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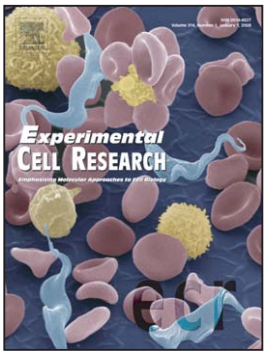

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S. Garcia, M.C. Martín, R. de la Fuente, J.C. Cigudosa, J. Garcia-Castro, A. Bernad

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PITFALLS IN SPONTANEOUS IN VITRO TRANSFORMATION OF HUMAN MESENCHYMAL STEM CELLS

Garcia S[1], Martín MC[2], de la Fuente R[3], Cigudosa JC[2], Garcia-Castro J[3,4,5] and Bernad A[1,3,5]

[1] Regenerative Cardiology Department. Centro Nacional de Investigaciones Cardiovasculares Carlos III. Melchor Fernández Almagro, 3. 28029 Madrid, Spain

[2] Cytogenetic Unit. Centro Nacional de Investigaciones Oncolologicas. Melchor Fernández Almagro, 3. 28029 Madrid, Spain

[3] Department of Immunology and Oncology. Centro Nacional de Biotecnología. UAM. Campus de Cantoblanco. 28049 Madrid, Spain

[4] Área de Biología Celular y del Desarrollo. Instituto de Salud Carlos III. Ctra Majadahonda-Pozuelo Km 2,200. 28220 – Majadahonda. Madrid, Spain

[5] **Corresponding authors**

Antonio Bernad. Regenerative Cardiology Department. Centro Nacional de Investigaciones Cardiovasculares Carlos III. Melchor Fernández Almagro, 3. 28029 Madrid, Spain
Tel.: 00 34 91 453 1274/ 12 77; Fax: 00 34 91 453 12 40; abernad@cnic.es

Javier García-Castro. Área de Biología Celular y del Desarrollo. Instituto de Salud Carlos III. Ctra Majadahonda-Pozuelo Km 2,200. 28220 – Majadahonda. Madrid, Spain
Tel: 00 34 918223288; Fax: 00 34 918223269; E-mail: jgcastro@isciii.es

Spontaneous *in vitro* transformation of human primary cells was, and continues to be, a scarcely described phenomenon. Only the description of the generation of the HaCAT cell line [1] is a canonical example, worldwide accepted. More recent examples included the emergence of tumorogenic populations upon *in vitro* culture of fetal human mesenchymal stem cells (hMSC), induced by GM-CSF and IL-4, [2] and bone marrow hMSC [3]. Other examples have also been reported after very long-term *in vitro* culture of telomerized hMSC-TerT [4]. In this scenario, our previous results [5-7] were only a new observation to be added to this list. Only very recently similar results have been published [8], reporting spontaneous malignant transformation in 46 % of bone marrow–derived hMSC long-term cultures (5–106 weeks). However, other authors reported opposite results [9].

Since our original publication, we continued to work on the definition and characterization of the hMSC spontaneous transformation phenomenon. Unexpectedly, however, we have been unable to obtain a single new human MSC transformation event (about 15 independent new adipose tissue samples –both adult and pediatric-, and 25 attempts of independent set of cultures). What we can, however, clearly state is that the *ex vivo* culture of human MSC under the conventional conditions promotes a variable but significant level of genomic instability, concurring with an important level of aneuploidy (Estrada et al., submitted). In any case, in all experiments, after a clear senescent phase the culture was exhausted. When we began to observe this central discrepancy we tried to evaluate several factors that could influence it, but finally, we carried out a STR (short tandem repeats) analysis to control the possibility of cross-contamination between the different TMC (for transformed mesenchymal stem cells) samples or between TMC and MSC cultures.

Surprisingly, none of the TMC populations obtained presented an STR profile compatible with the original human MSC samples and were quite similar among them. In addition, when we compared the TMC STR profile with a panel of human tumor cell lines, we found that is quite similar to the one corresponding for the HT1080 cell line. HLA genotype analyses confirmed these data. Finally, we repeated the karyotype analysis of old and new samples. We observed that although there was a high heterogeneity among the clones and samples selected after de co-culture, the most characteristic genomic alterations [der(5) and t(3;11)] present in TMC [5] are also common to the cell line HT1080 that we have available in our lab. This result again, supports the proposed working hypothesis (Figure 1, summarizes the most relevant results).

Currently, the most plausible explanation for the MSC spontaneous transformation phenomenon we described is thus an artifact mediated by an unnoticed minimal cross-contamination of some of the original samples with HT1080 cells.

Functional interaction between human MSC and HT1080 cells during long-term *in vitro* culture

The previously described data imply that there must be an effect of the human MSC on the tumor cells to control their expansion during the long-term cultures. TMC appeared in the last phases of the long-term cultures of human MSC. It is difficult to understand why the potential contaminating tumoral cells did not start to overgrow the cultures in the first weeks, but only 3-4 months later.

Prior to our initial communication, there were few and conflictive publications focused on this topic. Nakamura et al. reported an antitumor effect of rat MSC on glioblastoma [10]. On the contrary, Fierro et al. reported positive effects of MSC co-cultures of breast cancer cell lines [11]. Since then, the scientific literature continues to show similar results, strongly suggesting that probably there must be tumor cell-type specificity in the effect. In addition, it was recently demonstrated, both *in vitro* and *in vivo*, that MSC have the potential to migrate and intercalate in tumor capillary network, form Cx43-based gap junctions and increase the production of ROS, promoting capillary apoptosis and network degeneration [12].

Because the scientific literature was non-conclusive on the potential effect, we next designed a set of experiments in which we mixed HT1080-EGFP cells with hMSC obtained from different donors (in 3 dilutions: 1/10,000, 1/100,000 and 1/500,000) and maintained the mixed cultures until the end of the culture or the evident appearance of HT1080-like cells. Twice per week, presence of EGFP in the cultures was analyzed by flow cytometry, PCR and fluorescent microcopy. In most of the co-cultures, we detected a delay in the emergence of the HT1080-EGFP cells with respect to the expected kinetics in the case there were no functional interaction with MSC. A summary of the results obtained is included in Figure 2.

In conclusion, our results strongly suggest that the significative emergence of HT1080-EGFP cells was delayed respect the theoretical expected time and, in some cases, HT1080 were undetectable for weeks before they began to overgrow the culture. These data are compatible with our hypothesis that a minimal HT1080 contamination could have been present in some of our hMSC cell cultures since first weeks, and remained undetectable for months until hMSC advanced senescence manifests and further development of the crisis phase; then, the hMSC deterioration allowed HT1080 cells to invade the flask and growth with their normal duplication rates.

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FIGURE LEGENDS.

Figure 1. A) STR analyses of short-term MSC culture (MSC16), pre- and postsenescent (MSC-16 S, PS) and transformed (TMC-14 1-3, TMC-16 1-2) cell cultures. Several control cell lines (A549, Caco, H441, Hela, Saos, T98G and U87MG) were analyzed in parallel, including STR patterns of HT1080 and HT1080-eGFP cell lines cultured in our laboratory. **B)** HLA haplotypes of TMC-14, TMC-16 and HT1080. **C)** Karyotype of our HT1080 and HT1080-eGFP cell lines showing the same specific translocations of TMCs previously published.

Figure 2. Co-culture of the hMSC and HT1080 transduced with eGFP. Graphs show the eGFP percentage detected by flow cytometry in mixed cultures. Dilution showed are 1 HT1080 cell per 10,000 (red), 100,000 (blue) or 500,000 (green) hMSC generated from 3 different donors (PN17, PN32, PN33). Lower right panel show the mRNA relative expression of Cx43, quantificated by qRT-PCR, in short-term cultures of hMSC, postsenescent hMSC (MSC-PS) and TMC/HT1080 cultures.

A

	D3S1358		Vwa		D16S539		D2S1338		D8S1179		D2S11		D18S51		D19S433		TH01		FGA		XY	
MSC-16	14	15	17		12	13	23	25	13	15	31.2		12	13	14	15	7		21	23	X	Y
MSC-16 S	14	15	17		12	13	23	25	13	15	31.2		12	13	14	15	7		21	23	X	Y
MSC-16 PS	14	15	17		12	13	23	25	13	15	31.2		12	13	14	15	7		21	23	X	Y
TMC-14 1	16		14	19	9	12	25	26	13	14	28	30	12	18	13.2	15	6		22	25	X	
TMC-14 2	16		14	19	9	12	25	26	13	14	28	30	12	18	13.2	15	6		22	25	X	
TMC-14 3	16		14	19	9	12	25	26	13	14	28	30	12	18	13.2	15	6		22	25	X	Y
TMC-16 1	16		14	19	9	12	25	26	13	14	28	30	12	18	13.2	15	6		22	25	X	
TMC-16 2	16		14	19	9	12	25	26	13	14	28	30	12	18	13.2	15	6		22	25	X	
HT1080	16		14	19	9	12	25	26	13	14	28	30	12	18	13.2	15	6		22	25	X	Y
HT1080 eGFP	16		14	19	9	12	25	26	13	14	28	30	12	18	13.2	15	6		22	25	X	Y
A549	16		14		11	12	24		13	14	29		14	17	13		8	9.3	23		X	Y
Caco	13	17	16	18	12	13	17	25	12	14	30	32	12		14.2		6	7	19			
H441	17	18	17		9	13	17	19	8	14	32.2		18	19	14		7	9.3	24	25		
Hela	14	18	16	18	9	10	17		12	13	27	28	16		12.2	13.2	7		21		X	X
Saos	14	18	18		12	13	18		10	12	28	30	15		13		6	9	22	25		
T98G	16		17	20	23		19	24	13	14	28	32.2	13	16	12		7	9.3	21		X	Y
U87MG	16	17	15	17	12		20	23	10	11	28	32.2	13	14	14.2	15	9.3		18	28		

B

TMC-14	A*31DHJF,A*68EBCE	DRB1*03DRNV,DRB1*04DYMY	DRB3*01ANEX,DRB4*01ARYD
TMC-16	A*31DHJF,A*68EBCE	DRB1*03DRNV,DRB1*04DYMY	DRB3*01ANEX,DRB4*01ARYD
HT1080	A*31DHJF,A*68EBCE	DRB1*03DRNV,DRB1*04DYMY	DRB3*01ANEX,DRB4*01ARYD

C

HT1080	49-119, XY, der(5)t(5;5)(p15;?), der(11)t(3;11)(?;q25)
HT1080-eGFP	60-95, XY, der(5)t(5;5)(p15;?), der(11)t(3;11)(?;q25)