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AN EPITHELIAL CELL CULTURE SYSTEM FOR URETHRAL REGENERATION: *IN VITRO* PRECLINICAL ASSAYS AS ALTERNATIVE TO ANIMAL MODELS

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II. ABSTRACT

A plethora of animal models have been developed over years in order to evaluate advanced therapies and related medical devices for clinical application. Nevertheless the perfect animal model is still missing; there is often an incongruity when we switch to the human, as reported for example for cystic fibrosis (CF) mice models. In this case mice don't show the mucus-clogged lungs and persistent lung infections that characterize human CF sufferers. It could be because the lungs of mice are fundamentally different from those of humans and they have fewer mucus secreting glands and cells overall. This mismatch is so obvious that the European community has expressed, in the 1959, the famous three Rs (3Rs) guiding principles for more ethical use of animals in testing: replacement, reduction and refinement of and for animals.

In order to obtain more predictive results, human autologous cells should be used for *in vitro* preclinical assays mimicking the human condition. Only a consolidated system of cell culture, with standardized conditions can give strength to preclinical data, becoming an *in vitro* tissue culture model. In the XXth century, Rheinwald and Green pioneering work made possible the cultivation of human keratinocytes using a feeder layer of lethally irradiated 3T3-J2 cells, introducing a somewhat irreplaceable condition for epithelial stem cell-mediated cell therapy. Taking advantage from that discovery, over years our laboratory set a standardized system according to GMP (Good Manufacturing Practices) to cultivate keratinocytes. Furthermore recent breakthroughs in cell therapies, based on autologous culture of somatic stem cells, have generated many devices supporting this new stem cell-based regenerative medicine field; hence the increased need of assessing safety and efficacy of these materials. My thesis project of was focused on *in vitro* evaluation of safety of an advanced cell therapy for the surgical reconstruction of urethra, in case of stricture. This narrowing makes urination difficult can impair bladder function and allow infections leading to the need of a surgical reconstruction by urethroplasty. The procedure requires healthy tissue for the repair, such as autologous grafts taken from the oral cavity. In this study, taking advantage from these technologies, we produced an innovative scaffold for extensive urethral reconstruction. An

important step is the assessment of the safety and the efficacy of the cell culture model containing fibroblasts and epithelia from urethra and mucosa. Biopsies from bulbar urethra and oral mucosa were obtained from patients during reconstructive surgery, after informed consent. Over the last 5 years, tissue cultures of urethra and oral mucosa were characterized in our laboratory. Data suggest that there is a wide similarity among two epithelia, with the exception of a higher migration capacity of oral mucosa colonies, suggesting a more efficient wound healing capacity of oral mucosa respect to urethral epithelium.

These important data lead us to investigate a biocompatible scaffold for extensive urethral regeneration with oral mucosa stem cell. In order to develop the best tissue engineered therapy is really important to have a full knowledge of the pathology and the mechanisms regulating the epithelium homeostasis. Indeed, we were wondering what is the effect of the *BMI-1* transcription factor, that was regulated during clonal conversion from urethral and oral mucosa holoclone to meroclone and paraclone, as shown in previously published data (Corradini et all. 2015).

1. INTRODUCTION

1.1 From the animal models towards *in vitro* cell culture models

Animal models helped us to better understand the pathophysiology of some diseases, but they could not contribute so much to clinical practice. The discrepancies between animal experiments and clinical trials are yet not been solved. For this reason the development of alternatives to animal testing has been a subject of increasing research activity over the past 30 years (Piersma et all. 2006).

In 1959, Russel and Burch postulated the 3RS public concerns about the welfare of animals in research: Refinement, Replacement and Reduction. The concept involves refining animal use to lessen or avoid pain and distress and enhance animal well-being, reducing the total number of animals required for specific studies, and replacing animals with non animal systems and approaches. Nowadays countries have committees to establish animals that can be used in research in agreement with 3Rs (Stoke et all.2015). In addition to the ethical issue there is a problem concerning the reliability of data obtained because of frequent incongruities switching to the humans, as reported for example for Duchenne muscolar dystrophy (DMD) or cystic fibrosis (CF) mice models. In the latter case mice don't show the mucus-clogged lungs and persistent lung infections that characterize human CF sufferers. These findings could be due the fact that mice lungs are fundamentally different from those of humans, with fewer mucus secreting glands and cells overall (Clark et all.1992). Hence, in order to obtain more predictive results, *in vitro* preclinical assays, with human autologous cells, should be used to mimick the human condition. Only a consolidated system of cell culture with standardized condition can give strenght to preclinical data thus becoming an *in vitro* cell culture model allowing us to reduce the number of the animal used.

1.2 The long road of cell therapy from bench to bedside

Rheinwald and Green pioneering work made possible the cultivation of human keratinocytes using a feeder layer of lethally irradiated 3T3-J2 cells, introducing a somewhat irreplaceable condition for epithelial stem cell mediated cell therapy (Green et all.1980). Recent breacktroughs on *in vitro* manipulation of cells have generated the birth of the so called advanced therapy medicinal products (ATMPs), including cell/gene therapy and tissue engineering. These products are strictly regulated by the pharmaceutical sector, although they are not equal to the molecules chemically synthesized, having a very short shelf life and a limited period of time in order to make controls, before they are released (Pellegrini et all.,2014). Only few of the ATMPs entered the phase of clinical trials on humans. The first cell therapy used in humans was that using hematopoietic cells in hematologic diseases and in oncology, even though those cells were not exposed to extensive proliferation *ex vivo*. A real ATMP was proposed only in the ’90s, when severely burned patients were successfully treated with epithelial cells after an extensive *in vitro* manipulation due to support patient tissue long lasting regeneration (De Luca et all., 1989). Several years later, in 1997, the human limbo-corneal epithelium was reconstructed *in vitro* and transplanted in two patients. Immense expectation surrounds stem cells therapeutics, however, in order to approve a cell therapy on humans, lengthy studies are required to investigate the mechanisms controlling stable regeneration and enabling high reproducibility in the manufacturing process, but above all, the safety must be proven.

1.3 The safety issues: *in vitro* preclinical assessment

Clearly, stem cells-based therapies could not be addressed using standard analytical procedure thought for drugs. Inside the cell therapy then we must distinguish between intravenously administration of cells or local transplantation

with epithelial stem cell; since for the latter one it is not really important to monitor the cell biodistribution instead of the first case because the dissemination is unlike to occur, as the major concern is the tumorigenic potential. Indeed the successful implementation of cell-based treatment relies strongly on the ability to prove these safety concerns, at both preclinical and clinical stages. This evaluation includes the manufacturing process itself and the formal safety assessment of the finished product. Genetic stability and the capacity of malignant transformation are the key points to be unravelled. The product to be transplanted is made of cells that previously have been expanded *in vitro,* and it is imperative to verify their karyotype, because some cell types can show chromosomal instability during expansion in culture, and these are a hallmark of human cancer, even if any minor aberrations could not prevent clinical use of the finished product (Goldring et all.2011). This stem cell therapeutic product contain different cell types, at various stages of the differentiation process, and risk due to the fact that undifferentiated cells during the production process were becoming tumorigenic, must be avoided. The evaluation of cell anchorage and their growth factors dependency can provide detailed knowledge of the safety of the cell-based product.

1.4 Characteristics of the epithelial stem cells

The surface epithelia are renewed constantly during the life of an organism thanks to the presence of stem cells and progenitor cells that are the only proliferating cells in a normal adult tissue. In a specific tissue, adult stem cells have the capacity to generate and regenerate the cells of that tissue throughout the life of the organism.

These stem cells have the unique ability to self-renew and to generate progenitor cells "committed", known as "transient amplifying (TA) cells", which differentiate into cell lineages of the tissue of origin after a determined number of cell divisions (Figure 1).

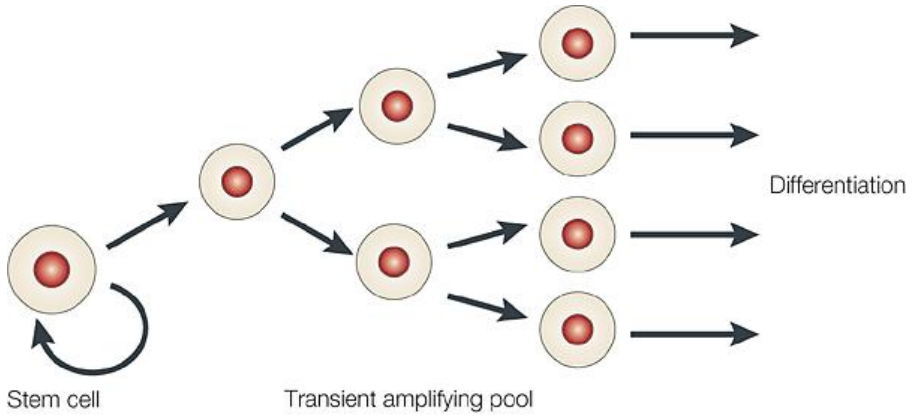


Figure 1. Self-renewal and differentiation of stem cells

The replication of a stem cell, in the asymmetric division, usually gives rise to another stem cell, which remains in this state, and to a TA cell that multiplies rapidly even if it has a limited proliferative capacity; however, in some circumstances (for example, wound healing or cultivation) the stem cell goes under a self-fate symmetric division, generating two identical stem cells, through which the stem cell gives rise to two TA cells addressed to terminal differentiation (Figure 2).

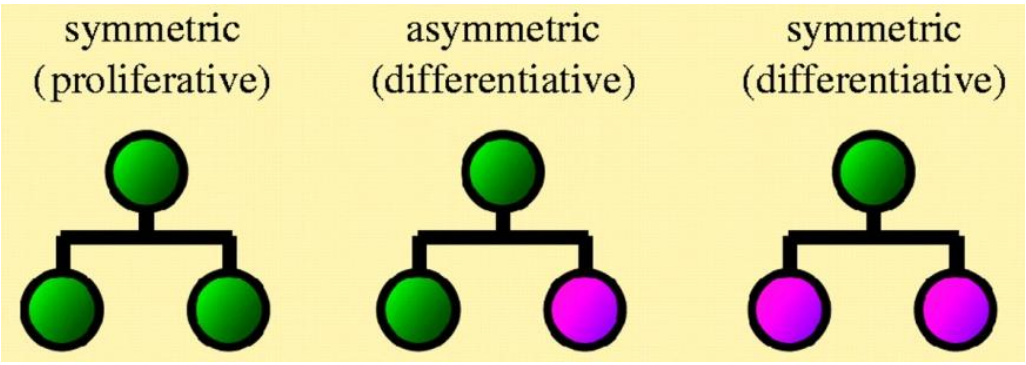


Figure 2. Modality of stem cell division

The most important function of TA cells is to increase the number of differentiated progeny produced by each division of a stem cell, thus allowing the stem cells to proliferate sporadically, at least in conditions of normal tissue

homeostasis. Adult stem cells have been identified in several tissues of the human body and show some special features:

- **Multipotency:** adult stem cells can generate only few cell types of the tissue of origin;
- **Self-renewal:** is the ability of a stem cell to self-renew, generating a cell identical to itself;
- **Clonogenicity:** indicates the ability of a stem cell to form a colony in culture, or a clone formed by cells identical to each other;

Human keratinocytes stem cells are usually characterized in relationship to their clonogenic capacity and growth potential by clonal analysis. The former indicates the capacity of a basal cell to generate a colony, the latter deals with the self-renewal potential, hence the 'stem-ness' of that cell. Different basal layer epithelial cells when cultured under particular conditions, can give rise to three clonal types: holoclone, meroclone and paraclone (Figure 3).

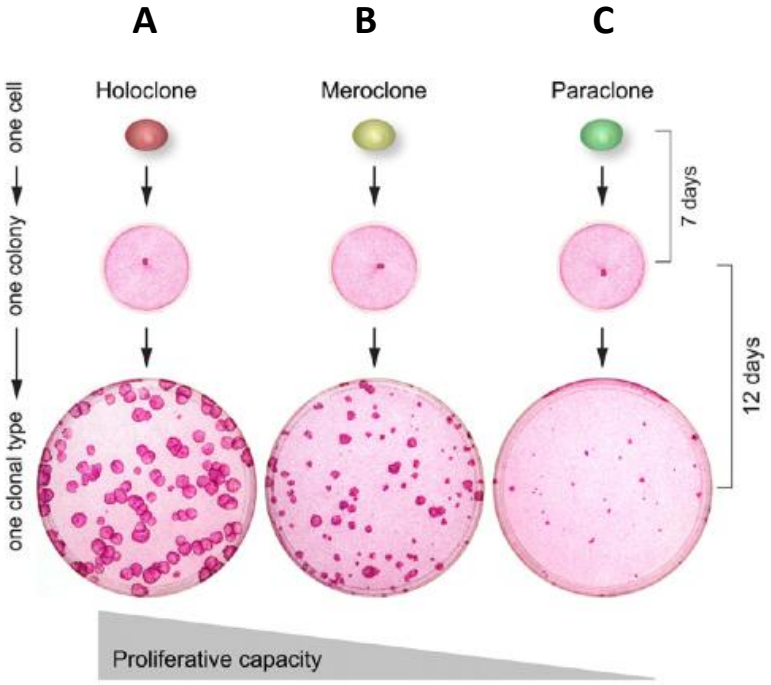


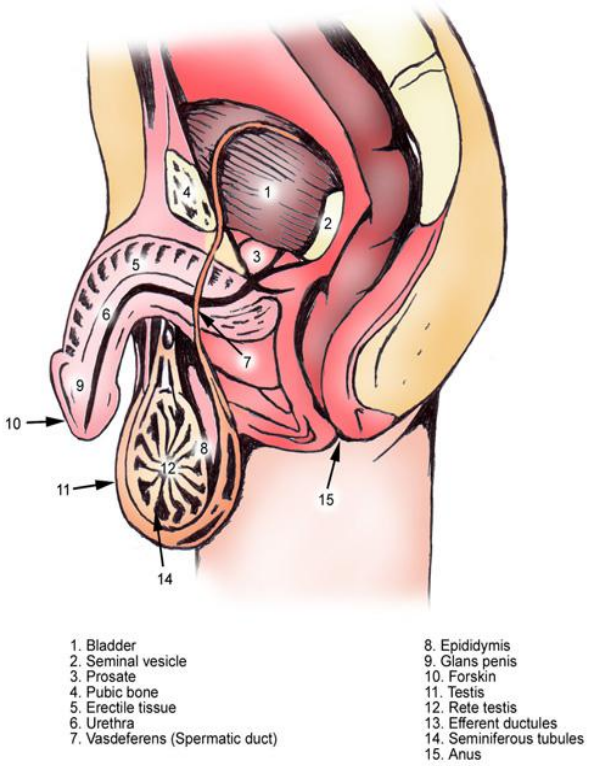
Figure 3. Clonal analysis. Single keratinocytes are isolated under microscope and individually cultured. After 7 days of cultivation, each clone is subcultured onto dish indicators. After 12 days, indicator dishes are fixed and stained with Rhodamine B. A) holoclone generates a progeny that

forms large progressively growing colonies and almost no terminal colonies (less than 5%). B) meroclone forms both large progressively growing and terminal colonies. C) paraclone generates only terminal colonies.

It is now widely accepted that holoclones are the phenotype of human keratinocyte stem cells in culture (Barrandon et all.1987). Indeed, holoclones have all the hallmarks of stem cells, including self-renewal capacity, telomerase activity with long telomere and an impressive proliferative potential (Claudinot et all.2005; Rochat et all.1994). A single holoclone can indeed generate the entire epidermis or the entire corneal epithelium of a human being (Pellegrini G. et all.1999). Holoclone-forming cells generate all the epithelial lineages of the tissue of origin permanently restoring massive epithelial defects (Gallico et all.1984), and they can be retrieved from human epidermis regenerated from cultured keratinocytes, many years after grafting (De Luca et all.2006). Meroclones derive from holoclones and generate a mixture of progressively growing colonies and terminal colonies. Paraclones are transient amplifying cells committed to a small number of divisions that generate only terminal aborted colonies containing large differentiated squame like-cells on indicator dishes. Paraclones are mostly generated by meroclones. The conversion of holoclones to meroclones and paraclones is termed clonal conversion and it is an irreversible phenomenon under normal circumstances (Wilson et all.2001).

1.5 Introduction to urethral anatomy

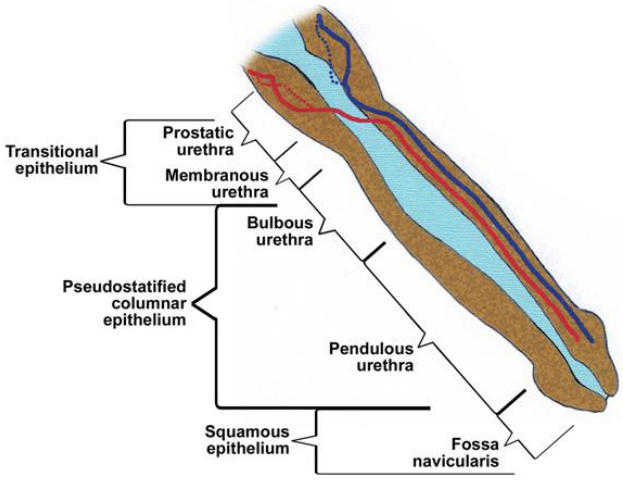
The male urethra is a narrow fibromuscular tube that conducts urine and semen from the baldder and ejaculatory ducts to the exterior of the body (Figure 4). The urethra originates at the bladder neck and terminates at the urethral meatus on the glans penis. It is roughly 15-25 cm long in the adult and forms an "S" curve when viewed from a median sagittal plane in an upright, in flaccid position.



http://emedicine.medscape.com/article/1972482-overview

Figure 4. Male urethra and its segments.

The urethra can be separated simply into anterior and posterior urethra, that forms from the urogenital sinus and it is often divided into 3 segments: prostatic urethra, membranous urethra, and spongy (or penile) urethra (Figure 5).



http://emedicine.medscape.com/article/1972482-overview

Figure 5. Posterior urethra and its segments.

Prostatic urethra

The prostatic urethra is the portion of the urethra that traverses the prostate. It originates in the region of the bladder neck, courses roughly 2.5 cm inferiorly, and terminates at the membranous urethra. It lies in a retropubic location and is bordered, superiorly by the bladder, and supported, inferiorly, by the sphincter urethrae externus muscle and the perineal membrane (formerly called the urogenital diaphragm). Inside the prostate there is a glandular and fibrostromal organ that secretes seminal fluids and has clinical relevance for men.

The urethra runs through the prostate eccentrically, with most of the prostatic tissue in a posterior and inferior location. The prostatic urethra is surrounded by an inner circular layer and an outer longitudinal layer of smooth muscle. The urethra forms an angle of roughly 45º (range, 0-90º) at the midpoint of the prostatic urethra. The segment proximal to this location is surrounded by the involuntary internal sphincter. It is also the area most commonly affected by benign prostatic hyperplasia (BPH).

The posterior wall of the prostatic urethra contains the urethral crest, which is bordered laterally by prostatic sinuses, into which the prostatic glands drain. The most prominent aspect of this crest is the seminal colliculus, or verumontanum, where the paired ejaculatory ducts and the opening of the prostatic utricle (a small midline paramesonephric duct remnant), meet the lumen of the urethra. The seminal colliculus has no functional significance but is a crucial landmark in urethroscopy and transurethral surgery.

Membranous urethra

The shortest and least distensible portion of the urethra is the membranous urethra. This region spans from the top of the prostate to the bulb of the penis. It is located in the external urethral sphincter muscle and the perineal membrane.

The external sphincter is related anteriorly to the dorsal venous complex and is connected to the puboprostatic ligaments and the suspensory ligament of the penis. The external urethral sphincter muscle and the perineal membrane fix the

urethra firmly to the ischial rami and inferior pubic rami, rendering this portion of the urethra susceptible to disruption with pelvic fracture.

Spongy urethra

The spongy urethra is the region that spans the corpus spongiosum of the penis. It is divided into the pendulous urethra and the bulbous (or bulbar) urethra. The pendulous urethra is situated in the corpus spongiosum of the penis in the pendulous portion of the penis. The urethra is located concentrically within the corpus spongiosum. In the distal urethra lies the fossa navicularis, a small dilation of the urethra just proximal to the urethral meatus. The meatus is a slit like orifice with its long axis in a midline sagittal plane. The urethral meatus is slightly ventral to the tip of the penis. The bulbous urethra is invested in the bulb of the penis, the portion of corpus spongiosum that lies between the split of the corpora cavernosa in the superficial perineal space.

Bulbourethral (Cowper) glands, a male homologue of the greater vestibular (Bartholin) glands, originate in the external urethral sphincter muscle but terminate in ducts that empty into the bulbous urethra. The spongy urethra lies closer to the dorsum of the penis in the bulb.

1.6 Bulbar urethral stricture: cell therapy an aid for surgery

Bulbar urethral stricture is common male disease and may results from trauma, infection, instrumentation and ischemia. This narrowing makes urination difficult, impairs bladder function and allows infections. Although endoscopic treatment can transiently improve urinary flow, open urethroplasty, that was first described in 1914 by Russell, is now considered the gold standard treatment of this disease. Nowadays in this trimmed tecnique, urethra is opened at the stricture site and its lumen is widened by applying an oral mucosa graft. Since Suprechko's first description of buccal mucosa used as a graft in 1886, it has become the tissue of choice for urethral reconstruction (Prabha et all.2015). Its popularity is due to the extensive work made by Braca and Barbagli, so that now

it is called the “The Barbagli tecnique”. Buccal mucosa was firstly chosen because it is hairless, and it has a thin, elastin rich epithelium giving it excellent handling characteristics and a highly vascular lamina propria, which facilitates harvesting and imbibitio. The buccal mucosa is harvested from the inner cheek (one or both sides, depending on the length required). To obtain a buccal graft of 2.5-3cm width and 6-7cm length the inner cheek is marked inside the labial angle up to the retromolar trigone, keeping 0.5cm away from the opening of the Stensen duct. Surgeons use a 26 gauge needle to infiltrate dilute (1:200000) adrenaline under the marked portion of the mucosa. The edges are incised, 2 stay sutures are placed at the distal corners of the graft using 3-0 chromic catgut, for traction. Once the graft is harvested, the raw area is allowed to epithelize secondarily. The buccal mucosal graft is trimmed to an appropriate size and is spread over the exposed half of the corpora then sutured to the corresponding edges of the opened urethral lumen using 4-0 polygalactin sutures over a 14French silicone Foley’s catheter (Barbagli et all.2008). The drawback of this technique is the suturing of the urethral margins to the second side of the graft, because the buccal mucosa is already fixed to the corpus cavernosum and the last line is sutured in the back side of the urethra out of sight (Barbagli et all.2013). In the case of dorsal meatotomy, incision allowed surgeon to widen the narrow meatus/fossa navicularis region and to draw the graft in through the glans from the distal urethrotomy and place it right up to the tip of the external meatus. After completion of anastomosis, the wound is closed in layers. The periurethral catheter is left *in-situ* for 3-4 weeks (Barbagli et all.2014). The tecnique above described, even though it is considered the gold standard treatment available, carries out a series of morbidity associated right to the harvesting of the buccal mucosa graft which it is the cause of postoperative pain, difficult in opening the mouth and change in salivary function. Furthermore the standard urethroplasty have approximately 20% of recurrences on the stricutures <4cm length and far higher in those strictures >5cm. The recurrency produce lack of donor tissue for further treatments. A stem cell therapy is indeed necessary to provide enough tissue and to reduce invasivness of the surgery, making a uretroplasty a procedure with a negligible risk of morbity and scarring thanks to a rapid healing,

and last but not least to have a less number of recurrences due to a sufficient number of stem cells that promote the long-term tissue renewal. The choice falls again on the oral mucosa keratinocytes instead of those of urethra because the first one require a less invasive surgery for biopsy retrieval and has better proliferative potential and clonogenic ability together with an higher migration capacity of colonies, suggesting an optimal wound healing capacity (Corradini et all.2015).

1.7 Urethra epithelium similarities and differences respect to the other epithelia

The urethra contains a squamous stratified epithelium and, as shown over time for other epithelia, its proliferative compartment is constitued by holoclones, meroclones and paraclones. These stem cells are uniformly distributed in the clonogenic layer, and their proliferative potential is approximately 80 doublings. This value is shorter than the ones reported for the epidermis, but it is comparable with those of the corneal epithelium, and also in this case, can drive a long term regeneration. The TA cell compartment, which is the most represented, is constitued by cells with higly variable proliferative potential. The holoclones residing in the basal layer have a cell size of 16 to 19 μm in diameter, instead of meroclones and paraclones that increse their size during clonal evolution. A molecular characterization revealed the expression of bmi-1, a member of the polycomb group of transcritpion factors, in cultures containing stem cells, positive for p63-α, on the contrary near senescence cells expressed low level of bmi-1 and p63-α (Corradini et all.2015).

1.8 Bmi-1 in human urethral keratinocytes

Bmi-1 is a protein of the polycomb group of transcription repressors that was initially identified as an oncogene in a murine model of lymphoma, hence the name B-cell-specific Moloney murine leukemia virus integration site 1. The members of the Polycomb group are known to be involved in epigenetics modification, such as chromatin remodeling, implicated in senescence. A pivotal role in the self-renewal of hematopoietic stem cells, HSC, was first discovered for *BMI-1* (Park et all.2003). Over the years scientists realized that it was involved in multiple biological processes, such as embryonic development, organ formation, tumorigenesis, telomerase expression (Reinisch et all. 2006; Jiang et all. 2009). This epigenetic regulator is thought to act by suppressing the locus ink4a/ARF, that encodes for proteins of the cell cycle, like $p16^{ink4a}$ and $p19^{arf}$ ($p14^{arf}$ in humans) leading to enhancement of a stem cell survival and proliferation (Iwama et all.2004). Indeed $p16^{ink4a}$ inhibits cell cycle progression by inhibiting cyclin D-dependent kinases, and thereby prevents phosphorylation of Retinoblastoma protein, prevents the degradation and inactivation of the p53 tumor suppressor protein by binding to MDM2 (Serrano et al., 1993; Weber *et al*., 1999). Taken together, these findings suggest an important role for *BMI-1* in regulating normal cell physiology (Vonlanthen et al., 2001; Dimri et al.,2002).

2. AIM OF THE THESIS

Some longstanding animal models failed to produce therapeutic advances in the clinical setting, such as mdx mice for Duchenne dystrophy, and kidney ischemia-reperfusion injury and diabetic nephropathy models. In this regard, most models are unsatisfactory and do not evolve into a reliable model for the disease study. The perfect animal model is still missing and there are frequent incongruities when the study switch to the human. This mismatch is so obvious that the European community has expressed, in the 1959, the famous three Rs (3Rs) guiding principles for more ethical use of animals in testing: replacement, reduction and refinement of and for animals. The scientific progress of the *in vitro* culturing of human cells have allowed researchers to assess preclinical evaluations without the use of animals. Only a consolidated system for cell culture, with standardized conditions can give strength to preclinical data, becoming an *in vitro* alternative tissue culture model. Over the years, in our laboratory was created a standardized system according to GMP (Good Manufacturing Practices) to cultivate human epithelial stem cells to support a new stem cell-based regenerative medicine. Advanced therapy medicinal products (ATMPs), including cell therapy products, form an innovative class of medicines in the European Union. Their importance was soon understood as well as their complexity, technical specificity and novelty, altogether necessitated tailoring rules to ensure safety. Hence the need of assessing safety and efficacy of these products was increased. The aim of my PhD project was the *in vitro* reconstruction of an urethral transplantable tissue, that mimicked the best *in vivo* conditions, to assess safety and efficacy of the produced engineered stem cells scaffold for extensive urethral reconstruction, avoiding the use of animal models, which carried out a series of limitation and incongruity related indeed to their animal being, that other where would remain incurable.

3. MATERIALS AND METHODS

3.1 Cell cultures

Human primary keratinocytes were obtained from biopsies and expanded by cultivation onto lethally irradiated 3T3-J2 cells (a gentle gift from H. Green's lab) in growth KNO medium, a DMEM and Ham's F12 media mixture (2:1) containing FBS (10%), penicillin-streptomycin (1%), glutamine (2%), insulin, adenine, hydrocortisone, cholera toxin, triiodotyronine. After 3 days KNO medium was replaced and KC medium (KNO medium containing 10ng/ml EGF) was added to the culture. For sub-culturing experiments, keratinocytes were trypsinized at sub-confluence and replated onto a new feeder-layer. Mouse 3T3-J2 fibroblast cell line was maintained in Dulbecco's Modified Eagle's medium (Euroclone), supplemented with 10% fetal calf serum.

3.2 Determination the Colony-forming Efficiency (CFE)

Cells (500-1500) from each cell passage of serially cultivated mass were inoculated into each of two indicator dishes containing irradiated 3T3-J2 cells. Colonies were fixed 12 days late and stained with Rodamine B and the number of colonies was determined separately in parallel dishes at the time of cell passage. The informations obtained after CFE analysis are the following: adhesion capacity of clonogenic cells, ratio between differentiated cells and proliferative clonogenic cells, regeneration capacity of the tissue (the colony was stratified in the middle), migration (diameter) and the number of abortive colonies is an early index of how many cells were differentiating.

3.3 Determination of N° of Cell Doublings (cell generations)

The number of cell generation was calculated using the following formula: x=3,322 log N/No. The number of cells (No) was the actual number of colony-forming cells plated, since they were the only cells capable of dividing. CFE data, determined separately at the time of cultures when passaged, were used for the calculation. N was the total number of cells obtained at passage.

3.4 Soft agar assay

3T3-J2 lethally irradiated (negative control) and MCF-7 (positive control) were used in this assay as control cell lines for anchorage independent growth in 96 well plates. Optimal cell concentrations to plate per well was 15,000 cells and then plates were incubated at 37°C and 5% CO2 over night. The growth kinetics chosen were 0, 5 and 9 days after which the plate was frozen at -70°C until the fluorimetric analysis. 200 ml/well of the cell lysis/dye solution were added to the cell and mixed by pipetting, without bubbles formation that might affect results. Plates were read at excitation: 480 nm and Emission: 520 nm on a Glomax plate reader.

3.5 Growth factor dependence assay

Keratinocytes were plated onto 6 well at 1×10^3 density in the presence or in the absence of feeder layer and of all the supplements of the culture medium, i.d the growth factors. Cells were all cultured for one passage and at the detachment was set up the colony forming efficiency assay (CFE) to monitored the behaviour of the cells at clonal density in response to that deprivation.

3.6 MTT assay

The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. MTT powder was used at 0,5% concentration in PBS 1X and added in each well, incubated for 2 hours. Later isopropanol was added to solubilized the resulting intracellular purple formazan, then they can be and quantified by spectrophotometric means.

3.7 Western blot analysis

Cells obtained from a confluent culture were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% deoxycolate, 1% Triton X-100, 0,1% SDS, 0,2% sodium azide, pH 7,5 containing protease inhibitors) for 30' on ice, and protein amount determined by Bradford assay (Biorad). Cell lysate samples (7-30 μg) were run on a 4-12% SDS-PAGE (100 V, 1-2 hrs) gel, transferred at 100 V at 4°C for 2 hours onto nitrocellulose membrane (Invitrogen) and immunoblotted using rabbit monoclonal antibody against p63-alfa protein (1:10.000, Primm), bmi-1 (1:1000, Cell Signaling), p16 (1:500, Santa Cruz), gapdh (1:1000, Abcam). Protein detection was carried out using a chemiluminescent labelling detection reagent (ECL, Thermoscientific).

3.8 Immunohistochemistry

For immunohistochemistry (IH), oral mucosa samples were embedded in paraffin and sectioned (7 μ m). IH was performed both on control oral mucosa keratinocytes cultured on fibrin and oral mucosa keratinocytes cultured on the innovative scaffold, using these antibodies: bmi-1 rabbit monoclonal antibody (Cell Signaling), rabbit purified anti-p63 α (PRIMM) (Di Iorio et al., 2005), guinea pig anti-cytokeratin-4 (Progen), guinea pig cytokeratin-13 (Progen). Cell nuclei were stained with hematoxylin and eosin. Images were captured with a Zeiss Zeiss Axio Imager A1.

3.9 Immunofluorescence

Urethral and oral mucosa cryo-sections included in OCT (optimal cutting temperature) and cultures cultivated on cover-glasses were fixed with (PFA 3%), permeabilized (0.5 % Triton X-100 in PBS), coated with 2% BSA for 30 minutes at 37°C. The following antibodies were used: rabbit anti-p63α (Primm), rabbit anti-bmi-1 (Cell signaling), mouse anti-gadph (Abcam), mouse anti alpha-sma actin (Sigma), mouse anti beta-tubulin (Sigma). Secondary FITC-labelled antibodies (Alexa-fluor 488) were obtained by Invitrogen. Confocal analysis was performed with confocal laser-scanning microscopy (LSM 510, Zeiss).

3.10 Plasmid constructs

The shRNA constructs were purchased from Open Biosystems; pTRIPZ-sh-Control-IRES-RFP was used as control for *BMI-1* knockdown.

Details of shRNA and oligonucleotide sequences used in this study.

shRNa Construct	Source	Homology	Oligo ID
pTRIPZ-sh-Control	Open Biosystems	-	-
pTRIPZ-H-shBMI-1#1	Open Biosystems	Human	V2THS_48576
pTRIPZ-H-shBMI-1#2	Open Biosystems	Human	V2THS_244779
pTRIPZ-H-shBMI-1#3	Open Biosystems	Human	V3THS_400015
pTRIPZ-H-shBMI-1#4	Open Biosystems	Human	V3THS_302126

3.11 Lentiviral production and primary human keratinocytes infection

Recombinant lentiviruses for: shRNA anti *BMI-1* and shRNA-scramble were produced by transient transfection in 293T cells using the calcium-phosphate method as described (Dull et al., 1998; Naldini et al., 1996b). Infectious lentiviruses were harvested at 48h post-transfection and filtered through 0.45 μ m-pore cellulose acetate filters. Recombinant lentiviruses were concentrated by ultracentrifugation (2 h at 50,000 × g) (Naldini et al., 1996a). Primary human keratinocytes were infected with the lentiviral particles in the presence of 8 μ g/mL polybrene (Sigma) 48h after plating. The cells were harvested 5-6 days after transfection for further analysis.

3.12 Senescence-Associated ß-Galactosidase Staining

Cells were washed in PBS, fixed for 3-5 min (room temperature) in 2% formaldehyde/0.2% glutaraldehyde, washed, and incubated at 37°C (no C02) with X-gal solution: 1 mg of 5-bromo-4-chloro-3-indolyl P3-D-galactoside (X-Gal) per ml (stock = 20mg of dimethylformamide per ml)/40 mM citric acid/sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium

ferricyanide/150 mM NaCl/2 mM MgCl2. Staining was evident in 2-4 hours and maximal in 12-16 hours.

3.13 FACS analysis

a) Apoptosis analysis

In order to evaluate the amount of apoptotic cells after the detachment the cells were pelleted and resuspended in 300 ul of a 20ug/ml solution of propidium iodide in PBS1x. Incubation time 15' at 4°C and then to proceed reading to the FACS without washing.

b) Anti feeder layer analysis

In order to evaluate the amount of the cell positive to the anti feeder layer antibody after the detachment the cell pellet was resuspended in 30 ul of a solution of PBS1x + 5% FBS with a anti-feeder layer APC conjugated antibody diluted 1:10. Incubation was 15' at 4°C and then centrifugation for 3' at 4°C; after resuspension in 500ul, cells were analysed by FACS.

c) Cell cycle analysis

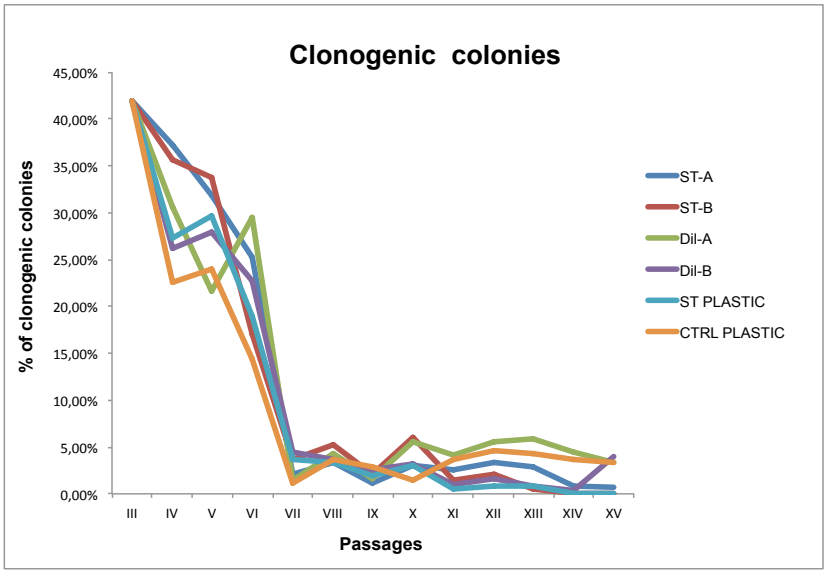
After the detachment, the cell pellet was stained with the above described protocol for feeder layer detection. Then it was fixed and permeabilized, before the hoechst staining, to analyse the cell cycle (FIX and PERM Invitrogen).

4. Results

4.1 Evaluation of a suitable scaffold for its use on urethral surgical reconstruction

Two main characteristics describe the quality of a cell cultures: one was the clonogenic ability and the second was the proliferative potential, each one standed for a different concept. In the first, the capacity of basal layer cells to found a colony was evaluated, in the latter the self-renewal was involved. Epithelial cells of oral mucosa were seeded on four different scaffolds and on plastic, as a control. Each condition was serially cultivated until its replicative senescence. Therefore the cultures were compared by colony forming efficiency assay (CFE) and by the number of cell doublings (Figure 1). The scaffolds designed for the surgical reconstruction of the urethra were made by two sides: the bottom was constant among the different scaffolds, consisting of a biological polymer acting as carrier for the cells. The top was a biological polymer, modulated among the scaffolds, and has the function of protective layer. The four scaffolds differed for concentration of the protective layer on the top and for their overall thickness. Our results indicated as best scaffold the protective layer Dilution A; since it gave rise to the highest number of clonogenic colonies and a number of abortive colonies comparable to the control grown on plastic. This scaffold has been designed for an innovative urethral surgical reconstruction, that did not require stitches, which aimed to use a Glubran 2 (GEM, Viareggio, Italy), an N-butyl-2-cyanoacrylate combined with a monomer (methacryloxy sulfolane), that was a glue with good adhesive and haemostatic properties. Indeed the first important thing to evaluate, that could eventually precluded further studies, was its cytotoxicity on the cell types with which it came in contact: fibroblasts and epithelial cells. Both the epithelium and the stroma were affected during the surgery. Moreover the following steps were to assess the safety of the suitable scaffold for clinical application and its tissue architecture by histology.

A



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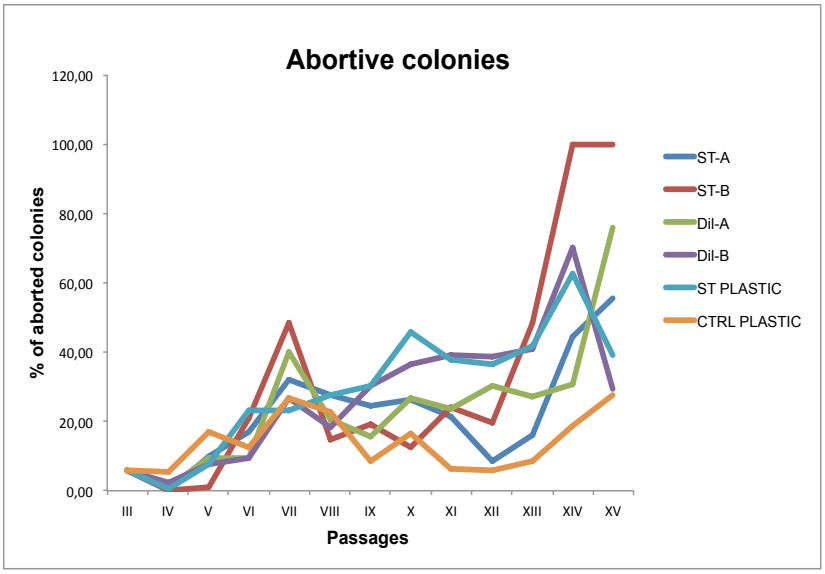


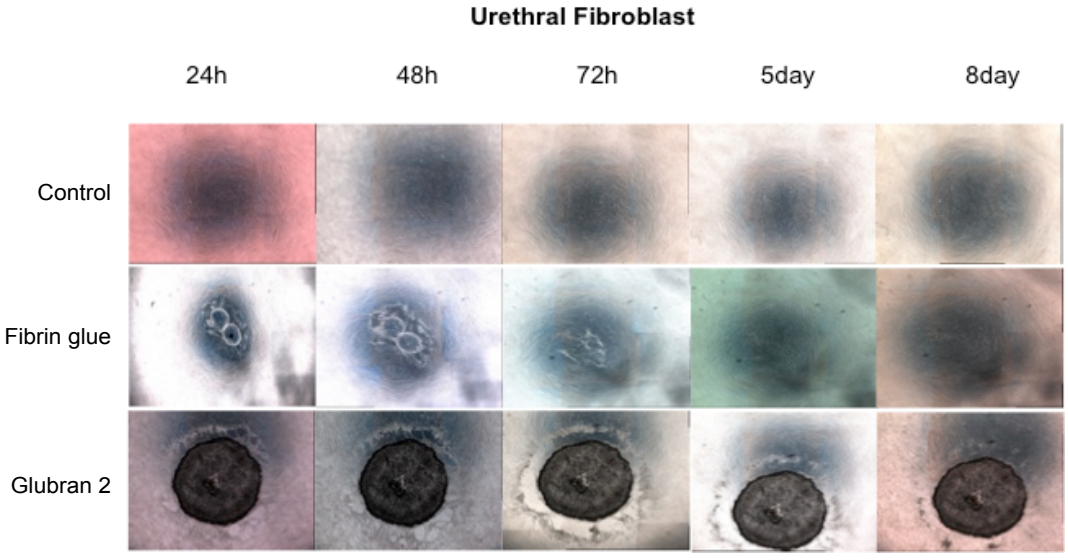
Figure 1: Keratinocytes from oral mucosa cultivated upon different scaffold.

A schematic representation of the behaviour of oral mucosa keratinocytes cultivated onto different scaffolds compared to the control one on plastic (Control plastic) and to the scaffold already used in clinic (ST-plastic). Standard A (ST-A) has a standard concentration of the polymer of the protective layer and it is thin, Standard B (ST-B) has a standard concentration of the polymer of the protective layer and it is thick, Diluted A (Dil-A) has a diluted concentration of the polymer of the protective layer and it is thin, Diluted B (Dil-B) has a diluted concentration of the polymer of the protective layer and it is thick.

4.2 A short term cytotoxic assessment: glubran 2 on urethral and oral mucosa fibroblasts

The application for urethral surgical reconstruction of the oral mucosa engineered scaffold, as described above, required the use of a glubran 2 to firmly placed the graft in the body. Biological effect of this cyanoacrilic-based surgical glue, was evaluated in a cell culture model of urethral and oral mucosa fibroblasts and keratinocytes, because both stroma and epithelium were involved in the urethral reconstruction. A biological tissue adhesive (fibrin glue), already used in clinic, was used as a control, only on urethral fibroblasts which were the most susceptible cell type. Biopsies from bulbar urethra and oral mucosa were obtained from patients during reconstructive surgery, after informed consent. Fibroblasts were isolated by explant and cultivated on plastic. Cytotoxicity was evaluated on both fibroblast cells by exposure over a confluent culture glubran 2 and fibrin glue using different approaches:

a) All cultures underwent digital image analysis soon after contact. Fibroblast cultures exposed to glubran 2 showed a halo devoid of cells surrounding the glue area, suggesting some cell toxicity on the contrary there was no effect in the control culture and in fibrin glue. As we can see the halo diminished over time in both urethral and oral mucosa cultures (Figure 2).



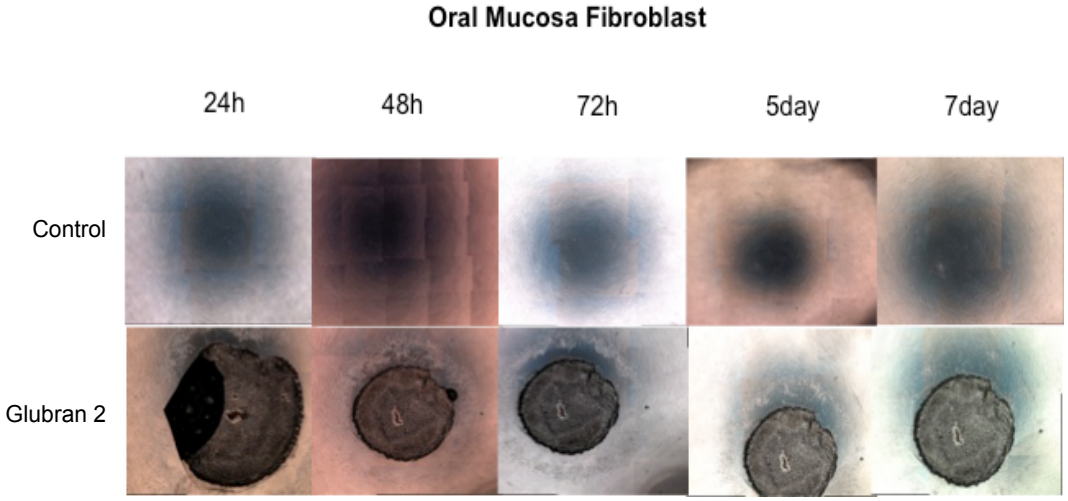
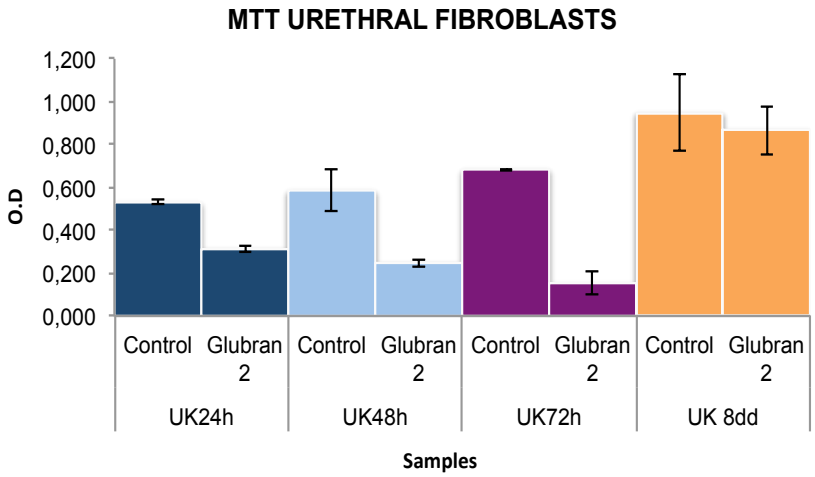


Figure 2: Digital Images of Cell Cultures. After contact with glues, urethral and oral mucosa fibroblasts were immediately monitored under Zeiss Axio Imager microscope to verify their cellular behaviour. A mosaic picture was obtained from each well to have a complete picture of the situation. As we can see control and fibrin glue cultures do not show any halo or toxicity instead of those in glubran 2 conditions.

b) The analysis of viability by the MTT assay showed that there was a short term toxicity effect, the greater one at 72h, as we can see in the graphics, on the other hand there was no effect in the control culture. Nevertheless in each case we observed an increase of the vitality consistent with that of a normal confluent culture. Finally we can conclude that the MTT assay showed a rescue of the viability reaching the same value of the control, respectively in 7 day for urethra and in 8 days for oral mucosa fibroblasts.



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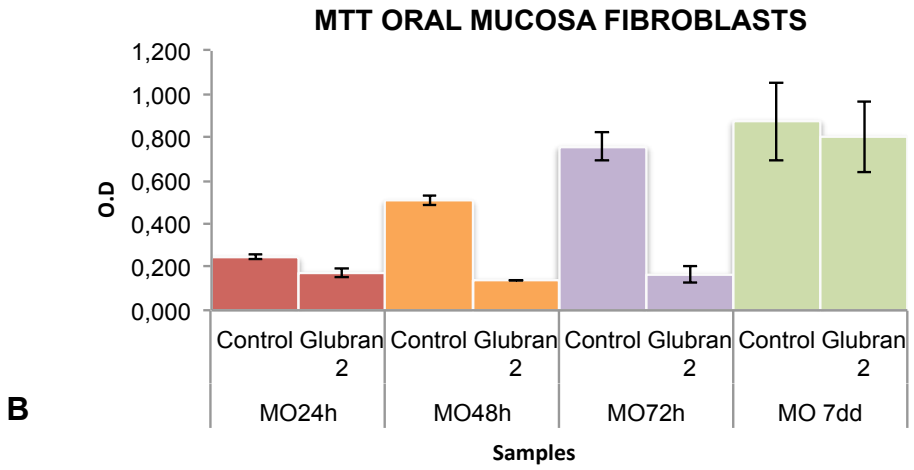
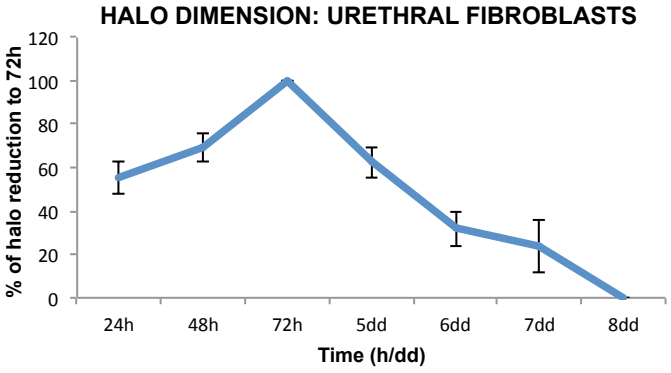


B

Figure 3. Cell proliferation assay (MTT). Optical density readings from luminometer Glomax during time in both type of fibroblasts.
A) The lowest vitality occurs at 72 hours for the urethral fibroblasts, and returns to the control level at day 8. B) The lowest vitality occurs at 72 hours, even though returns to control level one day before (at 7 day).

c) The daily measure of the halo area revealed that it reached the largest size in both urethral and oral mucosa fibroblasts at 72 hours after glue contact, but during a week, approximately, cells had the ability to repopulate the gap. These data were consistent with the MTT assay (Figure 3) in which a drop in vitality was highlighted at 72hours. The halo closed in 8 days and (Figure 4).



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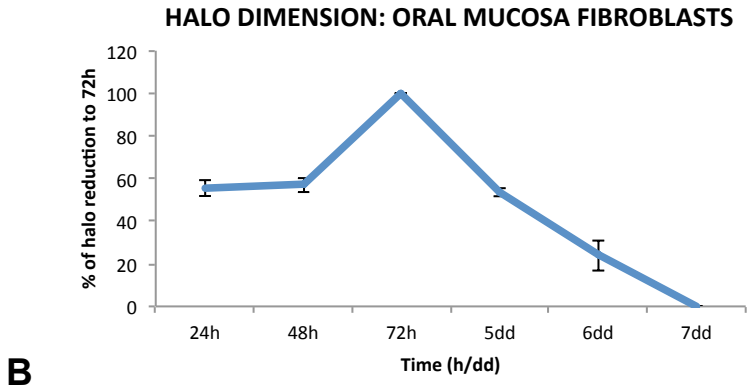
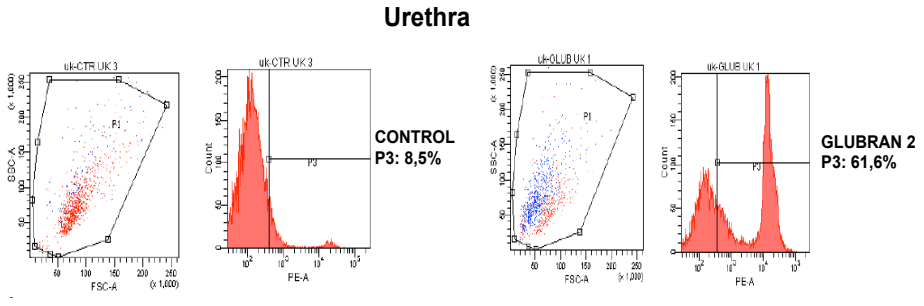


B

Figure 4. Halo size as marker of toxicity. A digital image analysis was performed by measuring the area of the halo through Zeiss Axio Vision software. (A) Urethral fibroblast halo takes 8 days to close. (B) Oral mucosa fibroblast halo takes 7 days to close.

d) To further confirm the previous results, on the cytotoxicity of the glue, we investigated the amount of apoptotic cells through FACS analysis in the culture treated with the glubran 2. As shown in the plots the percentage was significantly greater in the glubran 2 treated culture than in the control cultures (figure 5); both urethral and oral mucosa cultures suggested a short-term toxic effect of the glue.



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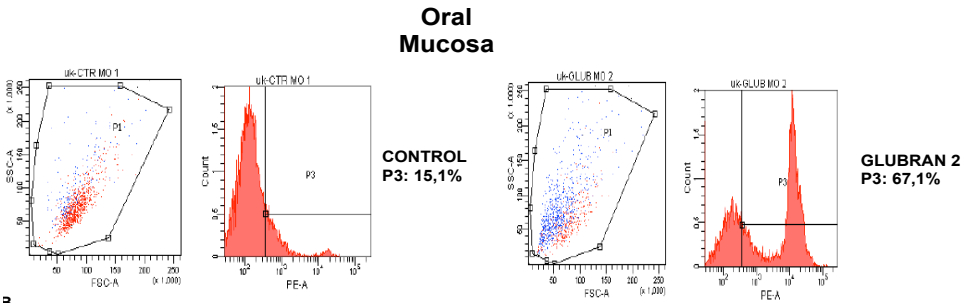


B

Figure 5. FACS analysis after glue contact. Control culture populations without any contact with glubran 2 and the culture of fibroblasts after contact with the glue. Each condition was detached at 72 hours post glubran contact as this was the time of maximum toxicity.
A) and B) The analysis shows that the percentage of apoptotic cells is significantly higher in both urethtral and oral mucosa treated fibroblasts than in the control cultures.

4.3 A long-term cytotoxic assessment: glubran 2 on urethral and oral mucosa fibroblasts

For long term assessment of glubran 2 cytotoxicity in urethral (Figure 6) and oral mucosa fibroblasts (Figure 7), we sub-cultured cells derived from two different culture conditions: in the first, cells were in contact with the glue for 24h and in the other for 7dd. Cells were detached in order to assess cell viability by quantifying dead cells at different time points after plating: 24h, 4dd, 8dd. The results in both groups of fibroblast showed no difference in cell proliferation among the three time points; moreover the three groups displayed, over time, an increase of cells number, as usually occurred in standard conditions.

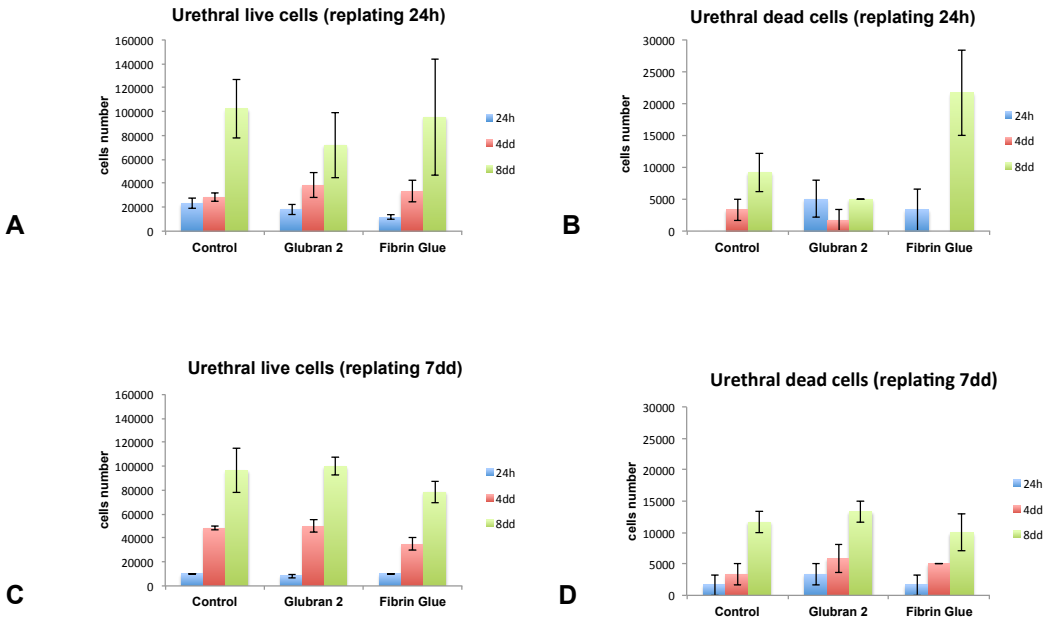


Figure 6. Long-term cytotoxicity assessement in urethral fibroblasts. The cell count at the detachment was represented on ordinate axis among the three groups on abscissae: control, glubran 2 and fibrin glue. A) No difference was shown in the trend of the three conditions. B) Control and glubran 2 are similar in spite of the fibrin control group. C) and D) In both case the three conditions have the same behaviour.

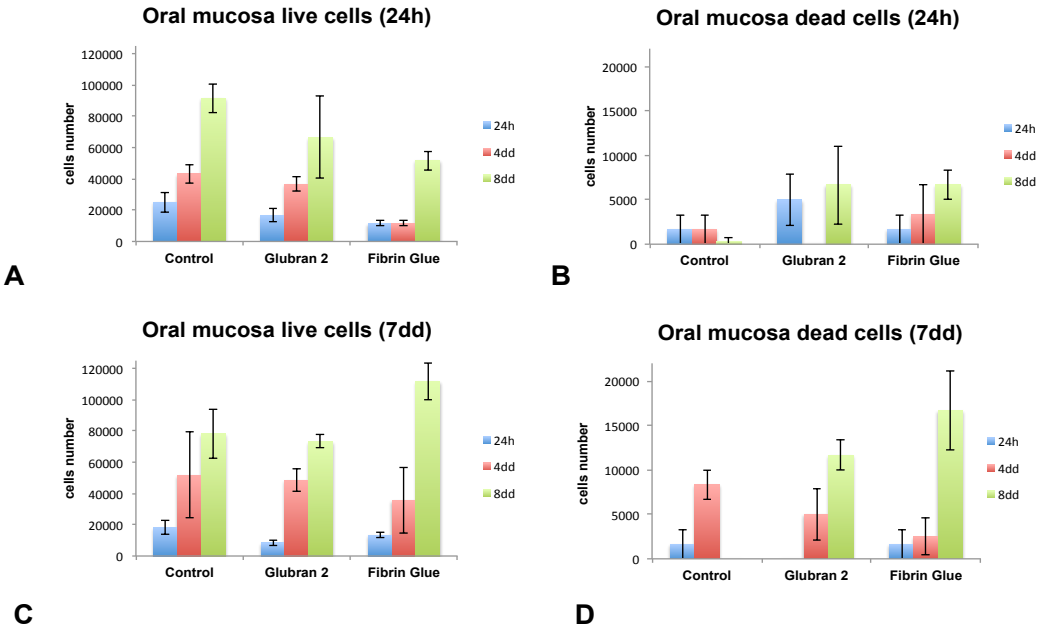


Figure 7. Long-term cytotoxicity assessment in oral mucosa fibroblasts. The cell count after detachment is represented on ordinate axis and the three groups on abscissae: control, glubran 2 and fibrin glue. A) Control and glubran 2 are similar, in spite of the fibrin group, having an higher number of viable cells at the day of detachment (8th). B) No significant differences among the conditions. C) No difference in the trend of the three conditions. D) No difference in the three different conditions.

Furthermore we performed an immunofluorescent staining with fibroblast specific markers of cytoskeleton, beta tubulin and alfa-sma at two different time point 24h and 7 day after the glubran 2 contact. As shown in figure 8, there was a significant difference between 24h and 7 day, not related to the cytoskeleton organization, normally stained in both cases, but rather to the number of cells

surrounding the perimeter of the glue which was low at 24h but was comparable to the control at 7 days. Results suggested a transient toxicity due to the glubran 2 and a cell recovery on the long term, as showed by data.

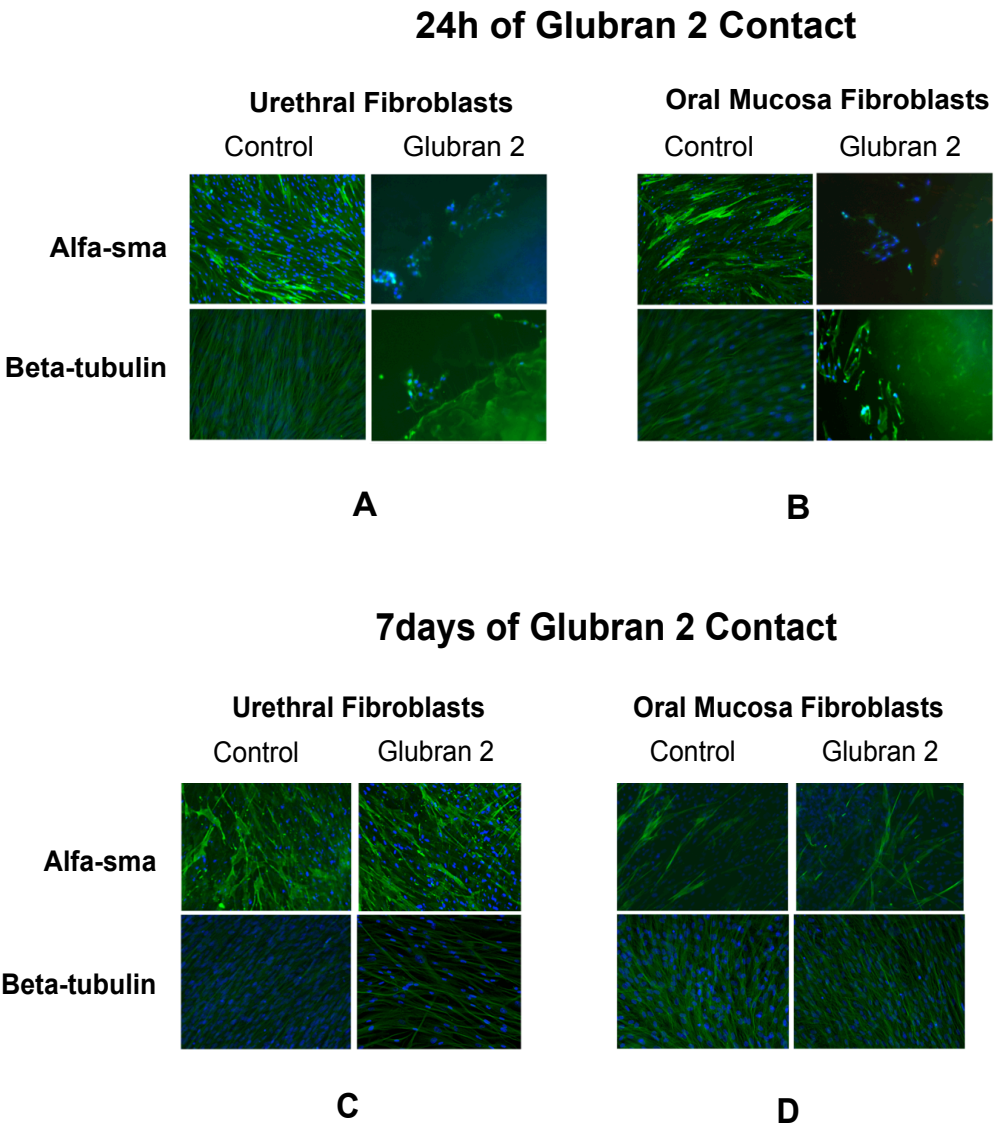


Figure 8. Immunofluorescence staining with alfa-sma and beta-tubulin on oral and urethral fibroblasts. Fibroblasts were fixed in paraformaldehyde and the following staining was set up with anti beta-tubulin and anti alfa-sma antibody. A) and B) Control of both urethral and oral mucosa fibroblasts does not show any damage, whereas glubran 2 condition in both groups reveals only few cells surrounding the glue area. C) and D) Control and glubran 2 appear comparable for beta tubulin expression, in both urethral and oral mucosa fibroblasts.

4.4 Cytotoxicity assessment of glubran 2 on epithelial cells

To investigate the effect of the glubran 2 on the epithelial cells was selected the same test used for fibroblasts. The glue was dropped over a confluent culture and the cytotoxicity was evaluated by different assays.

a) Digital image analysis of cell culture did not show any halo formation in this case .

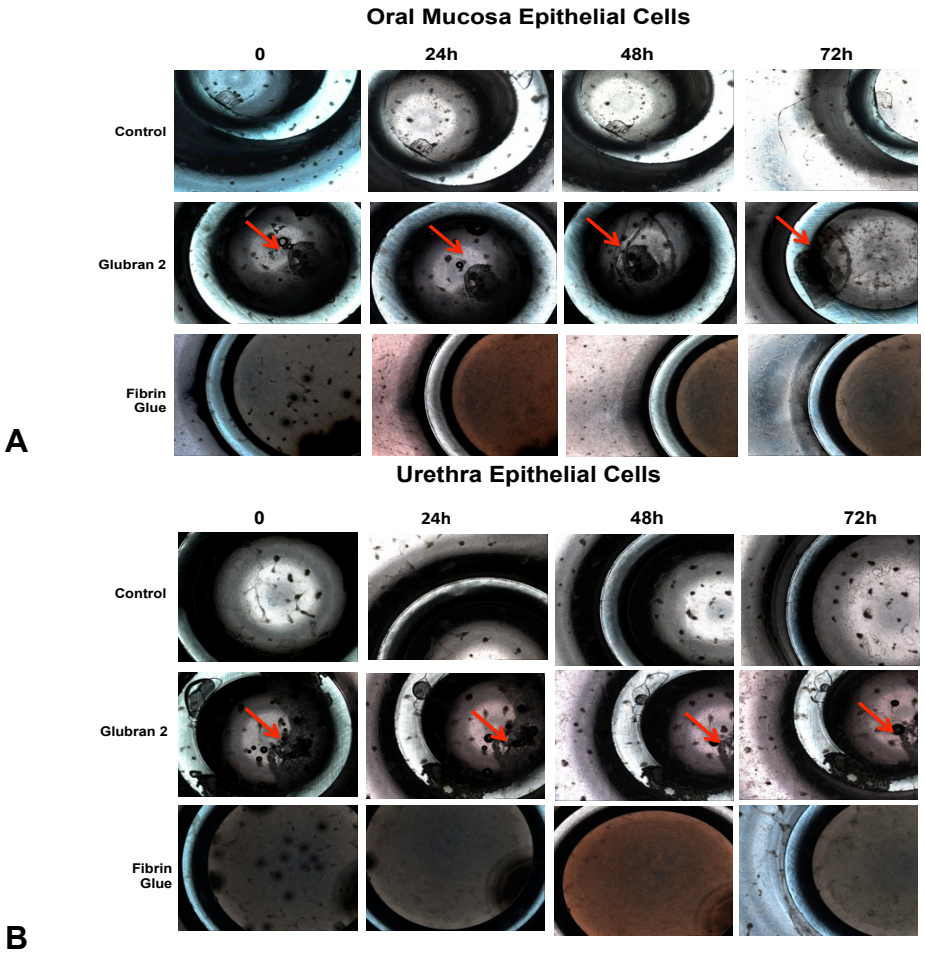


Figure 9. Epithelial cell behaviour after glue contact. Digital images taken, as a mosaic of the entire well, with Zeiss Axio Vision, soon after glue contact and up to 72 hours. A) and B) The glubran 2 condition do not show any halo formation surrounding the glue area.

b) In contrast with the absence of an halo devoid of cells, the cell count revealed a significant decrease in the viability after contact with glubran 2 when compared

to plastic and fibrin culture controls, suggesting a possible irreversible damage on the proliferation activity. The number of the cell counted was significant lower in glubran 2 condition whereas the other two condition were similar (Figure 10).

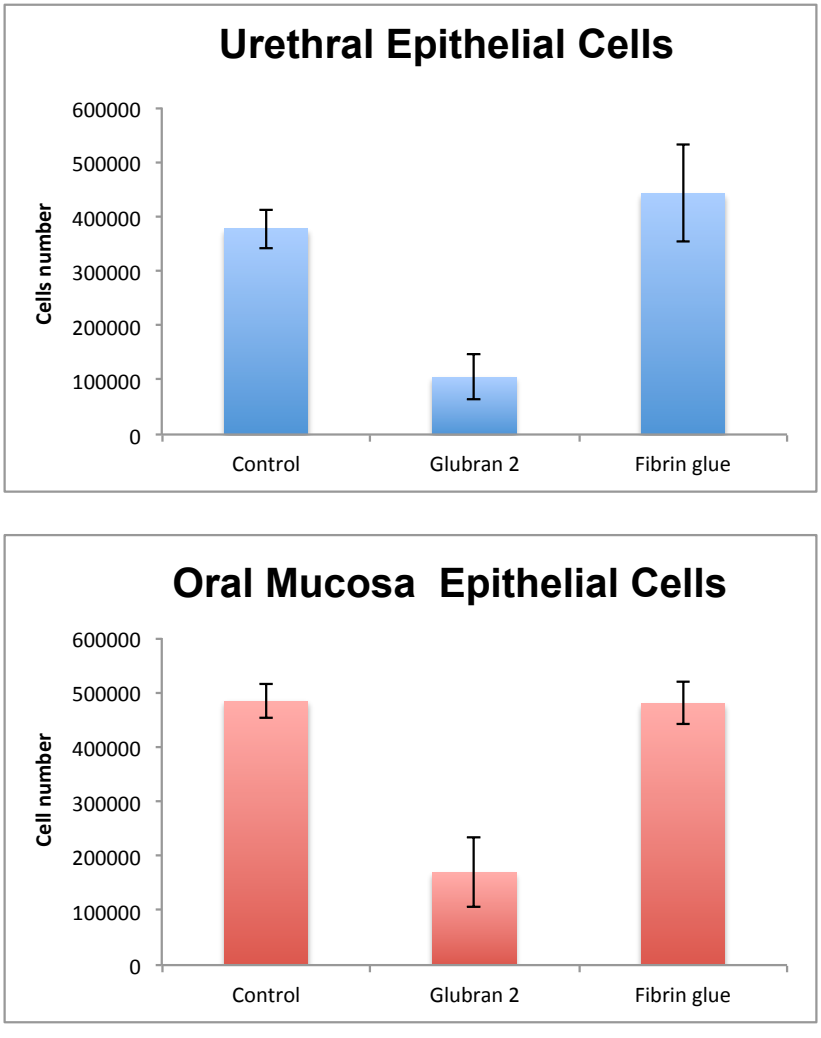


Figure 10. Cell count. At confluence, the cells of the three different groups were detached and counted. A) and B) Glubran 2 treated cultures provide a lower number of cells, compared with control and fibrin glue.

c) We have further investigated the cell behaviours, using a colony forming efficiency assay in order to understand the behaviour. The results did not confirm an irreversible injury on the proliferative compartment, since no differences were found in the percentage of clonogenic and aborted colonies among groups. The same findings resulted from comparison between urethra and oral mucosa epithelial cells (Figure 11). Indeed we could assert that there was only a short-term toxicity on viability in response of the surgical glue because on the long term the cells recovered from the toxicity effect.

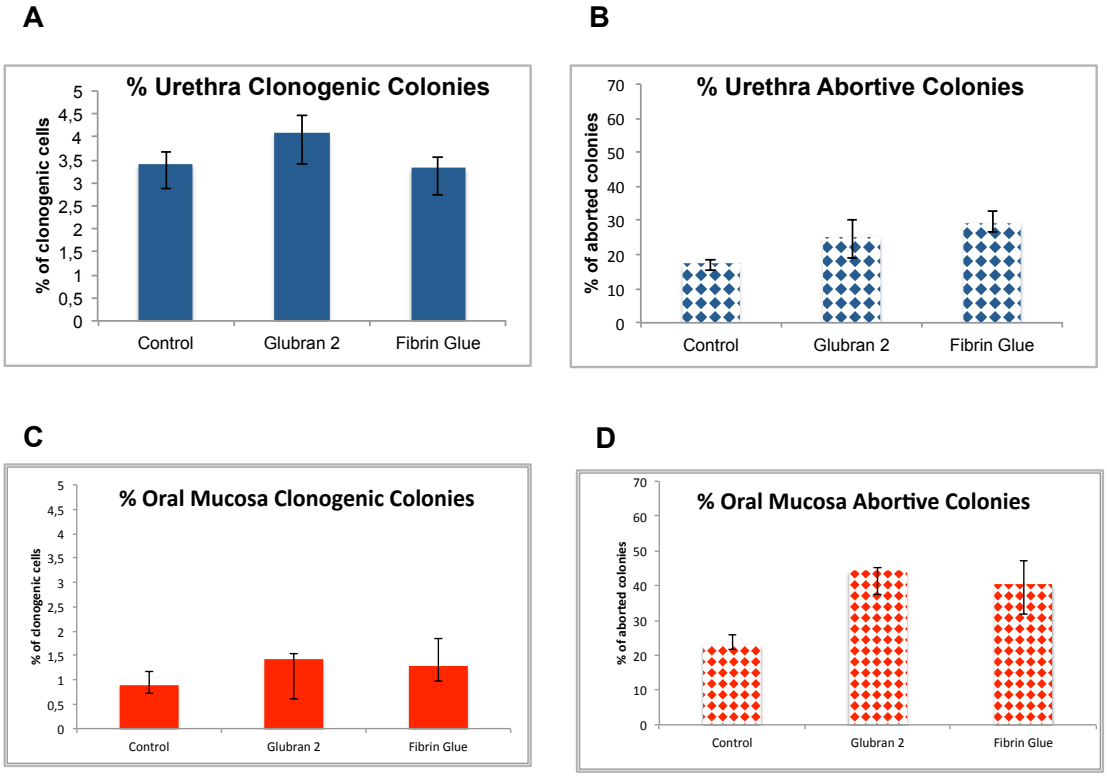


Figure 11. Colony forming efficiency assay of both epithelial cell. A) Urethral clonogenic colonies are comparable among the three conditions. B) Urethral aborted colonies are comparable among the three conditions. C) Oral mucosa clonogenic colonies are similar in the three groups. D) Oral mucosa aborted colonies show no significant differences among the groups.

4.5 Evaluation of the reduction of the polymer during time

Finally, has been evaluated the degradation rate of the glubran 2 due to the cells and to the glue itself. The glue absorption is mandatory in order to support any clinical use. We measured the decrease of the mass of polymerized cyanoacrilic-glue onto both urethral and oral mucosa fibroblasts and on plastic, by digital image analysis. The area of the polymerized glue, was reduced up to 20% by urethral fibroblasts, 10% by oral mucosa fibroblasts and approximately 10% on plastic. Indeed the analysis showed that there was a double-action in the reduction of the glue, one due to the cells and another one due to an intrinsic effect of polymerization (Figure 12). These data confirmed the safety of glubran 2 and the efficiency on supporting its clinical use for one-stage penile urethroplasty due to the capacity to be resorbable, Glubran 2 represented a surgical innovation in the management of urethral strictures.

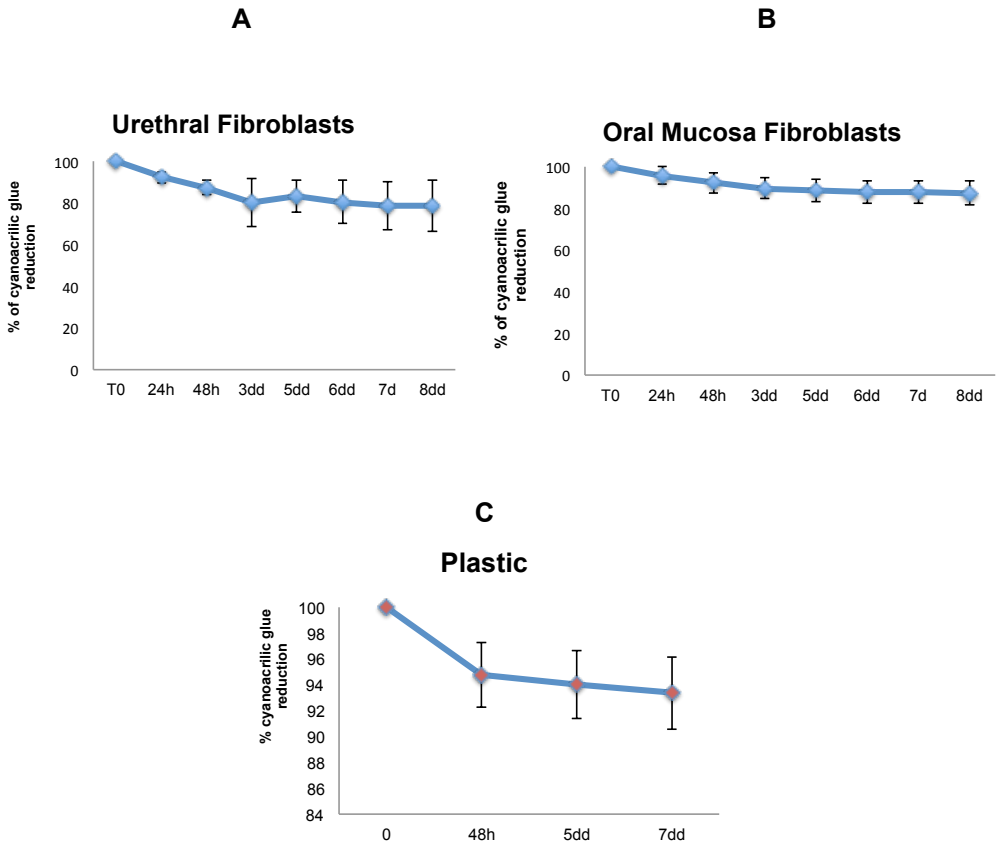


Figure 12. Reduction over time of the glue area in different conditions. The glue mass was monitored every two days and next analysed under Zeiss Axio Imager microscope. The different conditions were urethral and oral mucosa fibroblast cultures and plastic to measure any modification of the area. A) In urethral fibroblast cultures, the area of the polymerized glue is reduced up to 20% in 8 days. B) In oral mucosa fibroblast cultures, the area of the polymerized glue is reduced up 10% in 8 days. C) On plastic the area of the glue is reduced up to approximately 10% in a week.

4.6 Safety assessments of stem cells engineered scaffold: from the manufacturing to the finished product

A big concern with stem cells therapy was the risk of development of tumors due to the delivery of cells that had the ability to differentiate into other human cell types and due to their unlimited renewal potential. In order to minimize patient risks, the manufacturing process itself and the final product should be unravelled for potential safety concerns before transplantation into a human subject. It was essential to ensure that the cells, cultured *in vitro*, are fully characterized and selected, to avoid any problems. In order to achieve this goal we performed a series of preclinical assays, described below:

a) Soft agar migration assay

Anchorage-independent growth was one of the hallmarks of transformation; therefore the anchorage dependence was considered a reliable and stringent *in vitro* assay for detecting one of the first step of transformation.
The soft agar colony formation assay was a common method to monitor anchorage-independent growth, which measured proliferation in a semisolid culture media. Notably the oral mucosa keratinocytes (mo39) did not show any sign of growth respect to the other conditions, except 3T3 cells that were used as control.

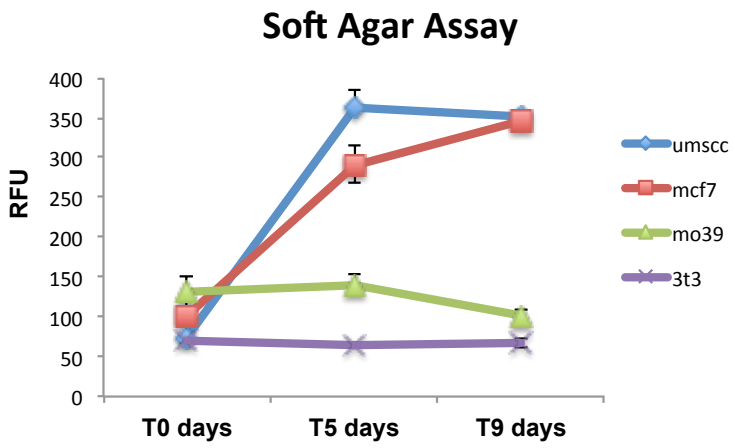


Figure 13. Soft agar assay. Anchorage independent growth of oral mucosa keratinocytes (mo39) in comparison with two cell lines: the umscc and the mcf7, in addition to 3t3, a lethally irradiated murine fibroblast cell line. Cells transformation is determined by measuring RFU (relative fluorescent unit) through dye detection. The figure shows that our cells and 3t3, after detachment from scaffold, do not have the capacity to grow, freeing themself from the anchorage as umscc and mcf7 cell line do

b) Growth factor dependence assay

Neoplastic transformation occurred via a series of genetic and epigenetic alterations that yield a cell population that was able to proliferate independently from external regulation. For example, transformed cells showed reduced requirements of extracellular growth promoting factors. For this reason was performed a growth factor dependent assay, in which the cells were grown without hormones, serum and feeder layer. Oral mucosa cells, detached from the engineered scaffold, were cultivated as above described, in comparison with oral mucosa cell line, the UMSCC. Notably, there was a significant difference among conditions: the oral mucosa cell line efficiently grew in three out of four conditions, whereas oral mucosa primary cells were able to grow maintaining the capacity to form colonies, only in standard conditions (+FL/+GF). In all the others culture conditions only keratinocytes scattered residual keratinocytes were found. To further investigate the effects of the growth factor deprivation, a colony forming efficiency assay (CFE) (Figure 14B) was produced from the different

cultures and no colonies were formed from oral mucosa epithelial cell in the absence of feeder layer or growth factor. On the contrary the mucosa tumor cell line grew also in deprivation conditions, with the exception of absence of feeder layer and grow factors (-FL/-GF).

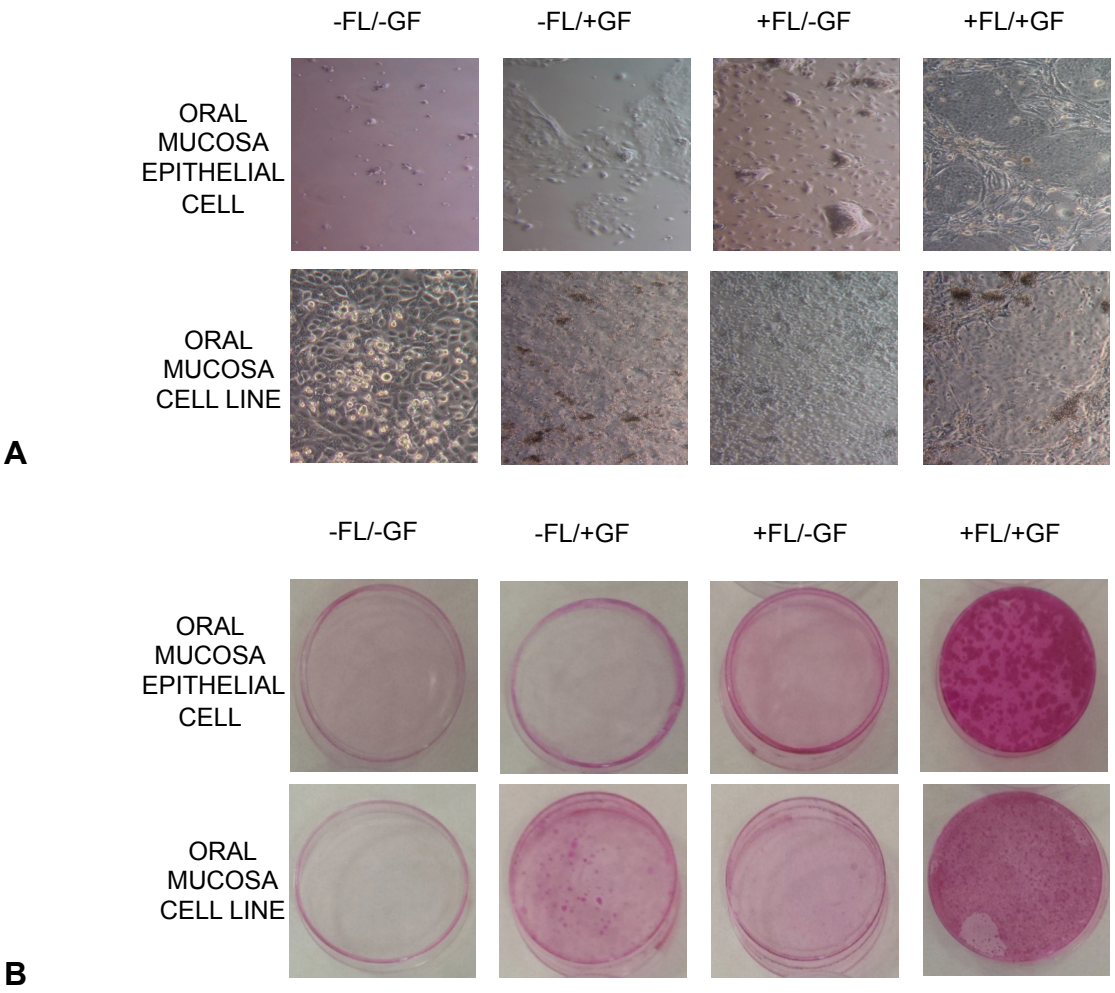


Figure 14. **Growth factor dependence assay. A)** Oral mucosa primary cells and oral mucosa tumor cell line are (UMSCC14C) plated in four different culture condition: standard condition (+FL/+GF), plus growth factor/minus feeder layer (+FL/-GF), minus growth factor/plus feeder layer (-GF/+FL), minus growth factor/minus feeder layer (-FL/-GF). B) Colony forming efficiency from each condition is performed in parallel. Oral mucosa keratinocytes are completely dependent from growth factors and feeder layer while tumor cells are able to grow even in condition of deprivation (-FL/+GF, +FL/-GF).

c) Karyotype analysis

In order to evaluate the number and structure of chromosomes and to detect possible abnormalities, karyotype analysis was performed. The analysis shows the chromosomes on metaphase plate and there was no evidence of chromosomal aberration or any possible chromosomal damage in none of the seven karyotypes analysed. The G-banding were consistent with a normal male phenotype (Figure 15).

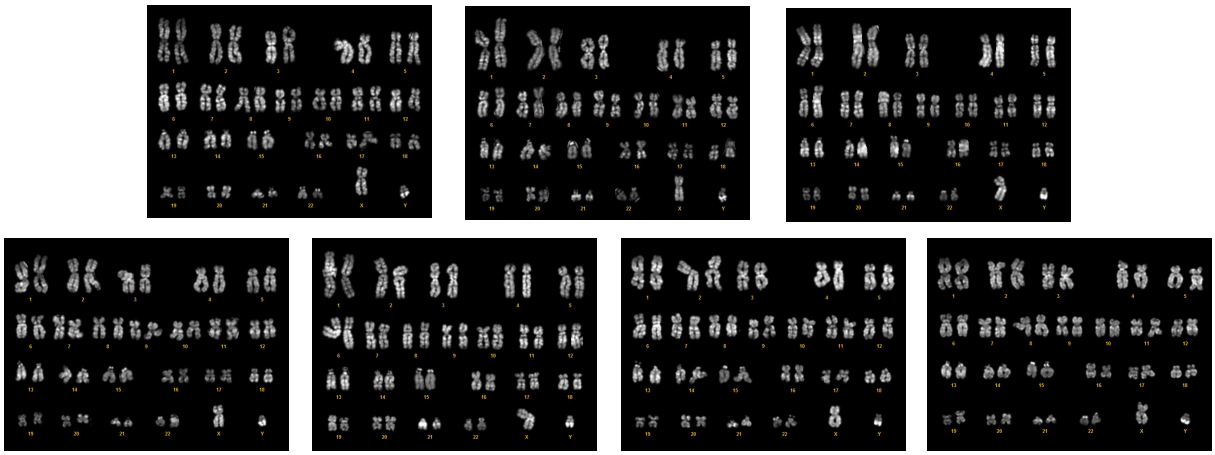


Figure 15. Karyotype. After the detachment of the oral mucosa cells from the scaffold 7 different cultures were cultured in order to analyse their karyotype.

4.7 Histologic evaluation of the stem cells engineered scaffold

Finally, in order to verify the normal tissue architecture of the stem cells engineered scaffold we performed histological evaluations of the oral mucosa primary cells grown within the two layers of the scaffold and we compared them with the oral mucosa cells seeded only on the biocompatible carrier.
Histological sections were analysed for two transcriptional factors, bmi-1 and p63-alfa as stemness markers, and similar positivity and localization for the two molecular markers was found in the two samples (Figure 16). Furthermore the

immunohistochemistry with haematoxylin and eosin revealed a normal architecture of the oral mucosa epithelial cells within the two layers of biological polymers. Some areas highlighted stratification.

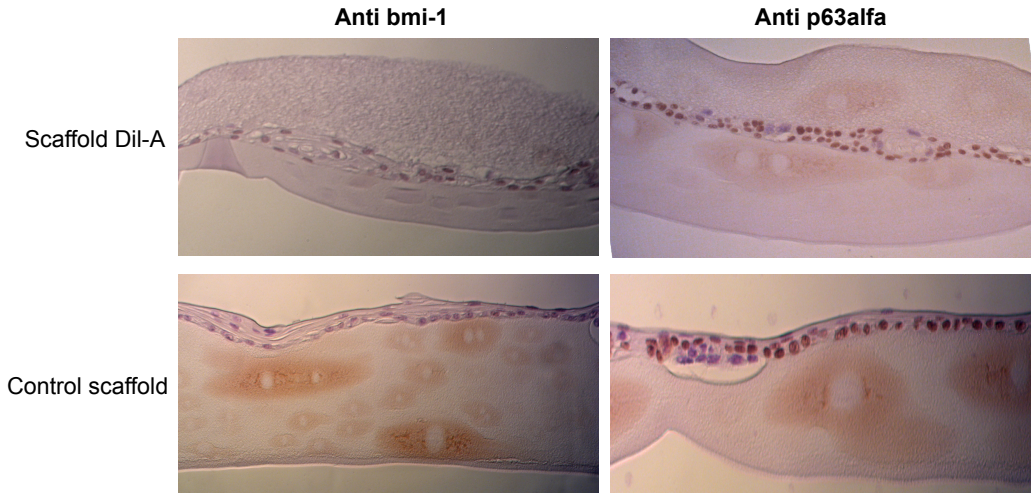
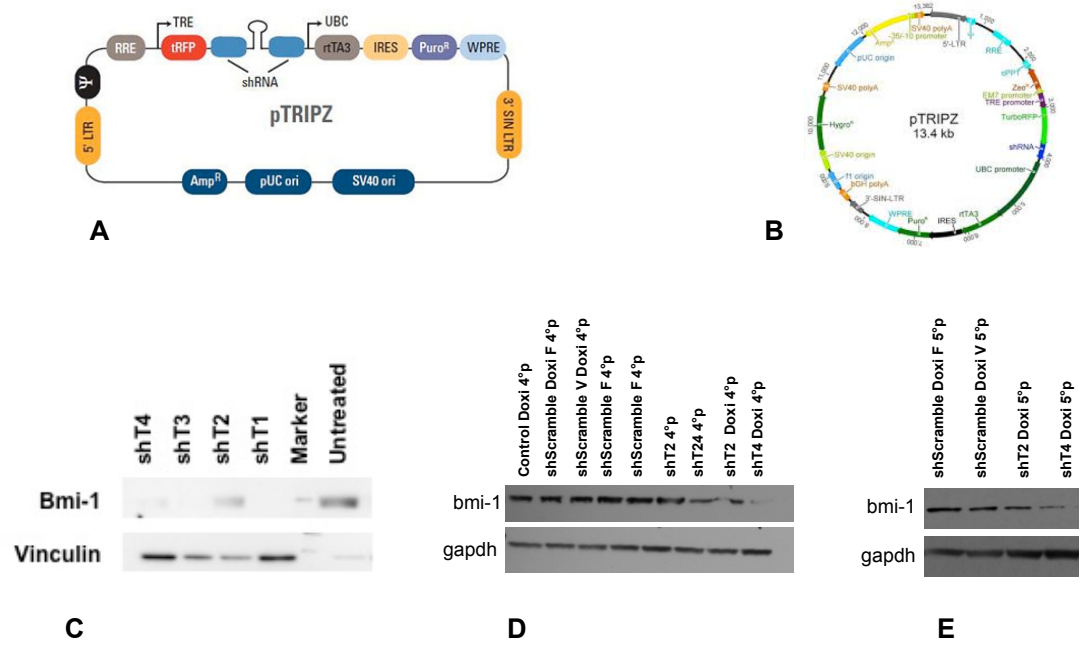


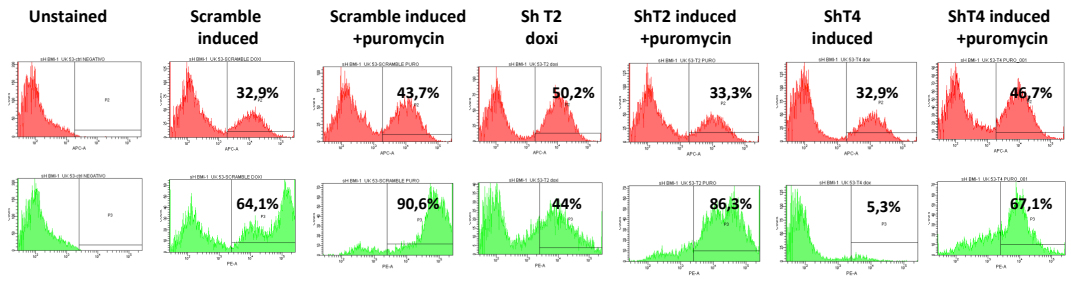
Figure 16. Scaffold tissue architecture.
The scaffolds were detached from the plastic when the cells were confluent and then were cut in several parts, fixed and paraffin-embedded before proceeding to immunohistochemistry analysis with anti-bmi-1 and anti-p63.

4.8 Investigation of *BMI-1* role in urethral epithelial cells

The clinical success of transplantation was based on the presence of stem cells. Previous study have revealed that the transcription factor *BMI-1* was abundantly expressed in urethral epithelial stem cells, suggested us that it had the potential to be what it was p63-alfa for the limbus, a marker of stem cells. In order to investigate the role of *BMI-1* in human urethral stem cell functions, we down-regulated bmi-1, in primary cultures of human urethral keratinocytes by inducible tripz lentiviral system (TRIPZ inducible shRNA vectors, ThermoScientific) (Figure 17A-B).

Two shRNA targeting *BMI-1* (here referred as shT2 e shT4) out of four, were chosen for silencing this interesting polycomb gene in primary keratinocytes culture of urethral epithelium. Knock-down was quantified by western blot analysis (Figure 17C). Primary urethral cells were infected with lentiviral vectors expressing a Tet-inducible shRNA-*BMI-1* or scrambled shRNA, and seeded in culture for a long-term evaluation. In order to determine the efficiency of gene silencing of the shRNAs sequences, two empty vector, here named as Scramble F and Scramble V, were co-transduced as control, one referred as shT2 and the other to shT4. Knock-down was quantified by western blot analysis (Figure 17D, E). Transduction efficiency was monitored through FACS analysis and proven to have high efficiency (Figure 17F). Microscopic evaluations of fluorescent red tag confirmed many fluorescent colonies, example given in figure 17L. After serial passages, monitoring the bmi-1 expression by western blot analysis , we noticed an higher colony forming efficiency in shScrambles samples than in sh*BMI-1* transduced cultures (CFE) (Figure 17G,H); furthermore we observed that shScrambles infected samples, performed a greater number of cell doublings during lifespan than sh*BMI-1* infected samples, as shown in Figure 17I.





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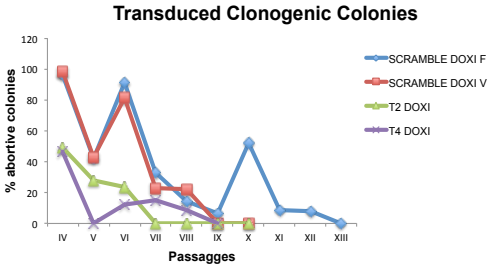

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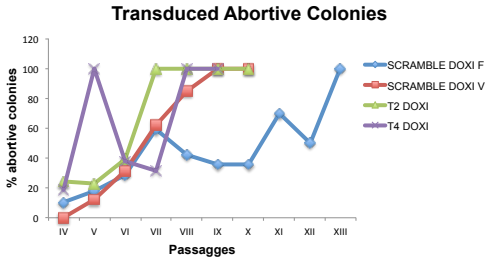
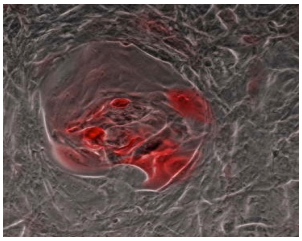


H

N.° Cell Doublings	
sh Scramble F	64,82
sh Scramble V	45,67
shT2	46,1
shT4	34,38

I



L

Figure 17. Silencing of *BMI-1*

A),B) TRIPZ inducible lentiviral shRNA maps. C) Western blot analysis one passage after transduction of urethral keratinocytes with anti *BMI-1-shRNA;* T4 is the one with the major silencing effect and T2 is the one with the mild effect. D), E) Western blot of one and two passages after transduction of urethral keratinocytes, as we can see in T2 and T4 induced with doxycycline, bmi-1 is down modulated. H) FACS analysis to quantify the number of colonies that have the RFP (transduced). First lane (in red) represents the percentage of the cell positive for the anti-feeder layer antibody; the second lane (in green) shows the percentage of transduced keratinocytes. G), H) Percentage of clonogenic and abortive colonies during a lifespan of the two scrambles and the two shRNA (T2 and T4). I) Number of cell doublings for each sample; shT2 and T4 resulted with a significant low number of cell doublings compared with the shScrambles. L) RFP keratinocytes colony.

4.9 p63-alfa and p16 during sh-silencing of *BMI-1*

The analysis throughout serial passages of urethral epithelial stem cells cultures transduced with shRNA targeting *BMI-1* revealed that there were any modulation of p63 and p16 between shScrambles and shT2 or T4 neither soon after the transfection or at the end of the lifespan, as shown in Figure 18.

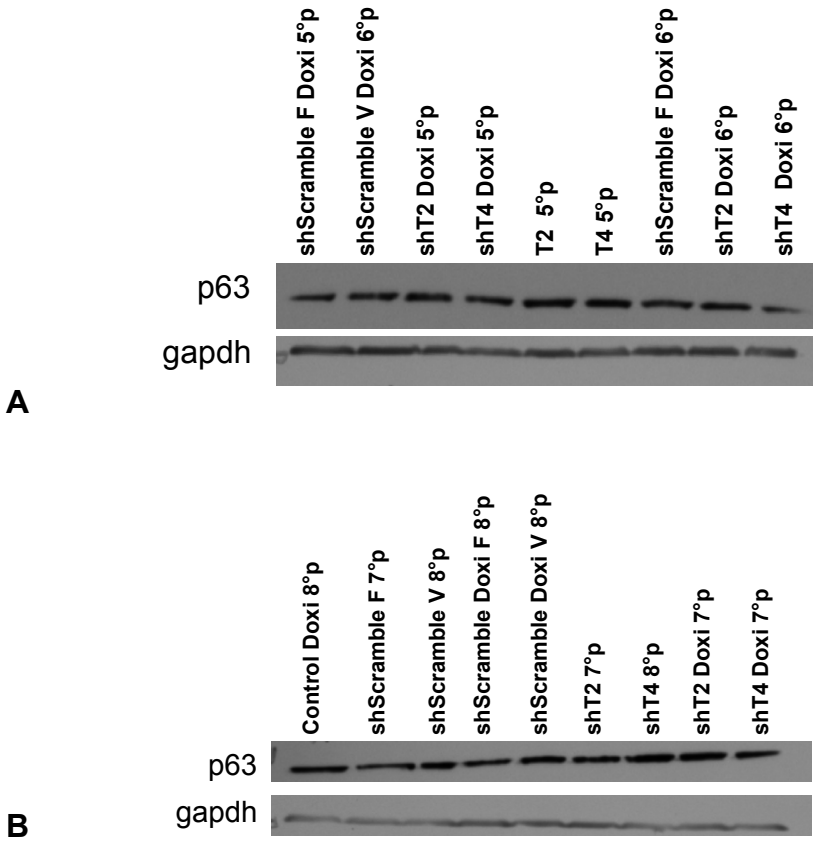


Figure 18. p63-alfa throughout silencing of *BMI-1*. Western blot of proteic lysates from the infection of the urethral primary cells. A) and B) The expression of p63 remains constant during the first steps in which we have the major effect of the silencing of *BMI-1*.

4.10 *BMI-1* silencing does not promote premature replicative senescence

To further investigate the effect of the silencing of *BMI-1* we analysed samples, from three passages after the silencing, for the expression of p16 and the Galactosidase staining. *BMI-1* transduced urethral keratinocytes did not display hallmarks of premature replicative senescence such as considerable increase of cell size and very low nuclear-cytoplasmic ratio, or expression of β-galactosidase, and presence/absence of p16INK4a (Figure 19A,B).

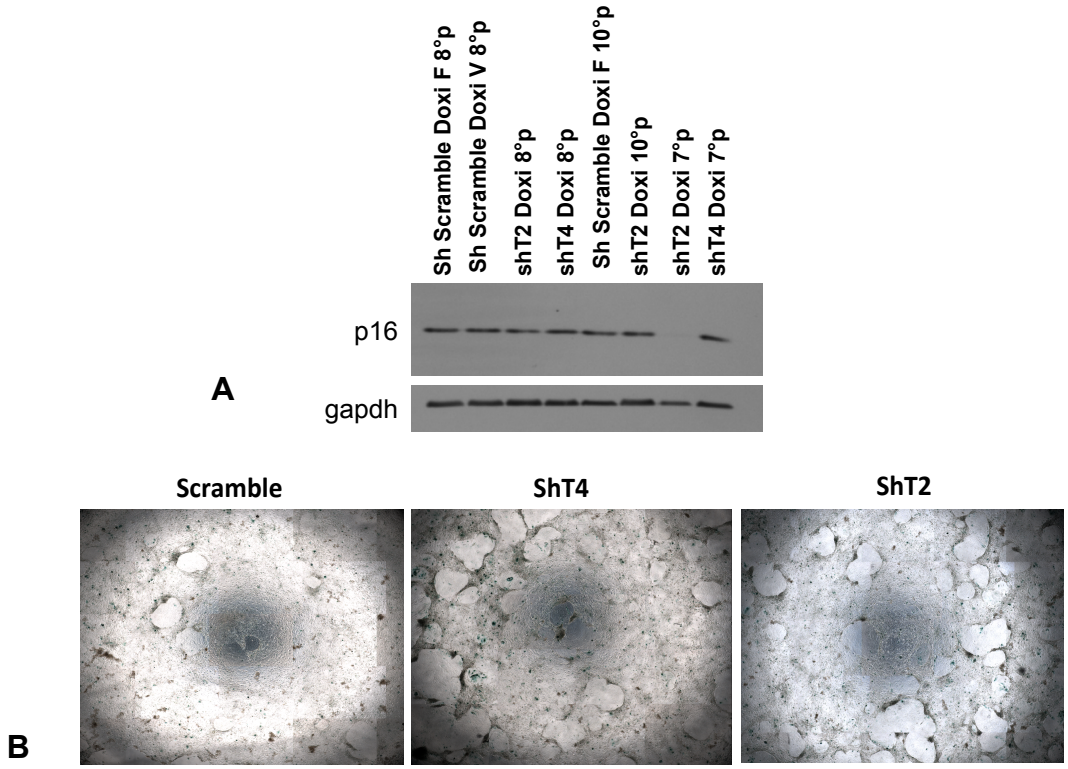


Figure 19. *BMI-1* silencing does not promote premature replicative senescence. A) p16 is continuously expressed during 7° and 8° passages independently from the *BMI-1* silencing. B) Photographs of transduced cells of scramble, shT4, shT2, stained for b-Gal, in which colonies do not take the blue colour.

5. Discussion

My PhD thesis is focused on the characterization of *an* vitro tissue culture, GMP standardized, as an altenative model to the animal one, in particular for the urethra regeneration. The bulbar urethral strictures were the objective on which test this new approch of *in vitro* preclinical evaluations. That pathology causes narrowing which makes urination difficult, impairs bladder function and allows infections leading to a surgical reconstruction, the urethroplasty. In this tecnique urethra is opened at the stricture site and its lumen is widened by applying an oral mucosa graft. The urethroplasty in addition to the complications due to the operation itself, carries a series of morbidity associated with the oral mucosa tissue biopsy. In our laboratory it has been developing an alternative approach to urethroplasty, based on tissue engineering and epithelial stem cells in order to reduce invasiveness of surgery and to improve functional and physiologic outcomes. Previous data have shown the potentiality of the oral mucosa stem cells for *in vitro* reconstruction of the urethra affected by large stenosis. Indeed, the biological parameters analyzed have determined the supremacy of the oral mucosa epithelium in term of proliferative potential and migration (Corradini F et all. 2014).

In this project was analysed a biocompatible oral mucosa stem cell engineered scaffold in order to verify if it was, first of all, safe and clinically effective for an extensive urethral reconstruction, making a urethroplasty a noninvasive procedure with a negligible risk of morbidity and scarring thanks to rapid healing. The surgical application of the oral mucosa engineered scaffold required the use of a surgical glue to firmly place the graft in the body. The glue used and tested in this study is Glubran 2 (GEM, Viareggio, Italy), an N-butyl-2-cyanoacrylate combined with a monomer (methacryloxy sulfolane) with good adhesive and haemostatic properties. Glue cytotoxicity was analyzed by dropping cyanoacrylic-based surgical glue on confluent fibroblast and keratinocyte cultures that were photographed daily and digitally analyzed immediately and at up to one week.

We observed the formation of an halo devoid of cells surrounding the glubran 2 area; the highest effect on vitality occurred at 72 hours, only in fibroblast cultures in contrast with those of keratinocytes in which there was any halo formation. Fibroblasts of both epithelia, urethral and oral mucosa, were however capable to fill out the halo in 8 or 7 days respectevely. In addition when subcultured after 24 hours or 7 days of contact with the glue, they displayed an increase in cell growth during the time, consistent with that it is normally observed in standard conditions.

The scaffold designed was composed of two layers, one at the bottom and the other one at the top, both made by a biological polymer. The first layer acted as a carrier for the cells and the other one functioned as a protective layer for the epithelium that had to be implanted during surgery. We tested the efficiency of four different scaffolds, differing in term of concentration of the protective layer on the top and in term of overall thickness of the scaffold itself. Standard A (ST-A) had a standard concentration of the polymer of the protective layer and was thin, standard B (ST-B) had a standard concentration of the polymer of the protective layer and was thick, diluted A (Dil-A) has a diluted concentration of the polymer of the protective layer and was thin, diluted B (Dil-B) has a diluted concentration of the polymer of the protective layer and was thick. The best results were obtained with scaffold Dil-A having the highest percentage of clonogenic cells in CFE assay, and the lowest number of aborted colonies in comparison with the control on plastic. Afterwards we proceeded to test the safety of the Dil-A scaffold in terms of not promoting tumorigenic transformation of stem cells, which usually were thought to be involved in the possible formation of tumors (Miller SJ et all. 1993). First of all, a soft agar migration assay was performed proving the total dependence of the epithelial cells from the anchorage, after contact with scaffold. Oral mucosa epithelial cells seeded on the scaffold did not show proliferation and migration capacity as the tumor cell line mcf7 and umscc-14. The second test, a growth factor dependent assay revealed that oral mucosa keratinocytes were unable of autonomous growth, and relied on autocrine and paracrine stimulation by growth factors. A third assay was the anaylsis of the karyotype from 7 replicates, resulted consistent with a

physiologically normal karyotype. Furthermore immunohistochemistry evaluations performed both on paraffin-embedded epithelial cell-based scaffolds and a control, demonstrated that the localization of previously defined stem cell markers, bmi-1 and p63alfa, was not affected by the culture in this innovative scaffold.

We also demonstrated the integrity of the cytoskelton by immunohistochemical analysis of alfa-sma and beta-tubulin. Our results indicated that there was only a short term effect of the glue on fibroblasts, which were capable in less then a week to totally recover from this transient cytotoxicity effect. The same was observed in keratinocytes colture in which even if there was no halo formation we observed a decrease in the viability of the glubran 2 cultures when compared to control and fibrin culture. It might suggest a possible irreversible damage, but further analysis such as the colony forming efficiency assay demonstrated there were no differences in the percentage of clonogenic and abortive cell among groups. We had the same results both for urethra and oral mucosa epithelial cells. Indeed we could assert that there was also on keratinocytes, as on fibroblasts, a short-term toxicity on viability in response of the surgical glue but on the long term the cells rescued from the toxic effect. These data supporting glubran 2 safe application for urethral reconstruction in humans (Barbagli et all.2016).

Previous study in our laboratory revealed that bmi-1 was abundantly expressed in urethral and oral mucosa epithelial stem cells as well as p63alfa, for urethra only, being a stem cell marker also in corneal epithelial stem cells. *BMI-1* was member of the polycomb group of transcription factors that was considered necessary for the maintenance of adult stem cells in normal human keratinocytes and, its expression together with telomerase activity, decreased during clonal evolution. Ablation of *BMI-1* in urethral epithelial cells led to a shortening lifespan, with a significantly lower number of cell doublings in comparison to that of the control. Moreover, CFE assay suggested that the silencing led keratinocytes to produce, a highest number of abortive colonies with a few clonogenic colonies, harming in this way the proliferative potential. Likely, the deficiency of *BMI-1* led stem cells to rapidly complete their clonal evolution,

giving rise only to transient amplifying cells that terminally differentiated, exhausting their proliferative potential (Maurelli et all.2006).

In this project we observed that persistent inactivation of *BMI-1*, in human urethral keratinocytes, did not produce a change in the expression of $p16^{Ink4A}$ during the lifespan among the different conditions. However this finding was consistent with previous data already showing that *BMI-1* could not affect $p16^{Ink4A}$ expression in oral epithelial cells (Lee et ll. 2007). Furthemore the beta galactosidase unstained cells suggested that silencing of *BMI-1* did not cause premature replicative senescence in keratinocytes. We hypothesize that *BMI-1* had a predominant role in cell cycle progression, regulating many molecules responsible of the progression to G_0/G_1 phase.

In conclusion in this thesis we have obtained a preclinical evaluation of the proposed cell based advanced therapy, as the regulatory authorities requested, assessing safety, toxicology and efficacy using an *in vitro* tissue culture model instead of animal models. Only a consolidated system of human cell culture, as the one here utilized, with standardized conditions could give strength to preclinical data, mimicking best the human condition allowing scientist to obtain more predictive results.

6. References

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