European Review for Medical and Pharmacological Sciences 2013; 17: 820-833

A novel DNA profiling application for the monitoring of cross-contamination in autologous chondrocyte implantation

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Abstract. – BACKGROUND: Autologous chondrocyte implantation (ACI) is a cell-based treatment that can be used to regenerate chondral defects. European legislation specifically classifies such produced chondrocytes as "medicinal for advanced cell therapy" that have to be manufactured in pharmaceutical factories according to specific rules, named Good Manufacturing Practices (GMPs). One main requirement of cell manipulation in advanced therapy is to prevent the risk of any contamination.

AIM: The aim of this study was to verify if chondrocyte cultures suitable for ACI were free of cross-contamination by means of DNA profiling techniques.

MATERIALS AND METHODS: Cell cultures were carried on in a Hospital Cell Factory in compliance with European current Good Manufacturing Practices. DNA profiling, by means of Short Tandem Repeats and miniShort Tandem Repeats analyses, was performed on expanded chondrocytes and their related control blood samples. Mitochondrial DNA was analysed to further confirm the results and to evaluate possible mutations occurred in the samples.

RESULTS: Our findings demonstrated the absence of cross-contamination between chondrocyte cultures and, thus, their identity maintenance until the end of the manipulation.

CONCLUSIONS: DNA profiling technique can be a suitable test for quality control not only for chondrocyte manipulation, but for cell therapy in general.

Key Words:
Advanced cell therapy, Autologous chondrocyte implantation, Good Manufacturing Practices, Cross-contamination, DNA profiling.

Abbreviations

ACI = Autologous Chondrocyte Implantation
AIFA = Agenzia Italiana del Farmaco
bp = base pair
BSE/TSE = Bovine Spongiform Encephalopathy/ Transmissible Spongiform Encephalopathy
DMEM = Dulbecco's Modified Eagle Medium
DNA = Deoxyribonucleic acid
EDTA = Ethylenediaminetetraacetic acid
EU-cGMPs = European current GMPs
EU/ml = Endotoxin Units per ml
FBS = Foetal bovine serum
FDA = Food and Drug Administration
GMP = Good Manufacturing Practices
HIV = Human Immunodeficiency Virus
HCV = Hepatitis C Virus
HBV = Hepatitis B Virus
LAL = Lymulus Amebocyte Lysate
PCR = Polymerase Chain Reaction
PES = probabilistic expert systems
QC = Quality control
RMP = Random Match Probability
rRNA = ribosomal Ribonucleic Acid
rRCS = revised reference sequence
STR = Short Tandem Repeat

Introduction

Cell-based therapies have existed since the first successful bone marrow transplantation in 1968[1,2]. The subsequent increased understanding of cell biology at protein, molecular and genetic levels and the development of techniques such as tissue engineering and concurrent studies in the field of scaffold design have expanded the horizon of likely therapeutic uses[3]. Cell-based therapies have evolved during last years and are now applied in an increasing numbers of fields and in several clinical trials for congenital and acquired disorders. Public expectation for such novel treatments is high, but there have been only few completed trials. Moreover, their full potential still remains to be clarified and aspects like long-

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term outcomes or tumorigenesis/cancerogenesis need to be more in depth investigated and completed. This complex situation including different stakeholders (academia, clinicians, patients, public structures, enterprises) has raised the need to develop regulatory frameworks to guarantee patient safety and efficacy[4].

In the musculoskeletal system, there is a wide variety of cell-based applications[5]. Cells are utilized to repair or regenerate injured tissues (cartilage, bones, tendons, ligaments, muscles, etc.), or to treat chronic conditions such as rheumatoid arthritis. From recent studies, it appears that, in Europe, in general, autologous cells are predominantly used[6,7]. Newly, advances in gene delivery technology have created the additional opportunity to treat genetic diseases like Duchenne muscular dystrophy (DMD) with gene therapy[8].

To date, proposed employments for cell-based medicinal products in regenerative medicine are quite impressive[9]. Autologous chondrocyte implantation (ACI) is a cell-based treatment that can be used as second-line measure to regenerate chondral or osteochondral defects in younger, active patients[10]. It is a widely diffused technique and many groups have reported good results both from the histological and clinical point of view. Nevertheless, there is still scepticism about ACI clinical and cost effectiveness, especially in comparison with other traditional treatments. Literature revisions highlights the need of further trials with long-term follow up[11,12].

It is known that hyaline articular cartilage injuries may lead to pain and loss of function due to tissue's limited capacity for self-repair[13]. Such lesions predispose individuals to osteoarthritis in later life and eventually to requirements for total joint replacement. This is in general associated with a significant impact on quality of life and represents a huge socioeconomic burden to society. ACI approach was firstly introduced by Brittberg et al[14] to treat full-thickness chondral defects of the knee. The treatment was later applied to the ankle[15] and it is now suitable also for other joints such as hip[16]. In the original procedure small grafts of normal cartilage removed from non weight bearing areas of the knee were treated in a proper laboratory to obtain chondrocytes. The cells were expanded in monolayer in suitable media and the suspension injected into the prepared defects a few weeks later. Clinical, radiological and histological results are available at 10 to 20 years after the implantation, suggesting that outcomes remain high, with relatively few complications[17]. Recent generation technique includes the additional step to culture chondrocytes onto scaffolds which act as carriers ensuring spatial cell distribution and phenotype stability. The engineered tissues are then cut to the correct size and shape of the defects. The scaffolds, which efficiently "mimic" the natural surroundings of cartilage cells, may have different origins (synthetic, natural) and characteristics (bi/three dimensional structures, gels, sponges, microspheres, etc.)[18].

It appears that *in vitro* chondrocyte manipulation is a crucial phase of ACI as long as the surgical one. Current European legislation specifically defines such manipulation as "extensive" and classifies such produced chondrocytes as "medicinal for advanced cell therapy" (Regulation (EC) No 1394/2007 of the European Parliament and of the Council on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004). Those are not only definitions, but imply specific technical and practical consequences. In fact, such cell-based products have to be manufactured in pharmaceutical factories and according to specific rules, named Good Manufacturing Practices (GMPs) (European Commission, The Rules Governing Medicinal Products in the European Union. Volume 4-Guidelines for good manufacturing practices for medicinal products for human and veterinary use. Current Edition) that are currently utilized for the pharmaceutical products. Moreover, in the European Union, manufacturing shall be authorised by the competent authority of each Member State.

Inside Rizzoli Orthopaedic Institute, Bologna, Italy, is located a "Cell Factory" authorized by the Italian Drug Agency (Agenzia Italiana del Farmaco, AIFA) since 2009, working according to current GMPs (cGMPs) and manipulating chondrocytes for clinical use (ACI). To reach cGMPs compliance and gain the Authorization we carefully designed and validated a chondrocyte manufacturing process ensuring products consistency (microbial and viral integrity, viability purity, identity, yield and stability) (Reflection paper on *in-vitro* cultured chondrocyte containing products for cartilage repair of the knee, London, 08 April 2010EMA/CAT/CPWP/568181/2009, Committee For Advanced Therapies). Finally, as specifically requested by GMPs, we had also to demonstrate that all the measures adopted to prevent the possibility of cross-contamination of each processing medicinal lot were effective.

In this paper we describe the study aimed to verify if chondrocyte cultures produced in our Cell Factory and suitable for autologous implantation were free of cross-contamination by means of a novel application of DNA profiling utilized in human identification for forensic purpose.

Materials and Methods

Manipulation Areas: Clean Rooms

For this study, chondrocyte manipulations were performed in a production facility (Cell Factory) located inside our Institute (Figure 1). The structure includes two clean rooms of different classification up to A (the local zone for high risk operations i.e. Biohazard Hood) in B work places (the background environment for the grade A zone), according to European current GMPs (EU-cGMPs). To minimize traffic and contamination entry into the clean room, raw materials are introduced separately from personnel through a clean pass box (Figure 1A). High risk operations includes manipulations where the cells are exposed to environment such as trypsinization, medium change, biomaterial seeding (Figure 1B). For lower risk operations, such as media warming or centrifugation, grade B areas are sufficient (Figure 1C and D). Only trained and equipped personnel is admitted in the structure. Operator's equipment includes protective and disposable clothes to wear. As required by EU-cGMPs, in the Cell Factory various factors, including airborne contaminants temperature, relative humidity and differential pressure and static electricity are kept under strict control by a real time measuring system (A&LCO Industries, Cologno Monzese, Milano, Italy; Pharmaceuticals Net 3.2 facility Monitoring Software, Boulder, CO, USA) (arrows in Figures 1B and C).

Reagents

Reagents choice was geared towards products suitable for cell therapy applications, ensuring high quality performances. In particular, we utilized a foetal bovine serum (FBS) that was certified to be produced in Australia and free from bovine spongiform encephalopathy/transmissible

Figure 1. Production facility (Cell Factory) of Rizzoli Orthopaedic Institute. ***A,*** Pass box to introduce raw materials into the clean room. ***B,*** Biohazard Hood (Grade A area) for high risk operations like cell manipulations. ***C,*** and ***D,*** Grade B work places for lower risk operations such as media warming and centrifugation. Air classification is monitored in real time by a measuring system as indicated by the arrows.

spongiform encephalopathy (BSE/TSE) (European Agency for the Evaluation of Medicinal products/Committee for Proprietary medicinal products. Note for Guidance on Use of Bovine Serum in the Manufacture of Human Biological Medicinal Product (CPMP/BWP/1793/02), 2002).

All reagents were all cross-checked by internal quality controls in the Cell Factory and, only after passing such tests, they were considered to be adequate for the manipulation process.

Donor Screening

We utilized cells from eight patients (here named Patient 1, Patient 2, Patient 3, Patient 4, Patient 5, Patient 6, Patient 7 and Patient 8) undergoing ACI. Informed consent was obtained from all patients who entered the study and the ACI procedure was approved by the Ethical Committee of Istituto Ortopedico Rizzoli, Bologna, Italy. Blood samples harvested from the patients during arthroscopy were used to evaluate the presence of transmissible pathologies. The same minimum set of testing requirements was applied as for allogeneic living donors: HIV-1/HIV-2 Antibody, Hepatitis B Surface Antigen, Hepatitis B Core Antibody, Hepatitic C Virus Antibody and test for syphilis. In addiction, Nucleic Acid Techniques (NAT) for HBV DNA, HCV RNA, HIV DNA detection were performed to reveal virus presence also during the window period (Directive 2004/23/EC Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells). Since our structure does not have the possibilty to manipulate the infected cells in separated areas positive patients must be excluded from our study.

Chondrocyte cGMPs Manipulation and Batch Release

Cartilage biopsies, removed arthroscopically from non-weight-bearing areas on the femoral condyles in the operating room, were introduced in the Cell Factory clean rooms for manipulation. After weighing, samples were minced with a scalpel and carefully washed with cell culture medium Dulbecco's Modified Eagle Medium (DMEM) high glucose (4.5 g/L) GMP Grade (Li StarFish, Carugate, Milan, Italy), supplemented with 10% FBS GMP Grade (Li StarFish) and L-Glutamine 4 mM (complete medium) (Li StarFish). No growth factors, cytokines or other supplements were added. The chondrocytes were then isolated by enzymatic digestion with Trypsin-EDTA (1:250) GMP Grade (Li StarFish) 15 minutes and 740 U/mL Collagenase II (Li StarFish) at 37°C, 5% CO_2, 95% RH, for 22 hours. Complete medium was added and, after centrifugation, cells were seeded at low density (BD Falcon™ vented cell culture flasks). Primary cultures were expanded in monolayer up to passage 3: medium was changed twice weekly and at confluence cells were trypsinized in larger flasks.

Cell morphology was monitored during expansion with inverted contrast phase microscope (Eclipse TE200, Nikon Instrument S.P.A., Florence, Italy) and pictures were taken at passage 0 and 3 with a DS-Fi1 digital camera.

Chondrocytes were then seeded onto a biomaterial derived from porcine collagen I/III, named Chondro-Gide® (Geistlich Biomateriale, Wollhusen, Switzerland)[19], at a density of $0.5x10^6$ cells/cm^2. This seeding low limit has been established by previous studies performed in our Laboratory. To assess cell number and viability a NucleoCounter® Automatic Cell Counting System (Sartorius Stedim, Biotech, Goettingen, Germany) was used. The system is able to detect signals from the fluorescent dye propidium iodide (PI) bound to cell nuclei. Cell viability acceptance limit was: ≥ 80%. After 3-5 days in culture inside the biomaterial the engineered tissues were double packaged and released.

Quality Control (QC)

QC analyses, complying with cGMPs and European Pharmacopeia (European Pharmacopoeia, Current Edition), were performed to assess the microbial load of the biopsy following collection, throughout the chondrocyte manipulation process and at the time of release for clinical use.

Sterility Testing

The technology used was BacT/Alert 3D, Automated Microbial Detection System (Biomerieux Industry, Marcy L'Etoile, Craponne, France) that allows to reveal the presence of aerobic, anaerobic bacteria and fungi. The System has been already validated by FDA for chondrocyte culture monitoring in autologous implantation[20]. At each testing point, 1 ml of cell culture surnatants was inoculated in bottles containing specific bacterial growth media. The bottles were then incubated in the BacT/alert System for 7 days. If there is a microbial growth inside the bottles, CO_2 will be produced (due to substrate metabolization) inducing a

colorimetric change in the culture medium (due to a change in pH medium) that is registered by the Detection System. If there is no microbial growth and thus no colorimetric change, the sample is considered negative.

Mycoplasma Testing

Analyses for Mycoplasma contamination detection were performed both on surnatants (0.5 ml) and on chondrocytes (50.000 cells) to even evaluate its presence inside the cells. Highly-sensitive Real Time Quantitative DNA PCR technology was the method used for detecting contaminating Mycoplasma. DNA extractions were performed with "High Pure PCR Template Preparation kit" (Roche, Applied Science, Basel, Switzerland), amplifications with Venor®GeM-qDual Mycoplasma Detection Kit for Real-Time PCR (Minerva Biolabs GmbH, Berlin, Germany). This Kit displays a detection range of more than 25 Mycoplasma species since primers are specific for a common region of the 16S rRNA gene. To detect amplicon a specific fluorescent SCORPION probe was utilized (Minerva Biolabs GmbH, Berlin, Germany). Real Time PCR reactions were carried out in a LightCycler® 2.0 instruments (Roche, Applied Science, Basel, Switzerland). All samples were tested in parallel with positive and negative controls, as well as with an internal amplification control.

Endotoxin Testing

Bacterial endotoxin assays were performed on final product surnatants (0.5 ml) by quantitative Lymulus Amebocyte Lysate *(LAL)*, test using the Endosafe®-PTS (Charles River Laboratoires, l'Arbresle, France). This System has been licensed by the FDA as a method for release testing of pharmaceuticals products. Test acceptance limit was < 0.5 Endotoxin Units per ml (EU/ml).

DNA Profiling Analysis

Samples

An aliquot of blood sample harvested from each patient during arthroscopy and a pelleted aliquot of chondrocytes (80.000 cells) from each culture before the seeding onto the biomaterial were frozen for genetic testing.

DNA Profiling

DNA was extracted by *QIAmp Mini Kit* following manufacturer's instructions (Qiagen, Hilder, Germany). Extracted DNA was visualized by electrophoresis on 1% agarose gel containing ethidium bromide. 4 ng of DNA template were submitted to fluorescent multiplex polymerase chain reaction (PCR) to amplify 15 tetranucleotide Short Tandem Repeat (STR) loci (molecular size: 107-358bp): D8S1179, D21S11, D7S820, CSF1PO, D3S1358, THO1, D13S357, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA and amelogenin included in the AmpFlSTR Identifiler PCR Amplification Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA)[21] according to the manufacturer's recommendations. Amelogenin locus for sex determination was also useful as internal control.

Nine miniSTRs of AmpFlSTR MiniFiler PCR Amplification Kit (Applied Biosystems), whose amplicons range from 70 to 283 nucleotides, were analyzed with 2 ng of DNA template, according to the manufacturer's recommendations[22]: D13S317, D7S820, D21S11, D2S1338, D16S539, D18S51, CSF1PO, FGA and amelogenin.

Mitochondrial DNA (mtDNA) analysis was performed in a reaction volume of 25 ml containing: 10 ng of genomic DNA, 1X PCR buffer, 1.5 μM $MgCl_2$, 200 mM of each dNTP, 1.5 U AmpliTaq DNA Polymerase (Applied Biosystems), 0.2 mM of each primer (L15997-H16401, L29-H408). The amplification was carried out for 35 cycles: 3 min at 94°C, 1 min at 94°C, 30 sec at 56°C and 1 min at 72°C, with a final extension of 10 min at 72°C. PCR products were run on a 2% agarose gel and stained with ethidium bromide. Amplicons were purified by ExoSAP-IT™ reagent (USB Corporation, Cleveland, OH, USA) according to the manufacturer's protocol. Sequencing was performed using BigDye Terminator Cycle Sequencing Kit v 1.1 (Applied Biosystems, USA) and sequence products were purified by ethanolic precipitation.

Dye-labeled PCR fragments were analyzed by capillary electrophoresis using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and sequences were aligned and compared with the revised reference sequence (rRCS) using the Sequence Navigator computer program (Applied Biosystems, Sequence Navigator version 1.0.1).

Statistical Analysis

The calculation of Random Match Probability (RMP) based on genotype frequencies[23] was estimated using the Italian database[24].

Mixed Samples

Three cultures were carried out in our Cell Factory in the same period of time. One chon-

drocyte culture was manipulated following GMPs rules, as described above, and released for clinical use (Patient 8). The others were two bone marrow Mesenchymal Stem Cell cultures (named Test 1 MSC and Test 2 MSC) utilized only for validation purposes and discarded at the end of processing. Briefly, bone marrow aspirates harvested from iliac crest with an optimized harvesting technique[25] were derived from two donors (median age 22) undergoing an orthopaedic treatment. Informed consent allowed us to use an aliquot of each bone marrow aspirates for our study. Mononuclear cells were isolated and MSCs cultures characterized as described elsewhere[26]. Donor screening and MSCs manipulations were performed in compliance with current directives and GMPs, as described above.

Cell samples were harvested at the end of manipulation of the three cultures, scheduled the same day. At first we manipulated the chondrocyte culture (Patient 8), harvesting the sample for DNA profiling. Then we manipulated Test 1 MSC culture harvesting two samples for DNA profiling one of which was maintained open (and named Test 1 MSC) under the laminar flow until the end of Test 2 MSC manipulation, the other was closed. Finally, we manipulated Test 2 MSC culture harvesting again two samples for DNA profiling, one of which was closed (and named Test 2 MSC), the other was mixed (same number of cells) with the closed sample of the previous culture and named Test 1 & Test 2 MSC.

Cell samples and their related blood samples were pelleted and analyzed for STRs, miniSTRs and mtDNA as described above.

Results

Donor Screening

None of the ten patients' blood samples harvested at the time of biopsy arthroscopy revealed the presence of transmissible pathologies. All the biopsies were allowed to enter the Cell Factory and thus manipulated.

Chondrocyte cGMPs Manipulation

Biopsies weight ranged from 238 to 352 mg.

Morphological observations of each patient's monolayer cultures revealed that, in general, chondrocytes were behaving similarly: at passage 0 the cells resemble more polygonal (Figure 2 A), while appeared in a more spindle/elongated morphology at passage 3 (Figure 2 B).

Chondrocyte viability ranged from 88% to 99% at the time of release, with a mean of 92.5% and a Standard Deviation of 6.36%.

Quality Control

All the cartilage biopsies were negative for sterility testing and Mycoplasma detection. Sterility was maintained throughout all chondrocyte manipulation processes and at the time of release for clinical use. Endotoxin tests were < 0.1 EU/ml in all cases.

DNA Profiling

The eight samples analysed from autologous chondrocytes cultures showed for STRs (Table I and Figure 3) typing a unique DNA profile matching the reference sample without extra peaks. The random match probability was esti-

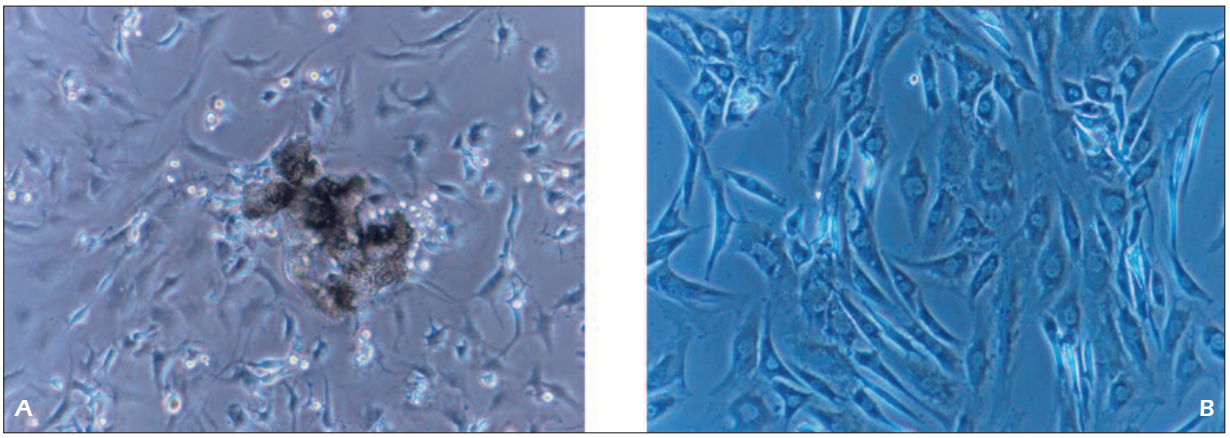

Figure 2. Morphological changes of monolayer expanded chondrocytes. Picture of a representative patient's chondrocytes monolayer. Morphological observations revealed that, in general, chondrocytes were behaving similarly. ***A,*** At passage 0 the cells resembled polygonal while appeared in a more spindle morphology at passage 3 ***(B)***. Inverted contrast phase microscope images were taken with a digital camera (magnification × 20).

mated in a range from 1×10^{-15} to 1×10^{-18}. Results were confirmed also by miniSTRs typing (data not shown).

Chondrocyte sequence of mitochondrial DNA HVI and HVII control regions showed an identical haplotype without heteroplasmic nucleotide positions neither de novo mutations compared to blood reference samples sequences and revealed the variations indicated in Table II, with respect to Anderson reference Sequence.

Mixed Sample

Test 1 and Test 2 MSC mixed samples showed a profile compatible with both its components (Table III and Figure 4). In particular, STRs analysis revealed a mixed genetic profile, enclosing both Test 1 and Test 2 MSC allelic components at 50% each. Applying a biostatistic calculation by means of the DNA mixture method (Fung and Hu, J. R. Statistic Society, 2000) we found a likelihood value of 6.5×10^{15}. This value strongly supports the hypothesis of a mixture of Test 1 and

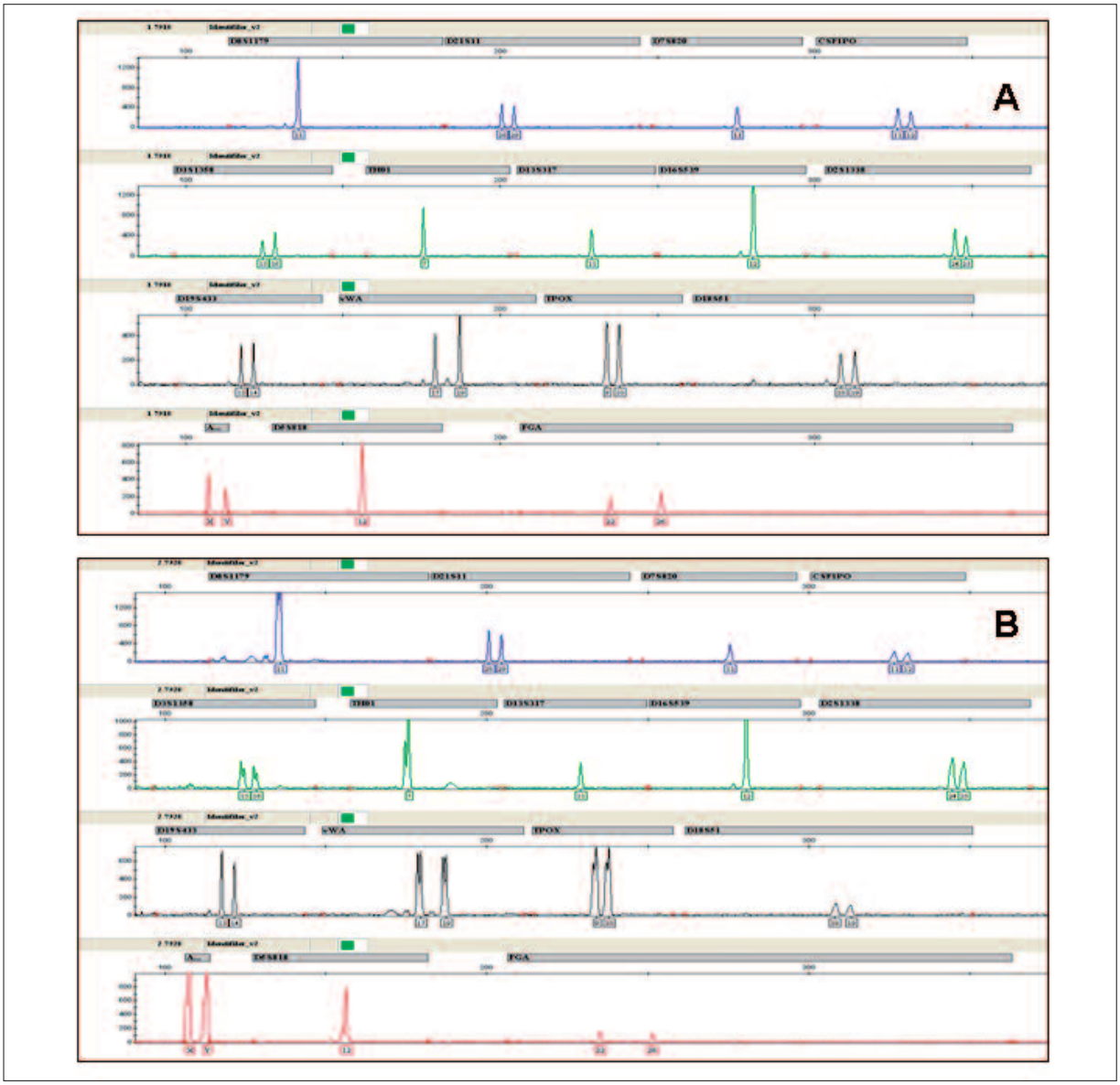

Figure 3. Electropherograms of PCR products in a representative patient (Patient 5). Eight engineered chondrocyte and related patient blood samples analyzed by means of DNA profiling in order to evaluate cross-contamination during GMPs manipulation. The picture of representative Patient 5 shows that engineered chondrocytes DNA profile ***(A)*** is unique matching the blood reference sample profile ***(B)*** without the presence of extra peaks corresponding to alleles revealing human exogenous DNA from other biological sources.

Table I. DNA profiling analysis by STRs on GMPs manipulated chondrocytes (cells) and related control samples (blood).

Autosomic markers	Patient 1		Patient 2		Patient 3		Patient 4		Patient 5		Patient 6		Patient 7		Patient 8	
	(cells)	(blood)	(cells)	(blood)	(cells)	(blood)	(cells)	(blood)	(cells)	(blood)	(cells)	(blood)	(cells)	(blood)	(cells)	(blood)
D8S1179	10-13	10-13	13-14	13-14	13-14	13-14	11-11	11-11	10-10	10-10	14-14	14-14	10-14	10-14	14-15	14-15
D21S11	30-31.2	30-31.2	28-30	28-30	28-29	28-29	28-29	28-29	30.2-32.2	30.2-32.2	28-28	28-28	28-30	28-30	28-31	28-31
D7S820	11-13	11-13	10-12	10-12	11-11	11-11	11-11	11-11	9-11	9-11	10-10	10-10	8-12	8-12	8-8	8-8
CSF1PO	10-10	10-10	10-10	10-10	11-13	11-13	11-12	11-12	11-13	11-13	7-10	7-10	9-11	9-11	10-12	10-12
D3S1358	15-18	15-18	15-15	15-15	15-18	15-18	15-16	15-16	16-17	16-17	15-17	15-17	16-18	16-18	15-17	15-17
TH01	6-9	6-9	8-9	8-9	6-8	6-8	7-7	7-7	7-9	7-9	7-7	7-7	8-9.3	8-9.3	6-9.3	6-9.3
D13S317	11-13	11-13	8-13	8-13	11-12	11-12	11-11	11-11	11-13	11-13	11-12	11-12	11-12	11-12	8-11	8-11
D16S539	11-13	11-13	11-11	11-11	12-12	12-12	12-12	12-12	9-13	9-13	9-11	9-11	10-10	10-10	11-12	11-12
D2S1338	17-23	17-23	17-17	17-17	24-25	24-25	24-25	24-25	19-25	19-25	17-18	17-18	20-25	20-25	19-23	19-23
D19S433	14-15	14-15	15-15.2	15-15.2	13-14	13-14	13-14	13-14	14-15	14-15	12.2-14	12.2-14	14-15	14-15	13-14	13-14
vWA	17-19	17-19	16-17	16-17	16-17	16-17	17-19	17-19	18-18	18-18	14-16	14-16	17-17	17-17	17-18	17-18
TPOX	8-8	8-8	8-8	8-8	8-11	8-11	9-10	9-10	8-9	8-9	8-9	8-9	9-11	9-11	8-9	8-9
D18S51	12-18	12-18	13-16	13-16	14-16	14-16	18-19	18-19	14-18	14-18	14-16	14-16	13-14	13-14	13-17	13-17
AMEL	XY	XY	XY	XY	XX	XX	XY	XY	XY	XY	XY	XY	XY	XY	XY	XY
D5S818	11-12	11-12	11-12	11-12	9-12	9-12	12-12	12-12	11-13	11-13	11-13	11-13	10-11	10-11	13-13	13-13
FGA	21-22	21-22	21-22	21-22	20-22	20-22	22-26	22-26	25-26	25-26	23-25	23-25	20-23	20-23	20-20	20-20

Test 2 samples rather that of two individuals taken at random in the population. Mitochondrial DNA sequencing revealed point heteroplasmies at the two hyper variable regions HV1 and HV2.

Patient 8 (chondrocytes), Test 1 MSC (the one left open during Test 2 MSC manipulation) and Test 2 MSC showed unique DNA profile both at nuclear and mitochondrial level (Table III A and B, respectively), in line with the results already obtained with the chondrocyte cultures.

Discussion

The term cross-contamination is used to indicate misidentification of one cell line by another, rather than contamination by microbiological organisms[27]. Recently, a role for gene profiling analysis in the authentication of human cell lines has been proposed[28,29]. Despite the still scarce literature, this is a serious and often unrecognized problem since it can compromise the reliability of scientific findings[30,31]. Such studies are mostly in the research field, concerning contamination coming from tumor cell cultures, while the possibility of mix-up between normal cells (from other types of cultures or from the same type but different patients) usually has little or no consideration. The risk of cross-contamination between cultures and the consequent loss of identity exists not only in the research field, but also for cell therapy, such as ACI, where it is obviously more relevant.

GMPs have been established by legislator precisely to guarantee the quality of pharmaceuticals products, including cell-based ones. The first step to this goal is the environment that must be built following specific and mandatory fees. A clean-room is a place where different parameters such as air filtration and ventilation conditions, temperature, relative humidity, differential pressure, number of air particles and Colony Forming Units are standardized and constantly monitored. Production operations must follow clearly defined procedures. Standardized processes, analytical method validations, defined cleaning procedure, personnel flow and training and process traceability should be developed and performed. High quality reagents and plastic ware, disposable materials, "closed systems" should be used. At every stage of processing, cells should be protected from microbial or other contaminants. Different cell-based products should not be manipulated simultaneously or consecutively in the same room, unless there is no risk of mix-up or cross-contamination.

Our facility is a public structure that was authorized for GMP Production for Advanced Cell Therapy by the Italian Drug Agency (Agenzia Italiana del Farmaco, A.I.F.A.) in 2009. We adopt all the appropriate and mandatory organizational and technical measures foreseen by the GMP to prevent cross-contamination between batches. However, our situation can be considered more subject to this risk in comparison with big enterprise's structure: small dimensions make difficult to have segregated areas and fully manual productions does not permit the use of "closed systems" (it has to be mentioned that the use of automated production lines does not solve every problem: instruments are considered a major source of cross-contamination during production). Even if we adopted other measures like the use of disposable materials and lots production by campaign (separation in time) followed by appropriate and validated cleaning, the Italian authority claimed an evidence that cross-contamination was not present in the manipulated chondrocytes cultures. We decided to apply the DNA profiling technique, a high sensitive and specific method already used in forensic caseworks for human identification[32] and also applied in hematopoietic stem cell transplantation to reveal and monitor microchimerism[33].

DNA profiling is the determination in an individual of unique genetic characteristics that make it distinguishable from of all other humans, including closely related ones. Identification is performed using validated commercial kits for specific molecular markers such as microsatellite, also known as STR loci, which represent hypervariable regions of DNA composed of tandem repeats of 4 bp core nucleotide sequences. These DNA markers display only a specificity for humans with no amplification of contaminant DNA such as fungal and bacterial sources[34]. Furthermore, in forensic field very low amounts of DNA can be more successfully typed with redesigned primers to obtain reduced sized PCR products (miniSTRs)[35]. Mitochondrial DNA, being a lineage marker since only maternally inherited, doesn't allow individual identification, but it can be used to further confirm DNA profiling results.

The statistical approach for the cell source attribution to the patient is based on the random match probability calculation equated with the probability that a match would occur by chance. This random match probability is the unlikely coincidence that an unrelated person would by chance have the same DNA profile and can be

Table II. Mitochondrial DNA HVI and HVII control regions analyses in both chondrocytes and blood samples.

Hypervariable regions	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
HV1	16234T, 16270T	16069T, 16126C, 16193T, 16217C	16169T, 16183C, 16189C, 16223T, 16278T, 16297C	16069T, 16126C	16126C, 16163G, 16186T, 16189C, 16294T	16126C, 16187T, 16189C, 16223T, 16264T, 16270T, 16276T, 16311C	Anderson	16304C
HV2	263G, 309.1C, 315.1C	73G, 150T, 152C, 263G, 295T, 315.1C	73G, 153G, 195C, 225A, 226C, 263G, 309.1C, 315.1C	73G, 185A, 228A, 263G, 295T, 315.1insC	73G, 263G, 309.1insC, 315.1insC	73G, 152C, 182T, 185T, 195C, 198T, 247A, 263G, 315.1insC, 357G	263G, 315.1insC	263G, 315.1C

HV: Hypervariable Region.

Table III. DNA profiling of three non-mixed samples and the mixed sample.

A. Nuclear DNA							
Autosomic markers	Test 1 MSC		Test 2 MSC		Test 1 and test 2 MSC	Patient 8	
	(cells)	(blood)	(cells)	(blood)	(cells)	(cells)	(blood)
D8S1179	10-13	10-13	14-14	14-14	10-13-14	14-15	14-15
D21S11	30-34.2	30-34.2	29-33.2	29-33.2	29-30-33.2-34.2	28-31	28-31
D7S820	10-11	10-11	9-12	9-12	9-10-11-12	8-8	8-8
CSF1PO	10-12	10-12	10-13	10-13	10-12-13	10-12	10-12
D3S1358	15-17	15-17	15-17	15-17	15-17	15-17	15-17
TH01	6-9	6-9	7-7	7-7	6-7-9	6-9.3	6-9.3
D13S317	12-12	12-12	11-11	11-11	11-12	8-11	8-11
D16S539	12-12	12-12	12-14	12-14	12-14	11-12	11-12
D2S1338	17-21	17-21	17-17	17-17	17-21	19-23	19-23
D19S433	14-15	14-15	13-14	13-14	13-14-15	13-14	13-14
vWA	16-16	16-16	15-16	15-16	15-16	17-18	17-18
TPOX	8-11	8-11	8-10	8-10	8-10-11	8-9	8-9
D18S51	13-18	13-18	14-16	14-16	13-14-16-18	13-17	13-17
AMEL	XX	XX	XY	XY	XY	XY	XY
D5S818	11-13	11-13	11-12	11-12	11-12-13	13-13	13-13
FGA	22-23.2	22-23.2	21-22.2	21-22.2	21-22-22.2-23.2	20-20	20-20

B. Mitochondrial DNA in both chondrocytes and blood samples				
Hypervariable region	Test 1 MSC	Test 2 MSC	Test 1 and test 2 MSC	Patient 8
HV1	16224C, 16261T, 16311C	16192T, 16256T, 16270T	16192C/T, 16224C/T, 16256C/T, 16261C/T, 16270T/C, 16311C/T	16304C
HV2	73G, 150T, 195C, 263G, 309.1C, 315.1C	73G, 263G, 309.1C, 315.1C	73G, 150T/C, 195T/C, 263G, 309.1C, 315.1C	263G, 315.1C

MCS: Mesenchymal stem cell; HV: Hypervariable Region.

determined by calculating the frequency of the observed profile in a reference population database. The estimated values for a set of 15 STRs are generally beyond one in billions or trillions to numbers that are not frequently used because they are so large[36], values of the same magnitude found in the present study.

Even if our results indicated the absence of cross-contamination during manipulation in our Cell Factory, we decided to test the strength of our system, as in general required by GMPs. We verified if the chondrocyte cultures were free of cross-contamination even when other cell types, such as MSCs, were present in the Cell Factory. For obvious ethical reasons and to preserve at the meantime the chondrocyte culture we manipulated MSCs in fully GMPs conditions and we waited until results before chondrocyte release for clinical use. The results indicated that cross-contamination was present only in the samples created by mixing on purpose the two MSCs cultures. The sample left open during manipulation of the other MSC culture (Test 1 MSC) was negative, confirming the validity of our system. These results do not mean that GMPs can be escaped, but that our process and our structure are robust enough to guarantee sterility and quality of the products, thus patient's safety. Moreover, they seem important for building a risk-based approach. In case of accidental contamination between batches there is no

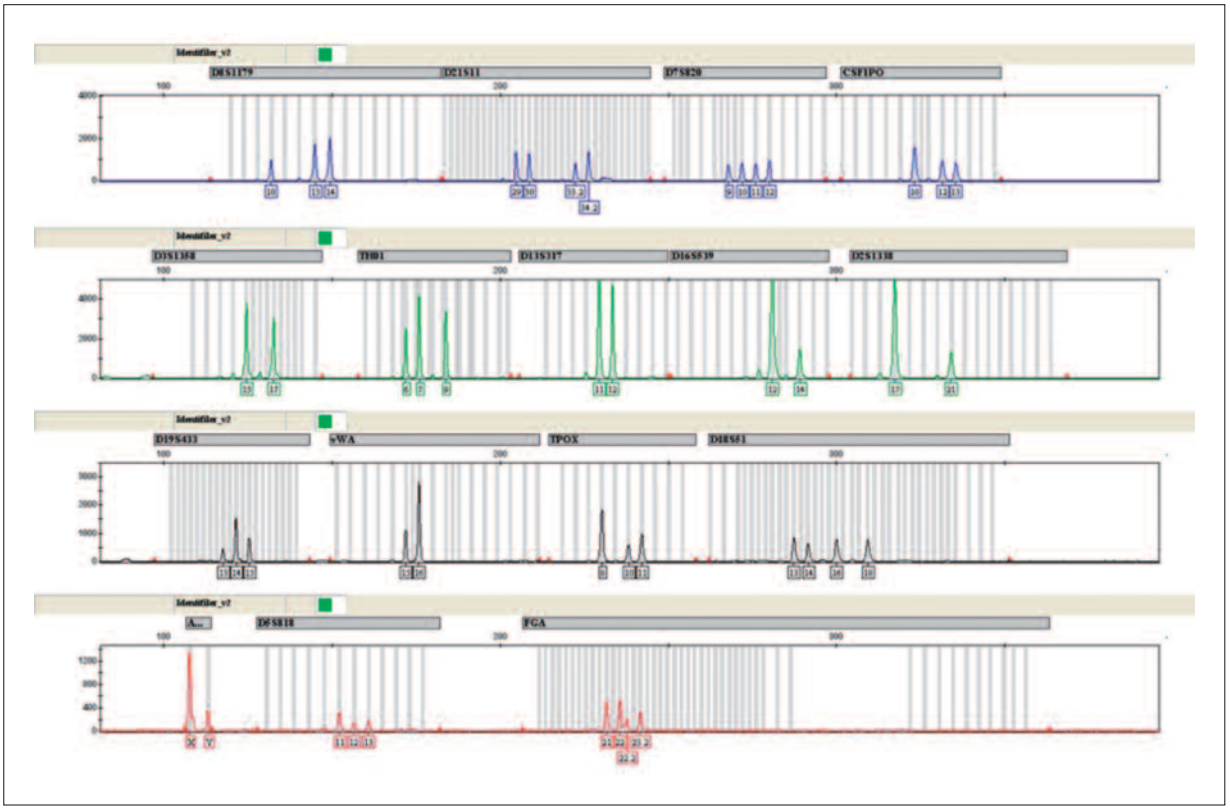

Figure 4. Electropherogram of PCR products in Test 1 & Test 2 mixed sample. STRs analysis performed on a mixed mesenchymal stem cells culture sample (Test 1 & Test 2 MSC) showing a DNA profile compatible with both components.

way to use them in patients, but they have to be eliminated. Special cleaning procedure should be applied and an investigation performed in order to find the causes, if any, and prevent or reduce this risk in the future.

This study suggests a novel application of DNA profiling in ACI procedure since it allows to detect cross-contamination between cultures and to verify the maintenance of their identity until the end of the *ex vivo* process. The technique has several advantages respect to the older ones such as chromosome banding: it is faster, giving the possibility to obtain the results before implantation, it is more precise allowing also the detection of small amounts of contaminating DNA and it needs only a few cells for the analysis.

Besides culture identification, our data showed also that the genetic profile of each patient detected in blood sample controls before manipulation remains unaltered in the cells at the end of the process. Thus it seems that, at list in the analyzed loci, our expansion process does not cause genomic alterations.

Further studies are needed in order to explore the potential of mtDNA mutations that seem to be related with rapidly diving cells in tumors[37]. Mitochondrial DNA, being a lineage marker since only maternally inherited, doesn't allow individual identification, but it can be used to further confirm DNA profiling results.

This could be important especially for stem cell-based therapies that are now used to treat different pathologies, but all the associated risks of in *vitro* or in *vivo* oncogenic transformations are still not well known or understood, especially for the long term follow-up[38].

Conclusions

Since advanced cell therapy is becoming increasingly diffuse all over the world, DNA profiling could be a method of choice ideal for quality control analysis in a Cell Factory working in cGMPs compliance.

Acknowledgements

This work was supported by the grant "Progetto di Medicina Rigenerativa" from "Regione Emilia Romagna" (Delibera di Giunta n. 2233/2008).

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