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Preclinical study for treatment of hypospadias by advanced therapy medicinal products

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Abstract

Purpose This paper explores the feasibility of a new therapy for the treatment of hypospadias patients. Hypospadias is a very common congenital malformation of male genitals, with very high rate of recurrences after surgery. The field of regenerative medicine, which offers innovative solutions for many pathologies, still does not offer reliable solution for this pathology. Here, we propose quality, safety, and clinical feasibility assessment for an oral mucosa advanced therapy medicinal product (ATMP) grown on a biocompatible scaffold for a clinical study on urethral reconstruction of hypospadias patients. **Methods** Urethral and oral mucosal epithelia from donor biopsies were cultivated between two fibrin layers, under clinical-grade conditions for cell and tissue characterization and comparison, aimed at tissue engineering. In addition, single-clone analyses were performed to analyze gene expression profiles of the two epithelia by microarray technology.

Results Oral mucosa appeared suitable for urethral reconstruction. The resulting ATMP was proven to maintain stem cells and regenerative potency. The preclinical safety studies were performed on human tissues to assess abnormalities and tumorigenicity, and confirmed the safety of the ATMP. Finally, the patient selection and the clinical protocol for the upcoming clinical trial were defined.

Conclusions Against this backdrop, in this paper, we are proposing a new reproducible and reliable ATMP for the treatment of hypospadias.

Keywords Hypospadias · Regenerative medicine · Oral mucosa · Clinical trial · Tissue engineering · Stem cells

Abbreviations

MO	Human oral mucosa keratinocytes
UK	Human urethra keratinocytes
CFE	Colony forming efficiency
DP	Final product
K	Cytokeratin
ATMP	Advanced therapy medicinal product
QFQ	Q band by fluorescence and quinacrine

Introduction

Hypospadias, one of the most common congenital malformations of male genitals with an incidence of 1 out of 1000 male births (Orphanet 440), is defined by an abortive development of the urethral corpus spongiosum, ventral prepuce, and arrest in the normal embryological development of the urethra [1]. Problems related to this disease include altered urination and ejaculation and psychological problems causing depression and disability. Foreskin is frequently used for one-stage urethral reconstruction, but it only provides one chance for application, and due to the high rate of treatment failure, skin flaps must later be removed from different body sites, with side effects such as hair growth inside the urethra,

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tissue retraction, and infection [2]. Oral mucosa tissue has been used in different surgical practices such as pharyngeal [3], maxillofacial [4], reconstructive vaginal surgeries [5], and urethral reconstruction. This is due to easy access to the donor site and its high regenerative capacity, as oral mucosa reacts to daily damage at its original anatomical site. In cases of large removal, as needed for hypospadias treatment, patients report pain, altered salivary function, a morbidity rate of 3–4% at the donor site [6], and scars and oral contractures in about 20% of cases [7]. Mechanical irritation from dental rubbing and parafunctional biting of the oral mucosa increases the chances of oral cancer development [8]. This practice only allows two big tissue removals from the oral mucosa, but the aforementioned procedures require repeated surgery in approximately half of the patients [9]. ATMPs, such as somatic cell therapy or tissue-engineered products, can enable tissue regeneration starting from a small biopsy. When supplied with stem cells, the resulting cultured tissue can engraft and persist at the desired location, self-renewing over the lifetime and restoring missing functionality [10].

Here, we planned a tissue-engineering-based clinical trial to (1) identify and characterize the cells able to restore the tissue in vitro and in vivo and (2) to select the appropriate biomaterials, "clinical-grade" culture medium, and controls for the different phases of the manufacturing process.

As requested by regulatory authorities, the cultured tissue was characterized by identity, purity, potency, and safety on in vitro models [11]. Under these conditions, the transplanted tissue can safely grow within the surrounding environment.

The pig animal model originally selected for preclinical studies revealed different sensitivities to these culture conditions compared to human cells, making it unsuitable for safety analysis. Alternatively, we performed preclinical tests in vitro using human cells. This process included the comparison of cultured urethra and oral mucosa to determine whether human oral mucosa keratinocytes (MO) could be used as substitute for urethral keratinocytes (UK) in tissue engineering.

Materials and methods

Donors

Samples were obtained in accordance with the Declaration of Helsinki, and informed consent was obtained from donors. Small oral mucosa biopsy samples (0.25 cm^2) were obtained from the inner cheek of patients undergoing surgery for urethral stricture treatment.

Urethra samples were obtained either from patients undergoing sexual transition surgery (healthy) or stricture treatment surgery (pathologic).

Cell culture and CFE

UK and MO were cultured and controlled as described [10, table 4 in 12].

Growth factor dependence assay

An aliquot of final Product (DP) was dissociated using 2 U/ml Dispase II at 37 °C for 15 min, followed by 0.05% trypsin and 0.01% EDTA at 37 °C for 10 min, and then plated in the absence of growth factors as described (Suppl. Mat.).

Soft agar assay

Keratinocytes obtained from DP were collected and seeded in a semisolid agar medium using the CytoSelect™ 96-Well Cell Transformation Assay Kit (Cell Biolabs, Inc.; Suppl. Mat.).

Karyotype analysis

After DP dissociation, MO were cultivated up to sub-confluence and shipped to the Medical genetics' laboratory of Modena Policlinic Hospital for karyotype analysis and Q band by fluorescence and quinacrine (QFQ) staining.

Immunofluorescence

Cultured epithelial cells were trypsinized, centrifuged, and fixed. Cytospins were permeabilized, blocked, incubated with anti-p63 (Ventana) or anti-BMI-1 (Cell Signaling) primary antibodies.

A portion of the cytospins was double-stained for anti-mouse Vimentin antibody (BioLegend) and anti-wide spectrum Cytokeratin antibody (AbCam) for the identity and purity assays.

Cells cultured on glass coverslips were fixed, permeabilized, and incubated with anti-K6 (AbCam), anti-K7 (Progen), anti-K8 (Progen), or anti-K18 (AbCam) primary antibodies.

Human tissues were embedded in an optimal cutting temperature (OCT) compound, frozen, and cut into 5–7 mm sections on a cryostat. The sections were then, fixed, permeabilized, blocked, and incubated with anti-K6, anti-K7, anti-Bmi1, anti-p63-α (PRIMM), or anti- K8 & K18 (Cell Marque) primary antibodies.

Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI). Further information in Suppl. Mat.

Microarray analysis

Stem-cell clones from sub-confluent primary cultures of oral mucosae ($n = 2$) and urethras ($n = 2$) were obtained to perform clonal analyses as previously described [13].

Subcultures from each clone were feeder-depleted using immunomagnetic beads (Miltenyi) and collected for RNA extraction and microarray analysis using HG-U133 Plus 2.0 Array (Affimetrix).

Differentially Expressed Genes (DEGs) were selected from Robust Multi-array Average-normalized data through a supervised analysis, using the ANOVA module supplied by the Partek GS. 6.6 Software Package (http://www.partek.com). The selected probe sets displayed a fold change contrast ≥ 2 and a false discovery rate ≤ 0.05 for DEGs in oral mucosa versus urethral clones. Upstream regulators were computationally predicted using the Ingenuity® Pathway Analysis software (IPA®, QIAGEN Redwood City, http://www.qiagen.com/ingenuity).

Results

In vitro reconstruction of oral mucosal and urethral epithelium

The biopsies were surgically removed from oral mucosa and/or penile urethra of the patients, were placed in primary containers with transport medium, and transferred to the laboratory within 24 h. Different tissues were derived from each biopsy, as described above. The culture conditions were modified according to regulatory requests for similar products and included clinical-grade hormones and growth factors. Reagents were extensively screened for the absence of contaminants and cytotoxic effects, and the serum was also irradiated. The final tissue constructs consisted of modified fibrin glue scaffolds (EP Patent 1451302) covered by adhering ex vivo expanded autologous human epithelia. The cultured tissue was a 30–35 cm^2 epithelial cell sheet containing oral stem cells, instrumental for the long-term regeneration of healthy, functional, and self-renewing epithelium. To reduce friction forces over the epithelium during and after surgery, the constructs were further covered with a different formulation of fibrin glue, easily resorbable (patent in preparation).

Oral mucosa vs. urethral epithelium in vivo and in vitro

Cytokeratins (K) expression is characteristic of epithelia, as they are part of the cytoskeleton and critical for the integrity and mechanical stability of cells and tissue as well as for cell function regulation. Indeed, some cytokeratins are involved in intracellular protection from stress, or wound healing, and apoptosis. Here, some of them were characterized in oral mucosal and urethral epithelia to investigate tissue-specific modulation in vivo and in vitro under mitogenic stimuli in the defined culture conditions. As shown in Fig. 1a, the two epithelia revealed very similar expression patterns, both in vivo and in vitro. K6, constitutively expressed in highly proliferative mucosae, was shown in both tissues in vivo and in vitro. The K18 primary epithelial keratin was activated in vitro, as it may change in reactive conditions [14], in both urethra and oral mucosa. K8, typically expressed in oral malignancies, was negative in oral mucosa in vivo and in vitro [15]. K7 exhibited a different expression in vivo and in vitro, as cultured cells exposed to mitogenic stimuli behave like cells in wound healing, and several functions are subsequently activated [14, 16]. Altogether, the cytokeratin expression maintained a physiologic tissue- and differentiation-specific location and regulation pattern, comparable in urethra and oral mucosa.

Clonogenic potential

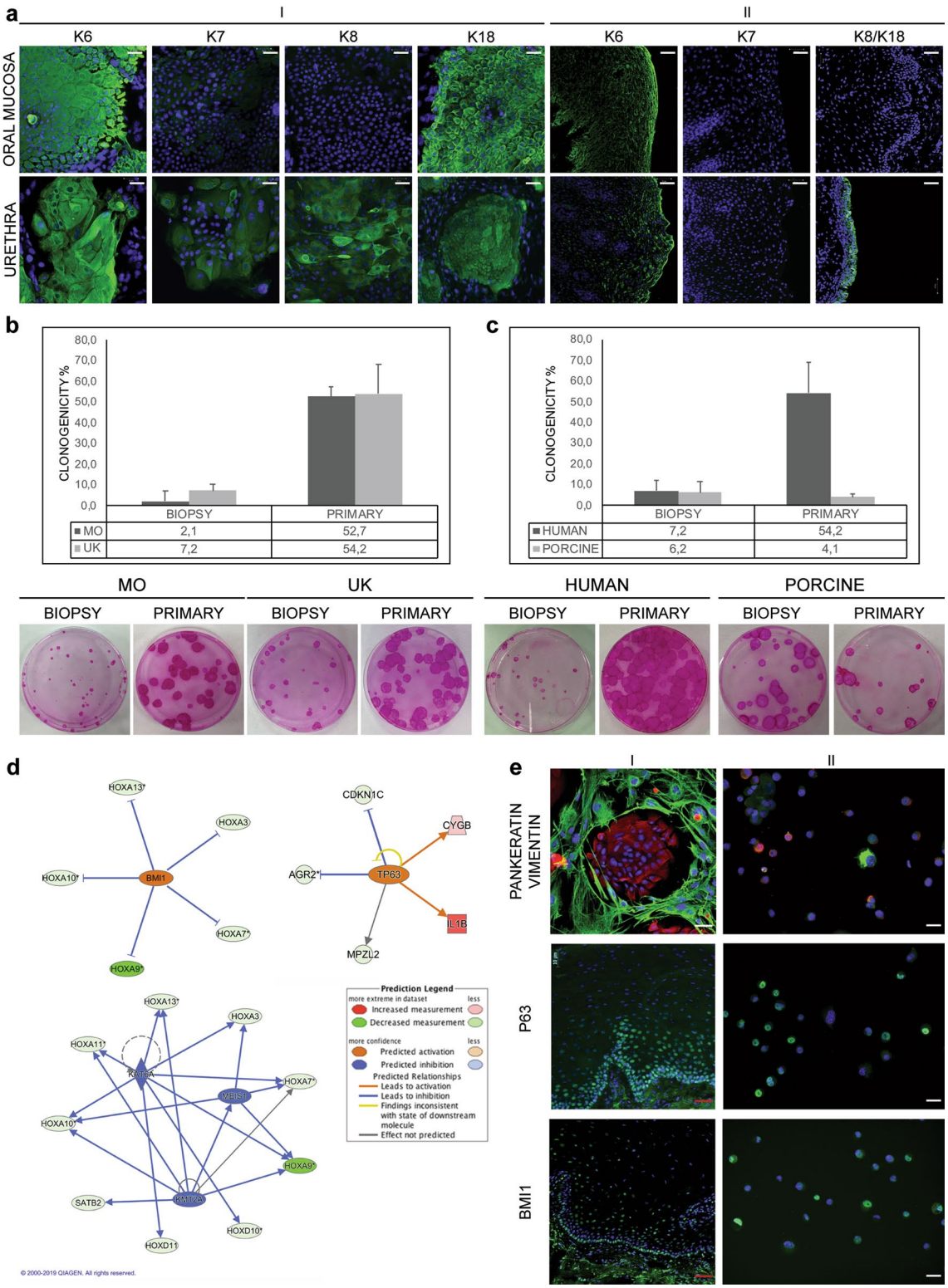
To assess the in vitro maintenance of tissue regenerative capacity, we considered: (i) the clonogenic potential, namely, the single-cell capacity to reconstitute the whole tissue; (ii) the comparative abundance of stem, progenitor, and transient-amplifying cells; and (iii) related marker expression, to identify subpopulations in culture.

Epithelial cells were isolated from 50 oral mucosae and 13 urethra biopsies from living donors, cultured using the same procedures described above. UK and MO had comparable clonogenic ability, respectively $7.2\% \pm 3.2$ and $2.1\% \pm 1.9$. After the primary culture, the colony-forming efficiencies of the two epithelia were still similar, showing a clonogenicity of $54.2\% \pm 14$ in urethra cells and $52.7\% \pm 29.9$ in oral mucosa cells (Fig. 1b). This clonogenic potential was maintained for several passages, and then progressively decreased until replicative senescence was reached. The end of proliferation occurred after an average of 88 cell doublings for oral mucosa ($N=5$), 167 for healthy ($N=4$), and 80 for affected urethras [17], respectively, proving the absence of any immortalization events (S1).

Stem, progenitor, and transient-amplifying cells

The proliferative compartment of lining epithelia is located in the basal layer and contains the three types of keratinocytes with different capacities for multiplication, holoclones, meroclones, and paraclones previously identified [13] and equally maintained after culturing oral mucosa and urethral epithelium under these "clinical-grade" conditions. Stem-cell clones (holoclones) appeared in a lower amount in urethra than in oral mucosa [17]. To evaluate differences between the two epithelia, the respective populations of stem cells were further investigated by microarray transcriptome analysis.

The gene expression profiles of 15 holoclones isolated from 2 strains of oral mucosa, and 4 holoclones from 2



strains of urethra were analyzed by microarray. 100 differentially expressed genes (DEGs) were identified in oral mucosa vs. urothelial holoclones (S2).

Based on the list of DEGs and according to current knowledge, IPA® upstream regulator analysis proposed transcriptional regulators that could underlie the differential gene expression of oral mucosa and urethra holoclones.

Interestingly, the activation of BMI1 was predicted to be responsible for the transcriptional repression of HOXA genes [18, 19] that were indeed, downregulated in oral

◂**Fig. 1** **a** Evaluation of cytokeratins expression of in vitro (I) and in vivo (II) in oral mucosa and urethral samples; scale bar 50 µm. **b** Comparison between biopsy and primary culture clonogenicity of oral mucosa ($N=50$) and urethral epithelium ($N=13$). The corresponding colony-forming efficiencies show a clonogenicity of 2.1% and 52.7% from biopsy and cultured oral mucosa, 7.2% and 54.2% from biopsy, and cultured urethra epithelium, respectively. **c** Percentage of clone developments from human (dark grey) and porcine (light grey) urethral biopsies and tissue cultures. The number of porcine clonogenic cells did not increase under these culture conditions. **d** Upstream regulators analysis results. IPA® software upstream regulators analysis predicted the transcriptional regulators that can underlay the different gene expressions detected between the holoclones from oral mucosa and those from urethra. BMI1 and TP63 were predicted to be activated, while KAT6A (i.e. MOZ), KMT2A, and MEIS1 were predicted to be inhibited in oral mucosa versus urethral holoclones. **e** Quality parameters and process controls defined by marker expression in vitro after culture: human pan-keratins staining (red) as control of epithelial cell identity and mouse vimentin (green) staining, as marker of process-related impurities by complementary number of human cells in vitro (I, II); P63 and BMI1 staining (green), for potency measure on in vivo cryosection (I) and on the Drug Product (scale bar 50 µm) (II). All the cells nuclei were counterstained with DAPI (blue)

mucosa vs. urethra holoclones. In addition, IPA highlighted the increased activity of TP63 in oral mucosa vs. urethra holoclones based on the modulation of its target genes (Fig. 1d).

The downregulation of HOX genes was partially confirmed in oral mucosa vs. urethra holoclones by a concurrent inhibition of KAT6A—i.e., MOZ, the catalytic subunit of a complex involved in the epigenetic regulation of HOX genes expression [19]—by KMT2A, an epigenetic transcriptional activator important for maintaining the expression of HOX genes, and by MEIS1, an HOX cofactor that promotes the transcription of HOX genes [20, 21].

The repression of HOX genes has been shown to preserve the undifferentiated state of stem cells [18] and these findings are consistent with the maintenance of stem cells in culture, as well as their higher content in oral mucosa than urethra. Finally, cyclooxygenase 2 appeared to be downregulated in MO, compared to urethra, where it increases in urethral strictures [22] and squamous cell carcinoma.

At protein level, no significant differences were detected in single cells for p63 and BMI1, highly expressed in holoclones [17]. The differential gene expression in clones could reflect a difference in the capacity for stem-cell maintenance of oral mucosa vs. urethra after cell expansion. BMI1 and p63's role in stem-cell function is also highlighted by their downregulation in progenitor and transient-amplifying cells, where loss of proliferative potential is associated with a reduced/null level of BMI1 and p63.

Altogether, these data suggest that BMI1 and p63 markers are related to regenerative properties needed for this treatment and have no significant differences between tissues except for a moderate advantage for oral mucosa.

Manufacturing process: quality assessment

Since ATMPs are subjected to an extensive assessment by the regulatory authorities, we considered the requirements for quality, safety, and efficacy, so that patients and practitioners can have high confidence in the product. We summarized the quality as follows.

Identity and purity of the cell culture

The cell identity was controlled during cell production and banking, and we confirmed that no segregation, major cross contamination, or 3T3 cell fusion could arise during manipulation in culture. The maintenance of cell identity was evaluated through positive and negative markers: keratins were constitutively expressed by oral mucosal cells, while murine vimentin is not produced by human epithelium, but was present in residual feeder cells only (Fig. 1e). The identity and the purity of the cell population were routinely identified by human pankeratin + and mouse vimentin + cells, excluding the presence of other unexpected contaminants in culture.

Potency

Potency measures the relevant biological function(s) of the tissue that are possibly related to clinical efficacy.

Maintenance and regeneration of the tissue over time rely on the presence of stem cells in self-renewing tissues such as epithelia. Thus, markers related to regeneration and proliferation of the oral mucosal stem cells were considered to be good candidate potency markers.

Accordingly, the product potency was evaluated by P63 and BMI1 expression in the cell nuclei (Fig. 1e). These transcription factors are related to the long-term capacity for proliferation and self-renewal in several cell types [23, 24], and oral mucosal stem cells (holoclones) maintain high levels of P63, unlike transient-amplifying clones (paraclones). This marker's expression decreases with replicative senescence and disappears with differentiation [17, 25].

Preclinical studies

Tests on the animal model

Traditional drugs are characterized by preclinical studies in vivo to evaluate toxicity, pharmacodynamics, and pharmacokinetics. It is not possible to perform these tests on ATMP such as the cultured oral mucosa. The pig is considered a common model for several studies about the digestive [26] and the cardiovascular system, and is also highly analogous to humans at a genomic level [27]. Therefore, we selected swine as a suitable animal model to test the safety and the efficacy of our treatment. The use of a xenograft transplant

on the animal model would trigger an immune response, requiring the co-administration of immunosuppressive therapy to prevent graft rejection. This practice is not representative of the clinical situation, since immunosuppressive drugs would alter the environment and cell survival, compromising the product's efficacy. On this basis, we cultured urethral porcine epithelia in our "clinical-grade" culture conditions to transplant the porcine cells onto a pig to mimic human treatment. Three different porcine biopsies showed a cell yield and clonogenic capacity comparable to human biopsies (Fig. 1c).

However, a wide divergence was observed in the subsequent cell expansion, and porcine cells revealed low responsiveness to culture conditions and mitogenic stimuli, maintaining a very low clonogenic capacity ($4.1\% \pm 1{,}6$) compared to human cells, which had a colony-forming efficiency (CFE) of $54.2\% \pm 14$. This divergent reaction to the culture conditions made the porcine model useless for safety studies, and therefore, its application was discontinued.

Thus, safety tests were performed on cultured cells as previously described [17], including soft agar colony formation assay to confirm the anchorage-dependent cell growth in vitro, growth factor dependence assay to predict uncontrolled cell growth, and finally, karyotype analysis of cultured cells. Data confirmed the absence of any abnormalities under the selected culture conditions (S3).

The treatment and ancillary products

This phase I trial was designed to demonstrate in-human safety at 7–60 days after transplantation, then efficacy and safety from 3 months to 1 year (short-term efficacy) and up to 6 years (long-term efficacy) after the first treatment. The administration of this construct will follow the surgical resection of scars in patients suffering from symptoms due to hypospadias treatment failure. All ancillary materials for surgery, as anesthetics, lubricants, bandages, etc., were previously tested for toxicity on epithelia, since they are critical for the engraftment of cultured tissues [28].

Patient selection

The clinical trial (EudraCT number 2017-000361-78) will involve male pediatric and adult patients suffering from reduced urethral functionality and/or distal and recurrent lesions in a very large part of urethra due to failure of primary hypospadias repair. Patients included will be divided into two groups, 12 months–12 years and 14–65 years, with a clinical history of the previous primary hypospadias repair during pediatric age, presenting recurrent obstructive or irritating symptoms (frequent day/night urination and burning) and recurrent Urinary Tract Infections (UTIs; fever) or epididymitis (fever, scrotal pain and edema, high residual urine volume > 100 cc). The following clinical parameters will also be considered:

- Maximum flow rate determined by uroflowmetry < 12 ml/s.
- Residual bladder urine > 100 cc.
- Evidence of penile urethra narrowing upon retrograde and voiding cystourethrography.
- Urethral meatus or fossa navicularis with caliber < 12 F.

Candidates for reconstructive urethroplasty by two-stage procedure to repair penile urethral stricture, related to a failed primary hypospadias treatment, will be selected based on need according to the clinical features described above. They should display a cooperative attitude following the study procedures, and they will be closely monitored throughout the trial, and evaluated by the investigator according to the Investigator brochure specifications. Briefly, success will be assessed by the presence and stability of the urethral canal, with no significant stenosis impairing the urinary function (see above). The take of the ATMP in the urethra will be evaluated at 1 week and up to 1 month after the first surgery on the open wound bed, by visual inspection and quantification of the epithelium engraftment; in the second step, urethra will be tubularized and the maintenance of a pervious canal will be assessed by endoscopy at 1, 2, and up to 5 year follow-up.

Histology and molecular markers will be investigated on a small biopsy of the treated area, obtained from compliant patients to further confirm whether the tissue derives from urethra, foreskin, or oral mucosa.

The trial will be conducted in compliance with the Declaration of Helsinki, current Good Clinical Practice, and all other applicable laws and regulations.

In summary, based on the previous clinical experience and scientific data, the expected therapeutic value combined with the safety profile of this procedure, provide an acceptable overall risk/benefit assessment for the proposed trial.

Discussion

Urethral reconstruction to address failed hypospadias repair has attracted considerable interest in the last decades, but has generated variable positive and negative outcomes [29]. The use of autologous oral mucosa tissue was thought to be preferable, as it avoids some complications, but doubts remained about whether the heterotopic application could efficiently mimic urethral tissue, or would instead result in adverse reactions. In this study, we first evaluated the oral mucosa as urethral substitute at cellular and molecular level, and then defined a standardized protocol to combine these cells between a double fibrin scaffold in a tissue-engineered

construct. Fibrin is a natural substrate in wound healing and is rapidly reabsorbed on the lesion, allowing the epithelium engraftment [30]. The double fibrin scaffold provides protection during transport and surgical manipulations; in addition, it enables continuous adhesion, preserving the proliferative potential of epithelia. Indeed, this construct was proven to provide a normal stratified epithelium populated by the long living stem cells of the tissue.

We addressed the potential problems of a future clinical trial by developing a GMP/clinical-grade method for deriving a functional oral mucosa construct, along with standards for safety evaluation and quality control assays. The analyses of different proteins and cell behavior confirmed that the tissues under investigation were comparable and undergo physiological senescence, providing safety reassurances.

The protocol promotes a remarkable cell expansion from a very small biopsy, enabling a clinical performance similar to other regenerative medicine applications. The maintenance of epithelial stem cells is highly relevant for wound healing and instrumental for long-term tissue renewal. Here, we provided two possible potency markers for their identification: p63 and BMI1. Many evidences support their role as epithelial stem-cell markers, as p63 KO mice revealed the disappearance of epithelial linings, and BMI1 mediated the extension of oral mucosa life span and was able to repress INK4A/ARF-encoded cell-cycle inhibitors. In addition, BMI1 was shown to inhibit cancer stem-cell functions.

The proposed protocol was proven to be reproducible and scalable, although some issues will be updated at the end of the clinical trial, as the optimal specification limits for the potency assay and the outcome of follow-up investigations.

The non-clinical testing strategy included an evaluation of the previous studies in conjunction with the proposed non-clinical human tissue testing for safety assessments, since suitable animal models showed significant inconsistencies with human cell behaviors, under these culture conditions.

The combined data from human cell characterization, stem-cell maintenance, standardization, and comparison with similar products indicate that the proposed treatment could represent an efficacious approach for the restoration of urethra, to address failed hypospadias cases.

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Compliance with ethical standards

Conflict of interest G. Pellegrini is member of the Board of Directors, R&D Director of Holostem Terapie Avanzate and J-TEC consultant. V. Sceberras is employee of Holostem Terapie Avanzate. E. Attico, M. Melonari, G. Galaverni, M. Fantacci, F. Corradini, G. Barbagli, S. Balò, C. Trombetta, M. Rizzo, L. Losi, E. Bianchi, R. Manfredini, A. Ribbene and M. Lazzeri declare that they have no conflict of interest.

Research involving human participants The study will be submitted to AIFA (Italian Drug Agency) for clinical trial approval.

Informed consent Informed consent was obtained from all donors and will be obtained by individual participants included in the study.

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