	▪	▪	▪	▪	▪
	CD24	▪						▪		▪		▪	▪	▪	▪	▪	▪
	GDNF									▪	▪	▪		▪		▪	▪
	PAX-2		▪			▪					▪		▪			▪	
	NEPHRIN						▪					▪	▪	▪	▪	▪	▪
	LIM1	▪	▪	▪		▪		▪		▪	▪	▪	▪	▪	▪		
	OCCLUDIN		▪							▪	▪	▪	▪	▪		▪	▪
	AQP-1	▪	▪	▪		▪	▪	▪		▪	▪	▪		▪	▪	▪	▪
	ZO-1	▪	▪	▪		▪	▪	▪	▪	▪	▪	▪		▪	▪	▪	▪

Figure 4 A-B-C –*Western* ***Blotting Analysis of Amniotic Fluid Total Cell*** *Population for markers of all the three germ layers and pluripotency (Figure 4A), tissue specific progenitor cells (Figure 4B) and kidney specific cell markers (Figure 4C). Colored areas are including the time range in which the expression is significant for different groups of correlated markers. Expression of Endodermal and Mesodermal markers decreases during the gestation while Ectodermal and pluripotency markers expression is preserved in older samples. By 17 weeks of gestation it's evident an increase in expression of several progenitor markers including renal specific proteins.*

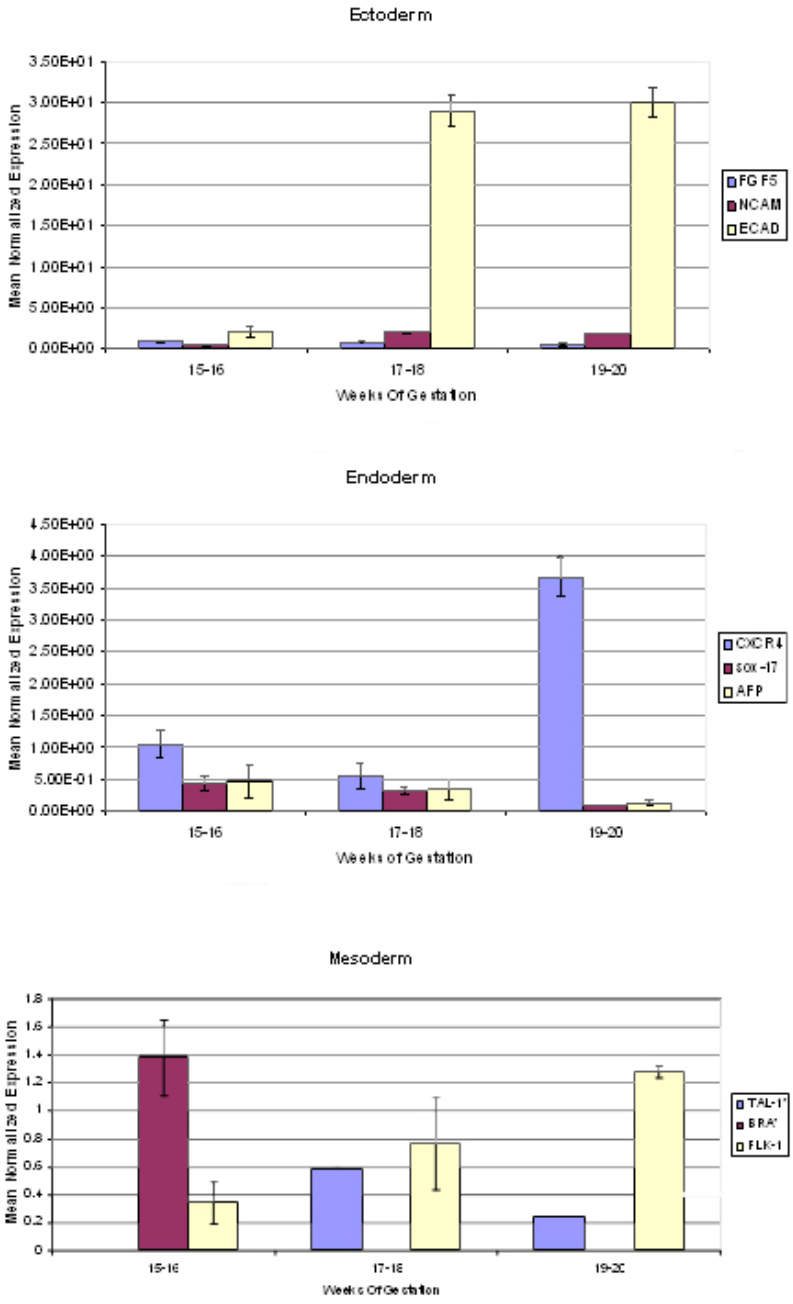


Figure 5 A - Real Time PCR for Mesoderm, Endoderm and Ectoderm markers *(Ectoderm, Endoderm, Mesoderm)*
Expression of markers for the three germ layers. Quantitative analysis of markers expression shows an increase of E-Cadherin (Ectoderm), Flk-1 (Mesoderm) and Cxcr-4 (Endoderm). While ectodermal markers FGF-5 and NCAM are constant over the time , endodermal and mesodermal markers are decreasing their expression in older samples.

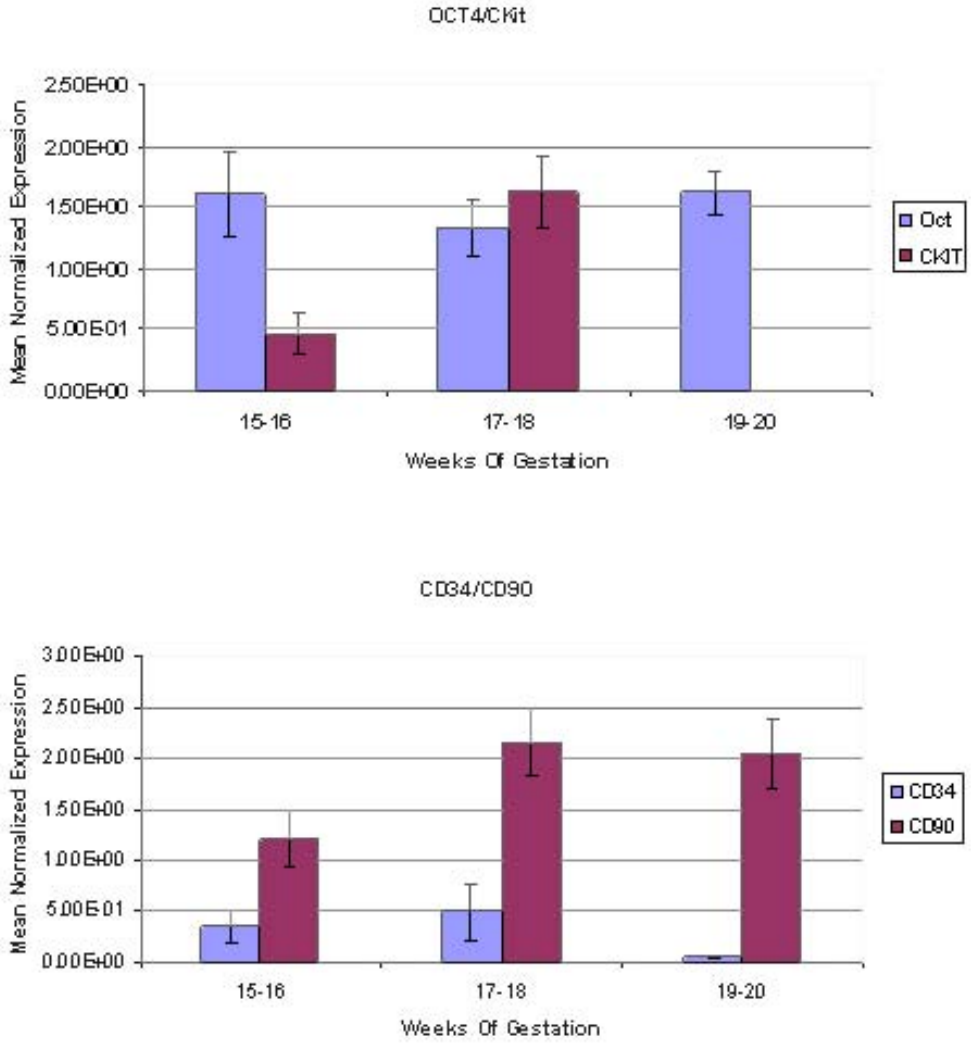
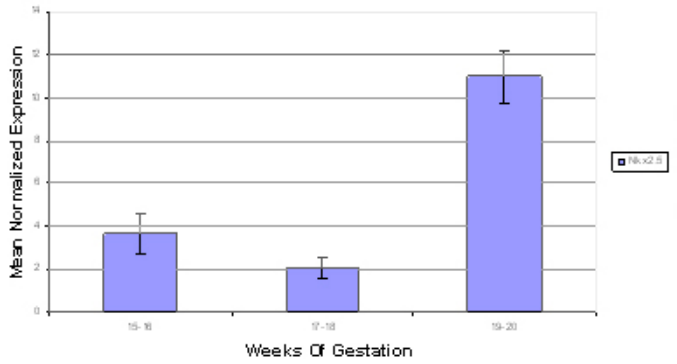
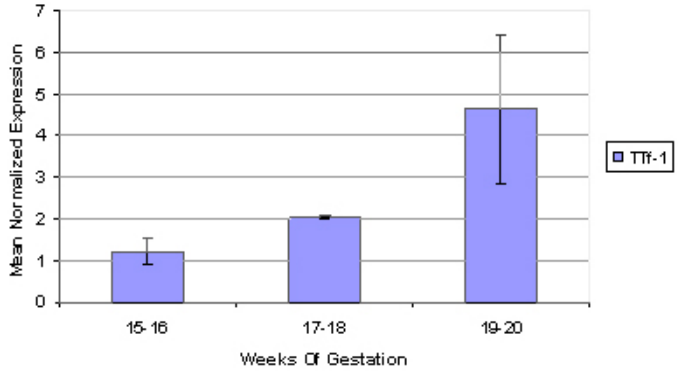


Figure 5 B – Real Time PCR for pluripotency, hematopoietic and mesenchymal markers. *(OCT-4, c-Kit, CD34, CD90)*
Expression of OCT-4 is not presenting any significant variation over the time while Ckit increases between 17-18 weeks and disappears at 19-20 weeks of gestation. Hematopoietic marker CD34 dramatically decreases as well at 19-20 weeks while CD90, marker for mesenchymal cells, is highly expressed in all the samples evaluated.

Nkx2.5



TTF-1



CEBPG

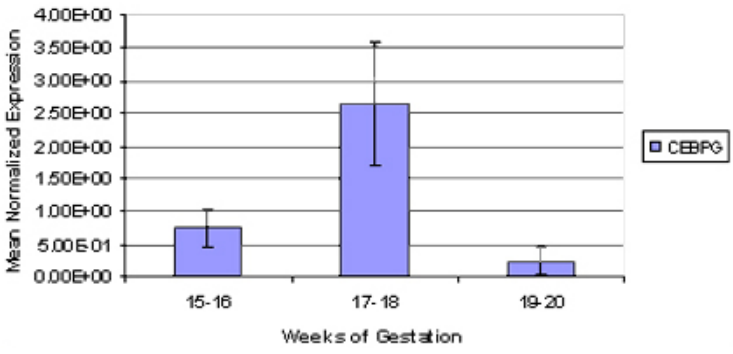


Figure 5 C – Real Time PCR for Progenitor markers *(Nkx2.5, TTF-1, CEBPG) Expression of Nkx2.5, early cardiac marker, and TTF-1, marker for lung differentiation increases at 19-20 weeks. CEBPG showed an increase between 17-18 weeks to return then to an expression comparable with the 15-16 weeks trend. PDX-1 is not reported. No data were retrieved in the samples analyzed.*

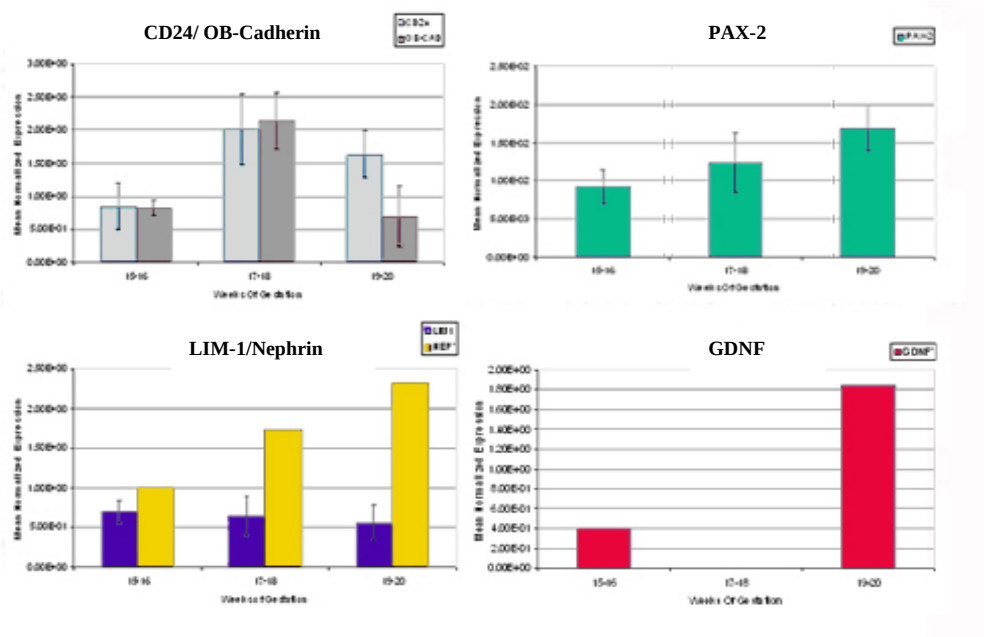


Figure 5D – Real Time PCR for Metanephric Mesenchyme markers in total amniotic fluid population. *(CD24, OB-Cadherin, PAX-2, LIM-1, Nephrin and GDNF)*
CD24 and OB-Cadherin, markers of the uninduced Metanephric Mesenchyme are increasing at 17-18 weeks. OB Cadherin is then decreasing to the previous level while CD24 maintain its high expression. Pax-2 shows a trend of increase over time like Nephrin, podocytes marker. LIM-1 maintains constant its expression. GDNF was detected in only two samples but shows a strong increase in the expression at 19-20 weeks of gestation.

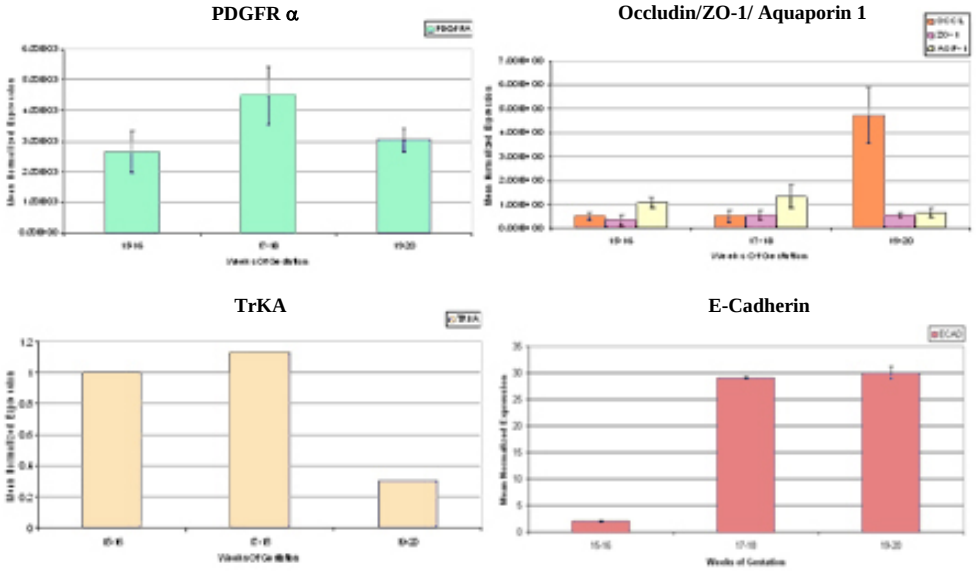


Figure 5 E - Real Time PCR for kidney markers in total amniotic fluid population*. (PDGFR*α*, Occludin, ZO-1, Aquaporin-1, TrKA and E-Cadherin)*
Expression of PDGFRA doesn't show a significant variation over the time while TrKA expression decreases at 19-20 weeks. Occludin expression increases at 19-20 weeks while AQP-1 and ZO-1 are constant over the time.

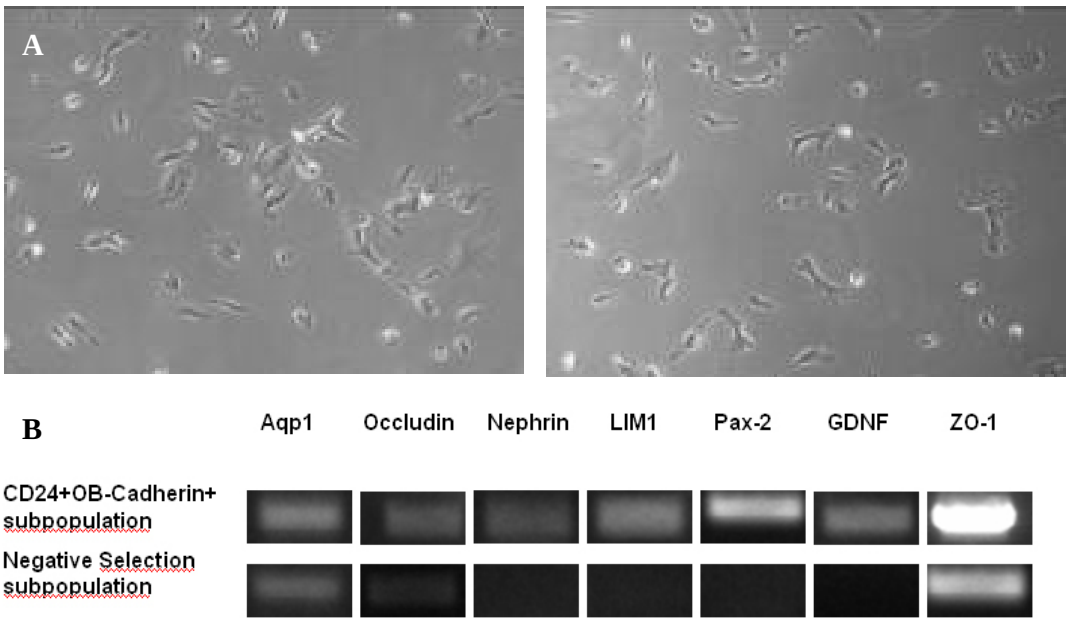


Figure 6 A-B – Morphology and Characterization of MMDC cells. A *Morphology of the MMDC selection. (x20)* ***B-*** *RT-PCR of MMDC population compared with a CD24 Negative $^{-}$ OB Cadherin^{-} negative selection. Expression of renal markers in the double positive selection, compared with the negative results for the negative selection, confirm the successful immunoseparation of cells with renal traits from the whole amniotic fluid population.*

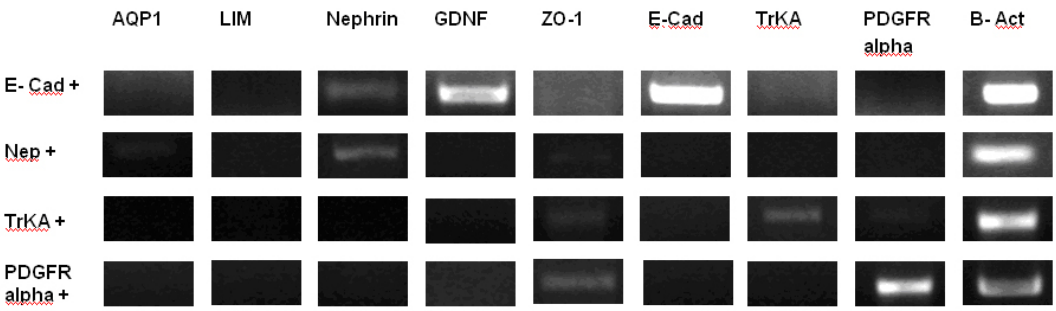


Figure 7 – *RT-PCR of CD24^{+}OBCadherin^{+} derived subpopulations (AKPC). Patterns of expression are showing that the selection retrieved four different populations with diverse characteristics.*

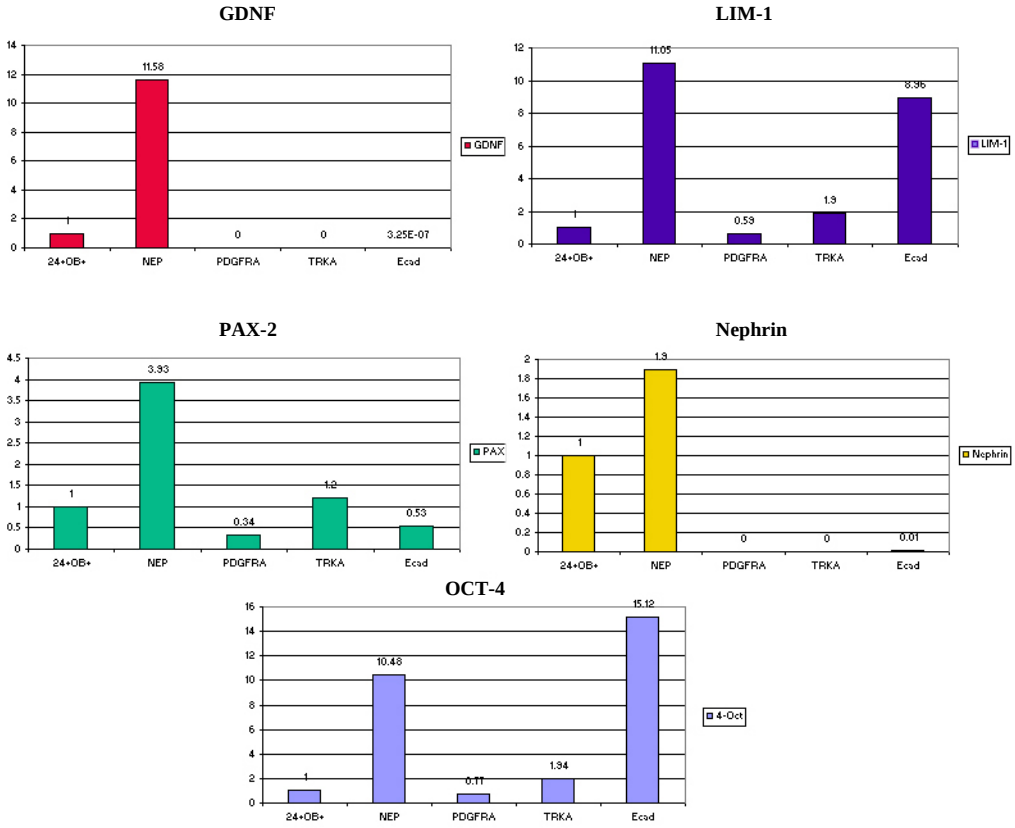


Figure 8A - ***Real Time PCR for AKPC populations***. *(GDNF, LIM-1, PAX-2, Nephrin and OCT-4). Expression of GDNF was found in the main population (AKPC) and in the Nephrin+ selection. LIM-1 was expressed by all the populations with a strong expression in Nephrin+ and E-Cadherin+ AKPC cells. PAX-2 was expressed highly by Nephrin+ cells but was present in all the selections. Nephrin was found expressed in the Nephrin population while OCT-4, highly expressed in Nephrin+ and E-Cadherin+ AKPC cells, was expressed broadly.*

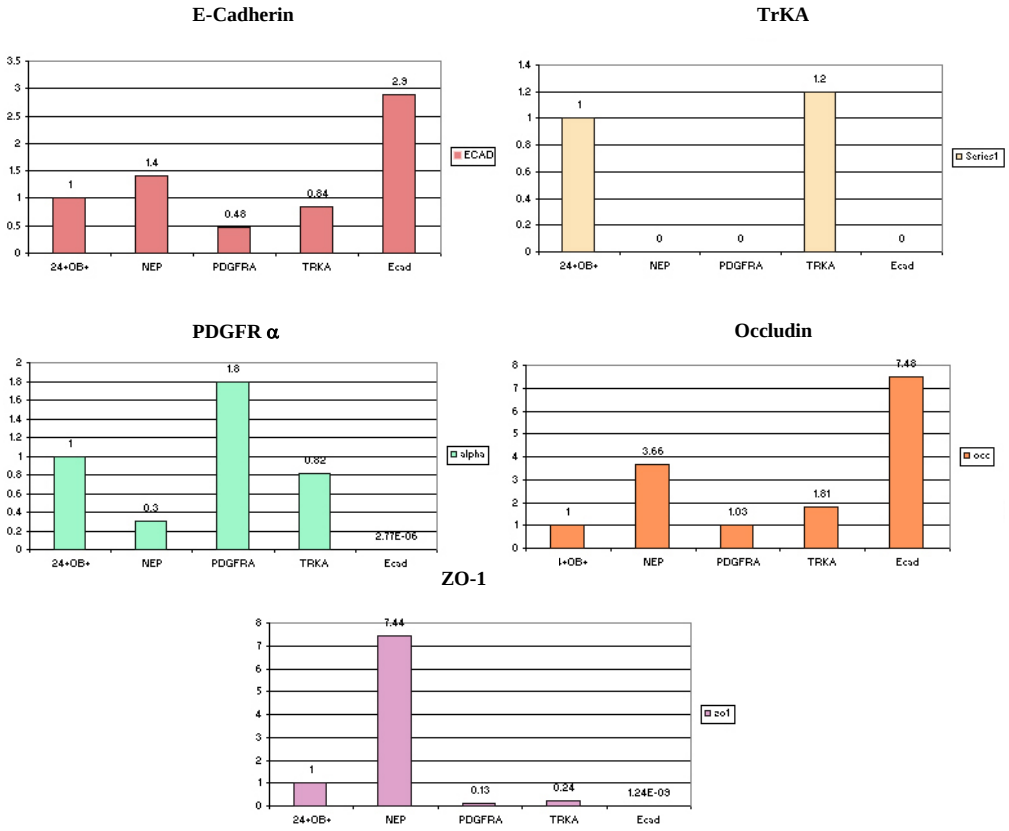


Figure 8B- Real Time PCR for AKPC populations. *(E-Cadherin, TrKA, PDGFRα,Occludin, ZO-1). Expression of E-Cadherin was found in all the samples, indicating E-Cadherin as a marker not specific for MET cells. TrKA was expressed solely by TrKA+ AKPC cells. PDGFR Alpha was expressed strongly by PDGFRα selection, under expressed in Nephrin+ and TrKA cells and barely detectable in E-Cadherin+ cells. Occludin was highly expressed By E-Cadherin and in minor degree by Nephrin+ AKPC and was present in PDGFR α+ and TrKA+ cells. ZO-1, molecule usually paired with Nephrin within podocytes, was highly expressed in Nephrin+ selection and was barely expressed in the other populations.*

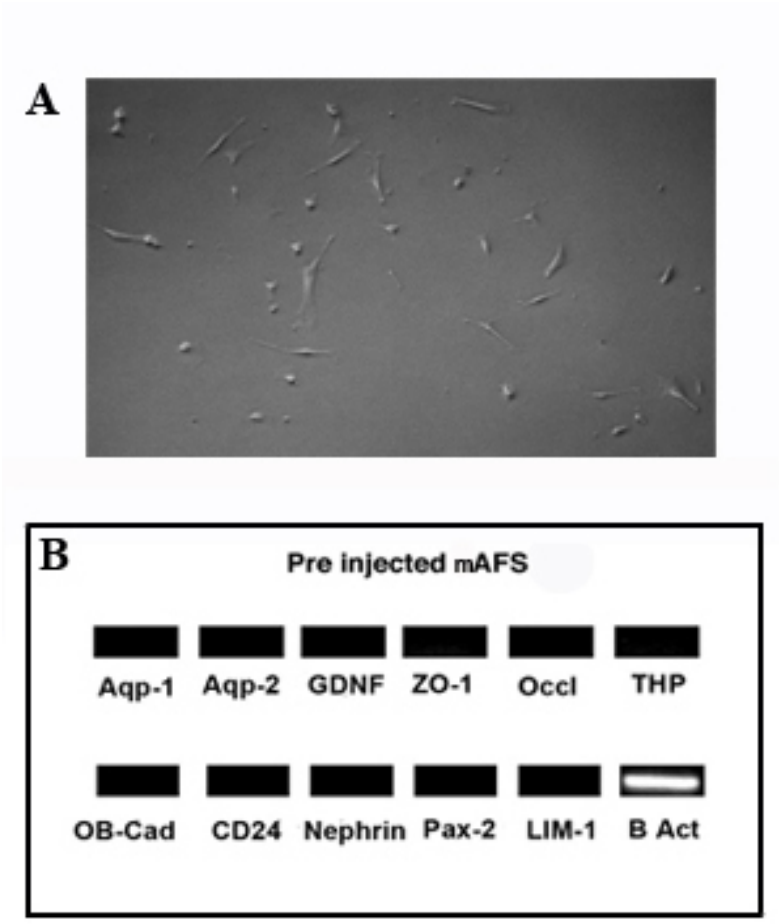


Figure 9 A-B – mAFSC analysis before the injection. *AFSC were investigated for morphology (FIG 9A, x20) and expression of renal markers such as Aqp-1 and 2 GDNF, ZO-1, Occludin, THP, OB-Cadherin, CD24, Nephrin, Pax-2 and LIM-1 resulting negative by RT-PCR (B);*

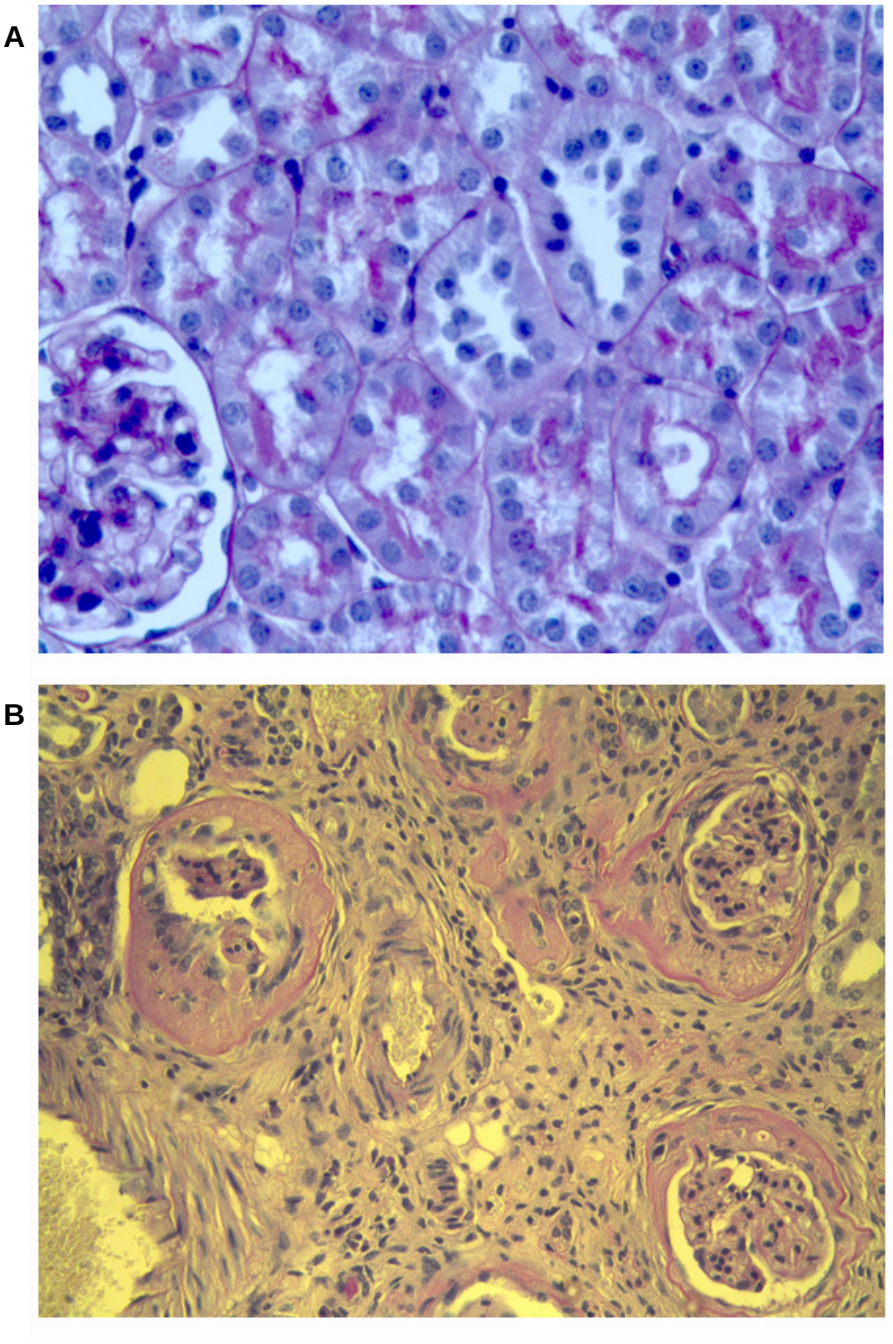


Figure 10 - PAS staining of paraffin embedded section of kidney in Wild Type (A) and Alport Syndrome mice (B) *In the WT kidney the renal structures are well defined (A) while in the AS kidney the glomerular space is damaged and it is not possible to clearly recognize the tubules (A,B x20).*

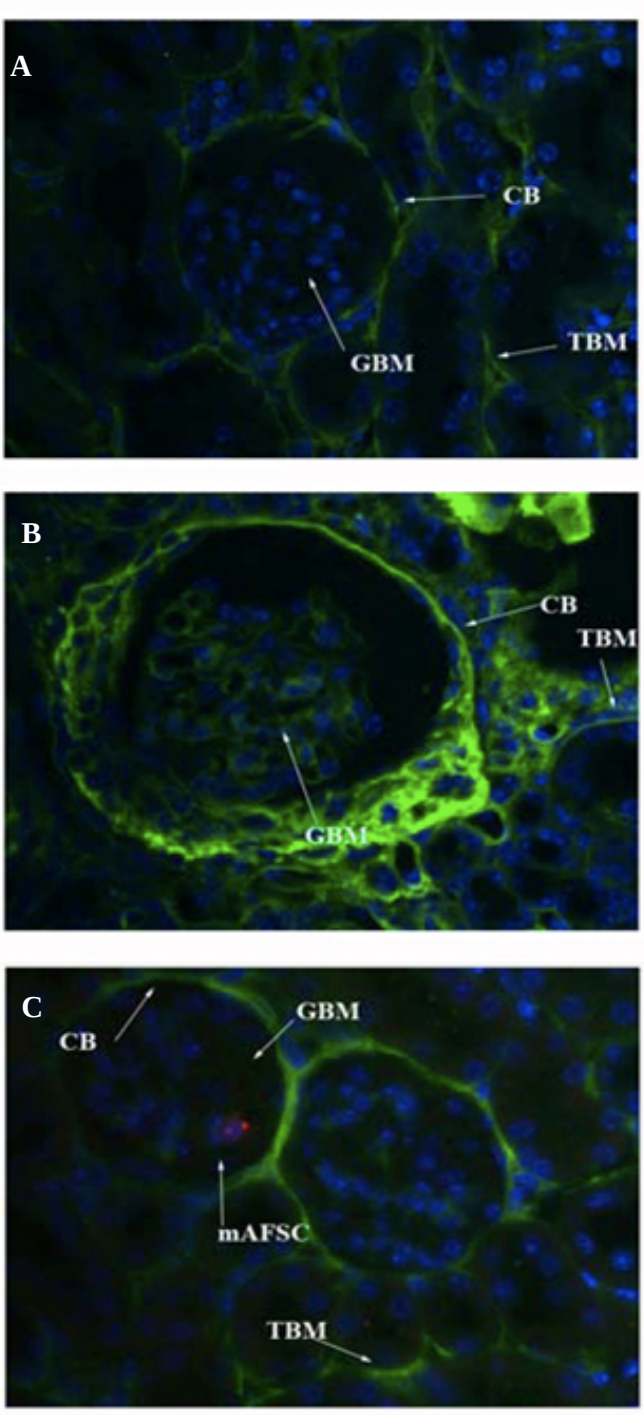


Figure 11 - Immunofluorescence staining of kidney glomeruli ***A.*** *In normal mouse kidney collIVα1 (green) is expressed in Bowman's capsule (CB -arrow), and basal membrane of the tubules (TBM-arrow) while it is absent from the GBM (arrow) (x20).* ***B.*** *In Alport mouse kidney stained for collIVα1, CB is thick due to substitution of collIVα5 with collIVα1 (arrow). The α1 chain signal is strongly present in GBM (arrow)(x20).* ***C.*** *Injected Alport kidney stained for collIVα1. The thickness of the CB is markedly decreased (arrow) and the α1 chain is absent from the GBM. In addition, the injected AFSC labeled red with CMDiI can be seen inside the glomeruli (arrow)(x20).*

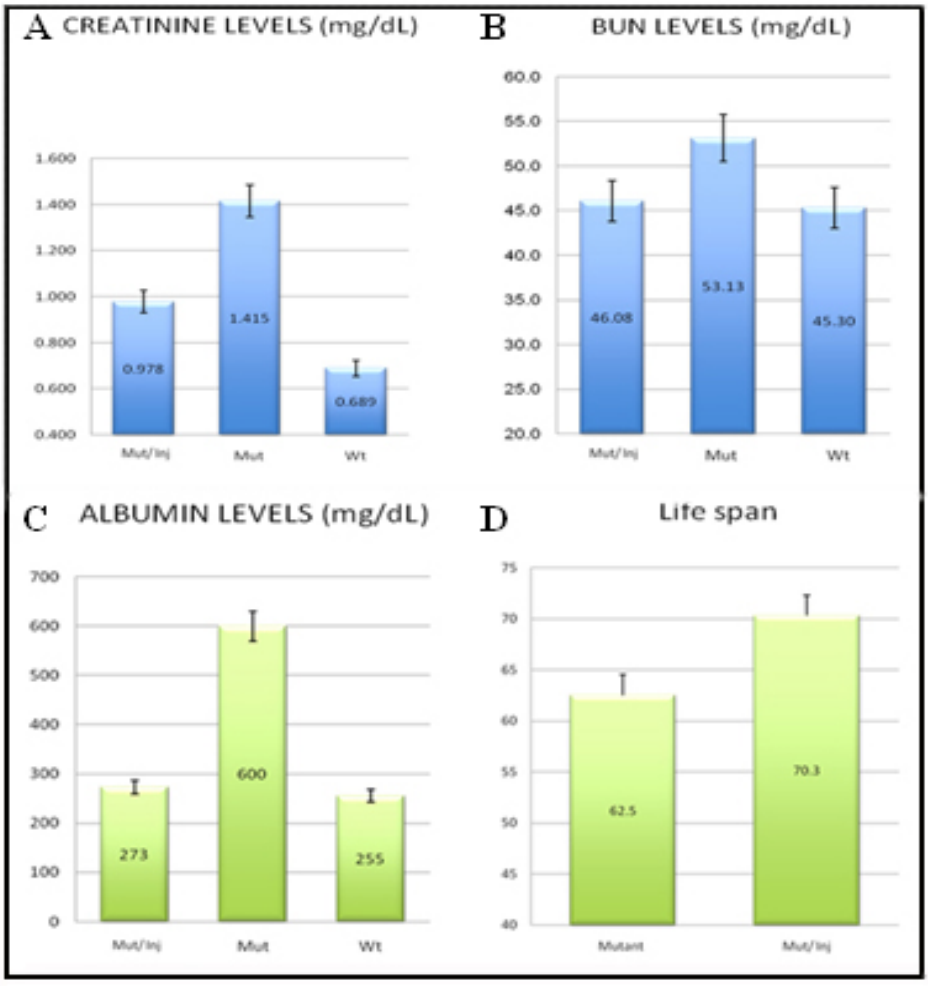


Figure 12 – ***Analysis of Renal Functionality*** *Levels of creatinine (**A**) , BUN (**B**) and albuminuria (**C**) in Alport mice injected with mAFSCs (n=5) compared with the wild type (n=5) versus non-injected (n=5). The levels of creatinine, BUN and albuminuria are decreased in mice injected with mAFSCs to close to normal.* ***D.*** *Life span of 5 Alport mice injected compared with 5 Alport mice non-injected: the injected mice have a 25% longer survival rate, (P<0.05)./*