COMPREHENSIVE HIGH-RESOLUTION GENOMIC PROFILING AND CYTOGENETICS OF HUMAN CHONDROCYTE CULTURES BY GTG-BANDING, LOCUS-SPECIFIC FISH, SKY AND SNP ARRAY

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Abstract

In the development of cell-based medicinal products, it is crucial to guarantee that the application of such an advanced therapy medicinal product (ATMP) is safe for the patients. The consensus of the European regulatory authorities is: "In conclusion, on the basis of the state of art, conventional karyotyping can be considered a valuable and useful technique to analyse chromosomal stability during preclinical studies".

408 chondrocyte samples (84 monolayers and 324 spheroids) from six patients were analysed using trypsin-Giemsa staining, spectral karyotyping and fluorescence *in situ* hybridisation, to evaluate the genetic stability of an ATMP named Spherox®. Single nucleotide polymorphism (SNP) array analysis was performed on chondrocyte spheroids from five of the six donors.

Applying this combination of techniques, the genetic analyses performed revealed no significant genetic instability until passage 3 in monolayer cells and interphase cells from spheroid cultures at different time points. Clonal occurrence of polyploid metaphases and endoreduplications were identified associated with prolonged cultivation time. Also, gonosomal losses were observed in chondrocyte spheroids, with increasing passage and duration of the differentiation phase. Interestingly, in one of the donors, chromosomal aberrations that are also described in extraskeletal myxoid chondrosarcoma were identified. The SNP array analysis exhibited chromosomal aberrations in two donors and copy neutral losses of heterozygosity regions in four donors.

This study showed the necessity of combined genetic analyses at defined cultivation time points in quality studies within the field of cell therapy.

Keywords: ATMP, CBMP, cell therapy, chondrocyte, GTG-banding, spectral karyotyping, SNP array.

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List of abbreviations

ACI	autologous chondrocyte implantation
ACT	autologous chondrocyte transplantation
AG	incorporated company
ATMP	advanced therapy medicinal product
CBMP	cell-based medicinal products
CEP	centromere-specific
CGH	comparative genomic hybridisation
ChAS	chromosome analysis suite
cn	copy neutral
CNV	copy number variation
ECM	extracellular matrix
EMA	European Medicines Agency

EMC	extraskeletal myxoid chondrosarcoma
FISH	fluorescence *in situ* hybridisation
GMP	good manufacturing practice
GTG	Giemsa staining
HIV	human immunodeficiency virus
IC	interphase cells
ICRS	International Cartilage Research Society
ISCN	International system for human cytogenetic nomenclature
LOH	loss of heterozygosity
Mbp	mega base pairs
MC	metaphase cells
MSC	mesenchymal stromal cells
NER	nucleotide excision repair
P	passage
PDL	population doubling level
SKY	spectral karyotyping
SNP	single nucleotide polymorphism
UPD	uniparental disomy
WHO	World Health Organization

Introduction

With the development of CBMP, the safety of cellular components is becoming increasingly relevant. Therefore, the potential tumorigenic risk *in vivo* due to the implantation of genetically unstable or modified cells should be identified in advance with *in vitro* studies regarding the quality – in particular, the stability – of the applied cells. Various genetic analytical techniques could be considered for this approach. In general, GTG-banding, complemented, if necessary, with locus-specific FISH, SKY and SNP array or CGH, is considered by the EMA as a useful analytical method to prove genetic stability of an ATMP candidate (Barkholt *et al.*, 2013).

A variety of numerical and/or structural genetic changes, including clonal changes, may occur in CBMPs. Considering potential neoplastic changes, clonal developments are defined as cell populations if they are derived from a single progenitor cell, as also described by the current ISCN criteria (McGowan-Jordan *et al.*, 2016). If several cells display the same – or highly similar – chromosomal aberrations, they are commonly considered to have a clonal origin. Therefore, these clonal cells do not have to occur homogeneously, as subclones could also have developed from these clonal cells. A clonal origin can be assumed when gained chromosomal material (*e.g.* polyploidy) or structural rearrangements are present in at least two different cells (McGowan-Jordan *et al.*, 2016). Ideally, these aberrations are discovered in distinct primary cultivation vessels.

Polyploidy is considered to be a disturbed distribution of a complete chromosome set, resulting in a more than diploid chromosome set in the corresponding cells. Healthy cells with a high transcription rate, for example liver cells, may sometimes show polyploidy. However, polyploidy is also found in tumour tissue (Andriani *et al.*, 2016). Endoreduplication is the replication of the chromosomes without chromatid separation or cytokinesis (McGowan-Jordan *et al.*, 2016). Higher frequencies of cells with endoreduplication are observed in human trophoblast cells and tumour cells (Therman, 1995).

ATMPs are medicines for human use based on genes or cells. A few advanced therapies, including two tissue-engineered products for articular cartilage repair, have successfully passed the centralised European marketing authorisation procedures (ChondroCelect® and MACI: matrix-applied characterised autologous cultured chondrocytes). ACT is a surgical treatment modality that repairs focal lesions and ideally regenerates the articular cartilage in the knee joint. ACT may provide pain relief, increase knee function and potentially prevent partial or total knee joint replacement surgery due to secondary osteoarthritis (Hunziker *et al.*, 2015). Independent of the generation of the ACT product, this cell-based articular cartilage repair procedure takes place in three stages. In a first stage, usually 200-300 mg of cartilage are sampled arthroscopically from an area of decreased weight-bearing, either from the intercondylar notch or the superior ridge of the medial or lateral femoral condyle of the patient. In a second stage, the extracellular matrix of the biopsy is removed enzymatically and the chondrocytes are isolated. These cells are grown *in vitro* in a GMP laboratory for up to 6 weeks in two-dimensional (2D) monolayer cultures and/or in various biomaterials in three-dimensions (3D). In a third stage, the patient undergoes a second surgical treatment, in which the respective (matrix-coupled) ACT products are applied to the debrided area of the lesion. Chondrocytes in the graft should adapt themselves to their new environment by forming new hyaline-like cartilage in the mid- and long-term (Brittberg, 2012). Spherox® (co.don® AG, Teltow, Germany), formerly known as Chondrosphere®, is an ACT-ATMP consisting of chondrocytes that are initially propagated in monolayer cultures until P 3 and, subsequently, transformed into spheroids containing chondrocytes and an endogenously produced extracellular matrix, with no use of an exogenous scaffold (Anderer and Libera, 2002). To obtain an EU-wide marketing authorisation, in 2013, Barkholt *et al.* performed further investigations – particularly regarding safety, including cellular stability – on Spherox®, to rule out any side effect and risk of the treatment method, as requested by the EMA.

Since osteoarthritis is mainly a disease of elderly patients, there might be a theoretical risk of age-, disease- and/or cultivation-dependent occurrence of chromosomal aberrations. After prolonged cultivation of cells, it is generally possible that numerical or structural chromosomal aberrations, mainly clonal changes, occur as cultivation-related events. Such genetic instability is shown for many different cell types after a prolonged cultivation (Gardner *et al.*, 2012). For those reasons, it is important

that only cells that are cultivated for a short time, with few PDLs, thereby remaining close to the *in vivo* situation, are used for ATMPs. In addition, this may also require comprehensive safety analyses of cells to be transplanted to rule out malignant changes or chromosomal aberrations (Boyle *et al.*, 2006). So far, there are no indications that cells with only a limited number of doublings outside the human body would cause tumorigenesis (Barkholt *et al.*, 2013). Comprehensive genetic data in cell therapy are limited but necessary to provide more detailed information about genetic stability. As previously demonstrated, the detection rate of chromosomal aberrations can be optimised by combining complementary cytogenetic and molecular techniques for tumour cell analyses (Xu *et al.*, 2015; Holland *et al.*, 2012).

Analyses of human chondrocytes of *post-mortem* donors and donors scheduled for scaffold-assisted ACT, using GTG-banding and FISH, are first described by Trimborn *et al.* (2012). In contrast to *post-mortem* donors, no clonal chromosomal aberrations are observed in the ACT donors. *Post-mortem* chondrocytes show up to 26.7 % numerical chromosomal aberrations, such as autosomal gains of chromosomes 5, 7 and 8 and gonosomal gains and losses, while chondrocytes of donors scheduled for ACT show up to 1.3 % numerical chromosomal aberrations. Single structural chromosomal aberrations in living donors scheduled for ACT include inversions, translocations, losses/gains of gonosomes, gains of chromosomes 5, 7 and 8 and marker chromosomes (Trimborn *et al.*, 2012). Stumm *et al.* (2012), in a study on genomic chondrocyte culture profiling by array-CGH and FISH, show autosomal stability but also variable loss of Y chromosome in all male samples in monolayer. Although losses of Y chromosomes may represent age-related processes, these data suggest some caution toward applying cultivation processes to chondrocytes from elderly patients for cell-based therapy. Williams *et al.* (2010), in a study on cytogenetic analyses of a progenitor cell sub-population in human articular cartilage, demonstrate that cartilage progenitors with a PDL of 31.3 are to the largest extent normal, but with some chromosomal aberrations that are likely linked to the high PDL and cultivation conditions. This was particularly apparent in the non-recurrent deletion of the short arm of the chromosome 20.

Combining different genetic techniques more precisely defines potential genomic instabilities and the resulting tumorigenic risks of ATMP products, which should be investigated during cell quality/

Table 1. Properties of biopsy material used for production of Spherox® and for subsequent (molecular) cytogenetic analyses.

Patient	Sample material	Gender	Age (years)	Diagnosis	Therapy
1	Femoral condyle, tibia plateau	Female	79	Gonarthrosis	Endoprothesis
2	Femoral condyle, tibia plateau	Male	73	Primary gonarthrosis	Endoprothesis
3	Femoral condyle, tibia plateau	Male	55	Primary gonarthrosis	Endoprothesis
4	Femoral condyle	Female	66	Gonarthrosis	Endoprothesis
5	Femoral condyle, tibia plateau	Female	50	Gonarthrosis	Endoprothesis
6	Femoral condyle	Female	69	Gonarthrosis	Endoprothesis

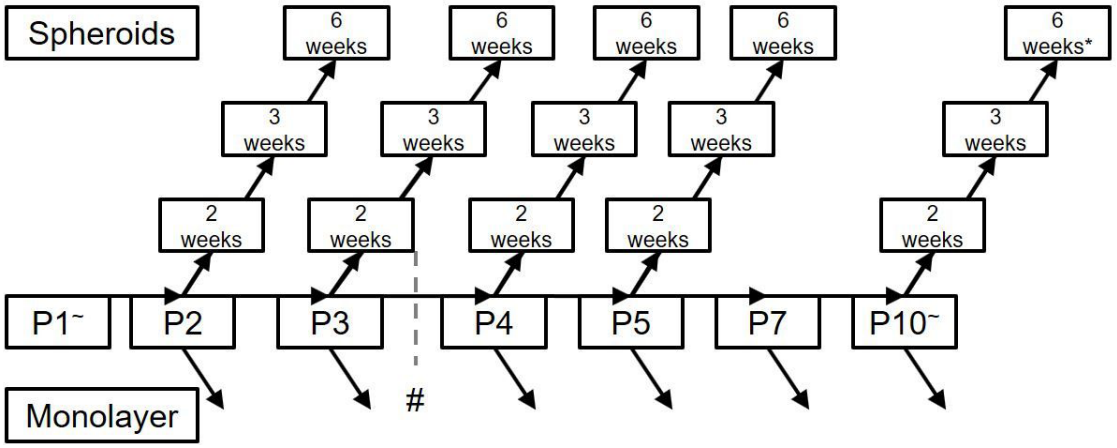

Fig. 1. Monolayer cultures and spheroids differentiated for a variable time were used. Cells at P 4 and higher P (#) are not utilised for the routine GMP production process of Spherox® by co.don® AG. The differentiation phase after P 10 was 7 weeks in patient 1 and 5 weeks in patient 6, instead of the standard 6 weeks (*). No data were available from monolayer culture of patient 1 in P 1 and P 10 (~).

Table 2. Cumulative PDL and P for patient 2 to 6. For patient 1, data were not recorded. * The GMP production of Spherox ® was only used until passage 3.

Passage	P 1	P 2	P 3*	P 4	P 5	P 6	P	P 8	P 9	P 10
Patient 1	n.a.									
Patient 2	1.94	3.78	6.14	8.17	10.02	12.49	14.24	15.88	17.53	18.67
Patient 3	1.84	3.70	6.05	8.38	10.41	11.83	13.91	15.80	17.70	20.14
Patient 4	6.19	8.27	10.17	12.01	13.86	15.03	15.40	16.03	16.64	16.99
Patient 5	2.35	5.55	8.07	10.23	12.06	13.85	14.88	16.39	17.82	19.25
Patient 6	2.58	5.68	8.08	10.07	12.24	13.50	14.86	15.81	17.14	18.30
Mean	2.98	5.40	7.70	9.77	11.72	13.34	14.66	15.98	17.37	18.67
SD	1.82	1.86	1.70	1.57	1.55	1.24	0.59	0.25	0.48	1.17

stability studies based on the envisaged GMP production process. Therefore, a comprehensive genetic analysis of multiple 2D and 3D cultivation stages of six chondrocyte donors using GTG-banding, SKY, FISH and genome wide SNP array (n = 5) was performed. The aim of the present study was to evaluate the genetic stability of these chondrocyte cultures that were processed in close analogy to the current GMP production process of Spherox®, which is intended for application in ACT.

Materials and Methods

Patient material

The chondrocyte monolayer cultures (84 samples) and the spheroids (324 samples) used for the analyses of genetic stability or chromosomal aberrations were derived from biopsies of residual joint samples of 6 patients, who underwent total knee joint replacement surgery, with written informed consent of the patient and ethics permission [AS 126(bB)/2017] for use of the material for research purposes only. Processed cartilage was taken from undamaged areas of elderly patients with degenerated osteoarthritic cartilage, which does not correspond to the healthy cartilage normally used during the manufacturing process of ACTs. The harvested biopsy material, patient characteristics, diagnosis and treatment are summarised in Table 1. With regard to the age of the patients, biopsy material does not comply with the specification for production of the Spherox® in the context of the approved production process of the co.don® AG.

Chondrocyte isolation and 2D and 3D cultivation of chondrocyte samples

Cells were cultured at co.don® AG under GMP-analogue conditions, including microbiological monitoring. At several time points during monolayer expansion and 3D cultivation, samples were collected for genetic stability analyses (Fig. 1). Chondrocyte isolation from articular cartilage, their monolayer expansion and the generation of spheroids were performed as described previously (Anderer and Libera, 2002).

In detail, cartilage tissue was removed from the residing knee joint sample and minced. The chondrocytes were released from the cartilage tissue by enzymatic digestion using collagenase/protease solution at 37 °C overnight. Cells were cultured in cell culture medium (Biochrom GmbH, now part of Merck), supplemented with 10 % human pool serum (pooled from about 15 donors), no growth factors, antibiotics or other supplements were added. The human serum was derived from the manufacturing department and each batch was tested for the presence of several viruses: HIV, Hepatitis B and C and Syphilis. Cell culture medium was refreshed twice a week. The cells were cultured to 100 % confluency. After reaching confluence, the cells were passaged by removing the cell culture medium followed by careful rinsing with phosphate-buffered saline (PBS) and by enzymatic release of the cells by adding papain for 5 min at 37 °C. Monolayer cells were cultivated until P 10. To produce spheroids, 2×10^5 cells of the respective monolayer passage (P 2, P 3, P 4, P 5 and P 10) were seeded onto coated non-adherent 96-well plates and cell culture medium supplemented with 10 % autologous serum (co.don® AG; the donors of the serum were patients who received a transplant from the company) was added. Under these conditions, the chondrocytes aggregated into spheroids. Medium was changed twice a week. The spheroids were cultured at 37 °C in a humidified atmosphere and 5 % CO_2 for 2, 3 and 6 weeks.

The cumulated PDLs per passage are given in Table 2.

Table 3a. Overview of samples for GTG and SKY analyses of chondrocyte monolayers.

Passage	P 1	P 2	P 3	P 4	P 5	P 7	P 10
Patient 1	n.a.	x	x	x	x	x	n.a.
Patient 2	x	x	x	x	x	x	x
Patient 3	x	x	x	x	x	x	x
Patient 4	x	x	x	x	x	x	x
Patient 5	x	x	x	x	x	x	x
Patient 6	x	x	x	x	x	x	x

Table 3b. Overview of samples for CEP 7/10 and CEP X/Y FISH or subtelomere FISH analyses of chondrocyte spheroid cultures.

Passage	**P 2**			**P 3**			**P 4**			**P 5**			**P 10**		
Weeks	**2**	**3**	**6**	**2**	**3**	**6**	**2**	**3**	**6**	**2**	**3**	**6**	**2**	**3**	**6**
Patient 1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	7 weeks
Patient 2	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Patient 3	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Patient 4	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Patient 5	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Patient 6	x	x	x	x	x	x	x	x	x	x	x	x	x	x	5 weeks

Table 3c. Overview of samples for SNP array analysis of chondrocyte spheroid cultures.

Passage	**P 2**			**P 3**			**P 4**			**P 5**			**P 10**		
Weeks	**2**	**3**	**6**	**2**	**3**	**6**	**2**	**3**	**6**	**2**	**3**	**6**	**2**	**3**	**6**
Patient 1			x						x						7 weeks
Patient 2			x						x						x
Patient 3			x						x						x
Patient 4			x						x						x
Patient 5			x						x						x
Patient 6			x						x						5 weeks

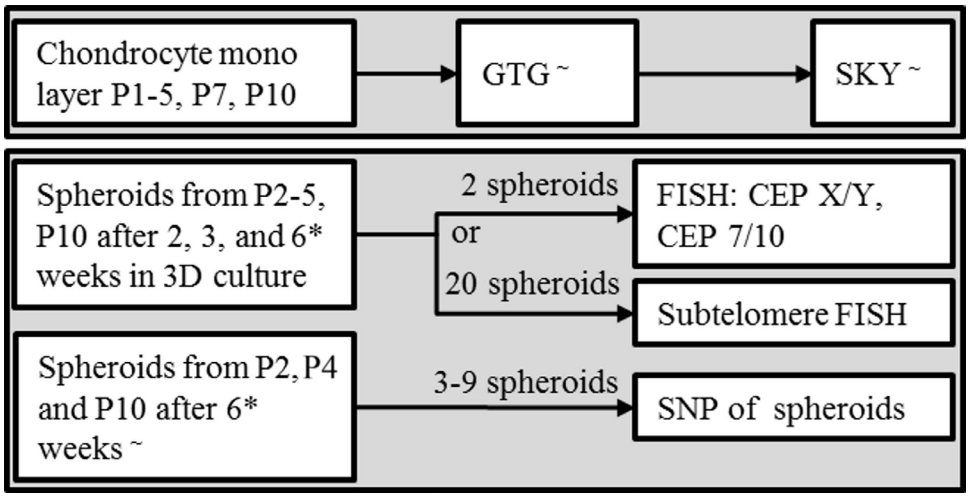

Fig. 2. Overview of the applied cytogenetic and molecular cytogenetic analyses. The differentiation phase after P 10 was 7 weeks in patient 1 and 5 weeks in patient 6 instead of the standard 6 weeks (*). No data were available from monolayer culture of patient 1 in P 1 and P 10 (~).

Chromosome preparation, GTG-banding, SKY and FISH analyses

According to the study design (Fig. 1, Tables 3**a**,**b**), chromosome preparation was performed on chondrocyte monolayer cells of multiple passages using standard cytogenetic techniques (colcemid treatment, hypotonic treatment and methanol/acetic acid fixation), GTG and SKY, according to manufacturer's instructions (ASI, Edingen-Neckarhausen, Baden-Württemberg, Germany) for karyotype analyses on chromosome spreads. The chondrocyte monolayer cultures were analysed by GTG-banding of 6-50 (mainly 25) MC per sample from two independent culture flasks. Additionally, 5-23 (mainly 10) metaphases were analysed by SKY (excluding patient 4, P 10 and patient 6, P 7) from one culture flask. For SKY analyses SKY-Paint DNA-H10 probes (ASI, Edingen-Neckarhausen, Baden-Württemberg, Germany) were used.

Chondrocyte spheroid samples, generated from different passages and three durations of the terminal differentiation phase of 2, 3 and 6 weeks, were analysed. If two spheroids were available, FISH analyses using the centromeric probes CEP X/Y

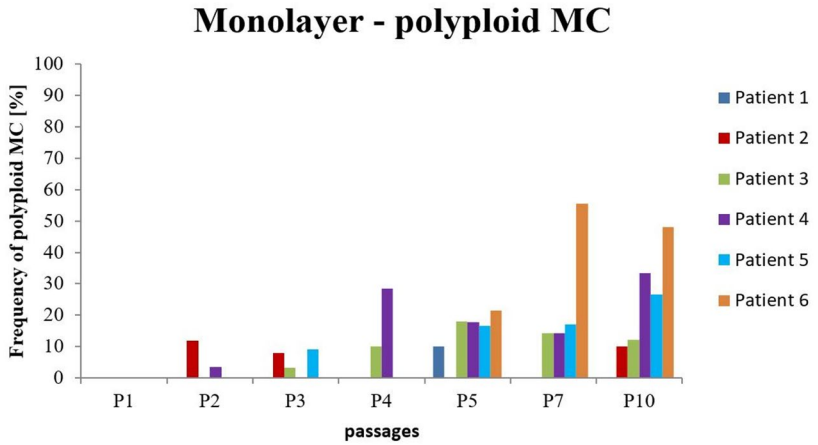

Fig. 3. Relative occurrence of polyploid MC with increasing cultivation time in monolayer cultures of 6 patients. Patterned columns indicate the frequency of polyploid MC from one culture batch, whereby these aberrations could not be confirmed in the parallel second culture batch. Completely stained columns depict the frequency of the sum of polyploid MC from both culture batches. Analyses were performed using GTG-banding according to ISCN guidelines (McGowan-Jordan *et al.*, 2016; Shaffer, 2013). No data were available from monolayer culture of patient 1 in P 1 and P 10.

(spectrum orange/green; Abbott, Wiesbaden, Hesse, Germany) and CEP 7/10 (spectrum green/orange; Abbott) were performed.

Subtelomere FISH (ToTelVysion Multi-colour DNA probe mixtures; Abbott) analysis was performed if 20 spheroids were available (Fig. 2). In total, 18,511 IC were analysed using FISH CEP X/Y (10,045 IC) and CEP 7/10 (8,466 IC) and 5,424 IC were analysed using subtelomere FISH. To exclude artefacts in the detection of gonosome aberration, a gonosomal chromosome loss was counted only if the other gonosome was detected.

DNA isolation and molecular karyotyping using SNP array

Depending on the availability, 3-9 chondrocyte spheroids of the 6 weeks differentiation group from 5 out of 6 patients (except patient 4) of P 2, P 4 and P 10 (Table 3**c**) were collected and subjected to genome-wide CNV analysis and assessment of copy number neutral loss of heterozygosity (cn-LOH) of chromosomal regions using SNP array (Affymetrix CytoScan® 750 Array; ATLAS Biolabs, Berlin, Berlin, Germany). Genomic DNA was extracted from chondrocyte spheroid samples according to the protocol "Isolation of total DNA from tissues" from the QIAamp® DNA Investigator Kit (QIAGEN). DNA quality was checked by agarose gel electrophoresis. For SNP array analyses, the Affymetrix Chromosome Analysis Suite (ChAS 2.0.0.195) *vs.* reference data file Affymetrix CytoScan750_Array.na32.3.v. using the copy number and LOH workflows with standard settings was used.

Results

The genetic analyses performed revealed no significant genetic instability in 3 monolayer culture passages, as well as IC from spheroid cultures differentiated for 2, 3 and 6 weeks within P 1-3.

Cytogenetics

Monolayer

Chondrocyte monolayer cultures from 6 patients in different passages, including P 1 (except patient 1), P 2 to P 5, P 7 and P 10 (except patient 1), were analysed cytogenetically using GTG-banding. In total, 1,233 MC were analysed by GTG-banding, of which 84 MC (6.8 %) showed clonal aberrations. Additionally, 393 MC were analysed by SKY (excluding patient 4, P 10 and patient 6, P 7). Using SKY on monolayer cultures, no additional recurrent chromosomal aberrations were identified.

All monolayer samples showed predominantly normal karyotypes, as seen by GTG-banding and SKY (patient 1: 46,XX; patient 2: 46,XY; patient 3: 46,XY; patient 4: 46,XX; patient 5: 46,XX; patient 6: 46,XX), with no indication of clonal aberrations until P 3. In addition to these predominantly inconspicuous cell populations, cell populations with polyploid MC from two cultures were present at a lower frequency, as measured by GTG-banding. Clonal occurrence of polyploid MC (≥ 10 %) was found in patients 3 and 4 from P 4 and in patients 5 and 6 from P 5 onwards. Furthermore, clonal occurrence of polyploid MC, larger than 10 %, was detected in the remaining three patient samples, with patient 1 at P 5 and patient 2 at P 10 (Fig. 3). In summary, a tendency towards the occurrence of polyploid chondrocyte cell populations over prolonged monolayer cultivation accompanied by higher passage levels was observed.

Monolayer cells of patient 2 and 3 (from two cultures) showed an increase in endoreduplications with increasing cultivation time from P 4 onwards (Fig. 4). Moreover, two endoreduplications of one cultivation batch were found in P 5 monolayer cells of patient 5. Interestingly, in monolayer cells of

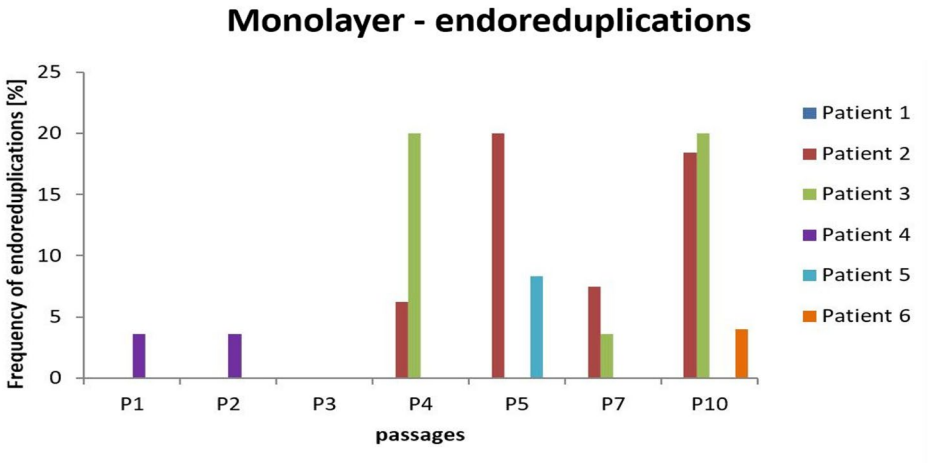

Fig. 4. Relative occurrence of endoreduplications with increasing cultivation time in monolayer cultures of six patients. Patterned columns indicate the frequency of endoreduplications from one culture batch, whereby these aberrations could not be confirmed in the parallel second culture batch. Completely stained columns depict the frequency of the sum of endoreduplications from both culture batches. In patient 1, no endoreduplications were detected throughout the entire monolayer cultures. Analyses were performed using GTG-banding according to ISCN guidelines (McGowan-Jordan *et al.*, 2016; Shaffer, 2013). No data were available from monolayer culture of patient 1 in P 1 and P 10.

Table 4. Numerical and structural chromosomal aberrations detected by GTG and/or SKY in monolayers of patient 2.

Passage	Aberration	Aberrant metaphases	Total metaphases
1	Trisomy of the chromosome 7	1	44
	Deletion of the chromosome 10: del(10)(q25)	1	44
2	Polyploid metaphases	3	35
	Trisomy of the chromosome 7	1	35
	Y chromosomal loss	2	35
3	Trisomy of chromosomes 7 and 12	1	39
4	Trisomy of the chromosome 7	1	42
	Y chromosomal loss	1	42
	Deletion of the chromosome 16: del(16)(q12)	1	42
5	Trisomy of the chromosomes 5,6,7,8,9, and 10	1	30
	Translocation of the chromosome 9 [der(9)t(9;?)(?;?)]	1	30
	Translocation of the chromosomes 7,9 and 12 [t(7;9;12)(q11.2;q34;q24)]	1	30
7	Y chromosomal loss	4	40
	Monosomy of the chromosome 22	3	40
	Trisomy of the chromosome 7	2	40
	Translocation [t(10;11)(q10;q10)]	1	40
	Marker chromosome	1	40
10	Polyploid metaphases	5	65
	Trisomy of the chromosome 1	1	65
	Trisomy of the chromosome 7	4	65
	Trisomy of the chromosome 8	3	65
	Trisomy of the chromosome 13	1	65
	Tetrasomy of the chromosome 7	1	65
	Deletion of the X chromosome [del(X)(q?)]	1	65
	Translocation of the chromosomes 22 and 21 [t(22;21)(?;?)]	1	65

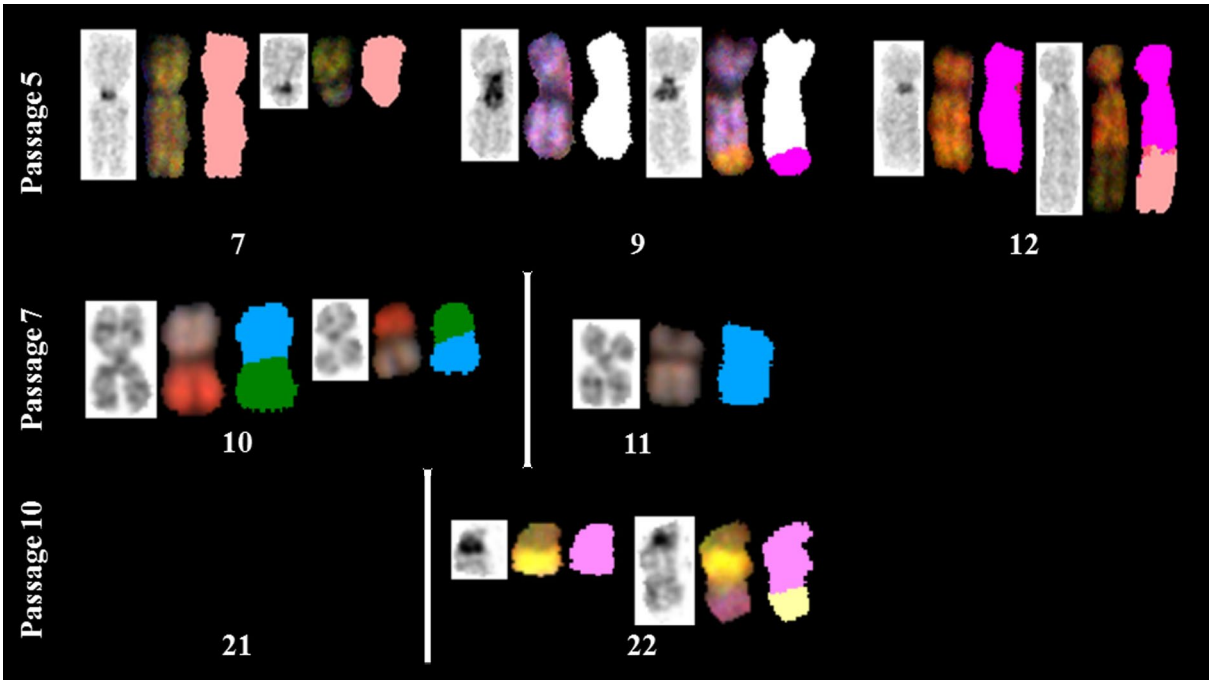

Fig. 5. Examples of detected structural aberrations using SKY technique. t(7;9;12)(q11.2;q34;q24), P 5; chromosomes 10 and 11 [t(10;11)(q10; q10)], P 7; chromosomes 21 and 22 [t(22;21)(?;?)], P 10 were found in different MC monolayer cultures of patient 2.

patient 2, indications of chromosomal instability were identified using both GTG-banding and SKY. As summarised in Table 4, numerical and structural aberrations were detected as single events from P 1 to P 5, but in P 7 and P 10, the following numerical chromosomal aberrations were found as clonal events: Y chromosome loss, monosomy 22, trisomy 7 and 8 and polyploid MC.

The SKY technique revealed the following structural chromosomal aberrations as single events: del(10)(q25) in P 1, del(16)(q12) in P 4, [der(9)t(9;?)(?:?)] and [t(7;9;12)(q11.2;q34;q24)] in P 5, t(10;11)(q10;10) and one marker chromosome in P 7, del(X)(q?) and t(22;21)(?;?) in P 10 (Fig. 5), which is partially described for EMC by Sjögren *et al.* (2003). Gonosomal losses were detected in patient 2 at P 2 (2/10 SKY-metaphases, from one cultivation batch) and at P 7 (4/25 GTG-metaphases, from one cultivation batch).

Spheroids

In parallel, 18,511 IC from 3D spheroid cultures were analysed using FISH with centromere probes CEP X/Y and CEP 7/10. In total, an average of 124 IC (20-356 IC; using CEP X/Y) and 105 IC (16-362 IC; using CEP 7/10) were detected (Table 5). 10,045 IC were available for FISH probe CEP X/Y. 167/10,045 IC (1.67 %) showed aberrant single signal patterns ≥ 10 % (51 IC with loss of one signal for the chromosome X, 116 IC with loss of the signal for chromosome Y). X chromosomal losses of more than 10 % in three spheroid differentiation phases were detected for the first time in P 10: 2 weeks; P 10: 3 weeks; P 10: 5 weeks, only in patient 6. In patient 2, significant Y chromosomal losses in spheroid cultures from P 4 onwards were identified, which increased over prolonged 3D cultivation [P 4: 6 weeks; P 5: 3 and 6 weeks; P 10: 2 (subteleromeric FISH), 3 and 6 weeks; Table 6**a**]. 8,466 IC were available for FISH probe CEP 7/10. 63/8,466 IC (0.74 %) showed aberrant single signal patterns ≥ 10 % [61 IC with three signals for chromosomes 7 and 10, respectively (patient 2, P 10: 3 weeks; patient 6, P 10: 2, 3 and 7 weeks, possible suggestions for polyploidy), and 2 IC with only one signal for chromosome 7 and two signals for chromosome 10 (patient 1, P 5: 2 weeks; 2/16 IC: limited appreciation)] (Table 6**b**). When 20 spheroids were available, subtelomere FISH analysis was performed instead of CEP FISH analysis (Fig. 2). The analysis of 5,424 IC revealed that 95 IC (1.75 %) were involved in aberrant single signal patterns ≥ 10 % [three signals for 4p, 7p/q, 9p, 14q, 20p; four signals for 16p/q, 19p/q; one signal for 13q, 17q, 18p, gonosomes (Table 6**c**)].

SNP array

SNP array analysis did not detect any CNV in all of the chondrocyte spheroids produced from P 2 and P 4 monolayer cells for the five analysed patients (chondrocyte spheroid sample from patient 4 was not analysed by SNP array) (Table 7). Furthermore, no abnormality was detectable in any spheroid group produced from P 10 monolayer cells of patients 1, 3 and 5. However, CNVs were identified in 5 (patient 6) and 6 (patient 2) week-old chondrocyte spheroids generated from P 10 monolayer cells of two patients. While cells of patient 2 showed gain of complete chromosomes 7 and 10, cells of patient 6 showed gain of complete chromosome 8 accompanied by clonal

Table 5. Mean values of analysed IC (FISH) generated from various P and differentiation phases. FISH using CEP X/Y and CEP 7/10.

Patient	Mean value of analysed interphase cells [range values]	
	CEP X/Y	CEP 7/10
1	101 [42-184]	68 [16-172]
2	188 [86-356]	157 [78-362]
3	162 [54-239]	134 [51-225]
4	102 [20-137]	88 [24-131]
5	86 [54-116]	83 [41-124]
6	104 [60-177]	97 [58-137]

loss of one complete X chromosome. Additionally, an indication of gain of chromosomal material was detectable for chromosomes 7 and 10 of P 10 in the 5 week-old spheroids of patient 6 (Table 7).

cn-LOH regions were detected in all spheroid variants of four out of five analysed donors, independently of the underlying monolayer passage levels (Table 8). The largest cn-LOH region with a size of > 7 Mbp was revealed in patient 5 at P 2, P 4 and P 10 at the chromosomal region Xq13.1-q21.1. A cn-LOH region of > 5 Mbp was detected at the chromosomal region 11p11.2-11.12 and 11q14.1 of patient 1 at P 2, P 4 and P 10 (Fig. 6). Germline material, such as peripheral blood of patients, was not available. SNP array analyses of patient 2 showed two cn-LOH regions (> 3 Mbp), one localised at 11p11.2-p11.12 and the other at 15q15.1-q21.1. In patient 6, SNP array analyses showed a cn-LOH region (> 3 Mbp) at 16p11.2-p11.1 (Table 8).

Discussion

Genetic stability is important in cell-based therapies due to a variety of (clonal) numerical and/or structural aberrations that may occur in genetically instable or altered cells. Therefore, a not previously described comprehensive genetic analysis of six chondrocyte donors was performed [using GTG-banding, SKY, FISH and genome wide SNP array ($n = 5$)] to evaluate the genetic stability of these chondrocyte cultures, processed in close analogy to the current GMP production of Spherox® – which is intended as ACT treatment.

Autosomal chromosomes

Cytogenetic and molecular profiling of 2D and 3D chondrocyte cultures by GTG-banding, SKY, (centromeric or subtelomere) FISH and SNP array did not give clear indications of chromosomal instability during cultivation up to P 3. With prolonged cultivation times, chromosomal aberrations occurred, partially also clonally present (*e.g.* polyploidy). According to the manufacturer of the ATMP-candidate Spherox®, co.don® AG, this prolonged 2D cultivation time is not included in the production process of Spherox®. Preferably, chondrocytes of P 2 are used to generate spheroids.

Cytogenetic and/or molecular cytogenetic publications on chondrocyte cultures are limited. Trimborn *et al.* (2012), analysing seven 12-48 h *post-mortem* human donors (22 to 62 year-old), report high frequency of aneuploidies (up to 26.7 %) by GTG-banding and gains and losses of gonosomes and gains of chromosomes 5, 7 and 8 (up to 10 %). These aberrations are already present in P 1, suggesting the occurrence in the harvested full-depth cartilage biopsy. However, five samples from younger patients (22 to 40 year-old and scheduled for ACI), without signs of osteoarthritic degeneration or traumatic injury, show mainly unremarkable karyotypes. Only single events of numerical and structural chromosomal aberrations are described. Williams *et al.* (2010), in a study on expanded full-depth normal human chondrocytes isolated from three patients undergoing knee surgery, show unremarkable karyotypes. Fuente *et al.* (2004) measure no effect on chromosomal stability after prolonged expansion time of expanded dedifferentiated adult human cartilage chondrocytes, karyotyping 3 cell samples from 25 donors (16 to 54 year-old) from 10 and 20

Table 6a. Single aberrant signal pattern during differentiation phase in spheroid cultures using CEP X/Y. Aberrant signal patterns ≥ 10 % are presented.

Patient	Differentiation phase	Aberrant IC	Analysed IC	Frequency of aberrant signal	Aberrant signal pattern
2	P 4, 6 weeks	15	135	11.11 %	X: x1; Y: x0
2	P 5, 3 weeks	28	235	11.91 %	X: x1; Y: x0
2	P 5, 6 weeks	16	110	14.55 %	X: x1; Y: x0
2	P 10, 3 weeks	41	187	21.93 %	X: x1; Y: x0
2	P10, 6 weeks	16	135	11.85 %	X: x1; Y: x0
6	P 10, 2 weeks	30	77	38.96 %	X: x1
6	P 10, 3 weeks	11	78	14.10 %	X: x1
6	P 10, 7 weeks	10	88	11.36 %	X: x1

Table 6b. Single aberrant signal pattern during differentiation phase in spheroid cultures using CEP 7/10. Aberrant signal patterns ≥ 10 % are presented.

Patient	Differentiation phase	Aberrant IC	Analysed IC	Frequency of aberrant signal	Aberrant signal pattern
1	P 5, 2 weeks	2	16	12.50 %	7: x1; 10: x2
2	P 10, 3 weeks	13	116	11.21 %	7: x3; 10: x3
6	P 10, 2 weeks	22	137	16.06 %	7: x3; 10: x3
6	P 10, 3 weeks	17	99	17.17 %	7: x3; 10: x3
6	P 10, 7 weeks	9	58	15.52 %	7: x3; 10: x3

Table 6c. Single aberrant signal pattern during differentiation phase in spheroid cultures using subtelomere FISH. Aberrant signal patterns ≥ 10 % are presented.

Patient	Differentiation phase	Aberrant IC	Analysed IC	Frequency of aberrant signal	Aberrant signal pattern
2	P 3, 2 weeks	5	50	10.00 %	7p/q: x2; 14q: x3
2	P 10, 2 weeks	6	57	10.53 %	1p/q: x2; Xp/Yp: x1
2	P 10, 2 weeks	6	51	11.76 %	4p: x3; 4q: x2; 21q: x2
2	P 10, 2 weeks	6	53	11.32 %	6p/q: x2; 13q: x1
2	P 10, 2 weeks	8	51	15.69 %	7p/7q: x3; 14q: x2
2	P 10, 2 weeks	7	54	12.96 %	9p: x3; 9q: x2; 17q: x2
2	P 10, 2 weeks	6	50	12.00 %	12p: x1; 12q: x2; 18q: x2
2	P 10, 2 weeks	6	53	11.32 %	16p/q: x4
2	P 10, 2 weeks	8	50	16.00 %	20p: x3; 20q: x2
3	P 10, 2 weeks	8	50	16.00 %	2p/q: x2; Xq/Yq: x1
3	P 10, 2 weeks	6	51	11.76 %	6p/q: x2; 13q: x1
3	P 10, 2 weeks	7	50	14.00 %	9p/q: x2, 17q: x1
3	P 10, 2 weeks	6	54	11.11 %	11p/q: x2, 18p: x1
3	P 10, 2 weeks	5	50	10.00 %	19p: x4, 19q: x2
3	P 10, 2 weeks	5	50	10.00 %	19p/q: x4

PDLs. Finally, Stumm *et al.* (2012), using human chondrocytes (articular cartilage tissue from patients with total knee replacement surgery and healthy cartilage from human organ donors) reveal autosomal stability, but losses of Y chromosomes.

Interestingly, in the current study, for patient 2, numeric and structural aberrations were detected as single events in all early passage levels from P 1 to P 5. In P 7 and P 10, the following chromosomal changes were present as clonal events: loss of Y chromosome, monosomy 22, trisomy 7 and 8 and polyploid MC. Further chromosomal aberrations, such as trisomy of chromosome 12 and involvement in translocations of chromosomes 7 and 9 (described for EMC) were also observed. Based on the presence of (initial) single aberrations already in P 1, it cannot be excluded that the harvested cells (from cartilage biopsy of an osteoarthritic knee) already carried these chromosomal aberrations. Thus, future stability studies should also investigate the properties of the starting material or, more specifically, the freshly isolated primary chondrocytes in addition to peripheral lymphocytes of whole blood samples, to discriminate whether the observed genetic alterations are due to the cultivation process or are inherent to the material itself. Usually, such autosomal chromosomal aberrations can be found in cartilaginous tumours. Cytogenetics in 21 benign chondromatous tumours show chromosomal aberration on chromosomes 5, 6, 7 and 12. The chromosomal regions 6q13, 12q13 and 17p13 are detectable in both malignant and benign cartilaginous tumours (Buddingh *et al.*, 2003). Noteworthy are the following chromosomal aberrations that were detected in different differentiation phases of monolayer cells of donor 2: deletion of chromosome 6, trisomy of chromosomes 1, 7 and 8, translocations der(9)t(9;?)(?;?) and t(7;9;12)(q11.2;q34;q24). Similar chromosomal aberrations are described in connection with EMC [*e.g.* t(9;15)(q22;q21), t(9;17)(q22;q11-12),

Table 7. Copy number variants and described cancer genes of patients dependent on increasing cultivation time. n.a.: not analysed, -: none, confirmation*: analyses of interphase cells using FISH with increasing cultivation time: P 2, P 4 and P 10 after 6 weeks of the differentiation phase. Donor 1, P 10, 7 weeks. Donor 6, P 10, 5 weeks.

Patient	Passage/Aberration (size)			Cancer genes	Confirmation*
	P 2	P 4	P 10		FISH
1	-	-	-	-	Yes
2	-	-	Chromosome 7: gain 159,076 Mbp	ABCB1, ABCB5, ACHE, ACTB, AKAP9, ANLN, ASNS, AUTS2, BCL7B, BRAF, CAV1, CLDN4, COL1A2, CREB3L2, CUX1, CYP2W1, DDC, DLX5, DLX6, DMTF1, EPHA1, EPHB6, ETV1, EZH2, FSCN1, GPER1, GPNMB, GRB10, HBP1, HGF, HIP1, HIPK2, HOXA11, HOXA9, HSPB1, HUS1, IKZF1, IL6, ING3, JAZF1, LIMK1, MET, MIR106B, MIR183, MIR196B, MNX1, MUC17, NAMPT, NPY, NRCAM, NRF1, PEG10, POU6F2, PTN, SEPT7, SFRP4, SHH, SPAM1, STEAP1, STEAP2, TAC1, TRIM24, TRPV6, TWIST1	Yes
	-	-	Chromosome 10: gain 135,327 Mbp	ABI1, ADAM12, ADD3, AFAP1L2, AIFM2, AKR1C3, ALOX5, ARID5B, BAG3, BLNK, BMI1, BNIP3, BTRC, CASP7, CCDC6, CELF2, CXCL12, DDIT4, DKK1, DMBT1, DOCK1, EIF3A, ERCC6, FAM107B, FAS, FGF8, FGFR2, GATA3, HELLS, HTRA1, KAT6B, KLF6, KLLN, LDB1, LGI1, LOXL4, MAPK8, MIR107, MIR146B, MKI67, MLLT10, MRC1, MXI1, NCOA4, NET1, NFKB2, NKX2-3, PAX2, PDCD4, PFKFB3, PIP4K2A, PSAP, PTEN, RET, RHOBTB1, SCD, SH3PXD2A, SIRT1, SNCG, TACC2	Yes
3	-	-	-	-	Yes
4	n.a.				
5	-	-	-	-	Yes
6	-	-	Chromosome 7: gain 157,201 Mbp	ABCB1, ABCB5, ACHE, ACTB, AKAP9, ANLN, ASNS, AUTS2, BCL7B, BRAF, CAV1, CLDN4, COL1A2, CREB3L2, CUX1, CYP2W1, DDC, DLX5, DLX6, DMTF1, EPHA1, EPHB6, ETV1, EZH2, FSCN1, GPER1, GPNMB, GRB10, HBP1, HGF, HIP1, HIPK2, HOXA11, HOXA9, HSPB1, HUS1, IKZF1, IL6, ING3, JAZF1, LIMK1, MET, MIR106B, MIR183, MIR196B, MNX1, MUC17, NAMPT, NPY, NRCAM, NRF1, PEG10, POU6F2, PTN, SEPT7, SFRP4, SHH, SPAM1, STEAP1, STEAP2, TAC1, TRIM24, TRPV6, TWIST1	Yes
	-	-	Chromosome 8: gain 145,881 Mbp	ADAM9, ASAP1, ASH2L, BAALC, BNIP3L, BOP1, CCAR2, CDH17, CLU, COX6C, CTHRC1, CTSB, CYP7A1, CYP7B1, DEFB1, DLC1, DUSP26, EBAG9, EIF4EBP1, ENPP2, EXT1, FABP5, FGFR1, GGH, HAS2, IDO1, IDO2, KAT6A, LOXL2, LZTS1, MAFA, MCPH1, MTUS1, MYBL1, MYC, NAT1, NAT2, NBN, NDRG1, NKX3-1, PIWIL2, PLAG1, PSCA, PTK2, RECQL4, RHOBTB2, RNF139, RUNX1T1, SDCBP, SNAI2, SULF1, TACC1, TNFRSF11B, TNKS, TP53INP1, TPD52, TRIB1, WHSC1L1, WRN, WWP1	n.a.
	-	-	Chromosome 10: gain 135,327 Mbp	ABI1, ADAM12, ADD3, AFAP1L2, AIFM2, AKR1C3, ALOX5, ARID5B, BAG3, BLNK, BMI1, BNIP3, BTRC, CASP7, CCDC6, CELF2, CXCL12, DDIT4, DKK1, DMBT1, DOCK1, EIF3A, ERCC6, FAM107B, FAS, FGF8, FGFR2, GATA3, HELLS, HTRA1, KAT6B, KLF6, KLLN, LDB1, LGI1, LOXL4, MAPK8, MIR107, MIR146B, MKI67, MLLT10, MRC1, MXI1, NCOA4, NET1, NFKB2, NKX2-3, PAX2, PDCD4, PFKFB3, PIP4K2A, PSAP, PTEN, RET, RHOBTB1, SCD, SH3PXD2A, SIRT1, SNCG, TACC2	Yes
	-	-	Chromosome X: loss 155,065 Mbp	AIFM1, AMER1, AMOT, ARMCX1, ARMCX2, ARMCX3, AR, BEX1, BEX2, BRWD3, BTK, CXCR3, DKC1, ELF4, ENOX2, FIGF, FOXO4, FOXP3, GATA1, GPC3, GRPR, IL3RA, INGX, L1CAM, LDOC1, MAGEA3, MIR221, MIR222, MSN, MTCP1, NONO, NR0B1, P2RY8, PASD1, PRDX4, RBBP7, REPS2, RPL10, RPS6KA6, SEPT6, SSX2, STAG2, TFE3, TRO, WAS, ZBTB33, ZFX	Yes

t(9;22)(q22;q12) and t(7;9;17)(q32;q22;q11)] (Sjögren *et al.*, 2003). Translocation t(9;22)(q22;q12) is a characteristic recurrent translocation in EMC, which is present in approximately in 75 % of the patients. An additional variant t(9;17)(q22;q11) is documented in approximately in 15 % of cases. Additional variants with t(9;15)(q22;q21) and t(3;9)(q12;q22) are also described. Also, in approximately 50 % of EMC, trisomies of chromosome 1 (partial trisomy), 7, 8, 12 and 19 are identified (Nishio *et al.*, 2011). Although the biological significance of these chromosomal aberrations is not yet fully known, a correlation between increase of aneuploidy and the progression of EMC from low to high WHO grades is observed (Hameed *et al.*, 2009). Other chondrosarcomas show polysomy 8 (Morrison *et al.*, 2005) or losses in chromosomes 5q, 6q and 9p (Hameed *et al.*, 2009).

Gonosomal chromosomes

X chromosomal losses of ≥ 10 % in spheroids first occurred from P 10 and only in patient 6, which is, according to co.don® AG, outside of the standard

Fig. 6. Example of cn-LOH detection using SNP array analysis. Three different spheroid cultures (P 2, P 4 and P 10) of patient 1 in the cn-LOH region (violet bars) of 11p11.2-p11.12 (5,259 Mbp, physical position: 46,304,337-51,563,636) are presented using SNP array. P 2 is shown in red, P 4 in light blue and P 10 in dark blue.

Table 8. Chromosomal regions (cn-LOH) and described cancer genes dependent on increasing cultivation time. Increasing cultivation time P 2, P 4, and P 10 after 6 weeks of the differentiation phase. Patient 1, P 10, 7 weeks and patient 6, P 10, 5 weeks.

Patient	Affected chromosomal area	Passage/size/physical position			Cancer genes
		P 2	P 4	P 10	
1	Chromosome 11p11.2-p11.12	5,259 Mbp 46,304,337-51,563,636 Mbp	5,259 Mbp 46,304,337-51,563,636 Mbp	5,259 Mbp 46,304,337-51,563,636 Mbp	DDB2, PTPRJ
	Chromosome 11q14.1	3,154 Mbp 81,224,285-84,377,856 Mbp	3,154 Mbp 81,224,285-84,377,856 Mbp	3,148 Mbp 81,229,936-84,377,856 Mbp	
2	Chromosome 11p11.2-p11.12	3,623 Mbp 47,940,924-51,563,636 Mbp	3,623 Mbp 47,940,924-51,563,636 Mbp	1,752 Mbp 47,933,842-49,686,065 Mbp	PTPRJ
	Chromosome 15q15.1-q21.1	3,499 Mbp 42,332,031-45,831,220 Mbp	3,518 Mbp 42,332,031-45,849,655 Mbp	3,124 Mbp 42,422,570-45,546,544 Mbp	
3	none	-	-	-	
4	n.a.				
5	Chromosome Xq13.1-q21.1	7,349 Mbp 71,523,649-78,872,361 Mbp	7,349 Mbp 71,523,649-78,872,361 Mbp	7,349 Mbp 71,523,649-78,872,361 Mbp	
6	Chromosome 16p11.2-p11.1	3,328 Mbp 31,892,235-35,220,544 Mbp	2,295 Mbp 32,926,025-35,220,544 Mbp	2,295 Mbp 32,926,025-35,220,544 Mbp	

production process for Spherox®. Gonosomal aneuploidies are described in connection with aging processes. Russell *et al.* (2007) analyse 19,650 cells of 655 female donors with an age spectrum from new-born to 80 year-old subjects, measuring the frequency of X chromosomal losses from 0.07 % (≤ 16 year-old) to 7.3 % (≥ 65 year-old) ($p \leq 0.00001$). Chapiro *et al.* (2014) describe a lower frequency of age-related occurrence of X chromosomal losses in elderly subjects. Furthermore, these X chromosomal losses, including clonal losses, are detected, for example, in patients with lymphoid neoplasia (Reimann-Berg *et al.*, 2011) and basal cell carcinomas (Kawasaki *et al.*, 1991). Similarly, a cumulative effect of cell cultivation conditions on the occurrence of losses of X chromosomes cannot be ruled out at present and is described, for example, in connection with chorionic villi (Gardner *et al.*, 2012). Results obtained for chondrocytes of patient 6 indicate such a cell cultivation effect.

The situation for Y chromosomal losses appears similar. Patient 2 was one of the older (73 year-old) patients in this study. Also, significant loss of the Y chromosome only occurred in spheroids derived from P 4 with a PDL of just 10.41 or subsequent passages, which lies outside of the production process for Spherox®. In *post-mortem* analyses of 7 patients, a high frequency of Y chromosome loss was observed in older patients (mean age of 58.1 years). However, this is not observed in younger patients (mean age of 24 years) (Trimborn *et al.*, 2012). In addition, several studies show that loss of the Y chromosome occurs at a relatively high frequency in the bone marrow and peripheral blood of older patients (Pierre and Hoagland, 1972; Guttenbach *et al.*, 1995). Results for patient 2 suggested the occurrence of this age-related and/or cell cultivation effect(s). Spontaneous loss of the Y chromosome is also observed in MSCs (Nikitina *et al.*, 2011). In contrast, loss of the Y chromosome is a disease-associated chromosomal aberration in patients with haematological tumorigenesis (Wiktor *et al.*, 2000).

In osteoarthritic tissue, using FISH analysis, Y chromosome deletions are found in ≥ 10 % of the analysed IC in 6 out of 10 male patients (Castellanos *et al.*, 2004). The lack of chromosomal aberrations in the healthy age-matched controls suggest that there is an association between chromosomal aberrations and osteoarthritis (Castellanos *et al.*, 2004). According to Stumm *et al.* (2012), the observed presence or absence of the Y chromosome does not appear to influence the transcriptional activity of chondrocytes.

Cn-LOH

Cn-LOH leads to LOH by duplication of a maternal (unimaternal) or paternal (unipaternal) chromosome or chromosomal region and concurrent loss of the other allele (O'Keefe *et al.*, 2010). In the current work, cn-LOH > 5 Mbp were considered as representing segmental and/or complete UPD. Using SNP array, cn-LOH regions were detected at 11p11.2p11.12 [5.26 Mbp, co.don® AG production-potential-analysis (PPA)-1: P 2, P 4 and P 10], at Xq13.1q21.1 (7.35 Mbp, patient 5: P 2, P 4 and P 10) and at 16p11.2-p11.1 (3.33 Mbp, PPA-6: P 2, P 4 and P 10). Described cancer genes within the chromosomal region 11p11.2-11p11.12 are damage-specific DNA binding protein 2 (DDB2) and receptor-type tyrosine-protein phosphatase eta (PTPRJ). DDB2 influences damage recognition prior to nucleotide excision repair (NER), since DDB proteins recognise many types of DNA lesions caused by UV damage and are inducible by treatment with DNA-damaging agents. DDB2 is involved in the protein ubiquitination pathway and in the NER pathway to initiate DNA repair together with DDB1, as UV-DDB complex (Yeh *et al.*, 2012). PTPRJ is a member of the protein tyrosine phosphatase (PTP) family. Since its expression is increased with

increasing cell density, it is strongly suggested that PTPRJ may contribute to the mechanism of contact inhibition of cell growth (Ostman *et al.*, 1994). It seems to be involved in protein tyrosine kinase signalling, cell-cell signalling, vasculogenesis and heart development (Takahashi *et al.*, 2003). PTPRJ plays a role in the β-type platelet-derived growth factor receptor (PDGFR-β) signalling pathway (Heldin, 2013). According to the study design of the SNP array analyses (analyses of spheroids of donors 1, 2, 3, 5, 6, P 2, P 4 and P 10), it could be excluded that the detected cn-LOHs were already present in the starting material. Therefore, they could represent constitutional events. However, it remains that most UPDs appear not to have any phenotypic consequence (Kotzot, 2002).

As an example for chromosome 11, mosaic segmental upd(11p)pat are described as the cause of about 20 % of sporadically occurring cases of Beckwith-Wiedemann-Syndrome (Li *et al.*, 1998; Engel *et al.*, 2000; Maher and Reik, 2000; Gardner *et al.*, 2012). This chromosomal region, located at 11p15, differs from the detected region 11p11.2p11.12. It is presumed that a segmental UPD, arising postzygotically and being karyotypically 46,XX or 46,XY, would imply no risk. Also, UPD due to rearrangement would have a risk according to the nature of the specific rearrangement (Gardner *et al.*, 2012).

Conclusions

GTG-banding, SKY, locus-specific FISH and SNP array analyses were applied to obtain profiles on different aspects of genetic stability of investigated 2D and 3D chondrocyte cultures. These techniques illuminated different aspects of the cell genomes. With the used sample size, it was not possible to derive statistically significant statements. Also, the average age (65 years) of the patients in this study was different from the Spherox® inclusion criteria´s (18-55 years). Despite these limitations, a combination of different (molecular) cytogenetic techniques was used to increase the knowledge and experience on potential cellular therapies and to shape the quality and safety of (combined) ATMPs. Genetic analyses identified chromosomal instability, especially for longer cultivation time, above P 3. With increasing cultivation time, both numeric and structural (patient 2, 73 year-old) chromosomal aberrations occurred, partially also clonally present in the form of polysomy. In patient 2, from P 1 onwards, chromosomal changes, already described for EMC, were identified. Based on the observations made, it is suggested that the influence of PDLs and age of donor on genetic stability should be evaluated when producing an ATMP. This should include starting materials entrance examination. Cytogenetic analysis of starting material may become a safety measure for each patient.

The meaning of gonosomal losses in older patients in connection with cellular therapies should be further investigated. However, this only partially applies to the GMP production process of Spherox®, for which the actual age range is of 18-55 years (Niemeyer *et al.*, 2013). Due to the occurrence of more than 10 % of polyploid cells in a culture vessel of P 2 cells from patient 2, it should be considered whether genetic safety controls at manufacturing and cultivation relevant time points should be performed. GTG-banding, FISH (locus-specific FISH), SKY and SNP array would be appropriate genetic methods, if possible. According to the current state of knowledge (Barkholt *et al.*, 2013) and the collected data in the present study, high-resolution genome wide SNP array analysis should be performed in the non-clinical research program at production-relevant time points as part of quality investigations. In general, in addition to the analyses of these cells, it is recommended that the peripheral lymphocytes of the donors are collected and analysed to rule out the potential presence of hereditary chromosomal events. In general, chromosomal aberrations were identified (as single events and/or clonally), suggesting that monitoring genetic stability should be part of the characterisation within the field of cell therapy.

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Discussion with reviewer

Sylvie Miot: Since biopsies of articular cartilage were harvested from an undamaged area of patients with osteoarthritis, can chondrocytes still be considered as healthy cells or is there a probability that these cells already present an abnormal genetic profile?

Authors: Only few cytogenetic reports from patients with osteoarthritis are published (Broberg *et al.*, 1997, additional reference; Mertens *et al.*, 1996, additional reference; Castellanos *et al.*, 2004). Trisomy X, Y, 5, and 7 are described in osteoarthritic cartilage of grade II to IV, according to the Kellgren and Lawrence Score (KLS) (Kellgren and Lawrence, 1957, additional reference). To the best of our knowledge, clonal chromosomal aberrations are not known for healthy as well as KLS grade I osteoarthritic cartilage. The majority of the chondrocyte-based ATMPs contraindicate the implantation of an (M)ACI product into joints with an KLS grade III and IV. Taken into account this exclusion criteria and the above stated literature, it might be possible that cartilage samples harvested from patients with KLS II exhibit a higher probability for numeric aberrations.

Sylvie Miot: Which additional quality control measure would you include during GMP manufacturing of a chondrocytes-based ATMP, in order to increase the patient safety?

Authors: Currently, many powerful genetic techniques are available for evaluating the genetic stability in cell therapy, including conventional GTG-banding, SKY, FISH, CGH or SNP, whole genome sequencing (NGS), gene expression analyses and epigenetics. Each of these techniques has its own advantages and limitations. Thus, it is frequently valuable to use a combination of conventional karyotyping and molecular (cyto)genetic techniques as complementary tools to evaluate genomic stability. In order to increase the patient safety, it is advised to include GTG-banding, SKY, SNP array, NGS and gene expression analyses (at different time points) as quality control measure system within the GMP-process validation study of the respective ATMP. Finally, a decision on the preferable genetic

stability quality control method(s) should be made, based on the observed product-specific (numeric and structural) genetic instabilities. Moreover, the optimal validated quality control method for product release shall be a fast and simple method that enables the immediate release of the cellular product. Based on the herein described case study on Spherox®, it is advised to include both, SKY and FISH, for the product release of Spherox®.

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