Chromosomal Abnormalities in Embryonic and Somatic Stem Cells

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Key Words

Aneuploidy · Chromosome instability · Stem cells

Abstract

The potential use of stem cells (SCs) for tissue engineering, regenerative medicine, disease modeling, toxicological studies, drug delivery, and as in vitro model for the study of basic developmental processes implies large-scale in vitro culture. Here, after a brief description of the main techniques used for karyotype analysis, we will give a detailed overview of the chromosome abnormalities described in pluripotent (embryonic and induced pluripotent SCs) and somatic SCs, and the possible causes of their origin during culture.

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Stem cells (SCs) are undifferentiated cells capable of self-renewal and of differentiation into specific terminally-differentiated cell types. Based on their source of derivation, they can be classified into embryonic stem cells (ESCs) or somatic stem cells (SSCs).

The first mouse ESC (mESC) lines were derived in 2 independent laboratories at the beginning of the 1980s from preimplantation embryos at the blastocyst stage

[Evans and Kaufman, 1981; Martin, 1981]. About 15 years later, the first primate non-human ESC lines (rhesus monkey, *Callithrix jacchus*) were generated [Thomson et al., 1995, 1996], followed by the first human (hESC) [Thomson et al., 1998], rabbit [Fang et al., 2006; Wang et al., 2007], canine [Hayes et al., 2008], and rat [Buehr et al., 2008; Li et al., 2008] lines. Once established in vitro, ESCs are able to differentiate into almost all mature fetal and adult cell types, and thus they are defined as pluripotent cells. When transferred into a blastocyst, they participate in the formation of both somatic and germ lines of the growing individual [Rossant, 2001; Smith, 2001; Cockburn and Rossant, 2010]. In 2006, Takahashi and Yamanaka demonstrated the feasibility to obtain pluripotent ESC-like cells by reprogramming somatic terminally-differentiated mouse or human fibroblasts [Takahashi and Yamanaka, 2006; Takahashi et al., 2007]. These authors showed that the induced ectopic expression of 4 genes (*Oct4*, *Sox2*, *Klf4*, and *cMyc*) was sufficient to reprogram fibroblasts into the so-called induced pluripotent stem cells (iPSCs), which show similar developmental potential and pluripotency as ESCs [Takahashi and Yamanaka, 2006; Okita et al., 2007; Wernig et al., 2007].

The first SSCs were identified long before ESCs or iPSCs. In the early 1960s, hematopoietic SSCs were puri-

fied from mouse bone marrow [Till and McCullough, 1961]. Since then, SSCs have been identified in many different organs (i.e. skeletal muscle, heart, liver, fat, umbilical cord blood, or placenta). SSCs exert a crucial role in the maintenance of tissue homeostasis and participate in the repairing processes within their specific tissue. In vivo, SSCs can differentiate either in one, few or multiple cell lineages and thus are classified as unipotent (e.g. spermatogonia, oogonia), oligopotent (e.g. neural stem cells, NSCs) or multipotent (e.g. hematopoietic SCs). In vitro, SSCs display greater plasticity, showing higher differentiation potential than in vivo [Jiang et al., 2002; Franco Lambert et al., 2009]. Among SSCs, human mesenchymal stromal, hematopoietic and neural SCs already find an application in the clinic [Bajada et al., 2008; Thomsen et al., 2014; Porada et al., 2015].

Both ESCs and SSCs have boosted existing and, sometimes, given rise to novel and interdisciplinary research fields such as tissue engineering [Katari et al., 2015], regenerative medicine [Grompe, 2012], disease modeling [Merkle and Eggan, 2013], toxicological studies, and drug delivery [Seiler and Spielmann, 2011; Liu et al., 2013]. Furthermore, ESCs are an in vitro model for the study of basic developmental processes [Neri et al., 2011; Rebuzzini et al., 2013].

If, on one side, this interdisciplinarity allowed a quantum leap in our basic knowledge of the biology of SCs, it has also brought out a number of criticalities that limit their clinical use. For example, the potential risk of forming tumors, the migration far away from the site of infusion and colonization of other tissues, the dedifferentiation of SC-derived differentiated cells, the establishment of an incorrect epigenetic and genetic status, and an abnormal chromosome complement.

The reflection paper on SC-based medicinal products – EMA/CAT 14 January 2011 – defines, among other requirements, that ‘stem cell preparations undergoing extensive in vitro manipulation such as prolonged cell culture, as well as those derived from hESCs or iPSCs are evaluated for both their tumorigenicity and chromosomal stability before their initial clinical use’.

During the last decade, several papers evidenced the difficulty of SCs to maintain a correct chromosome complement during prolonged expansion [for reviews see Rebuzzini et al., 2011; Oliveira et al., 2014]. After a brief description of the main techniques used for the karyotype analysis, this review will give a detailed overview of the chromosome abnormalities described in both pluripotent SCs (ESCs and iPSCs) and SSCs, and the possible causes of their origin during prolonged culture.

Techniques for Karyotype Analyses

Several techniques are currently available to investigate the integrity of the chromosome complement of a cell line. Each method has advantages and disadvantages in terms of sensitivity, resolution and costs [Catalina et al., 2007].

Conventional banding techniques (G-, Q- or DAPI-banding) allow a snapshot of the entire chromosome complement and the ordinary gross karyotype control of a cell line. These techniques, providing 300–400 stained bands, facilitate the detection of incorrect chromosome numbers (aneuploidies), mosaicism and large structural chromosome abnormalities, such as translocations, deletions or insertions, with a resolution of 5–10 Mb.

Spectral karyotyping (SKY) and multicolor FISH represent an advancement of the conventional cytogenetic banding analysis [Liyanage et al., 1996; Schröck et al., 1996]. These techniques, using chromosome-specific fluorescently labeled probes, allow the detection of submicroscopic deletions, insertions or amplifications of DNA, with a resolution of ~1–2 Mb.

To detect smaller genetic imbalances, the array-based comparative genomic hybridization (array-CGH) [Sanlaville et al., 2005] and the single nucleotide polymorphism (SNP) array [Peiffer et al., 2006] are useful techniques. They allow the identification of tiny aberrations, comprising homo- or hemizygous deletions, copy-neutral loss of heterozygosity, duplications, and amplifications. Their resolution ranges from 1 Mb to <100 kb. Both CGH and SNP-array are unable to evaluate the frequency of a specific abnormality within a cell population.

Pluripotent Stem Cells

Human Embryonic Stem Cells

Variations in the chromosome complement of hESC lines, detected by conventional banding techniques or by CGH assays, comprise both numerical and structural aberrations. Chromosomes 1, 12, 17, 20, and X are most frequently involved (~50% of the aberrations found involved these chromosomes). Duplication of 1q11q32, whole or partial gain of chromosomes 12 and 17, duplication of the 20q11.21 region or aneuploidy of chromosome X have been reported by many independent laboratories (table 1) [Brimble et al., 2004; Draper et al., 2004; Inzunza et al., 2004; Mitalipova et al., 2005; Imreh et al., 2006; Baker et al., 2007; Lefort et al., 2008, 2009; Spits et al., 2008; International Stem Cell Initiative, 2011]. The recurrence

Table 1. Frequency of pluripotent SC lines with recurrent numerical and structural aberrations

Cell type	Main recurrent aberrations		Cell lines with aberrations, %	References
hESCs	numerical structural	gain of chr. 1, 12, 17, 20, or X	32–34	Brimble et al., 2004; Draper et al., 2004; Inzunza et al., 2004; Mitalipova et al., 2005; Imreh et al., 2006; Baker et al., 2007; Lefort et al., 2008, 2009; Spits et al., 2008; International Stem Cell Initiative, 2011
		duplication 1q11q32	10–25	
		partial gain of chr. 12	13	
		partial gain of chr. 17	20	
		duplication 20q11.21	10	
mESCs	numerical	trisomy 8	16	Liu et al., 1997; Rebuzzini et al., 2008a, b, 2012; Gaztelumendi and Nogués, 2014; Luft et al., 2014
		trisomy 11	8	
	structural	deletion 10qB	14	
		deletion 14qC–14qE	5	
Human iPSCs	numerical	trisomy 8	5	Aasen et al., 2008; Mayshar et al., 2010; Boulting et al., 2011; Martins-Taylor and Xu, 2012; Peterson and Loring, 2014
		trisomy 12	12	
	structural	amplification 1q31.1	>25	
		amplification or deletion 17q21.1	>25	
		amplification 20q11.21	10	
		deletion 8q24.3	12	

of these specific types of mutations, linked to the presence of additional copies of some specific genes, may confer carrier cells with a selective and/or proliferative advantage, higher culture adaptation and resistance to apoptosis [Clark et al., 2004; Rosler et al., 2004; Caldas and Brenton, 2005; Maitra et al., 2005; Baker et al., 2007; Lefort et al., 2008; Navarro et al., 2008; Spits et al., 2008; Blum and Benvenisty, 2009; Werbowetski-Ogilvie et al., 2009; International Stem Cell Initiative, 2011; Oliveira et al., 2014].

Mouse Embryonic Stem Cells

A general feature of mESCs is the alteration of the chromosome number. Extensive cytogenetic studies on 540 [Sugawara et al., 2006] and 97 [Kim et al., 2013] mESC lines described high heterogeneity in numerical aberrations, detected by conventional banding techniques, SKY or CGH assays, with recurrent trisomies of chromosomes 8 and 11. Similar results were obtained in our and other laboratories [Liu et al., 1997; Rebuzzini et al., 2008a, b, 2012; Gaztelumendi and Nogués, 2014; Luft et al., 2014]. Also, several random subchromosomal variations were detected by CGH in mESC samples, among which the most recurrent were deletions of chromosomes 10qB and 14qC–14qE and the duplications of specific regions on chromosomes 1, 2 and 12 (table 1) [Ben-David and Benvenisty, 2012; Liang and Zhang, 2013]. However, the analysis until passage 22–34 of the chromosome complement of 4 mESC lines derived from B6C3F1 blastocysts

showed a very heterogeneous spectrum of abnormalities that varied during the culture period, suggesting for these specific cell lines a lack of selective pressure in favor of specific chromosome complements [Rebuzzini et al., 2008a, b].

Parthenogenetic Embryonic Stem Cells

Parthenogenetic ESCs (pESCs), derived from non-human primate embryos [Cibelli et al., 2002; Vrana et al., 2003], display a normal diploid karyotype set of 42 chromosomes, even after 80–120 passages, analyzed using G-banding [Wei et al., 2011]. On the contrary, human pESCs [Kim et al., 2007; Revazova et al., 2007; Brevini et al., 2009, 2012] appear to be more variable. Some cell lines are stable, showing a normal karyotype after long-term culture (>50–100 passages) after standard G-banding analysis [Lin et al., 2007; Mai et al., 2007]; others lose 1 copy of the X chromosome with a frequency that increases during passages [Liu et al., 2011]. Chromosome deletions and translocations have also been reported in the hPES-2 cell line, when cultivated for 120 passages [Mai et al., 2007]. Few cytogenetic data are available on mouse pESC lines, reporting a stable karyotype during culture (up to passage 120), analyzed by standard G-banding [Shao et al., 2007; Ju et al., 2008; Shan et al., 2012].

Induced Pluripotent Stem Cells

iPSC lines seem to maintain a normal karyotype during early (~5) culture passages [Takahashi et al., 2007];

Table 2. SSC lines in which numerical and structural aberrations were described

Cell type	Recurrent aberrations		Cells with aberrations, %	References
Human bone marrow-derived MSCs	numerical	monosomy 13 or X trisomy 5, 8 or 20	≤1 15–20	Ben-David et al., 2011; Nikitina et al., 2011 Tarte et al., 2010; Grigorian et al., 2010
Human adipose-derived MSCs	numerical	trisomy 8 or 17 tetrasomy 11 or 17	8–12 (passage 2); 18–28 (passage 20)	Estrada et al., 2013
Human dental pulp-derived MSCs	numerical structural	loss of chr. 2, 3, 5, 6, 7, 8, 9, and 16 44,XX,t(13,14),–22	70 3	Duailibi et al., 2012 Suchánek et al., 2007
Murine bone marrow-derived MSCs	numerical structural	clonal gain of chr. 2; non-clonal gains or losses of several chromosomes double-minutes; chromosomal imbalances	40–70	Miura et al., 2006; Josse et al., 2010; Fan et al., 2011
Hematopoietic SCs	numerical structural	tetraploidy 13; monosomy X 46,XX,t(2;8); 45,XX,–8; 45,XX,–7,6q+	10–15	Ge et al., 2011
EPCs	numerical	92,XXYY +77,XX +55,XX gain of chr. 11 and 14	33 68 100 74	Corselli et al., 2008
Human NSCs	numerical	trisomy 7 or 19 trisomy 10, 18 monosomy 18	5–24 5 5	Sareen et al., 2009 Ben-David et al., 2011
Mouse NSCs	numerical	clonal monosomy 17 trisomy 1	6 29 (passage 10); 62 (passage 40)	Diaferia et al., 2011

whereas, during long-term culture (~60 passages), large-scale screenings have evidenced the presence of several chromosomal (detected by standard karyotyping and FISH) and subchromosomal abnormalities such as translocations, duplications or deletions and point mutations (detected by array-CGH and SNP-arrays) [Takahashi et al., 2007; Mayshar et al., 2010; Gore et al., 2011; Martins-Taylor et al., 2011; Martins-Taylor and Xu, 2012]. Full trisomy of chromosomes 8 and 12, amplification of portions of chromosome 1, 17 or 20 (1q31.1, 17q21.1 and 20q11.21, respectively) and deletion of chromosome 8 and 17 (8q24.3 and 17q21.1, respectively) are the most frequent aberrations observed (table 1) [Aasen et al., 2008; Mayshar et al., 2010; Boulting et al., 2011; Martins-Taylor and Xu, 2012; Peterson and Loring, 2014]. Up to date, it is unclear whether the chromosomal and subchromosomal variations detected in iPSC lines originate early in individual cells and are propagated during the prolonged culture or are caused by the reprogramming process itself. The presence of recurrent aberrations may facilitate the adaptation process during reprogramming [Mayshar et al., 2010].

Somatic Stem Cells

Mesenchymal Stromal Stem Cells

Most studies on chromosome variability have been performed on human mesenchymal stromal cells (MSCs) derived from bone marrow. Independent laboratories reported contrasting results on the accumulation of chromosomal aberrations during in vitro culture, a debate that still remains open [Ben-David et al., 2011, 2012; Sensebé et al., 2012]. Some authors showed that human bone marrow-derived MSCs remain chromosomally stable throughout long-term culture [Pittenger and Martin, 2004; Soukup et al., 2006; Bernardo et al., 2007; Zhang et al., 2007; Sensebé et al., 2012], whereas others claimed the occurrence of spontaneous numerical and structural chromosome aberrations within 10 passages of in vitro culture (table 2). To this regard, monosomy of chromosomes 13 and X (in 0.3–1.3% of cells analyzed) [Ben-David et al., 2011; Nikitina et al., 2011], trisomies of chromosome 5 and, less frequently, of chromosomes 8 and 20 (in 15–20% of cell analyzed) [Grigorian et al., 2010; Tarte et al., 2010] were reported after conventional G-banding.

Similarly, discordant results have been obtained for MSCs derived from other tissues. Human adipose-derived MSC lines cultivated for more than 6 months seem to maintain chromosome stability [Meza-Zepeda et al., 2008; Ra et al., 2011; Yang et al., 2011]. Minor deletions and pericentromeric instabilities were detected by high-resolution microarray-based CGH analyses in few cells, but they were spontaneously eliminated from the population [Meza-Zepeda et al., 2008]. More recently, trisomy of chromosomes 8 or 17 and tetrasomy of chromosomes 11 or 17, acquired within few passages of in vitro culture, were described in human adipose-derived MSCs (at passage 2, 8–12% of cells were aneuploid, at passage 20, 18–28%) [Estrada et al., 2013]. Human dental pulp MSCs showed a normal karyotype until the Hayflick limit (passage 65) [Gronthos et al., 2000; Suchánek et al., 2007; Tamaki et al., 2013]. Beyond this limit, some chromosome abnormalities have been described [2n = 44,XX,t(13;14),-22 in 3% of cells analyzed; Suchánek et al., 2007]. More recently, polyploid aberrant metaphases (loss of chromosomes 2, 3, 5, 6, 7, 8, 9, and 16 in ~70% of the cells) were found in 4 human dental pulp-derived MSC lines, in association with the presence of ring chromosomes (table 2) [Duailibi et al., 2012]. Finally, data on chorionic villi-, human umbilical cord- and amniotic fluid-derived MSCs showed normal chromosome complements after 6–10 passages [Poloni et al., 2011; Wang et al., 2013; Ruan et al., 2014] or after 150 days of cryopreservation [Angelo et al., 2012].

High chromosomal instability is characteristic of murine bone marrow-derived MSCs [Fan et al., 2011]. Several papers showed the accumulation of both clonal numerical and structural chromosome abnormalities in different cell lines since the very early passages from their isolation (table 2). Double-minute chromosomes, clonal gain of chromosome 2, chromosomal imbalances, non-clonal gains or losses of several chromosomes were extensively described [Miura et al., 2006; Josse et al., 2010; Fan et al., 2011].

Hematopoietic Stem Cells

Expanded umbilical cord blood (UCB)-derived CD34⁺ SCs showed karyotype stability even after 20 and 43 days of culture [Tian et al., 2005]. A recent comprehensive analysis of 55 CD34⁺ hematopoietic SC samples derived from bone marrow, peripheral blood or UCB of healthy donors revealed that these cells preserved their correct chromosome complement [Ben-David et al., 2011]. However, a paper published in 2011 reported that already after 7–14 days of in vitro culture ~10–15% of UCB-derived

CD34⁺ SCs displayed various chromosomal abnormalities [i.e. 46,XX,t(2;8); 45,XX,-8; 45,XX,-7,6q+; tetraploidy of chromosome 13, loss of an X chromosome]. After 28 days of culture, loss of entire chromosomes was described (i.e. 45,XX,-19 in 10% of cells analyzed) (table 2) [Ge et al., 2011].

Endothelial Progenitor Cells

To our knowledge, few papers reported the karyotype analysis of endothelial progenitor cells (EPCs). Although samples of cord blood-derived EPCs showed a normal karyotype throughout the ex vivo expansion, some cord blood EPC samples showed a high incidence of chromosomal alterations during culture, such as tetraploidy (92,XXYY; 33% of cells at passage 2), several types of aneuploidy (+77,XX, 68% of cells at passages 2 and 4; +55,XX, 100% of cells from passage 2 to 8) including gain of chromosomes 11 and 14 (74% of cells at passage 10) (table 2) [Corselli et al., 2008]. On the contrary, peripheral blood-derived EPCs showed a normal karyotype during in vitro culture (40–60 cell population doublings) [Untergasser et al., 2006].

Neural Stem Cells

Neural progenitor stem cells (NSCs) show a limited capacity of mitotic expansion in vitro; for this reason, few papers tackled the analysis of their chromosome complement. NSCs display high variability in the chromosome complement when cultured. For example, neither numerical nor structural chromosome abnormalities were detected in both rat and human NSC samples, cultivated up to 70 passages and analyzed with G-banding [Bai et al., 2004; Jiang et al., 2008; Sareen et al., 2009]; on the contrary, using the same technique, other samples (human NSCs derived from the striatum fetal) displayed different numerical (from 60–70 chromosomes per metaphase) and structural chromosome aberrations [Wu et al., 2011]. Also, the analysis of more than 70 other human NSC samples identified trisomy of chromosomes 7, 10, 18, or 19 [Sareen et al., 2009; Ben-David et al., 2011] and monosomy of chromosome 18 [Ben-David et al., 2011] in long-term cultures (table 2).

The occurrence of chromosome aberrations in mouse NSCs after extensive passaging was also reported. NSCs derived from fetal mouse brain (analyzed within 39 passages) showed no clonal structural aberrations but clonal monosomy 17 in 6.1% of cells (at passage 21). NSCs derived from the subventricular zone displayed a higher frequency of clonal aneuploidies than structural aberrations. Trisomy of chromosome 1 was detected in 29% of

subventricular zone-derived NSCs at passage 10 and in 62% at passage 40. Also, trisomies of chromosomes 18 and 19 and duplication of chromosome 19q were reported with a lower frequency (table 2) [Diaferia et al., 2011].

Effect of Culture Conditions on Chromosome Stability

The effect of in vitro environmental conditions on genomic stability of cells received much attention during the last years. However, the variability among culture protocols applied in different laboratories for derivation and cultivation of SCs complicates the identification of the source of such variations.

The techniques used for cell detachment and disaggregation seem to be a major factor affecting the maintenance of genome integrity during long culture. Mechanical (i.e. pipetting in and out and flushing the medium until the colonies are detached and disaggregated) or manual (i.e. colonies are cut and removed using a blade) methods are more gentle passaging techniques for subculturing than the use of enzymes (i.e. trypsin or collagenase), being less aggressive and able to better preserve genome integrity [Buzzard et al., 2004; Mitalipova et al., 2005; Lefort et al., 2008]. A modified enzymatic dissociation solution (0.25% trypsin, 0.1% collagenase IV, 20% KSR, and 1 mM CaCl₂ in PBS), in combination with manual dissection for bulk passaging, has been proposed for hESC dissociation, demonstrating the maintenance of a normal chromosome complement even after more than 100 passages [Suemori et al., 2006].

Several studies focused their attention on oxygen concentration during culture although with contrasting results. In some studies, physiological concentrations of O₂ (ranging from 1 to 7%) seemed to significantly reduce the incidence of aneuploidies in hMSCs and promote the maintenance of genetic integrity [Holzwarth et al., 2010; Li et al., 2011; Tsai et al., 2011; Estrada et al., 2012]; whereas, in others, 1–5% O₂ concentrations were found to increase the risk of aneuploidies, chromosome and microsatellite instability in mouse NSCs, human bone marrow MSCs and human adipose SCs, even at early passages [Oliveira et al., 2012; Ueyama et al., 2012]. Also, high rates of aneuploidy, gaps and breaks were reported in hESC lines cultured with air concentration of O₂ (21%), in comparison with lower concentrations [Forsyth et al., 2006; Lim et al., 2011].

A fundamental component of the SC medium is the serum. However, its animal (calf or bovine) or artificial (knockout serum replacement of defined composition,

only used for ESC culture) origin does not seem to play a role in the maintenance or in the alteration of the genome stability [Inzunza et al., 2005; Ludwig et al., 2006]. Similarly, the choice of a cell feeder layer (mouse embryonic or immortalized fibroblasts) or of supportive matrixes (i.e. gelatin, fibronectin or matrigel™) during the derivation and maintenance of ESC lines does not seem to influence either the onset or the restraint of aberrations in the ESC genome [Cowan et al., 2004; Draper et al., 2004; Guo et al., 2005; Maitra et al., 2005; Mitalipova et al., 2005; Imreh et al., 2006; Sugawara et al., 2006; Rebuzzini et al., 2008a, b].

It should not be forgotten that various ESC lines have been derived from different strains and at different time points of preimplantation embryonic development, when gene expression and DNA methylation vary significantly. Hence, a differing tolerance to genetic instability might be also a characteristic defined at the time of ESC derivation. Unfortunately, up to date, there are no studies that have undertaken these comparisons.

Concluding Remarks

As evidenced in this review, SCs accumulate during long-term culture both numerical and structural aberrations. This genomic instability does not affect their stemness and, upon challenging, SCs achieve a differentiated phenotype [Rebuzzini et al., 2012, 2013; Gaztelumendi and Nogués, 2014; Luft et al., 2014]. Strikingly, there is a lack of studies that analytically investigate if and which type of aneuploidies is permissive for cell differentiation, an aspect that is central to the use of SCs in the clinic. Given the enormous investments in terms of human and economic resources undertaken for the use of SCs in regenerative medicine and the recent approval of their use as medicinal products, we foresee a timely discussion among scientists, clinicians and regulatory agencies to produce appropriate guidelines for SC derivation and culture.

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