REETTA SARTONEVA

Tissue Engineering in Urological and Gynaecological Applications

ACADEMIC DISSERTATION
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University of Tampere, BioMediTech
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Supervised by
Docent Suvi Haimi
University of Tampere
Finland
Docent Susanna Miettinen
University of Tampere
Finland

Reviewed by
Professor Minna Hakkarainen
KTH Royal Institute of Technology
Sweden
Docent Mika Matikainen
University of Tampere
Finland

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Abstract

The tissue engineering applications has emerged as a new potential treatment method for urological and gynaecological applications. Hypospadia is the most common congenital malformation of penis, where the urethra opens ventrally to the shaft of penis or even perineum. The hypospadias are traditionally reconstructed using patients' own genital tissue, or in severe cases non-urological graft tissue is used. However, the operations are susceptible to complications, especially in severe cases and when non-urological tissue is used. The most common complications are strictures, fistula formation and poor cosmetic outcome. Therefore, the development of alternative treatment methods is essential. Thus far, different natural and synthetic biomaterials, such as different acellular collagen based membranes, polylactide (PLA) and polyglycolide (PGA) have been studied as a growth surface for urothelial cells. However, the optimal biomaterial for urothelial applications has not been found yet. The biomaterial should be biodegradable, biocompatible, elastic, easy to handle and suture, support the urothelial cell growth and the structure of the de novo urethra. Further, the biomaterial should not evoke inflammatory tissue reaction.

Nowadays, the first-line surgical treatment for urinary incontinence is the mid-urethral sling operation, though, the development of injectable bulking agents is also important in order to obtain less invasive treatments. Previously, collagen and polyacrylamide hydrogel have been used as a bulking agent to treat stress urinary incontinence. Nevertheless, the sustainability of the treatment effect

has been a major problem and additional injections have been required. Therefore, tissue engineering based injection therapies using stem cells, aiming regeneration of the damaged tissue, could solve the sustainability problem. Adipose stem cells (ASCs) isolated from adipose tissue are an attractive cell source due to their abundance. Further, ASCs are known to differentiate towards myogenic cell lineages, and therefore being potential to regenerate the muscle tissue and treat urinary incontinence.

This thesis is composed of two different parts. First, we studied the use of human amniotic membrane (hAM) and different synthetic biomaterial membranes, smooth (s) poly-(L-lactide-ε-co-caprolactone) (PLCL), textured (t) PLCL and knitted PLA mesh with compression moulded PLCL (cPLCL) as a growth surface for urothelial cells *in vitro*. In the second part, we evaluated the suitability of ASCs in combination with collagen gel to treat urinary incontinence in a clinical pilot study.

In the first part, the hAM did not support the hUCs proliferation, viability and phenotype compared to the PLCL. However, on all the studied synthetic biomaterials the hUCs maintained their viability and phenotype. Further, the cPLCL supported the hUCs proliferation slightly poorer compared to the sPLCL and tPLCL. In our clinical pilot study we demonstrated that ASCs in combination with collagen gel is a safe and moderately effective treatment method for female urinary incontinence. Further, ASCs derived from the treated patients were confirmed to differentiate towards myogenic, adipogenic, osteogenic and chondrogenic cell lineages *in vitro*.

In conclusion, PLCL membrane could be a potential biomaterial for urothelial tissue engineering. However, further research is needed to

evaluate the *in vivo* applicability and biocompatibility of PLCL. Finally, tissue engineering based injection treatments with ASCs could be potential to treat female urinary incontinence in the future.

Tiivistelmä

Kudosteknologisista hoitomuodoista on kehittymässä potentiaalinen vaihtoehto urologisiin ja gynekologisiin sovellutuksiin. Virtsaputken alahalkio (hypospadia) on yleisin peniksen synnynnäinen epämuodostuma, jossa virtsaputken kehitys on jäänyt kesken niin, että virtsaputki avautuu ventraalisesti terskan ja välilihan väliselle alueelle. Nykykäytäntö on, että hypospadia hoidetaan muotoilemalla potilaalle puuttuva osa virtsaputkea genitaalialueen kudoksista esimerkiksi esinahasta. Vakavimmissa tapauksissa voidaan käyttää genitaalialueen ulkopuolisia kudoksia, kuten posken limakalvosiirrettä, ja etenkin nämä toimenpiteet ovat erittäin herkkiä leikkauksen jälkeisille komplikaatioille kuten arpeutumiselle, ihoavanteille ja huonolle kosmeettiselle lopputulokselle. Tämän vuoksi uusien hoitomenetelmien ja vaihtoehtoisten siirrematerіaalien kehittäminen on tärkeää. Tähän mennessä uroteelin kudosteknologisiin sovellutuksiin on eri tutkimuksissa testattu erilaisia luonnonperäisiä ja synteettisiä biomateriaaleja kuten soluttomia kollageenipohjaisia materiaaleja, polylaktidia ja polyglykolidia. Kuitenkaan optimaalista biomateriaalia uroteelisovellutuksiin ei ole vielä kehitetty. Uroteelisovellutuksia varten biomateriaalin pitäisi olla biohajoava, bioyhteensopiva, elastinen, helposti käsiteltävä leikkauksen yhteydessä ja sen tulisi tukea uroteelisolujen kasvua sekä virtsaputken rakennetta. Kriittistä myös on, että biomateriaali ei aiheuta haitallista tulehdus reaktiota kudoksessa.

Nykyisin virtsainkontinenssin kirurgisessa hoidossa käytetään ensisijaisesti nauhaleikkauksia, jossa polypropyleeninauha asetetaan

virtsaputken alle. On kuitenkin tärkeä kehittää hoitokeinoja, jotka olisivat vähemmän invasiivisia kuin kirurgiset tekniikat. Ponnistusinkontinenssin hoidossa on aiemmin käytetty myös virtsaputkea ja virtsaputken sulkijan aluetta ahtauttavia ja tiivistäviä injektiohoitoja, joissa esimerkiksi kollageeni- tai polyakryyliamidi hydrogeeliä ruiskutetaan virtsaputken alle. Ongelmana näissä hoidossa on ollut hoitotuloksen heikkeneminen ajan myötä, joten uusintainjektioita on tarvittu ylläpitämään hoitotulosta. Kudosteknologinen injektio, jossa biomateriaaligeeliä ja soluja käytetään yhdessä, voisi olla potentiaalinen tulevaisuuden hoitokeino virtsainkontinenssista kärsiville potilaille. Rasvasta eristetyt kantasolut ovat houkutteleva solulähde inkontinenssihoitoihin, sillä rasvakudosta on saatavilla runsaasti. Rasvakudoksen kantasolujen on myös todettu erilaistuvan lihassolujen suuntaan, mikä lisää niiden soveltuvuutta solulähteenä lihaskudoksen korjaamiseen ja ponnistusinkontinenssin injektiohoitoihin.

Tämä väitöskirjatyö koostuu kahdesta erillisestä osiosta. Ensimmäisessä osiossa tutkittiin luonnonperäisen vesikalvon ja synteettisten polykaprolaktoni-polylaktidi komposiittikalvon sekä sileän ja teksturoidun polykaprolaktonikalvon soveltuvuutta uroteelisolujen, eli virtsateiden epiteelisolujen, kasvatusalustaksi *in vitro*. Työn toisessa osuudessa tutkimme soveltuvatko rasvakudoksen kantasolut yhdistettynä kollageenigeeliin virtsainkontinenssin hoitoon kliinisessä pilotti potilastutkimuksessa.

Työn ensimmäisessä vaiheessa havaitsimme, että amnionkalvo ei tukenut uroteelisolujen jakautumista, elinkykyä ja fenotyyppin pysyvyyttä verrattuna polykaprolaktonikalvoon. Kaikilla tutkituilla synteettisillä kalvoilla uroteelisolut olivat elinkykyisiä ja säilyttivät fenotyyppinsä. Kuitenkin komposiitti kalvolla uroteelisolut jakautuivat

hieman hitaammin kuin sileällä ja teksturoidulla polykaprolaktonikalvolla. Kliinisessä pilottitutkimuksessa totesimme, että rasvakudoksen kantasolut yhdessä kollageenin kanssa ovat turvallinen ja kohtalaisen tehokas hoitokeino käytettäväksi virtsainkontinenssin hoitoon. Lisäksi totesimme, että potilailta eristetyt rasvakudoksen kantasolut erilaistuvat lihas-, rasva-, luu- ja rustosolujen suuntaan *in vitro*.

Yhteenvetona voidaan todeta, että tutkimusten perusteella polykaprolaktoni on potentiaalinen biomateriaali uroteelin kudosteknologisiin sovelluksiin. Lisätutkimuksia kuitenkin tarvitaan, jotta voidaan arvioida polykaprolaktonin käytettävyyttä ja bioyhteensopivuuttaa *in vivo*. Lisäksi myös kudosteknologiset injektiohoidot ovat lupaavia uusia menetelmiä virtsainkontinenssin hoitoon tulevaisuudessa.

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List of abbreviations

5-aza	5-azacytidine
ALCAM	Activated leukocyte cell adhesion molecule
ALP	Alkaline phosphatase
APC	Allophycocyanin
α-SMA	Alpha smooth muscle actin
ASC	Adipose stem cell
AUM	Asymmetric unit membrane
ATP	Adenosine triphosphate
BM	Basal medium
BMP-4	Bone morphogenetic protein 4
BMSC	Bone marrow derived stem cell
CBSC	Umbilical cord blood stem cell
CD	Cluster of differentiation
DFV	Discoidal/fusiform-shaped vesicle
DIS	Detrusor instability score
CK	Cytokeratin
Cy	Cyanine
DPBS	Dulbecco's phosphate buffered saline
DMEM/F-12	Dulbecco's modified Eagle's Medium: nutrient mixture F-12
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EpCAM	Epithelial cell adhesion molecule
EthD-1	Ethidium homodimer-1
FACS	Fluorescence activated cell sorter
FGF-1	Fibroblast growth factor 1

GMP	Good Manufacturing Practice
hAM	Human amniotic membrane
HBSS	Hank's balanced salt solution
HLA-ABC	Human leukocyte antigen class I
HLA-DR	Human leukocyte antigen class II
HEPES	N'-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HS	Human serum
hUC	Human urothelial cell
IBMX	Isobutylmethylxanthine
IGF	Insulin-like growth factor
IIQ-7	Incontinence impact questionnaire-short form
ITS+1	Insulin, transferrin, selenous acid, bovine serum albumin, linoleic acid
IV	Inherent viscosity
MAb	Monoclonal antibody
MDSC	Muscle derived stem cell
MSC	Mesenchymal stem cell
MUI	Mixed urinary incontinence
NaCl	Sodium chloride
PCL	Polycaprolactone
PDGF-A	Platelet derived growth factor A
PE	Phycoerythrin
PFA	Paraformaldehyde
PGA	Polyglycolide
PLDLA	L-lactide/D-lactide copolymer
PLA	Polylactide
PLCL	Poly (L-lactide-ε-co-caprolactone)
PLGA	Poly-lactide-co-glycolide
PLLA	Poly-l-lactide
PS	Polystyrene

P/S	Penicillin/streptomycin
PTFE	Polytetrafluoroethylene
RGD	Arginine-glysine-aspartic acid peptide sequence
SEM	Scanning electron microscope
SIS	Small intestine submucosa
SM22-α	Smooth muscle 22-alpha
SM-MHC	Smooth muscle myosin heavy chain
SUI	Stress urinary incontinence
SVF	Stromal vascular fraction
Tg	Glass transition temperature
TGF-β1	Transforming growth factor beta 1
UDI-6	Urogenital distress inventory-short form
UISS	Urinary inventory stress test
UP	Uroplakin
VAS	Visual analogue scale

Original publications

The present study is based on the subsequent original articles, which are referred to by their Roman numerals (I-III) in the text.

I. **Sartoneva R***, Haimi S*, Miettinen S, Mannerström B, Haaparanta A-M Sándor G, Kellomäki M, Suuronen R, Lahdes-Vasama T, Comparison of poly(lactide-ε-caprolactone) membrane and amniotic membrane for urothelium tissue engineering applications. J R Soc Interface. 2011 May 6; 8 (58): 671-7.

II. **Sartoneva R*,** Haaparanta A-M*, Lahdes-Vasama T, Mannerström B, Kellomäki M, Salomäki M, Sándor G, Seppänen R, Miettinen S, Haimi S, Characterizing and optimizing poly-l-lactide-co-e-caprolactone membranes for urothelial tissue engineering. J R Soc Interface 2012 Dec 7; 9 (77): 3444-54.

III. Kuismanen K, **Sartoneva R**, Haimi S, Mannerström B, Tomas E, Miettinen S, Nieminen K, Autologous adipose stem cells in treatment of female stress urinary incontinence; result of a pilot study, Stem Cells Translational Medicine, *in press*.

* These authors contributed equally

1 Introduction

Hypospadia, where the urethral meatus opens ventrally in the shaft of the penis or even perineum, is one of the most common congenital anomalies, the annual number of new patients being approximately 100 patients in Finland (Aho *et al.* 2000). The reconstructive surgery of hypospadias is challenging and prone to complications, especially in severe cases and in cases where non-urological graft tissues are used for reconstruction of undeveloped urethra (Baskin and Ebbers 2006). This has initiated the development of alternative treatment methods.

Tissue engineering has emerged as a potential method to treat urothelial defects. In this study the main focus was on hypospadia, although, the tissue engineered urothelium can be utilized for all the patients lacking urothelium. Currently, the major challenge has been the development of optimal matrix material for the engineered urothelium. Both synthetic and natural biomaterials have been studied for the urohtelial tissue engineering applications (Atala 2009, Pariente *et al.* 2001). The advantage of natural derived biomaterials is their excellent biocompatibility, however, they are generally allogenic or xenogenic, which can cause problems, such as high batch to batch variation and immunological reactions. Further, the mechanical properties of natural biomaterials are poor (Kim *et al.* 2000, Nair and Laurencin 2006). These concerns can be avoided using synthetic biomaterials such as polylactide (PLA), polyglycolide (PGA), and polycaprolactone (PCL). These polymers have been widely studied synthetic biomaterials in various tissue engineering applications, as in urinary applications due to their biocompatibility and controllable mechanical properties. Though, compared to PLA and PGA, the PCL is more elastic and therefore demonstrates a good potential for soft tissue applications (Nair and Laurencin 2006, Pariente *et al.* 2001, Rohman *et al.* 2007).

The urinary incontinence is a highly common health issue and approximately 30 % of females 30-60 years of age are suffering from the involuntary loss of urine (Magon *et al.* 2011). Further, the prevalence of urinary incontinence increases with age (Nuotio *et al*. 2003). The most common type of incontinence, and the focus of our research project is a stress urinary incontinence (SUI), which is characteristic with involuntary loss of urine during physical effort (Magon *et al.* 2011). At the moment, the mid-urethral slings are the primary treatment method for SUI. However, complications such as bleeding, bladder injury or voiding dysfunction are related to the sling operations (Gilchrist and Rovner 2011, Novara *et al*. 2010). Additionally, injectable bulking agents, being a less invasive treatment method, have also been widely studied as a SUI treatment. However, the sustainability of the treatment result has not been optimal and additional injections are needed (Magon *et al.* 2011, Mohr *et al.* 2013). Tissue engineering and cell based injection therapies are also emerging for SUI treatments, aiming to achieve more stable treatment results by adding the cells to the biomaterial carrier. In addition to potential *in vivo* results, few clinical studies have also shown the potential of cell based injection therapies for SUI treatment (Ho and Bhatia 2012, Lin and Lue 2012). Different cell sources such as muscle derived cells, bone marrow derived mesechymal stem cells (BMSCs) and adipose stem cells (ASCs) have been tested *in vivo* or clinically (Lin and Lue 2012). Compared to ASCs the major limitations of these cell types are the donor site morbidity and yield of sufficient amount of cells for clinical applications (Lindroos *et al.* 2011, Wu *et al.* 2010). Adipose tissue provides an attractive and abundant cell source for adipose derived mesenchymal stem cells (ASCs). The adipose tissue derived mesenchymal stem cells can be relatively easily isolated and expanded, and are therefore advantageous for tissue engineering applications (Gimble and Guilak 2003).

2 Review of the literature

2.1 Urinary tract, urothelium and female continence system

The urinary tract consists of the paired renal pelvises and ureters, bladder, and urethra. The renal pelvises are located inside the kidney, and the urine is convoyed via ureters to the bladder. The urethra excretes the urine exterior to the body (Drake *et al.* 2005). The urinary tract is considered as an inert tissue, which conveys and stores urine from the kidneys to the exterior of the body. The luminal wall of the upper urinary tract, the bladder and proximal and middle part of the urethra is covered by a transitional epithelium, urothelium. The urothelium functions as a barrier between the lumen, the external environment, and the underlying tissue. It prevents the reabsorption of the urine and protects the surrounding tissues from the urine waste products (Drake *et al.* 2005, Ross and Pawlina 2006).

The female continence system is mainly consisting of sphincter unit and the surrounding support system, and it is ought to prevent the incontinence during the increase in abdominal pressure during the daily activities. In order to maintain the continence the urethral closure pressure has to be above the bladder pressure, but then allow the voiding when it is suitable. The overall mechanism of female continence system is still poorly understood and several theories have been presented over the decades, even though it would be crucial in order to develop the new treatment methods for urinary incontinence (Cundiff 2004, Delancey and Ashton-Miller 2004, Norton and Brubaker 2006).

2.1.1 Anatomy of urinary tract

Urinary tract extends from renal pelvises inside kidneys to the exterior of body via ureters, bladder and urethra. Ureters are approximately 25-35 cm long tubes that convey urine from renal pelvises to bladder with the aid of peristaltic contraction of the underlying smooth muscle layer. The ureters form a smooth S curve, descending retroperitoneally alongside psoas major muscle, entering obliquely to the base of the bladder. The ureters are narrowed in ureteropelvic junction, while the ureters cross the iliac artery, and at the point where ureters enter through the bladder wall. The proximal parts of the ureters derive their circulation from the renal arteries. The internal ovarian or spermatic arteries, branches of the common iliac artery and the internal iliac artery, supply the mid and lower parts of the ureters. The veins and lymphatic drainage are following the arteries (Drake *et al.* 2005, Krstic 1997).

The bladder is a hollow muscular organ serving as a reservoir for urine, changing its size and shape depending on the intraluminal urine volume. The bladder is located in the pelvic cavity posterior to the pubic bone, while distended the bladder extents to the lower abdomen. The empty bladder resembles a three-sided pyramid that lies on its margin, its apex facing the pubis. The triangular area, called trigone forms the base of the bladder. Two openings for ureters and one for the urethra are located at the corners of the trigone. The neck of the bladder is the most inferior part of the bladder, which is attached to its position with fibromuscular ligaments and surrounding the urethral opening. The smooth muscle layer of the bladder constitutes the detrusor muscle, which voids the bladder while contracting. Also, the detrusor muscle forms the involuntary internal urethral sphincter around the origin of the urethra. The branches of internal iliac artery are taking care of the blood blood supply to the bladder. A rich venal plexus that drains in to the internal iliac veins surrounds the bladder. Vesical, external iliac, internal iliac and common iliac lymph nodes receive the lymphatics from the

bladder (Krstic 1997, Ross and Pawlina 2006, Tanagho and McAninch 2008).

The urethra is a fibromuscular tube conveying urine from the bladder to the exterior of the body. The urethral size, structure and function differ significantly in women and men. The female urethra is short, about 4 cm and the diameter is about 8 mm. The urethra is slightly curved and the opening is anterior to the vagina. Each side of the urethral orifice there is openings of the paraurethral mucous gland, Skene's glands, which produce an alkaline secretion. The male urethra is approximately 20 cm long and the diameter is 8-9 mm. The male urethra has three distinctive segments; prostatic urethra, membranous urethra, and penile urethra. Prostatic urethra extends from the bladder through the prostate, the following membranous part of the urethra is surrounded by skeletal muscle of the external urethral sphincter. Penile urethra is surrounded by the corpus spongiosum of the penis. The ejaculatory duct empty in to the prostatic urethra and the ducts of bulbourethral glands and Littre glands drain in to the penile urethra. The inferior vesical, vaginal, and internal pudendal arteries supply the blood to the female urethra, the male urethra is supplied by the internal pudendal arteries. The lymphatic drainage is into the inguinal and subinguinal lymph nodes or into the internal iliac lymph nodes (Krstic 1997, Ross and Pawlina 2006, Tanagho and McAninch 2008).

2.1.2 Histological structure of the urinary tract and urothelium

The urinary tract has the same general structure as all the other excretory canals; a mucosa, submucosa, muscularis, and adventitia (Figure 1). The mucosa consists of urothelium, basal lamina, supporting connective tissue called lamina propria, and muscularis mucosae in the bladder wall. The lamina propria in urinary tract consists of connective tissue, vascular plexuses, nerves, occasional adipose tissue and lymphatic tissue (Paner *et al.* 2007, Ross and Pawlina 2006). The muscularis mucosa is irregularly arranged and forms a discontinuous layer, which may

explain why the muscularis mucosae bundles in bladder wall were not recognized until year 1982 (Dixon and Gosling 1983, Paner *et al.* 2007, Ross and Pawlina 2006). The highly vascularised urethral submucosa comprising of connective tissue, elastic tissue and mucous secreting periurethral glands is supporting the overlying mucosa (Tanagho and McAninch 2008).

The smooth muscle layer below the mucosa is arranged in two layers. In the inner longitudinal layer the smooth muscle cells are arranged as a loose spiral and in the outer circular layer as a tight spiral. In the distal end of the ureters the smooth muscle is apparent in three different layers: an inner longitudinal layer, a middle circular layer, and an outer longitudinal layer. In the bladder wall, the detrusor muscle fibres are arranged randomly as longitudinal, circular, and spiral manner. Additionally, the smooth muscle of the urinary tract forms bundles rather than muscular sheets, since the muscular layer is mixed with the surrounding connective tissue (Ross and Pawlina 2006, Tanagho and McAninch 2008).

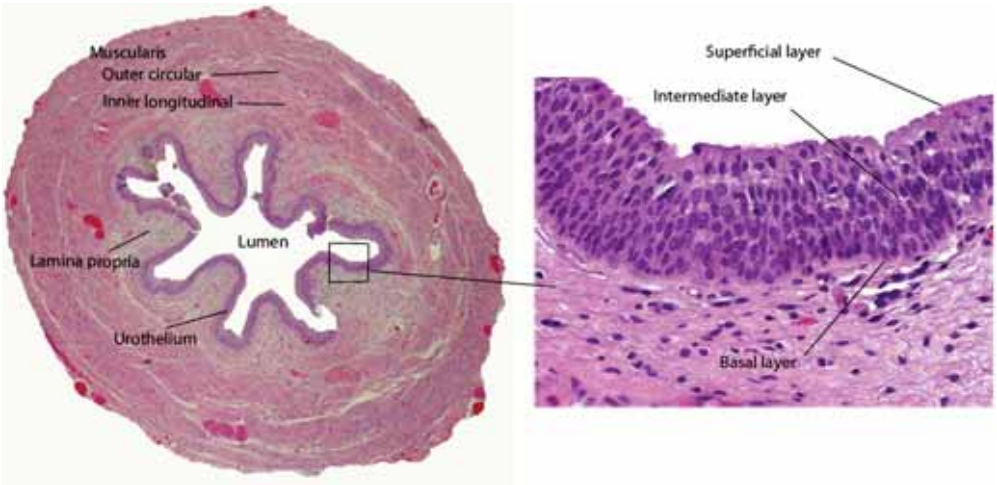

Figure 1. Histological structure of urinary tract (left image) and urothelium (right image). The mucosa; urothelium and lamina propria and muscularis are lining the lumen. The urothelium is a transitional epithelium consisting of superficial, intermediate and basal layer. The urothelium thickness varies depending on the intraluminal volume. Images modified from (http://www.pathologyoutlines.com/topic/bladderhistology.html).

The adventitia, a loose connective tissue, surrounds the muscularis. Expect the posterior surface of bladder and some regions of the urethra are covered by a serous membrane lining the peritoneum. The adventitial layer in bladder wall is richly vascularised and contains a well-developed network of autonomic nerve fibres. A thin adventitial layer covers the ureters and the urethra (Krstic 1997, Ross and Pawlina 2006, Tanagho and McAninch 2008).

The epithelium in the urinary tract is called urothelium, which is a transitional epithelium consisting of 3-7 cell layers, depending on the intraluminal volume, and three distinctive zones; basal, intermediate, and superficial cell zones (Figure 1) (Jost *et al.* 1989, Lewis 2000). In the distal part of the urethra the epithelium gradually changes to a stratified squamous epithelium. The urothelium functions as a barrier restraining the unregulated exchange of substances between the lumen and the underlying tissue. It is the tightest and the most impermeable barrier in the body, restraining the unregulated exchange of substances between urine and blood. Usually, substances such as water, ions and urea cross the membranes readily, however, via urothelium the permeability of these substances is extremely low (Kreft *et al.* 2010). The urothelium is capable of modulating the movement of ions, solutes, and water across to urothelium. Moreover, it has been discovered that the urothelium releases different neurotransmitters and mediators, such as adenosine and adenosine triphosphate (ATP) from its basal surface for information transmission across the urothelium (Apodaca 2004, Jost *et al.* 1989, Ross and Pawlina 2006, Southgate *et al.* 2002).

2.1.3 Anatomy and function of female continence system

The female continence indicates the ability to maintain urine storage with voluntary emptying of the bladder requiring a complex coordination of muscle contraction and relaxation, connective tissue support, and integrated innervation modulated

by the pontine micturation centre. To maintain the continence, the urethral closure pressure has to be greater than the bladder pressure both at rest and during the increase in abdominal pressure (Delancey and Ashton-Miller 2004, Magon *et al.* 2011, Norton and Brubaker 2006). Several theories have been introduced in order to explain urinary incontinence. The early theories predominantly focused on anatomic abnormalities. Thereafter, the importance of urethral sphincter dysfunction was recognized (Cundiff 2004). Petros and Ulmsten have published their integral theory for urinary incontinence in 1990. Accordingly, in addition to spesific structures the effect of age, hormones, and iatrogenically induced scar tissue are involved in female urinary incontinence. They hypothesized that the symptoms of both stress and urge incontinence derive from anatomic laxity of anterior vaginal wall due to the defects in wall itself or in the supporting ligaments and muscles (Petros and Ulmsten 1990). Recent theories are focusing on combining the anatomical and functional causes (Cundiff 2004).

Urethral sphincter and surrounding support system composed of connective tissue with interspersing smooth muscle and the levator ani muscle are consisting the female continence control system (Figure 2) (Delancey 2010). The striated urogenital sphincter muscle (rhabdosphincter) is the dominant element of the urethral sphincter. Smooth muscle and the vascular plexus inside the mucosa are also contributing to resting urethral closure pressure (Delancey and Ashton-Miller 2004). The detrusor muscle of the bladder extends slightly below the vesicle neck and a circular striated urethral sphicter muscle is initiating at the end of the detrusor muscle fibres. In the midurethra, the striated muscles of the urogenital diaphragm, the compressor urethrae and the urethrovaginal sphincter are detected. The distal part of the urethra is passing beside the bulbocavernosus muscle (Ashton-Miller and DeLancey 2007, Delancey and Ashton-Miller 2004).

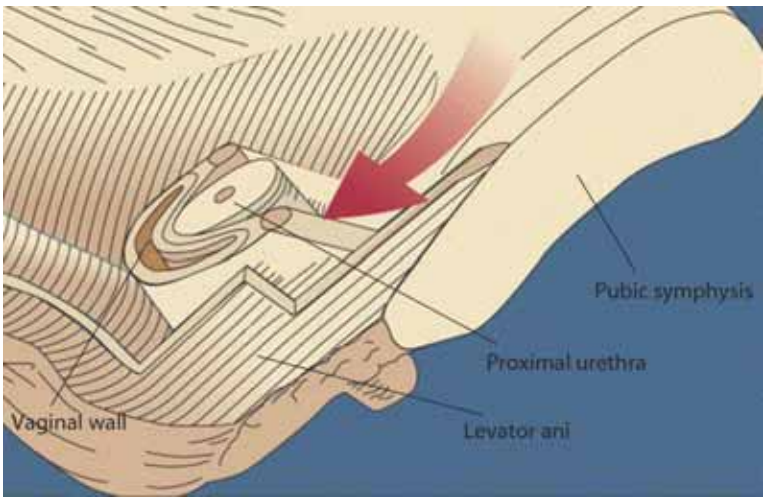

Figure 2. View of the structures supporting the continence. Arrow is indicating the direction of force during the increased intra-abominal pressure, for instance cough. The figure is modified from (Norton and Brubaker 2006).

The urethral support system is composed of all the support providing structures exterior to the urethra. The levator ani muscle, anterior vaginal wall, endopelvic fascia, arcus tendineus fasciae pelvis are the main components of this support system. The contraction of the levator ani, supporting the urethra via endopelvic fascia, increases the urethral compression and it is required for the normal function of urethral support system. The arcus tendineus fasciae are located bilaterally beside the urethra and vagina suspending the urethra on the anterior vaginal wall. Further, the vaginal wall is attached to the medial surface of levator ani. The contraction of levator ani is creating a backstop for urinary track by pulling the vagina towards pubic symphysis (Ashton-Miller and DeLancey 2007, Delancey and Ashton-Miller 2004, Norton and Brubaker 2006). This compresses the urethra and prevents the urine leakage during physical exertion or cough (Norton and Brubaker 2006). This urethral support system is arranged as a complex three-dimensional construct, which is exposed to substansial forces and controlled by an inadequately understood neural mechanism (Ashton-Miller and DeLancey 2007, Delancey 2010).

2.2 Urothelial defects

Different trauma, cancer, infections, iatrogenic reasons and congenital disorders can cause defects to the protecting urothelium requiring reconstructive surgery (Atala 1998, Wunsch *et al.* 2005, Yamzon *et al.* 2008). Nowadays, the urothelial defects are remedied using traditional reconstructive surgery techniques. The patient's genital tissues such as foreskin or nonurological tissues such as skin or buccal mucosa are used as transplant for urethral reconstruction (Yamzon *et al.* 2008). However, in severe cases these operations are extremely susceptible to complications especially when nonurological tissues are used. Thus, there is a high demand on alternative treatment methods, tissue engineering serving as a potential method for future urothelial reconstruction (Atala 1998, Atala 2009).

2.2.1 Hypospadia

Hypospadia, defined as an abnormal development of ventral prepuce and urethral spongiosum, is the major congenital cause for urethral defects (Leung and Robson 2007, Yamzon *et al.* 2008). Furthermore, it is one of the most common congenital abnormality, incidence being 0.3-0.5 % of all live births (Yamzon *et al.* 2008). In Finland there are approximately 100 new hypospadia patients every year (Aho *et al.* 2000).

Hypospadias can be divided according to their severity in to the distal, the most common, middle and proximal hypospadias (Figure 3). The proximal hypospadia, 10-15 % of hypospadias, is the most severe and the urethral meatus opens in the base of the penile shaft or even in the perineum. Depending on severity, the hypospadias may cause both cosmetic and functional difficulties; penis is typically small and ventrally curved which may prevent normal sexual behaviour in adulthood. Also the direction of the urine flow is abnormal (Baskin and Ebbers 2006, Leung and Robson 2007).

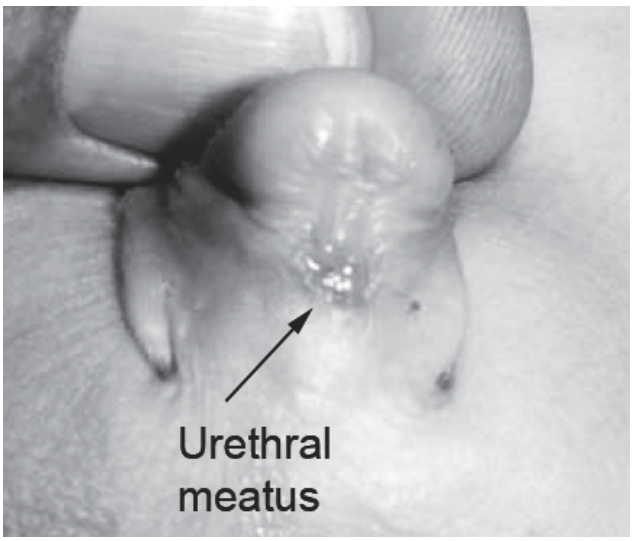

Figure 3. The middle hypospadia. The urethral opening (arrow) is locating in the ventral surface of penis. Image modified from (Baskin and Ebbers 2006).

Nowadays, the hypospadias are operated with traditional reconstructive surgeries and the severe cases may require two operations. At least 300 surgical techniques are described due to the susceptibility to complications (Baskin and Ebbers 2006). The general complications are infections, urinary retention, fistula formation, meatuses, strictures and poor cosmetic outcome and the complication frequency increases with the hypospadia severity (Fossum *et al*. 2007). In the most severe cases where additional tissue graft is needed, the complication rate is even 50 % (Baskin and Ebbers 2006, Mouriquand and Mure 2004). Due to the high complication frequency, the development of biocompatible and biodegradable graft material is important.

2.2.2 Urothelial regeneration and tissue healing

The renewal time of the intact urothelium is rather slow when compared to the regeneration rate of epidermis or intestinal epithelium. The source of urothelial stem cells still remain uncertain, however, it has been thought that these cells exist in

the urothelial basal cell layer (Khandelwal *et al.* 2009). However, after injury the urothelium has a tremendous capability to regenerate within days of damage. Rapid regeneration of the urothelium is critical in order to maintain the barrier function and preventing the admission of urine products and pathogens into the underlying tissues (Khandelwal *et al.* 2009, Kreft *et al.* 2005).

Primarily the urothelium reacts to the injury by desquamation and necrosis or apoptosis of the urothelial cells (Kreft *et al.* 2005). If the barrier function has been interfered by a bacterial infection or experimental manipulation, the uppermost cells of intermediate layer are able to rapidly differentiate into umbrella cells. After the injury the cells are increasing the expression of uroplakins (UP) and after 48 h the immature tight junctions are developed. The urothelium regenerates in 5 days, and after 10 days of injury the newly developed umbrella cells have reached their normal size (Khandelwal *et al.* 2009, Lavelle *et al.* 2002).

In thicker wounds, where the lamina propria has been exposed the remaining basal cells start to proliferate and 1-3 layered de novo urothelium is evident after 24 hours. At the same time the remaining umbrella cells begin to stretch and flatten, being approximately 3 times larger in length compared to the normal size umbrella cells. The extensive cell proliferation during the healing results a hyperplastic urohtelium with multiple cell layers after 3-5 days of the injury. Thereafter, the upper cell layer begins the differentiation process towards the superficially cells and the urothelium has regained its normal structure after 10 days of the damage. During the urothelial regeneration at least the expression of transforming growth factor beta 1 (TGF-β1), fibroblast growth factor 1 (FGF-1), insulin-like growth factor 1 (IGF-1), IGF-2, and platelet derived growth factor A (PDGF-A) increases indicating the involvement of these growth factors in urothelial repair (de Boer *et al.* 1994). Further, de Boer *et al*. detected that the alterations in GF or receptor expression were mainly detected in urothelium and no major changes were detected in submucosa or muscular layer implicating that the regeneration of urothelium is mainly

actuated by urothelium itself (de Boer *et al.* 1994, Kreft *et al.* 2005).

2.3 Cell sources for urothelial defect regeneration

Various cell sources have been studied for urothelial tissue engineering (Fu *et al.* 2007, Pariente *et al.* 2001, Shi *et al.* 2012). The primary urothelial cells have been the mostly studied cell types for urothelial applications (Pariente *et al.* 2001, Rohman *et al.* 2007, Wunsch *et al.* 2005). Additionally, urinary derived cell have emerged as a potential cell source for urothelial tissue engineering avoiding the donor site morbidity (Chun *et al.* 20012).

Furthermore, the non-urological primary cells such as foreskin epidermal cells and oral keratinocytes have been used. However, the major problem using these cell types in addition to non-urologic origin is the donor site morbidity (Fu *et al.* 2007, Fu *et al.* 2008, Li *et al.* 2008). Adult stem cells such as bone marrow derived stem cells (BMSC) and adipose stem cells (ASC) have been studied for urological applications as well. Both of these stem cell types have been demonstrated in few studies to differentiate towards urothelial cells (Shi *et al.* 2012, Shi *et al.* 2012, Tian *et al.* 2010). However, further studies are needed with adult stem cells before the use in urological applications and at the moment the UCs are the most potential choice.

2.3.1 Urothelium and urothelial cells

In this project we chose human urothelial cells since those are widely studied in urothelial tissue engineering applications and are relatively easily isolated and expanded in the laboratories (Atala 2011, Pariente *et al.* 2001). The hUCs (Figure 4) have been cultivated at least three decades and the first long-term cultures were established in the eighties (Southgate *et al.* 2002, Wu *et al.* 1982). The urothelial cells have been isolated succesfully from urinary track by biopsy (Magnan *et al.* 2006) or by tissue removal

during a routine surgery (Southgate *et al.* 2002). Further, considering the future clinical application, the donor site morbidity can be avoided since the urothelial cells can be isolated using bladder lavage (Fossum *et al.* 2007, Nagele *et al.* 2008).

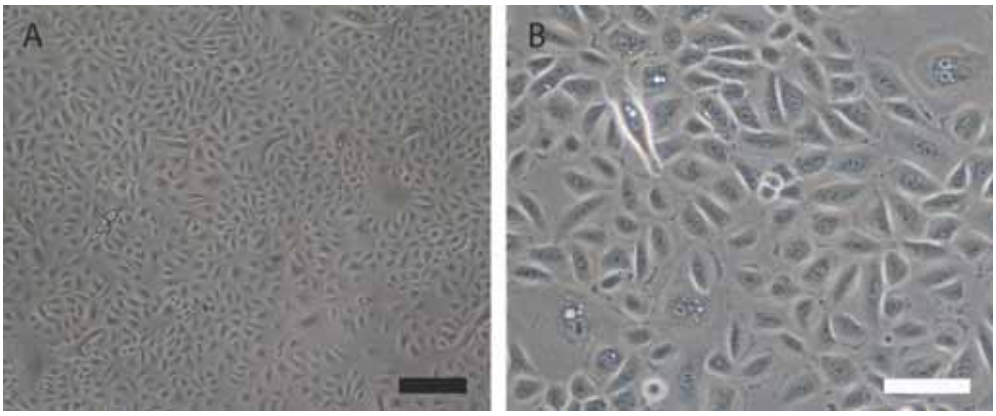

Figure 4. Freshly isolated hUCs. The images are indicating the typical hUCs morphology, being small and roundish or cuboidal. These cells were isolated and expanded in our laboratory facilites. Black scale bar 200 μm, white scale bar 100 μm.

The urothelial cells are derived from the embryologic endoderm (Lewis 2000). As all epithelial cells, the urothelial cells are characteristic with frequently small size and tight junctions to adjacent cell. The basal precursor cells are mononucleated, small, approximately 5-10 μm in diameter, and cuboidal in shape. These cells are arranged perpendicular to basement membrane, and are attached to the basal lamina by hemidesmosomes and to the intermediate cells by desmosomes. The intermediate cell layer consists of variable number of cell layers depending on the intraluminal urine volume. The intermediate cells are typically pyriform shaped mononuclear cells with a diameter of 10-20 μm. The cells in intermediate layer adhere to umbrella cells and to each other by desmosomes, further, occasionally the intermediate cells appear to attach to the basal lamina by cytoplasmic projections (Jost *et al.* 1989). Beneath the superficial layer, the intermediate cells are starting to resemple the umbrella cells, being partially differentiated. If the overlying umbrella cells are damaged, the

uppermost intermediate cells can rapidly differentiate to umbrella cells (Apodaca 2004, Khandelwal *et al.* 2009, Lewis 2000).

The superficial cell layer is a single cell layer with highly differentiated umbrella cells arranged perpendicular to the basement membrane. The umbrella cells are attached to each other by tight junctions, which together with their parallel combination have the major responsibility of the barrier properties of urothelium. The umbrella cells are bi- or multinucleated, polyhedral in shape, and between 25-250 μm in diameter. The size and shape of umbrella cells changes depending on the intraluminal urine volume, while distended the cells become stretched and squamous in morphology. The umbrella cells have distinctive apical surface, covered with scalloped-shaped plaques, which are separated by plasma membrane ridges called the “hinge”. The plaques are covering 70-90 % of the umbrella cells apical surface, the hinge membrane covers the remaining domains. Each plaque is composed of approximately 1000 subunits that contain different uroplakin (UP) proteins organized in a six-folded symmetry. The outer leaflet plasma membrane appears to be twice as thick in the plaque regions as the inner leaflet, forming the asymmetric unit membrane (AUM) plaque regions. Furthermore, the umbrella cells contain a high number of discoidal/fusiform-shaped vesicles (DFV), which consist of 2 apposing plaques connected by the hinge membrane. During the increase of intraluminal volume, the DFVs accumulate under and fuse with the apical surface of umbrella cells (Apodaca 2004, Jost *et al.* 1989, Khandelwal *et al.* 2009, Lewis 2000).

2.3.2 Urothelial cell characterization

In tissue engineering applications, the cell characterization is used for instance to verify the accurate cell type, or the stability of the phenotype during the cell culture. Specific surface or intracellular protein, such cytokeratins (CK), uroplakins (UP) or cluster of differentiation molecules (CD) are frequently used to characterize the urothelial cells. The flow cytometry, which is widely used for

instance cell counting and cancer diagnostic and immunostaining with specific antibodies are the frequently used characterization methods also in urohtelial applications (Chun *et al.* 2012, Nagele *et al.* 2008, Southgate *et al.* 1999, *Zhang et al.* 2008).

2.3.2.1 Cytokeratins

The CKs are a cluster of intermediate filament proteins characteristic with epithelial cells, their molecular mass varying from 40 000 to 68 000 Mr (Southgate *et al.* 1999). Nearly all the epithelial cells express some CK proteins, however, the expression profile is characteristic with epithelial differentiation level. The CK expression pattern changes also in malignant tumors, thus CKs may give additional information for diagnosing urothelial cancer (Achtstatter *et al.* 1985, Southgate *et al.* 1999).

In urothelial cells, the CK expression profile changes in different urothelial layers. The isotypes CK7, CK8, CK18, and CK19 are expressed through all layers in normal urohtelium. Additionally, the basal cells express the CK5 and 17 proteins. The CK13 is expressed in all urothelial layers except in the superficial layer, whereas, the CK20 is present solely in umbrella cells indicating differentiated urothelial cells (Khandelwal *et al.* 2009, Moll *et al.* 1982, Moll *et al.* 1988, Southgate *et al.* 1999, Veranic and Jezernik 2006).

2.3.2.2 Uroplakins

The UPs are group of transmembrane proteins. Five UP proteins; UPIa, UPIb, UPII, UPIIIa, and UPIIIb have been identified, their molecular mass is ranging from 15 to 47 kDa. The UPIa and UPIb have four transmembrane regions, while, the UPII, UPIIIa, and UPIIIb pass the lipid bilayer once. The UPs are the major element of AUM, which covers the majority of the apical membrane of the umbrella cells (Apodaca 2004, Khandelwal *et al.* 2009).

The UPs are expressed in superficial cell layer, in umbrella cells, however, the UPs are also expressed in upper intermediate cell layers. Additonally to the urothelium, the UPIb expression has also detected in the cornea and conjunctiva. The uroplakins have several functions, including serving as a barrier to water flow and solutes over the AUM (Khandelwal *et al.* 2009), alterating the bladder function (Aboushwareb *et al.* 2009), and serving as a receptor for E. coli (Apodaca 2004, Khandelwal *et al.* 2009). Furthermore, the UPs may have a major part in a urogenital development (Jenkins *et al.* 2005).

2.3.2.3 Cluster of differentiation molecules

Cluster of differentiation molecules are a group of cell surface proteins, which have a role for instance in immunology, cell signaling and adhesion. For urothelial cell characterization, at least the mesenchymal stem cell markers CD44, CD73, CD90, and CD105 and hematopoietic markers CD31, CD34, and CD45, and CD117 (Chun *et al.* 2012, Zhang *et al.* 2008) and epithelial marker CD133 have been studied. Generally, the hUCs are lacking the expression of hematopoietic markers CD31, CD34, CD45 and CD117 (Zhang *et al.* 2008), whereas, the hUCs are expressing the extracellular matrix adhesion marker CD44, which is though to be a marker for basal layer cells (Desai *et al.* 2000). The endothelial and mesenchymal stem cell (MSC) markers CD73, CD90, and CD105 are also expressed by hUCs. Moreover, the hUCs are shown to express CD133, a marker of various epithelial cells (Zhang *et al.* 2008).

2.4 Biomaterials for urothelial tissue engineering

A biomaterial can be defined as a nonviable material used in medical applications in order to interact with biological systems to treat, augment, or replace any tissue, organ, or body function (Williams 2009).

Biomaterials can be divided as synthetic and natural materials, furthermore, they can be classified according to the chemical composition as polymers, metals, ceramics and composites. Natural biomaterials highly resemble the macromolecules in the body and are therefore easily recognized and metabolized by biological environment. Due to the enzymatic degradation, the degradation rate of natural biomaterials is higher compared to the synthetic biomaterials and it also varies between the patients and body parts (Nair and Laurencin 2007). Additionally the natural biomaterials are extremely biocompatible and in contrast with the synthetic biomaterials, the toxicity and chronic inflammation is rarely a problem. However the high variations in material quality, risk of viral infections and immunological reactions are the major disadvantages concerning natural biomaterials (Rattner *et al.* 1996). For urothelial applications the collagen based matrices such as acellular bladder submucosa, small intestine submucosa (SIS), human amniotic membrane (hAM) and different commercial collagen matrices have been previously studied (Atala 2009, Kim *et al.* 2000, Pariente *et al.* 2001).

The synthetic biomaterials are generally less bioactive when compared to the natural biomaterials. However, as a main advantage they can be manufactured in large-scale and the bach-to-bach variations are minimal. Additionally, the mechanical properties and degradation rates are relatively easy to control (Nair and Laurencin 2006). The synthetic biomaterials and especially aliphatic polyesters also known as poly(α-esters) have been abundantly studied in the field of urothelial tissue engineering for instance, polylactides (PLA), polyglycolide (PGA), polycaprolactone (PCL) and their copolymers have been studied with promising results (Atala 2011, Drewa *et al.* 2006, Pariente *et al.* 2001, Rohman *et al.* 2007). The main disadvantage of the poly(α-esters) is their hydrophobic nature and a lack of cell recognition sites, leading to weaker cell attachment (Burks *et al.* 2006, Nair and Laurencin 2007). Additionally, one challenge with synthetic biomaterials has been tissue scarring and stricture formation. However, the use of cells in combination with

biomaterials could prevent these problems and allow more rapid tissue regeneration (Atala 2000, Drewa *et al.* 2006).

For tissue engineering applications the optimal biomaterial should be biodegradable, biocompatible, noncarcinogenic and nontoxic. It should degrade without disadvantageous tissue reactions while the new tissue regenerates, moreover, the degradation product should be nontoxic. Further, the biomaterial ought to promote cellular interactions, vascularization, tissue regeneration, and have an adequate mechanical properties depending on the application. Generally, the biomaterial membranes or scaffolds should mimic the functional and mechanical properties of the cell supporting extracellular matrix (ECM) as closely as possible (Atala 2009, Fu *et al.* 2012, Kim *et al.* 2000, Nair and Laurencin 2007). In addition to the general requirement, the biomaterial for urothelial tissue engineering should be elastic, flexible, suturable, and easy to be made as a tube like structure. Even though various different biomaterials have been studied with successful results, the optimal biomaterial for urothelial tissue engineering has not been found yet.

2.4.1 Aliphatic polyesters

Aliphatic polyesters are thermoplastic polymers with hydrolytically labile ester bonds. These polymers are known to be highly biocompatible synthetic biomaterials. Additionally, Food and Drug Administration (FDA) has approved PLA, PGA and PCL for human use in several applications which has increased their popularity also in tissue engineering field (Nair and Laurencin 2006).

2.4.1.1 Polylactides

Lactic acid (2-hydroxypropanoic acid) monomer is the simplest and smallest chiral molecule existing as two optically active stereoisomers: D-lactic acid and L-lactic acid (Figure 5). The fact that L-lactide acid is formed during anaerobic energy production in

human body has increased the use of polylactic acid-based polymers clinically.

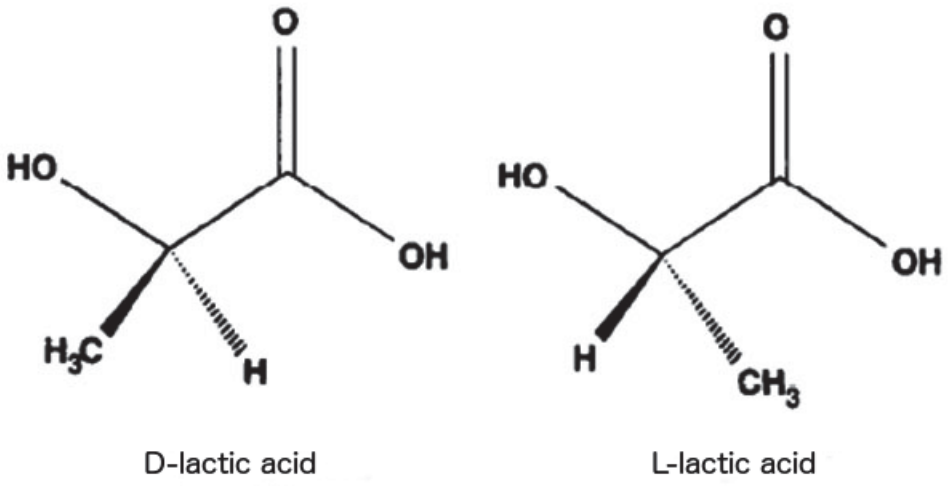

Figure 5. The schematic images of lactic acid stereoisomers. Image modified from (Lunt 1998).

PLA degrades hydrolytically via bulk erosion forming lactic acid, which is easily eliminated by the body metabolism. Due to its excellent biocompatibility and readily tailorable properties, PLAs have been studied in many fields in tissue engineering from bone tissue engineering to soft tissue engineering applications, including urothelium tissue engineering (Haimi *et al.* 2009, Kim *et al.* 2000, Nair and Laurencin 2006).

2.4.1.2 Polycaprolactone and Poly(L-lactide-ε-caprolactone)

PCL is manufactured from the ε-caprolactone monomer by ring-opening polymerization (Figure 6) (Sinha *et al.* 2004). It degrades via hydrolysis, however, enzymes may also affect the degradation at the latter stage (Kellomäki *et al.* 2002). The mechanical properties of PCL are suitable for soft tissue engineering, since the PLC is soft and pliable, additionally, the PCL has an excellent

biocombatibility. However, the degradation rate of PCL is rather slow even 2-3 years (Kellomäki *et al.* 2002, Nair and Laurencin 2007). To achieve a pliable polymer, with excellent biocompatibility, PCL is often used as a copolymer with PLA resulting in a copolymer with a higher degradation rate than for either homopolymer alone (Kellomäki *et al.* 2002).

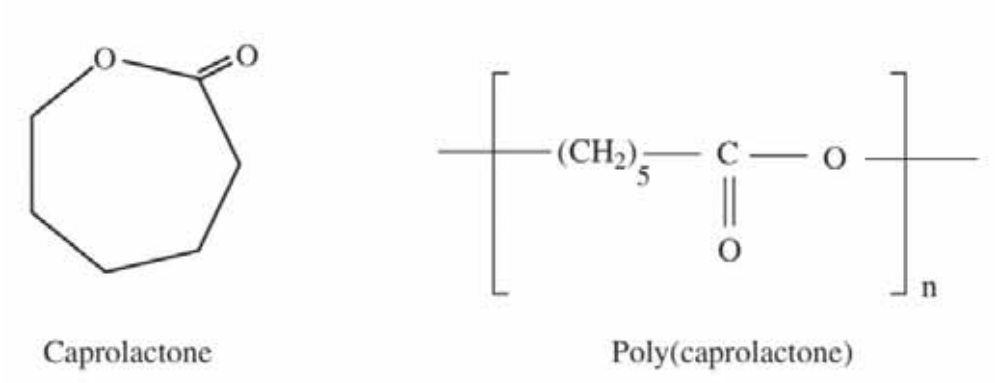

Figure 6. Schematic illustrations of ε-caprolactone monomer and poly(caprolactone) polymer. Image modified from (Nair and Laurencin 2007).

Poly (L-lactide-ε-co-caprolactone) (PLCL) is a copolymer of L-lactide and ε-caprolactone, further, the properties of PLCL can be modified by changing the proportions of monomers. Increasing the amount of PCL results in a more flexible and rubbery copolymer whereas PLA makes the copolymer harder and increases the glass transition temperature of the copolymer (Burks *et al.* 2006). Due to its elastic properties PLCL has been widely studied especially for soft tissue engineering applications, including vascular, neural and esophageal with promising results (Burks *et al.* 2006, Zhu *et al.* 2007). Zhu *et al.* studied electrospunned nanofibrous scaffold 84/16 poly(L-lactide-co-caprolactone) (PLLC) for regeneration of esophageal epithelium. They demonstrated that PLLC grafted with fibronectin promotes regeneration of esophageal epithelial cells (Zhu *et al.* 2007). Additionally, Jung *et al.* studied porous 50/50 PLCL scaffolds for cartilage tissue engineering demonstrating that the chondrocytes proliferated on PLCL scaffolds. They concluded

that porous PLCL scaffolds could also promote cartilage regeneration *in vivo*, being potential also for cartilage tissue engineering applications (Jung *et al.* 2008). There are few research, studying the PCL with urothelial cells (Kundu *et al.* 2011, Sun *et al.* 2012), whereas, at least to our knowledge there is no previous reports concerning the suitability of PLCL for urothelial tissue engineering.

2.4.2 Human amniotic membrane

The hAM is the innermost placental membrane composed of a single epithelial layer, a basement membrane and an avascular stroma. The basement membrane is mainly composed of collagen I, III, and IV, laminin, and fibronectin. Due to its composition, biocompatibility and biodegradability it has been widely studied in tissue engineering, especially in corneal application (Lim *et al.* 2009, Wilshaw *et al.* 2006) but also as a matrix for endothelial cells, epidermal keratinocytes, and UCs (Tsai *et al.* 2007, Sharifiaghdas *et al.* 2007, Yang *et al.* 2006). It has been detected that the epithelial cells grow better on denuded AM compared to intact AM and the acellular hAM has shown to remain its biocompatibility after enzymatic denuding (Koizumi *et al.* 2007). Therefore, hAM is typically denuded for instance with dispase prior to the use as a growth matrix for cells (Sippel *et al.* 2001). Tsai *et al.* studied amniotic membrane, where the epithelial layer wad denuded with scraper, for vascular tissue engineering. They demonstrated that the endothelial cells proliferated well on AM and concluded that AM could be a potential matrix for vascular tissue engineering (Tsai *et al.* 2007). Further, Yang *et al.* demonstrated that AM overlaid on living skin equivalent embedded with fibroblasts enhanced the growth of epidermal keratinosytes compared to the plain fibroplast embedded living skin equivalent (Yang *et al.* 2006). The main advantages of hAM are that it is inexpensive and readily available since it can be separated from the placenta after the labour, and in Finland, the hAM is available as a tissue bank product. Furthermore, it is an allogenic

biomaterial, whereas, the other acellular matrices are mainly of animal origin alias xenograft (Hopkinson *et al.* 2008, Wilshaw *et al.* 2006).

2.5 Urothelial tissue engineering

Tissue engineering is a multidisciplinary field of science aiming to restore, maintain or improve tissue function using combination with cells, biomaterial and tissue inducing substances such as growth factors (Langer and Vacanti 1993). The urge for tissue engineering has emerged from the substantial need of organs and tissues that the organ replacement surgery cannot satisfy. However, there are major challenges concerning tissue-engineering application in urology. Finding an optimal biomaterial for the application and the selection of appropriate cell source for each application are critical. Additionally, establishing a blood supply to tissue-engineered organ is one of the major challenges (Atala 2009, Dissaranan *et al.* 2011, Langer and Vacanti 1993, Matoka and Cheng 2009).

2.5.1 *In vitro* studies

For urothelial tissue engineering rather many synthetic and natural biomaterials have been studied even though the amount of studies is relatively low. Pariente *et al*. studied the biocompatibility of naturally derived and synthetic biomaterials; bladder submucosa, small intestin submucosa, collagen sponge, alginate, alloderm. PGA, poly-l-lactide (PLLA), poly-lactide-co-glycolide (PLGA) and silicone, on hUCs and detected that all the tested biomaterials, expect alginate, demonstrated an excellent biocompatibility *in vitro* (Pariente *et al.* 2001). The different commercially available biomaterials (Biogide®, Ethisorb®, Lyoplant®, SIS®, Vircyl® covered with matrigel and Xenoderm®) were compared by Wünsh *et al*. They detected that the morphology of hUCs cultured on Biogide®, Lyoplant®, Vircyl®

covered with matrigel and Xenoderm® resembled native urothelial cells, whereas, the hUCs on Ethisorb® and SIS® showed a spherical cell morphology indicating a less favourable attachment between the cells and biomaterial (Wunsch *et al.* 2005). Furthermore, the Rohman *et al*. demonstrated a good biocompatibility of PLGA with hUCs during 14 d assessment period (Rohman *et al.* 2007).

Additionally, some 3D scaffolds have been tested as a matrix for urothelial cells. Kundu *et al*. studied different scaffolds; electrospun (e) PCL and ePLLA and composite scaffolds ePCL on thin film (tf) PCL and ePLLA on tfPCL and SIS were compared as a growth matrix for hUCs (Kundu *et al.* 2011). The study demonstrated that the proliferation of hUCs cultured on thin film side of the composite scaffold and on luminal side of SUS was superior on composite scaffolds compared to SIS. Furthermore, they demonstrated that the hUCs proliferated significantly more on smooth composite scaffold than on fibrous ePCL or ePLLA scaffolds (Kundu *et al.* 2011). The composite scaffold of collagen and PCL with different compositions has also been studied with co-culture of bladder smooth muscle and urothelial cells by Sun *et al*. demonstrating a more homogenous cell distribution inside the low collagen content scaffold (Sun *et al.* 2012).

2.5.2 *In vivo* studies

In order to study the biocompatibility and behaviour of engineered urothelial tissue construct in a physiological environment, the *in vivo* studies give crucial information in addition to *in vitro* research. Atala *et al*. have showed over a decade ago that the rabbit urothelial cells seeded into PGA mesh remained viable in a rabbit model and after 20 or 30 days a 1 to 3 layered urothelium was detected. Further, their study suggested that autologous urothelium could be a potential cell source for urothelial applications (Atala *et al.* 1992). Further, the Drewa *et al*. demonstrated in a rat study that the fibroblasts could facilitate urothelial cell invasion and proliferation *in vivo* in PGA scaffold

(Drewa *et al.* 2006). A composite scaffold composed of PLLA and collagen has been shown to support the viability of urothelial cells after 2 weeks of implantation in to the mouse subcutis. Additionally, a novel centrifugal seeding method was shown to lead to more homogenous cell distribution and better cell ingrowth in these composite scaffolds when compared to static cell seeding (Fu *et al.* 2012).

Li *et al*. showed that an acellular bladder matrix seeded with oral keratinocytes was superior to acellular bladder matrix alone when used to reconstruct the rabbit urothelial defect. The formation of multilayered epithelium was evident in cell-seeded matrix after 6 months, whereas no single or stratified epithelium was detected with non-cellular matrix. Also, stricture formation was detected in the non-cellular matrix group (Li *et al.* 2008). Fu *et al*. studied that the foreskin epidermal cells seeded on a acellular bladder matrix enhanced the urothelial reconstruction compared to control acellular bladder matrix. Additionally, the amount of side effects such as strictures was lower on a cell seeded matrix group compared to control group (Fu *et al.* 2007). During their 1 year follow up study in a rabbit model Fu *et al*. demonstrated that the epidermal keratinocytes transform towards transitional cells and therefore suggested that the urethral environment actuates the transformation (Fu *et al.* 2008).

2.6 Urinary incontinence

Urinary incontinence is a common health issue among female, an estimated prevalence being 30 % for females 30-60 years of age (Magon *et al.* 2011). The urinary incontinence can be divided predominantly to stress, urge and mixed incontinence. The focuses in this thesis are on stress urinary incontinenece (SUI) and mixed urinary incontinence (MUI), which are the most common type of inontinence (Haylen *et al*. 2010). SUI can be defined as an involuntary loss of urine during physical exertion or effort (Magon *et al.* 2011). The MUI is a combination of SUI and urge incontinence, meaning an urgent need for bladder emptying. The

major factors increasing the risk of urinary incontinence are age, parity, obesity, hysterectomy and smoking (Delancey and Ashton-Miller 2004, Delancey 2010, Magon *et al.* 2011).

The primary treatment methods for incontinence are conservative, lifestyle intervention, pelvic floor muscle training, medication, and restoring estrogen deficiency. Furthermore, over 200 surgical procedures have been described for SUI treatment. The mid-urethral slings have emerged as first-line surgical methods since they are minimally invasive operations and their long-term success rates are good (Magon *et al.* 2011). Even though the mid-urethral sling operations are generally well tolerated the adverse effects such as bleeding during the surgery, urethral injuries, infection or difficulties in micturation are detected (Gilchrist and Rovner 2011, Lin and Lue 2012). Additionally, there are patients who are not suitable for mid-urethral sling operations or the treatment is ineffective. Therefore the development of novel and noninvasive treatment methods is essential (Magon *et al.* 2011).

2.6.1 Biomaterials for incontinence injection therapies

Injection therapies with bulking agents have been used for SUI treatment for decades. The main advantages of the injection therapies are that they are mini-invasive and easy to use. Also, it is safe even for weak elderly patients. Additionally, the amount of adverse effects is low and the side-effects are mild. However, many studies have revealed that the sustainability of treatment effect is not optimal and additional injections are needed to maintain the bulking effect (Kirchin *et al.* 2012, Mohr *et al.* 2013).

Several bulking agents both non-biodegradable and biodegradable have been used for injection therapies. Collagen, especially bovine collagen (Contigen®) is the most studied and clinically used biomaterial for the injection therapies due to its biocompatibility and abundant history in clinical use (Rovner and Goudelocke 2010, Kirchin *et al*. 2012). Previously, fat tissue has been used as a bulking agent to treat females SUI patients,

though, the results had a high variation depending on the study and the reported success rate have a variation from 22 % to 65% (Lee *et al.* 2001, Palma *et al.* 1997, Tsai *et al.* 2006). Palma *et al*. treated 30 female patients with single or douple periurethral adipose tissue injections and after 1 year follow-up reported a cure rates 31 % and 64 % respectively (Palma *et al.* 1997). However, in a douple-blinded study performed by Lee *et al*. 68 female patients were treated with autologous fat (n= 35) and saline (n=33). After the 3 months follow-up 22.2% in fat group and 20.7% in saline group were cured or had less symptoms (Lee *et al.* 2001). Furthermore, silicone, polytetrafluoroethylene (PTFE), polyacrylamide hydrogel and hyaluronic acid have been used to treat female urinary incontinence (Dmochowski and Appell 2000, Kirchin *et al*. 2012, Mohr *et al.* 2013).

2.6.1.1 Collagen

Collagen is a natural biomaterial with unique biological, physiochemical and mechanical properties (Nair and Laurencin 2006, Rattner *et al.* 1996). Nowadays more than 22 different collagen types have been identified, the most common being types I-IV. Type I collagen is the most studied collagen type for urological tissue engineering applications (Mohr *et al.* 2013, Shi *et al.* 2012), and also the most common collagen type in the human body. Further, it is considered as a promising biomaterial due to the fact that it is a major component of ECS, and functions as a natural matrix for cell attachment, proliferation and differentiation in the human body (Shi *et al.* 2012). Additionally it contains the arginine-glysine-aspartic acid (RGD) peptide sequence, which is known to be important for cell adhesion properties. The collagen is enzymatically degraded by the body enzymes such as as collagenases and metalloproteinases (Nair and Laurencin 2007).

Bovine dermal collagen (Contigen™) consists of 95 % of type I and 5 % of type III collagen (Bent *et al.* 2001). Prior to use collagen is purified, crosslinked with glutaraldehyde solution and

dispersed in a phosphate buffer saline solution. The resulting Contigen™ gel is a sterile and homogenous gel. After an injection, the Contigen™ condenses into a fibrous network where the adjacent connective tissue cells and vessels are migrating (Tsai *et al.* 2006). The reported cure rates in different studies have varied from 7-83 %, however in most studied the cure rates have been approximately 40-60 % (Dmochowski and Appell 2000, Eckford and Abrams 1991, Homma *et al.* 1996, Smith *et al.* 1997). Haab *et al*. compared fat (n=45) and collagen (n=22) injections for treatment of SUI. After the follow-up 43 % of the patients from fat group and 86 % of collagen group were cured or the symptoms were improved. Their conclusion was that the collagen is more effective bulking agent for incontinence compared to fat (Haab *et al.* 1997). However, the sustainability of the gollagen bulking effect is not optimal and additional injections are needed which diminishes the cost effectiveness of this treatment method (Dmochowski and Appell 2000). The Contigen™ injections are generally well tolerated, however, it can cause allergic reactions for approximately 4 % of females (Bent *et al.* 2001).

2.7 Cell based therapy for urinary incontinence

The cell based tissue engineering techniques are emerging methods to treat female incontinence. The cell source for injection therapies should be autologous, readily expanded and the isolation procedure should be minimally invasive. Using the patients' own cells diminishes the risks of tissue rejection and transfer of infectious agents (Gimble and Guilak 2003). The future tissue engineering based therapies include two phases; first a tissue biopsy will be taken from the patients following cell isolation and expansion at good manufacturing practice (GMP) level laboratories. After the expansion, the patient will receive autologous cell injections transurehtrally under a local anesthesia to regenerate the spincter musculature (Wang *et al.* 2011). The first clinical study to treat SUI patients was done by Mittenberger

et al. in 2007 (Miggenberger *et al*. 2007). They used primary muscle derived cells in their study. However, the primary cells have a limited potential for self-reneval and cell division and thus are not ideal for cell based therapies (Wu *et al.* 2010).

2.7.1 Stem cells for urinary incontinence

Stem cells are defined as cells having ability to self-renewal and differentiate towards multiple lineages (Gimble *et al.* 2007, Mizuno 2009). The stem cells can be classified to totipotent, pluripotent, multipotent or unipotent, according to their ability to differentiate into other cell types. Adult mesenchymal stem cells (MSC) present in the human body are either multipotent such as bone marrow derived stem cells (BMSC) or adipose derived stem cells (ASC) or unipotent such as MDSCs. These cells are characteristic with limited self-renewal and differentiation capability compared to embryonic stem cells (Behr *et al.* 2010, Choumerianou *et al.* 2008).

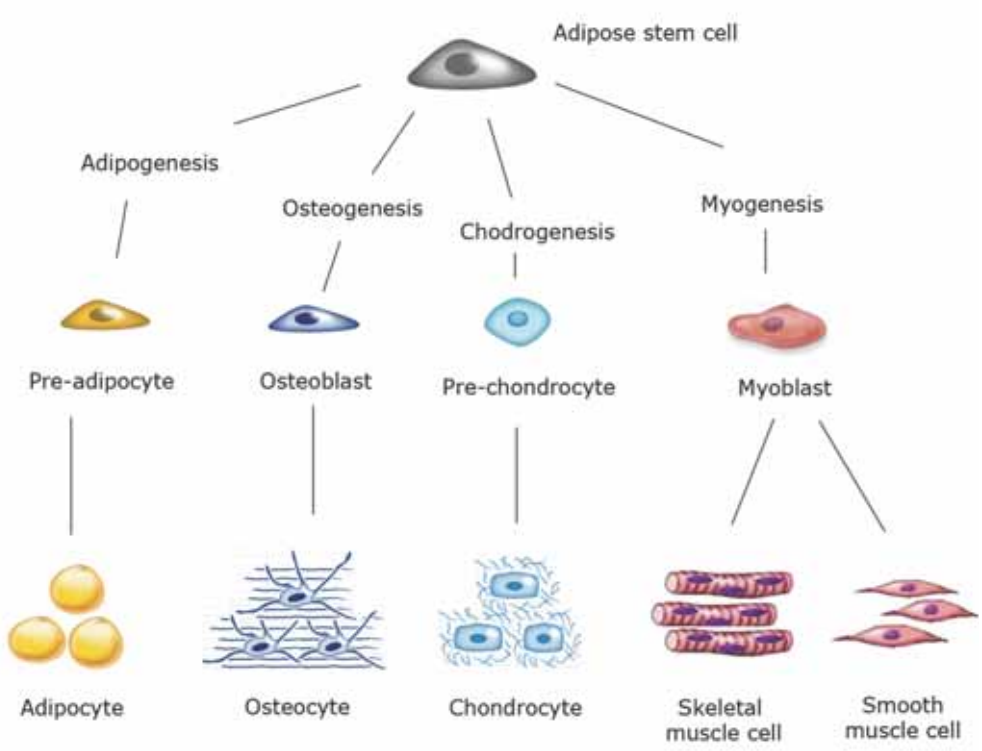

Figure 7. The ASCs are shown to differentiate towards myogenic, adipogenic, osteogenic and chondrogenic cell lineages (Zuk *et al.* 2001).

BMSCs and ASCs are shown to differentiate at least towards to myogenic, adipogenic, osteogenic and chondrogenic cell lineages (Figure 7) (Lindroos *et al.* 2011, Zuk *et al.* 2002). Previously, BMSCs, ASCs and MDSCs have been studied for urinary incontinence applications using animal models with promising results (Ho and Bhatia 2012, Lin and Lue 2012). Additionally, MDSC and umbilical cord blood SCs (CBSC) have been used in few clinical studies to threat female stress urinary incontinence (SUI) with favourable results (Carr *et al.* 2008, Lin and Lue 2012, Sebe *et al.* 2011). The MDSCs are considered as potential MSCs for SUI therapies. The MDSCs are relatively easily isolated from the muscle tissue, and are shown to possess a similar properties compared to other MSCs with regards to their proliferation, differentiation potential, and self-renewal capability (Jackson *et al.*

2010, Wu *et al.* 2010). However, the MDSCs are isolated by muscle biopsy, which may cause donor site morbidity. Also, the extraction and expansion of sufficient amount of MDSCs for clinical applications is challenging (Wu *et al.* 2010).

2.7.2 Adipose stem cells

Recently, ASCs have been under extensive research and emerged as an interesting cell source for several tissue engineering applications. Compared to BMSCs the major advantage of ASCs is their abundance. In addition, the ASCs can be readily isolated from the adipose tissue with minimal isolation site injury and the expansion of cells is relatively easy (Ho and Bhatia 2012, Mesimaki *et al.* 2009) which are important cell source characteristics for clinical applications.

Subcutaneus fat is the major reservoir for ASCs. The adipose tissue, deriving from the mesodermal layer of the embryo, is composed of adipocytes and heterogenous cell population called stromal vascular fraction (SVF). The SVF contains ASCs, endothelial cells, fibroblasts, circulating blood cells and pericytes (Gimble *et al.* 2007). The ASCs can be relatively easily isolated from the adipose tissue with enzymatic degradation with collagenase (Gimble *et al.* 2007), furthermore; several isolation methods are established and shown to be repeatable (Gimble *et al.* 2007, Mesimaki *et al.* 2009, Sándor *et al.* 2013). The International Society for Cellular Therapy has defined that MSC including ASCs need to express the markers CD73, CD90 and CD105. Whereas, markers CD31, CD45, and CD133 representing hematopoietic and angiogenic lineages are usually absent on ASCs as well as HLA class II antigen. In addition to these, the surface markers CD9, CD10, CD13, CD14, CD19, CD29, CD44, CD49d, CD106, CD146, CD166 and human leukocyte antigen (HLA) class I are used to characterize the ASCs (Dominici *et al.* 2006, Lindroos *et al.* 2010, Lindroos *et al.* 2011, Zuk *et al.* 2002).

Previously, the ASCs have been studied in clinical applications of bone tissue engineering and demonstrated to be safe and well

tolerated (Lindroos *et al.* 2011, Mesimaki *et al.* 2009, Sándor *et al.* 2013). For urinary incontinence applications ASCs have been studied in few *in vivo* models with promising results (Lin *et al.* 2010, Wu *et al.* 2011). Lin *et al*. treated SUI induced rats with periurethral injections of 300 μl ASCs mixed with medium and demonstrated an improvement of voiding function compared to the injection 300 μl of medium (Lin *et al.* 2010). Additionally, Wu *et al*. detected an improvement in sphincter function, urethral closure pressure, maximum bladder capacity and abdominal leak point pressure with ASC treated SUI induced rats (Wu *et al.* 2011). The results of these *in vivo* studies indicate that ASCs are an effective cell source to treat female SUI (Lin *et al.* 2010, Wu *et al.* 2011).

2.7.3 Myogenic differentiation potiential of adipose stem cells

The ASCs have been established to differentiate towards adipogenic (Zuk *et al.* 2002), osteogenic (Mesimäki *et al.* 2009, Zuk *et al.* 2002), chondrogenic (Zuk *et al.* 2001) and myogenic (Mizuno *et al.* 2002, Zuk *et al.* 2001) cell lineages. The myogenic differentation capability of ASCs is crucial for clinical treatments of urinary incontinence. The ASCs have established to differentiate both *in vitro* and *in vivo* towards smooth muscle cells (Jack *et al.* 2009, Mizuno 2009, Wang *et al.* 2010) and skeletal muscle cells (Choi *et al.* 2012, Di Rocco *et al.* 2006, Fu *et al.* 2010, Mizuno *et al.* 2002), which are both present in urinary sphincter.

The ASCs have been detected to express myogenic markers including early myogenic markers alpha smooth muscle actin (α-SMA), smooth muscle 22-alpha (SM22-α), calponin, and late marker smooth muscle myosin heavy chain (SM-MHC) when stimulated with TGF-β1 and bone morphogenetic protein-4 (BMP-4) either alone or in combination with culture medium (Wang *et al.* 2010). Wang *et al*. demonstrated that in combined stimulation of TGF-β1 and BMP-4 the ASCs expressed both early markers α-SMA,

calponin SM22-α, and late marker SM-MHC of smooth muscle cell differentiation (Wang *et al.* 2010). Furthermore, several studies have indicated that TGF-β1 can induce the differentiation of bone marrow derived stem cells towards myogenic cell lineages (Narita *et al.* 2008, Wang *et al.* 2010, Zhao and Hantash 2011).

Wu *et al*. demonstrated that ASCs induced with 5-aza and horse serum *in vitro* differentiated towards myoblasts significantly better compared to the non-induced control cells (Wu *et al.* 2010). Moreover, Fu *et al.* demonstrated in a rat model that similarly induced ASCs injected periurethrally increased the amount of myoblasts under the mucosa, after the 3 months follow-up period (Fu *et al.* 2010). Additionally, the hydrocortisone and horse serum induced ASCs have been demonstrated to express myogenic markers including MyoD1, which is an early marker and MHC indicating the myogenic potential of ASCs (Mizuno *et al.* 2002, Zuk *et al.* 2001, Zuk *et al.* 2002). Furthermore, co-culturing the ASCs with myoblast has been shown to enhance myogenic cell differentation further demonstrating the myogenic potential of ASCs (Di Rocco *et al.* 2006, Lee and Kemp 2006). Moreover, Di Rocco *et al*. demonstrated that some of the ASCs spontaneously differentiated towards myoblasts in fibronectin coated wells (Di Rocco *et al.* 2006).

3 Aims of the study

During this thesis we evaluated the potential of different biomaterial membranes for urothelial tissue engineering applications *in vitro*. Secondly, we studied the tissue engineering based injection therapies for urinary incontinence application. Specific aims for each study are described below.

I. To compare the effects of natural human amniotic membrane, synthetic PLCL membrane and PS on hUCs viability, proliferation and phenotype maintenance *in vitro*.

II. To further verify the potential of PLCL based membranes *in vitro* by comparing the effect of different mechanical texturing on hUCs attachment, viability, proliferation and phenotype maintenance.

III. To evaluate the efficacy and safety of injection therapy using ASC in combination with collagen gel, Contigen™ on female urinary incontinence in a clinical pilot study. The *in vitro* multidifferentiation potential of ASCs was also studied.

4 Materials and methods

4.1 Materials

The hAM, used in the study I, was obtained after routine caesarean section at Tampere University Hospital with the approval of the Ethics Committee of Pirkanmaa Hospital District (Tampere, Finland, R06045). Briefly, hAM was detached from chorion, rinsed with 0.9 % NaCl and incubated in Dulbecco's phsospatase saline solution (DPBS, Sigma-Aldrich, St. Louis, MO, USA) containing 2.5 μg/ml of amphotericin B (Sigma-Aldrich), 5.0 μg/ml penicillin, 50 μg/ml streptomycin and 100 μg/ml neomycin (Sigma-Aldrich). To detach the epithelial cells, 4 % dispase was used (Life Technologies, CA, USA), which after acellularity of hAM was confirmed with a light microscope.

The 70/30 PLCL (Purac Biochem BV, Gorinchem, The Netherlands) was used to manufacture the PLCL membranes (I and II). The polymer was highly purified, with residual monomer < 0.5 % and with an inherent viscosity of 1.6 dl/g. Polylactide (96/4 L-lactide/D-lactide copolymer (PLDLA), Purac, Biochem BV) with an inherent viscosity of 2.12 dl/g was used to manufacture knitted PLDLA for composite membranes in the study II. In the study I, polystyrene (PS, Corning CellBIND, Sigma-Aldrich) cell culture wells were used as a control material.

For the pilot patient study III, the commercially available bovine collagen gel (ContigenTM, Bard Medical, Covington, USA) was used in combination with ASCs for SUI treatment.

4.2 The sample manufacturing

The biomaterial membranes used in these studies were manufactured at the Tampere University of Technology, Department of Biomedical Engineering.

The sPLCL membranes were manufactured by compression moulding of PLCL granules with NIKE hydraulic press (Hydraulics Ab, Eskilstuna, Sweden). The preliminary moulding of PLCL granules was done between the polytetrafluoroethylene (PTFE) – taped moulds at 10 MPa, 110 °C for 90 s. The final moulding was done at the 20 MPa, 130 °C for 30 s between the plain steel moulds. The produced smooth (s) PLCL membranes (Figure 8) had a final thickness of 150 μm (I) or 190 μm (II). The primary moulding of textured (t) PLCL (Figure 8) was done as with sPLCL. Followed by a final moulding between PTFE-taped moulds at 20 MPa, 130 °C for 45 s. The tPLCL membranes used in study II had a final thickness of 120 μm.

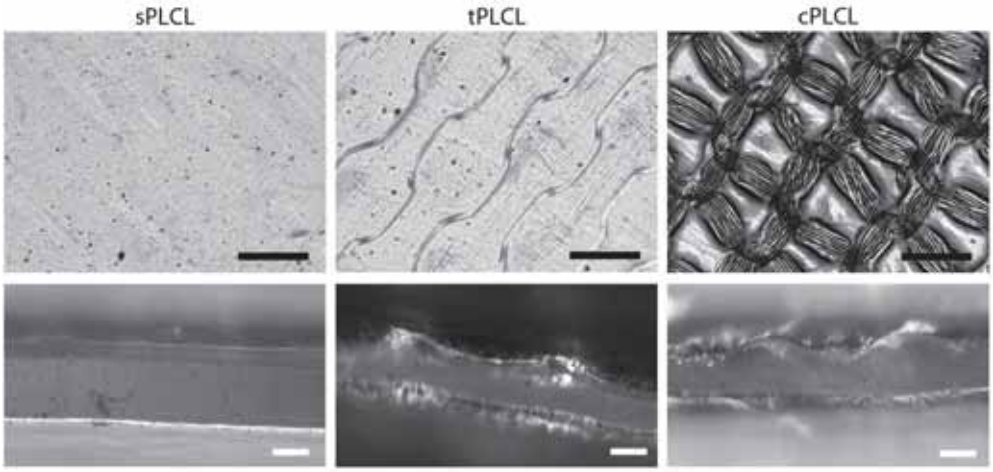

Figure 8. The illustration of sPLCL, tPLCL and cPLCL membrane surfaces (upper images) and cross section (lower images). The black scale bar is 500 μm and the white scale bar is 100 μm.

For composite (c) PLCL membranes (Figure 8, II) the tubular single jersey knitting made of PLDLA was produced with a circular knitting machine (ELHA R-1s, Textilmaschinenfabrik Harry Lucas GmbH & co. KG, Neumünster, Germany) from 16-filament fibres with a filament thickness of 10-20 μm.

Figure 9. The knitted PLDLA mesh strained into the frame.

The knitted PLDLA (Figure 9) was strained into the frame and compression moulded at 10 MPa, 95 °C for 60 s. After, the PLDLA mesh and preliminary moulded PLCL were compression moulded together at 10 MPa, 95 °C for 60 s to obtain a composite membrane with a final thickness of 150 μm. All the manufactured membranes were cut in to samples and washed with ethanol (Etax Aa, Altia Oyj, Rajamäki, Finland). The gamma sterilization with 25 kGy was used to sterilize the manufactured samples prior to further characterization or *in vitro* cell experiments.

4.3 Sample characterization

4.3.1 Inherent viscosity and glass transition temperature (I, II)

The inherent viscosity (IV) measurements (I) were done with Lauda PVS viscometer (Lauda Dr R., Wobser GmbH&Co, Königshofen, Germany). The samples were dissoluted into 1 mg/ml chloroform solution (Sigma-Aldrich) and the capillary viscometer was used to determine the IV.

The glass transition temperature (Tg) was determined to raw 70/30 PLCL (I, II) and to manufactured samples after the gamma sterilization (I, II) and after hydrolysis (II) using a differential scanning calorimeter (DSC Q 1000; TA Instruments, New Castle, DE, USA).

4.3.2 Hydrolysis study (II)

During the study II hydrolysis of the samples was performed in a phosphate buffer solution (pH 6.1) at 37 °C to mimic the pH of urine. The buffer volume was above the required 10 ml for each sample, according to the International Standard, ISO 15814, 1999. The samples weighed approximately 100 mg and were 10 x 50 mm in size. The samples (n=6) were incubated in a buffer solution for 0, 2, 4, 6, 8, 10, and 12 weeks. The pH was measured weekly with a SevenMulti pH meter (Mettler-Toledo GmbH, Schwerzenbach, Switzerland) and the buffer solution was changed every 2 weeks. After incubation, the samples were weighed wet and then tensile tested. The samples were dried in a fume chamber for a week following vacuum drying for a week at room temperature, which after the dried samples were weighed again.

4.3.3 Tensile test

The tensile testing (n=6) was performed with dry samples prior to hydrolysis (0 week samples) and after hydrolysis for wet samples in the study II. The Instron 4411 materials testing machine (Instron Ltd, High Wycombe, UK) was used for tensile testing at a cross-head speed of 30 mm/min. The sheep bladder samples (n=6) were used as a control material for tensile testing. Before testing, the sheep bladder was washed with 0.9 % NaCl and cut into 10 x 50 mm pieces.

4.4 Cell isolation and culture

4.4.1 Urothelial cells (I, II)

The human urothelial tissue was obtained from the elective surgery of dilated ureters from child patients (details in Table 1) in Tampere University Hospital with the approval of the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (R071609).

Table 1. The general information of the tissue donors. The anatomical place and indication are referring to the anatomical origin of the tissue sample and reason for the surgical operation.

Donor	Study	Passage	Age	Sex	Anatomical place	Indication
1	I	3	4 months	Female	Ureter	Obstructive megaureter
2	I	3	6 years 1 month	Female	Ureter	Urethral atresia and broad distal ureter
3	I	2-3	8 years	Male	Ureter	Not known
4	I,II	4	1 years	Male	Ureter	Vesicourethral reflux
5	II	3	11 years 11 months	Male	Ureter	Pyonefros
6	II	3	4 years 4 months	Male	Ureter	Wilms tumor
7	II	3	7 years 1 month	Male	Renal pelvis	Pyelourethral obstruction

The hUCs were isolated according to the protocol demonstrated by Southgate *et al*. with minor modifications (Southgate *et al.* 2002). Briefly, the tissue samples (Figure 10) were cleaned, cut into smaller pieces and incubated over night in a stripping solution containing 0.01 % buffer 1 M, N'-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES; Sigma-Aldrich), 4 x 10^-3 % aprotin (1 kIU/μl, Sigma-Aldrich), 0.1 % ethylene diamine tetra-acetic acid (EDTA; Sigma-Aldrich) and 0.01 % penicillin/streptomycin (P/S, Lonza, Verviers, Belgium) in Hank's balanced salt solution (HBSS without Ca2+ and Mg2+, Life Technologies) to loosen the urothelial layer. The following day, the urothelial sheets were separated from the tissue samples and incubated in 0.1 % trypsin (Lonza) to detach the hUCs. To inactivate the trypsin, 10 % human serum type AB (HS, PAA laboratories, Pasching, Austria) in HBSS was used. After centrifugation, the resulting pellet was suspended in a defined urothelium medium (EpiLife, Life Technologies) and cultured in CellBIND T75 flasks (Sigma-Aldrich) at 37 °C in a humified athmosphere of 5 % CO2 in air. Overall, the hUCs from 7 donors at passages 2-4 were used in the studies I and II.

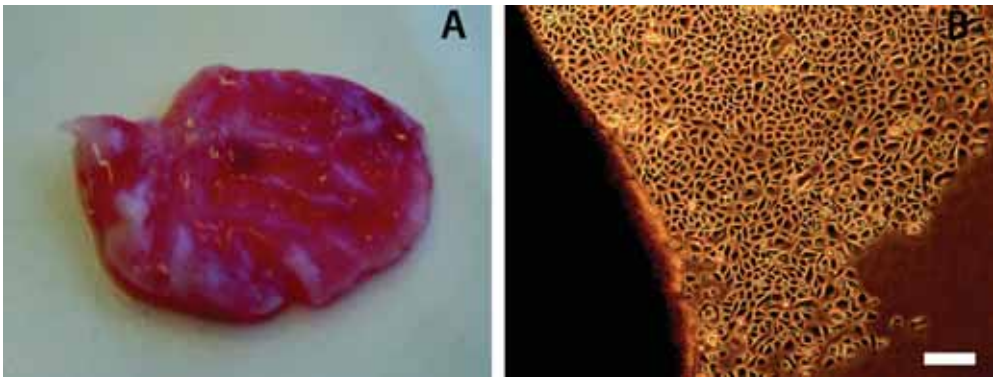

Figure 10. The tissue sample and the isolated hUCs. The left figure illustrates the ureter, which is cut open, and the urothelium appears at the surface of the tissue piece. The right image representing the freashly isolated hUCs proliferating from the small tissue piece. Scalebar 200 μm.

Prior to cell seeding, the biomaterial samples, sPLCL (I and II), hAM (I), tPLCL (II) and cPLCL (II) were attached to the biomaterial holders (CellCrown 24 (I) or 48 (II); Scaffdex, Tampere, Finland). The samples with biomaterial holders were placed to the wells of cell culture plates (Corning® CellBIND® , Sigma-Aldrich) and preincubated in EpiLife at 37 °C for 48 h to improve the cell attachment. In the study I, 20 000 cells were seeded onto each membrane, previously supplied with 1 ml of EpiLife medium. For the study II, the cells were seeded in a medium volume of 30 μl with a density of 30 000 cells/cm^2 and the after 2 h attachment 0.4 ml of urothelium medium was added. The hUCs were cultured at 37 °C until subsequent characterization and *in vitro* experiments.

4.4.2 Adipose stem cells (III)

The ASCs were isolated from 0.3-0.5 dl of subcutaneus fat collected from lower abdomen under local anesthesia from 6 female patiens (age 59.2 ± 13.8 years, n=5, details in Table 4; page 67). These patients also later received the injection threatment of clinical pilot study. Additionally, 50 ml of autologous serum was obtained. The cell isolation and expansion was performed in good manufacturing practice (GMP) class clean rooms in BioMediTech, University of Tampere according to the Standard Operation Procedures with the approval of Pirkanmaa Hospital distric Ethics Committee (R09179) as described previously (Mesimaki *et al.* 2009, Sándor *et al.* 2013).

Briefly, the adipose tissue was minced in to small pieces and digested with collagenase NB-6 (Life Technologies; GMP grade; SERVA Electrophoresis GmbH, Heidenlberg, Germany) in 37 °C incubator for 60 min while mixing every 20 min. After centrifuging and lysing the red blood cells, the pellet was suspended in to the medium containing 15 % of autologous serum in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12, Life Technologies). The isolated cells were expanded for 3-4 weeks. When confluent, the cells were mechanically detached with cell scraper (Nunc, Life

Technologies) and passaged. For the injection therapy, the ASCs were at passage 3-4. Prior to the injection therapies, the ASC underwent chromosome, sterility, endotoxin, and mycoplasma testing. Further, the viability of ASCs was confirmed with live/dead staining before the injections to the patient.

For the following *in vitro* experiments the cells were further expanded in BM containing DMEM/F12, 15 % of HS (Lonza), and 1 % of GlutaMAX (Life Technologies).

4.5 Flow cytometric analysis (I, II, III)

The hUCs (n=4-7, passage 3-4; **I, II**) were analyzed after primary culture with a fluorescence-activated cell sorter (FACSAria; BD Biosciences, Erembodegem, Belgium). Monoclonal antibodies (MAb) against CD44-PE, CD73-PE, CD105-PE, CD133-PE (ALCAM), CD166-PE (EpCAM) (BD Bioscience), CD326-APC (Miltenyi Biotech, Bergisch Gadbach, Germany), and keratin8/18 (Cell Signaling Technology, Dancers, MA, USA) were used. Keratin 8/18 was conjugated with IgG-alexa488 (Life Technologies; Table 2).

Furthermore, the ASCs (n=5, passage 5-6) used for patient study were analyzed with FACS. MAbs against CD14-PECy7 (BD Biosciences), CD19-PECy5 (BD Biosciences), CD34-APC (Immunotools GmbH, Friesoythe, Germany), CD45-PE, CD49d-PE, CD73-PE (BD Biosciences), CD90- PE, CD105-PE, MHC class I isotype (HLA-ABC) PE, and MCH class II isotype (HLA-DR) PE (Immunotools) were used to characterize the ASCs in III study.

Table 2. A list of used flow cytometric markers in all publications.

Marker	Label	Antigen name	Study
CD14	PE-Cy7	Serum lipopolysaccharide binding protein	III
CD19	PE-Cy5	B lymphocyte-lineage differentiation antigen	III
CD34	APC	Sialomucin-like adhesion molecule	I, II
CD44	PE	Extracellular matrix receptor III	III
CD45	PE	Leukocyte common antigen	III
CD49d	PE	Integrin α2, VLA-4	I, II, III
CD73	PE	Ecto-5'-nucleotidase	III
CD90	PE	Thy-1	I, II, III
CD105	PE	SH-2, endoglin	I, II
CD133	PE	AC133	I, II
CD166	PE	ALCAM	I, II
CD326	APC	EpCAM	I, II
Keratin 8/18	IgG-alexa488	-	III
HLA-ABC	PE	Major histocompatibility class I antigen	III
HLA-DR	PE	Major histocompatibility class II antigen	III

CD, Cluster of differentiation; PE, Phycoerythin; Cy, Cyanine; APC, Allophycocyanin; VLA-4, Very late antigen 4; Thy-1, T cell surface glycoprotein; ALCAM, Activated leukocyte cell adhesion molecule; EpCAM, Epithelial cell adhesion molecule.

The analysis was performed for 10 000 cells per sample and the background autofluorescence level was compensated with unstained control samples.

4.6 The scanning electron microscopy imaging (II)

The hUCs cultured on biomaterials for 2 h, 7 d or 14 d were analyzed with scanning electron microscopy (SEM) to assess the attachment and morphology. The samples were carefully washed with DPBS and fixed for 48 h at room temperature with 5 %

glutaraldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.4, Sigma-Aldrich). The samples were dehydrated through a sequence of increasing concentrations of ethanol, followed by a critical point drying with liquid CO2. The samples were coated with gold sputtering and examined with SEM device (Jeol JSM 6335F; Jeol Ltd, Japan). The SEM imaging was repeated twice with hUCs from 2 donors.

4.7 Live/dead staining (I, II, III)

The viability of cells was evaluated with live/dead fluorescence staining at 3 (I), 7 (I, II), and 14 (I, II) d time points for hUCs and for ASCs prior to injection therapy (III) after blending the cells with ContigenTM gel. Briefly, the cells were incubated in a mixture of calcein AM (green fluorescence; Life Technologies) and ethidium homodimer-1 (red fluorescence; EthD-1; Life Technologies) in DPBS at room temperature. The cells were imaged with fluorescent microscope (Olympus IX51S8F-2; Camera DP71), the green fluorescence indicating the viable cells and the red fluorescence the dead cells.

4.8 WST-1 proliferation assay (I, II)

The proliferation of hUCs on different biomaterial membranes was assessed using PreMix WST-1 Cell Proliferation Assay System (Takara Bio Inc., Otsu, Shiga, Japan), measuring the mitochondrial activity of viable hUCs after 2 h (II), 3 (I), 7 (I, II), and 14 (I, II) d of cell culture. Briefly, after careful washing, the cells were incubated with 50 μl of premix WST-1 and 500 μl of DPBS at 37 °C for 1 h (II) or 4 h (I). The microplate reader (Victor 1420 Multilabel Counter, Wallac, Turku, Finland) was used to measure the absorbanse at 450 nm.

4.9 The phenotype characterization of human urothelial cells (I, II)

The phenotype of hUCs on biomaterial samples was evaluated with immunostaining, using CK7 (II; 1:400; Epitomics, CA, USA), CK7/8 (I; Lab Vision, Fremont, CA, USA), CK19 Ab-1 (I, II; Lab Vision) and uroplakin III (UPIII; I, Santa Cruz Biotechnology, Heidelberg, Germany) as primary antibodies (Table 3) at 7 and 14 d time points.

Table 3. Detailed information of primary antibodies and secondary antibodies.

Antibody	Study	Origin	Dilution	Secondary antibody and dilution
CK7	II	Rabbit	1/200	Donkey, anti-rabbit, IgG, alexa 488, 1/400
CK7/8	I	Mouse	1/200	Goat, anti-mouse, IgG, alexa 488, 1/400
CK19	I, II	Mouse	1/500	Donkey, anti-mouse, alexa 488, 1/400
UPIII	I	Goat	1/200	Donkey, anti-goat, IgG, alexa488, 1/400

CK, Cytokeratin; UP, Uroplakin.

The hUCs were fixed with 4 % paraformaldehyde fixative (PFA; Sigma-Aldrich) and incubated in primary antibody dilutions. The following day, the secondary antibodies (Table 3; Life Technologies) were conjugated to primary antibodies and the cell nuclei stained with Vectashield (DAPI; blue fluorescence, Vector Laboratories, Peterborough, UK). Finally, the cells were imaged with fluorescence microscope (Olympus IX51S8F-2; Camera DP71).

4.10 Differentiation analyses (III)

The myogenic, adipogenic, osteogenic and chondrogenic differentiation potiential of ASCs was verified after 14 d of cell

culture in differentiation conditions. The BM supplemented with antibiotics (P/S; Life Technologies) was used as a control.

4.10.1 Myogenic differentiation

To induce myogenesis, ASCs were plated onto fibronectin (Sanquin, The Netherlands) coated wells with a density of approximately 2.6x10^^{3} cells/cm^2. The cells were cultured in a myocenic medium containing BM supplemented with 5 ng/ml of transforming growth factor beta 1 (TGFβ1; hBA-112, Santa Gruz Biotechnology). The antibodies SM22-α (1:100; Abcam, Cambridge, UK), α-SMA (1:100; Abcam) and MHCII (1:200; Thermo Scientific, Illinois, USA) were used to demonstrate the myogenic differentiation of ASCs after 14 d. Briefly, the cells were fixed with 4 % PFA, incubated in primary antibody dilutions and thereafter, conjugated with secondary antibodies from goat, donkey, and donkey (1:200; Life Technologies), respectively. The ASCs were mounted with Vectashield (blue fluorescence) to stain the cell nuclei and imaged with fluorescent microscope (Olympus IX51S8F-2; Camera DP71).

4.10.2 Adipogenic differentiation

For adipogenic differentiation was done as described previously (Sándor *et al.* 2013), the ASCs were plated at a density of 2x10^^{4} cells/cm^2 in BM. The following day, adipogenesis was induced with adipogenic medium containing 10 % HS (Lonza), 1 % GlutaMax (Life Technologies), 1 % P/S (Lonza), 1 μM dexamethasone (Sigma-Aldrich), 100 nM insulin (Life Technologies), 17 μM pantothenate, 33 μM biotin (Sigma-Aldrich) in DMEM/F12 and 0.25 μM isobutylmethylxanthine (IBMX; Sigma-Aldrich) for 24 h. After 24 h, the adipogenic differentiation was continued in apipogenic medium without IBMX.

After the 14 d of cell culture, the adipogenic differentiation was detected with Oil Red O staining (Sigma-Aldrich) demonstrating

the intracellular lipid accumulation. Briefly, the cells were washed with DPBS, fixed with 4 % PFA for 1 h and rinsed with Milli-Q water (Millipore/Merck). Thereafter, the cells were incubated in 60 % isopropanol (Merck, Frankfurt, Germany) for 2-5 min followed by incubation in Oil Red O solution for 15 min at room temperature. Finally, the cells were washed with Milli-Q water and imaged with light microsope (Nikon eclipse TE2000-S, Tokyo, Japan).

4.10.3 Osteogenic differentiation

For osteogenesis, performed as previously described by Sandor *et al.* (Sándor *et al.* 2013), the ASCs were plated at the density of 2.5x10^3 cells/cm^2. After 24 h of incubation in BM, the osteogenesis was initiated using osteogenic medium containing DMEM/F-12 supplemented with 10 % HS (Lonza), 50 μM L-ascorbic acid 2-phosphate, 10 mM β-glyserophosphate (Sigma-Aldrich), 100 nM of dexamethasone (Sigma-Aldrich), 1 % GlutaMax and 1 % P/S.

The osteogenic potential of ASCs was confirmed with alkaline phosphatase (ALP) staining. Briefly, after the osteogenic differentiation the cells were fixed with 4 % PFA. Subsequently, the cells were rinsed with Milli-Q water and incubated in ALP staining kit (Sigma-Aldrich) for 15 min protected from light. Finally, the cells were rinsed and imaged with light microscope (Nikon).

4.10.4 Chondrogenic differentiation

To induce the chondrogenic differentiation the cells were cultured with a micromass culture method as described previously (Mesimaki *et al.* 2009, Zuk *et al.* 2001). The 1x10^5 ASCs were plated as a droplets onto the cell culture well in a medium volume of 10 μl. After 3 h of cell adhesion in the incubator, the chondrogenic medium containing 1 % ITS+1 (Sigma-Aldrich), 50 μM of L-ascorbic acid 2-phosphate (Sigma-Aldrich), 55 μM of

natriumpyruvate (Lonza), 23 μM of L-proline (Sigma-Aldrich), 1 % of GlutaMax, 0.3 % of P/S in DMEM/F12 was added.

After the chondrogenic differentiation for 14 d, the micromasses were fixed with 4 % PFA, embedded in paraffin and cut into 5 μm thickness. The chondrogenic potential of ASCs was evaluated using histological Alcian blue (Sigma-Aldrich) staining demonstrating the sulphated proteoglycans being substantial in cartilaginous matrices followed by counterstaining with Nuclear Fast Red (Biocare Medical, CA, USA). The samples were imaged with microscope (Zeiss Axio, AxioCam MRc5, Jena, Germany)

4.11 Clinical pilot study (III)

We treated 5 incontinent female patients (59.2 ± 13.8 years, Table 4) with the injection of ASCs mixed with Contigen™ or saline. The study was approved by The Ethics Committee of Pirkanmaa Hospital District (R09179) and the patients were enrolled from the Tampere University Hospital outpatient clinic. These patients did not primarily want the mid-urethral sling operation and 2 patients were operated before with unfavourable results. For 2 patients, the primary diagnosis was SUI and for 3 patients MUI according to the anamnesis, a positive cough test and urodynamic evaluations. The patients were followed 3, 6 and 12 months after the treatment.

Table 4. Detailed information of patients treated with transurethral injections.

Patient	Age	BMI	Previous incontinence operation	Incontinence type	MUCP (mmHg)	24h pad test (g)
Patient 1	50	27		SUI	57	0*
Patient 2	61	25	TVT -03	MUI	21	5
Patient 3	59	41		MUI	38	10
Patient 4	45	27		SUI	72	28
Patient 5	81	31	TVT-O -07, Bulkamid -09, TVT -10	MUI	20	70

*, No exercise at the time of testing due to the hip problem. BMI, Body mass index; MUCP, Maximal urethral closure pressure; MUI, Mixed urinary incontinence; SUI, Stress urinary incontinence; TVT, Tension-free vaginal tape; TVT-O, Trans-obturator tabe.

After cell isolation and expansion ASCs were mechanically detached and the amount of cells used for injections varied from 2.5x10^6 to 8.5x10^6 depending on the patient (Figure 11). The half of the ASCs were mixed with 2.1 ml of Contigen™ and the other half with 0.9 % NaCl. The mixture of ASCs and Contigen™, volume being 2.4-4 ml per patient, was injected transurethrally through cytoscope under the mucosa 1.5 cm distal from the urethral neck at 3 and 9 o'clock. In addition, 2 injections with the blend of ASCs and NaCl were done.

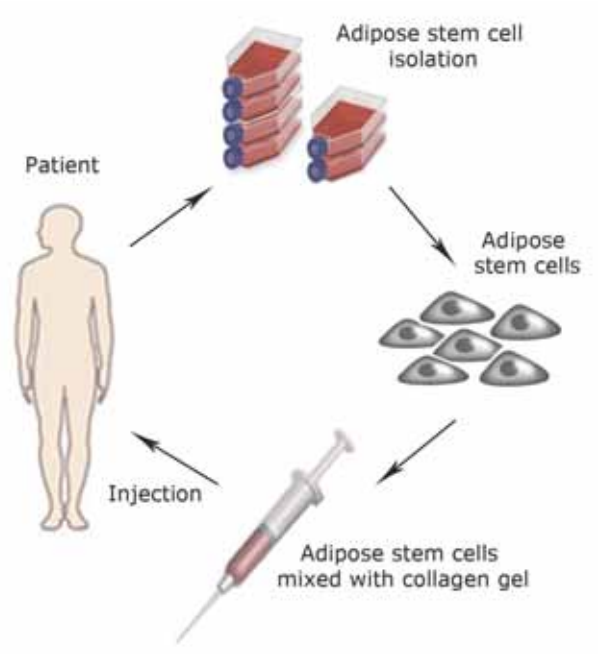

Figure 11. Schematic illustration of ASCs based injection therapy to treat female incontinence.

Prior to the injection therapies and at each following point the gynaecological examination was performed. The patients filled the validated questionnaires; urinary inventory stress test (UISS), detrusor instability score (DIS), incontinence impact questionnaire-short form (IIQ-7), urogenital distress inventory-short form (UDI-6), and the visual analogue scale (VAS) to demonstrate the bother of incontinence. Additionally, vaginal ultrasonography, cough test and 24-h pad test were done at each following point. The urodynamic evaluations were done after 6 moths of the injection treatment. The cough test was defined as primary outcome measure.

4.12 Statistical analyses (I, II)

The statistical analyses were performed with SPSS, v. 13 (SPSS, Chicago, IL, USA). The one-way analysis of variance (ANOVA) was

used to study the effect of the different materials and the culturing periods after verifying normal distribution and homogeneity of the variance. The significant differences between the different biomaterials and culturing periods (I: 3 versus 7 days and 7 versus 14 days, II: 2 h versus 7 days and 7 versus 14 days) were studied using post hoc tests. The data was reported as the mean ± standard deviation (SD), $p < 0.05$ vas considered significant and $p < 0.01$ highly significant.

5 Results

5.1 Material characterization

5.1.1 Inherent viscosity and thermal properties

There were minor changes in the PLCL properties after processing and sterilization. The IV values (I) decreased from 1.58 to 1.03 dl/g indicating that there was some polymer degradation during the membrane manufacturing.

After the sample manufacturing and sterilization there was a minor change in Tg value, decreasing from 18.1 °C to 15.7 °C (I). In the study II, the Tg values of different samples were merely identical. After the sterilization a minor decrease from 23 °C to 22 °C was detected. Furthermore, during the hydrolysis the Tg values decreased constantly and after 6 wk the Tg values were dropped to 20 °C. After the 6 wk the Tg values decreased more rapidly being approximately 16 °C after 12 wk of hydrolysis.

5.1.2 Hydrolysis study (II)

The dimensional stability, weight change and mechanical properties were studied during hydrolysis study at the 0, 2, 4, 6, 8, 10, and 12 wk timepoints (Figure 12). Through the hydrolysis, the pH values remained within the limits (6.05-6.15) given in the standard.

Moderate changes in the samples dimensional stability could be detected; mostly increase or decrease of thickness. The sPLCL, tPLCL, and cPLCL demonstrated an increase of 7 %, 11 %, and 14 % in their thickness after 8 wk hydrolysis. Thereafter, the thickness began to decrease approaching their original values

after 12 wk of hydrolysis. The structural stability of the samples was retained during the hydrolysis, however, after the 10 wk of hydrolysis the sPLCL and tPLCL became fragile to handle and after 12 wks the samples were substantially degraded. The cPLCL maintained the structural stability the best due to the fact that the knitted PLA mesh remained stable through the 12 wk hydrolysis. However, after the 12 wk hydrolysis the PLCL component was fragmented between the PLA mesh loops.

After 6 wk of hydrolysis the weight of tPLCL began to decrease, while the weight of sPLCL and cPLCL began to decrease after 8 wk. After 12 wk of hydrolysis, the weight of the sPLCL, tPLCL, and cPLCL decreased 17.3 %, 41.5 %, and 29.2 %, respectively.

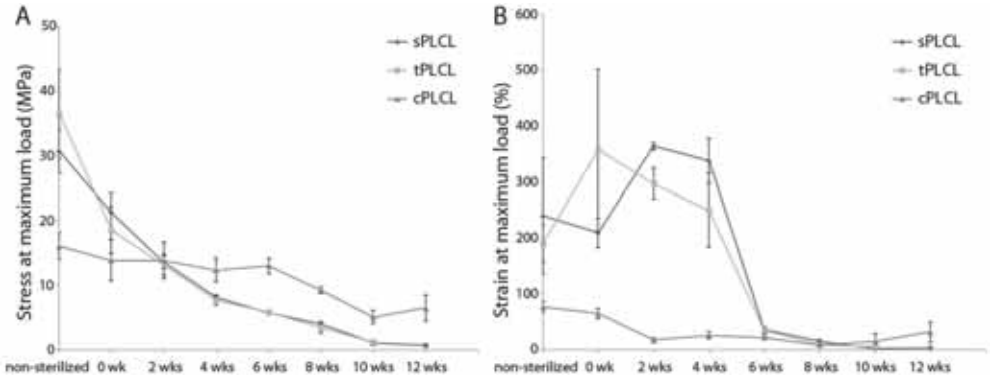

Figure 12. The mechanical properties of samples during hydrolysis. A. Stress at maximum load (MPa). B. Strain at maximum load (%). The tPLCL was not tested after 10 wks of hydrolysis due to the degree of degradation.

There was a decrease in the mechanical properties after sterilization. The sPLCL and cPLCL were affected the most (Figure 12). After the sterilization, the stresses at maximum loads were 21.3, 18.6, and 13.9 MPa for sPLCL, tPLCL, and cPLCL, respectively. The stress at the maximum load remained constant with cPLCL until the 6 wk, therefore, the value started to decrease being 5.1 MPa after 10 wk of hydrolysis. With the sPLCL and tPLCL there was a constant decrease in the stresses at the

maximum loads during the hydrolysis and after 6 wks the stress values were reduced to 5.7 MPa for sPLCL and to 5.9 for tPLCL. The stress values for sheep bladders at maximum load were 0.16 ± 0.03 MPa.

After the sterilization, the strain values for sPLCL and tPLCL were 200 % to 350 %. Interestingly, after 4 wk of hydrolysis the strain values started to decrease, being approximately 35 % after 6 wks. The strain value for cPLCL after the sterilization was 70 %, after which it decreased to 20 % at 2 wk time point and maintaining the 20 % strain value through the 12 wks hydrolysis study. For the control sheep bladder, the strain value at maximum load was 85.1 ± 32.7 %.

5.2 Surface marker expression of hUCs (I, II) and ASCs (III)

The surface marker expression profile of hUCs was verified before the cell seeding onto biomaterials. In I and II study, the population of isolated hUCs was shown to be homologous according to the cell size and surface complexity demonstrated by FACS analysis. The hUCs were shown to express the extracellular matrix adhesion molecule CD44, endothelial molecules CD 73 and CD105, and molecules CD133, CD166, and CD326, which are also detected in epithelial cells. Moreover, the isolated hUCs expressed the intracellular molecule keratin 8/18 being a specific marker for epithelial cells (Figure 13).

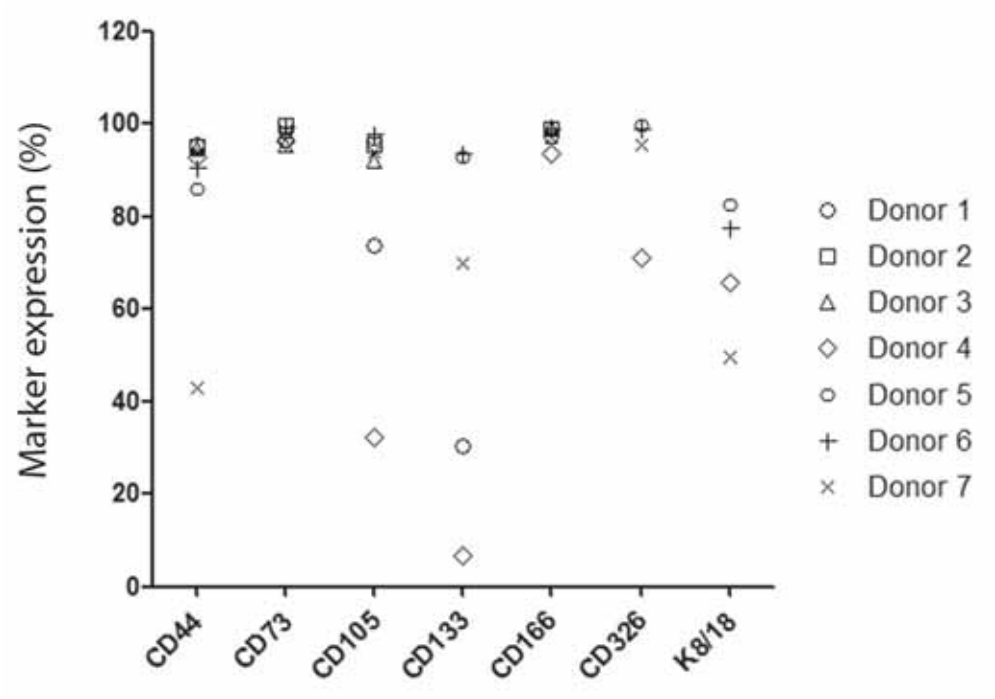

Figure 13. The figure is representing the percentual amount of hUCs expressing the different cluster of differentiation (CD) markers. The last column in each marker is indicating the average expression ± standard deviation.

In the study III, the ASCs were demonstrated to express (Figure 14) adhesion molecules CD49d, CD73 and CD105; extracellular matrix molecule CD90; and cell surface molecule HLA-ABC. The ASCs expression of molecules CD14, CD19, CD34, CD45, and HLA-DR was considered as minor to moderate indicating a low amount of angiogenic or hematopoietic cell lineages.

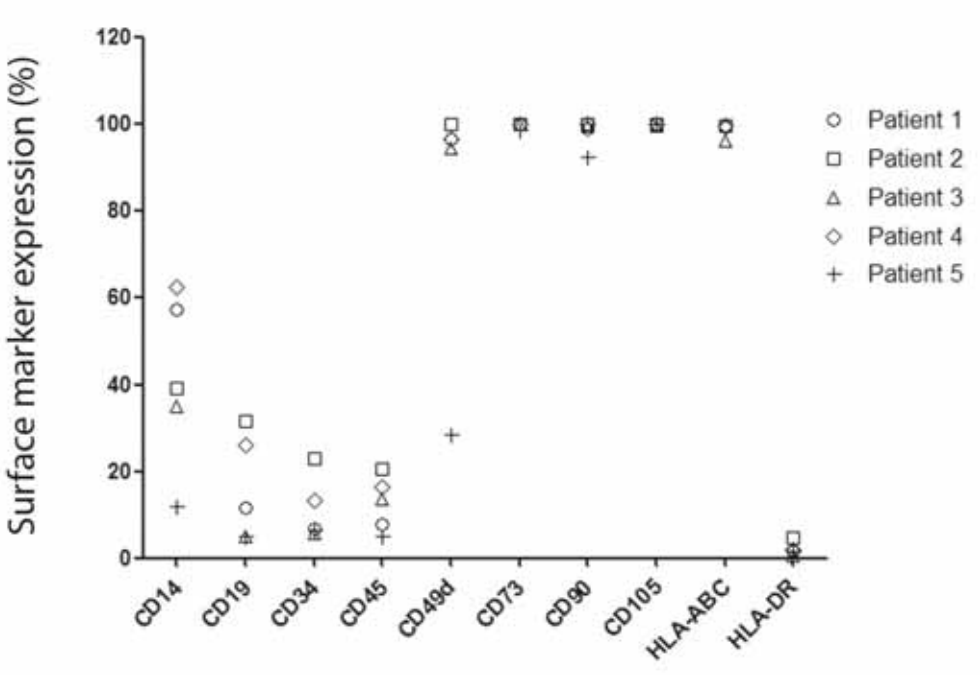

Figure 14. The percentual amount of ASCs expressing the different CD markers. The last column in each marker is indicating the average expression ± standard deviation

5.3 The viability of hUCs (I, II) and ASCs (III)

The viability of hUCs was determined after 3 d (I study), 7 d (I and II study), and 14 d (I and II study) of cell culture (Figure 15). In the study I, the hUCs were viable on the hAM, sPLCL and PS after 3 d, furter, the amount of dead cells was minor. On the sPLCL and PS the majority of cells were viable after 7 and 14 d even though more dead cells were detected on PS compared to PLCL. Interestingly, on the hAM tha amount of dead cells increased over the culturing period and at the 14 d time point the majority of the hUCs were dead.

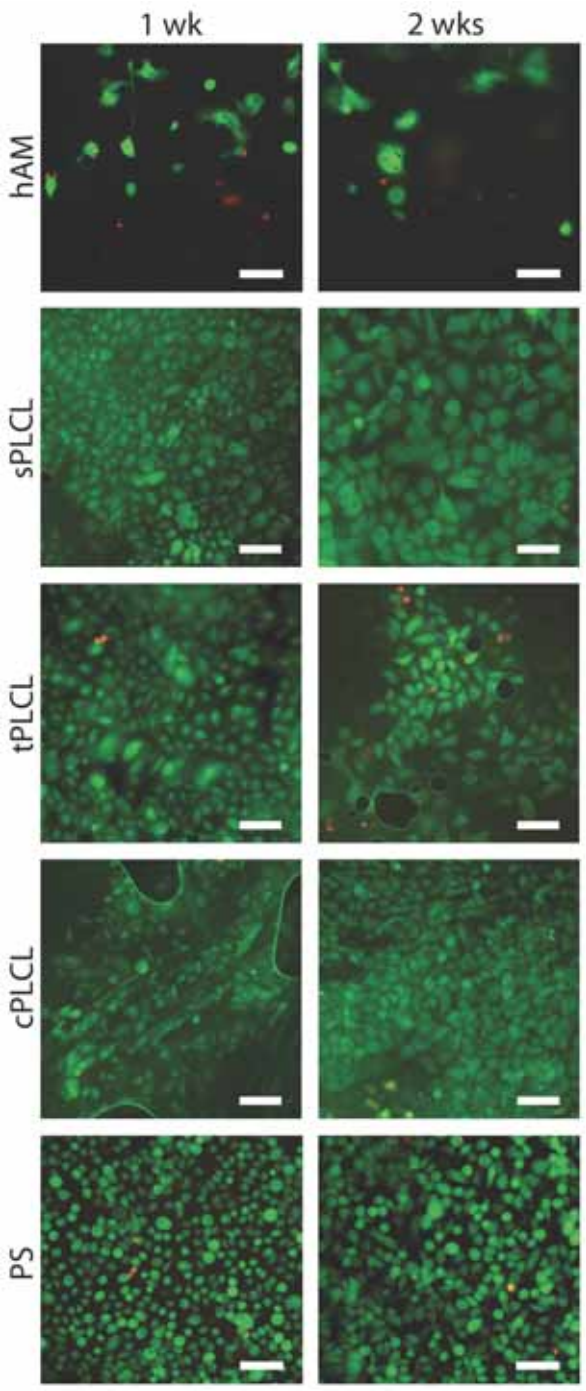

Figure 15. The representative images depicting viable (green) and dead (red) hUCs cultured on hAM, sPLCL, tPLCL, cPLCL and PS after 7 and 14 d of cell culture. Scale bar 100 μm.

The II study showed that the hUCs were viable after 7 d of cell culture on sPLCL, tPLCL, and cPLCL. The number of dead cells was indistinguishable. Moreover, the cells sustained the viability during the 14 d culturing period and there was no detectable increase in dead cell amount on any studied biomaterials.

The ASCs viability (study III) was verified before the injection therapy after the ASCs were mixed with Contigen™ gel (Figure 16). The live/dead staining demonstrated the majority of the cells were viable and the amount of the dead cells in the blend was minor.

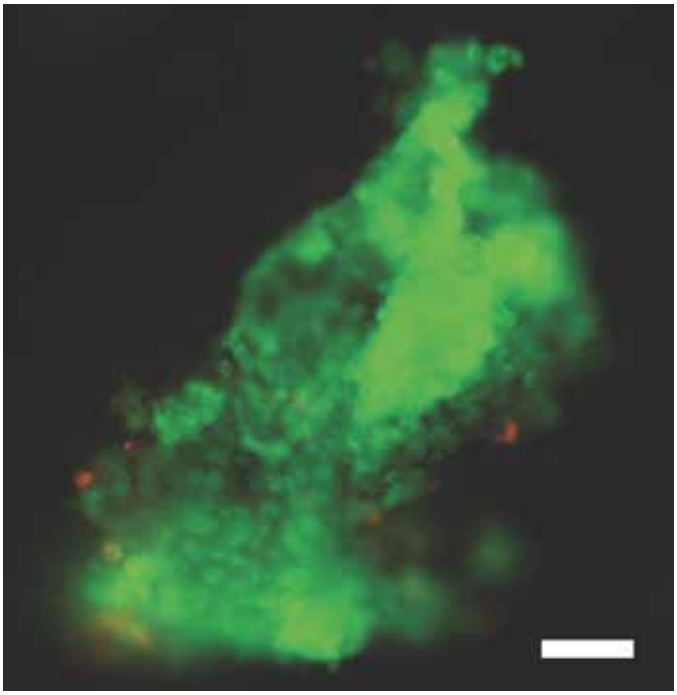

Figure 16. The viability of ASCs was on Contigen™ prior to the injection therapy. Scale bar 200 μm.

5.4 The hUCs attachment and morphology (II)

In the study II, the SEM imaging was used to evaluate the attachment and morphology of the hUCs (Figure 17). After 2 h of initial cell attachment, the hUCs attachment was lower on the PLA fibre regions compared to the PLCL regions on the cPLCL. On the other hand, there were no substantial differences in the hUCs attachment between the sPLCL and tPLCL.

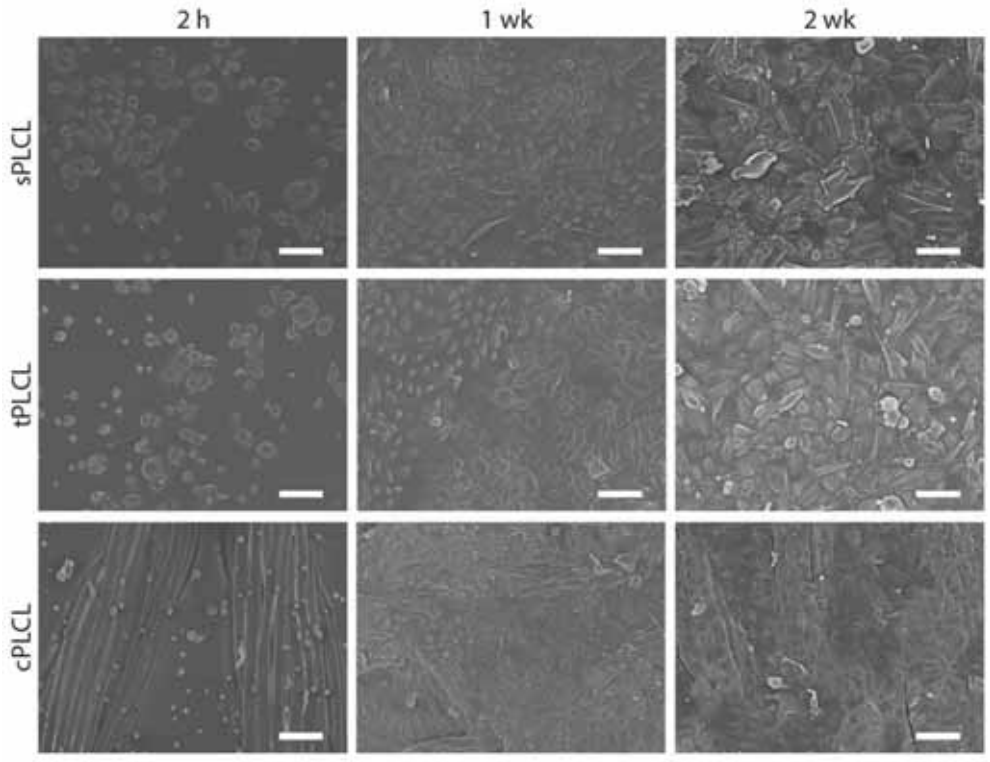

Figure 17. The SEM images illustrating the attachment and morphology of hUCs on sPLCL, tPLCL and cPLCL at 2 h, 7 d and 14 d time points. Scale bar 100 μm.

At the 7 d timepoint the formation of hUCs cluster onto studied biomaterials could be detected with SEM imaging. There were no substantial differences in the cell morphology after 7 d between the sPLCL, tPLCL and cPLCL; the cells were small and oval or roundish. After the 14 d of cell culture, the hUCs on the all studied biomaterials adhered to adjacent cells and formed a confluent cell layer over the whole cell culture area. On the cPLCL, the hUCs distributed more irregularly in the PLA regions compared to PLCL regions. Further, after 14 d the morphology of hUCs was fairly similar on the sPLCL, tPLCL, and cPLCL, varying from roundish or oval to cubic or angular.

5.5 The hUCs proliferation (I, II)

In the study I and II the WST-1 measurement was used to evaluate the cell proliferation at 2 h (II), 3 d (I), 7 d (I,II) and 14 d (I, II) time points (Figure 18). The study I revealed that the hUCs proliferated increasingly with sPLCL and after 14 d the cell amount was significantly higher on sPLCL compared to hAM ($p<0.001$) and PS ($p=0.003$). However, on the PS and hAM the hUCs reached their proliferation maximum at 7 d time point. The hAM supported the proliferation of hUCs poorly and at the 14 d time point the amount of hUCs on hAM was significantly lower compared to other biomaterials.

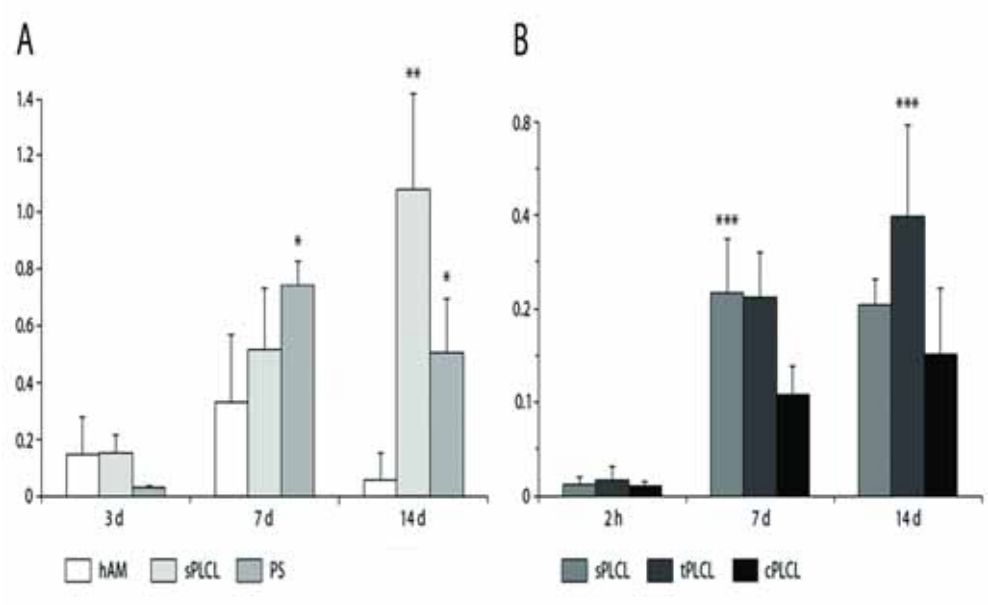

Figure 18. A: The cell number of hUCs (n=5-6) cultured on hAM, sPLCL and PS for 3, 7 and 14 days. Results are expressed as means ± SD. *p < 0.05 with respect to hAM; **p < 0.01 with respect to hAM and PS. B: The number of hUCs cultured on sPLCL, tPLCL and cPLCL after 2 h, 7 d and 14 d of cell culture. The results are expressed as means ± SD. ***p < 0.05 with respect to cPLCL

The proliferation assay demonstrated no significant differences in cell attachment between the sPLCL, tPLCL, and cPLCL at 2 h time point (study II). However, at the 3 d to 7 d assessment period the amount of hUCs increased significantly on all the studied biomaterials. The tPLCL and sPLCL supported the hUCs proliferation superiorly compared to the cPLCL at the 7 and 14 d time points, though, the statistically significant difference was detected after 7 d merely with sPLCL ($p=0.042$ with respect to cPLCL) and after 14 d with tPLCL ($p=0.011$ with respect to cPLCL).

5.6 The phenotype characterization (I,II)

The immunnostaining (Figure 19) with the markers CK7 (II; green fluorescence), CK7/8 (I; green fluorescence), CK19 (I, II; green fluorescence) and UPIII (I) was used to characterize the phenotype of hUCS after 7 and 14 d (I, II) of cell culture.

In the study I, the phenotype of hUCs on sPLCL remained unchanged, whereas, the marker expression on hAM was decreased at 14 d timepoint when compared to 7 d timepoint. The expression both CK7/8 and CK19 on sPLCL on both timepoints was considered as moderate. However, the UPIII was not expressed on any time point or any studied biomaterial. The CK7 and CK19 expression of hUCs in the study II was considered intensive at both 7 d and 14 d time points on sPLCL, tPLCL, and cPLCL. Further, the phenotype of hUCs was maintained during the culture as the intensity of the staining remained from the 7 d to 14 d culturing period.

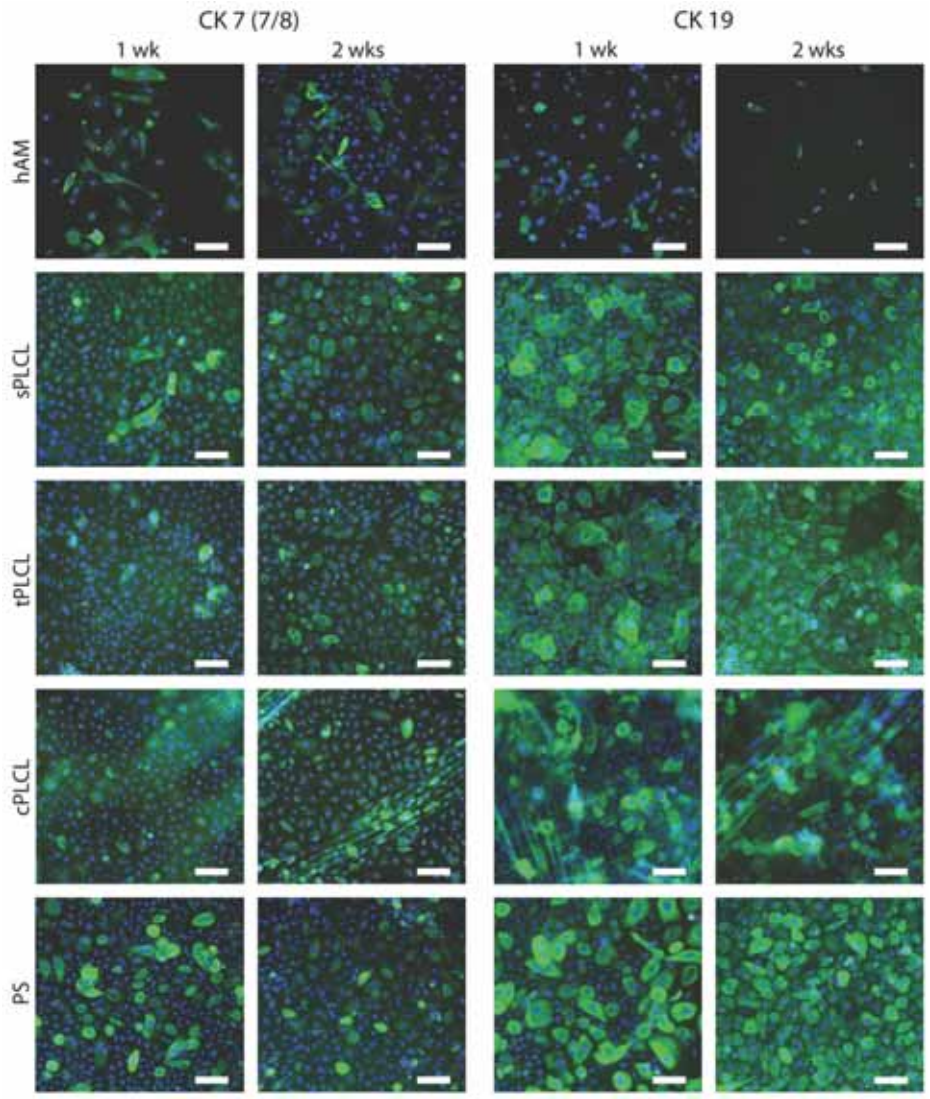

Figure 19. The representative immunofluorescence images of hUCs cultured on hAM, sPLCL, tPLCL and cPLCL at 7 and 14 d time points. The markers CK 7 (or 7/8 with hAM; green) and CK 19 (green) were used to confirm the urothelial phenotype maintenance. The DAPI (blue) was used to stain the cell nuclei. Scale bar 100 μm.

5.7 Differentiation analyses of ASCs (III)

The myogenic, adipogenic, osteogenic, and chondrogenic differentiation potential of ASCs was confirmed to demonstrate the multidifferentiation capacity (Figure 20 and 21). The differentiation analyses were performed after 14 d of cell culture in differentiation conditions. The myogenic potential was confirmed using immunostaining with myogenic markers; SM22-α, α-SMA and MHCII. The expression of all the studied markers was detected, indicating the myogenic potential of ASCs.

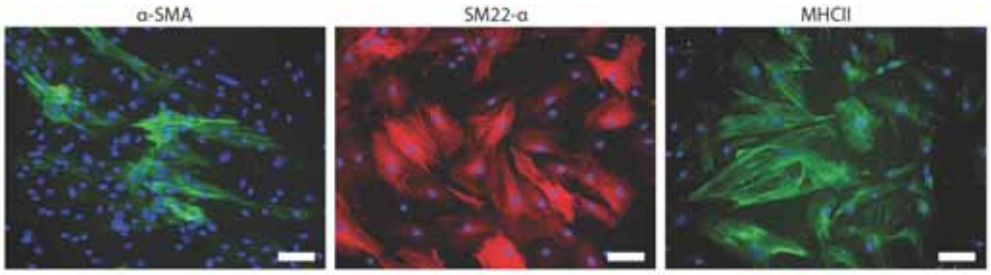

Figure 20. The myogenic potential of ASCs was verified using immunostaining with markers α-SMA (green), SM22-α (red), and MHCII (green). Scale bar 100 μm.

The Oil Red O staining was used to demonstrate the adipogenic capability of ASCs. The staining showed the evident lipid droplets indicating the adipogenic differentiation. Further, the ALP staining was used to indicate the osteogenesis of the induced ASCs. The chondrogenic potential was verified using Alcian blue staining, which indicated the cartilage specific glycosaminoglycans after the chondrogenic differentiation.

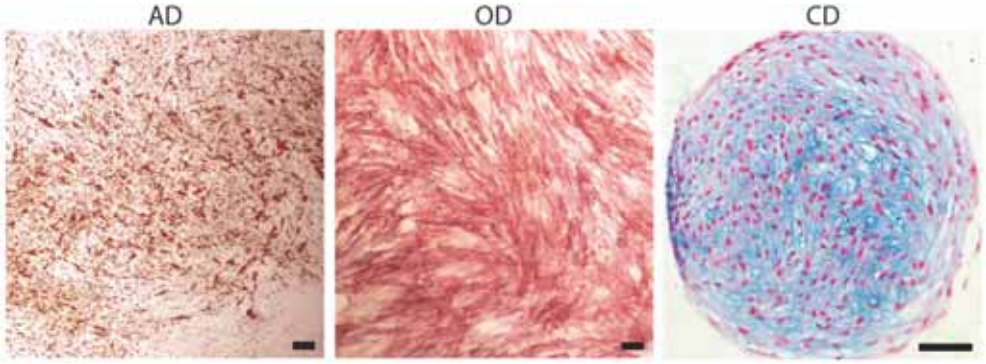

Figure 21. The ASCs were depicted to differentiate towards adipogenic, osteogenic and chondrogenic cell lineages. Adipogenesis (AD) was detected using Oil red O staining, osteogenic differentiation (OD) with ALP staining and chondrogenic differentiation (CD) with Alcian blue staining. Scale bar 150 μm.

5.8 The data of clinical results (III)

The adipose tissue for the clinical study was collected from 6 female patients under local anaesthesia and no adverse effects, with the exception of small haematomas, were detected. However, due to the bacterial contamination, the treatment was refrained from 1 patient and 5 patients were treated with the injection therapy. The patients were followed for one year. After the injections, no major complications for instance urinary retention, heamaturia, or urinary infection, were detected. After 3, 6, and 12 m the follow-up tests were performed. After the 6 months, the cough test, were the bladder was filled with 500 ml of saline, was negative with one patient. After the follow-up period two of the treated patients were satisfied with the treatment. The cough test was negative with three patients; however, one of the patients had symptoms during her daily activities. Additionally, with the objectively cured patients there was also improvement based on the 24 h pad-test.

Table 5. The results of the quality of life questionnaires of patients before and after 1 year follow up of transurethral injections of ASCs+Contigen™.

	UISS		IIQ-7		UDI-6		VAS	
Patient	Preoperative	At 1-year	Preoperative	At 1-year	Preoperative	At 1-year	Preoperative	At 1-year
Patient 1	65	35	48	29	61	17	4	1
Patient 2	50	40	19	29	27	50	5.1	4.7
Patient 3	45	45	5	10	38	33	5.8	5.5
Patient 4	40	10	76	10	44	11	7.5	1
Patient 5	74	18	62	0	72	17	9.4	4.5

ASC, Adipose stem cell; UISS, Urinary inventory stress test; IIQ-7, Incontinence impact questionnaire short form; UDI-6, Urogenital distress inventory short form; VAS, Visual analogue scale.

According to the UISS, IIQ-7, UDI-6 and VAS questionnaires there was improvement with all five treated patients, whereas, the DIS did not illustrate the effect of the treatment (Table 5). Further, the two last treated patients showed an improvement consistently in UISS, IIQ-7, UDI-6 and VAS questionnaires, whereas, the first treated patients showed an improvement merely in part of the questionnaires. After the follow-up period, three of the patients were treated with TVT operation.

6 Discussion

6.1 The cell characterization of urothelial cells and ASCs

The flow cytometric characterization can be used to verify the isolated cell type, which is highly important especially prior to the clinical therapies. The flow cytometry was used to characterize hUCs and ASCs during this thesis. Contrary to hUCs, the ASCs have been extensively characterized with flow cytometry and the expressed markers are known.

To characterize hUCS in the studies I and II we used surface markers CD44, CD 73, CD105 and CD133 and intracellular marker Keratin 8/18 since these markers have previously been used to characterize hUCs (Magnan *et al.* 2006, Zhang *et al.* 2008). The surface markers CD166 and CD326 have not previously been used to characterize hUC, however, the epithelial cells are known to express these surface proteins (Swart 2002, Trzpis *et al.* 2007, Trzpis *et al.* 2008). Though, only few studies have been done to characterize hUCs with flow cytometric method, therefore, the assessment of used marker is difficult. More systematic study is required to determine the markers that are adequate to characterize the hUCs. Our characterization results illustrated that the hUCs expressed all the studied markers, as expected, and the results were mainly parallel with the previous characterization results for hUCs (Chun *et al.* 2012, Magnan *et al.* 2006, Zhang *et al.* 2008). Compared to the previous results of Zhang et al., who studied the urine derived cells, our characterization results for hUCs showed more strong expression of marker CD133. This difference may have been due to the different isolation techniques or differences in patient ages (Zhang *et al.* 2008). The results between patients in our studies were mainly concordant,

indicating the repeatability and reliability of our isolation and characterization methods.

Additionally, in order to be classified as mesenchymal stem cells ASCs need to express specific markers defined by The International Society for Cellular Therapy (Dominici *et al.* 2006).

Table 6. The characterization results for ASCs cultured in autologous serum. In our study (III) the ASCs expressed hematopoietic markers (CD14, CD34 and CD45) more strongly compared to the other studies. Otherwise, no substantial differences in expression levels were detected (Mesimaki *et al.* 2009, Sándor *et al.* 2013, Wolff *et al.* 2013).

Surface Protein	Study III (n=5)	Mesimäki *et al.* 2009 (n=1)	Sandor *et al.* 2013 (n=1)	Wolf *et al.* 2013 (n=3)
CD14	41.2 ± 20.0	-	16	21.5 ± 9.0
CD19	15.9 ± 12.3	-	14	9.9 ± 5.0
CD34	11.1 ± 7.4	0.9	1	1.7 ± 2.1
CD45	12.8 ± 6.3	0.5	2	3.1 ± 1.8
CD49d	83.2 ± 30.6	26.60	99	-
CD73	99.7 ± 0.5	-	100	99.9 ± 0.1
CD90	98.2 ± 3.2	99.5	96	98.5 ± 2.3
CD105	99.9 ± 0.1	99.3	100	99.9 ± 0.2
HLA-ABC	98.8 ± 1.8	-	91	-
HLA-DR	2.0 ± 1.5	-	6	2.7 ± 2.7

ASC, Adipose stem cell; CD, Cluster of differentiation; HLA-ABC, Human leukocyte antigen class I; HLA-DR, Human leukocyte antigen class II.

The characterization results for ASCs (III, Table 6) were parallel with the previously described results for ASCs (Dominici *et al.* 2006, Mesimaki *et al.* 2009, Sándor *et al.* 2013). The ASCs strongly expressed markers CD73, CD90 and CD105, which are

characteristic for mesenchymal stem cells (Dominici *et al.* 2006). Compared to the other expression results done for ASCs cultured in autolgous serum the cells in our study expressed more strongly the hematopoietic cell markers CD14 (41.2±20.0), CD34 (11.1±7.4) and CD45 (12.8±6.3) (Mesimaki *et al.* 2009, Sándor *et al.* 2013, Wolff *et al.* 2013). However, a high patient variation is common with ASCs, which was evident also in our study (Lindroos *et al.* 2010, Lindroos *et al.* 2011). The high variation may be partly due to the high variation of patient ages (Lindroos *et al.* 2011).

6.2 The biomaterial selection for urothelial tissue engineering

The requirements for biomaterial used in urothelial tissue engineering were described in the review of the literature section (see pages 36-38). Generally, the optimal biomaterial should mimic the urethral submucosa as closely as possible. In the study I, the applicability of hAM, sPLCL, and PS were studied for hUCs. The hAM is an acellular matrix containing natural cell recognition sites and similar ECM proteins compared to matrix of natural urothelium, therefore, it has been considered as an interesting biomaterial also for urothelial applications (Niknejad *et al.* 2008). Further, Sharifiaghdas *et al*. compared hAM, human omentum and peritoneal membranes as a material for mouse urothelial cells, concluding that UCs preferred hAM to omentum and peritoneum. Interestingly, UCs growth rate on hAM was low during the first week; however, during the second week UCs proliferated and formed intercellular connections characteristic to urothelium (Sharifiaghdas *et al.* 2007). These results were contradictory to our study, which demonstrated that the hUCs did not grow or proliferate on hAM. The majority of hUCs were dead on hAM (study I) after 14 d culturing period. Moreover, according to the WST-1 measurement, the amount of hUCs started to decrease after 7 days of cell culture on hAM. Additionally, the cell morphology was not characteristic to UCs. The cells were either extremely small or large with cytoplasmic projections. Our

contradictory results compared to Sharifiaghdas *et al*. studies may have been partly due to the used cell source. Sharifiaghdas *et al*. used mouse UCs, whereas, we used hUCs obtained from child patients. Additionally, the decellularization process might also have an effect on the biocompatibility of hAM, since in our study dispase was used to enable the decellularization, whereas, Sharifiaghdas *et al*. used trypsinization (Sharifiaghdas *et al.* 2007, Zhang *et al.* 2013). In the recent study of Zhang *et al*. the dispase and trypsin were shown to affect the structure and chemistry of the hAM, which may decrease the cell attachment and proliferation (Zhang *et al.* 2013). However, previously the dispase has been used to remove the epithelial cells without decreasing the biocompatibility (Koizumi *et al.* 2007, Lim *et al.* 2009). In the future it would be interesting to study different decellularization method or how hUCs behave on a cellular hAM.

The PLCL based biomaterials were studied further in our project, since the sPLCL shown to promote UCs proliferation and viability over hAM and PS in the study I. Furthermore, to the best of our knowledge, the PLCL has not been evaluated as a growth surface for hUCs. The effect of topographical texturing of PLCL was studied, as it is generally known that surface texturing has an effect on cell adhesion and behaviour on biomaterial (Garkhal *et al.* 2007, Ma *et al.* 2007, Vasita *et al.* 2008). Interestingly, according to the quantitative WST-1 measurement and qualitative SEM imaging, the topographical texturing did not increase the initial attachment or proliferation of hUCs, as we hypothesized. Consistently with the study I, sPLCL supported proliferation of hUCs as tPLCL and cPLCL, and the hUCs reached confluency on all the PLCL based membranes after 14 d culturing period. In the study II, the sPLCL and tPLCL supported the hUCs proliferation significantly better compared to cPLCL at 7 and 14 d time points, respectively.

Our studies (**I, II**) demonstrated that hUCs remained viable on sPLCL, tPCLC and cPLCL over the whole culturing period indicating a good biocompatibility of PLCL. In addition, hUCs (**I, II**) exhibited normal hUC morphology; the cells were small, polyhedral or

cuboidal on all the tested PLCL based membranes (Khandelwal *et al.* 2009). However, the hUCs were slightly larger on PLA fibres compared to PLCL regions. Furthermore, after 7 d of cell culture a part of the hUCs were small and oval-shaped, which may demonstrate recently divided hUCs. Our study demonstrated that hUCs on cPLCL seemed to prefer PLCL areas over PLA fibres. This finding was not expected as PLA is highly biocompatible and it has been widely studied for tissue engineering applications as well as urothelial tissue engineering (Atala 2009, Nair and Laurencin 2006, Wunsch *et al.* 2005). We though that the mechanical structure of composite biomaterial may have affected on the hUCs growth. The thickness of PLA fibres may have been too high for hUCs growth.

6.3 Maintaining the urothelial phenotype on different biomaterials

The maintenance of urothelial phenotype was confirmed with immunostaining method. The markers CK7 (II), CK7/8 (I), CK19 (I and II) were selected as these markers are known to be expressed through all the urothelial layers (Southgate *et al.* 1999) and the expression was also evident in our study with *in vitro* expanded hUCs. The positive expression strongly denotes that the cultured cells are particularly urothelial origin even though these cytokeratins are also demonstrated in other epithelial types (Moll *et al.* 1982, Southgate *et al.* 1999). Additionally we tested the expression of UPIII (I), which is evident merely in terminally differentiated umbrella cells (Khandelwal *et al.* 2009, Nagele *et al.* 2008). During our study, the expression of UPIII was not detected with hUCs, the similar results were obtained by Nagele *et al*. (Nagele *et al.* 2008). The terminally differentiated urothelial cells may not attach and proliferate on cell culture surfaces as the cells from basal or intermediate layers, which may explain the lack of UPIII expression.

In the study I, the hAM did not support the urothelial phenotype as the CK7/8 and CK 19 expression decreased with time, whereas, on PS and sPLCL the hUCs maintained their phenotype. This further confirmed the unsuitability of hAM as a matrix for urothelial applications. The expression of markers CK7 and CK19 in the study II demonstrated, that the hUCs maintained their phenotype when cultured on sPLCL, tPLCL and cPLCL during the assessment period, which also substantiates the potential of PLCL based biomaterials for urothelial applications.

6.4 Future perspectives for hypospadia repair

In the future, the tissue engineering methods could be potential to treat severe hypospadia. Thus far, one research group has treated 6 patients suffering from severe hypospadia by the means of tissue engineering (Fossum *et al.* 2007, Fossum *et al.* 2012). The autologous hUCs were isolated using bladder lavage and expanded in laboratories. After expansion, the hUCs were seeded on an acellular dermis obtained from human donors. The patients were followed until 6-8 years post operatively, after which 5 out of 6 patients voided in a standing position and the cosmetic outcome was good with all treated patients. However, as a complication two patients developed fistula and two other had a urethral stricture. Further, after the follow-up period the neourethra had a transitional epithelium only with 2 patients (Fossum *et al.* 2007, Fossum and Nordenskjold 2010, Fossum *et al.* 2012). However, Fossum *et al*. used allogenic acellular matrix in their study, which may cause certain problems relating to the allogenic origin of the biomaterial such as tissue rejection, infection or donor variation. These problems could be avoided using synthetic biomaterials such as PLCL (Fossum *et al.* 2007).

In order to accomplish a rapid implantation of the cellular graft the good adhesion and rapid proliferation of hUCs is crucial. PLCL, as the other polylactide based polymers, are hydrophobic biomaterials leading to weaker cell adhesion compared to natural biomaterials (Ma *et al.* 2007, Nair and Laurencin 2006).

Therefore, increasing the hydrophilic nature of PLCL could improve the initial cell attachment and lead to more rapid cell confluence. This may be achieved by manufacturing a composite biomaterial of PLCL and hydrophilic polymer or photochemical modifications of the biomaterial surface (Ma *et al.* 2007, Vasita *et al.* 2008). Additionally, the graft for clinical use should be manufactured as a tube-like structure or alternatively should be easily formed as tubular for instance using PLCL mesh (Kanatani *et al.* 2007) or extrusion and salt leaching (Park *et al.* 2009). In order to maintain the viability of hUCs after the implantation, the graft should also allow the transportation of nutrients through the biomaterial. Though, owing to patients' young age the transition from *in vitro* studies to clinical trials requires remarkably careful consideration. Therefore, before clinical applications, the assessment of specific biomaterial constructs, biocompatibility and applicability of the PLCL requires further *in vivo* research.

6.5 Multidifferentiation potential of ASCs

The multidifferentiation potential of ASCs was evaluated in order to further confirm the mesenchymal origin of the cells in the study III. ASCs isolated from the patients were shown to differentiate towards myogenic cell lineages. The myogenic differentiation of ASCs has also been reported previous studies using different inducing factors (Gimble and Guilak 2003, Mesimaki *et al.* 2009, Zuk *et al.* 2002). To induce myogenic differentiation we used TGF-β1 and fibronectin coating, which have been detected to differentiate adult stem cells toward myogenic cell lineages. Though, the most studies have been done using BMSCs (Narita *et al.* 2008, Rowlands *et al.* 2008, Zhao and Hantash 2011) some implications concerning the myogenic inductivity of TGF-β1 and fibronectin have also demonstrated with ASCs (Di Rocco *et al.* 2006, Wang *et al.* 2010). However, the development of standardized method for ASCs myogenic differentiation requires further research. In our study III we used

myogenic markers; SM22-α, α-SMA and MHCII, which are characteristic to smooth muscle cells (Wang *et al.* 2010). Lack of skeletal muscle markers was one deficiency in our study. However, the purpose of this study was not particularly to identify the differentiated muscle type but to confirm that ASCs are able to differentiate towards myogenic cell lineages.

In addition to myogenic differentiation, the adipogenic, osteogenic, and chondrogenic differentiation of ASCs are widely recognized (Mesimaki *et al.* 2009, Sándor *et al.* 2013, Zuk *et al.* 2002), as verified also during our study III. The differentiation of ASCs towards adipocytes was confirmed with Oil Red O staining, which is a widely used indicator for apipogenic differentiation, demonstrating lipid vacuoles accumulated in the ASCs (Sándor *et al.* 2013, Wang *et al.* 2010, Zuk *et al.* 2002). ALP staining was used as a marker for osteogenic differentiation since it is widely used and considered as a reliable marker for early osteogenesis (Kyllönen *et al.* 2013, Park *et al.* 2007, Zuk *et al.* 2001). Further, the micromass culture system is routinely used to differentiate ASCs towards chondrogenic cell lineages, which was demonstrated using Alcian blue staining. The chondrogenic differentiation has been widely acknowledged in previous studies with ASCs as demonstrated also in our study III (Huang *et al.* 2004, Mesimaki *et al.* 2009, Zuk *et al.* 2002). In addition, there are some studies, which indicate that ASCs could differentiate towards other cell lineages such as neurons or epithelial cells (Shi *et al.* 2012, Zuk *et al.* 2002). However, these were not studied during this project.

6.6 Clinical patient study

Previously, few research groups have used cellular based injection therapies for treatment of urinary incontinenece in clinical studies with encouraging results (Table 7). During this pilot research (III), we studied the effect of collagen gel (Contigen™) mixture with ASCs on female urinary incontinence treatment. Collagen gel was selected as a carrier material since it is extremely biocompatible and widely studied biomaterial for tissue engineering applications

(Nair and Laurencin 2007). Further, the Contigen™ is proven to be safe and no severe adverse effects have been demonstrated in clinical use (Mohr *et al.* 2013). Moreover, the Contigen™ is previously widely studied for urinary incontinence injection therapy as a bulking agent (Dmochowski and Appell 2000, Homma *et al.* 1996) and also in combination with cells (Mitterberger *et al.* 2007). Mittenberger *et al*. treated 123 female patients with the injections of autologous myoblasts and fibroblasts mixed with collagen. The myoblasts were injected into the rhabdosphincter and the fibroplasts mixed with collagen were injected into urethral submucosa. After 1-year follow-up 79 % of the patients were continent and did not need pads in their daily life (Mitterberger *et al.* 2007). In their further study, 20 female SUI patients were treated with the same method and followed up to 2 years (Mitterberger *et al.* 2007). After the follow-up; 16 patients were cured and 2 patients had less SUI symptoms (Mitterberger *et al.* 2008). Furthermore, Stangel-Wojcikiewics *et al*. studied the safety and efficacy of muscle derived cells to treat female urinary incontinence with 16 patients. After the 2-year follow-up 50 % of the patients reached complete improvement, further the treatment was considered safe (Stangel-Wojcikiewicz *et al.* 2013).

In addition to primary cells, adult stem cells have also been studied for incontinence injection therapy. Sebe *et al*. used autologous MDSCs isolated from deltoid muscle to treat 12 female patients, who had undergone an ineffective surgical treatment, with intrasphincteric injections. The patients were followed 12 months, which after 3 patients were dry, 7 patients had an improvement in pad test and 2 were slightly worse (Sebe *et al.* 2011). Carr *et al*. demonstrated an improvement in 5 of 8 female SUI patients after 1 year of injection therapy with MDSCs isolated from a thigh, however, only 1 patient was totally continent and 3 patients were withdrawn from the study (Carr *et al.* 2008). Altogether, these studies demonstrated that the injection therapies with MDSCs are safe and adverse effects are minimal when used with female SUI treatment (Carr *et al.* 2008, Mitterberger *et al.* 2007, Sebe *et al.* 2011). In addition to these

studies with MDSCs transurethral injections with allogenic CBSCs have been used to treat female SUI patients. After 1-year follow-up 36.1% of 39 patients were totally cured and according to the Patient's Satisfaction results 72.2 % of patients were more than 50 % satisfied (Lee *et al.* 2010).

To our knowledge ASCs have not been previously used to threat female SUI patients. However, 2 previous studies by the same reseach group have been published on treating male SUI with ASC injections. Yamamoto *et al*. treated 3 male SUI patients with periurethral ASC and adipose tissue injections with favourable results. The patients were followed 6 months, after which 1 patient had a negative pad test and the other 2 had an improvement in SUI symptoms according to the pad test (Yamamoto *et al.* 2012). Further, Gotoh *et al*. treated 11 male patients with the above-mentioned method. After 1 year follow-up the leakage volume in 24-h pad test was decreased 59.8 % on average (Gotoh *et al.* 2013). Contrary to our study, Yamamoto *et al*. or Gotoh *et al*. did not use any biomaterial carrier.

Table 7. The clinical results of the studies using cell based therapies to treat female urinary incontinence. Compared to the other studies we used adipose stem cells (ASC) in combination with Contingen™ gel. BMI: Body mass index. - No information available. MDSC: Muscle derived stem cells. CBSC: Umbilical cord blood stem cell. MDC: Muscle derived cells (Carr *et al.* 2008, Lee *et al.* 2010, Mitterberger *et al.* 2007, Mitterberger *et al.* 2008, Sebe *et al.* 2011, Stangel-Wojcikiewicz *et al.* 2013).

First author / Publication year	Number of patients	Mean age	BMI	Cell type	Cell expansion time	Cell number	Follow-up time	Cure rate
Mitterberger/2007	123	62.8 ± 10.5	-	Autologous myoblasts and fibroblast mixed with Contigen™	6-8 wks	Myoblasts: $5.1x10^{6}$ - $3.6x10^{7}$ Fibroblasts: $5.4x10^{6}$ - $6.0x10^{7}$	1 year	79 %
Mitterberger/2008	20	49.8 ± 9.2	-	Autologous myoblasts and fibroblast mixed with Contigen™	Approximately 7 wks	Myoblasts: $1.0x10^{6}$ - $3.0x10^{7}$ Fibroblasts: $1.4x10^{7}$ - $6.08x10^{7}$	1 year	90 %
Carr/2008	8	54 (range 42-65)	< 25 with 6 patient, all < 30	Autologous MDSC	3-5 wks	$1.8 - 2.2x10^{7}$	1 year	20 %
Lee/2010	39	51.0 ± 7.6	-	Autologous CBSC	No cell culture	$4.3 \pm 1.9 \times 10^{8}$	1 year	38.1 %
Sebe/2011	12	58 ± 13.5	27.6 ± 4.7	Autologous MDSC	3-4 wks	$1.0 - 6.0x10^{7}$	1 year	25 %
Stangel-Wójcikiewicz/2013	16	56.75 ± 7.63	46.06 ± 6.94	Autologous MDC	8-10 wks	$0.6 x10^{6} - 2.5 x10^{7}$	2 years	50 %
Study III	5	59.2 ± 13.8	30.2 ± 6.4	Autologous ASC mixed with Contigen™	3-4 wks	$2.5 - 8.5 x10^{6}$	1 year	40 %

In our study III we treated 5 female patients suffering from urinary incontinence with a mixture of ASCs and Contigen™ and detected a negative cough test with 3 patients after 1 year of follow-up. Furthermore, 2 of those patients were satisfied with the treatment results and did not want any additional treatments for SUI. Thus, after the follow-up period 40 % of the patients were totally cured. Furthermore, subjective improvement in SUI symptoms was detected according to the questionnaires with all treated patients. Interestingly, the patients who benefited from the treatment the most were the last treated patients. This might have been partly due to the learning curve of injection technique and the processing of ASCs and Contigen™.

In the study III, the used cell amount varied from 2.5x10^6 to 8.5x10^6 ASCs depending on the patient. Interestingly, Carr *et al*. demonstrated that the effect of injection therapy may enhance by increasing the amount of used cells (Carr *et al.* 2013). They treated 38 patients with muscle derived cells isolated form quadriceps femoris muscle with high (32, 64, or 128 x10^6 cells) or low cell dose (1, 2, 4, 8, or 16 x10^6 cells). In high dose group a greater amount of patients had over 50 % reduction in pad weight (88.9 % vs 61.5 %) and diary reported (77.8 % vs 53.3 %) leaks (Carr *et al.* 2013). Therefore, increasing the cell amount for injection therapy could lead to more effective results also when using ASCs as a cell source.

The Contigen™ was not the optimal carrier for the application owing to its inadequate mechanical properties. After blending the ASCs with Contigen™ gel, the blend became more liquid and the gellous characteristics diminished notably, which most probably decreased the bulking effect of the injection treatment. However, the Contigen™ gel was the only commercially available collagen gel accepted for clinical use, however, the manufacturing of Contigen™ has been completed. Developing an appropriate biomaterial gel for the application could further enhance the effect of the injection therapy. An ideal biomaterial for the application should be biodegradable, gellous and injectable though, possess an adequate mechanical properties in order to achieve the

bulking effect. Additionally, the mechanical properties should not diminish after blending the cells with the biomaterial. Further, the ideal biomaterial should also promote the myogenic differentiation of ASCs. Even though, in our study we hypothesized that the environment would partly enhance the ASCs differentiation toward the myogenic cell lineage. The effect of the environment has been previously detected when ASCs were injected in rat's ureter and bladder *in vivo* (Jack *et al.* 2005). In summary, the tissue engineering based injection therapies with ASCs could be potential in the future to treat urinary incontinence. The treatment is minimally invasive, easy to conduct and could be performed in outpatient clinic circumstances. Therefore it is a good treatment option even for elderly patients.

6.7 Ethical issues related to clinical pilot study

Tissue engineering and cellular therapies are under extensive research and becoming more common, thus, laboratories following good manufacturing practise (GMP) are essential to control the laboratory processes. Our clinical pilot study (III) was done in GMP class laboratories to verify the safety of cell based therapy. The European Medicines Agency (EMA) and the Committee for Advanced Therapies (CAT) have defined regulations of the stem cell based therapies including tissue engineering therapies under the regulations of Advanced Therapy Medicinal Products (ATMP) (Committee for Advanced Therapies (CAT) 2010, European Medicines Agency 2011). At the moment, when searching the clinical trials utilizing adipose derived stem cells, altogether 109 ongoing or completed studies can be found. However, only 3 clinical trials where ASCs are used to treat urinary incontinence can be found. In 2 of these studies patients are currently recruited. The third study, where male urinary incontinence was treated with ASCs after radical prostate cancer surgery, has been completed. Unfortunately, the results of that study are not available yet (http://clinicaltrials.gov).

The use of ASCs for clinical applications requires throughout evaluation of advantages and risks. Tumorigenity of ASCs is under extensive debate and contradictory results regarding to this topic have been published (Devarajan *et al.* 2012, Kucerova *et al.* 2010). Cousin *et al*. studied the effect of ASCs on pancreatic ductal adenocarcinoma cell response demonstrating a strong inhibitatory effect on cancer cell viability and growth both *in vitro* and *in vivo* (Cousin *et al.* 2009). Furthermore, Kucerova *et al*. reported that ASCs had an antitumorigenic effect on glioblastoma cells, whereas, on melanoma cells the ASCs seemed to have a tumor-promoting effect (Kucerova *et al.* 2010). It has also been suggested that ASCs may increase the number of breast cancer cells in a paracrine fashion (Devarajan *et al.* 2012). The tumorigenity or antitumorigenity of ASCs may be dependent on a studied cancer cell type. However, further research is required prior to verify the fundamental effect of ASCs cancer progress. The patient selection and careful assessment of cancer risk is therefore crucial when using ASCs in clinical patients studies.

Our clinical study was approved by the Ethics Committee of Pirkanmaa Hospital District. The injection therapy is minimally invasive and during our study no severe side effects were detected. However, ethical concerns, patient information and welfare have high importance. At least two articles where urinary incontinence has been treated with cell based injection therapy have been retracted due to the ethical concerns (Kleinert and Horton 2008, Strasser *et al.* 2007, Strasser *et al.* 2007). In these retracted studies, autologous myoblast and fibroblast injections to collagen injection for incontinence treatment were compared. After the 1-year follow up, potential results with autologous cell injection compared to collagen injection were gained (Strasser *et al.* 2007, Strasser *et al.* 2007). Though, the studies did not have ethical approval and the studies were not performed according to the Good Clinical Practise (GCP). Furthermore, there were critical deficiencies how the patients' consent was collected in addition to source data documentation (Kleinert and Horton 2008).

At the moment, the cellular therapies using MSCs are emerging rapidly and high hopes have been generated for tissue engineering based therapies. However, extensive research, especially related to the safety and efficacy, is still required prior to the development of cell based therapies as an everyday treatment.

7 Conclusions

Firstly, this study demonstrated that PLCL is a potential biomaterial for urothelial tissue engineering applications and should be further studied in the urothelial field. Secondly, a tissue engineering based injection therapy was shown to be a safe and beneficial method to treat urinary incontinence. Based on these studies, the following conclusions can be drawn:

I. The PLCL supported the viability, proliferation and phenotype maintenance of hUCs over hAM. Based on this study, the hAM was revealed to be an unsuitable matrix for hUCs, whereas, the PLCL was demonstrated to be beneficial biomaterial matrix for urothelial applications *in vitro*.

II. The potential of PLCL for urothelial tissue engineering was futher demonstrated, as hUCs proliferated and maintained their phenotype on all the studied PLCL based membranes. Interestingly, the mechanical texturing of PLCL membranes did not increase the attachment or proliferation of hUCs *in vitro*.

III. The injection therapy with ASCs mixed with collagen gel, Contigen™ was shown to be a safe method to treat urinary incontinence. Further, after 1-year follow-up, the cough test was negative for 3 out of 5 patients. The ASCs were also demonstrated to differentiate towards myogenic, adipogenic, osteogenic and chondrogenic cell lineages.

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10 Original communications

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Comparison of a poly-L-lactide-co-ε-caprolactone and human amniotic membrane for urothelium tissue engineering applications

Reetta Sartoneva[1,2,*,†], Suvi Haimi[1,2,†], Susanna Miettinen[1,2], Bettina Mannerström[1,2], Anne-Marie Haaparanta[3], George K. Sándor[1,2], Minna Kellomäki[3], Riitta Suuronen[1,2,3,4,†] and Tuija Lahdes-Vasama[2,5,†]

[1]*Regea–Institute for Regenerative Medicine, University of Tampere, Tampere, Finland*
[2]*Science Centre, University of Tampere, Tampere, Finland*
[3]*Department of Biomedical Engineering, Tampere University of Technology, Tampere, Finland*
[4]*Department of Eye, Ear and Oral Diseases, Tampere University Hospital, Tampere, Finland*
[5]*Paediatric and Adolescent Surgery Unit, Pediatric Research Centre and Tampere University Hospital, Tampere, Finland*

The reconstructive surgery of urothelial defects, such as severe hypospadias is susceptible to complications. The major problem is the lack of suitable grafting materials. Therefore, finding alternative treatments such as reconstruction of urethra using tissue engineering is essential. The aim of this study was to compare the effects of naturally derived acellular human amniotic membrane (hAM) to synthetic poly-L-lactide-co-ε-caprolactone (PLCL) on human urothelial cell (hUC) viability, proliferation and urothelial differentiation level. The viability of cells was evaluated using live/dead staining and the proliferation was studied using WST-1 measurement. Cytokeratin (CK)7/8 and CK19 were used to confirm that the hUCs maintained their phenotype on different biomaterials. On the PLCL, the cell number significantly increased during the culturing period, in contrast to the hAM, where hUC proliferation was the weakest at 7 and 14 days. In addition, the majority of cells were viable and maintained their phenotype when cultured on PLCL and cell culture plastic, whereas on the hAM, the viability of hUCs decreased with time and the cells did not maintain their phenotype. The PLCL membranes supported the hUC proliferation significantly more than the hAM. These results revealed the significant potential of PLCL membranes in urothelial tissue engineering applications.

Keywords: urothelium tissue engineering; poly-L-lactide-co-ε-caprolactone; amniotic membrane; urothelial cell characterization

1. INTRODUCTION

Urothelial defects are fairly common and these defects are mainly caused by congenital malformation, trauma or stricture. For instance, the incidence of congenital hypospadia is approximately 1 of 200 male births [1,2]. The urothelial defects are nowadays repaired by traditional reconstructive surgery using, for instance, the patient's own genital tissue. The more severe cases, when the patients' genital tissue is inadequate, are repaired using non-urological tissue, such as buccal mucosal grafts [1–3]. However, these operations, particularly those where non-urological grafts are used, are prone to complications, such as fistula formation or urethral strictures [3,4]. Thus, alternative methods are needed and tissue engineering may be a potential method for remedying severe urothelial defects in the future.

Native urethra has a tubular structure; therefore an ideal biomaterial for urothelial tissue engineering should be elastic to form the tube-like structure; further, the basement membrane of urothelium is elastic and, therefore, the elastic biomaterial would mimic the natural growth surface of human urothelial cells (hUCs). Additionally, the biomaterial should be biodegradable, biocompatible, promote urothelial tissue regeneration and degrade without disadvantageous tissue reactions [5–7]. Furthermore, the ideal tissue-engineered urothelium should have a urothelial-specific surface structure: the cells in the superficial cell layer of native urothelium are large and frequently binucleated umbrella cells characterized by compact tight junctions

*Author for correspondence (reetta.sartoneva@regea.fi).
†These authors contributed equally to the study.

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and the presence of defined plaques of asymmetric unit membrane (AUM). The native urothelium expresses intermediate filament proteins, cytokeratins (CKs) and uroplakins (UPs) and these proteins should also be present in the ideal tissue-engineered urothelium.

Various natural biomaterials, such as collagen, small intestinal submucosa, human amniotic membrane (hAM) and different synthetic biomaterials, such as polyglycolide (PGA), polylactide (PLA) and polycaprolactone, have proven to be suitable for urothelial tissue-engineering applications [5,7–9]. Additionally, Wünsch *et al.* [7] demonstrated that porcine urothelial cells on natural matrices were more similar to native urothelium when compared with synthetic matrices. Furthermore, a recent study showed that the hAM was a potential matrix for mouse urothelial cells (mUCs) when compared with collagen, peritoneum and omentum [10]. The major problems concerning natural biomaterials have been their poor mechanical properties, xenograft origin and fabrication difficulties [5,9]. These problems may be avoided using synthetic biomaterials, but the absence of bioactive molecules makes the utilization of synthetic biomaterials in cell culture more challenging compared with natural biomaterials [5]. PGA and PLA have shown to possess an excellent biocompatibility for hUCs [11]; however, the disadvantage of using PGA and PLA is their hardness and inelasticity. Additionally, PGA degrades and loses its mechanical properties rapidly [12,13]. Even though many different biomaterials have been tested with promising results, the optimal biomaterial has not yet been found.

In this study, we compared the effects of synthetic poly-L-lactide-co-ε-caprolactone (PLCL) membrane to natural hAM matrix on hUC proliferation and differentiation. We hypothesized that especially PLCL would serve as a suitable matrix for hUCs because of the elasticity of PLCL and the potential of PLA-based polymers in various tissue-engineering applications [5,9].

2. MATERIALS AND METHODS

2.1. Materials

The hAM was separated from the chorion, rinsed with 0.9 per cent NaCl and incubated in 2.5 $\mu g\,ml^{-1}$ amphotericin B, 5.0 $\mu g\,ml^{-1}$ penicillin, 50 $\mu g\,ml^{-1}$ streptomycin and 100 $\mu g\,ml^{-1}$ neomycin in Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich). The epithelial cells on hAM were removed with 4 per cent dispase (Invitrogen, Paisley, UK) solution. The hAM was rinsed to completely remove dispase traces and the acellularity of hAM was confirmed with a microscope.

The copolymer, poly-L-lactide-co-ε-caprolactone (70/30) (PURAC biochem bv, Gorinchem, The Netherlands), used for manufacturing the PLCL membranes, was medical grade and highly purified (residual monomer <0.5%) with an inherent viscosity of 1.58 $dl\,g^{-1}$. The PLCL membranes were fabricated by compression moulding of PLCL granules, which was performed with NIKE (Hydraulics Ab, Eskilstuna, Sweden) device as follows. First, preliminary moulding was performed at 10 MPa, 110°C for 90 s followed by the final moulding into 150 μm thick membranes at 20 MPa, 130°C for 30 s. The membranes were cut into samples with a diameter of 16 mm, washed with ethanol and sterilized at 25 kGy before the cell culture experiments. Cell culture plastic (PS) wells (Sigma–Aldrich/Corning CellBIND, St Louis, MO, USA) served as a control material.

2.2. Material characterization

Material characterization was performed to the raw 70/30 PLCL material and to the fabricated samples after sterilization. Differential scanning calorimeter (DSC; DSC Q 1000, TA Instruments, New Castle, Delaware, USA) was used to determine the glass-transition temperature (T_g) of the material.

Inherent viscosity (IV) measurements were performed with Lauda PVS viscometer (Lauda Dr R. Wobser Gmbh&co, Königshofen, Germany). Samples were prepared by dissolution of the sample in chloroform with a concentration of about 1 $mg\,ml^{-1}$. The IV was determined with capillary viscometer (Ubbelohde type 0c, Schott-Geräte, Mainz, Germany).

2.3. Cell isolation and culture

Cell isolation was performed as represented by Southgate *et al.* [14] with minor modifications. Briefly, the tissue samples were cleaned, cut and incubated in stripping solution containing 0.01 per cent HEPES buffer (1 M, Sigma–Aldrich), 4×10^{-3} per cent aprotin (1 KIU μl^{-1}, Sigma–Aldrich), 0.1 per cent EDTA (Sigma–Aldrich), 0.01 per cent penicillin/streptomycin (Lonza, Verviers, Belgium) in Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (Invitrogen). The urothelial sheets were separated from tissue samples and incubated in 0.1 per cent trypsin (Lonza). To inactivate trypsin, 10 per cent human serum of the clot type AB (HS, PAA Laboratories, Pasching, Austria) in HBSS was added. The solution was centrifuged and the resulting pellet was suspended in a defined urothelium medium (EpiLife, Invitrogen). The hUCs were cultured in cell bind T75 flasks (Sigma–Aldrich) at 37°C in a humidified atmosphere of 5 per cent CO_2 in air. Overall, hUCs from three patients, passages 2 and 3, were used in the experiments.

2.4. Flow-cytometric surface marker expression analysis of human urothelial cells

The hUCs were harvested and analysed after primary culture by a fluorescence-activated cell sorter (FACS) (FACSAria: BD Biosciences, Erembodegem, Belgium). Monoclonal antibodies against CD44-PE, CD73-PE, CD105-PE, CD133-PE, CD166-PE (BD Biosciences), CD326-APC (Miltenyi Biotech, Bergisch Gladbach, Germany) and Keratin 8/18 (Cell Signaling Technology, Danvers, MA, USA) were used. MAb Keratin 8/18 was conjugated with IgG-alexa488 (Molecular Probes, Eugene, OR, USA). A total of 10 000 cells per sample was analysed; positive expression was defined as a level of fluorescence which was 99 per cent of the corresponding unstained cell sample.

2.5. Cell seeding

Before cell seeding, PLCL membrane and hAM were attached to the membrane fixation devices, cell crowns (CellCrown24, Scaffdex, Tampere, Finland); later, these were attached to the wells of a 24-well plate. The sample materials were preincubated in urothelium medium at 37°C for 48 h. On to each membrane, previously supplied with 1 ml of medium, 20 000 cells were seeded. The hUCs were cultured at 37°C in a humidified atmosphere until analysis.

2.6. Cell viability evaluation

Cell viability was studied using qualitative live/dead fluorescent staining after 3, 7 and 14 days of cell culture. Briefly, the cells were rinsed with DPBS and incubated at room temperature for at least 30 min with a mixture of 0.25 µM Calcein AM (green fluorescence, Molecular Probes) and 0.3 µM Ethidium homodimer-1 (red fluorescence, EthD-1, Molecular Probes). A fluorescence microscope (Olympus, IX51S8F-2, camera DP71) was used to image the viable cells (green fluorescence) and dead cells (red fluorescence). PLCL, hAM and PS without cells were used to exclude false-positive staining caused by materials alone.

2.7. Cell proliferation assay

After 3, 7 and 14 days of culture, the proliferation of hUCs was determined by WST-1 analysis by measuring the mitochondrial activity. The cells were incubated at 37°C for 4 h with 50 µl of premix WST-1 cell proliferation assay system (Takara Bio Inc, Otsu, Shiga, Japan) and 500 µl of DPBS. The absorbance was measured with a microplate reader (Victor 1420 Multilabel Counter, Wallac, Turku, Finland) at 450 nm.

2.8. The phenotype characterization of human urothelial cells using immunostaining

The phenotype of hUCs was verified by immunostaining after 7 and 14 days using CK7/8 (1 : 200, Lab Vision, Fremont, CA, USA), CK19 Ab-1 (1 : 500, Lab Vision) and UPIII (1 : 200, Santa Cruz Biotechnology, Heidelberg, Germany) as primary antibodies. The cells were fixed with 4 per cent paraformaldehyde fixative (Sigma–Aldrich) and incubated in primary antibody dilutions. Thereafter, secondary antibodies from mouse and goat (1 : 400, Alexa-488, green fluorescence, Molecular Probes) were conjugated to primary antibodies. Finally, the cells were mounted with Vectashield (DAPI; blue fluorescence, Vector Laboratories, Peterborough, UK) and imaged with the fluorescence microscope (Olympus). False-positive staining caused by materials alone was excluded using membrane materials without cells.

2.9. Statistical analysis

Statistical analyses were performed with SPSS, v. 13 (SPSS, Chicago, IL, USA). After verifying normal distribution and homogeneity of variance, the effects of different materials were compared using one-way analysis of variance (ANOVA). Post hoc (Bonferroni) tests were performed to detect significant differences between the materials. The effect of culturing period (3 versus 7 days and 7 versus 14 days) was also studied using one-way ANOVA and *post hoc* tests. Data were reported as mean $\pm$ s.d., $p < 0.05$ was considered significant and $p < 0.01$ highly significant.

3. RESULTS

3.1. Material characterization

Material characterization studies showed minor changes in the PLCL properties after processing and sterilization. The T_g of PLCL samples was reduced from 18.1°C to 15.7°C, which indicated that there was a certain degree of polymer degradation during membrane manufacturing. Also, IV measurements supported these results as they decreased from 1.58 to 1.03 dl g^{-1}.

3.2. Flow-cytometric surface marker expression analysis of human urothelial cells

Based on the FACS analysis, the isolated hUC population was homogeneous. The hUCs expressed the extracellular matrix adhesion marker CD44, endothelial markers CD73 and CD105, and epithelial markers CD133, CD166 (ALCAM) and CD326 (EpCAM). The hUCs also expressed the intracellular marker Keratin 8/18, which is a specific marker for epithelial cells (figure 1).

3.3. Cell viability

Live/dead staining illustrated that the majority of cells adhered to all the studied materials and were viable at the 3 day timepoint (figure 2). After a week of cell culture, more dead cells were detected on the hAM and PS than after 3 days but on the PLCL there were very few dead cells. Additionally, after two weeks of cell culture, the majority of cells were viable on the PLCL. On the PS, more dead cells were detected, although the majority of cells were viable. However, on the hAM, the majority of cells were dead after 2 weeks of culture. The visual qualitative analysis revealed that the number of cells attached on the PS and PLCL membranes was notably higher than on the hAM at the 7th and 14th days of culture. In addition, on the PLCL and PS, the number of cells increased from the 3rd day of culture to the 14th day of culture as opposed to the hAM.

3.4. Cell proliferation

WST-1 analysis revealed significant differences between the material types and culturing periods (figure 3). On the PLCL membranes, the cell number significantly increased in all timepoints, and after 14 days of culture the cell number was significantly higher than on the other materials. Only on the hAM, the cell number did not increase after 7 days of culture when compared with 3 days. Also, the hAM supported hUC proliferation less well at the 7 and 14 day timepoints when compared with the PLCL and PS. Interestingly, the

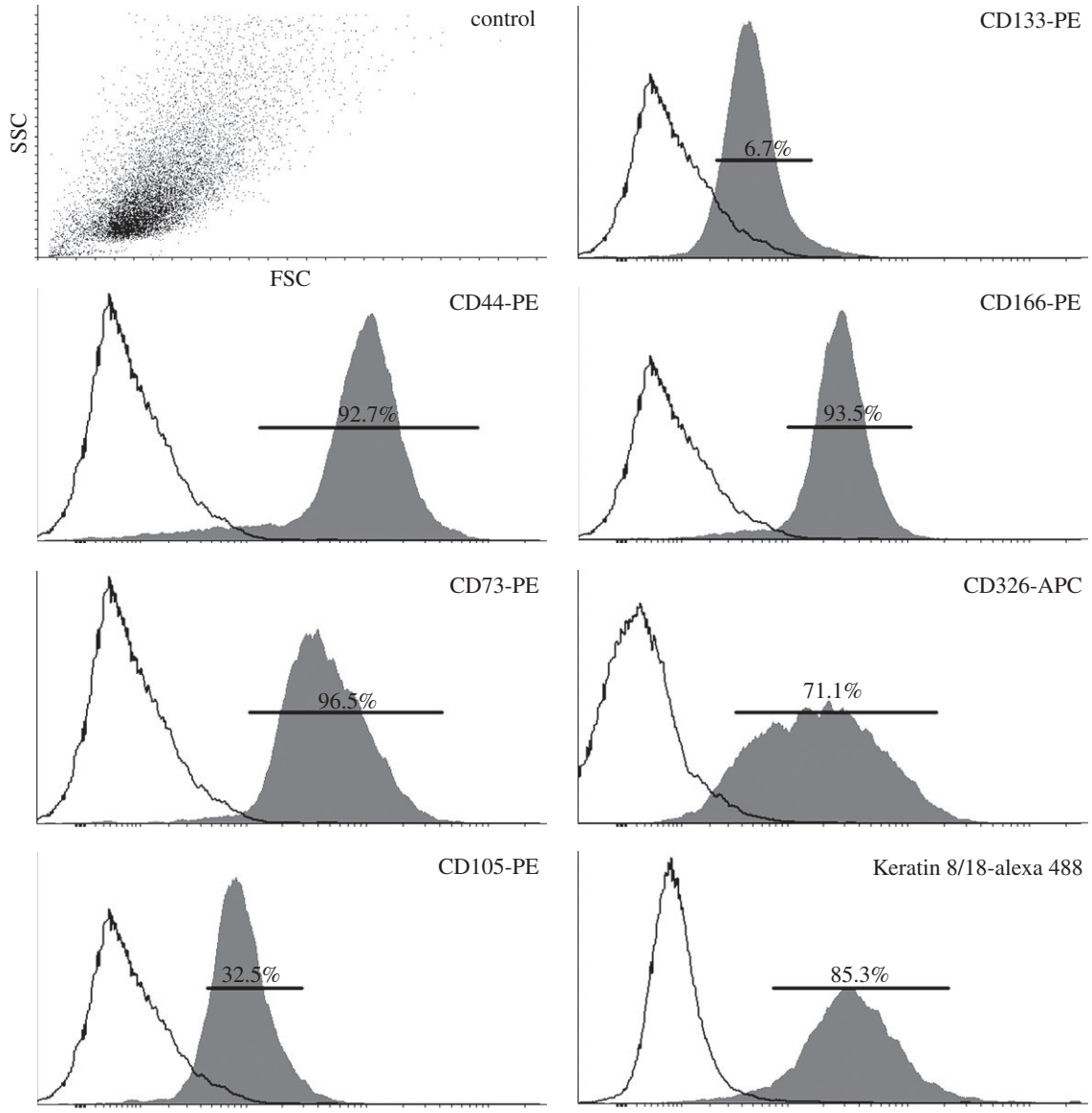

Figure 1. Surface marker expression characteristics of human urothelial cells (hUCs) as analysed by flow cytometry. Dot plot demonstrating homogeneity of cell size (FSC) and surface (SSC) complexity of unstained control cells. Histograms depicting relative cell number (*y*-axis) and fluorescence intensity (*x*-axis), with unstained control cells (empty histograms) and cells stained with antibodies against the surface proteins (filled histograms).

hUCs reached maximum proliferation at the 7 day timepoint when cultured on the PS and hAM, and the cell number significantly decreased at the 14 day timepoint when compared with the 7 day timepoint.

3.5. The phenotype characterization of human urothelial cells using immunostaining

The phenotype of hUCs was assessed after 7 and 14 days of cell culture by determining the expression of CK7/8, CK19 and UPIII proteins. Moderate expression of CK7/8 and CK19 (green fluorescence) was detected after 7 and 14 days of cell culture on the PS and PLCL (figure 4). The phenotype of hUCs on the PLCL and PS was unchanged at 14 days when compared with 7 days. However, on the hAM, the CK19 and CK7/8 expression of hUCs decreased during the assessment period. UPIII was not expressed by hUCs on any material or at any timepoint (data not shown).

4. DISCUSSION

In this study, we used hUCs taken from the ureter during routine surgery. These cells are fairly similar to cells in the proximal part of the urethra, as the whole urinary tract except the distal part of urethra is covered by the urothelium and therefore these cells can be used in future urethral reconstruction applications [15]. Urothelial cells have also been widely studied *in vitro* for urothelial tissue engineering purposes; further, Fossum *et al.* [16] used bladder urothelial cells for clinical urethra reconstruction. Moreover, urethral stratified squamous epithelial cells could not be used for this since it would have been unethical to take tissue from the urethra because the urethra is susceptible to scarring [17].

The identity of hUCs was confirmed using FACS analysis. The markers CD44, CD73, CD105, CD133 and Keratin 8/18 were selected for this study as they have been previously used to characterize hUCs.

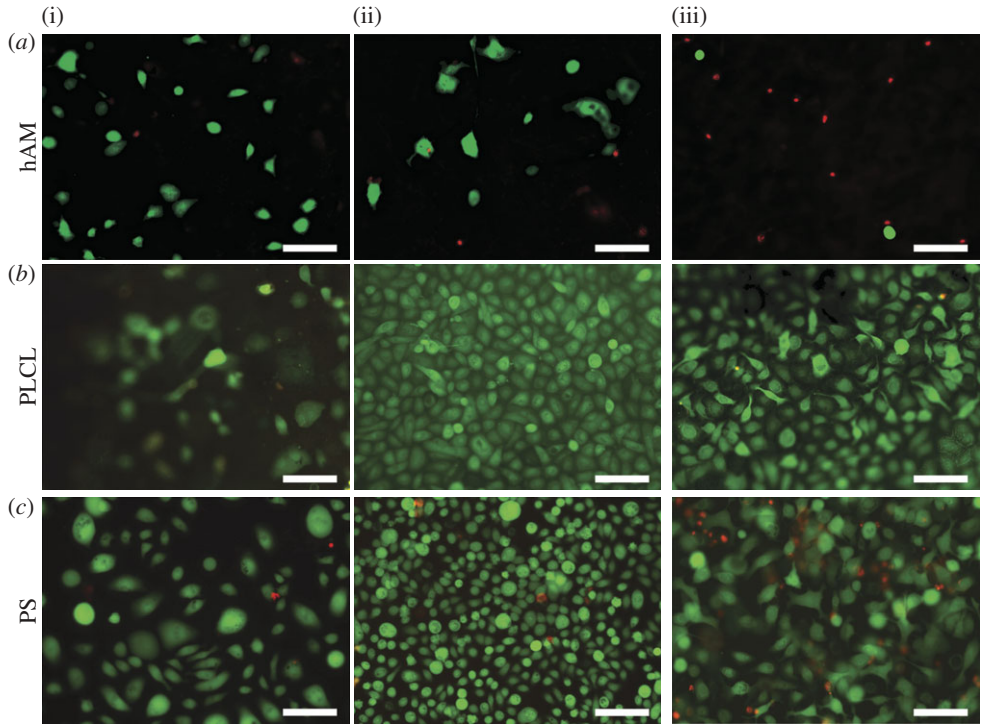

Figure 2. Representative images of viable (green fluorescence) and dead (red fluorescence) hUCs attached to (*a*) hAM, (*b*) PLCL and (*c*) PS after (i) 3, (ii) 7 and (iii) 14 days of culture. Scale bar, 100 μm.

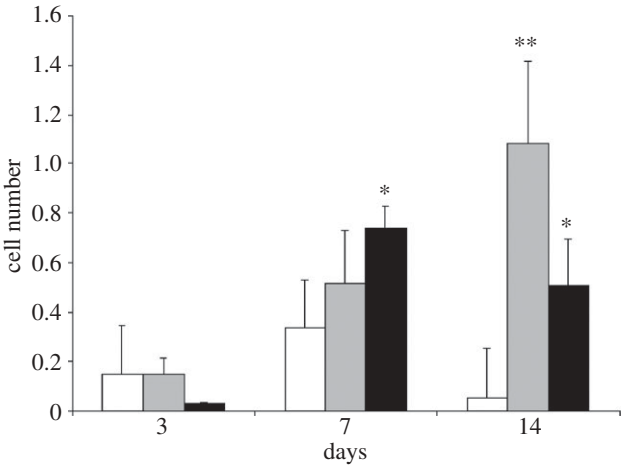

Figure 3. The number of hUCs cultured for 3, 7 and 14 days on the hAM (white), PLCL (grey) and PS (black). Results are expressed as means + s.d. cell number in two experiments ($n = 5$–6). *$p < 0.05$ with respect to hAM; **$p < 0.01$ with respect to hAM and PS. The number of cells increased significantly ($p < 0.05$) on PLCL in all timepoints. On PS, the cell number increased significantly ($p < 0.01$) after 14 days of culture when compared with 7 days. Cell number on PS and hAM decreased significantly ($p < 0.05$) at day 14 when compared with day 7.

Our characterization results were consistent with previous results for urothelial cells [18,19]. In addition to these previously tested markers, we wanted to test CD166 and CD326, as those markers are known to be expressed in epithelial cells [20,21], which was also demonstrated for hUCs in our study.

The immunostaining results demonstrated that on PLCL and PS, CK7/8 and CK19 staining was evident during the whole cell culture period as expected, because those markers are present in all layers of normal urothelium [14,22–24]. In contrast, on the hAM, the CK19 expression of hUCs decreased with time. Although UPIII is expressed in native urothelium, previous studies have demonstrated that UPIII is not expressed in human urothelium primary cultures, as our results also indicated [14,25,26]. Only one study, carried out with mouse cells, has demonstrated the expression of UPIII in urothelial primary cultures [10]. The lack of expression may have been because UPIII is present only in terminally differentiated umbrella cells in AUM [14,24]. After the isolation of hUCs, the umbrella cells may not attach and grow as well as the other hUCs from the intermediate and basal layers.

To our knowledge, this is the first study comparing the capacity of PLCL membrane and hAM to maintain the viability, proliferation and phenotype of hUCs. Choosing a suitable biomaterial is critical for successful urothelial tissue engineering application. We selected PLCL, a copolymer of lactic acid and ε-caprolactone, owing to the application requirements, and because the ε-caprolactone makes the material softer, more elastic and easier to suture and construct as a tube-like structure [27,28]. Furthermore, PLCL was a safe choice since synthetic lactic acid-based polymers are widely used in different tissue engineering applications, including urology, with promising results [6,9,11].

Our WST-1 measurement results showed that the PLCL membranes supported the proliferation of hUCs during the 14 day culture period, as expected. In addition, according to the live/dead staining, the majority of hUCs were viable and the cell morphology corresponded to the native hUCs with compact and round shapes at all timepoints. The live/dead staining

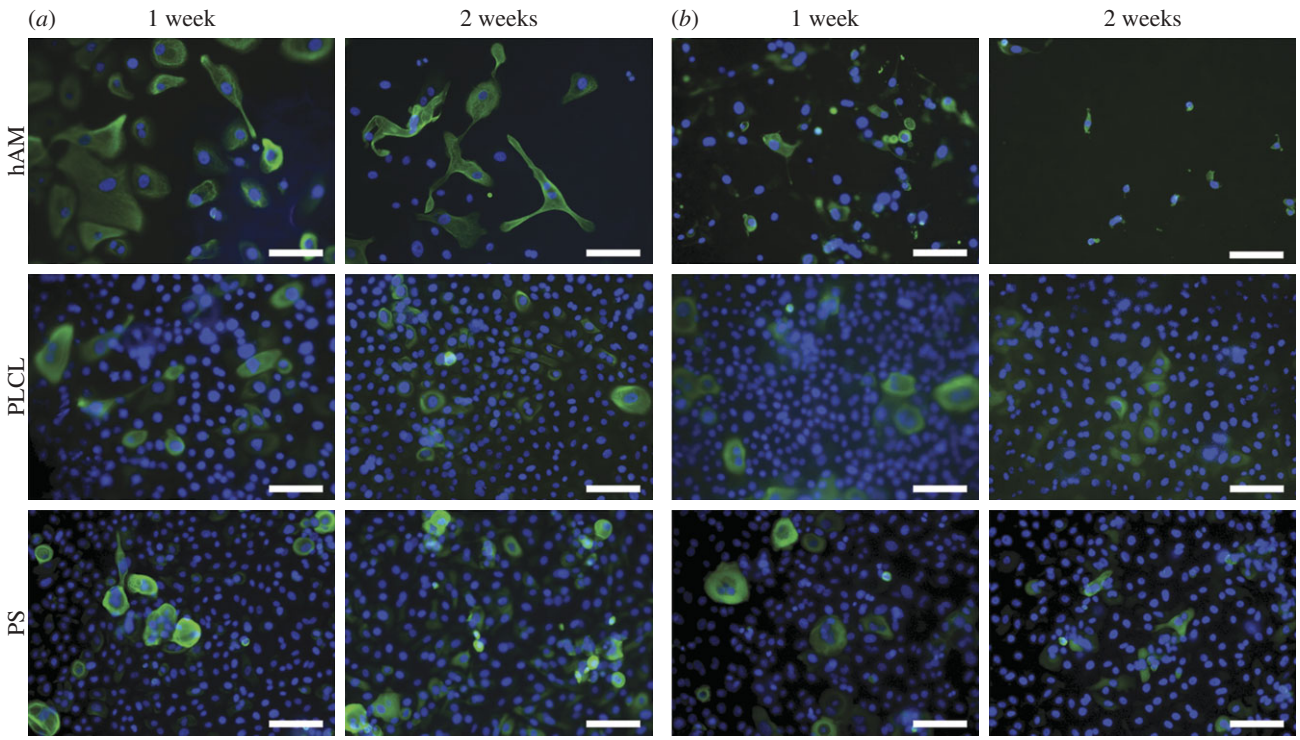

Figure 4. The immunofluorescence images of hUCs at the 7th and 14th day when cultured on hAM, PLCL and PS. Antibodies (*a*) CK7/8 and (*b*) CK19 (green fluorescence) were used to confirm urothelial phenotype. Scale bar, 100 μm.

and WST-1 measurement results were consistent, showing the increase in cell number on the PLCL with time. These results are also concordant with studies where PLCL has proven to be a biocompatible material for different tissue engineering applications [29,30].

The second material, acellular hAM, was chosen for this study as the previous reports have shown the hAM to be a potential matrix material for corneal epithelial cells [31] and mUCs [10]. hAM is a highly collagenous natural biomaterial, containing mainly collagen I and III; collagenous biomaterials are generally known to facilitate cellular adhesion [5,32]. Furthermore, Koizumi *et al.* [33] showed that epithelial-based cells grew better on denuded hAM than on cellular hAM. As expected, the attachment of hUCs on the hAM was good. However, in contrast to the previous study of mUCs [10], our live/dead staining and WST-1 measurement both verified that the cell number significantly decreased after 7 days, demonstrating that hAM is not a suitable biomaterial for hUC growth. Furthermore, the cell morphology on the hAM differed notably from the normal hUC morphology on the PLCL membranes and on the PS; the cells were large, irregularly shaped and formed projections. Results contradicting those of Sharifiaghdas *et al.* [10] may be due to the differences between the species and cell types, as in this study we used urothelial cells isolated from human tissue.

5. CONCLUSIONS

The results of this work demonstrated that hUCs preferred to proliferate on the synthetic PLCL membranes. On the other hand, hAM appeared to be an unsuitable matrix material for the hUCs. Based on these results, the PLCL membranes show significant potential in future urothelial tissue engineering applications. Additionally, owing to the promising results, PLCL will be further studied in an *in vivo* model.

Amniotic membranes were obtained following routine caesarean section at Tampere University Hospital in accordance with the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (R06045). Human urothelial tissue samples were isolated from dilated ureters, which were resected or tapered for neoimplantation from child patients in Tampere University Hospital with the approval of the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (R071609).

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Characterizing and optimizing poly-L-lactide-co-ε-caprolactone membranes for urothelial tissue engineering

Reetta Sartoneva[1,2,3,*,†], Anne-Marie Haaparanta[2,5,†], Tuija Lahdes-Vasama[6], Bettina Mannerström[1,2,3], Minna Kellomäki[3,5], Minna Salomäki[1,2,3], George Sándor[1,2,3], Riitta Seppänen[1,2,4,5], Susanna Miettinen[1,2,3,†] and Suvi Haimi[1,2,3,7,†]

[1]*Institute of Biomedical Technology, University of Tampere, Tampere, Finland*
[2]*BioMediTech, Tampere, Finland*
[3]*Science Centre, and* [4]*Department of Eye, Ear and Oral Diseases, Tampere University Hospital, Tampere, Finland*
[5]*Department of Biomedical Engineering, Tampere University of Technology, Tampere, Finland*
[6]*Paediatric and Adolescent Surgery Unit, Paediatric Research Centre and Tampere University Hospital, Tampere, Finland*
[7]*Department of Biomaterials Science and Technology, University of Twente, Enschede, The Netherlands*

Different synthetic biomaterials such as polylactide (PLA), polycaprolactone and poly-L-lactide-co-ε-caprolactone (PLCL) have been studied for urothelial tissue engineering, with favourable results. The aim of this research was to further optimize the growth surface for human urothelial cells (hUCs) by comparing different PLCL-based membranes: smooth (s) and textured (t) PLCL and knitted PLA mesh with compression-moulded PLCL (cPLCL). The effects of topographical texturing on urothelial cell response and mechanical properties under hydrolysis were studied. The main finding was that both sPLCL and tPLCL supported hUC growth significantly better than cPLCL. Interestingly, tPLCL gave no significant advantage to hUC attachment or proliferation compared with sPLCL. However, during the 14 day assessment period, the majority of cells were viable and maintained phenotype on all the membranes studied. The material characterization exhibited potential mechanical characteristics of sPLCL and tPLCL for urothelial applications. Furthermore, the highest elongation of tPLCL supports the use of this kind of texturing. In conclusion, in light of our cell culture results and mechanical characterization, both sPLCL and tPLCL should be further studied for urothelial tissue engineering.

Keywords: urothelial tissue engineering; poly-L-lactide-co-ε-caprolactone; PLCL characterization; urothelial cell characterization

1. INTRODUCTION

Urothelial tissue engineering may be a future method in the reconstructive surgery of urothelial defects caused by, for instance, strictures, traumas or congenital abnormalities, such as hypospadias [1]. Traditionally, these defects have been repaired surgically using the patient's genital tissue as a graft but, in more severe cases, additional distant graft tissue, such as buccal mucosa, is needed. These techniques are prone to complications; therefore, the development of novel reconstruction techniques by means of tissue engineering is essential. In future clinical treatments, autologous human urothelial cells (hUCs) obtained, for instance, from bladder washing [1,2] could be used. After cell expansion *in vitro*, the hUCs are seeded on the biomaterial and the graft is used to reconstruct the urothelium that is lacking. Although these techniques would require two separate operations, it would be acceptable because patients with severe hypospadias usually require more than one surgical procedure [3].

The selection of an appropriate biomaterial for urothelial tissue engineering is critical, because the biomaterial should mimic the natural basement membrane of the urothelium as closely as possible and allow the underlying stroma to attach to the biomaterial. The mechanical properties should be adequate to prevent collapse of the constructed urethra, while being elastic enough to form tubular structures. Further, the biomaterial should be biocompatible, and degrade while the urothelium regenerates without excessive inflammation reaction. Also, from

*Author for correspondence (reetta.sartoneva@uta.fi).
†These authors contributed equally to the study.

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Table 1. Details of heat pressing parameters used to manufacture the different membrane structures.

	pressure (MPa)	temperature (°C)	time (s)	used moulds	membrane thickness (μm)
preliminary moulding					
PLCL	l0	110	90	PTFE	
PLA 96/4	l0	95	60	plain steel	
final moulding					
sPLCL	20	130	30	plain steel	190
tPLCL	20	130	45	PTFE	120
cPLCL	20	130	45	plain steel	150

a surgical perspective the biomaterial should be suturable and relatively easy to handle [4,5]. Lately, poly-α-hydroxy-acid-based biomaterials, such as polylactide (PLA), polyglycolide (PGA) and polycaprolactone (PCL), have been used in urothelial tissue engineering, with favourable results [6–8]. However, the mechanical properties of PGA or PLA membranes are not optimal because these materials are inelastic and have too high strength for the intended application. Additionally, the degradation rate of PGA is relatively rapid for urothelial tissue engineering [9–11]. Although PCL is highly elastic and strain is more than 700 per cent at breakage [10], the degradation rate of PCL is rather slow for urothelial tissue engineering, taking up to 2 years [10,12]. Various poly-L-lactide-co-ε-caprolactone (PLCL) compositions have been studied in tissue engineering applications [13–18], but the main focus has been on producing electrospun nanofibrous membranes. In our recent study [19], we showed that compression-moulded smooth PLCL membranes supported the proliferation and differentiation of hUCs better than human amniotic membrane. To the best of our knowledge, apart from our previous study, there are no other publications using this compression-moulded highly elastic PLCL copolymer for urothelial tissue engineering.

The aim of this study was to further develop and characterize the PLCL matrix for urothelial tissue engineering applications. In this study, we compared the mechanical properties of different lactide-based biomaterial membranes: smooth PLCL (sPLCL) and textured PLCL (tPLCL), and knitted PLA mesh with compression-moulded PLCL (cPLCL). In addition, we compared the effects of these materials on hUC morphology, viability and phenotype maintenance *in vitro*. Furthermore, we hypothesized that the topographically textured membranes tPLCL and cPLCL would enhance the hUCs' attachment and proliferation *in vitro* owing to the fact that surface texturing is generally known to facilitate cellular adhesion and proliferation [20–22]. The PLCL membranes are highly elastic and flexible; however, it has not been shown whether plain PLCL has adequate mechanical stability for urothelial applications. Therefore, we also studied the effect of a knitted PLA mesh on the degradation and mechanical behaviour of PLCL, and the usability of this kind of structure for urothelial applications [10].

2. MATERIAL AND METHODS

2.1. Materials

The polymer used for PLCL membranes was manufactured from 70/30 PLCL (Purac Biochem BV, Gorinchem, The Netherlands), with an inherent viscosity of 1.60 dl g^{-1}. The polymer used for knitted composite membranes was manufactured from polylactide (P(L/D)LA 96/4) (Purac Biochem BV), with an inherent viscosity of 2.12 dl g^{-1}.

2.2. Sample manufacturing

The tubular single jersey knitting made of PLA (P(L/D)LA 96/4) was produced on a circular knitting machine ELHA R-1s (Textilmaschinenfabrik Harry Lucas GmbH & Co. KG, Neumünster, Germany) from 16-filament fibres with a single filament thickness of 10–20 μm. The PLCL membranes were produced by compression moulding of PLCL granules using a NIKE hydraulic press (Hydraulics Ab, Eskilstuna, Sweden). Also, the PLA knitting used for composite membranes underwent preliminary heat pressing as described in table 1. The preliminary moulding of PLCL was performed between polytetrafluoroethylene (PTFE)-taped moulds. The preliminary moulding was followed by the final moulding into sPLCL (figure 1) with a final thickness of 190 μm; tPLCL (figure 1) with a final thickness of 120 μm; and cPLCL (figure 1) with a final thickness of 150 μm (table 1). The cPLCL samples were produced in three steps as the two preliminarily moulded components (the PLCL and PLA membranes) were compression moulded together into the composite membranes (table 1). Afterwards, the membranes were cut into samples, washed with ethanol and sterilized at 25 kGy before further *in vitro* experiments and characterization.

2.3. Material characterization

2.3.1. Hydrolysis

The hydrolysis was carried out at 37°C in a phosphate buffer solution (pH 6.1) to mimic the pH of urine. The size of samples was 10 × 50 mm (see thicknesses in table 1), and the weight was approximately 100 mg. The volume of buffer was above the required minimum (10 ml) for each sample (according to the International Standard, ISO 15814, 1999). The samples ($n = 6$) were incubated for 0, 2, 4, 6, 8, 10 and 12 weeks. The buffer solution was changed every two weeks, and pH was measured weekly using a SevenMulti pH meter (Mettler-Toledo GmbH, Schwerzenbach, Switzerland). Once the samples were removed from the solution, they were weighed wet and then tensile tested. After tensile testing, the samples were dried first in a fume chamber for a week and subsequently in a vacuum for

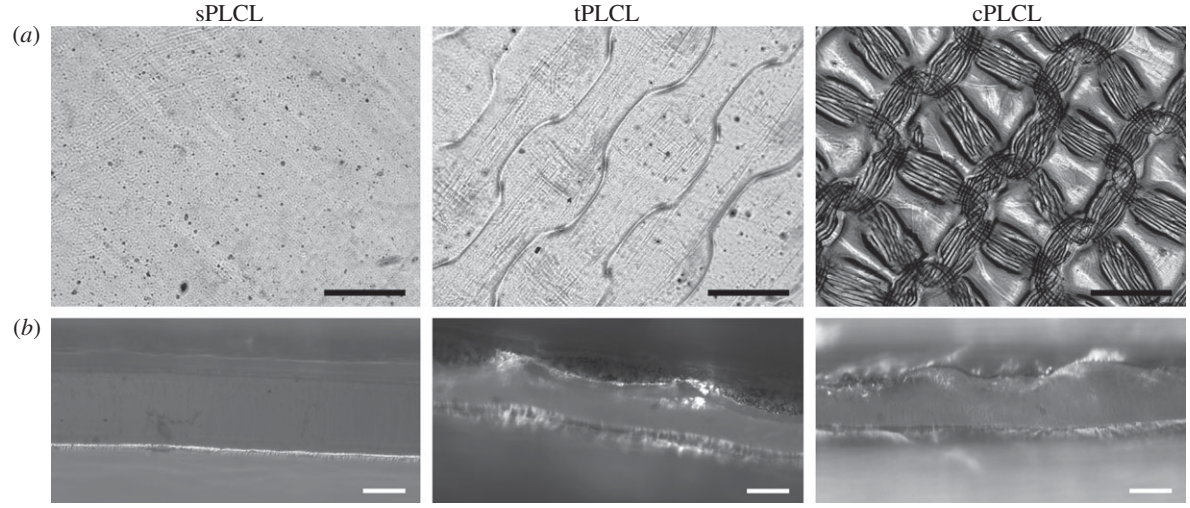

Figure 1. Light microscopic images of membranes without cells. (*a*) Images representing the surface topography of membranes. (*b*) The cross section of different membranes, depicting the surface alterations of the membranes studied. Black scale bars, 500 μm; white scale bars, 100 μm.

a week at room temperature, after which the samples were weighed again when dry.

2.3.2. Tensile testing

For wet samples ($n = 6$), after hydrolysis, the tensile testing was performed—and for dry samples prior to hydrolysis (zero-week samples). The tensile testing was performed with an Instron 4411 materials testing machine (Instron Ltd, High Wycombe, UK) at a cross-head speed of 30 mm min^{-1}. Pneumatic grips were used, and the gauge length was 25 mm.

As controls, sheep bladder samples ($n = 6$) were also tensile tested. The sheep bladder was washed with physiological saline and cut into 10 × 50 mm samples, prior to testing.

2.3.3. Differential scanning calorimetry

A differential scanning calorimeter (DSC Q 1000; TA Instruments, New Castle, DE, USA) was used to determine the glass transition temperatures (T_g) of the samples. Samples (weight 5 mg) were heated from −50°C to 150°C at a heating rate of 20°C min^{-1} ($n = 2$).

2.4. Cell isolation

The protocol by Southgate *et al.* [23] was used for cell isolation with minor modifications, and the isolation was performed as described previously [19]. Briefly, the tissue samples were cleaned and cut into small pieces and incubated overnight, to loosen the urothelial layer, in a solution containing 0.01 per cent HEPES buffer (1 M, *N*′-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; Sigma-Aldrich), 4×10^{-3} per cent aprotin (1 kIU μl^{-1}; Sigma-Aldrich), 0.1 per cent EDTA (Sigma-Aldrich), 0.01 per cent penicillin/streptomycin (Lonza, Verviers, Belgium) in Hank's balanced salt solution (Invitrogen, Paisley, UK) without Ca^{2+} and Mg^{2+}. The next day, 0.1 per cent trypsin (Lonza) was used to detach the cells from the urothelial sheet. The isolated cells were suspended in a defined urothelium medium (EpiLife, Invitrogen) and cultured in CellBIND T75 flasks (Sigma-Aldrich) at 37°C under a humidified atmosphere of 5 per cent CO_2 in air. The hUC passages 3 and 4, from three male donors, were used in the experiments.

2.5. Flow cytometric marker expression analysis of human urothelial cells

The hUCs were harvested and analysed after primary culture by a fluorescence-activated cell sorter (FACSAria; BD Biosciences, Erembodegem, Belgium), as described previously [19]. Monoclonal antibodies (MAbs) against CD44-PE, CD73-PE, CD105-PE, CD133-PE, CD166-PE (BD Biosciences), CD326-APC (Miltenyi Biotech, Bergisch Gladbach, Germany) and keratin8/18 (Cell Signaling Technology, Danvers, MA, USA) were used. MAb keratin8/18 was conjugated with IgG-alexa488 (Molecular Probes, Eugene, OR, USA). The analysis was performed on 10 000 cells per sample, and unstained cell samples were used to compensate for the background autofluorescence levels. The positive expression was defined as more than 50 per cent expression level.

2.6. Cell seeding

Before seeding the cells, biomaterial membranes were attached to the cell crowns (CellCrown48; Scaffdex, Tampere, Finland), after which the samples with cell crowns were attached to a 48-well plate leading to a 0.4 cm^2 cell culture surface area. The membranes were preincubated in urothelium medium at 37°C for 48 h. The cells were seeded onto each membrane at a density of 30 000 cells cm^{-2} in a medium volume of 30 μl. The cells were allowed to attach for 2 h, which after 0.4 ml of medium was added to each well.

2.7. Scanning electron microscopy imaging

Scanning electron microscopy (SEM) was used to evaluate the attachment and morphology of hUCs after 2 h, 7 days and 14 days of cell culture. After washing with Dulbecco's phosphate-buffered saline (DPBS), the cells were fixed with 5 per cent glutaraldehyde

(Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.4, Sigma-Aldrich) at room temperature for 48 h. Thereafter, the samples were dehydrated through a sequence of increasing concentrations (30%, 50%, 70%, 80%, 90%, 95% and 100%) of ethanol for 5 min. The final dehydration in 100 per cent ethanol was repeated, followed by critical point drying with liquid CO_2. A gold coating was sputtered on the sample, and the coated samples were examined with an SEM (Jeol JSM 6335F; Jeol Ltd, Japan) device. The SEM imaging was repeated twice, using two different cell donors.

2.8. Cell viability and proliferation

Live/dead fluorescent staining was used to evaluate cell viability after 7 and 14 days of cell culture, as described previously [19]. Briefly, the cells were incubated at room temperature for at least 30 min with a mixture of 0.25 µM calcein AM (green fluorescence; Molecular Probes) and 0.3 µM ethidium homodimer-1 (red fluorescence; EthD-1; Molecular Probes) in DPBS. A fluorescence microscope (Olympus IX51S8F-2; camera DP71) was used to image the viable cells (green fluorescence) and dead cells (red fluorescence).

The cell proliferation was determined by WST-1 analysis, measuring the mitochondrial activity of viable hUCs, after 2 h, 7 days and 14 days of cell culture. Briefly, the cells were washed with DPBS and incubated with 50 µl of premixed WST-1 (Premix WST-1 Cell Proliferation Assay System; Takara Bio Inc., Otsu, Shiga, Japan) and 500 µl of DPBS at 37°C for 1 h. Absorbance was measured with a microplate reader (Victor 1420 Multilabel Counter; Wallac, Turku, Finland) at 450 nm.

2.9. Phenotype characterization of human urothelial cells using immunostaining

Immunostaining with primary antibodies, cytokeratin (CK) 7 (1 : 400; Epitomics, CA, USA) and CK19 Ab-1 (1 : 500; Lab Vision, Fremont, CA, USA) was used to confirm the phenotype of hUCs after 7 and 14 days of cell culture, as previously described [19]. Briefly, the hUCs were fixed with 4 per cent paraformaldehyde (Sigma-Aldrich) and incubated overnight in primary antibody dilutions. Thereafter, secondary antibodies from donkey (1 : 400; Alexa-488; green fluorescence; Molecular Probes) were conjugated to primary antibodies. Finally, cell nuclei were stained with Vectashield (DAPI; blue fluorescence; Vector Laboratories, Peterborough, UK), and the cells were imaged with a fluorescence microscope (Olympus).

2.10. Statistical analysis

Statistical analysis was performed with SPSS v. 13 (SPSS, Chicago, IL, USA). After verifying normal distribution and homogeneity of variance, the effect of the culturing period and the effects of different materials on cell number were compared using one-way ANOVA. *Post hoc* (Tukey) tests were performed to detect significant differences between the culturing periods (2 h versus 7 days and 7 days versus 14 days) and between the different materials. Data were reported as the mean ± standard deviation (s.d.); $p < 0.05$ was considered significant.

3. RESULTS

3.1. In vitro *degradation* (*and tensile testing*)

The pH of the samples remained within the limits (6.05–6.15) that are given in the standard, throughout the hydrolysis for 12 weeks.

3.1.1. Dimensional stability during hydrolysis

The samples retained their structural stability during the hydrolysis. Minor variations in their dimensions could be detected after 12 weeks of hydrolysis (table 2). However, the sPLCL and tPLCL samples became fragile to handle after 10 weeks in hydrolysis. Also, both sPLCL and tPLCL were greatly degraded after the 12-week hydrolysis, and the samples became fragile and difficult to handle without complete disintegration. The composite samples (cPLCL) retained their structure best, as the knitted PLA mesh remained stable throughout the hydrolysis for 12 weeks. However, the PLCL membrane component in the composite samples was degraded at the 12-week time point, and fragmentation in the cPLCL membrane was observed between the PLA mesh loops.

Moderate dimensional changes (2–3%) were observed by week 6 (data not shown). Most of the dimensional changes detected during the hydrolysis were due to an increase or a decrease in thickness. After eight weeks of hydrolysis, the samples showed an increase of 7, 11 and 14 per cent from their original thickness for sPLCL, tPLCL and cPLCL samples, respectively. After eight weeks, the thickness started to decrease and, at the end of the hydrolysis at week 12, the thickness of the samples was close to their original values.

3.1.2. Weight change during hydrolysis

The weight of the tPLCL samples started to decrease after six weeks of hydrolysis and, for the sPLCL and cPLCL samples, after eight weeks of hydrolysis (data not shown). After 10 weeks of hydrolysis, the weight of the samples had decreased by 10 per cent for sPLCL, by 30 per cent for tPLCL and by 20 per cent for cPLCL samples. At the end of the hydrolysis, the weight of the samples had further decreased by 10 per cent for all samples when compared with the 10-week values.

3.1.3. Mechanical properties during hydrolysis

As the graphs in figure 2 indicate, the initial mechanical properties of all the samples decreased after sterilization. The sterilization affected the mechanical properties of sPLCL and tPLCL samples to the greatest extent.

The stresses at maximum loads of the samples were 21.3, 18.6 and 13.9 MPa for sPLCL, tPLCL and cPLCL samples, respectively, after sterilization (figure 2). During the hydrolysis for 12 weeks, the stresses at maximum loads of the sPLCL and tPLCL samples decreased steadily and, after six weeks of hydrolysis, the maximum loads of the samples had decreased to 5.7 MPa for the sPLCL and 5.9 MPa for the tPLCL samples. Owing to the degree of degradation, 10 weeks

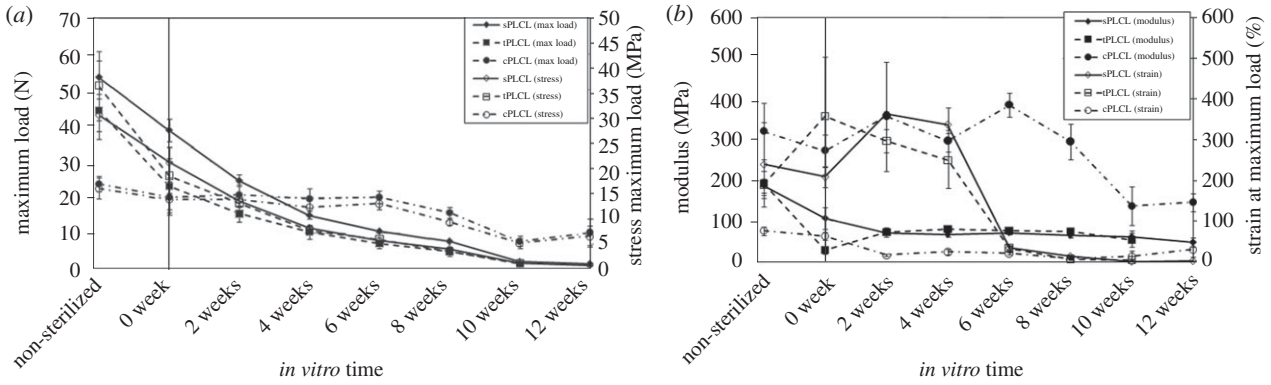

Figure 2. The mechanical properties of samples during hydrolysis. (*a*) Maximum load and stress at maximum load values and (*b*) modulus and strain at maximum load values.

Table 2. A summary of mechanical characterization and cell study results. T_g, glass transition temperature; minor change, maximum of 5%; +++, good; ++, average; +, low.

sample	dimensional change during hydrolysis	weight change during hydrolysis	T_g change during hydrolysis	mechanical properties (max load; stress at max load) compared with natural model[a]	strain compared with natural model[a]
hydrolysis studies					
sPLCL	only minor changes during 12 weeks	only minor changes until week 8	steady drop of T_g during 12 weeks	better than natural model until week 10	better than natural model until week 4
tPLCL	only minor changes until week 6	only minor changes until week 8	steady drop of T_g during 12 weeks	better than natural model until week 10	better than natural model until week 4
cPLCL	only minor changes until week 6	only minor changes until week 6	steady drop of T_g during 12 weeks	better than natural model during 12 weeks	lower than natural model even prior to hydrolysis

sample	cell adhesion	cell viability	cell morphology and confluency	proliferation	phenotype maintenance
cell studies					
sPLCL	++	+++	small, roundish or angular, confluent	+++	+++
tPLCL	++	+++	small, roundish or angular, confluent	+++	+++
cPLCL	+	+++	small, roundish or angular, fewer cells on PLA fibres	++	+++

[a]Tested native sheep bladder.

was the last time point for mechanical testing of tPLCL. The stresses at maximum loads of the cPLCL samples remained steady until week 6, but, after that, the values also started to decrease and, after 10 weeks, the stress was 5.1 MPa. The stress at maximum load of the sheep bladder samples was 0.16 ± 0.03 MPa.

The initial maximum loads of the samples were 38.7, 23.2 and 20.1 N for the sPLCL, tPLCL and cPLCL samples, respectively, after sterilization (figure 2). During the hydrolysis for 12 weeks, the maximum load of all the samples studied followed the same trend as the stress at maximum load values. After 10 weeks, the maximum load values were 2.0 N for the sPLCL and 1.3 N for the tPLCL samples. The maximum load values of the cPLCL samples at week 10 was 7.7 N and remained constant until week 12. The maximum load of the sheep bladder samples was 3.6 ± 0.6 N.

After sterilization, the modulus values of the sPLCL and tPLCL samples remained steady at 70 MPa throughout the hydrolysis (figure 2). However, the cPLCL samples had a modulus value of 300 MPa until week 8. After week 10, the modulus values of the cPLCL samples also dropped to 137.9 MPa. The modulus of the sheep bladder samples was 0.45 ± 0.12 MPa.

The strain values at maximum loads of sPLCL and tPLCL samples were between 200 per cent and 350 per cent at the beginning of the hydrolysis. Interestingly, the strain values at maximum load decreased after week 4, and, at week 6, the strains were 35 per cent for both samples. After that point, the strain values of sPLCL and tPLCL samples dropped steadily,

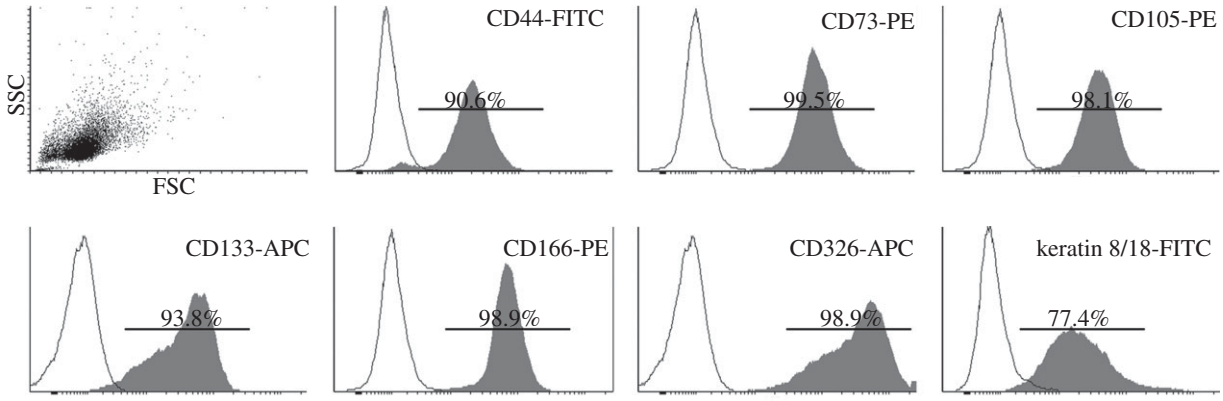

Figure 3. Flow cytometric marker expression analysis of hUCs after cell isolation. The dot plot image illustrates the homogeneity of the unstained cell population with regard to cell size (FSC) and surface complexity (SSC) of the unstained control samples. Relative cell number (y-axis) and fluorescence intensity (x-axis) of unstained control cells (empty histograms) and antibody-stained cells (filled histograms). The position of the filled histogram, representing the stained sample, illustrates the positive expression of hUCs; the more separate the empty and filled histograms are, the more positive the cells are for the specific marker. The bar demonstrates the average percentage of cells which express the specific antibody, and the expression level more than 50% was considered as a positive expression.

and, after the hydrolysis, the values were only 3.8 per cent for sPLCL and 0 per cent for tPLCL. On the other hand, the strain values at maximum load of the cPLCL samples remained steady, being 20 per cent during the hydrolysis, except for the zero-week samples, which had mean strain values of 70 per cent. The strain at maximum load of the sheep bladder samples was 85.1 ± 32.7 per cent.

3.1.4. Thermal properties during hydrolysis

The T_g values of the different sample series were almost identical (data not shown). A minor decrease in T_g values was seen after sterilization, from 23°C to 22°C. The T_g values of the samples dropped steadily during the hydrolysis, and, after week 6, the values were dropped to 20°C. After week 6, the T_g values started to decrease more, and at the end of the hydrolysis at 12 weeks, the T_g values were 16°C.

3.2. Flow cytometric analysis

Prior to cell seeding, the hUCs were identified using flow cytometry (figure 3). The flow cytometric analysis showed that the population of isolated hUCs was homogeneous with regard to cell size and surface complexity of unstained control samples. The hUCs expressed extracellular matrix adhesion marker CD44, endothelial markers CD73 and CD105, and epithelial markers CD133, CD166 and CD326. Furthermore, the hUCs expressed the intracellular marker keratin 8/18, which is a specific marker for epithelial cells. These studied markers have previously been used to characterize hUCs [19,24–26].

3.3. Attachment and morphology of hUCs

According to the SEM imaging (figure 4), there were no remarkable differences in cell attachment or morphology between the sPLCL and tPLCL at the 2 h time point. However, on the cPLCL the attachment of hUCs was inferior on the PLA fibres compared with the plain PLCL regions (table 2).

After 7 days of cell culture, the hUCs on the sPLCL, tPLCL and cPLCL were morphologically similar: small and oval or roundish. Further, the hUCs had already formed clusters despite not being spread homogeneously over the whole cell culture area. At the 14 day time point, the hUCs on the sPLCL, tPLCL and cPLCL had formed a confluent cell layer and adhered to the adjacent cells. However, on the cPLCL, the hUCs spread unevenly, preferring the PLCL regions. On all the membranes, the hUCs were small, and the morphology of cells varied from roundish or oval to cubic or angular. Moreover, no substantial differences were detected in the hUCs' morphology, regardless of culture surface.

3.4. Viability and proliferation of human urothelial cells

The live/dead staining (figure 5*a*) demonstrated that the cells were viable on all the membranes studied, and the number of dead cells was negligible after 7 days of culture. Furthermore, the hUCs maintained their viability during the 7–14 days culturing period, and no increase in dead cell number was detected (table 2).

The WST-1 measurement revealed significant differences between the materials (figure 5*b*). The number of hUCs increased significantly from the 2 h time point to the 7 days time point in all the biomaterials studied; sPLCL ($p < 0.001$), tPLCL ($p < 0.001$) and cPLCL ($p = 0.005$). The WST-1 measurement showed no significant differences in cell attachment between the biomaterials studied at the 2 h time point. At the 7 and 14 days time points, however, the sPLCL and tPLCL supported the hUCs' proliferation better than the cPLCL, albeit that the difference was statistically significant compared with sPLCL at the 7 days time point ($p = 0.042$) and compared with tPLCL at the 14 days time point ($p = 0.011$).

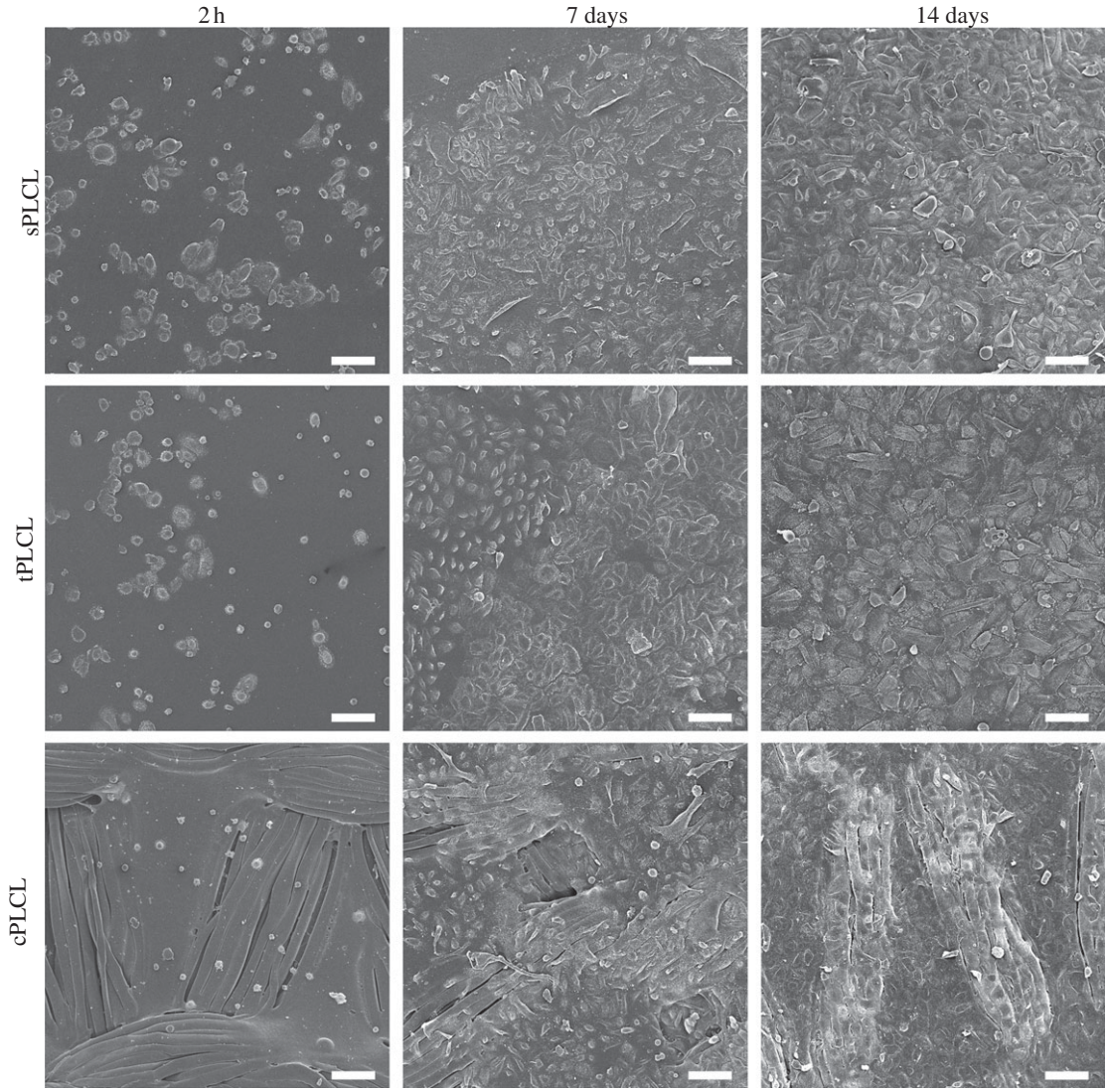

Figure 4. SEM images of hUCs cultured on sPLCL, tPLCL and cPLCL for 2 h, 7 days and 14 days representing the adhesion and morphology of cells. Scale bars, 100 μm.

3.5. Phenotype characterization of human urothelial cells using immunostaining

The hUCs expressed CK7 and CK19 (green fluorescence) after 7 days (data not shown) and 14 days of cell culture (figure 6). The CK19 and CK7 expression of hUCs on the sPLCL, tPLCL and cPLCL was considered intensive at both time points; moreover, the intensity of expression did not substantially change during the 7–14 days assessment period, and the hUCs maintained their phenotype (table 2).

4. DISCUSSION

To the best of our knowledge, this is the first study to characterize the mechanical properties of compression-moulded PLCL membranes for urothelial tissue engineering and to compare the effects of these membranes with regard to the viability, morphology, proliferation and phenotype maintenance of hUCs. The influence of topographical texturing of PLCL using sPLCL, tPLCL and cPLCL membranes was also studied, which has not yet been reported with hUCs.

The *in vitro* degradation studies indicated that the PLCL samples exhibited moderate dimensional stability and mass loss, relatively high elongation and also moderate thermal property changes until week 6. Therefore, these results demonstrate that these highly elastic and pliable membranes theoretically meet the prerequisites to function properly in urothelial tissue engineering applications and also in other tissue engineering applications in which elasticity and pliability are important [15]. Compared with the study by Eberli *et al.* [27] of mechanical properties of native porcine bladder, i.e. tensile stress at break less than 1 MPa and strain approximately 130 per cent, our results indicate that PLCL possesses similar mechanical properties. Furthermore, our tensile tests on native sheep bladder show that the values of PLCL samples maintain sufficient tensile strengths at least until week 4, after which the plain PLCL samples showed decreased strain values compared with the natural tissue. The modulus values of all of the studied PLCL samples were higher than the respective modulus values of natural sheep bladder, indicating more rigid behaviour of the PLCL samples. The tensile strength and modulus values decreased

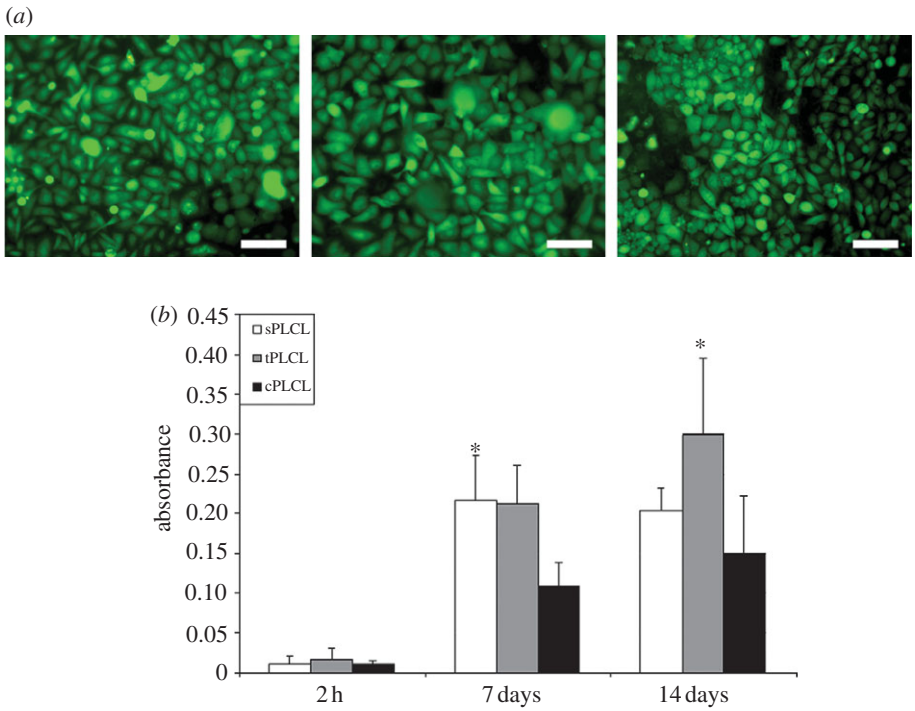

Figure 5. (*a*) Representative images of viable (green fluorescence) and dead (red fluorescence) hUCs attached to sPLCL, tPLCL and cPLCL at the 14 days time point. (*b*) The number of hUCs cultured for 2 h, 7 days and 14 days on sPCLC (white bars), tPLCL (grey bars) and cPLCL (black bars). Results are expressed as the mean ± s.d., $n = 6$. $^*p < 0.05$ with respect to cPLCL. (*a*) Scale bars, 100 μm.

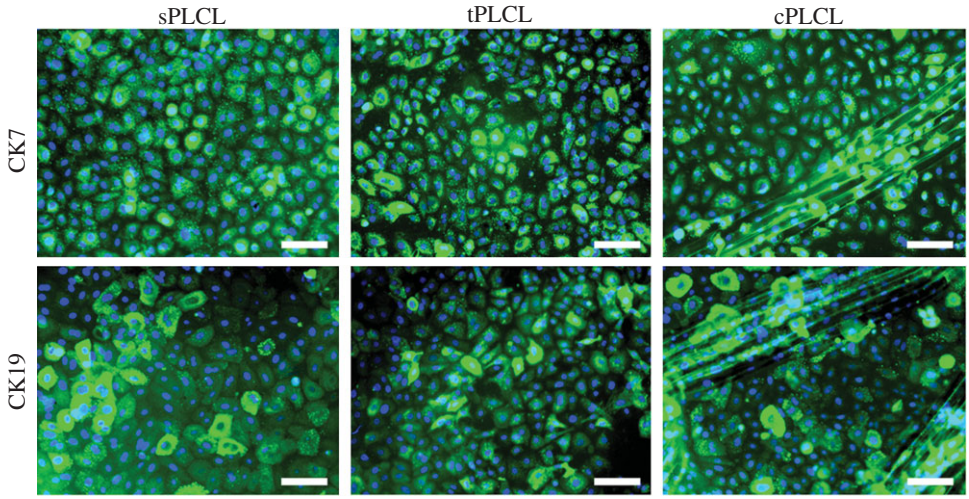

Figure 6. Immunofluorescence images of hUCs cultured on sPLCL, tPLCL and cPLCL for 14 days. Antibodies CK7 and CK19 (green fluorescence) were used to confirm the urothelial phenotype. Scale bars, 100 μm.

after the sterilization, and it is assumed that the tensile strength and the modulus values of these samples would also highly decrease *in vivo* when the cells start to affect the PLCL matrix [28].

As a manufacturing method, compression moulding was selected because relatively thin membranes, but possessing adequate mechanical strength, can be fabricated. Also, no solvents are needed for compression moulding; therefore, no solvent residues are involved. The different manufacturing methods of the membranes had an effect on the degradation behaviour of the samples studied. The results showed that the tPLCL samples were the first to start to degrade and also had lower mechanical properties than the sPLCL samples. This may be due to the fact that the tPLCL samples only had 63 per cent of the original thickness of the sPLCL samples. This was due to the processing method in which the structuring of the tPLCL samples between the PTFE moulds led to thinner sample structures. In addition, the tPLCL samples had a textured surface, and therefore the surface area of the samples was greater than in the sPLCL membranes, leading to higher degradation rates [10,29].

The cPLCL samples showed degradation results similar to those of the sPLCL and tPLCL samples within the PLCL matrix; therefore, the difference, for example, in mechanical properties was mainly affected by the PLA mesh in the structure. The PLA mesh in

the composite samples made the samples tougher than expected and with less ductility than the plain PLCL samples [29,30]. In our application, the elastic properties of the samples are preferable to the toughness of the cPLCL samples. Therefore, the cPLCL samples did not give any additional value compared with the plain PLCL samples. Because of the textured surface, the tPLCL samples were the most pliable samples and the easiest to handle before hydrolysis. The texturing was one of the major reasons that these samples degraded first because texturing increases the surface area of the membrane. It also accounted for the mechanical properties of the tPLCL membrane when compared with the other membranes studied [12].

What should be taken into account is that the samples started to degrade more noticeably after six to eight weeks of hydrolysis and thereafter showed a relatively rapid degradation process. The urothelium is known to regenerate rapidly after injury [31,32], thus the degradation rate is probably adequate for the urothelial tissue engineering applications; nevertheless, in the future this also has to be verified *in vivo* [5,12]. During surgical implantation into a patient, a biomaterial gives the regenerating urothelium mechanical support. Moreover, the biomaterial functions as a basement membrane for urothelial cells. While the biomaterial degrades and the mechanical properties decrease, the urothelial cells should secrete extracellular matrix, forming cells supporting the basement membrane. The biomaterial should also attach to the underlying stromal layer, which also gives mechanical support for the urothelium while the biomaterial degrades [10,33].

A pH of 6.1 most probably affected the degradation time of the membranes studied, as PLCL degradation occurs by hydrolysis of an ester bond, forming lactic acid and caproic acid. This is catalysed by acidic conditions [17]. We chose this pH because it mimics the pH in the lumen of the native urethra and therefore it can be assumed that the degradation rate of these membranes most probably is similar to that in the urinary tract. At the beginning of graft implantation, the urothelial barrier has not been completely developed; therefore, the biomaterial will be in contact with acidic urine. In addition, the pH value of 6.1 has previously been used to study the mechanical characteristics of biomaterials for urothelial applications [34].

For urothelial applications, several cell sources have been used. In addition to primary urothelial cells, oral keratinocytes and foreskin epidermal cells have been studied for urethral reconstruction [35–37]. Fossum *et al.* [33] used urothelial cells on acellular dermis for clinical studies to reconstruct urethras for paediatric patients. In this study, we used hUCs taken from ureters, because these cells are similar to the cells in the proximal parts of the urethra. Although the distal part of the urethra is covered by squamous epithelium, the urothelial cells are a potential cell source for future clinical urethral reconstruction applications, as demonstrated by Fossum *et al.* [33]. We used flow cytometry to characterize the hUCs after isolation and also to verify the repeatability of our isolation protocol. The hUC characterization data were consistent with our previously reported results [19], which further indicates the reliability of our method. Otherwise, the hUCs have been characterized in only a few studies. These results were in parallel with the earlier results obtained for urothelial cells [24,38], and hUCs expressed all the markers studied as expected.

The attachment of cells was studied 2 h after the initial cell attachment, using SEM imaging and quantitative WST-1 measurement, indicating that the mechanical texturing using tPLCL or cPLCL provided no additional advantage to hUC attachment. This result contradicted our hypothesis, which was that the mechanical texturing would facilitate cell attachment owing to the well-known advantages of surface structuring [20,21]. On the basis of SEM imaging and live/dead staining, the hUCs on the cPLCL samples seemed to prefer PLCL over PLA areas, because the hUCs attached scantily and covered the PLA fibres unevenly, suggesting the weaker adhesion of hUCs to the PLA fibres.

According to the live/dead staining, all the biomaterials supported the viability of hUCs. No significant change in hUC viability was detected between the 7 and 14 days time points, suggesting good biocompatibility of the studied biomaterials as expected, because PLCL with different compositions has previously been studied for tissue engineering applications, with encouraging results [19,39,40]. The morphology evaluated by SEM imaging was consistent with the live/dead staining. The hUCs on the sPLCL, tPLCL and cPLCL samples were morphologically similar, exhibiting the normal shape of UCs (small and roundish or cuboidal), further indicating good biocompatibility for hUCs.

An increase in cell number was detected during the culturing period with both SEM imaging and WST-1 measurement. After 14 days of cell culture, the hUCs were confluent on the sPLCL, tPLCL and cPLCL samples. Additionally, the WST-1 measurement demonstrated that the cell number on the cPLCL samples was lower than on the sPLCL and tPLCL samples. Interestingly, topographical texturing with tPLCL or cPLCL yielded no additional advantage in cell proliferation compared with sPLCL, suggesting that the material selection has more effect on hUC attachment and proliferation than mechanical texturing.

The immunostaining demonstrated the expression of CK19 and CK7 on all the biomaterials studied, as expected, because both CK7 and CK19 are present in all layers of the native urothelium: basal, intermediate and superficial layers [23,41]. These results are in concordance with our earlier results, in which CK19 expression was also demonstrated [19]. The marker expressions remained intensive during the culturing period, which indicates a stable phenotype during the assessment period [2,19,23,42].

The main limitation of this study was that it only demonstrates the *in vitro* effects of different PLCL-based membranes on urothelial cell response. Furthermore, the *in vitro* hydrolysis conditions used always differ from the natural environment. Therefore, *in vivo* studies are needed to verify the potential of PLCL for urothelial tissue engineering. Despite these limitations, our results showed that both sPLCL and tPLCL exhibited suitable properties for urothelial

tissue engineering applications. From a surgical point of view, the texturing of tPLCL made the handling of the membrane easier than that of sPLCL; therefore, tPLCL membrane will be selected over sPLCL membrane for future *in vivo* studies.

5. CONCLUSION

Complementing our previous results, this study further verifies the potential of PLCL for urothelial tissue engineering applications. As the cell studies indicated, the sPLCL and tPLCL membranes supported the hUCs' attachment and proliferation better than the cPLCL membranes. Surprisingly, the material itself, rather than the mechanical texturing, appeared to have more effect on hUC growth. Furthermore, the *in vitro* degradation and mechanical properties of the PLCL membranes, especially tPLCL, showed a capability to function properly in urothelial applications.

Human urothelial tissue samples were obtained from normal ureters of child donors, aged 1, 4 and 12 years, during routine surgery in Tampere University Hospital, with the approval of the Ethics Committee of Pirkanmaa Hospital District (Tampere, Finland, R071609).

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Autologous adipose stem cells in treatment of female stress urinary incontinence; results of a pilot study

Kuismanen K[1,2], Sartoneva R [2,3,4,], Haimi S [3,5], Mannerström B [3,4,], Tomas E[1], Miettinen S [3,4,], Nieminen K[1]

1. Tampere University Hospital, department of Obstetrics and Gynaecology, Tampere, Finland
2. University of Tampere, School of Medicine, Tampere, Finland
3. University of Tampere, Adult Stem Cell Group, BioMediTech, Tampere, Finland
4. Tampere University Hospital, Science Center, Tampere, Finland
5. University of Twente, Department of Biomaterials Science and Technology, Enschede, The Netherlands

Corresponding author: Kuismanen Kirsi, Tampere University Hospital, department of Obstetrics and Gynaecology, PL2000, 33521 Tampere, Finland.

Tel. +358 3 3116 9812, +358 40 5348024.

E-mail kirsi.kuismanen@pshp.fi

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Abstract

Purpose

The purpose of our study was to find out if transurethral injections of autologous adipose stem cells (ASCs) are an effective and a safe treatment for female stress urinary incontinence (SUI).

Materials and methods

We treated five SUI patients with ASCs combined with bovine collagen gel (Contigen™) and saline. Prior to the treatment, the ASCs were isolated from subcutaneous fat and expanded for three weeks in a Good Manufacturing Practice (GMP) level laboratory. The mixture of ASCs and collagen (Contigen™) was injected transurethrally via cystoscope. Additionally, viability, multipotency and surface marker profile of ASCs was analysed *in vitro*. Patients were followed 3, 6 and 12 months after the injections. The primary end point was a cough test to measure objectively the effect of the treatment. Validated questionnaires were used to determine the subjective cure rate.

Results

After six months, one out of five patients displayed a negative cough test with full bladder filled with 500ml saline. At one year, the cough test was negative with three patients; two of them were satisfied with the treatment and didn't wish for further treatment for SUI. Validated questionnaires showed some subjective improvement in all five patients.

Conclusions

This is the first study describing the use of autologous ASCs in combination with collagen gel for female SUI treatments. Thus far, the treatment with autologous ASCs has proven safe and well tolerated. However, the feasibility and efficacy of the treatment were not optimal; therefore additional research is needed to develop SUI injection therapies.

Introduction

Urinary incontinence is a common health problem affecting a large number of women. Approximately 35% of women over 18 years in Europe reported involuntary urine loss [1]. Stress urinary incontinence (SUI) is the most common type and it is defined as involuntary loss of urine on effort or physical exertion. Urgency urinary incontinence (UUI) is associated with feeling of urgency, and mixed urinary incontinence (MUI) is a combination of the two aforementioned types [2]. SUI is further categorized as urethral hypermobility, intrinsic sphincter deficiency (ISD), or both [3]. Furthermore, the prevalence of incontinence increases with age [4].

The urethral continence control system is a vital component in stress urinary incontinence. It consists of the sphincteric unit and the vaginal support system. The urethra is a multilayered structure consisting of striated muscle, smooth muscle, connective tissue, submucosal vascular plexus, and a lining epithelium. The combined actions of these tissues serve to create a wall tension that compresses the lumen closed. Striated muscle has been shown to be responsible for one third of the total intraurethral pressure, other third being exerted by the urethral vascular bed, and the remaining third by the smooth musculature and connective tissues [5 6].

Mid-urethral slings have become first-line surgical treatments for SUI because they are minimally invasive, have excellent short-term success rates and good long-term success rates, and a short learning curve [7]. However, there are patients who have a SUI unresponsive to operative treatment, and patients who are not suitable for mid-urethral sling operation. Additionally, there are patients who do not want artificial synthetic material as their treatment.

Tissue engineering offers an attractive treatment method to regenerate sphincter muscle. Previously, various different cell sources, such as skeletal muscle-derived stem cells (SkMSCs), mesenchymal stem cells (derived from bone marrow, BMSCs) and adipose stem cells (ASCs), have been studied for treating urinary incontinence. The SkMSCs and BMSCs would be a potential alternative for incontinence therapy. However, when compared to ASCs, the major limitation of SkMSCs and BMSCs is the difficulty to obtain these cells in large quantities. Furthermore, the isolation site injury for ASCs is minimal and they are readily expanded *in vitro*. These features are important when considering the cell source for clinical applications. ASCs, like BMSCs, have been shown to undergo myogenic, adipogenic, osteogenic and chondrogenic differentiation [8]. Moreover, the ASCs have been used for clinical applications in different surgical areas and are considered safe for clinical use [9].

The aim of this study was to find out whether the adipose stem cells could provide a new and a possibly effective alternative to invasive surgical treatment of SUI. To our knowledge, this was the first study of transurethral injections of autologous ASCs in combination with collagen (Contigen™) in treatment of SUI or MUI.

Materials and methods

Patients were recruited from the Tampere University Hospital outpatient clinic and they were patients who did not primarily want the mid-urethral sling operation. Two of them had been operated previously **(table 1)** with unsuccessful results. The Ethics Committee of Pirkanmaa Hospital District approved the study pilot. Furthermore, all patients signed a written informed consent of the study.

After gynaecological examination, diagnosis of either pure SUI or predominantly stress MUI was made according to anamnesis, a positive cough test and urodynamic evaluations. Validated questionnaires – Urinary Inventory Stress test UISS, Detrusor Instability Score DIS, Incontinence Impact Questionnaire-short form IIQ-7 and Urogenital Distress Inventory-short form UDI-6 (with scores 0-100, lower scores reflecting better QoL) and the bother of incontinence in patients life according to Visual Analogue scale VAS (ranging 0-10)[10 1112] – were filled, and exclusion of infectious diseases (hepatitis B and C, HIV, syphilis) was conducted.

The subcutaneous fat was collected from patients´ lower abdomen under local anaesthesia. Approximately 0.3-0.5 dl of fat was obtained from six patients. However, because of bacterial contamination (p*ropionibacterium acnes* in repeated samples), the treatment was refrained from one patient. In addition, 50 ml of autologous serum was obtained for the expansion of clinically used ASCs. The ASCs were then isolated and augmented as described later in this article. Mixture of ASCs and collagen (Contigen^{TM}, Bard Medical, Covington, USA) was injected transurethrally via cystoscope under local anaesthesia. The injections were placed directly under mucosa: 1,5 cm distal from the urethral neck at 3 and 9 o′clock, injected volume being 2.4-4 ml per patient. Additional two concomitant injections of ASCs mixed with saline solution (volume 2 ml) were performed 2 mm more distally in order to bring the ASCs in contact with the urethral musculature.

The patients were followed at 3, 6 and 12 months after the injections by a gynaecological examination, a vaginal ultrasonography, a cough test, a 24 h pad test, standardized questionnaires and urodynamic evaluations (at 6 months).

The primary outcome measure was the cough test. Other outcome measures were the 24-h pad test, urodynamic evaluations (maximal urethral pressure MUCP, urethral stress profile), and patients' evaluations on their quality of life.

Stem cell isolation and preparation for injection

The isolation and expansion of ASC was done in a validated cleanroom (Biomeditech, University of Tampere) following EU GMP quality system guidelines.
The cell isolation, expansion, karyotyping, sterility, endotoxin and mycoplasma testing were performed as described previously [9 13]. Briefly, the adipose tissue was minced in to small pieces and digested with collagenase NB-6 (Life Technologies, CA, USA; GMP grade; SERVA Electrophoresis GmbH, Heidenlberg, Germany) in a 37°C incubator for 60 min while mixing by pipeting up and down every 20 min. After centrifuging and lysing the red blood cells, the pellet was suspended in to the basal medium (BM) containing 15 % of autologous serum in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12, Life Technologies). The isolated cells were expanded for 3 to 4 weeks in a BM. When nearly (90 %) confluent, the cells were mechanically detached using cell scraper (Nunc, Life

Technologies) and passaged. For the injection therapies we used passages 3 to 4, which were the lowest possible passages to get adequate amount of cells.

Half of the freshly isolated ASCs, were blended with 2.1 ml of collagen (Contigen™) and the rest of the ASCs were blended with 0.9% NaCl. The amount of cells used for injection therapy varied from 2.5x10^6 to 8.5x10^6 cells depending on the patient. The live/dead staining was used to evaluate the viability of ASCs in Contigen™ prior to the injection therapy as previously described [13].

In vitro analyses

Cell expansion

For the following *in vitro* analyses, the cells were expanded *in vitro* in basal medium (BM) consisting of DMEM/F12 supplemented with 15% human serum (Lonza, Basel, Switzerland) and 1% GlutaMAX (Life Technologies).

Flow cytometric surface marker expression analysis

The ASCs (n=5) at passages 5 to 6 were analysed with a fluorescence-activated cell sorter (FACSAria; BD Biosciences, Erembodegem, Belgium). Antibodies against CD14-PECy7 (BD Biosciences), CD19-PECy5 (BD Biosciences), CD34-APC (Immunotools GmBH Friesoythe, Germany), CD45-, CD49d-PE (BD Biosciences), CD73-PE (BD Biosciences), CD90-, CD105-, HLA-ABC-PE (Immunotools), and HLA-DR-PE (Immunotools) were used. The analysis was performed on 10 000 cells per sample, and unstained cell samples were used to compensate for the background auto-fluorescence levels. The surface marker expression > 2 % was defined as a positive expression.

Differentiation analyses

The myogenic, adipogenic, osteogenic and chondrogenic differentiation analyses were carried out in order to verify the multidifferentiation potential of ASCs (n=5, passages 7 to 10). All the cultures were maintained for 14 d in differentiation conditions. The BM supplemented with antibiotics (P/S; 100 U/ml penicillin and 0.1 mg/ml streptomycin; Life Technologies) served as a control medium.

For myogenic differentiation, the ASCs were plated onto fibronectin (Sanquin, The Netherlands) coated wells with a density of 2631 cells/cm^2. The ASCs were cultured in myogenic medium, containing 5 ng/ml of transforming growth factor beta 1 (TGFβ1; hBA-112, Santa Cruz Biotechnology, Heidelberg, Germany) in a BM for 14 d. The myogenic differentiation was verified by immunostaining using smooth muscle protein 22-alpha (SM22-α, 1:100, Abcam, Cambridge, UK), alpha smooth muscle actin (α-SMA, 1:100, Abcam), and myosin heavy chain II (MHCII, 1:200, Thermo Scientific, Illinois, USA) as primary antibodies. The cells were fixed with 4 % paraformaldehyde (PFA; Sigma-Aldrich, St. Louis. US) and incubated in primary antibodies. Thereafter, secondary antibodies from goat, donkey and donkey (1:200, Life Technologies), respectively, were conjugated to primary antibodies. Finally, the cells were mounted with Vectashield (Vector Laboratories, Burlingame, US) and imaged with the fluorescent microscope (Olympus).

The adipogenic, osteogenic and chondrogenic differentiation have been described in detail previously [13]. Briefly, the adipogenic differentiation was performed by culturing ASCs in adipogenic medium at the density of 2x10^^{4} cells/ cm^2. After 14 d the adipogenesis was verified with Oil Red O staining (Sigma). For the osteogenic differentiation, 2500 cells/ cm^2

were incubated in osteogenic medium, and the osteogenic capacity was studied using alkaline phosphatase (ALP) staining (Sigma). A micromass culture method was used for chondrogenic differentiation, and the chondrogenic potential was verified by Alcian blue staining (Sigma).

Results

Clinical results

Five patients reached one-year follow-up. At six months, one out of five patients displayed a negative cough test with full bladder filled with 500 ml saline. At one year, the cough test was negative with three patients and two of them were satisfied and did not wish for further treatment for SUI. There was also improvement according to the 24 h pad-test with the objectively cured patients **(table 2)**. There were no changes in either urodynamic parameters or in urine residual volume in any of the patients.

There was subjective improvement with all five treated patients according to the UISS, IIQ-7, UDI-6, VAS **(table 3),** however not in all questionnaires of the first three patients**.** The two patients who benefited from the ASC treatment were consistent in their answers and were subjectively cured or improved according to the UISS-, IIQ-7-, UDI-6- and VAS-questionnaires. Three of the patients have been operated on after the one-year follow-up.

With the exception of small haematomas, there were no adverse events from the adipose tissue collection. There were no major complications (urinary retention, heamaturia, urinary infection) after the transurethral injections. One patient displayed mild pollacis- and dysuria that resolved spontaneously within a week.

In vitro results

After ASC isolation, the cells proliferated rapidly in autologous serum containing medium. The live/dead analysis prior to injection therapy confirmed the viability of ASCs in the Contigen^{TM}: the majority of cells were viable and only a few dead cells were detected (**Figure 1).**
The surface marker expression analysis of the ASCs showed a positive expression for adhesion molecules CD49d, CD73 and CD105; extracellular matrix protein CD90; and MHC class I isotype HLA-ABC. Minor or moderate expression of markers CD14, CD19, CD34, CD45, and MHC class II isotype HLA-DR were detected, which suggested a low number of cells of hematopoietic and angiogenic lineages **(table 4).**
The multidifferentiation potential of ASCs was demonstrated by their capability to differentiate towards myogenic, adipogenic, osteogenic and chondrogenic cell lineages (**Figure 2**). After 14 d of exposing the ASCs to adipogenic medium, the lipid droplets were evident, which confirmed the ASCs potential to differentiate towards adipocytes. The positive ALP staining verified the osteogenic potential of ASCs. The chondrogenic differentiation was detected with Alcian blue staining for cartilage specific glycosaminoglycans. The ASCs showed positive expression for all the used myogenic markers; SM22-α, α-SMA and MHCII, thus verifying the myogenic potential of these cells.

Discussion

The purpose of this study was to find out whether transurethral injections of autologous ASCs with collagen could be safe and effective for SUI or MUI treatment. In this study, two out of five patients had a recurrent SUI, while two patients had mixed urinary incontinence. Two patients had low MUCP, and the BMI of all the patients in the study was ≥25. All of these factors (overweight, mixed incontinence, previous continence surgery and intrinsic sphincter deficiency) are significant independent predictors for mid-urethral sling failure [14]. Recurrent incontinence is not an ideal target for any method of treatment. The first three patients did not benefit from the cellular therapy, which may be at least partly due to the introduction of a new method.

Prior to the injection therapy, the adipose tissue was readily collected under local anaesthesia. The tissue collection caused some discomfort for the patients, and with the patient who was not eventually treated because of bacterial contamination in repeated samples there was postoperative haemorrhage that was managed conservatively. The transurethral injections were relatively easily implemented, and the patients were able to return to their everyday activities within the same day. The injections under local anaesthesia were well tolerated and there were no severe complications.

To the best of our knowledge, ASCs have not been previously used clinically for female SUI treatments. However, nowadays stem cell therapies have been under extensive research [15], and prior to this study some clinical studies on cell-based injection therapies have been published. Mitterberger et al demonstrated an efficacy of cell-based injection therapy using myoblasts and fibroblasts mixed with Contigen to treat female SUI [16 17]. However, the primary cells are not the optimal choice due to the difficulties in high yield expansion of cells. In addition to primary cells, autologous muscle-derived stem cells (MSCs) and allogenic umbilical cord blood SCs (CBSC) have been both used in a few clinical trials to treat female SUI patients with promising results [8 15]. Carr *et al*. used autologous MDSCs to treat 8 female SUI patients. They demonstrated an improvement of SUI symptoms in 5 patients after one-year follow-up, though only one patient was totally continent [18]. Furthermore, Sebe *et al.* treated 12 female patients with autologous MDSCs, and after one-year follow-up 3 patients were dry and an improvement of SUI was detected in 7 patients [19]. Additionally to MDSCs, Lee *et al*. used allogenic CBSCs in one clinical study to treat 39 female SUI patients. They demonstrated a 36.1 % cure rate after the one-year follow-up [20]. These treatments were demonstrated as a safe method and no severe adverse effects were detected. When compared to the aforementioned cell sources, the main advantage of ASCs is that the yield of stem cells from adipose tissue is much higher, and the isolation and expansion of ASCs is also relatively easy [15 21]. Furthermore, the injection therapy with ASCs was detected as a potential method to treat male urinary incontinence [22], and therefore we wanted to study the efficacy of ASCs for female urinary incontinence. Our approach was to use multipotent autologous ASCs with bulking agent Contigen™ for transurethral injection therapy.

The mesenchymal origin was confirmed prior to the clinical injections using FACS analysis after the subsequent cells expansion of ASCs in laboratory facilities. The fact that the analysed ASCs were at higher passages compared to the ASCs used for injection therapies could have slightly affected the expression results; however, the expression results were concordant with the previous results for ASCs [13 9 23]. Compared to the previous studies, the ASCs in our study expressed the hematopoietic markers; CD14, CD

34, and CD 45 slightly more. This difference in expression results could be explained with the patient variation, which is typically high in ASCs [21] [23]. In addition to adipogenic, chondrogenic and osteogenic differentiation, several studies have exhibited the ASCs' capability to undergo myogenic differentiation [24] [25].The ASCs used in this study were verified to be multipotent, as they also had a myogenic capacity. Compared to other cell sources, the main advantage of ASCs is that the yield of stem cells from adipose tissue is much higher [15].

Previously, ASCs have been used in several *in vivo* studies to treat SUI with potential results. Lin G et al. showed that transplantation of ASCs via urethral or intravenous injection was an effective treatment and/or prevention of SUI in a preclinical setting in rats [25]. Fu G et al. induced rat ASCs to differentiate into myoblasts *in vitro*, prior to transurethral injection therapy and showed that the myoblasts served an important function in improving the urine controlling ability [24]. Furthermore, Wu G et al showed that ASCs transplanted under the urethral mucosa of pudendal nerve mutilated rats improved maximum bladder capacity, abdominal leak point pressure, maximum urethral closure pressure, and functional urethral length in urodynamic testing compared to the control group [26].

Although traditionally the ASCs regenerative capacity was associated with their plasticity, their therapeutic effects appear to be particularly due to their paracrine function through the secretion of a broad range of bioactive molecules. Their potential mechanism of action might be related to immunomodulation, support of growth and differentiation of local stem and progenitor cells, proangiogenic action, chemo-attraction, anti-scarring and anti-apoptosis effects [27].

New techniques in many tissue regeneration applications are emerging, but mid-urethral slings are still the first-line surgical treatments for SUI. Reported cure rates of mid-urethral sling operations rise even up to ninety percent [28]. Furthermore, other treatment methods such as bulking agents are an option especially for patients who are unresponsive to traditional treatments or who are not suitable for surgical operation. However, injections with bulking agents have not been as effective as surgical methods, and the main problem has been the relatively poor sustainability of the bulking effect [29]. The short term subjective cure rate with plain Contigen™ has been low in women with severe sphincteric incontinence [30]. Several investigators have produced variable results from 23% to 83% cure rates at 1 and 2 years of follow-up after collagen therapy [31].

In our pilot study, we chose bovine collagen (Contigen™) to be the carrier of the autologous ASCs, since it is a widely studied biomaterial in SUI injection therapy. Furthermore, it is the only commercially available collagen gel accepted for clinical use. Additionally, collagen is shown to be biocompatible and biodegradable [32]. Our results also showed that ASCs remain viable when combined with Contigen™. However, Contigen™ was not the ideal carrier for cells due to its poor mechanical properties. When Contigen™ was combined with ASCs, the collagen gel became more liquid, which may have affected the overall bulking efficacy of the injection therapy. More preclinical studies are needed to develop an ideal carrier material for the stem cells.

Conclusions

In this pilot study, we studied the effect of ASCs in combination with injectable bulking agent to treat female SUI. During the one-year follow-up period, the treatment with autologous ASCs was shown to be safe and well-tolerated and reasonably effective in two out of five patients. Tissue engineering techniques hold the potential to provide an efficient treatment for SUI in the future, but more research is needed to reach this goal. Especially, developing more optimal biomaterial carriers for cells may increase the efficiency of the stem cell based injection therapies for SUI.

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Figure legends

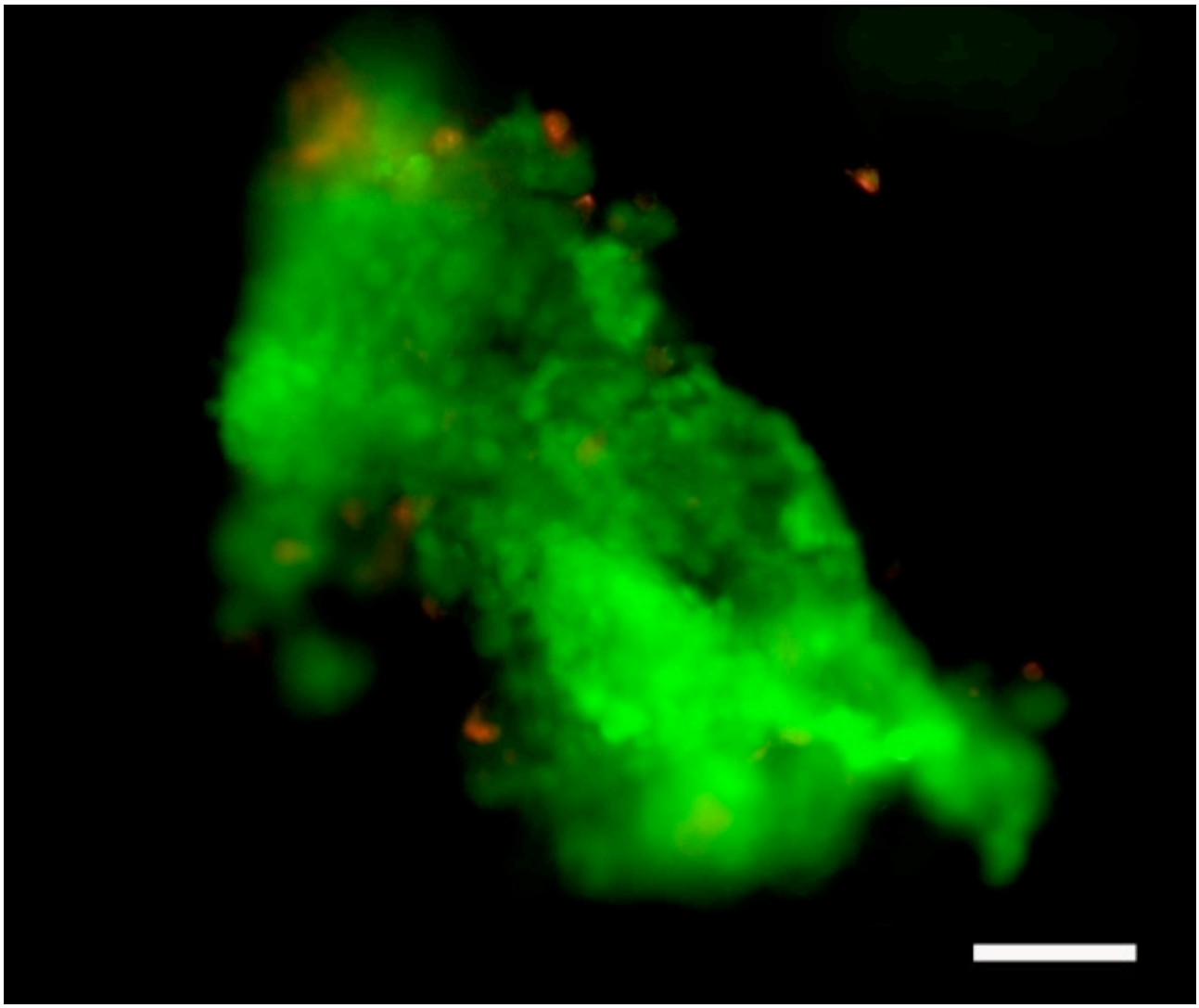

Figure 1

Representative image of viable (green) and dead (red) ASCs mixed with Contigen^{TM}. Scale bar 100 μm

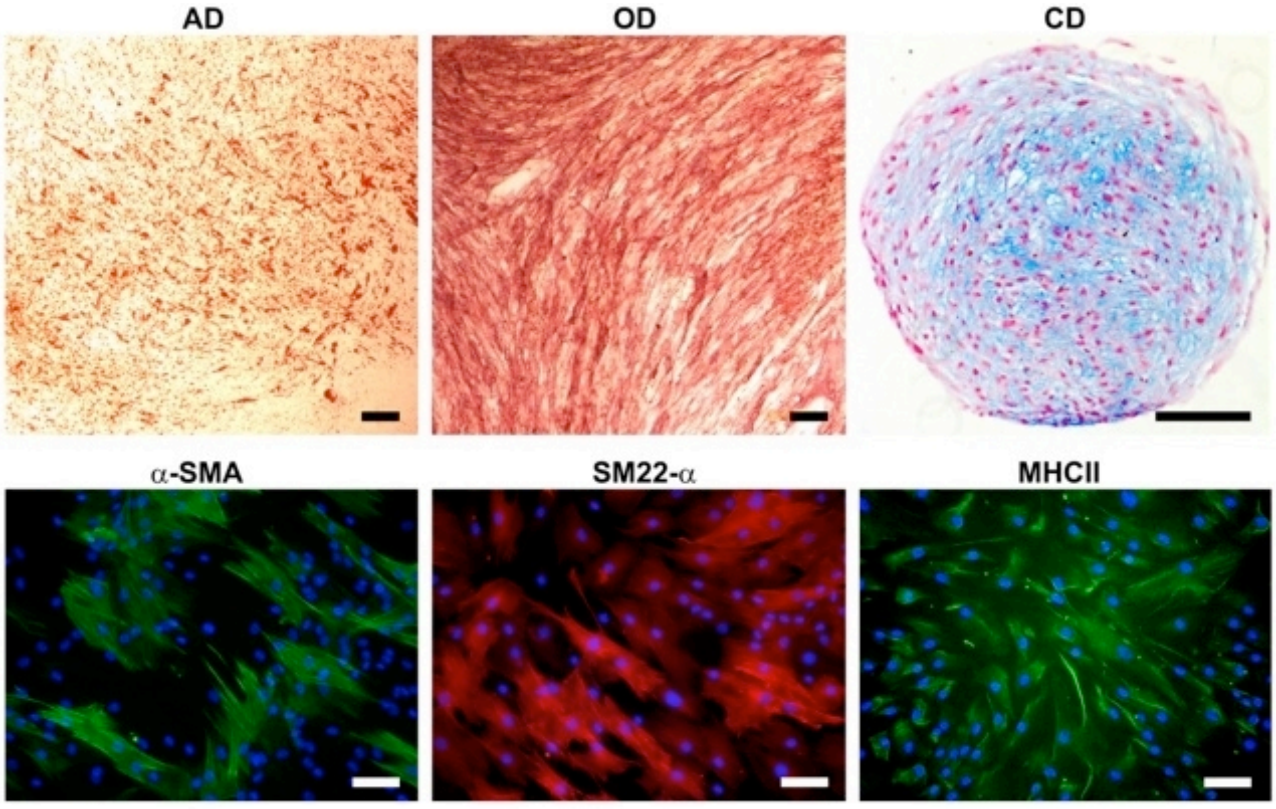

Figure 2

Multipotency of apipose stem cells. Adipogenesis (AD) was detected using Oil red O staining, The ALP staining ensuring the osteogenic differentiation (OD) and the Alcian blue staining confirming the chondrogenic differentiation (CD). The myogenesis was verified using three immunostaining markers; α-SMA, SM22-α, and MHCII. Black scale bar 150 μm, white scale bar 100 μm.

Table 1

Characteristics of the patients treated with transurethral injections of ASCs+Contigen™

	age	BMI	Previous incontinence operation	Incontinence type	MUCP (mmHg)
Patient 1	50	27		SUI	57
Patient 2	61	25	TVT -03	MUI	21
Patient 3	59	41		MUI	38
Patient 4	45	27		SUI	72
Patient 5	81	31	TVT-O -07 Bulkamid™ -09 TVT -10	MUI	20

ASCs Adipose stem cells
BMI Body mass index
SUI Stress urinary incontinence
MUI mixed urinary incontinence
MUCP maximal urethral closure pressure
TVT tension free vaginal tape
TVT-O trans-obturator tape

Table 2

Objective findings of urinary incontinence in patients treated with transurethral injections of ASCs+Contigen™

	Pre cough test	Cough test at 6 months	Cough test at 12 months	Pre 24-h pad test (g)	24-h pad test at 12 months (g)
Patient 1	+	+	+	0*	0
Patient 2	+	+	-	5	3
Patient 3	+	+	+	10	9
Patient 4	+	-	-	28	4
Patient 5	+	-	-	70	28

*no exercise due to a hip problem

Table 3

Quality of life/subjective findings of patients treated with transurethral injections of ASCs+Contigen™ according to UISS (Urinary Inventory Stress test), IIQ-7 (Incontinence Impact Questionnaire short form), UDI-6 (Urogenital Distress Inventory short form) and VAS (Visual Analogue scale).

	Pre UISS (0-100)	UISS 12months (0-100)	Pre IIQ-7 (0-100)	IIQ-7 12months (0-100)	Pre UDI-6 (0-100)	UDI-6 12months (0-100)	Pre VAS (0-10)	VAS 12months (0-10)
Patient 1	65	35	48	29	61	17	4,0	1,0
Patient 2	50	40	19	29	27	50	5,1	4,7
Patient 3	45	45	5	10	38	33	5,8	5,5
Patient 4	40	10	76	10	44	11	7,5	1,0
Patient 5	74	18	62	0	72	17	9,4	4,5

Table 4

Surface marker expression of undifferentiated ASCs analysed by flow cytometry.

Surface Protein	Antigen	Surface marker expression (n=5)
CD14	Serum lipopolysaccharide binding protein	41,2 ± 20,0
CD19	B lymphocyte-lineage differentiation antigen	15,9 ± 12,3
CD34	Sialomucin-like adhesion molecule	11,1 ± 7,4
CD45	Leukocyte common antigen	12,8 ± 6,3
CD49d	Integrin α2, VLA-4	83,2 ± 30,6
CD73	Ecto-5'-nucleotidase	99,7 ± 0,5
CD90	Thy-1	98,2 ± 3,2
CD105	SH-2, endoglin	99,9 ± 0,1
HLA-ABC	Major histocompatibility class I antigens	98,8 ± 1,8
HLA-DR	Major histocompatibility class II antigens	2,0 ± 1,5

Data was was reported as a mean ± standard deviation in percent.
CD, cluster of differentation
Thy-1, T cell surface glycoprotein.
VLA-4, Very late antigen 4