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Influence of Culture Environment and *Mollicutes* Contaminations on CD133 Modulation in Cancer Stem Cells

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1. Introduction

During last years great effort has been addressed towards the research area of cancer stem cells (CSC), also called tumour-initiating cells. The intriguing hierarchical model underlying the CSC theory, has been extensively described: it implies that only a very small cellular compartment within a neoplastic tissue is able to maintain indefinitely tumour growth (Bomken, 2010; Sottoriva, 2010). This cellular nucleus is made up of self-renewing stem cells, connoted by a more aggressive phenotype, that give rise to all differentiated populations forming the tumour (O'Brien, 2010; Zhou, 2009).

Increasing experimental evidences highlight the key role of CD133 (prominin-1) as CSC marker in several human cancers, such as colon carcinoma as well as neural and hepatic tumours (Vermeulen et al., 2008).

The aim of this section is to describe, in details, how environmental factors, culture conditions and mycoplasma infections can play a relevant role in CD133 modulation.

It was clearly demonstrated that environmental variables can determine a dramatic increase of CD133 expression in several human cellular models such as primary culture of tumor cells, neurosphere cultures and continuous cancer cell lines. In particular, it has been shown that a reduction in oxygen tension, during the culture, both in neurosphere cultures (Bar et al., 2010) and in human glioma cell lines (Soeda et al., 2009) can be promptly translated in a reversible over-expression of the stemness marker.

Recently, the involvement of transforming growth factor β (TGF β) in up-regulation of CD133 expression has been clearly demonstrated in human hepatoma, suggesting the epigenetic mechanism through TGF β influences CD133 modulation (You et al., 2010).

Additionally, we found evidence that the percentage of CD133+ cells in human colorectal cancer cell lines is considerably increased, in reversible manner, in presence of *Mycoplasma hyorhinis* infection (Mariotti et al., 2010).

In conclusion, all these observations suggest, the need to maintain CSC cultures in a suitable microenvironment, faithful to the hypoxic condition that is “physiologically” found in tumors. In addition, it is fundamental to handle cells in good tissue culture practices in order to prevent dangerous mycoplasma infections. In this respect, it is essential to constantly monitor contamination status of the cultures, by specific detection methods.

2. Cancer stem cells and CD133

Stem cells represent the cell population within a tissue, characterized by a longer lifespan compared to its more differentiated progeny. This feature makes progenitor cells more exposed to genotoxic damage, leading more easily to the induction of oncogenetic mutations, and so to cancer development (Pardal, 2003; Reya, 2001).

The CSC theory implies that cells in tumour have the same hierarchical organization that is generally present in the normal tissue counterpart. Therefore, CSC, can be considered the population responsible for maintaining and growing the tumour, while other cancer cells can only contribute to tumour bulk (Lobo et al., 2007).

The CSC population within a tumour, due to its “auto-protective” nature, is frequently resistant to chemotherapy and radiotherapy and thus it can be responsible for disease relapse, or for metastasis generation; for these reasons the targeting and the eradication of CSC represent the next frontier in cancer therapy (Dean et al., 2005).

Currently, there are increasing evidences that CSC, constitute a small compartment of distinct cells, characterized by a peculiar phenotype in comparison with other cells that form the tumour. Presently, one of the most investigated CSC markers is the CD133. This molecule, also called prominin-1, was the first to be identified in the family of prominin membrane proteins, both in humans and mice and it was initially classified as a marker of primitive haematopoietic and neural stem cells (Mizrak et al. 2008). From a structural point of view, CD133 is a cell surface glycoprotein, formed by five trans-membrane domains, two cytoplasmic loops, two glycosylated extracellular domains, and a cytoplasmic C-terminal domain (Corbeil, 2000; Miraglia, 1997; Yin, 1997). Biological function of CD133 has not yet been elucidated, but its peculiar localization suggests its involvement in the organization of plasma membrane protrusion (Maw et al., 2000). Recently, it has been confirmed that CD133 antigen plays a key role as CSC marker in several human cancers, such as colon (O’Brain, 2007; Ricci-Vitiani, 2007) and hepatocellular (Yin, 2007; Zhang, 2011) carcinoma as well as neural (Pallini, 2011; Singh, 2004) and renal (Bruno, 2006; Florek, 2005) tumours. In particular, it has been demonstrated the ability of CD133 positive cellular fraction, isolated from human tumours, (i) to proliferate *in vitro*, (ii) to differentiate *in vitro* and (iii) to develop a neoplasia with the original phenotype, when it is transplanted in immunodeficient mice, unlike its CD133 negative counterpart (Neuzil, 2007; Singh, 2003).

In conclusion, CSC can be purified on the basis of their specific cell membrane immunophenotypes and in this context, the correct individuation of a peculiar antigenic paradigm can become a fundamental target for CSC detection and isolation, aimed at the development of future innovative therapeutic strategies.

3. Culture environment and CD133 modulation sustained by *Mollicutes*

The employment of human continuous cell lines is essential for studying biological and functional alterations of tumour cells (Hayashi, 2011; van Staveren, 2009). In the previous

paragraph, the relevance of CD133 marker as well as its role in detecting and isolating CSC from several kinds of human solid tumour has been elucidated. Unfortunately, the correct determination of CD133 expression intensity, on cellular surface, can be prejudiced by several environmental factors. Our aim is to describe how modifications of culture microenvironment, induced by a strong reduction in oxygen tension or growth factors exposure or even by *Mollicutes* infections, can play an important role resulting in a dramatic increase of CD133 marker expression. This occurrence has been shown in primary culture of tumour cells (Sheehan, 2011; Soeda, 2009), neurosphere cultures (Bar et al., 2010) and in several human cellular models such as continuous cancer cell lines.

3.1 Culture environment

In standard culture conditions, cancer cells are exposed to the ambient oxygen tension, that is of about 20%. This percentage is far from the mean oxygen tension generally described for *in vivo* tissues, that ranges from 2% to 9% (Brahimi-Horn, 2007; Studer, 2004). Thus, in conventional cell cultures, the oxygenation parameter does not reflect a physiological condition and it can be responsible for function alterations, decrease of proliferation and loss of stem cells undifferentiated state (Ezashi, 2005; Mohyeldin, 2010).

These negative effects of hyperoxygenation on stem cells can be explained by the generation of dangerous reactive oxygen species, that in turn can cause aberration in DNA structure (Busuttill et al., 2003); on the other hand, it has been demonstrated the involvement of hypoxia in the activation of the molecular pathways for the regulation of two fundamental modulators of stemness, such as Oct-4 and Nocht (Simon & Keith, 2008).

Interestingly, the hyperoxic condition can reversibly affect the CD133 intensity of expression in neurosphere (Bar et al., 2010) and human glioma cell lines cultures (Soeda et al., 2009). In particular, human primary tumor neurosphere culture exhibited higher CD133 percentage at 3% of oxygen compared to control culture maintained at 20% of oxygen (Platet et al., 2007); in addition, in hypoxia that is about 1% of oxygen, the human U251MG glioma cells showed a time dependent CD133 increase, that was reversed when cells were exposed to standard normoxic conditions that is about 21% of oxygen (Griguer et al., 2008).

These important data have methodological and conceptual implications. Firstly, the standard culture condition, under atmospheric oxygen, could represent one of the variables involved in the decrease of CD133 cellular phenotype; secondly, CD133 up-regulation could be interpreted as an event occurring when tumor cells are exposed to a non-physiological condition or rather as a stress response.

The environment necessary to obtain stem cells cultures requires several cytokines and growth factors use. In this regard, it was demonstrated that TGF β is involved in the up-regulation of CD133 expression in the Huh7 continuous cell line, a model for studying human hepatocellular carcinoma. Unexpectedly, TGF β influenced the marker expression in a dose- and time-dependent manner, triggering specific epigenetic events such as the inhibition of DNA methyltransferase 1 and DNA methyltransferase3 β expression, and subsequent demethylation of promoter-1 (You et al., 2010).

Finally, it was also established that epidermal growth factor (EGF) heightened the CD133-positive subpopulation in primary brain tumor stem cell cultures, determining an increase of the stemness marker in a growth factor concentration-related manner (Soeda et al., 2008).

3.2 *Mollicutes* contaminations

Mollicutes contamination still remains one of the major problems tightly correlated to the manipulation of human continuous cell lines. *Mollicutes* infections are extremely dangerous for two main aspects. Firstly, the contaminations are not evident and they can be revealed exclusively by the application of specific detection techniques, such as bioluminescence assays (Mariotti et al., 2008), Polymerase Chain Reaction (PCR), by using specific primer sequences (Uphof, 2002; Shahhosseiny, 2010) and aerobic and anaerobic microbiological agar culture (Uphoff et al., 1992). Secondly, the chronic infections can determine several negative consequences on the cultures, including significant morphological changes, induction of chromosomal aberrations, modification in protein concentration and alterations in DNA and RNA synthesis (Drexler, 2002; Uphoff & Drexler, 2005). In the most serious events *Mollicutes* contaminations lead to the production of unreliable data or to the irreversible lost of the culture itself (Drexler & Uphoff, 2002).

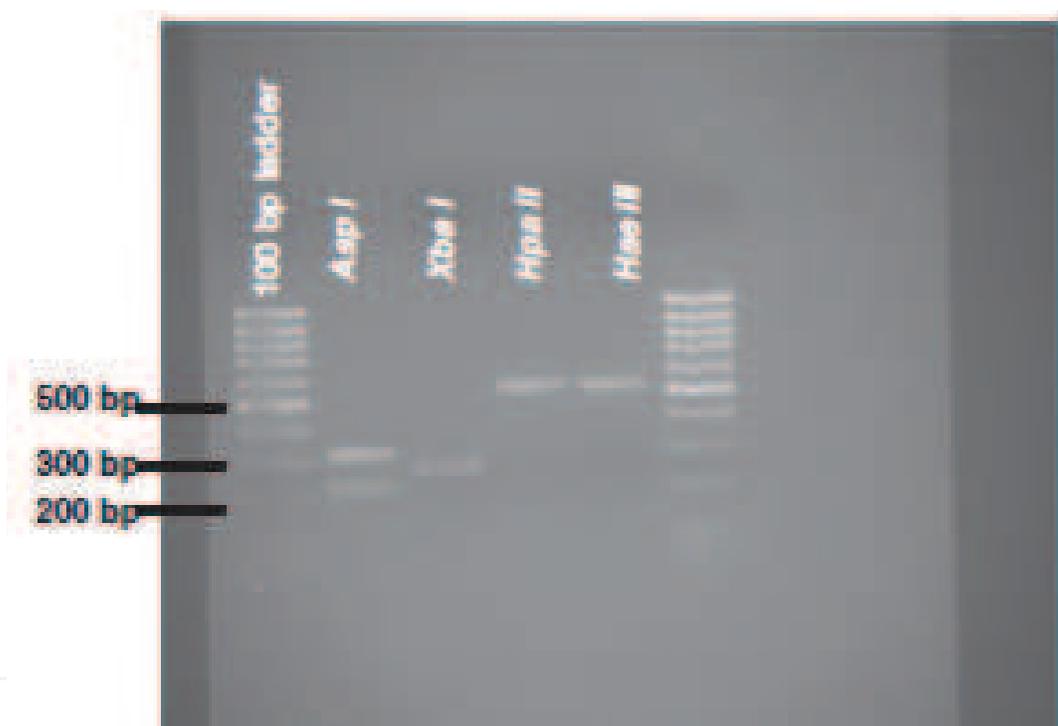


Fig. 1. Agarose gel with the pattern of restriction fragment length polymorphisms that demonstrates the presence of *Mycoplasma hyorhinitis* species in cell culture supernatants

In this context, our group demonstrated the heavy influence of *Mycoplasma hyorhinitis* contamination on CD133 expression in the CSC colon carcinoma compartment (Mariotti et al., 2010). In particular, our research project was focused on the isolation and characterization of CD133 positive CSC fraction, belonging to the three human colorectal cancer continuous cell lines such as GEO, SW480 and HT-29. All cell lines, as suggested by good tissue culture practice (UKCCR, 2000), were tested for *Mollicutes* contaminations by two independent methods: the biochemical assay MycoAlert® (Cheong, 2010; Mariotti, 2008) and the aerobic and anaerobic microbiological agar culture (Young et al., 2010). All supernatants, derived from the three tested cultures, resulted unequivocally *Mollicutes* positive to the above mentioned applied assays. With the aim of establishing the species of

the *Mollicutes* contaminant, we performed on every continuous cell line culture the extraction of mycoplasmatic DNA, the amplification by PCR using specific primer sequences and the digestion of the amplicons with the restriction endonucleases Asp I, Xba I, Hpa II and Hae III. Finally, the analysis of modified restriction fragment length polymorphisms (Uphoff & Drexler, 2005) demonstrated that GEO, SW480 and HT-29 cell lines were infected by *Mycoplasma hyorhinitis*, as shown in figure 1.

It is well known that this mycoplasma species is one of the most common infectious agent found in continuous cell lines, with a frequency of contamination ranging from 10% to 40%. *Mycoplasma hyorhinitis* is a frequent pathogenic agent, isolated from the upper swine respiratory tract (Friis & Feenstra, 1994) even if it is thought to derive from bovine source. In fact, as swine and cattle are processed through the same abattoirs, the swine strain of *Mycoplasma hyorhinitis* may be introduced into bovine serum, commonly used in cell culture practice (Drexler & Uphoff, 2000).

All cell lines for CD133 cytometric expression were then characterized, in confluent culture of *Mycoplasma hyorhinitis* GEO, SW480 and HT-29 infected cells, after adequate detachment with trypsin/EDTA. The monoclonal antibody used in the study was the anti-CD133/1-PE, AC133 clone purchased from Miltenyi Biotec company; an unstained control was prepared for each cell line in order to establish the degree of background cellular autofluorescence (Figure 2, panel A). All samples were analyzed by a FACSAria flow cytometer with the FACS-Diva software.

As shown in panel B of figure 2, we observed the marked separation of the CD133 positive CSC population that was equal to 5.7%, 52.5% and 92.5% in case of GEO, SW480 and HT-29 mycoplasma infected cell lines, respectively.

After mycoplasma decontamination, according to the United Kingdom Coordinating Committee on Cancer Research guidelines (UKCCR, 2000), CD133 expression was re-evaluated in the rescue cell lines, in another set of cytometric experiments. As evidenced by panel C of figure 2, a noteworthy decrease in CD133 expression occurred, resulting in 2.3%, 0.2% and 75.6% in case of GEO, SW480 and HT-29, respectively. These interesting data confirmed the reversibility of the surprising antigenic variation.

In order to confirm the exclusive *Mycoplasma hyorhinitis* ability to increase the percentage of CD133 positive cells in human colon carcinoma cell lines, we re-infected GEO model by aliquots of the same contaminant purchased from the American Type Culture Collection (ATCC) international cell bank.

On the day of the inoculation, the medium was completely replaced with fresh medium containing an established dilution of viable *Mycoplasma hyorhinitis*, at a concentration of about 10 Colony Forming Unit/ml. The infected cells were then incubated at 37°C and 5% CO₂ and tested with MycoAlert® every seven days. Only in case of positive results they were detached with trypsin/EDTA and processed by flow cytometry, for the evaluation of CD133. The contamination was detectable only after 3 weeks of culture exposition to the specific infective agent.

This experiment set confirmed that the increase of CD133 expression was closely related to *Mycoplasma hyorhinitis* presence in GEO cells, and demonstrated that this “side effect” became more severe with the chronicization of the infection. In particular, 3.4%, 4.3%, 26.5% and 37.2% CD133 positive cells were found on days 21, 28, 49 and 56 post infection respectively, whereas 0.3%, 0.7%, 0.8% and 0.9% were detected on days 21, 28, 49 and 56 respectively, in *Mycoplasma hyorhinitis* free GEO cells (figure 3 panel C and B).

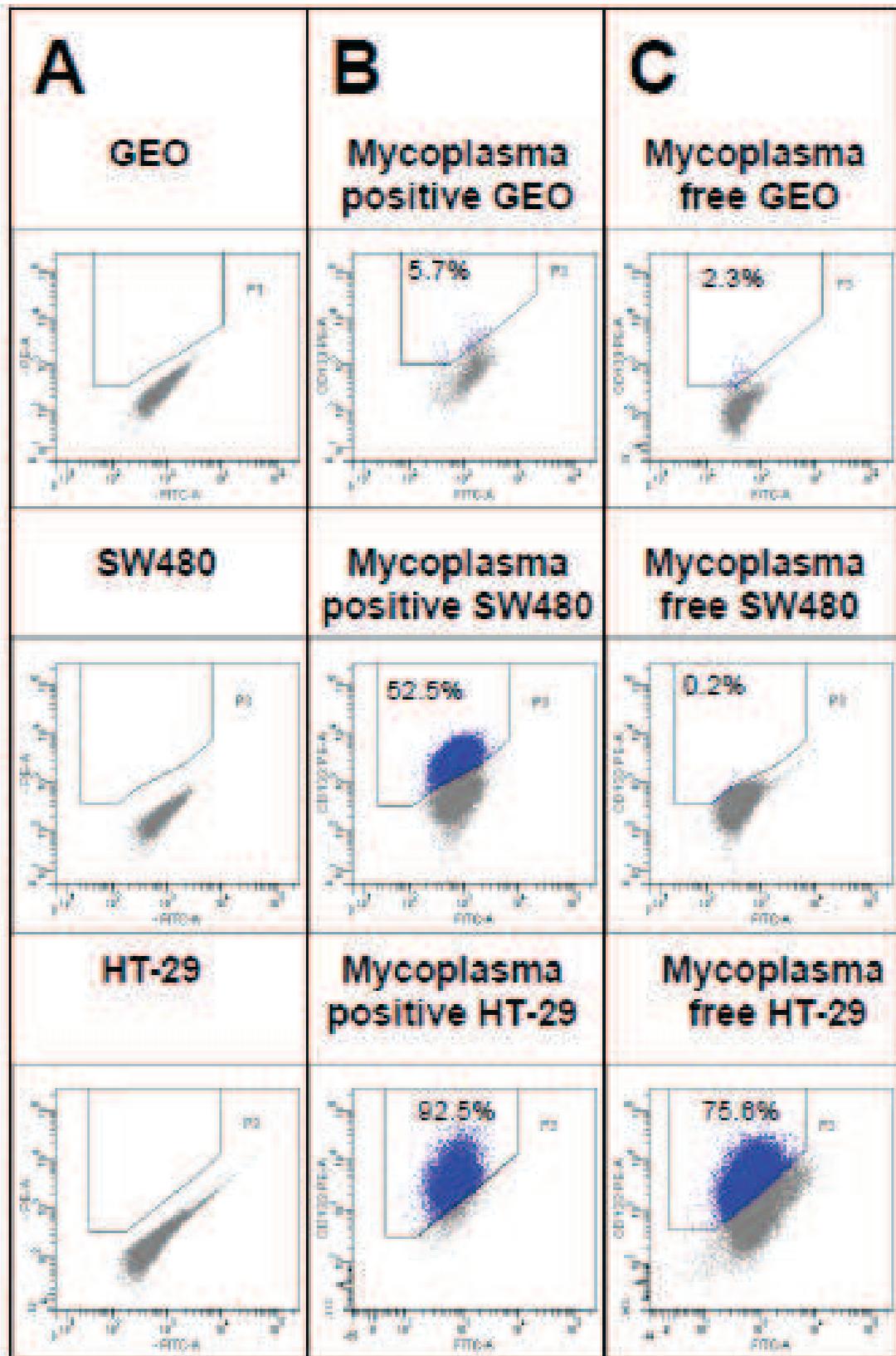


Fig. 2. Cytometric evaluation of CD133 expression in GEO, SW480 and HT-29 cell lines in presence of *Mycoplasma hyorhinis* (panel B), or decontaminated (panel C). Panel A represents the negative control

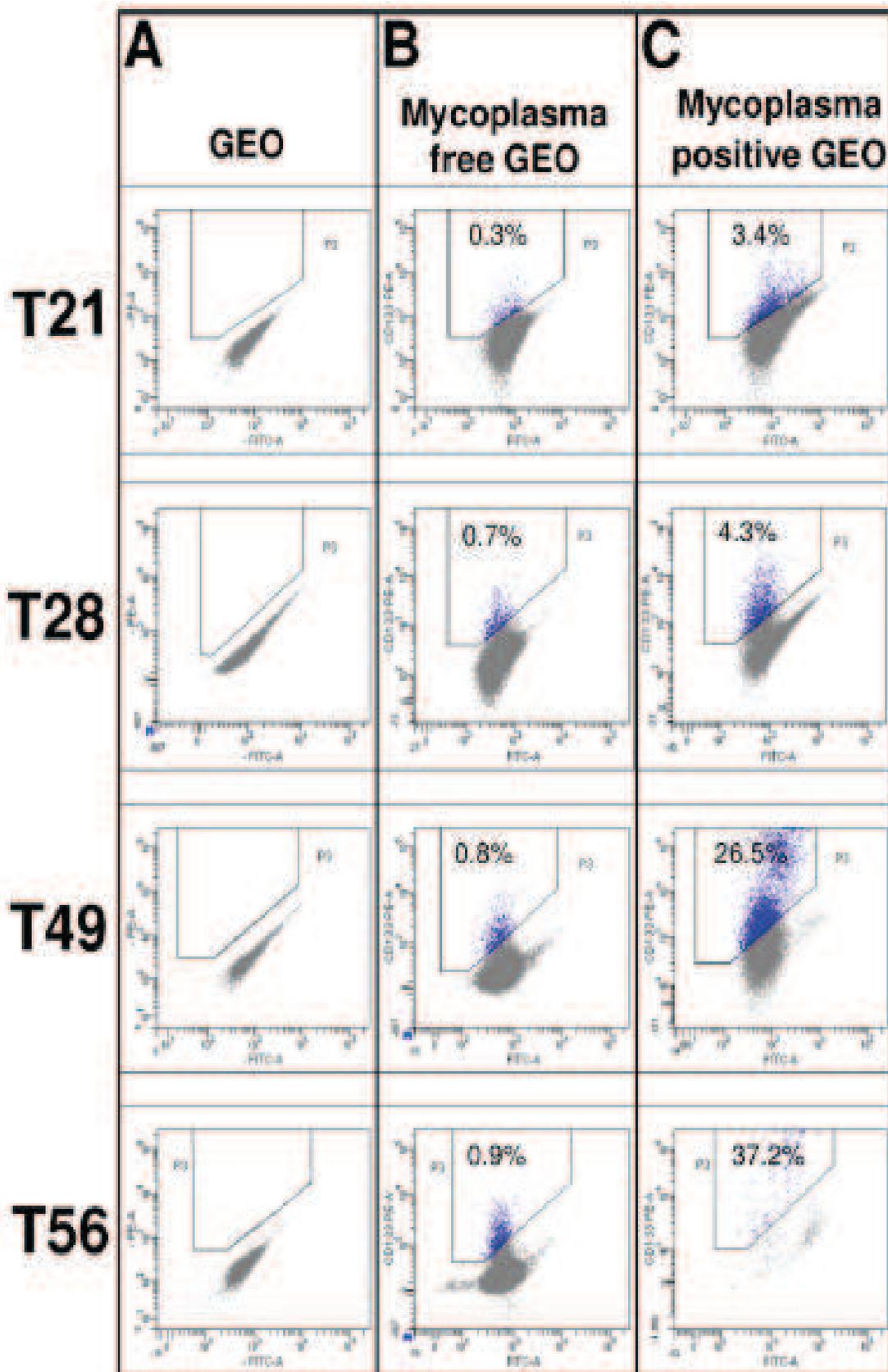


Fig. 3. Cytometric evaluation of CD133 expression in mycoplasma free GEO cell lines (panel B), and (panel C) infected. Panel A represents the negative control

In recent times, the direct *Mycoplasma hyorhinitis* involvement in carcinogenesis of human prostatic cells has also been shown (Namiki et al., 2009) as well as its significant role in the increase of mesenchymal stromal cells anti-proliferative action in standard lymphocyte proliferation assay (Zinocker et al., 2011).

4. Conclusion

The aim of this section is to describe the relevant role of environmental factors, culture conditions and *Mollicutes* contamination in CD133-positive CSC compartment.

First of all, even though standard culture condition implies the use of about 20% of oxygen tension, this “non-physiological condition” seems to be involved in the decrease of CD133-positive CSC cellular fraction. It is important to highlight the need to maintain CSC cultures in a more suitable microenvironment, faithful to the hypoxic condition that is “physiologically” found in tumors. This is desirable in order to work with an *in vitro* cellular model that is, as similar as possible to the original tumor from a biologic and physiologic point of view.

In addition, growth factors as TGF β and EGF are able to increase CD133 expression in CSC population in a time- and concentration-dependent manner. It is evident that a culture medium containing much more appropriate concentrations of cytokines and growth factors could be another fundamental tool that enables CSC obtained *in vitro* to show a phenotype which can be better compared to the fresh tumor.

Finally, it is interesting to notice the effects exerted by *Mycoplasma hyorhinitis* infection on the up-regulation of CD133 positive CSC fraction. This occurrence may be justified by: i) the influence of the contaminant on monoclonal antibody binding sites, or ii) the selective pressure on the CD133-positive CSC fraction, in response to mycoplasma infection. We further demonstrated that the extent of CD133 modulation grew with the increase of the exposure time to the specific infectious agent.

We are confident that a tighter control of microenvironmental variables combined with prevention measures, detection methods and eradication of *Mollicutes* infections, will be an integral part of the basic panel of CSC manipulation techniques.

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Over the last thirty years, the foremost inspiration for research on metastasis, cancer recurrence, and increased resistance to chemo- and radiotherapy has been the notion of cancer stem cells. The twenty-eight chapters assembled in *Cancer Stem Cells - The Cutting Edge* summarize the work of cancer researchers and oncologists at leading universities and hospitals around the world on every aspect of cancer stem cells, from theory and models to specific applications (glioma), from laboratory research on signal pathways to clinical trials of bio-therapies using a host of devices, from solutions to laboratory problems to speculation on cancers' stem cells' evolution. Cancer stem cells may or may not be a subset of slowly dividing cancer cells that both disseminate cancers and defy oncotoxic drugs and radiation directed at rapidly dividing bulk cancer cells, but research on cancer stem cells has paid dividends for cancer prevention, detection, targeted treatment, and improved prognosis.

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