PRIMARY HUMAN UROTHELIAL CELL CULTURE ON A MATRIX OF SPIDER SILK; A NOVEL TECHNIQUE IN BLADDER RECONSTRUCTION

Master thesis Technical Medicine by

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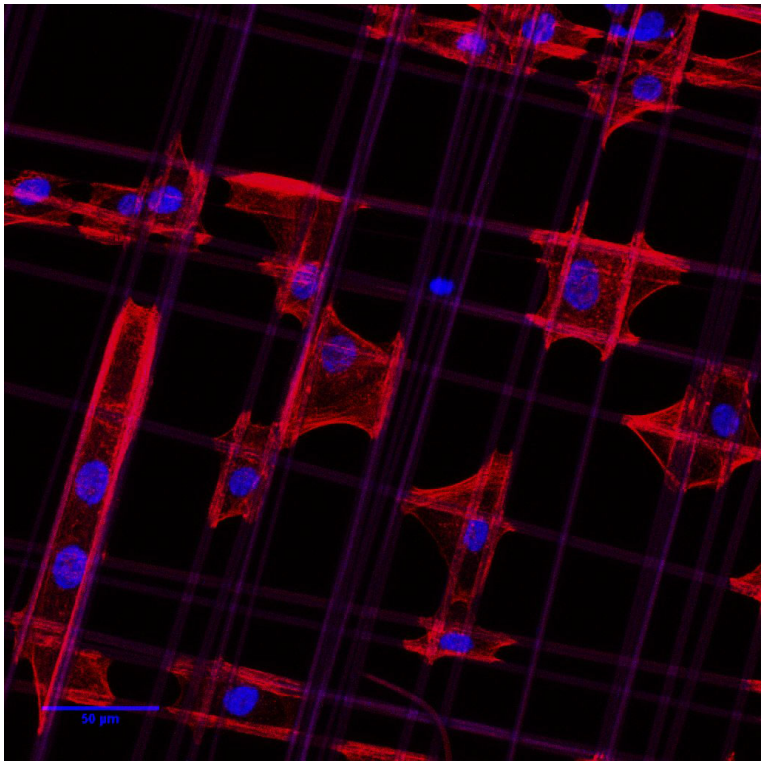

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ABSTRACT

Various diseases of the genitourinary tract can cause damage to the bladder and result in urinary retention or incontinence. Treatment options of affected bladders include augmentation cystoplasty, in which the bladder is enlarged by using a piece of intestine. Since the use of intestine in the urinary tract can cause serious adverse effects, new (bio)materials are being tested for bladder reconstruction. However, the ideal (bio)material for reconstruction of the bladder has not yet been discovered, mostly due to mechanical failure *in vivo*. A promising natural biomaterial is spider silk, which is outstanding in its slow degradation rate and mechanical properties. In this study, we assess the *in vitro* potential of spider silk as a suitable biomaterial for bladder reconstruction by cultivating primary human urothelial cells (HUCs) on native spider silk matrices. Here, we manufactured dental wire frames which were woven with the dragline silk of female *Nephila Clavipes* spiders under standardized conditions. Upon sterilization of spider silk frames, HUCs were cultivated on spider silk matrices and evaluated for adhesion, expansion, viability and cellular differentiation. Analysis of cell morphology and filamentous actin in HUCs revealed abundant adhesion sites and bridging of HUCs to spider silk matrices. In addition, qRT-PCR-based analysis of adhesion molecules present in HUCs revealed down-regulation of certain adhesion molecules when HUCs were cultured on spider silk. Expansion of HUCs on spider silk matrices was determined over time using Presto Blue and DAPI cell count in fields of vision and showed that spider silk supports expansion of HUCs. *In vitro* biocompatibility of spider silk was assessed by culturing HUCs in an extract of spider silk and revealed no cytotoxic effects on HUCs. This is supported by live/dead staining which demonstrated that HUCs were viable when cultured on spider silk matrices. QRT-PCR-based analysis of epithelial-mesenchymal transition, fibrosis and cellular differentiation markers expressed in HUCs when cultured on spider silk showed, besides down-regulation of CD44, no differences compared to control. Flow cytometry-based analysis of urothelial cellular differentiation markers CD90, CD44 and CD49f demonstrated a small subpopulation lower in CD44 expression at protein level when HUCs were cultured on spider silk. These results indicate that spider silk causes HUCs to mature slightly faster, but has no major effects on cellular differentiation of HUCs. Together, results describe a novel biomaterial which supports adhesion, expansion and viability of HUCs. Our study gives new understanding of cellular mechanisms occurring in HUCs when cultured on spider silk and demonstrates that spider silk is a promising biomaterial for bladder reconstruction.

ACKNOWLEDGEMENTS

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CONTENTS

ACRONYMS

CIC	clean intermittent catheterization
Ctk	cytokeratin
EMT	epithelial-mesenchymal transition
FBS	fetal bovine serum
FOV	fields of vision
HUC	primary human urothelial cell
MSC	human mesenchymal stem cell
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PB	presto blue
PBS	phosphate buffered saline
PGA	polyglycolic acid
PLA	polylactic acid
RT	room temperature
SIS	small intestine submucosa
UCM	urothelial cell medium

1 INTRODUCTION

1.1 CLINICAL RELEVANCE

Various diseases of the genitourinary system can cause damage to the bladder. These diseases include congenital abnormalities such as bladder exstrophy and meningomyelocele [1,2]. Further, structural damage to the bladder can be caused by trauma, bladder cancer, iatrogenic injuries and interstitial cystitis (painful bladder syndrome) [3,4]. In addition, diseases and injuries to nerves which innervate the bladder can cause dysfunction of the bladder. This condition is termed 'neurogenic bladder' and is solely caused by a true neurologic deficit [5]. Here, neurogenic bladder can be caused by pathologies at the level of the spinal cord (e.g. sacral agenesis, tethered spinal cord, trauma and multiple sclerosis) or at the level of the brain (e.g. spastic diplegia, stroke, Parkinson's disease and brain tumors) [6]. Of all conditions, meningomyelocele, which is the most common form of spina bifida, is the most common cause of neurogenic bladder in children and is found in approximately one per 1000 births [1,5,7].

The normal function of the urinary bladder is to store and excrete urine, which is coordinated by the central and peripheral nervous system. In neurogenic bladder, this coordination is impaired resulting in dysfunction of the bladder. Symptoms of neurogenic bladder range from detrusor muscle and urethral sphincter under activity to over activity. This manifests in problems with either urine storage in the bladder or emptying of the bladder by means of severe urinary retention or incontinence [1,5,8,9]. The greatest risk is in the group of patients with an overactive sphincter which can result in high detrusor leak point pressure. If the intravesical pressure is higher than 40 cm H_2O, external detrusor-sphincter dyssynergia and elevated detrusor filling pressure occurs and patients have a $\geqslant$50% risk for upper urinary tract damage. Complications include sepsis, hydronephrosis, renal insufficiency and renal failure, thus giving rise to significant morbidity [5,6,8,10].

In addition, urinary disorders have a major social and psychological impact on patients, affecting their quality of life and producing dependence on others [11–15]. Since survival rates of infants with spina bifida have increased from 83% in 1983 to 92% in 2001, it is of great importance to manage urinary retention or incontinence [5].

1.2 TREATMENT OPTIONS

In order to achieve continence, prevent urinary tract infections and preserve upper urinary tract integrity, multiple treatment options are possible these days. Clean intermittent catheterization (CIC) and medication are preferred in most patients to facilitate bladder emptying. However, CIC can result in urinary tract infections, damage to the urethra and decreased quality of life. Further, only two-third of patients benefit from CIC treatment, whereas the remaining one-third will require surgery. This includes minimal invasive surgery procedures (e.g. the use of Botulinum Toxin (Botox) or bladder stimulation and neuromodulation) or more invasive and reconstructive surgery procedures (e.g. augmentation cystoplasty) [5,8,16,17].

In augmentation cystoplasty, the bladder is reconstructed by enlargement of the bladder with a piece of intestine to increase bladder capacity and lower the intravesicular pressure permanently in order to prevent upper tract damage (fig 1) [5]. Mikulicsz was the first to describe the use of small intestine to augment the bladder in 1899 [18]. Currently, tissues used for augmentation cystoplasty are stomach, large bowel, small bowel, ureter and engineered tissue [19–26]. Often, bladder augmentation with small intestine is the procedure of choice for urologists [27]. Even though the use of gastrointestinal segments is the gold standard for bladder replacement or repair, major postoperative complications occur since gastrointestinal tissues are designed to absorb specific solutes whereas the bladder is designed to excrete these solutes [26]. Complications include metabolic disorders, hematuria syndrome, mucus production, enteric fistulas, bladder rupture, intestinal obstruction and malignancy [28–38] To avoid the use of intestine in the urinary tract and its complications, a

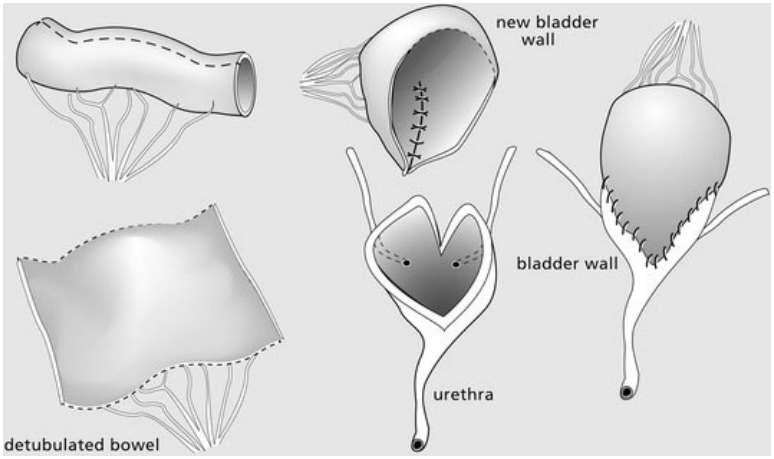

Figure 1: ***Overview of augmentation cystoplasty***
When the bladder capacity is too small, augmentation cytsoplasty is performed in which a piece of intestine is used to enlarge the bladder [39].

number of strategies have been developed for patients in whom conservative therapy (i.e. CIC and pharmaceuticals) is not effective. Bladder autoaugmentation by means of detrusorectomy is a minor surgical procedure in which the bladder is changed in a physiological way by partial excision of detrusor muscle, thus increasing bladder capacity and normalizing compliance [40–42]. Further, ureterocystoplasty has been introduced as an alternative reconstructive procedure in augmentation cystoplasty. By using a dilated ureter to augment the bladder, it avoids the complications associated with intestinal mucosa [27, 38, 43]. Even though these strategies look promising, doubts remain about the long-term efficiency, limitation of bladder expansion and the need to perform re-augmentation [27,38,43]. In addition, ureterocystoplasty is limited to cases in which a dilated ureter is already present. It seems that, for patients with good bladder capacity where the aim is not to augment the bladder but to improve compliance and cure detrusor overactivity, detrusorectomy is a good approach [44].

To overcome these problems and complications, there is need for a new approach to develop alternative tissues for augmentation cystoplasty. Therefore, novel methods on the field of regenerative medicine are being explored such as cell transplantation and tissue engineering.

1.3 BLADDER HISTOLOGY

In regenerative medicine, often autologous cells are used to reconstruct affected tissues. The urinary bladder consists of four layers, from inside to outside these are mucosa (i.e. urothelium or transitional epithelium and lamina propria), submucosa, detrusor muscle and adventitia which covers the organ (fig 2) [45]. The bladder submucosa is a connective tissue collagen-rich layer supporting the mucosa. The detrusor muscle, which consists of longitudinal and circular layers of thick muscle bundles, allows for urine storage and miction by means of contraction. The bladder urothelium lines the vesical lumen and consists of a basal, intermediate and superficial (i.e. umbrella) cell layer. It is a highly specialized structure involved in numerous processes (e.g. metabolic secretion and micturation reflex) and pathological conditions such as aberrant cell growth, differentiation and cancer [46–48]. For tissue engineering of the bladder, both urothelium as detrusor muscle cells should be present since both structures act in a synergistic way in bladder function [47, 49–51].

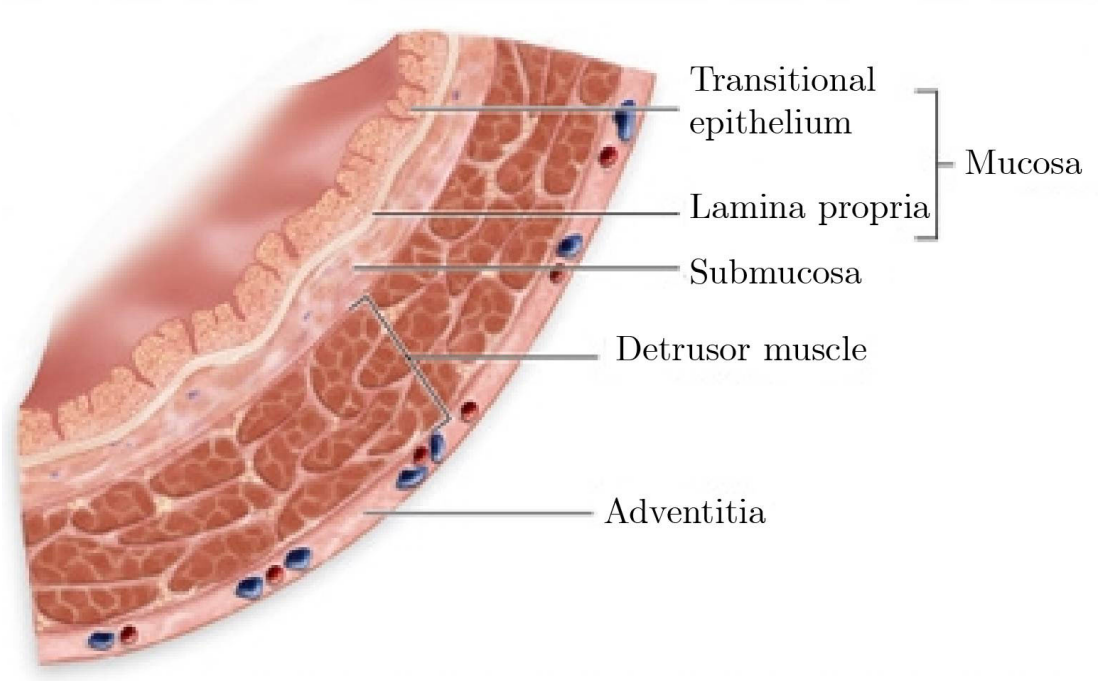

Figure 2: ***Overview of bladder layers***
The bladder consists out of 4 layers, the mucosa (i.e. transitional epithelium and lamina propria), submucosa, detrusor muscle and adventitia. Image copyright© The McGraw-Hill Companies, Inc.

1.4 TISSUE ENGINEERING

Regenerative medicine replaces or regenerates damaged human cells, tissue or organs to restore or establish normal function [52]. This can be achieved by means of injection of functional cells into a non-functional site to stimulate regeneration or by creating new tissues on natural or synthetic biocompatible materials (i.e. scaffolds). Scaffolds can support the (direction of) growth of new tissues and stimulate regeneration, in

which scaffolds can be implanted unseeded or seeded with cells and the success depends on the natural regeneration capacity of the bladder.

In augmentation cystoplasty, the biomaterial should be biocompatible and biodegradable to support the reconstruction without inflammation. Furthermore, it should function as extracellular matrix by bringing cells together, controlling the tissue structure and regulating the phenotype. It should have mechanical and physical properties able to withstand forces resulting from urine storage, emptying and filling of the bladder and forces exerted on it by the pelvic muscle. In addition, it should be degraded and replaced completely by newly grown tissue without influencing the tissue environment and the biomaterial should have properties which enable it to be processed in specific shapes.

Different kinds of biomaterials have been tested both *in vitro* and *in vivo* in animals for engineering of genitourinary tissues. In general, there are three types of biomaterials; naturally derived materials (e.g. collagen, alginate and keratin), acellular tissue matrices (e.g. decellularized submucosa of for instance intestine of bladder) and synthetic polymers (e.g. polyglycolic acid (PGA), silicone and polylactic acid (PLA)). However, up until now, the optimal biomaterial has not been discovered yet. The use of collagen as a biomaterial has as drawback that it can lose its mechanical properties by proteolytic acitivity, whilst the use of alginate as a biomaterial can induce toxic effects on urothelial cells [53]. Further, synthetic materials are often subject to structural, mechanical, functional and biocompatible failure since no natural recognition sites are available on the synthetic matrix surface [54–57]. To overcome these problems, studies aim at combining both synthetic and natural biomaterials. Striking is a study performed by Atala et al. which demonstrates a biodegradable composite scaffold made of collagen and PGA seeded with autologous urothelial and muscle cells, showing promising results when implanted in patients [26]. However, further research and improvements are needed to study proper mechanical function, angiogenesis and other long-term effects.

Of all biomaterials used in augmentation cystoplasty, small intestine submucosa (SIS) is the most thoroughly studied biomaterial and most promising scaffold for bladder regeneration. Here, after removal of mucosal, serosal and muscular cells from porcine small intestine, a strong collagenous matrix remains (i.e. SIS). Even though studies of bladder reconstruction by means of cell seeded SIS look promising since they support cell ingrowth and regeneration, such acellular collagen matrices show poor long-term results. This is due to complications such as donor site morbidity and segmental fibrosis leading to graft shrinkage and infection [58–62].

1.5 SPIDER SILK

Therefore, there is need for alternative tissue engineering biomaterials in order to replace the integration of bowel segments into the urinary tract while maintaining mechanical and biocompatible properties of native bladder tissue. A promising natural biomaterial is silk, which is outstanding in its slow degradation rate and, most of all, its mechanical properties [63–67]. In addition, silk shows resistance against enzymatic cleavage, has a high oxygen and drug permeability and is able to perform under a wide humidity and temperature range [66]. The most widely studied silk is cocoon silk from the silkworm *Bombyx Mori* (*B. Mori*), since it can be produced on a large scale because of the domestication of silkworms which allows farming. However, problems with respect to the biocompatibility and allergic reactions have been

reported regarding *B. Mori* silk, due to the sericin coating of their fibers [65, 68–70]. The sericin coating is a family of glue-like proteins that holds two fibroin fibers together to form a composite fiber. To overcome this problem, *B. Mori* fibers are cleaned from their coating (i.e. degummed) by boiling the silk with a soap alkali solution or protease treatment [71, 72]. Even though degumming prevents immunologic reactions, a substantial disadvantage is the fact that degumming might cause additional alterations in chemical composition of the fibers. The extend of sericin removal is not known and chances are that other components of the fibers are removed as well [73, 74].

This is in contrast with spider silks, where these glue-like sericin proteins are generally absent making it an attractive biomaterial in terms of biocompatibility [65, 75–77]. In addition, tensile strength, toughness, extension and Young's modulus of spider silk fibers are almost twice as big as *B. Mori* fibers, in which spider silk is estimated to be at least five times as strong as steel and twice as elastic as nylon.

The strength of dragline spider silk arises from highly conserved poly-Glycine and Alanine amino acid sequences, each containing a N and C terminal domain at the extremities. In the spinning passage, under the influence of low salt concentration and abundant sheer forces, the C terminal domain becomes sticky and causes the chains to overlap forming β-sheet nanocrystals. Each nanocrystal employes a dense network of hydrogen bonds and nanocrystals cross-link into a polymer network with great toughness. In addition, under the influence of a more acidic environment in the spinning passage, the N terminal domain ends connect to each other forming an amorphous network chain which embeds the nanocrystals and gives spider silk its tensile strength (fig 3) [67, 78–80].

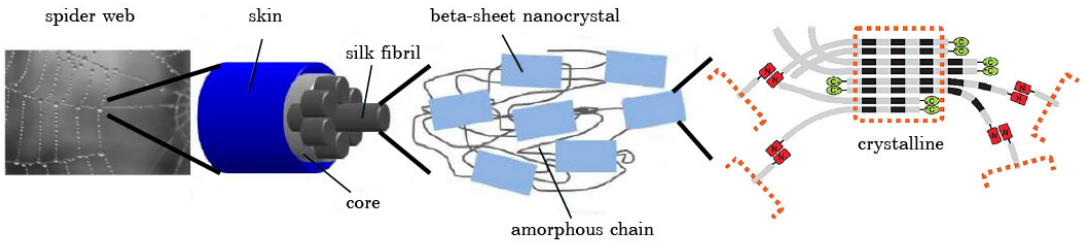

Figure 3: ***Hierarchical structure of spider silk***
Spider silk consists of fibrils containing poly-Glycine and Alanine amino acid sequences which form beta-sheet nanocrystals and amorphous chains by the bonding of respectively C and N terminal domains and hydrogen bonds [78–80].

In vitro studies show great potential for native spider silk in the reconstruction of various organs. Gellynck et al. show that chondrocytes cultured on both spider silk fiber of the dragline and egg sac are able to attach and grow without losing their phenotype and being cytotoxic [81]. Culture of Schwann cells on spider silk shows enhanced cell migration, axonal regrowth and remyelination resulting in functional discovery of nerves [76]. In addition, spider silk appears to be a promising biomaterial for skin regeneration since fibroblasts and keratinocytes adhere to the silk and proliferate [82]. *In vivo* studies testing the biocompatibility of spider silk in animal models show a mild to low inflammatory reaction similar to other clinically used ma-

terials (e.g. collagen), which can be enhanced by pretreating spider silk with trypsin without affecting mechanical properties [75, 77, 83–85]. In addition, degradation of spider dragline silk of *Nephila Clavipes* implanted in rats is approximately 6 months without cytotoxic by-products [75].

Even though spider silk has exceptionally good characteristics, little is known about the use of spider silk for biomedical applications because of its low availability. Commercial spider silk production can not easily be achieved since the cannibalistic nature of the spiders prevents them from being farmed and spiders produce relatively low levels of silk. Therefore, alternative methods to produce spider silk have been studied and major efforts have been directed to the production of recombinant spider silks. Here, the recombinant miniature spider silk protein 4RepCT, which is produced in a gram-negative host (i.e. *E.coli*), shows promising results [86–89]. Not only can it be produced at high levels in soluble form when fused to a protein, it can also spontaneously form fibers in physiological buffer. In addition, *in vivo* studies performed in rats demonstrate that 4RepCT is cyto- and biocompatible when implanted subcutaneously and has angiogenic properties [90–92]. Further, studies show that 4RepCT supports growth, attachment and functionality in human primary fibroblasts and neural stem cells [89, 93]. This gives great perspective for the future (commercial) use of spider silk as a biomaterial. Encouraged by these results, this study aimed at pioneering the potential of native spider silk as a biomaterial for bladder reconstruction (e.g. augmentation cystoplasty or total bladder substitution in cystectomy) by seeding primary human urothelial cells (HUCs) on native spider silk matrices from *Nephila Clavipes* and assess cells on viability, adherence, growth and cellular differentiation.

We believe that native spider dragline silk has the potential of being an interesting biomaterial for bladder reconstruction in future. For this reason, the main goal of this study was to determine whether spider dragline silk of *Nephila Clavipes* is a suitable biomaterial for primary human urothelial cell cultivation. To achieve this goal, the following primary goals must be met first;

- Develop a method for native spider dragline silk collection, frame manufacturing and weaving which is standardized, reproducible and suitable for cell cultivation.
- Develop a method for cell seeding of HUCs on spider silk.

After achieving the primary goals, suitability of spider silk for HUC cultivation must be researched *in vitro*, in which it has to meet the following prerequisites;

- Spider silk should support adhesion and expansion of HUCs.
- Spider silk should have no cytotoxic effects on viability of HUCs.
- Spider silk should not alter the phenotype (i.e. cellular differentiation) of HUCs.

Based upon previous studies performed on spider silk, we hypothesize that spider silk will support, but not enhance, adhesion and growth of HUCs and will have no cytotoxic effects on HUCs. In addition, we expect spider silk to have effects on expression of adhesion molecules but not on cellular differentiation molecules.

2

MATERIALS AND METHODS

2.1 MATERIAL COLLECTION & PREPARATION

Breeding of spiders
Nephila Clavipes spiders were bred at the Medical School Hannover, dept. of Plastic, Hand and Reconstructive Surgery, under animal specific conditions in separate rooms, temperature $\geqslant$17°C and 70% humidity (first animals were obtained from BTBE; Germany). Male and female spiders were kept together, received water every day and spiders were fed with *Acheta domestic* every week.

Harvesting of spider dragline silk
Spider silk was collected from female spiders only by fixation on a foam platform by means of a non-harmful procedure at the Medical School Hannover [94]. Briefly, spiders were put on their back, head-down, and fixated on the platform with gauze bandages and needles to prevent the legs from interfering with the spinneret. Dragline silk was pulled out of the spinneret by stimulation of the Major ampullate gland and spun onto a frame (fig 4a). Upon silk collection, spiders were fed and given water.

Frame design, weaving and culturing
Weaving frames were manufactured by hand. Stainless steel dental wire (REF 527-070-00, Remanium® Dentaurum) with a diameter of 0.7 mm was bent into square frames with a side length of 0.5-0.8 cm. One side was left longer to handle frames without damaging the silk. Subsequently, frames were clamped into a special tuning device, which allowed spider silk to be woven onto the frame at a fixed rate (i.e. 30 revolutions per minute). Spider silk was woven for 3.5 and 7.5 min on each side of the frame resulting in a cross weave mesh on the upper side of the frame and one on the lower side of the frame due to the diameter of the thickness of the steel. Different weaving times (referred to as; weaving density) were used per side to determine if HUCs preferred a bigger or smaller pore size to adhere and grow on. Spider silk frames woven for 3.5 min and 7.5 min contained an average pore size of respectively 21 µm and 13 µm. Frames with spider silk were stored separately in 24-well plates at room temperature (RT) until they were used. Frames were autoclaved (Sanyo Labo Autoclave) at 120°C for 1 hour and placed separately in 24-wells plates in sterile PBS at 37°C, 5% CO_2, for several days prior to use.

Cell culturing (HUCs, T24, RT112, MSCs)
HUCs were cultured in serum-free Urothelial Cell Medium (UCM) supplemented with 1% urothelial cell growth supplement and 1% Penicillin/Streptomycin (penstrep) solution (all ScienCell). Cells were detached with equal volumes of 0,25% Trypsin/EDTA solution (ScienCell) and PBS for 3 min, 5 ml Trypsin Neutralization Solution (ScienCell) was added and cells were collected in 5 ml fetal bovine serum (FBS, PAA Cell culture Company), counted and centrifuged for 5 min at 1000 rpm and resuspended in UCM. To enhance cell adhesion, culture flasks were pretreated

with Poly-L-Lysine (1,5 µl/ml UCM) (ScienCell) for 1 hour at 37°C, 5% CO_2 and washed twice with PBS prior to culturing.

T24 cells, a high-graded bladder cancer cell line (kindly provided by the dept. of Microbiology, UMC Utrecht), were cultured in IMDM medium (Gibco) supplemented with 1% penstrep (Gibco) and 10% FBS (PAA Cell culture Company).

RT112 cells, a low-graded bladder cancer cell line (kindly provided by the dept. of Urology, UMC St. Radboud Nijmegen), were cultured in RPMI+ Glutamax medium (Gibco) supplemented with 1% penstrep (Gibco) and 10% FBS.

Human mesenchymal stemcells (MSCs) were cultured in α-MEM medium (Gibco) supplemented with 1% penstrep, 0,2% Heparin (LEO Pharma) and 5% platelet lysate (Sanquin) which was freshly added to the cells each time. T24, RT112 and hMSCs were detached with 0,05% Trypsin-EDTA 1X (Gibco), collected, counted and centrifuged for 5 min at 1500 rpm and resuspended in medium.

Seeding cells on spider silk frames; the general principle
Frames were transferred from sterile PBS into new 24-well plates and washed twice with sterile PBS. Next, all remaining droplets of PBS were aspirated from the frame leaving it as dry as possible and 1×10^4 HUCs at passage 5-6 were pipetted on each frame in a small volume of UCM (i.e. 20 µl) to promote cell attachment. After 1,5 hours incubation at 37°C, 5% CO_2, 1 ml of UCM was added to each well and left to incubate overnight. The next day each frame was transferred into a new well with 1 ml UCM. Subsequently, media were changed every three days.

2.2 BIOCOMPATIBILITY OF SPIDER SILK

To determine cytotoxicity of the spider silk frame itself, a biocompatibility assay was performed by culturing HUCs in an extract of spider silk compared to other extracts. Extracts were obtained by immersing a spider silk frame and a piece of SIS (Biodesign Cook Medical) measuring 0,5x0,5 cm in UCM for 2 days at 37°C, 5% CO_2. 5×10^3 HUCs/well at passage 2 were plated in triplo in a 96-wells plate for 24 hours at 37°C, 5% CO_2. Extract from 1 mM H_2O_2 (VWR International), which is considered toxic, was used as a positive control. Culture medium without extract was used as a negative control. After 24 hours, culture medium was removed and replaced with extract medium for 1, 3 and 7 days at 37°C, 5% CO_2 and extract medium was changed every three days. At the end of the incubation the extracts were discarded and cytotoxicity in terms of loss of viable cells was measured with Vibrant® MTT cell proliferation assay (Molecular Probes). For this, 100 µl of a 1,2 mM (0,5 mg/ml) MTT solution was added to each well for four hours at 37°C, 5% CO_2. After aspirating the MTT solution, 100 µl DMSO (Sigma) was added to each well, resuspended and left on a plate shaker for 10 min. Subsequently, absorbance was measured of each well with a Microplate Reader (model 3550, Bio-Rad) at 540 nm.

2.3 IMMUNOFLUORESCENCE AND HISTOLOGY

To determine cell attachment, shape and motility, immunofluorescence was performed. 1×10^4 HUCs were seeded on the spider silk frame as described above and 1×10^3 HUCs were seeded on a 13 mm round glass coverslip (Thermo Scientific) in a 24-wells plate as positive control, all in triplo for each time-point. After 1, 4, 8 and 12 days of incubation, frames and controls were fixed in 4% formaldehyde (Merck) for 10 min

at RT. After washing each sample twice with PBS, 1 ml of PBS containing 5% Saponin (Sigma) and 1% BSA (Sigma) (1x Saponin blocking buffer) was added to each sample for 15 min at RT. After washing the samples thrice with PBS, 350 µl Phalloidin (Sigma; 1:200 in 1x Saponin buffer) was added to each sample and incubated for 45 min at RT in the dark. Subsequently, each sample was washed 3x5 min with PBS and 1 ml DAPI (Sigma; 1:1000 in PBS) was added and incubated for 5 minutes at RT in the dark. Samples were stored in 1% formaldehyde at 4°C and imaged by means of confocal microscopy (LSM700, Zeiss) and an EVOS®FL fluorescence microscope.
In addition, histology was performed on 2 spider silk frames that were seeded with 1×10^4 HUCs as described earlier. After 7 days of culturing frames were fixed in 4% formaldehyde for 24 hours at RT. Frames were dehydrated in a series of increasing alcohol concentrations, cleared in Isopropyl Alcohol and embedded in paraffin. Prior to paraffin-embedding, the steel frames were removed. For this, the paraffin on the spider silk was hardened to prevent supercontraction of the silk and the silk was cut away from the frame. After embedding the silk in a paraffin block, 4 µm thick sections were cut with a microtome and sections were left on a heat-plate at 57°C overnight. Slides were deparaffinated in Xylene (Merck), rehydrated by a series of decreasing alcohol concentrations and stained with Haematoxylin (Klinipath) for 2 min, rinsed and stained with Eosin (Merck) for 1 min. Slides were dehydrated in a series of increasing alcohol concentrations, cleared in Xylene for 10 min and mounted with Pertex Mounting Media (Leica Biosystems). Samples were imaged with a Nikon DXM1200 digital camera.

2.4 CELL EXPANSION MEASUREMENTS

To determine expansion of HUCs when cultured on spider silk, two approaches were used to quantify the amount of cells in time; Presto Blue (PB), where frames could be quantified in time without fixation, and DAPI, where different frames where fixed at different time points. PB is a resazurin-based solution which is modified to red fluorescence by the reducing environment of viable cells. For expansion measurements by PB, 2 experiments were performed; on the one hand proliferation of HUCs on spider silk versus plastic. On the other hand proliferation of HUCs on unprocessed spider silk versus spider silk coated with Fibronectin or Poly-L-Lysine. The same method was used for both experiments. For coating of spider silk, 1 ml fibronectin (Sigma; 1:100 in PBS (10µg/ml)) or 1 ml Poly-L-Lysine (ScienCell; 1:667 in PBS) was added to each frame, incubated at 37°C, 5% CO_2, for 2 hours and washed twice with PBS prior to use. 1×10^4 HUCs were seeded on each spider silk frame as described earlier, 1×10^3 HUCs/well were seeded on plastic in a 24-wells plate and each condition was performed in triplo. In addition, three wells containing medium only and a spider silk frame without cells were analyzed as well to subtract background. On day 1, 4, 8 and 12, 50 µl PB (Molecular Probes) was added to 500 µl UCM and incubated in aluminum foil for 30 min at 37°C, 5% CO_2. The amount of red fluorescence (i.e. viable cells) was measured with the SpectraMax (Molecular Devices) at ex/em: 560/590 nm. Subsequently, PB solution was removed, wells were washed with PBS twice and replaced with UCM for further cultivation. Data was processed in MS Excel 2007.
For quantification with DAPI, only unprocessed spider silk frames were used. The exact same method was used as described earlier in 'immunofluorescence' since these frames and controls were, besides phalloidin staining, also stained for DAPI. DAPI images were made with EVOS® where 5 fixed fields of vision (FOV, enlargement

10x) were imaged on each coverslip and frame (fig 4b and c). For quantification of cells on the frame, multiple images in z-direction were made and images were processed with ImageJ. Nuclei were counted automatically with the 'analyze particles' function in most samples. Some samples, for which automatic counting was not accurate enough, manual counting was performed with the 'cell counter' function. The 5 FOV's of each frame and control were averaged and the average represented the relative amount of cells on the corresponding sample.

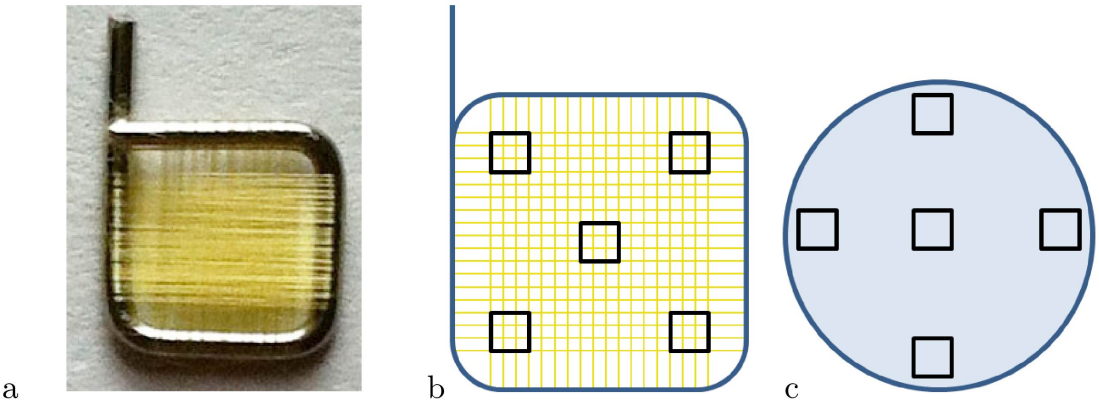

Figure 4: (a) Steel frames were woven with spider dragline silk of *Nephila Clavipes*. For quantification of cells on spider silk matrices (b) and glass coverslips (c), 5 fixed fiels of vision were counted of which a schematic indication is given by the black frames.

2.5 LIVE/DEAD ASSAY

To determine the amount of living cells on the spider silk frames a live/dead cell imaging assay (Molecular Probes) was performed. 1×10^4 HUCs were seeded on the spider silk frame as described above and 1×10^3 HUCs/well were seeded on plastic in a 24-wells plate as positive control, all in triplo. HUCs cultured for 3 hours in 100% DMSO (Sigma) were used as negative control. After seven days of culturing HUCs on plastic or spider silk, media was aspirated and 350 µl live/dead solution (1:2 in PBS) was added to each well. After 15 min of incubation at RT the live/dead solution was replaced by 1 ml of PBS. Living cells were imaged at 488 nm (FITC) and dead cells at 570 nm (Texas Red) with EVOS®. Of each spider silk frame and control well, pictures were taken of the same areas in order to compare conditions.

2.6 RNA ISOLATION AND QPCR

RNA isolation and qPCR were performed to determine differentiation, adhesion, fibrosis and epithelial-mesenchymal transition (EMT) markers in HUCs cultured on spider silk. Two spider silk frames were seeded with 1×10^4 HUCs at passage 5 as described above and 1×10^3 HUCs/well were seeded in duplo in a 24-wells plate as positive control. The experiment was repeated with the same conditions, only HUCs were at passage 6. After six days of culturing RNA was isolated from the frames and control wells. To compare gene expression of HUCs, the following control cell lines were analyzed as well; T24, RT112, hMSCs and HUCs at time-point 0 before seeding on frames. Cell lines were trypsinized as described earlier and 100.000 cells per cell line were pelletted for RNA isolation. Total RNA was isolated from all samples using

the RNeasy® mini kit as directed by manufacturer (Qiagen, Hilden, Germany) and samples were treated with DNase I. Quantification of total extracted RNA in each sample was measured with a Nanodrop (Thermo Scientific).Subsequently, 100 ng RNA was used per sample for cDNA synthesis using the iScript^{TM} cDNA Synthesis kit (Bio-Rad) as directed by manufacturer in a T3000 Thermocycler (Biometra) under the following thermal conditions; 25°C for 5 min; 42°C for 45 min and 85°C for 5 min. Primer pairs for human Beta-2-microglobulin (B2M, housekeeper), GAPDH (housekeeper), N-cadherin, Vimentin, E-cadherin, Collagen 1, Collagen 3, Uroplakin 3, Cytokeratin 7 & 17-20, Integrin $\alpha 2$, $\alpha 5$, $\alpha 6$ & $\beta 1$ and CD44 were purchased from Sigma (appendix A). QPCR was performed using SYBR green Supermix (Bio-Rad). Here, per qPCR sample, 10 µl Mastermix was used (7,5 µl SYBR green + 2,5 µl ddH2O + 0,075 µl primer fwd + 0,075 µl primer rv) and 5 µl cDNA (1:10 diluted in ddH_2O). Analysis was performed using the MyiQ Single-Color RT-PCR detection system (Bio-Rad) under the following thermal conditions; 95°C for 3 min, 40 cycles of 95°C, 60°C and 72°C for 30 sec, 95°C for 1 min, 65°C for 1 min and 61 cycles of 65°C for 10 sec. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

2.7 FLUORESCENCE-ACTIVATED CELL SORTING

To determine the differentiation markers of HUCs cultured on spider silk, cells were analyzed by flow cytometry. Spider silk frames were seeded with 1×10^4 HUCs at passage 5 as described above and 1×10^3 HUCs/well were seeded in a 24-wells plate as positive control, all in triplo. After six days of culturing, HUCs were trypsinized from both the frames and control wells in the same way. Each sample was washed twice with 0,05% trypsin-EDTA, subsequently 500 µl 0,05% trypsin-EDTA was added and incubated at 37°C, 5% CO_2, for six min. Cells were collected, centrifuged and resuspended in 50 µl PBS + 2% FBS (FACS buffer). To compare expression patterns of FACS antibodies, the following control cell lines were analyzed; T24, RT112, HUCs and HUCs, which have been cultured in UCM+FBS for 7 days. All cell lines were trypsinized as described earlier in the section 'cell culturing' and 100.000 cells per cell line were resuspended in 50 µl FACS buffer. CD90 (BD Pharmingen; 1:800; FITC), CD44 (Immunotools; 1:100; APC) and CD49f (BD Pharmingen; 1:150; PerCP-Cy5) were added to the FACS buffer of each sample and incubated for 30 min at 4°C in the dark. Samples were washed twice with FACS buffer, resuspended in 200 µl FACS buffer containing Sytox Blue (Invitrogen; 1:400; Pacific Blue) and measured on a FACSCanto II (BD Biosciences) flow cytometer. Data analysis was performed using FlowJo version 7.6 after exclusion of dead and doublet cells.

2.8 STATISTICAL ANALYSIS

The mean and standard deviation (SD) were calculated using MS Excel 2007 and Graphpad Prism 5. Statistical analysis was performed using the unpaired student's t-test and differences with $p \leqslant 0,05$ were considered statistically significant.

3

RESULTS

3.1 SPIDER SILK IS BIOCOMPATIBLE

As an initial test to assess if spider silk has the potential of *in vivo* application for bladder reconstruction, the cytotoxicity of spider silk to HUCs was evaluated. Viability of HUCs exposed to an extract of spider silk or SIS as a function of time was measured by MTT assay. Over time, absolute absorbance values showed that the number of viable cells present in control was increased and growth of HUCs occured (fig 5a). The same counted for HUCs cultured in an extract of spider silk or SIS. When comparing spider silk and SIS to control, in which absorbance was normalized for negative control (set at 100%), it showed an increase of cell numbers exposed to spider silk (126±27%) and SIS (124±9%) extract at day 1 (fig 5b). At day 7, cell number is somewhat decreased when exposed to spider silk (85±55%) and SIS (78±10%) compared to control. However, no statistical significant differences were found at each time point between spider silk or SIS and negative control. Together, these results show that spider silk has no cytotoxic effects on HUCs and indicates *in vitro* biocompatibility.

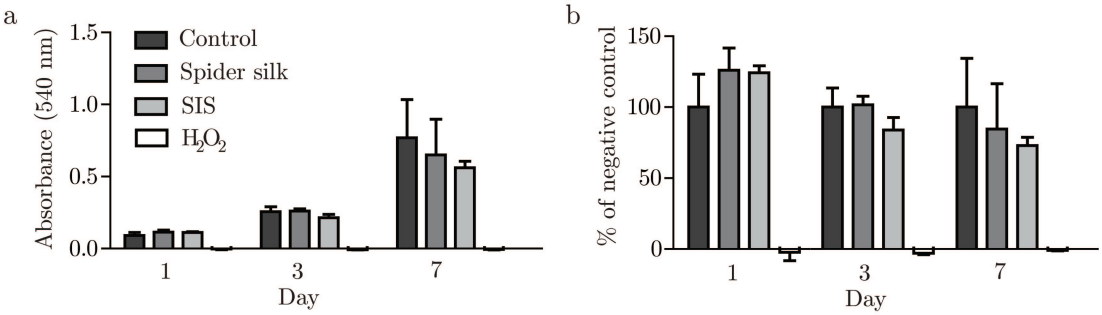

Figure 5: ***Spider silk has no cytotoxic effects on HUCs***
MTT assays showing the effects of urothelial cell viability and proliferation when exposed to extracts of spider silk, SIS, medium (negative control) and H_2O_2 (positive control) after 1, 3, and 7 days of culturing. Absolute values of MTT absorbance (a) and values normalized to negative control values (b) are shown for each sample at each time point. Data is shown as mean ±SD and no statistical significant differences were seen in absorbance between spider silk or SIS extract and negative control samples.

3.2 SPIDER SILK SUPPORTS HUC ADHESION

To assess which weaving density is most optimal for cell culture, adhesion of HUCs was determined on both densities. Light microscopy already showed adhesion of HUCs to spider silk matrices at 1.5 hours after seeding, in which cells are more capable of adhering to the spider silk matrices with a weaving density of 7.5 minutes per side (referred to as 'spider silk 7.5') (fig 6b). Seeding of HUCs on spider silk matrices

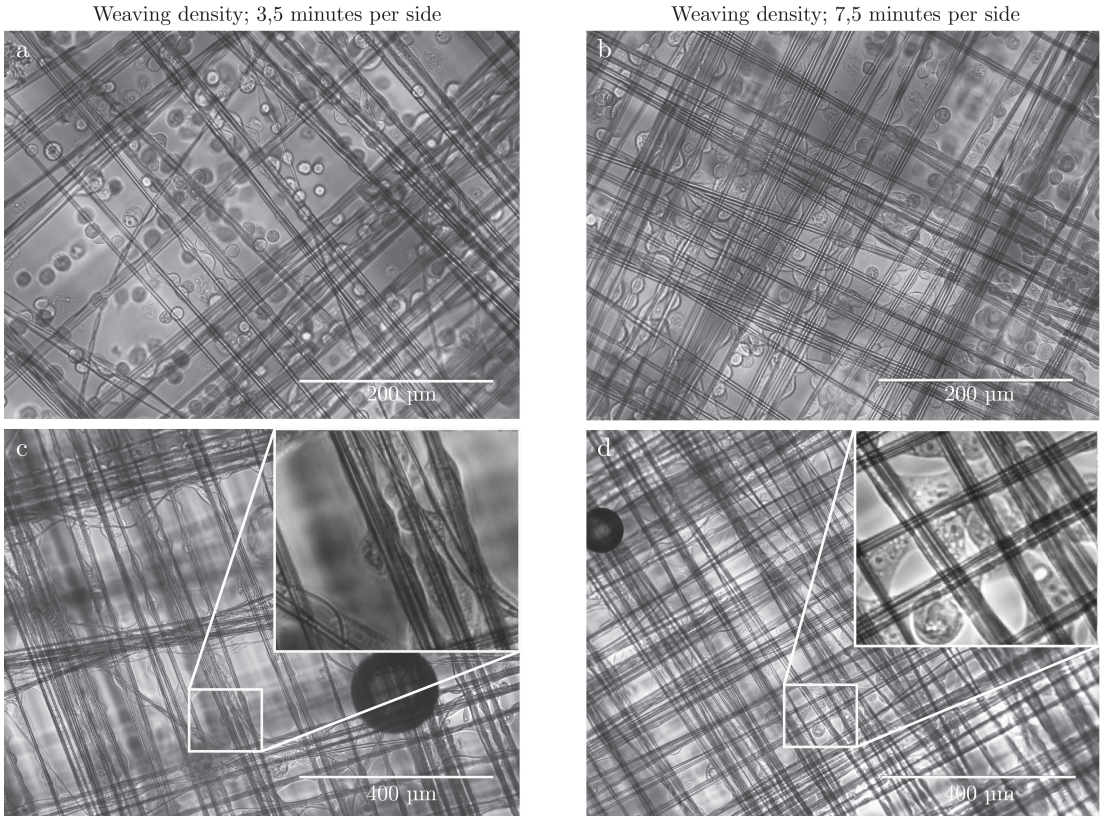

Figure 6: ***HUCs adhere better to spider silk with a smaller pore size***
Light microscopy of spider silk matrices at 1,5 hours after seeding 2,5 x 104 HUCs on spider 3,5 (a) and 7,5 (b), and at 3 days after seeding on spider silk 3,5 (c) and 7,5 (d). Insert shows a zoom-in of a small area (c+d) to elucidate cell adherence and growth. HUCs cultured on spider silk 7,5 indicate increased initial adherence (b) and were more capable of bridging the spider silk matrix due to smaller pore size (d).

woven for 3.5 minutes per side (referred to as 'spider silk 3.5') resulted more cells falling through the spider silk matrix on the bottom of the well due to the larger pore size (fig 6a). Morphology of cells that adhered to the spider silk was stretched longitudinally, cells not attached to the spider silk appeared rounded up. After three days of culturing it showed that HUCs cultured on spider silk 3.5 were not able to bridge the distance between spider silk fibers (fig 6c), as opposed to spider silk 7.5 on which HUCs were able to fill up the pores (fig 6d). Therefore, further experiments in this study were performed with spider silk 7.5 frames only.
Since it was difficult to visualize cell morphology and adherence to spider silk fibers with light microscopy alone, other approaches were used. Histological analysis revealed cell attachment and the capacity of HUCs to bridge the spider silk pores. The H&E stained paraffin cross-sections showed spider silk fibers in pink as perfectly round structures and an occasional longitudinal cross-section (fig 7a). Fibers were surrounded by cells and demonstrated conclusively that HUCs were capable of bridging the distance between several spider silk fibers (fig 7a, blue arrows).
As a confirmation, we also stained HUCs seeded on spider silk matrices with DAPI and phalloidin to detect the nucleus and filamentous actin respectively. Intracellular filamentous actin is linked to the membrane bound receptors that mediate adhesion. Staining of HUCs with filamentous actin can thus, indirectly, visualize the adhesion between cells and the underlying material. Actin filaments were detected through-

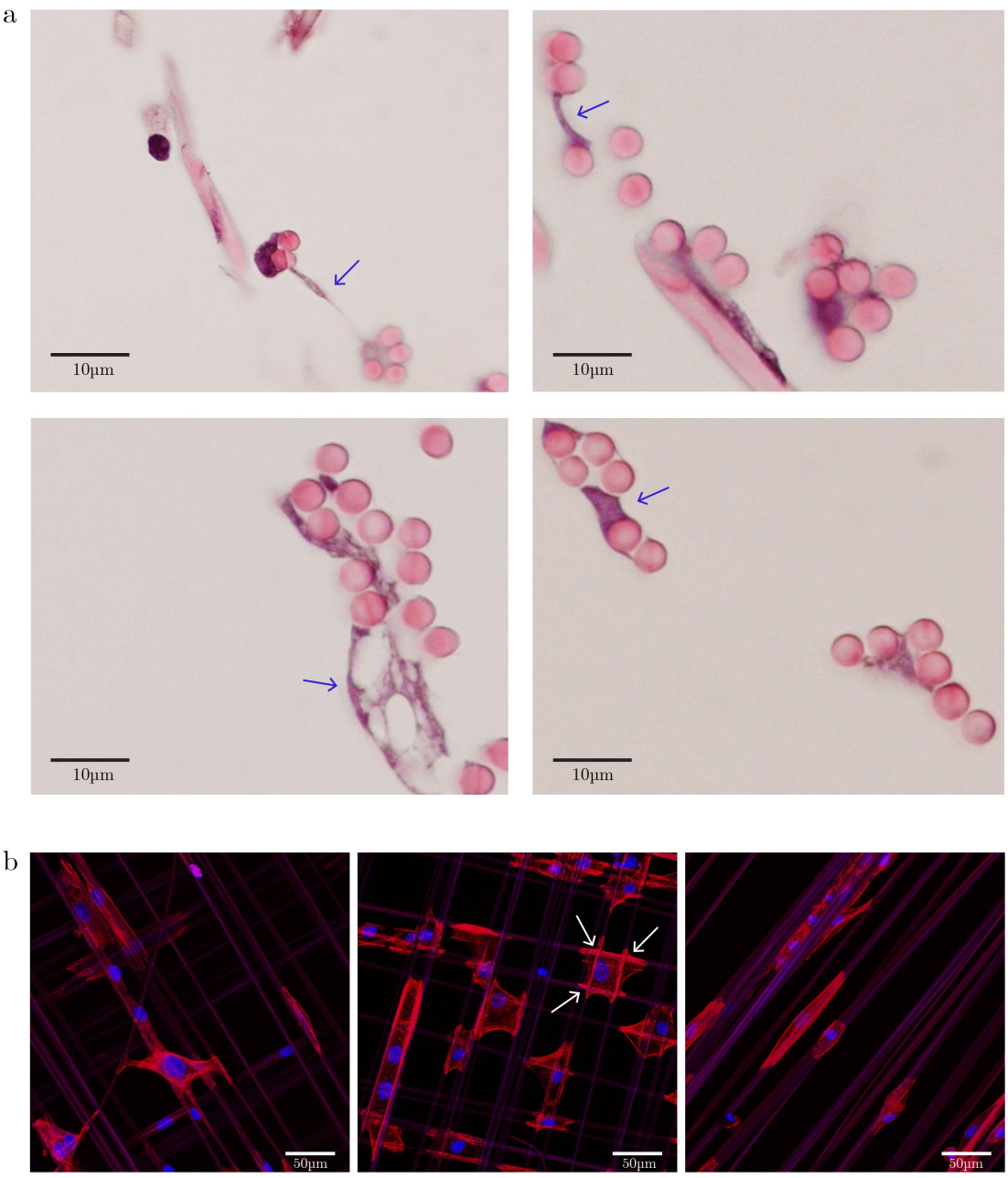

Figure 7: ***Histology of spider silk matrices seeded with HUCs***

(a) Spider silk frames were seeded with 1×10^4 *HUCs and stained with H&E after 6 days of culturing. Histological analysis shows adherence and orientation of HUCs on spider silk fibers (colored pink in images) and their capacity to bridge between spider silk fibers (blue arrows).*

(b) Spider silk frames seeded with 1×10^4 *HUCs were stained for DAPI and phalloidin for the detection of filamentous actin (red) and nuclei (blue) at day 1 post seeding. Z-stacks were made of frames and slices were merged to show orientation of filamentous actin on spider silk fibers. Attachment sites are indicated with white arrows and show filamentous actin concentrating around spider silk fibers. Spider silk fibers were autofluorescent in both channels and colored purple.*

out the cell body by confocal microscopy and showed cells stretching along and also between spider silk fibers in every direction (fig 7b). Actin filaments were mostly aligned with spider silk fibers indicating concentrated adherence of cells to spider silk matrices. Z-stacks showed that the cells grew at different depths of the spider silk matrix but mostly adhered to the cross weaved mesh at the lower side of the frame. HUCs spread out on the surface of the spider silk matrix in which phalloidin was stained remarkably bright near spider silk fibers, indicating abundant attachment sites of HUCs to the fibers (fig 7b, white arrows). Together, results demonstrate that HUCs are able to adhere to spider silk matrices and bridge the distance in between fibers.

3.3 HUCS ARE ABLE TO GROW ON SPIDER SILK

To assess the growth of HUCs seeded on spider silk matrices, cells were seeded on spider silk frames and quantified on day 1, 4, 8 and 12 by means of two approaches. The first approach of cell growth evaluation was to manually count the amount of nuclei per FOV for each sample using DAPI staining. Immunofluorescence with DAPI and phalloidin staining of spider silk frames seeded with HUCs already indicated expansion of cells over time (fig 8a). Quantification of nuclei per FOV revealed that cell growth of HUCs on spider silk is similar to control from day 1 untill day 8. However, expansion of HUCs is significantly decreased on spider silk ($p \leqslant 0.01$) at 12 days post-seeding compared to control (fig 8b).
The second approach was to monitor fluorescence as a measure for cell viability by means of Presto Blue in time, in which the main difference between both approaches is the fact that Presto Blue can quantify the same spider silk frames in time, whereas DAPI requires fixating new frames each time. In the initial phases (day 1-4) cell growth on spider silk was more or less similar to control and showed no significant differences using Presto Blue (fig 4c). However, in the later stages (day 4-12) spider silk showed significantly lower cell numbers ($p \leqslant 0.01$) compared to control.
Even though control showed significantly better cell growth over time than HUCs cultured on spider silk over time, cells were capable of expanding on spider silk. Together, the results of both approaches indicate that spider silk matrix is able to support expansion of HUCs.

3.4 SPIDER SILK DOES NOT INFLUENCE VIABILITY OF HUCS

To determine whether the difference in HUCs growth on spider silk could be caused by silk-induced death, we evaluated viability of HUCs seeded on spider silk by means of a live/dead assay in which live (green fluorescent cytoplasm) and dead (red fluorescent nuclei) cells were stained after 6 days of culturing. Spider silk matrices (fig 9b) showed a cell density and live/dead ratio that were similar to those for control (i.e. plastic containing a round glass cover slip) (fig 9a). Dead cells were only detected occasionally in both control as spider silk samples (white arrows). Negative control showed that the live/dead staining was sensitive for dead cells (fig 9e). By merging the channels with bright field, living and dead cells could be depicted showing the spider silk matrix (fig 9c). Together, results suggest that the viability of HUCs is not affected when they are cultivated on spider silk matrices.

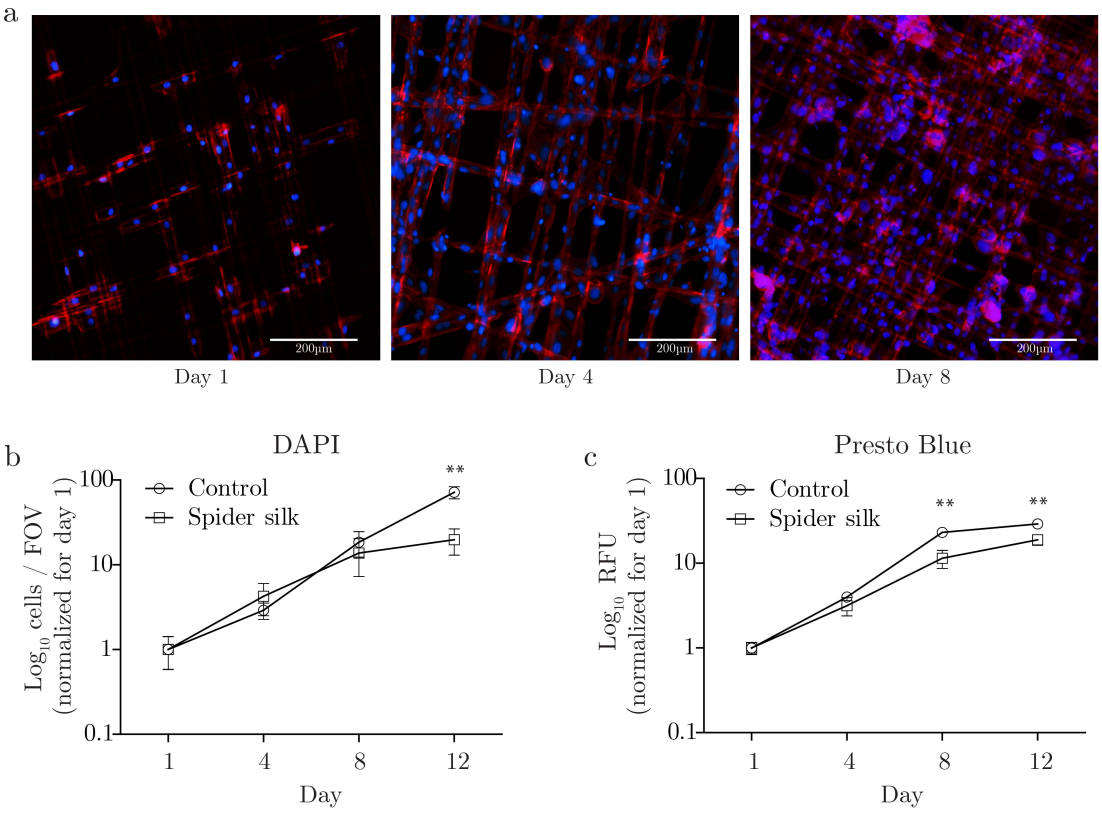

Figure 8: ***Spider silk supports expansion of HUCs***

(a) Spider silk frames were stained for DAPI and phalloidin as described earlier at day 1, 4 and 8 post seeding to show cell proliferation on spider silk matrices in time (channels were merged, 10x magnification).

(b) Spider silk frames were seeded with 1×10^4 *HUCs and controls (i.e. plastic) with* 1×10^3 *HUCs. Frames and controls were fixed and stained with DAPI at day 1, 4, 8 and 12 and nuclei were counted in fields of vision (FOV) to assess expansion of cells. Significantly more HUCs were growing on controls at day 12 compared to spider silk. Data is shown as mean* $\pm$*SD with n=3 for each time point and asterisks mark significance level where* $**$ *denotes* $p \leqslant 0.01$ *compared to control.*

(c) Viable cells growing on spider silk and controls were also quantified in time by means of Presto Blue at the same time points, depicted as relative fluorescence units (RFU). Significantly more HUCs were present in controls at day 8 and 12 compared to spider silk specimens. Data is shown as mean $\pm$*SD with n=3 for each time point and asterisks mark significance level where* $**$ *denotes* $p \leqslant 0.01$ *compared to control.*

3.5 COATED SPIDER SILK COMPROMISES GROWTH OF HUCS

Literature shows that pretreated/coated (bio)materials could enhance cell adherence of endothelial and urothelial cells [95, 96]. To determine if this is also the case for HUCs cultivated on spider silk matrices, we quantified the amount of viable HUCs by means of Presto Blue on spider silk coated with either Poly-L-Lysine or Fibronectin, and unprocessed spider silk after 1, 4, 8 and 12 days of culturing. Cells were adherent to all specimens after 1 day of cultivation displaying a significantly higher amount of viable cells adhering to spider silk coated with Poly-L-Lysine ($p \leqslant 0.01$) compared to Fibronectin coated and uncoated spider silk (fig 10a). In addition, initial adherence of HUCs seemed better in Fibronectin coated spider silk compared to unprocessed spider silk but no statistical significant difference was observed. Monitoring cell growth

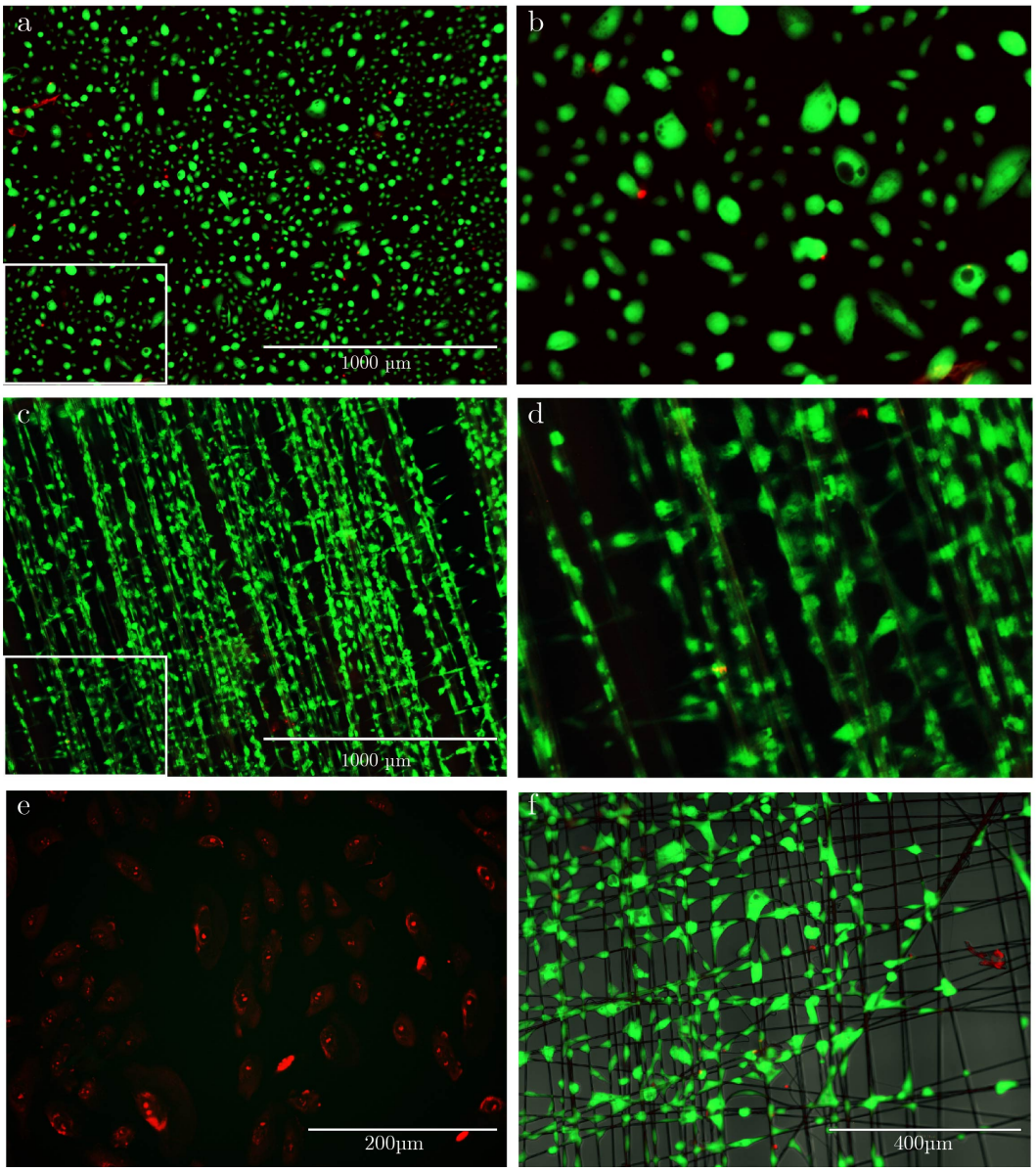

Figure 9: ***Spider silk does not enhance cell death in HUCs***
Spider silk frames (a) were seeded with 1×10^4 *HUCs and controls (c) (i.e. plastic containing a round cover slip) with* 1×10^3 *HUCs and stained with live/dead for detection of living (green) and dead (red) cells at day 7 post seeding. Channels were merged and pictures were taken with EVOS fluorescence microscopy. Insert shows a zoom-in of a small area (b+d) and shows similar amounts of living/dead cells on spider silk compared to control. HUCs cultivated in DMSO showed proof of principle that live/dead staining was sensitive for dead cells (e). To elucidate living and dead cells on spider silk matrices, a bright field image was superimposed to fluorescence live/dead images (f). Images are representative for all samples.*

on each specimen revealed that HUCs cultured on unprocessed spider silk grew at a higher rate than on coated spider silk (fig 10b). After 12 days of culturing significantly more viable cells were present on unprocessed spider silk compared to Fibronectin coated silk ($p \leqslant 0.05$) and Poly-L-Lysine coated silk ($p \leqslant 0.01$). Together, these results implicate that spider silk coated with Poly-L-Lysine or Fibronectin enhances HUC adhesion but impairs growth of HUCs.

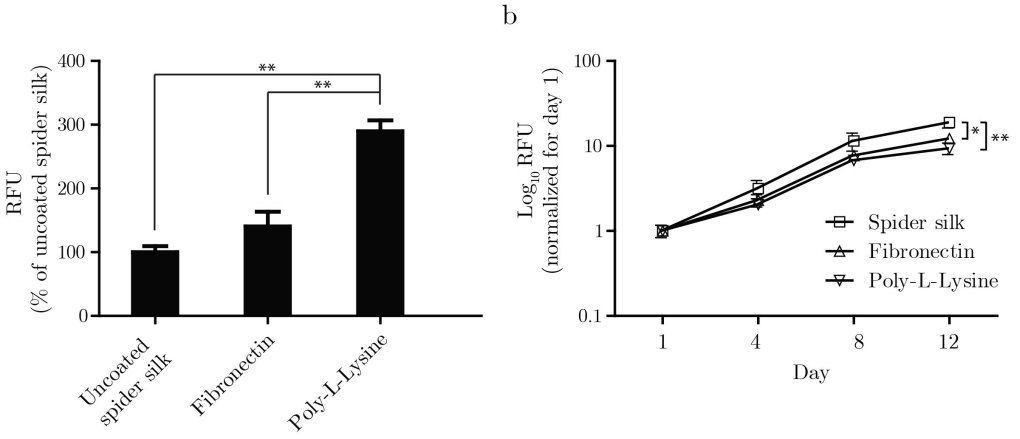

Figure 10: ***Coated spider silk enhances cell adherence but diminishes growth***
Spider silk frames were coated with Poly-L-Lysine or Fibronectin and seeded with 1×10^4 *HUCs, uncoated spider silk was used as control and viable cells were monitored with Presto Blue on day 1, 4, 8 and 12. In (a), the amount of viable cells in RFU at day 1 is shown for each specimen representing adherence of HUCs to different coatings. Significantly more HUCs adhere to spider silk coated with Poly-L-Lysine compared to Fibronectin and uncoated spider silk. When monitoring viable cells on specimens over time (b), significantly more cells were growing on uncoated spider silk at day 12 compared to coated spider silk. All data is shown as mean ±SD with n=3 for each time point and asterisks mark significance level where ∗ denotes p<0.05 and ∗∗ denotes p⩽0.01 compared to control.*

3.6 CERTAIN ADHESION MOLECULES ARE DOWN-REGULATED AT MRNA LEVEL IN HUCS CULTURED ON SPIDER SILK

To determine whether HUCs cultured on spider silk are molecularly altered, qPCR was used to determine mRNA expression levels of several genes correlated with epithelial-to-mesenchymal transition (EMT), fibrosis, differentiation and adhesion were determined in HUCs cultured on spider silk. This was compared to HUCs cultured on plastic, high- and low-graded bladder cancer cell lines T24 and RT112 and MSCs, which are a non-urothelial cell type. RT112, T24 and HUCs all showed a very aberrant expression pattern of most genes compared to MSCs gene expression, in which urothelial cellular differentiation genes stood out the most (fig 11a). T24 showed the most agreements with MSCs losing most of its urothelial differentiation gene expression.
Interestingly, results also showed substantial differences in expression levels of RT112 and T24 compared to HUCs. As expected, low graded bladder cancer cell line RT112 is more comparable to HUCs in gene expression but contained aberrant expression values for Vimentin (lower) and Ctk 20 (higher) indicating a more differentiated type of the urothelial cell. High graded bladder cancer cell line T24 has the least similarities with HUCs expressing lower levels of Vimentin, E-cadherin and most Cytokeratins indicating considerable changes in genotype.
Importantly, relative to MSCs, only fractional differences are present between HUCs cultured on plastic and HUCs cultured on spider silk. The expression pattern of primary urothelial cells of most genes was as expected. Urothelial cell differentiation molecules (i.e. all Cytokeratins and Uroplakin 3) and epithelial/adhesion marker E-cadherin were expressed high relative to MSCs. In contrast, low levels of mes-

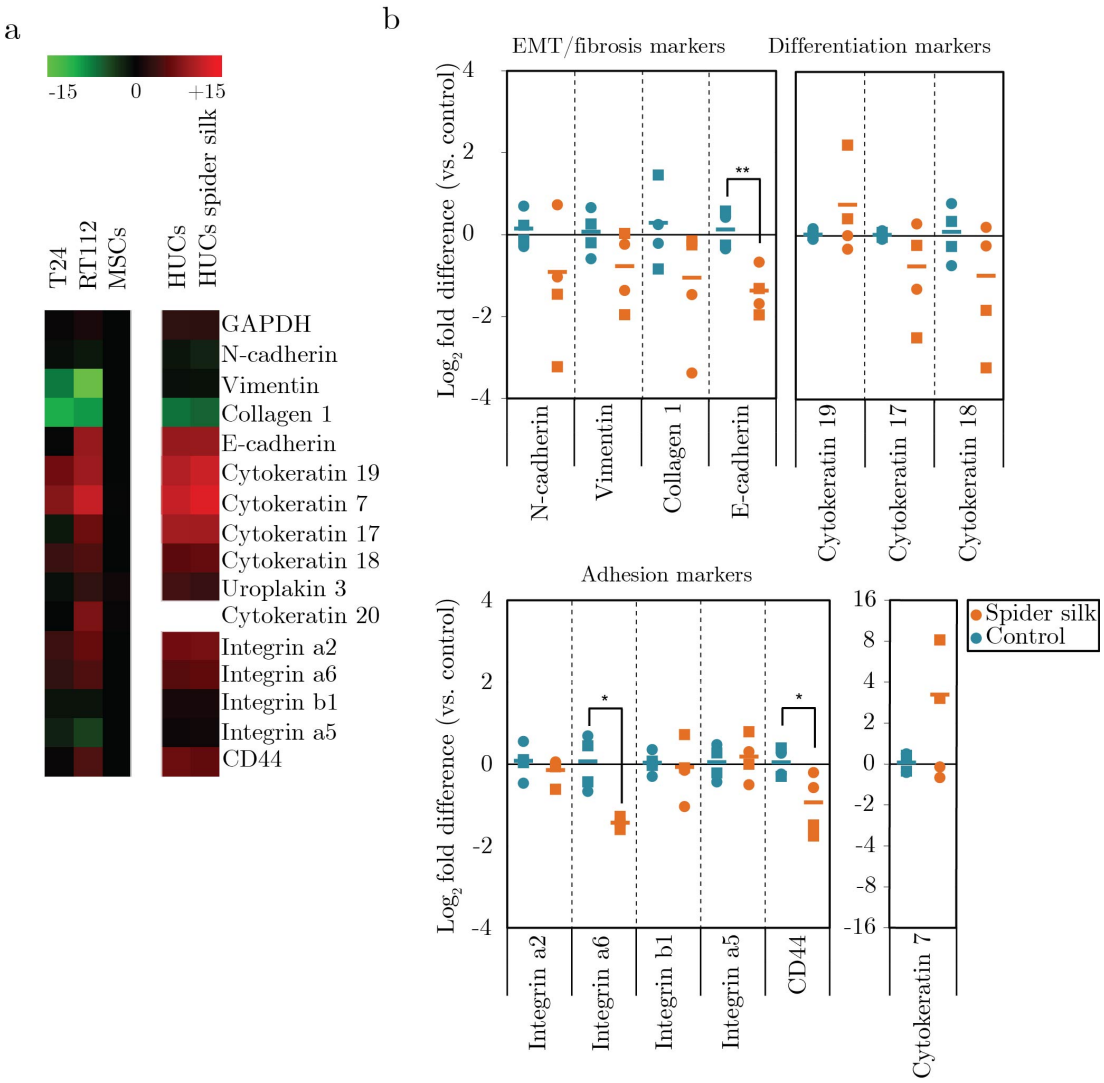

Figure 11: ***Down-regulation of certain adhesion molecules in HUCs when cultured on spider silk***

RNA was isolated from MSCs, T24, RT112 and HUCs, which were cultured on plastic or spider silk for 6 days, and qPCR was performed to compare gene expression levels of all samples. Expression is relative to housekeeper B2M. In (a), qPCR results are represented of all samples in a heatmap detailing the genes involved in EMT, fibrosis, differentiation and adhesion. HUCs cultured on plastic or spider silk show high similarities, whilst RT112 and T24 show aberrant mRNA expression levels. Data are expressed as log_2 fold change relative to average level in MSCs (set to 0). In (b), detailed qPCR results are represented of HUCs cultured on spider silk compared to plastic. E-cadherin, Integrin α6 and CD44 were expressed significantly lower in HUCs cultured on spider silk. Expression is relative to housekeeper B2M and data is expressed as log_2 fold change relative to average level in HUCs cultured on plastic (set to 0). Data is shown as mean ±SD and asterisks mark significance level where $*$ denotes $p \leqslant 0.05$ and $**$ denotes $p \leqslant 0.01$ compared to control.

enchymal markers N-cadherin and Vimentin and fibrosis marker Collagen 1 were expressed by HUCs on plastic and spider silk. Regarding adhesion, Integrins were expressed at higher or similar levels compared to MSCs and expression of Ctk 20 was not detectable in HUCs.

Looking more detailed at mRNA expression levels of HUCs compared to HUCs cul-

tured on spider silk, revealed that HUCs cultured on spider silk express significantly lower levels of E-cadherin ($p \leqslant 0.01$), Integrin $\alpha 6$ ($p \leqslant 0.05$) and CD44 ($p \leqslant 0.05$) with an approximately 1.5-fold change (fig 11b). Interestingly, all differentially expressed genes are involved in cell-cell or cell-matrix interaction (i.e. adhesion). Genes involved in EMT, fibrosis and differentiation showed no statistically significant differences between HUCs cultured on plastic and spider silk. These results indicate that spider silk has some effects on down-regulation of various adhesion molecules at the mRNA level of HUCs.

3.7 SPIDER SILK SLIGHTLY CHANGES CELLULAR DIFFERENTIATION OF HUCS

Ho et al. recently show the process of cellular differentiation in normal urothelium in which expression of certain proteins can indicate urothelial cell type; i.e. urothelial stem cell (CD90+ CD44+ CD49f+), basal cell (CD90- CD44+ CD49f+), intermediate cell (CD90- CD44- CD49f+) and umbrella cell (CD90- CD44- CD49f-) [97]. The observation that CD44 mRNA level could be indicative of increased cellular differentiation in HUCs cultured on spider silk. To test this, and to determine the differentiation states of the cells cultured on spider silk, cells were stained for CD90, CD44 and CD49f. As a first test and proof of principle, the expression pattern of these proteins was determined in HUCs and compared to HUCs cultivated in FBS, which stimulates differentiation of HUCs. Results showed that HUCs were positive for both CD44 and CD49f and negative for CD90, indicating a basal cell type (fig 12a). When cultured in FBS, HUCs were lower in CD44 expression and showed both higher and lower CD49f expression, confirming increased differentiation of HUCs.
Subsequently, high- and low-graded cancer cell lines T24 and RT112 were analyzed and compared to HUCs. Results revealed that T24 was higher in CD49f and CD44 expression, whilst RT112 cells were higher in CD49f but lower in CD44 expression. The effects of spider silk cultivation on cellular differentiation were analyzed and compared to HUCs cultured on plastic, in which results demonstrated that HUCs cultured on spider silk formed a small subpopulation lower in expression of CD44. In addition, overall expression of CD90 was significantly higher ($p \leqslant 0.05$) in HUCs cultured on spider silk (fig 12b). Together, these results suggest that spider silk might slightly induce differentiation (i.e. maturation) of HUCs at the protein level.

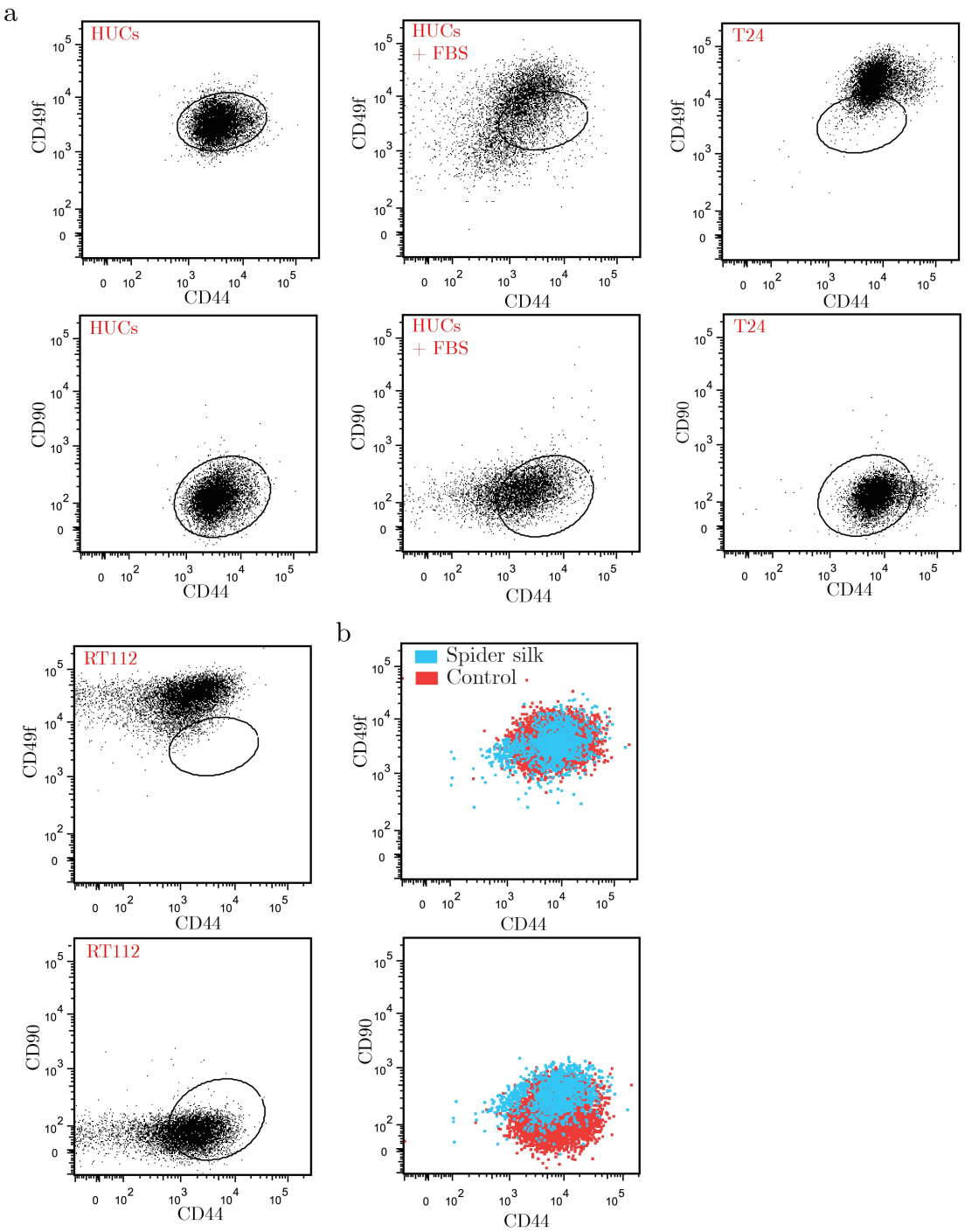

Figure 12: ***Cellular differentiation of HUCs is altered when cultured on spider silk***
(a) T24 cells, RT112 cells, HUCs, HUCs + FBS were stained for CD90, CD44, CD49f and Sytox Blue and analyzed on cellular differentiation with FACS (after exclusion of dead or doublet cells).
(b) Spider silk frames were seeded with 1×10^4 *HUCs and controls (i.e. plastic) with* 1×10^3 *HUCs. After six days of culturing cells were stained for CD90, CD44 and CD49f and analyzed by FACS. CD90 is expressed significantly higher in HUCs cultured on spider silk compared to control* ($p \leqslant 0.05$). *A small subpopulation lower in CD44 expression could be seen in HUCs cultured on spider silk, but showed no significance. Data is representative for all samples and statistical analysis was based upon mean* $\pm SD$ *and* $n=3$.

4

DISCUSSION

Summary

Previous studies investigating the potential of spider silk as a biomaterial already described promising results regarding adherence and proliferation of chondrocytes, fibroblasts, keratinocytes and Schwann cells on spider silk [76, 81, 82, 98]. This study shows that spider dragline silk from *Nephila Clavipes* could potentially be used as a biological matrix for reconstruction of the bladder. Performing studies with HUCs, we demonstrate that we were able to create native spider silk matrices, which support adhesion and proliferation of cells. Our results demonstrate that HUCs remained viable and spider silk had no cytotoxic effects on HUCs. In addition, qPCR and flow-cytometry analysis showed small changes in certain adhesion molecules but no major alterations in cellular differentiation.
To assess the potential of native spider silk as a biomaterial for bladder reconstruction, spider silk has to meet the following requirements; the spider silk matrix should (1) be biocompatible for HUCs, (2) support adherence of HUCs, (3) support expansion of HUCs and (4) not induce alterations in characteristics of HUCs.

Biocompatibility

This study is performed to test the fundamental cellular processes that occur in HUCs when cultivated on native spider silk. Ultimately, in future we would want to incorporate spider silk into the human body for reconstruction purposes. It is of great importance that spider silk should be biocompatible in order to prevent immunological reactions and rejection of the construct. One of the great advantages of spider silk, compared to other silks, is the absence of the immunogenic sericin coating. In addition, recent studies show that *in vivo* application of spider silk in rats and pigs demonstrates promising results regarding biocompatibility [66, 77, 90].
In our study, we tested the *in vitro* biocompatibility of the spider silk frame on viability of HUCs in terms of toxic effects of spider silk or steel frame substances on cells. This was performed by culturing cells in an extract of spider silk using a method described in other studies [53]. Since SIS is the present gold standard for bladder reconstruction using a biomaterial, it was used as a positive control.
Results in biocompatibility testing showed a high variability, especially in the negative control (i.e. medium) and spider silk samples at day 7, resulting in a high standard deviation (fig 5). However, raw data at day 7 showed, in both control and spider silk triplicates, one extreme outlier with a lower absorbance value than medium only control, suggesting potential pipetting errors (i.e. the potential omission to pipet cells or presence of an air bubble). If we would exclude these outliers, cell viability of HUCs cultured in spider silk extract at day 7 would be similar to negative control and cell viability of HUCs cultured in SIS extract would be significantly lower. This assumption is supported by a study investigating the cytotoxicity of SIS *in vitro* revealing SIS-conditioned cell culture medium to be cytotoxic for HUCs [99].
Importantly, irrespective of this potential pipetting error, in either case cultivation of HUCs in an extract of spider silk was not toxic to HUCs. Since this study is the first

to describe culture of HUCs on native spider silk, it is difficult to draw a comparison with other studies using spider silk and urothelial cells. Wang et al., however, already demonstrate that spider silk conditioned-medium has no toxic effects on endothelial cells, indicating that spider silk itself is not toxic to cells [100].
Also, the results of the live/dead staining indicated that spider silk has no toxic effects on HUCs, since HUCs cultured on spider silk showed similar, or even better, viability of cells compared to cells cultured on plastic (fig 9). This indicates that, even though spider silk is an aberrant substrate for cells to adhere and grow on, cells remain viable even after 7 days of culturing. This is supported by studies researching the *in vitro* toxic effects of natural spider silk on different cell types in which similar results are demonstrated and cells remain viable [75, 82, 98]. However, to give our statistical analysis and hypothesis more power, the biocompatibility assay should be repeated. Promising is the fact that recent studies show that anionic and hydrophilic substrates, both characteristics of native spider silk, are more biocompatible *in vivo* [101–103].

Adhesion mechanisms

Since the bladder is one of the most dynamic organs of the body enduring relatively large mechanical forces, it is of great importance that spider silk should support cell adhesion. In this study, we aimed at developing a method to achieve and optimize primary urothelial cell adhesion. Here, we used primary human urothelial cells that have the capacity to expand *in vitro* and generate all various urothelial cell lineages that constitute the human bladder to mimic *in vivo* behavior of urothelial cells more closely [104, 105]. Drawback of using primary cells is the fact that they have a finite life span, are very sensitive to culture conditions and require careful handling [106]. For this reason, seeding and initial adherence of HUCs on the spider silk frame turned out to be challenging and the method had to be optimized. It turned out that the ideal cell seeding density was 500 cells/µl applied in 2 droplets of 10 µl, bigger droplets caused cells to fall through the spider silk matrix. The time of incubating these droplets, prior to adding medium, should not exceed 1,5 hours since cells then died potentially due to lack of nutrients and/or increased osmolarity due to drying out of the droplets.
To improve initial cell adhesion, different approaches were used that were tested successfully in other spider silk culture studies. Gellynck et al. describe the use of sterile agarose gel on the bottom of the well to prevent cells from adhering and stimulate adhesion to the spider silk fibers [81]. In our study, the use of agarose while culturing HUCs on spider silk negatively influenced cell morphology and viability, probably due to osmolarity and primary cells being more sensitive to culture condition variations (data not shown). Wendt et al. prevent cell attachment to the bottom of culture dishes by coating it with Pluronic F-127 [82]. We tested the use of non-treated cell culture well-plates, to prevent cell adhesion to the bottom, but it did not enhance adhesion of HUCs to spider silk fibers.
Another approach of improving cell-matrix adhesion would be to coat the spider silk matrix with matrix-protein such as Fibronectin. Fibronectin is expressed in and near the urothelial basement area and is mostly associated with superficial urothelial cells [107]. Assuming that extracellular matrix components can mimic the natural microenvironment of urothelial cells more closely and thus enhance cell adhesion, we coated spider silk matrices with Fibronectin. In addition, we coated spider silk ma-

trices with Poly-L-Lysine, a synthetic compound which is highly positively charged, thus enhancing cell adhesion, and is recommended for normal primary urothelial cell culture. Our study demonstrated an increase in initial cell adhesion of HUCs on Poly-L-Lysine and fibronectin coated spider silk, as expected (fig 10a). However, expansion rates after 12 days of culturing was significantly lower when HUCs were grown on coated spider silk matrices suggesting that, even though initial adhesion is improved, coating of spider silk matrices does not seem beneficial for HUCs due to impaired expansion (fig 10b). This is supported by a recent study which shows significantly increased initial adherence of primary urothelial cells to fibronectin coated silk biomaterials, but relatively lower expansion rates after seven days cultivation compared to uncoated silk biomaterial [95]. It remains to be determined whether this relative inhibition of cell growth of HUCs on both Fibronectin and Poly-L-Lysine coated spider silk matrices is the result of decreased proliferation, increased differentiation or increased cell death.
Eventually, cell adhesion was most optimal when the spider silk matrices where incubated in PBS for several days thus removing the 10-20 nm outermost lipid coat of the fibers revealing the glyco layer of spider silk containing fine fibrils [108]. Results revealed that filamentous actin staining was specifically localized to areas where the HUCs were in contact with the spider silk (fig 7b). Actin filaments are known to contribute to cell adhesion via focal adhesions. Here, large bundles of actin filaments form stress fibers and these anchor the cell against the substrate via interactions with integrins [108]. The numerous actin filaments present in HUCs on spider silk indicate the formation of stress fibers adhering to spider silk matrices via integrins. The fact that filamentous actin was aligned along spider silk fibers suggests that contacts were not random. In addition, HE staining demonstrated cell adhesion to spider silk fibers as well. This is in contrast to SIS, the gold standard in bladder reconstruction, where *in vitro* adhesion of HUCs was only possible when cultivated in 5% FCS [99]. However, the use of FCS is not in agreement with GMP laboratory setting and therefore these seeded SIS constructs are not allowed for clinical application. This suggests that spider silk is an attractive biomaterial for *in vivo* use since HUCs are easily capable of adhesion without the use of serum.

Expansion

Another important requirement of spider silk is to support expansion of HUCs, thus developing a monolayer of cells on a spider silk matrix for incorporation in the bladder. Different approaches have been used to quantify cell numbers in time on spider silk varying from Alamar Blue assays to Tubulin immunostainings [89, 95, 98]. However, drawback of these approaches is the fact that all these quantification methods are end-point measurements at each time point. Since there is a relatively large variation in the amount of HUCs that initially adhere to each frame, the variance between cell numbers per spider silk matrix in time shall be even higher due to exponential growth. By performing end-point measurements at each time point, no corrections can be made for initial cell adhesion numbers.
Therefore, our study aimed at developing a method to quantify cell numbers on the same spider silk matrix in time, thus demonstrating relative cell growth in time normalized for the amount of cells that initially adhered to the matrix. Presto Blue has these features and can quantitatively measure cell viability over time. Alongside, we also quantified cell numbers using an end-point analysis method by counting nuclei

per FOV by means of DAPI as described by Kuhbier et al. [98]. We demonstrated with both methods that HUCs were capable of expanding on spider silk matrices (fig 8). The end-point analysis, as hypothesized, showed a much higher standard deviation per time-point in cell numbers when HUCs were grown on spider silk matrices. In addition, by counting five FOV's we assumed cell numbers to be representative for the whole spider silk frame, but it still remains an estimate. Presto blue quantification, on the contrary, demonstrated very small standard deviations and represented cell numbers on the whole spider silk frame. Thus, indicating a more trustworthy and reproducible approach for cell quantification of HUCs on spider silk and recommended for future quantification on spider silk matrices.
Even though HUCs were capable of expanding on spider silk matrices, controls showed in both methods significantly better expansion after 8-12 days of cultivation. This is in accordance with studies demonstrating lower expansion rates of fibroblasts when cultured on spider silk matrices as compared to cover slips [98]. This could be due to the fact that HUCs on spider silk matrices have a lower growth rate (a), differentiate earlier (b), reached confluence earlier in our experiments (c) or HUCs die on spider silk (d). The surface interaction between a cell and a spider silk fiber is considerably smaller (i.e. ± 4 µm) compared to a cover slip and cells have to bridge the fiber gaps to reach confluency. This could affect cell growth rate, especially since primary cells in general are sensitive to cultural conditions. Also, DAPI and phalloidin staining at day 8 already revealed that HUCs were reaching confluence on the spider silk frames indicating reduced growth rates due to an overcrowded frame. In addition, our study also revealed a slight increase in differentiation of HUCs when cultured on spider silk perhaps impairing growth.
In a study performed with HUCs cultured on uncoated recombinant silk matrices, cells are capable of expanding on the matrix but results are less convincing compared to our study [95]. In addition, HUCs cultured on SIS show hardly any cell growth at all *in vitro* [99, 109]. Therefore, even though the exact reason for the slightly aberrant expansion of HUCs on spider silk compared to control remains to be elucidated in this study, the key element is that HUCs showed compelling expansion and are capable of creating a monolayer on spider silk *in vitro*.

Cellular differentiation at mRNA level

Since the ultimate goal is to generate constructs with spider silk and HUCs to be used *in vivo*, the biomaterial should not functionally alter HUCs regarding cell type and differentiation. The substrate on which cells are cultured can modulate *in vitro* and *in vivo* cellular responses such as adhesion, proliferation, viability, cell cycle progression and expression of differentiated celltypes [110, 111]. Keselowsky et al. demonstrate that surface chemistry in terms of integrin expression can modulate osteoblastic differentiation and matrix mineralization [111]. In addition, matrix stiffness can influence proliferation and differentiation as well [112]. Studies show that cells (i.e. human dermal fibroblasts, bone marrow stem cells and bone-derived cells) cultured on stiffer collagen matrices can significantly affect gene-regulation and proliferation of cells [113, 114]. At worst, substrate material can cause dedifferentiation of cells which has also been implicated in cancer [115].
Therefore it is of great importance to monitor differentiation of HUCs when cultured on spider silk. Here, we monitored differentiation by means of mRNA expression changes by qPCR. Our study focused on intracellular cytokines (Ctk) 7, 18, 19 (all

expressed in all cell layers of the urothelium), Ctk 17 and CD44 (expressed by basal urothelial cells only) & Ctk 20 and Uroplakin-3 (expressed by superficial urothelial umbrella cells only). Interestingly, aberrant expression of most of these genes have been associated with carcinogenesis of the bladder [116, 117]. In addition, we also monitored genes involved in the EMT. EMT confers mesenchymal properties to epithelial cells to generate mesenchymal cell types by up regulation of N-cadherin, Vimentin and Fibronectin and is associated with carcinogenesis as well [118]. Collagen 1 expression was determined to give an impression of fibrosis of urothelial cells when cultured on spider silk matrices, and eventually give a prediction of the *in vivo* mechanical functionality of the construct. Lastly, adhesion molecules known to be involved in urothelial cell-cell and cell-matrix interactions (i.e. E-cadherin, Integrin $\alpha 2$, Integrin $\alpha 6$, Integrin $\alpha 5$, Integrin $\beta 1$ and CD44) were examined to reveal potential adhesion mechanisms [119].

We demonstrated that, compared to HUCs, bladder cancer cell lines T24 and RT112 showed considerable differences in expression of EMT genes (fig 11a). The loss of E-cadherin and small increase of N-cadherin by T24 cells compared to HUCs indicates a loss of epithelial phenotype. Even though an increase in vimentin is expected when EMT occurs, we observed that T24 and RT112 are low in vimentin expression which is in line with previous studies [120]. RT112 showed more similarities with HUCs than T24, in terms of equal levels of E-cadherin and N-cadherin expression, confirming a lower graded carcinoma type. On the contrary, Ctk 20 was (strongly) up-regulated in RT112 and T24 which is as expected for transitional-cell carcinomas [117]. Further, mRNA expression patterns of Cytokeratins for T24 and RT112 are in accordance with other studies [120]. Together, these results confirmed the differentiation status of the control cell lines and the quality of the primers used.

QPCR results comparing HUCs cultured on plastic to HUCs cultured on spider silk, when normalized for MSCs, demonstrated that these cells show way less dramatic gene expression changes as compared to the gene expression changes in the bladder cancer cell lines. This is in line with a study demonstrating that recombinant silk supports expression of differentiation markers in HUCs [95].

We only observed small changes including down-regulation of E-cadherin, Integrin $\alpha 6$ and CD44 in HUCs cultured on spider silk (fig 11b). Interestingly, these genes are all involved in cell-cell and cell-matrix interaction and aberrant expression levels of adhesion molecules are not alarming when using a new substrate. Even though changes are at mRNA level, on protein level it could potentially indicate that; (1) same amounts of adhesion sites are being overall reduced in HUCs on spider silk, (2) reduced amounts of adhesion sites of corresponding genes in HUCs each still remain the same expression as controls or (3) other adhesion pathways are being activated in the interaction between HUCs and spider silk. Therefore, these aberrant genes require investigation at protein level in future. To elucidate alternative adhesion mechanisms and pathways, we also suggest screening a wide range of cadherins and integrins on mRNA expression. In addition, blocking antibodies against up- or down-regulated genes could reveal adhesion mechanisms.

As described earlier, E-cadherin, Integrin $\alpha 6$ and CD44 are all involved in cell-cell and cell-matrix interaction. However, E-cadherin is also the keystone of epithelial state and down-regulation of E-cadherin could also indicate a potential loss of epithelial phenotype. On the other hand, mesenchymal markers N-cadherin and Vimentin showed a tendency to be down-regulated as well, suggesting no indication of EMT occurring. Future research should be performed in which HUCs are cultured on spi-

der silk matrices and implanted in an animal model to monitor *in vivo* changes over a longer period of time and to ensure no loss of epithelial phenotype.
CD44, besides being a cell-cell adhesion molecule, is also predominantly expressed in the basal layer of the urothelium [116]. The significantly lower expression of CD44 in HUCs cultured on spider silk could suggest that HUCs lose basal cell phenotype indicating a shift of cells into a more differentiated celtype (e.g. intermediate or umbrella cell) or a more immature celtype (e.g. progenitor or stemcell). Therefore, flow cytometry was performed to determine the differentiation status of HUCs cultured on spider silk matrices and elucidate CD44 expression on a protein level.

Cellular differentiation at protein level

Recently, Ho et al. described the flow cytometry-based identification of different cell types within the normal urothelium by means of the espression of the differentiation marker CD90, CD44 and CD49f [97]. In this scheme, urothelial stem cells are positive for CD90, CD44 and CD49f. During the process of differentiation, basal cells lose expression of the marker CD90 (CD90- CD44+ CD49f+), followed by loss of expression of CD44 in intermediate cells (CD90- CD44- CD49f+) and eventually loss of expression of CD49f in umbrella cells (CD90- CD44- CD49f-). In our study, we use these markers to determine the status of cellular differentiation of T24, RT112 cells, HUCs, HUCs + FBS and HUCs seeded on spider silk matrices.
As expected, normal HUCs expressed molecules associated with a basal cell type (fig 12a). To test proof of principle, HUCs were cultured in serum since this induces differentiation of urothelial cells [119]. Accordingly, we observed that HUCs cultured in serum were lower in CD44 and CD49f, indicating increased differentiation of cells to the intermediate and umbrella cell types. Protein expression patterns of T24 and RT112 cells indicated a basal and intermediate cell type respectively. This is in accordance with Ho et al. implicating basal subtype cancers with poor clinical outcomes (i.e. T24) and better outcomes in intermediate subtype cancers (i.e. RT112) [97]. Strikingly, both T24 and RT112 expressed CD49f extremely high potentially suggesting a correlation between CD49f expression and carcinogenesis.
Eventually, we determined expression levels of markers in HUCs cultured on spider silk matrices. The changes we observed in these genes are nowhere near as dramatic as differences observed between HUCs and urothelial cancer cell lines. The changes we observed revealed a small subpopulation low in CD44 expression, indicating that the low CD44 mRNA levels were of a small population showing low CD44 expression rather that a decrease of CD44 on all spider silk exposed HUCs (fig 12b). This suggests that a small group of HUCs tend to differentiate while the other HUCs remain their basal phenotype. On the contrary, all HUCs cultured on spider silk move slightly higher in expression of CD90, which is suggestive of a more stem cell like phenotype. However, CD90 has also been associated with cell-cell and cell-matrix interactions and can thus implicate higher adhesion interactions in HUCs on spider silk.
All together, we observed various subtle changes at mRNA and protein expression levels of HUCs cultured on spider silk. Experiments have to be repeated in future and over a longer period in time to draw definite conclusions regarding spider silk altering cellular differentiation and functionality of HUCs.
For future use it is of interest to philosophize about the composition of the construct prior to implantation in terms of cellular differentiation. Here, the question is whether

one should implant an already *in vitro* fully differentiated construct, or an immature construct (i.e. proliferating HUCs) in patients. We believe that spider silk matrix containing a monolayer of immature HUCs and smooth muscle cells should have the most functional *in vivo* potential, since proliferating immature cells should adjust and incorporate better into other bladder structures (i.e. increased regeneration). This theory is supported by Atala et al., demonstrating successful engraftment of an a-cellular scaffold seeded with urothelial and smooth muscle cells in patients [26]. Here, urothelial cells are seeded on scaffolds at passage 5 and are implanted in the human body within 4 days post-seeding, indicating that the scaffold contains immature urothelial cells. Results from our study demonstrate that it is achievable to develop a spider silk construct *in vitro* containing a monolayer of largely immature HUCs. Promising is the fact that immunohistology of biopsies of the engineered bladders in patients, at 2.5 years post-implantation, reveals a phenotypically normal tri-layered structure (i.e. urothelial cells, submucosa and smooth muscle cell) [26]. This indicates that a (bio)material containing a monolayer of cells is capable of regenerating into normal bladder tissue.

Conclusion

In conclusion, we have shown that native spider silk has the potential of being a promising biomaterial for bladder reconstruction, and potentially other urinary tissues. In this study, we choose to use native spider silk to benefit from its unique features and test the most fundamental cellular questions in putting forward a new biomaterial for *in vivo* use. Major drawback of native spider silk for *in vivo* use is supercontraction and not being able to produce silk on a large scale. However, ongoing advances in recombinant spider silk and all its properties and possibilities will probably solve these issues. It is likely that recombinant spider silk can be genetically modified overcoming issues such as supercontraction. In addition, RGD cell-binding domains can enhance adhesion of cells to spider silk even more, which is of great interest since a dynamic organ such as the bladder endures considerable tensile forces [121]. In order to make a functional construct, similar experiments should be performed with smooth muscle cells in future and, eventually, co-culture both cell types on spider silk matrices. Long term effects on cellular functionality (e.g. are HUCs capable of building up a glycosaminoglycan layer) and differentiation *in vivo* merit future investigation. In addition, effects of bladder environment on spider silk (e.g. degradation due to ureum and mechanical forces) and cells (e.g. tensile forces affecting adherence and functionality) should be investigated *in vivo* as well. When these steps have been taken, possibilities of spider silk as a reconstructive biomaterial (i.e. filler, scaffold or regenerative gauze) for urinary tissues would be endless.

A

APPENDIX A

Gene	Primer
Vimentin	Fwd: ACCAACGACAAAGCCCGCGT
	Rv: CAGAGACGCATTGTCAACATCCTGT
E-cadherin	Fwd: CACCACGTACAAGGGTCAGGTGC
	Rv: CAGCCTCCCACGCTGGGGTAT
N-Cadherin	Fwd: AGTCACCGTGGTCAAACCAATCGA
	Rv: TGCAGTTGACTGAGGCGGGTG
Collagen 3	Fwd: GTTGCACGAAACACACTGGG
	Rv: AAAAGCAAACAGGGCCAACG
Uroplakin 3	Fwd: GACTTTCGCCACCAACAACC
	Rv: TTCCTGGAAATGGCTGAGTCG
Collagen 1	Fwd: CCCCAGCCACAAAGAGTCTAC
	Rv: TGATTGGTGGGATGTCTTCGT
Cytokeratin 18	Fwd: CATGCAAAGCCTGAACGACC
	Rv: ATTTGCGAAGATCTGAGCCCT
Cytokeratin 19	Fwd: TGAGGAGGAAATCAGTACGCTG
	Rv: TTGGCTTCGCATGTCACTCA
Cytokeratin 20	Fwd: CCTACACAAGCATCTGGGCA
	Rv: AGAACTGCAGTCTGTCTCTCA
Cytokeratin 17	Fwd: GGAGCAGCAGAACCAGGAATA
	Rv: CTTGTACTGAGTCAGGTGGGC
Cytokeratin 7	Fwd: TCTTTGAGGCCCAGATTGCTG
	Rv: GCAGCATCCACATCCTTCTTC
Integrin $\alpha 2$	Fwd: TTAGCGCTCAGTCAAGGCAT
	Rv: CGGTTCTCAGGAAAGCCACT
Integrin $\beta 1$	Fwd: CCGCGCGGAAAAGATGAATTT
	Rv: CCACAATTTGGCCCTGCTTG
Integrin $\alpha 6$	Fwd: CAAATGCAGGCACTCAGGTTC
	Rv: AGCCTTGTGATATGTGGCATCA
Integrin $\alpha 5$	Fwd: AAGACTTTCTTGCAGCGGGA
	Rv: GCCACCTGACGCTCTTTTTG
CD44	Fwd: AGCAACTGAGACAGCAACCA
	Rv: AGACGTACCAGCCATTTGTGT

Table 1: Overview of all primers used in this study and their corresponding sequences

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