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Does renal GCDH expression contribute to the explanation of the two excretor types in glutaric aciduria type I?

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Table of content

Acknowledgments 3
Introduction 4
Background 4
Specificities of PTECs as an in vitro culture system 6
Aims of the project 7
Material and methods 8
Ethic statement 8
Urine sediment harvest and pellet seeding 8
Cell culture 9
Culture analyses 9
pRNS-1 amplification 13
Results 14
Efficacy of different culture strategies 14
Details of urinary sediment cultures 17
Discussion 24
Discussion 24
Bias 25
Conclusions 26
References 27

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Introduction

This Master Thesis was carried out at the University of Lausanne and is part of the master's curriculum in the School of Medicine(1). All manipulations have been done at the laboratory of the "Centre des Maladies Moléculaires" (CMM) which is part of the "Département Médico-Chirurgical de Pédiatrie" (DMCP) at the "Centre Hospitalier Universitaire Vaudois" (CHUV) (2).

One of the research groups of the CMM works on fundamental and translational research on organic acidurias, mainly on glutaric aciduria type 1 (GA-1) and methylmalonic aciduria with a special focus on the understanding of pathophysiological mechanisms leading to brain damage in these diseases (2–5).

The research exposed below is part of a subproject of the research on GA-1 which aims to understand the physiological role of GCDH in proximal tubular cells of the kidney and to elucidate whether its presence in these specific cells might be the explanation for the existence of two excretor types (low and high excretors) in GA-I patients (6).

Background

Glutaric aciduria type I in brief

GA-1 is an organic aciduria, which means a defect in the catabolism of the amino acids lysine, hydroxylysine and tryptophan. In this disease, the gene encoding for glutaryl-CoA dehydrogenase (GCDH) is deficient. Its lack of function leads to the accumulation of glutaric acid (GA), 3-hydroxyglutaric acid (3-OHGA), glutaconic acid and glutarylcarnitine in body fluids (6–8).

Although patients can present mild neurologic symptoms during the first months of life, the pathology appears in most cases between the ages of 6 to 18 months with the first metabolic crisis. Vaccinations, common infant illnesses or other catabolic states are usually the trigger of a first encephalopathic crisis. Patients regress in their psychomotor development and can secondary develop failure to thrive. Most of them do not recover from the first encephalopathic episode, even with appropriate treatment and diet. They usually develop an irreversible dystonic-dyskinetic movement disorder with preserved cognition (3,6).

Typically, patients already present a macrocephaly during the first months of life marked by mild neurologic impairment such as hypotonia, irritability or feeding difficulties associated with frontotemporal atrophy and enlarged Sylvian fissures at neuroimaging (3,9). In addition, they are at risk of several other problems such as subdural haemorrhages, retinal haemorrhages, rhabdomyolysis and renal failure (3,6,8,9).

Genetic transmission of GA-1 follows an autosomal recessive pattern and more than 200 different mutations have been reported (7). This disorder has an estimated prevalence of 1 in 100'000 life births with higher prevalence in several ethnic communities such as Canadian Oij-Cree natives, the Amish community, Irish travellers and black South Africans (3).

Lysine, Hydroxylysine and tryptophan metabolism and the function of GCDH in amino acid metabolism

Lysine, Hydroxylysine and tryptophan have a common metabolic pathway from α-acetoadipate to acetoacetate. *De facto*, those amino acids are ketogenic. Because of previous steps in its metabolism, tryptophan can also be named as glucogenic. Indeed, catabolism of tryptophan generates alanine and acetoacetate.

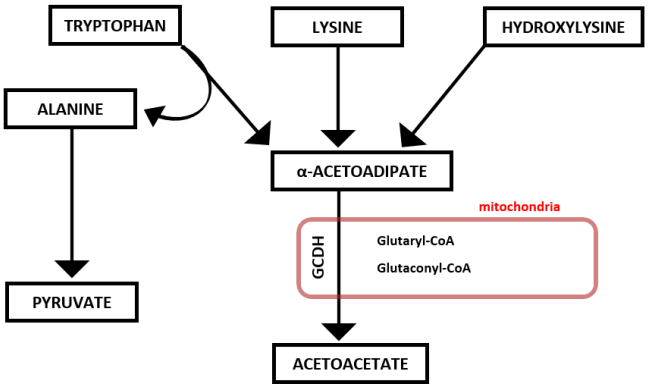


Figure 1: lysine, hydroxylysine and tryptophan metabolism

GCDH is an obligatory step in their common pathway. It allows the transformation of glutaryl-CoA to glutaconyl-CoA in mitochondria (3,10). Those reactions take place mostly in liver. The significance of GCDH expression in kidney in the context of the different GA-1 phenotypes is currently unclear (11).

The proximal tubule of the nephron

Nephrons can be divided in numerous functional units, typically in glomerulus, Bowman's capsule, proximal tubule, Henle's loop, distal tubule and a connective part to the collecting duct. Each part can vary in size depending on the cortical or juxtamedular position of the nephron (12,13). Following the observation of juxtamedular expression of GCDH (11), we hypothesized that proximal tubular epithelial cells (PTECs) might have different functions in amino acid metabolism depending on their place in the renal cortex. This affirmation requires further investigations.

The proximal tubule is coated with highly specialized epithelia showing numerous specificities linked to their functions. PTECs or nephrocytes are polarized cells (14). To share the maximal surface with primary urine they display a large apical brush border and have processes that interdigitate cells at the basal lamina pole. To support their intense metabolic function, they contain a large amount of mitochondria, which make them acidophilic. Finally, these cells are joined at the apical pole by tight junctions (12,13).

GA-1 and the kidney

Significant co-expression of GCDH and organic anion transporter 1 (OAT1) was found in the juxtamedular area of rat kidney (11). The same co-localisation was found in neurons, which let to the hypothesis of a functional coupling of both proteins (11). This hypothesis is supported by the possibility that residual GCDH activity leads to the "low excretor" phenotype and complete absence of enzyme activity to the typical urinary pattern of "high excretor" GA-1 patients (3).

In the nephron, the proximal tubule is specialized in reuptake, excretion and transformation of several components of primary urine and in particular of amino acids (12,14). This explains why PTECs are thought to display a particular role in GA-1.

Urinary sediment microscopic analysis of healthy volunteers

Urine is commonly seen as a complex mix of body waste. In urine, we can find many precipitated crystals. They have no real relevance for the culture of PTECs, but they can give much information on urine quality. Following the same idea, direct microscopic visualisation of the pellet can show fungal, bacterial or parasitic infection of the subject. In urine sediment, besides PTECs some other cells types can be found such as squamous epithelial cells that are the most common cells in a normal pellet and transitional epithelial cells. Tubular epithelial cells, erythrocytes and lymphocytes are pathologic if they are present in large amounts. We can also see abnormal cells in urine sediment, for example neoplastic cells or caudate epithelial cells (15–23).

Specificities of PTECs as an in vitro *culture system*

Techniques to collect and study PTECs over a long time

Some groups harvested cells from nephrectomised kidneys and used different protocols to collect more or less specifically PTECs (24–27). However, harvesting PTECs from fresh urine is a valuable alternative (24,28–31). A possibility to use PTECs over a longer period for research is to transform them secondarily into immortalized cell lines (24,25,28,32,33).

The validity of cell lines derived from fresh urine has been questioned. It was thought that an apoptosis-induced mechanism was responsible for the exfoliation. However, it has been shown that cells keep their normal morphology and function. The actual explanation is a regulation of overcrowding by exfoliation due to a constant proliferation and migration of PTECs (24).

As a conclusion, cultivation of PTECs derived from fresh urine seems to be a non-invasive and equivalent technique to PTECs derived from nephrectomy (24).

Techniques to isolate PTECs using flow cytometry

Van der Hauwaert *et al*. exposed a way to isolate PTECs using flow cytometric technics (26). As immunostaining is involved, they proposed the use of CD10 and CD13 as specific labelling for PTECs. Indeed, double positive cells seem to be derived from all segments of the nephron's proximal tubule. The use of fluorescent activated cells sorter (FACS) permits the selection of cell populations based on their labelling. They thus proposed a standard protocol for the establishment of pure PTEC lines.

Regeneration and stem cells of the proximal tubule

Tubular cells have a large regenerative capacity and can recreate the normal microanatomy of the nephron after severe tubular injury. Recently, a new cell type called robust cells was discovered showing specific morphology and several stem cell markers. They seem to be involved in regeneration of tubules (25).

Due to their robustness to injury and their high potential of regeneration, especially in regeneration of the proximal tubule, those cells could be an alternative to the use of PTECs for our project (26). The creation of PTECs derived from stem cells is theoretically also possible.

Epithelial to mesenchymal transition (EMT)

After some passages, morphological and immunohistochemical modifications of PTECs can be observed. The moment of these changes can vary (34).

Those modifications include loss of polarity, reduction in cell density and a myofibroblatic aspect. In addition, the appearance of vimentin, a mensenchymal marker, can be observed by immunohistochemistry (27,34–36).

Several studies tried to understand the mechanisms involved in EMT (27,34–37). The same mechanism is thought to be involved in renal fibrosis after chronic injuries inducing persistent proteinuria (34). As a result, PTECs seem to start EMT when they are in contact with components of pathologic primary urine, particularly with inflammatory mediators such as C3a (27).

Possibility of culturing children's cells and its yield

Historically, PTECs were tried to be cultured to study amniocentesis. It was thought that the cells used to build a karyotype were of renal origin (38).

Further research focused on describing the evolution of cultured epithelial cells from fresh urine (30,39). Several groups used cells harvested from urine to build *in vitro* models for numerous diseases and escaping invasive biopsy to get pathological cells (28,29,31).

Many studies used children's urine, from new-borns to young adults, and surprisingly, the yield of children's urine is higher than for adults (28–31). An average number of 3 (range of 0.3 to 19) colonies per 1ml of urine is described to be obtained for children while for adults the yield is only at 1-10 colonies per 100 ml of urine (30).

As a conclusion, PTEC cultures derived from fresh children's urine give a higher yield per urine volume compared to cultures derived from adults and are an excellent model to study different diseases in these cells thus avoiding renal biopsy.

Contamination risks

Due to the non-sterile origin of the sample, PTEC cultures derived from fresh urine are at high risk for contaminations. Moreover, many antifungals and antibiotics usually used in cells cultures are toxic for PTECs and should be avoided, such as gentamycine and non-liposomal amphotericine B. As a consequence, there is a need for a close microbiological follow-up of samples.

Aims of the project

As a middle term objective, the research group in which this master thesis was performed wants to generate an *in vitro* model of immortalized PTECs derived from low and high excretor GA-1 patients and healthy aged-matched controls. This biologic tool will provide a useful model for further research on physiological GCDH function in PTECs and its pathophysiological consequences in GA-1 patients.

On the other hand, the development of protocols for collection and culture of urinary sediment cells leading to pure single-cell cultures by specific selection is a powerful tool for multiple laboratory investigations e.g. the establishment of cultures from fresh urine samples could avoid skin biopsies in patients for which biochemical, enzymatic or genetic analyses have to be performed on fibroblasts.

The aim of this master thesis was to establish a protocol for the collection, culture and immortalization of PTECs from fresh urine of healthy volunteers.

Material and methods

Ethic statement

This experiment has been approved by the "Commission cantonale d'éthique de la recherche sur l'être humain" (CER-VD).

Urine sediment harvest and pellet seeding

Protocol

As described below, two conditions of basal medium were tested. As the contamination risk was relatively high, several precautions were required, such as using sterilized collecting bottles and sterile wipes moist with non-alcoholic chlorhexidine (Braun Medical, #19286) to disinfect genitalia. In addition, several other precautions were tested such as screening of urine portions with urinary sticks (Zambon© Monulab) for nitrite and leucocytes or the avoidance of females as volunteers. An early antifungal treatment with Amphotericin-B (Sigma-Aldrich: A2942-20ML) was also tried.

Urinary sediments were centrifuged at 1'600 rpm (280 N) for 15 min at room temperature. Pellets of 0.5 to 1ml were seeded in TC-treated 12 well-plates (Costar® 12 Well Clear TC-Treated Multiple Well Plates, #3512) with 3 ml of standard medium. Culture plates were placed in an incubator at 37 °C and 5 % CO_2.

Basal medium 1:

DMEM/Ham's F-12 liquid medium with stable glutamine (BIOCHROM #FG 4815) supplemented with: T3, epinephrine, rhEGF, Hydrocortisone, insulin, transferrin from REGM SingleQuot Kit Suppl. & Growth Factors (Lonza #CC-4127), 5 ng/ml Sodium selenite (Catalog Number S5261 Sigma-Aldrich Co), 3.4 µg/ml β-Nicotinamide adenine dinucleotide hydrate (Catalog Number N3014 Sigma-Aldrich Co), 100 U/ml-100microg/ml penicillin-streptomycin (Catalog Number 15140122 Life Technologies) and 10 % heat-inactivated Foetal Calf Serum (FCS) (BioConcept Ltd. Amimed 2-01F100-I).

Basal medium 2:

DMEM/Ham's F-12 standard medium (Gibco® 11330-032) basically supplemented with: 100 U/ml-100microg/ml Penicillin-Streptomycin (Gibco® 15140-122), 10 ng/ml EGF (Sigma-Aldrich: E9644-2MG), 35 ng/ml Hydroxycortisone (Sigma-Aldrich H0888-1G), 5 ng/ml insulin (Sigma-Aldrich I-5500), 6.4 ng/ml T3 (Sigma-Aldrich T-2752), 5 ng/ml sodium selenite (Catalog Number S5261 Sigma-Aldrich Co) and 1.25 µg/ml Fungizone (Amphotericin-B) (Sigma-Aldrich A2942-20ML) until first medium change.

Cell culture

Protocol

The first medium change was performed at 24 h. It was a 5/6[1] medium removal. If a lot of waste was observable (crystals, squamous cells…), full medium removal with PBS (1x; 37°C) washing was performed. Before disposal in the incubator, 3 ml of medium were added.

Standard 5/6 medium changes were performed every 3 days until cells started to detach from the ground of the first colony.

When cells started to detach from the ground of the colony, the medium was collected and seeded in another well. This new culture followed exactly the same protocol as the primary culture. The harvest of cells from culture medium can occur every 3 days.

Culture maturity was achieved when cells covered the whole plate surface, when cells stopped growing or when the first signs of EMT appeared.

Culture analyses

Daily follow-up

Cell morphology, colony biodynamics and health of cultures were analysed by direct microscopy with photo documentation as follow-up (Jenoptik easyshot and Leitz Labovert) completed by personal notes.

Immunostaining

Cellular characterisations were studied using immunohistochemistry and immunofluorescence technics. Several protocols were tested.

General protocol

For each tested cellular substrate, the following manipulations were performed. After fixation in paraformaldehyde 4% (PAF) (P6148 Sigma-Aldrich,) cells were washed $3X5$ min in PBS 1x, endogenous peroxidases were inactivated using a bath of H2O2 1.5% associated with Triton 0.1 % to permeabilize cell membranes during 15 min. After a second run of wash, epitopes were blocked using PBS-Bovine Serum Albumin 1 % (PBS-BSA). Primary antibodies were incubate overnight at 4 °C. Before incubation with the secondary antibodies at the recommended dilution, a third run of wash was performed.

For immunohistochemistry, secondary antibodies coupled with horseradish peroxidase (HRP) were incubated 1h30 at room temperature. Revelation of labelled cells was performed using AEC Chromogen substrate.

For immunofluorescence, secondary antibodies coupled with fluorochrome were incubated 1h30 at room temperature in the dark. A DAPI fluorescent staining was performed when possible.

Cellular substrate alternatives

Direct immunostaining in the culture well:

Immunostaining was directly performed in the culture well after fixation with PAF

[1] Remove 5 of 6 parts of old medium.

Immunostaining on coverslips:

We added sterile glass coverslips (microscope cover glasses (Assistant: 1001/20)) to the culture wells in order to let cells grow on it and use them thereafter for immunostaining. Glass coverslip were sterilized by placing them in Becher containing pure ethanol before burning them on a bec Bunsen flame.

Immunostaining of a monolayer cell membrane:

Cell monolayers were scratched from culture wells using sterile scalpels and tissue plier to get cell membranes. They were fixed in PAF after their disposal on immunostaining glass slides.

Immunostaining of cells in suspension:

Culture medium was harvest in 15 ml Falcons®. Cell monolayers were washed with PBS 1x, at 37 °C. The washed product was transferred into Falcons®. Trypsinisation using TrypLE™ Express solution (LifeTechnologies 12604013) until cell detachment was performed. Cells suspensions were harvested in Falcons® where 1 ml FCS was added. Volume was completed with PBS 1x. It underwent a centrifugation at 1'600 rpm (280 N) for 10 min. Supernatant was removed. After a PAF incubation of the pellet in the Falcon® during 1 h, a centrifugation at 1'600 rpm for 10 min was performed. The supernatant was then removed, followed by an incubation with 12 % sucrose overnight. After centrifugation at 1'600 rpm for 10 min, the supernatant was again removed. A second incubation in 18% sucrose during 4 h was performed. The Falcon® underwent a last centrifugation at 1'600 rpm for 10 min. The supernatant was removed leaving 0.5 ml of liquid. The pellet was suspended in the residual volume. The suspension was then transferred into a 1 ml Eppendorf® tube.

In a 200 µl PCR Eppendorf® tube, 100 µl of optimal cutting temperature compound (OCT) was added. Cell suspensions were briefly quick spinned. Supernatant was removed. The pellet was carefully disposed in OCT by pipetting. The volume of the 200 µl PCR Eppendorf® tube was competed with OCT. PCR Eppendorf® tubes containing cell solutions in OCT were disposed in a liquid nitrogen bath to be cryogenized. Products were stored in a -80°C freezer waiting to be cut with a Cryostat.

During cutting with the Cryostat, a regular control of cell presence was performed by the help of DAPI labelling.

Markers

Primary antibodies:

- Pancytokeratin (PCK) (Santa-Cruz 15367) as epithelial marker.
- Gamma-glutamyltranspeptidase 1 (GGT1) (Santa-Cruz 166908) to label a brush-border enzyme. It gives the indication of polarity, cell phenotype preservation and tubular origin of cells.
- Zonula occuldens 1 (ZO-1) (Santa-Cruz 8147) as epithelial marker and polarity marker.
- Glutaryl-CoA dehydrogenase (GCDH) (Sigma AV43559) to label central protein in GA1 pathogenesis.
- CD10 (eBioscience 17-0106-42), almost specific markers for PTECs[2]
- Vimentin (V4630, Sigma) as epithelial-to-mesenchymal transition marker

[2] cf flow cytometry results

Secondary antibodies:

- Goat anti-mouse 555 (Lifetechnology A21422)
- Goat anti-rabbit 350 (Invitrogen A21068)
- Donkey anti-rabbit 555 (Invitrogen A31572)
- Goat anti mouse HRP (BIO RAD 1706516)
- Donkey anti-goat HRP (Santa-Cruz sc-2020)
- Goat anti-rabbit HRP (BIO RAD 1706515)

Flow cytometry

This experiment was realized at the Ludwig Institute in Epalinges with the precious help of Anne Wilson, Stefanie Siegert and Catherine Fumey.

In order to determine the proportion of PTECs in culture, we passed suspended cells labelled with anti-CD13 conjugated with PE (CD13-PE) and anti-CD10 conjugated with APC (CD10-APC) on a *fluorescent activated cells sorter (FACS).*

Standards marker dilutions were obtained by titration on human peripheral blood lymphocytes (human PBL).

A cell suspension was obtained using TrypLE™ Express (LifeTechnologies 12604013) to spare epitopes from enzyme digestion. Trypsinization was stopped using standard medium. After centrifugation at 1650 rpm for 5 min, the supernatant was removed and EDTA 5 mmol/l with PBS-FCS 2 % buffer was added to avoid aggregation. Markers were added at standard dilution (1:100 for CD10-APC and 1:200 for CD13-PE). After an incubation of 20 min at 4 °C two rounds of washing with PBS 1x were performed. The final cell suspension was stained with DAPI.

Samples were analysed using the BD LSR 11b engine for flow cytometry analysis and Amnis Mark II to combine flow cytometry and microscopy analysis.

We analysed 12 samples of urinary sediment cells cultured with the standard protocol. All samples derived from the same subject.

For each sample, 20'000 cells were analysed and named as events. The proportion of living cells was determined by subtracting DAPI-positive cells (DAPI+) to all the events. In DAPI- population, subpopulations of cells were determined by their labelling in order to get subpopulations of double positive cells CD10+/CD13+, simple positive cells CD10+/CD13-, simple positive cells CD10-/CD13+ and double negative cells CD10-/CD13-. All observations were materialized on graphics (Figure 2) and on numeric values (cf annexes).

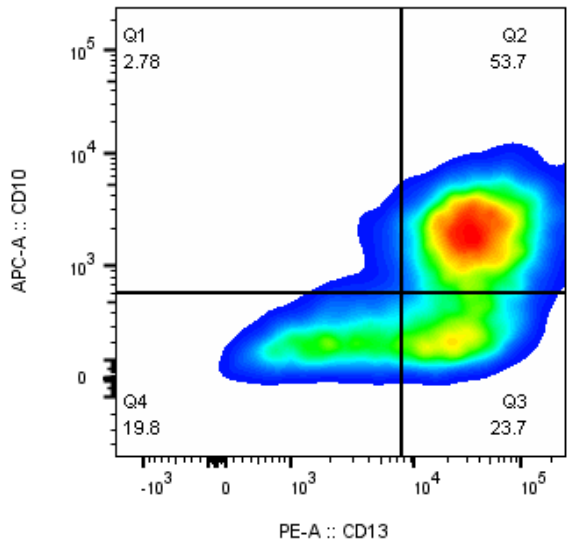


Figure 2: example of FACS analysis. The axes show the labelling intensity signal on cells for CD10 and CD13 labelling. Colours express the density of cells in this range of labelling intensity. Blue expresses the lowest density and red the highest. The horizontal line expresses the level of CD10 labelling intensity where the signal is higher than the background. The vertical line expresses the same for CD13 labelling. As consequence, we obtained four subpopulation groups which correspond to Q1=CD10+/CD13-, Q2=CD10+/CD13+, Q3=CD10-/CD13+ and Q4=CD10-/CD13-. In this example, we saw three cell subpopulations corresponding to Q2, Q3 and Q4. The biggest and most homogenous subpopulation group is in Q2 which corresponds to CD10+/CD13+.

In addition to these observations, flux cytomorphometry analysed several geometrical characteristics (cf annexes) and generated cell pictures (figure 3). Ten samples were analysed by this modality.

Flow cytometry coupled to microscopy with CD10 and CD13 labelling

Unstained

CD10+/CD13+

CD10-/CD13+

CD10-/CD13-

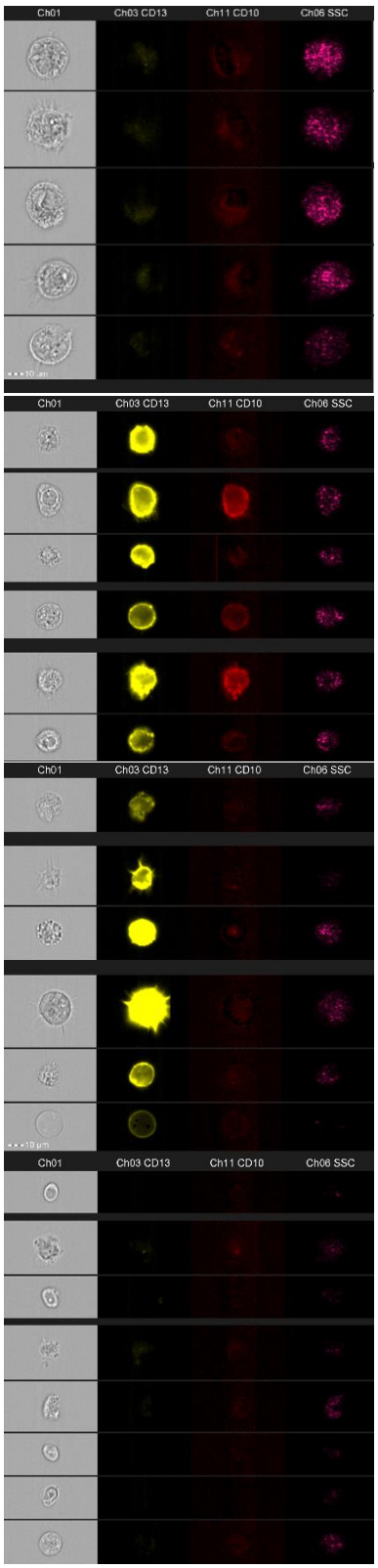


Figure 3:CD10/CD13 labelling. First column: native cells. Second column: CD13 labelling. Third column: CD10 labelling. Fourth column: side scatter fluorescence that shows cell granularity. No CD10+/CD13- subpopulation was observable.

pRNS-1 amplification

Getting immortalized PTECs lines was one of the objectives of this master thesis. To do so, a transfection by electroporation of genetic material in PTECs was thought to be a valuable possibility. Even if this step lacks in realisation in this master thesis, I finally got the material necessary to start immortalization.

pRNS-1 is a plasmid containing an immortalization gene for eukaryotic cells. It contains also a resistance to ampicillin and an insertion domain. This plasmid was kindly provided by Petra Novotna Zavadokova.

We performed a bacterial amplification using E. coli. Bacteria were submitted to a heat-shock transfection with pRNS-1. Transfected bacteria were selected by an ampicillin-supplemented medium. After bacterial amplification, pRNS-1 was harvested using the Maxiprep Kit (Promega).

Results

Efficacy of different culture strategies

In this section, I present the different culture methods that we tried and which parameters we chose for our standard protocol.

Harvest of urine sediment

The aim of the harvest protocol was the reduction of the risk for microbial contamination. In this part of the project, I analysed all seeded pellets, without discrimination of cellular development, from the harvest until the second medium change. Even if this period was chosen arbitrarily, a contamination at harvest could reasonably be excluded after this time period.

The harvest protocol gave me an overall yield of non-contaminated cultures after the second medium change in 89.1% of all cultures.

Several conditions were tested. We avoided female samples, used urinary sticks to screen for urinary infection and documented urinary pellet microbiology by microscopy. None of these measures seemed to have a convincing effect. We also tried to use Amphotericin-B as an anti-fungal drug, but its toxicity prevented the growth of urinary sediment cells.

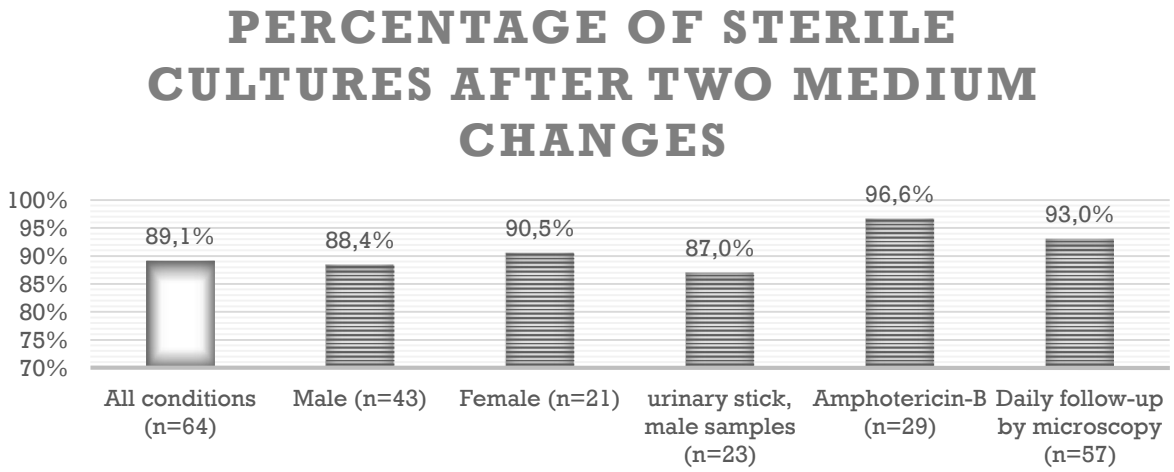


Table 1:Percentage of sterile cultures after the second medium change

Bests conditions for urinary sediment cultures

Basal medium

Two basal media were tested. We analysed the cellular development for both basal media measuring cellular growth as an endpoint. No discrimination of growth speed or growth surface was performed. A major difference between basal media 1 and 2 was detected. In fact, 63.2 % of pellets cultured in basal medium 1 showed a cellular development while only 5.9% of pellets started to grow in basal medium 2.

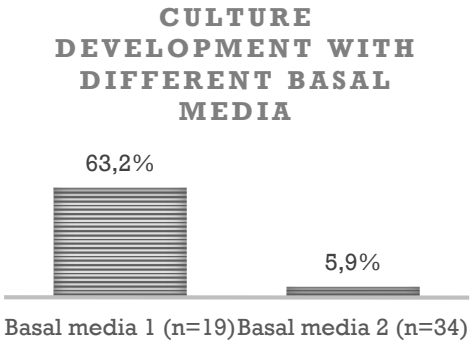


Table 2: percentage of urinary pellets that developed in basal medium 1 and 2.

Moreover, we observed a major difference of growth speed and growth surface in basal medium 1.

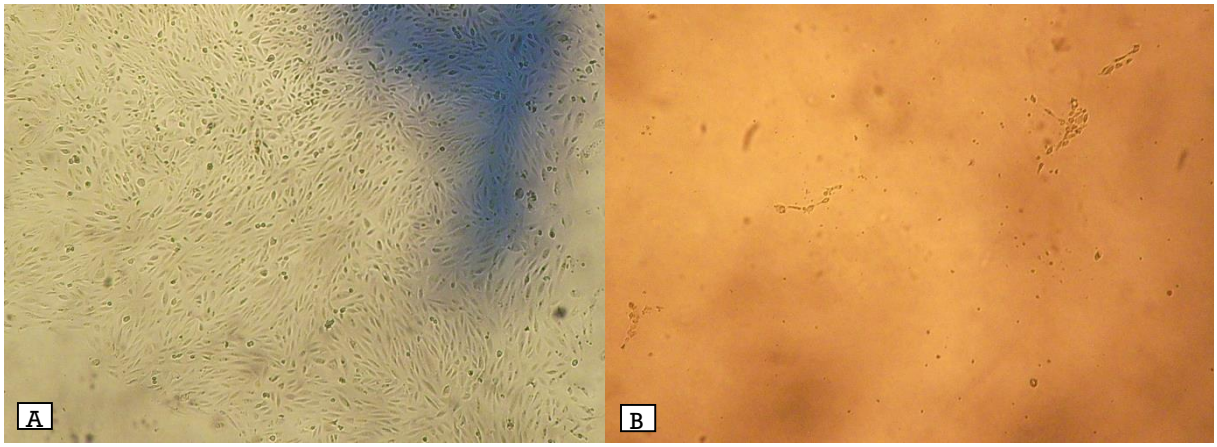


Figure 4: (A) Basal medium 1; Day in vitro (DIV) 16. It shows large amounts of cells disposed in a cobblestone manner / (B) Basal medium 2; DIV 17. It shows four limited cell proliferations

Seeding from medium

In order to increase the cellular growth surface, we tried to harvest cells in suspension from cultured medium. In fact, there was a time when cells in the middle of colonies continued to have mitoses, but had no more space to grow as a monolayer. We named this period the active proliferation. During this period, some cells could be harvest directly from the medium.

Surprisingly, the yield of cellular development with this manipulation was even higher than collection from fresh urine. We obtained a cellular development in 84.2% of seeded pellets from culture medium versus 72.7% of those derived from fresh urine.

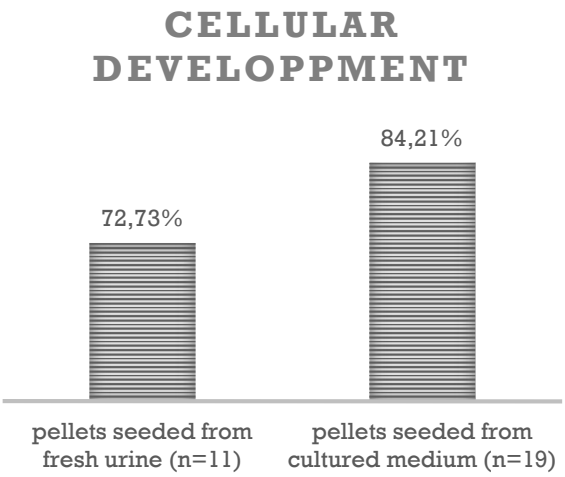


Table 3: cellular development in cultures derived from fresh urine versus cells seeded from culture medium.

We approximately determined the growth area visually by giving the fraction of the well that was covered by cells (approximate well surface: 3.8cm² (40)). The use of this protocol increased dramatically our monolayer surface per urinary pellet. Without the re-seeding protocol cultures showed an approximate median growth surface of 50% while this could be increased to approximately 116.7% by addition of the re-seeding protocol (Table 4).

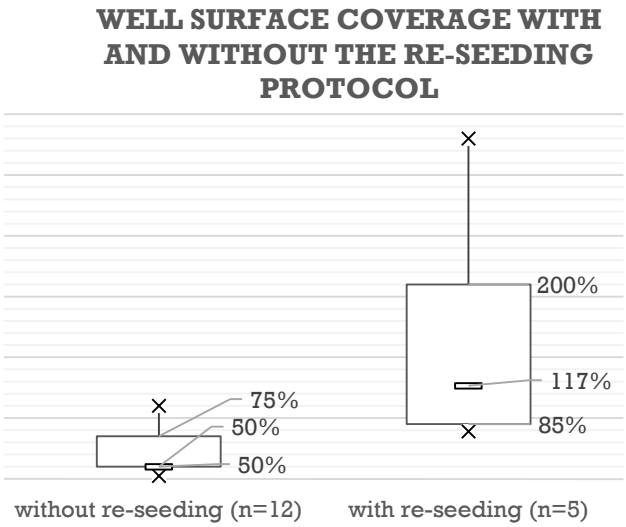
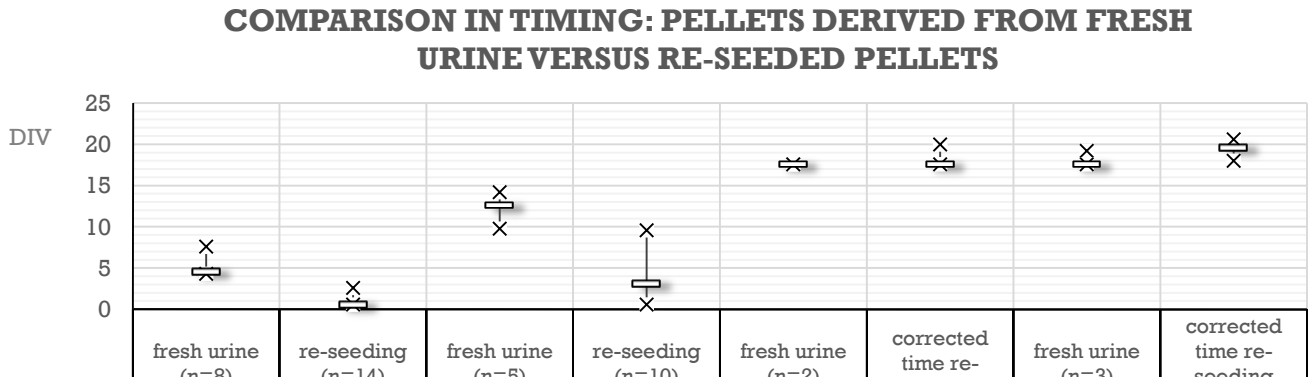


Table 4: The re-seeding protocol increased significantly the final number of cultured cells started from one pellet.

Another interesting particularity of cultures derived from the re-seeding protocol was that their development over time was identical to the primary culture derived from fresh urine. In fact, they did not seem to go through the latency period (cf Developmental steps of urinary sediment cultures). While it took a median of 5 days to see the first colonies in pellets derived from fresh urine, only 1 day was necessary in those derived from the re-seeding protocol. Moreover, when colonies appeared in re-seeded cultures, they were rapidly confluent, while it took a median time of 13 days to have the first confluences of colonies in urinary pellet cultures.

At the opposite, the entrance in the dedifferentiation period seems to be somehow coordinated. In fact, when we analysed the corrected time (time since re-seeding + age of the culture when the medium was collected) we saw a surprising similarity (Table 5).



	fresh urine (n=8)	re-seeding (n=14)	fresh urine (n=5)	re-seeding (n=10)	fresh urine (n=2)	corrected time re-seeding (n=3)	fresh urine (n=3)	corrected time re-seeding (n=13)
	time untill first colony (DIV)		time untill first confluence (DIV)		time untill coverage of whole surface (DIV)		time untill dedifferenciation (DIV)	
P90	8	3	14,6	10	18	20,4	19,6	21
P10	4,7	1	10,2	1	18	18	18	18,4
P50	5	1	13	3,5	18	18	18	20

Table 5: Cultures derived from re-seeding protocol develop first colony and first confluence of colony faster than primary culture derived from urine pellets. However, when corrected time is used to analyze moment of whole surface coverage or dedifferentiation, cultures seem to be coordinate.

Details of urinary sediment cultures

In this section I analysed qualitatively what I observed in urinary sediment cultures under standard conditions.

Developmental steps of urinary sediment cultures

Following my observations, PTEC cultures took an average of 19.9+/-2.5 days to reach maturity. This is the moment when cells reached their highest level of confluence before undergoing an Epithelial-to-Mesenchymatous transformation (EMT) or stopping their growth. The time-line of a culture can be divided into 4 periods as follows:

1. Latency

This initial period took an average of 5.6+/-3 days. During this time, no cell development was seen. Only a decrease of pellet waste was observable. This period came to an end when the first colony was observed.

Figure 5: Latency. Waste of urinary sediment at DIV 1

2. Primary colonies

This period started at the discovery of the first primary colony and finished when first cells started to detach from primary colonies, after an average of 8.5+/-3.8 days. It was observed to be a short period of approximately 3 days.

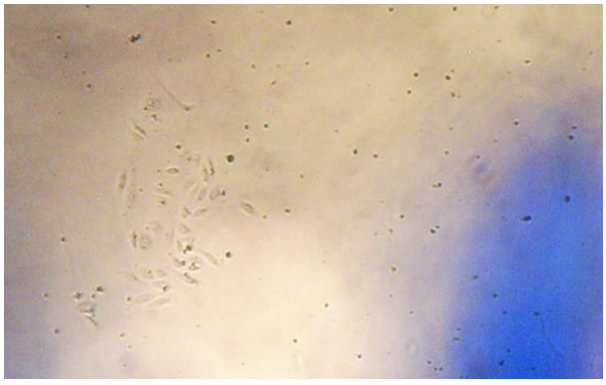


Figure 6: Primary colony. Apparition of cells at DIV 5

3. *Active proliferation*

This period started when the first cells went into suspension and finished with the firsts sign of EMT or when colonies stopped their growth, after an average of 19.9+/-2.5 days. It was during this period that the re-seeding protocol could be successfully performed.

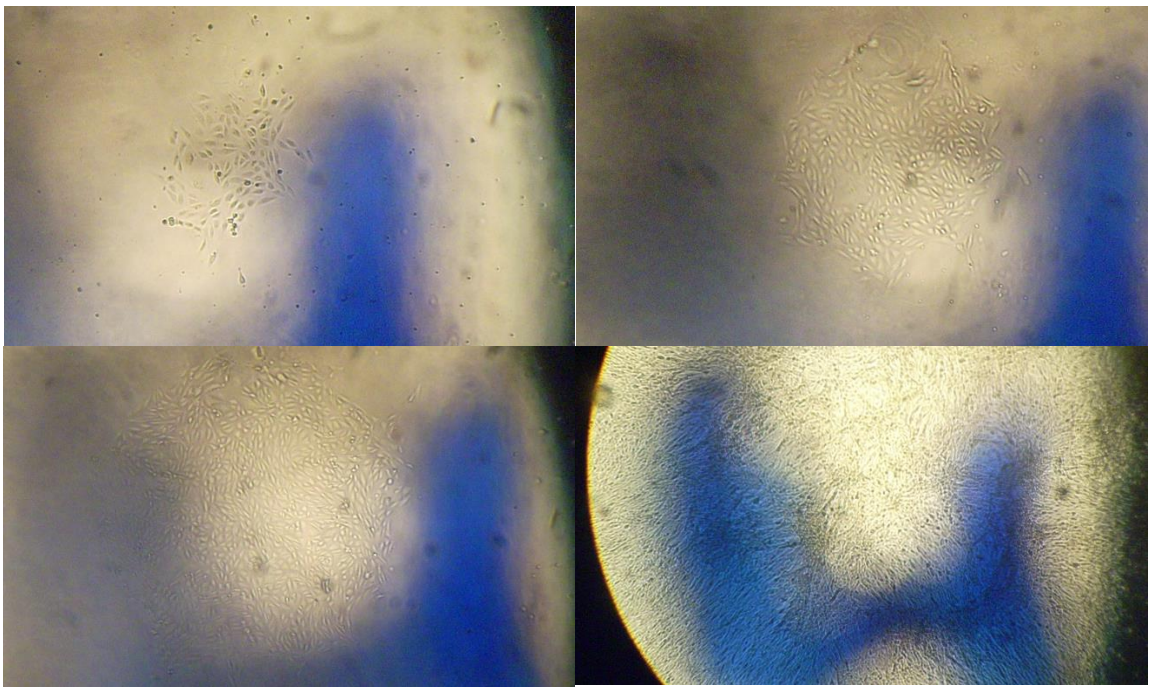


Figure 7: Active proliferation. We see the development of the same colony from DIV 6 to DIV 14

4. *Dedifferentiation*

This period is the post mature period. Cells showed a limited expansion with growth arrest and underwent EMT. When EMT occurred, cells showed a significant increase of vimentin intermediate filament and morphological changes.

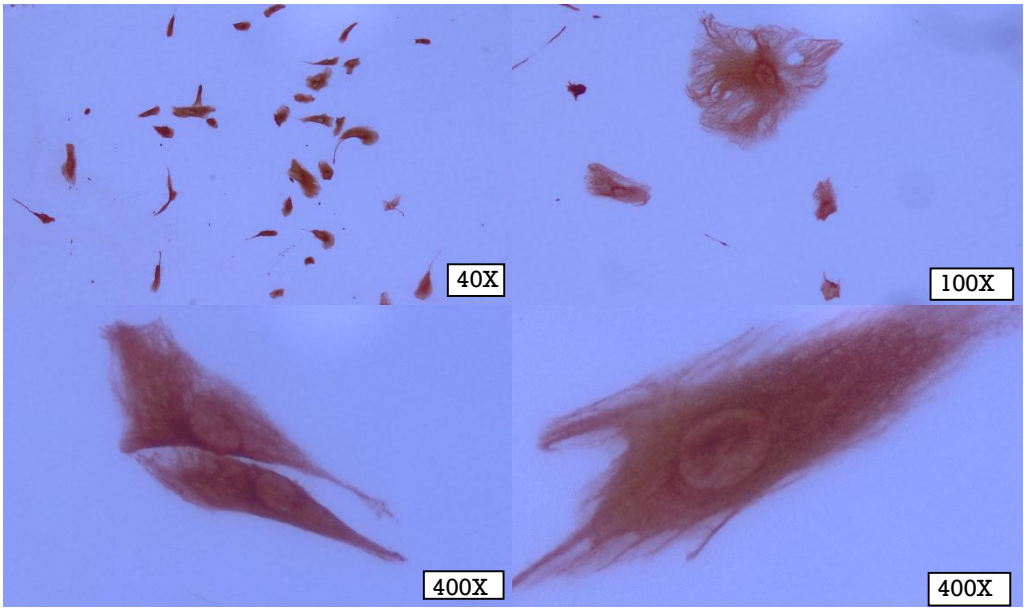
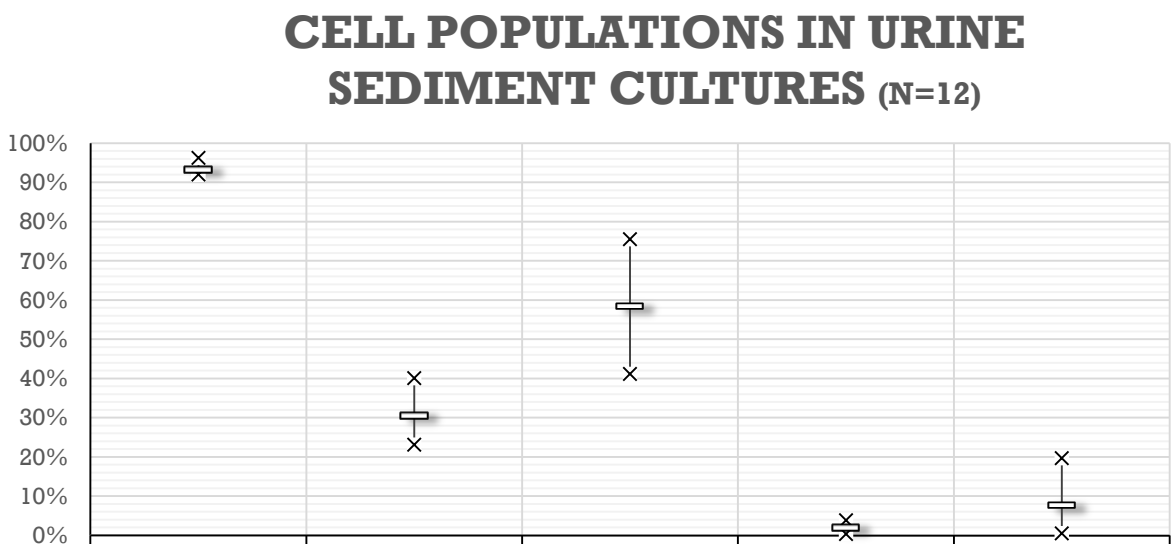


Figure 8:Dedifferentiation. Immunohistochemistry, vimentin labelling, DIV 66

FACS results

In the 12 analysed samples, a mean of 92.3% (SD=5.5%) cells were alive (DAPI-) in the sample.
In the DAPI- population, a mean of 57.3% (SD=15.7%) cells were double positive (dp) for CD10 and CD13, a sample median of 58.45%. The CD10 simple positive (sp) population in the DAPI- population represented a mean of 2.2% (SD=1.9%) and a sample median of 1.99%. The CD13 sp population in the DAPI- population represented a mean of 32.3% (SD=10.1%) and a sample median of 30.55%. The double negative (dn) population in the DAPI- population represents a mean of 9.4 % (SD=7.9%) and a sample median of 7.73%.
Following those results, a high proportion of cells were DAPI- at the time of analysis, which means that a large proportion of cells were alive and thus that the FACS results were interpretable. The highest subpopulation of cells in those samples were the double positive CD10/CD13 cells which are known to be PTECs. Two smaller subpopulation groups were also detected which represent other cells.
In conclusion, the protocol of PTEC cultures derived from fresh urine pellets used for this experiment gave mixed cellular subpopulations with a large predominance of PTECs. Unfortunatelly, cytomorphometry did not show any interpretable results.



	DAPI- (total of living cells)	DAPI- CD13+ CD10-	DAPI- CD13+ CD10+	DAPI- CD13- CD10+	DAPI- CD13- CD10-
P90	96,25%	40,06%	75,51%	3,82%	19,69%
P10	91,98%	23,07%	41,16%	0,34%	0,52%
P50	93,25%	30,55%	58,45%	1,99%	7,73%

Table 6: Four different cell populations could be observed in urine sediment cultures derived from fresh urine.

Immunohistochemistry and immunofluorescence results

In this section, I expose briefly my results for immunostaining. Manipulations were performed on several cellular substrates, but the protocol used for immunostaining was identical. Every cellular substrate was a valuable alternative except immunostaining directly done on cultures wells. Indeed, the plastic of the well-plate showed an important fluorescence at 350 nm which interfered with microscopic analysis (Figure 9).

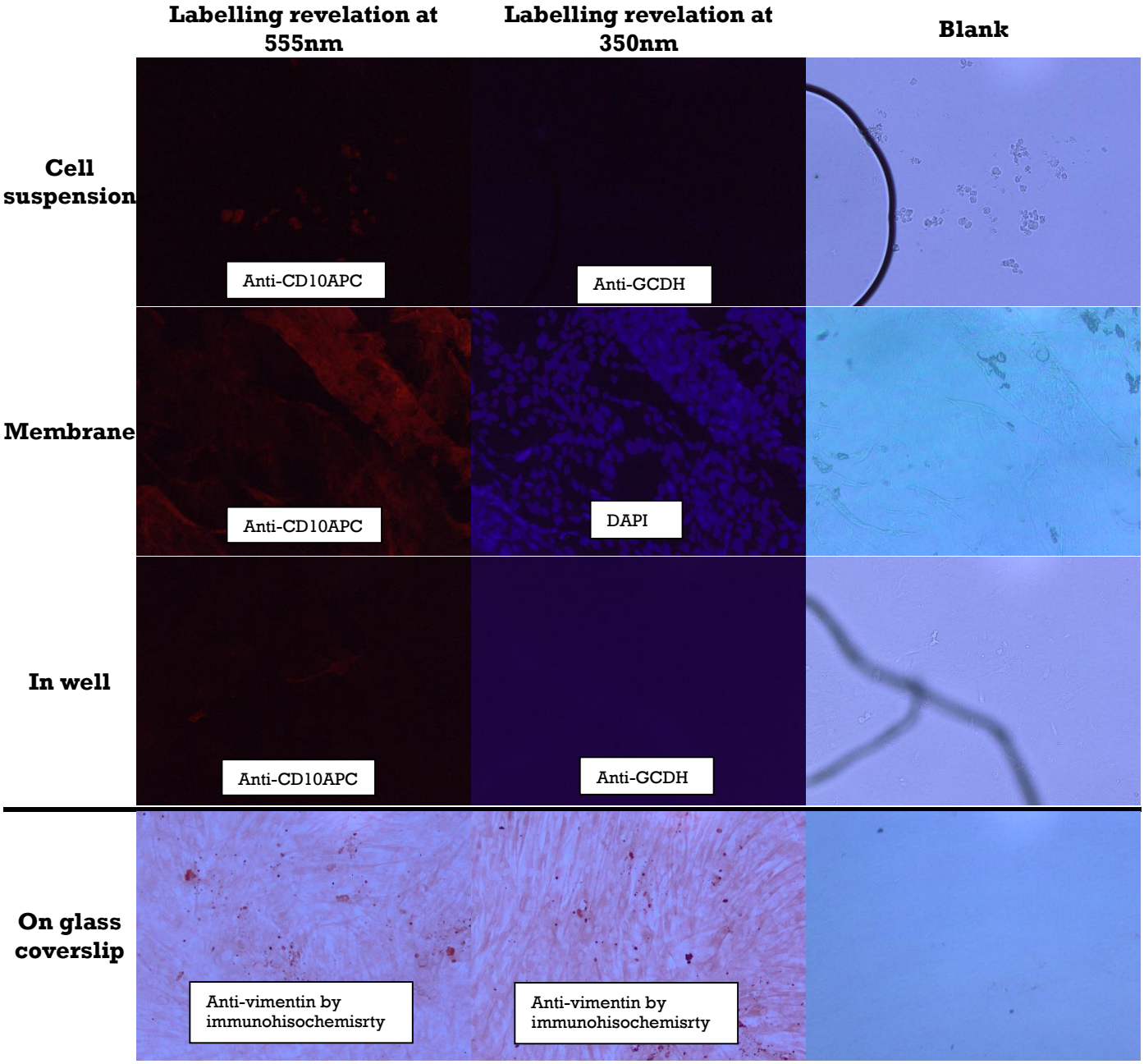


Figure 9:This figure shows the feasibility of immunostaining on different cellular substrates. Cell suspensions and cell membranes can be used as cellular substrate for labelling with 555nm and 350nm fluorochromes. Plastic fluorescence in immunostaining done directly in well culture prevent its use for 350nm labelling. Unfortunately, no immunofluorescence from our experiments was available for immunostaining on glass coverslip. But an immunohistochemistry performed on fibroblasts labelled for vimentin show the potential of this technic.

One of the aims of this master thesis was the determination of GCDH expression in PTECs. As we proved the presence of PTECs using immunostaining technics during FACS analysis, a similar experiment was set up in immunofluorescence.

Co-labelling of CD10 and CD13 was tried without success. PE fluorochrome of CD13-PE was not effective in immunofluorescence. But, based on our FACS results, which showed a population of CD10+/CD13+ cells, but a negligible population of CD10+/CD13- in our sample, I can assume that CD10+ stained cells were in majority the same as the double

positive population. Thus, a simple labelling of CD10 allowed us to show the presence of PTECs.

Immunofluorescence on membrane bound cells and cells in suspension were performed. It showed a co-staining for CD10 and GCDH (Figure 10).

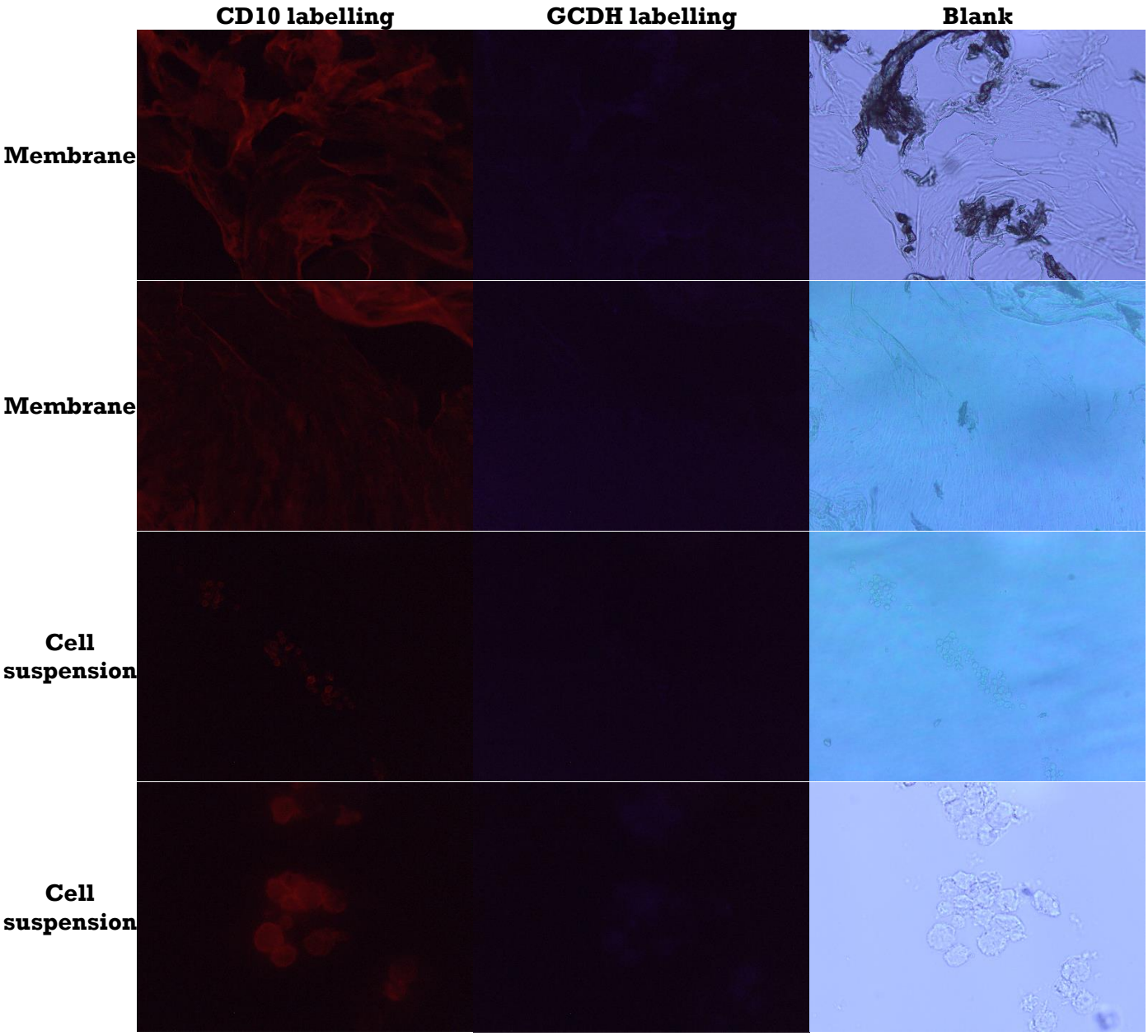


Figure 10: co-staining of CD10/GCDH by immunofluorescence, DIV 27. It shows a co-labelling for those two markers.

We performed an immunohistochemistry on glass coverslip during late dedifferentiation (DIV 66). It showed populations of cells without cohesion, showing pathologic morphologies such as giant cells and presenting mesenchymatous markers such as vimentin. Unfortunately, we did not have a valid control earlier in culture development (figure 9). However, the same manipulations were performed on fibroblasts and showed a similar staining for vimentin and gammaglutamyltranspeptidase-1.

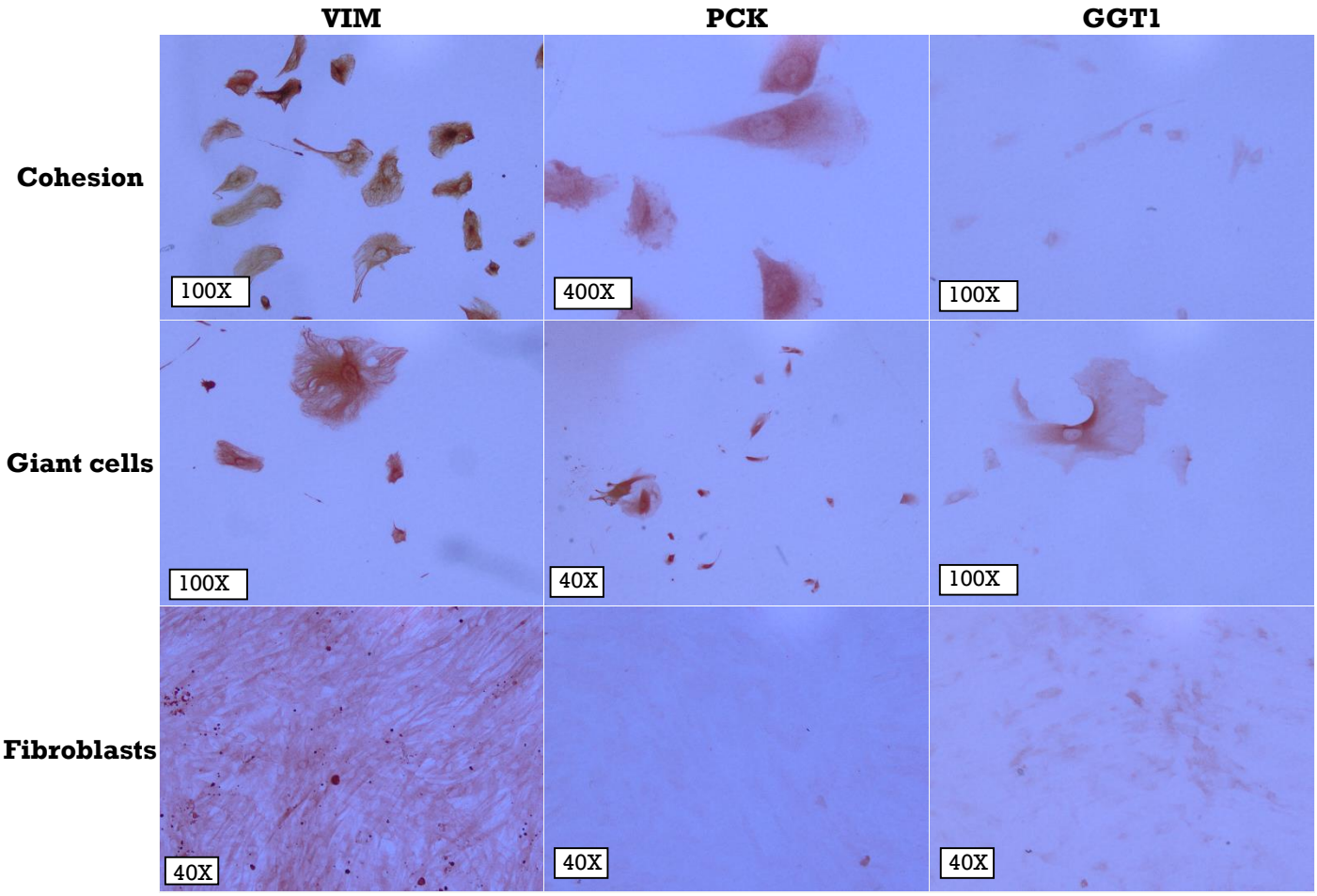


Figure 11: Immunohistochemistry with labelling for vimentin (VIM), pancytokeratin (PCK) and gammaglutamyltranspeptidase-1 (GGT1) at DIV 66 and on fibroblasts.

pRNS-1

Our vector amplification gave two aliquots with DNA concentrations of respectively 1,6 μg/μl and 1,4 μg/μl. Unfortunately, I have been unable to verify the quality of the amplified DNA by restriction corresponding to the vector card that was in our possession. This probably did not work because we lack knowledge on the exact pRNS-1 topography or a spontaneous mutation has abolished one of the restriction sites.

Observed particularities

Using our standard protocol produced a mysterious phenomenon. In 7 out of 30 cultures (23.3%) under standard conditions, some cell populations started to roll on the surface of the well and to build a polypus formation (Figure 12). When they were re-seeded, cells grew as monolayer. That probably indicate the conservation of original cell differentiation. Indeed, neoplastic formation would grow in three dimension. Moreover, cells kept CD10 as immunological marker.

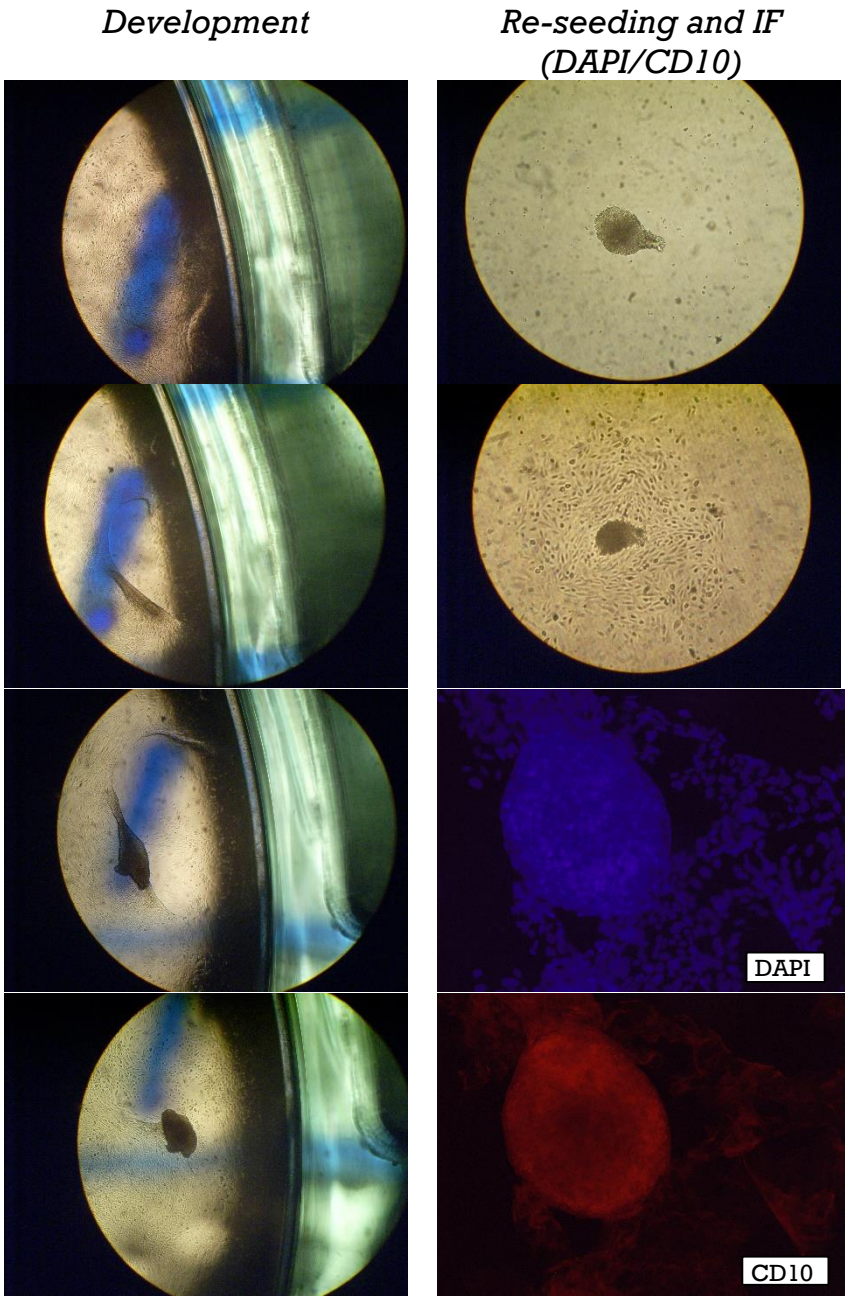


Figure 12: First column shows development of polypus formation over 2 days. Second column show the re-seeding of a polypus formation and its labelling for immunofluorescence using DAPI and CD10. The membrane technic was chosen as cellular substrate. After re-seeding, cellular growth follows a monolayer fashion. Immunostaining shows a regular formation of cells expressing CD10.

Discussion

Discussion

The long-term aim of this master thesis was to define standard protocols for the research on PTECs from GA-1 patients. The specific aim was the establishment of a valid protocol for the harvest from fresh urine, culturing conditions, PTEC selection and immortalisation.

Several experiments were performed for the proof of concept that a PTEC culture derived from fresh urine can be realized in the CMM laboratory of the CHUV and to determine the best protocols and culture conditions.

During this master project I successfully developed a protocol for the harvest of cells from fresh urine. The microbiological contamination risk at two days was at 10.9% of seeded pellets, which represents a non-negligible problem. Daily follow-up by microscopy avoided transmission of contamination and other modifications reduced the contamination risk at two days to 7%.

Even if literature says that the amount of tubular epithelial cells is poor in urine sediment from healthy volunteers (23), we obtained a cellular development in 72.7% of seeded pellets by using our harvest protocol and culture condition in our standard medium. To follow culture development, we divided the time-line of the cultures into 4 periods. This division did not follow a classical definition, but reflects our own observations. In order to describe the evolution of culture, it allows an optimized timetable for the application of several protocols, such as the re-seeding protocol or FACS analysis.

Our standard conditions permitted a mean area of cellular confluence of 50% of well surface in T-12 well-plates (approximate well surface: 3.8cm^2 (40)) per harvested urinary pellet. As trypsinisation disturbed normal development of tubular epithelial cells and triggered EMT, we developed a re-seeding protocol. Its use increased 2.3 times the area of cellular growth without disturbing the cell's morphology.

Following our FACS results, we obtained a median of 58.45% of CD10+/CD13+ cells, which are assumed to be PTECs. In regard with Van Der Hauwaert results (26), who obtained 4% of double positive cells, we observed a similar morphology of epithelial cells. Those results showed us that our culture is not a pure PTEC culture, but our culture conditions seem to be partially selective for PTECs.

We observed the development of domes, which are characteristics for epithelial cell cultures. Cells in our culture developed tubular and polypus formations. Even if we can already say that those particularities show similar markers as the other cultured cells, they require further investigations to determine their nature.

Our cultures were analysed by immunohistochemistry, immunofluorescence and FACS. Even if those protocols should be improved, they can already be used.

For a better selection to create pure PTEC cultures, a good alternative could be the use of the FACS. Indeed, we already showed that the selection of PTECs is possible using CD10 and CD13 markers. Further investigations should focus on the re-seeding of selected cells to observe their development after trypsinisation by TrypLE™ Express, exposition to antibodies and manipulation in FACS.

Unfortunately, very little progress could be obtained on the immortalization of PTECs during this master thesis.

Bias

Selection bias

Subjects selected for this research were all young healthy adult volunteers. This does not correspond to the target population of further research on GA1 cell lines as this is predominantly an infantile population with a chronic disorder.

We analysed only cells derived from fresh urine. We did not have a control cell line. We analysed selected cell lines from subjects who loose kidney cells in their urine. We do not know if this is the case in other population groups.

Moreover, most of our observations were derived from a single individual.

Analytical bias

Most of our numerical observations were made by visual numeration and approximation. It is notably the case for all data about colony numbers, time and growth surface.

All measures and analyses were performed by a student who gathered a certain experience during this master thesis. Modelling of qualitative observations was performed without any theoretical bases.

Frequently, only few data were available from our experiments, therefore affecting the validity of our data.

Observer bias

Follow-up technics changed along the research. Observations can differ between series.

Bias of an estimator

Observations were performed by a single person. This could affect the objectivity of the results.

Conclusions

This research has numerous biases and weaknesses, which are listed and detailed above. Nevertheless, numerous elements have been demonstrated.

It was clearly shown that it is possible to cultivate urinary sediment cells using urine pellets and to get an important fraction of PTECs in these cultures. Suitable protocols to seed and cultivate urine sediments cells were established. Optimization of cultures without trypsinisation passages was also effective by using our re-seeding protocol.

The daily observation of culture relieved different periods of urinary sediment cell cultures. Even if these observations would need further investigations to characterize them more precisely, they can already be used to prepare and document new cultures. Protocols for immunostaining, notably immunostaining for cells in suspension and FACS, are already available for use in further experiments.

Even if several questions were solved in this master thesis, numerous steps in the creation of PTEC immortal lines remain unexplored. This is the case for questions about PTEC selection, cultivation of pure PTECs lines and immortalisation. In fact, we do not know if FACS is a suitable alternative to select living PTECs and re-seed them to obtain a pure PTEC culture. As immortalisation could not be tried in this master thesis, nothing can be said about the feasibility of electroporation of PTECs cultures or even the use of pRNS-1 in this target. GA1 patients are still the target population. We are actually unable to say if our protocol will lead to the establishment of such immortalized patient cell lines.

As a conclusion, we can say that despite methodological weaknesses, this research can conclude on usable protocols to cultivate PTECs. Further investigations with a bigger population are required to get further data on selection and immortalisation of PTEC lines.

The understanding of the pathophysiological role of GCDH in PTECs derived from the urine of GA1 patients is still far away from now. But this master thesis was the first step on the way to this goal defined by the CMM laboratory.

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