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Mycoplasma Infection Alters Cancer Stem Cell Properties in Vitro

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Abstract

Cancer cell lines can be useful to model cancer stem cells. Infection with *Mycoplasma* species is an insidious problem in mammalian cell culture. While investigating stem-like properties in early passage melanoma cell lines, we noted poorly reproducible results from an aliquot of a cell line that was later found to be infected with *Mycoplasma hyorhinis*. Deliberate infection of other early passage melanoma cell lines aliquots induced variable

and unpredictable effects on expression of putative cancer stem cell markers, clonogenicity, proliferation and global gene expression. Cell lines established in stem cell media (SCM) were equally susceptible. *Mycoplasma* status is rarely reported in publications using cultured cells to study the cancer stem cell hypothesis. Our work highlights the importance of surveillance for *Mycoplasma* infection while using any cultured cells to interrogate tumor heterogeneity.

Keywords

Mycoplasma

Cancer stem cells

Cancer cell lines, CD133

Abbreviations

CSC Cancer stem cell

SCM Stem cell media

Introduction

The cancer stem cell (CSC) hypothesis (Ailles and Weissman 2007) posits that intratumoral epigenetic heterogeneity is hierarchical, and is ideally studied using fresh human tumors. Cancer cell lines are useful models to supplement the low availability of fresh *ex vivo* samples, and though older cell lines have been criticized as models for CSC (van Staveren et al. 2009), early passage cell lines are very useful as they maintain *ex vivo* characteristics in vitro (Pollard et al. 2009). Occult infection of mammalian cell lines by *Mycoplasma* species is pernicious and widespread. The detection (Uphoff and Drexler 2005a) and eradication (Uphoff and Drexler 2005b) of *Mycoplasma* infection have vastly improved since identification of this problem 50 years ago. While the prevalence of *Mycoplasma* cell line infection was as high as 80 % (Drexler and Uphoff 2000), contemporary estimates of 15–30 % may be more accurate (Timenetsky et al. 2006). Up to 7 % of samples in the Thousand Genome Project are infected (Langdon 2014). Infection with *Mycoplasma* is frequently occult, and while the influence of infection on cellular behavior and gene expression can be substantial (Miller et al. 2003), infections are eminently

curable (Romorini et al. 2013).

During our examination of stem-like properties in early passage melanoma cell lines (Aggelis et al. 2009) we noted inconsistent results from an aliquot of one cell line, which was discovered to be infected by *Mycoplasma*. Infection appeared to induce the expression of the putative CSC marker CD133. Eradication of *Mycoplasma* infection resulted in a complete loss of CD133 expression and markedly altered proliferation and clonogenicity. We deliberately infected other early passage melanoma cell lines grown in quarantine to safely compare with uninfected samples, finding diverse and unpredictable effects on stem-like properties.

Materials and Methods

The LM-Mel-14, -24 and -34 cell lines established in our laboratory (Gibbs et al. 2000) were cultured and passaged as previously described (Gedye et al. 2009). The LM-Mel-53, -56, -62 and -93 cell lines were established *de novo* from patient tumors in stem cell media (SCM) as previously described (Anaka et al. 2012). All work was under approval of the Austin Health Human Research Ethics Committee. Cell line identity was established by HLA typing versus patient's peripheral blood haplotype (Victorian Transplantation and Immunogenetic Service, Australian Red Cross Blood Service, Melbourne, Australia) and verified routinely using STR profiling (QIMR Berghofer Scientific Services Support Unit, Herston, Queensland.

Australia). *Mycoplasma* infection was detected by the MycoAlert Kit (Lonza), and by nested polymerase chain reaction detecting the 16S rRNA sequence expressed by the eight most common *Mycoplasma* species (Wong-Lee and Lovett 1993). DNA was extracted using QIAamp DNA Mini Kit (QIAGEN) from cells grown to confluence in antibiotic-free media. DNA template or water controls (5 µL) were incubated with 10X buffer (2.5 µL), MgCl₂ (2 µL), dNTPs (2 µL), PCR water (12.875 µL), AmpliTaq Gold (0.125 µL; Applied Biosystems) and forward and reverse primers (forward 5'-GGCGAATGGGTGAGTAACACG-3' and reverse 5'-CGGATAACGCTTGCGACCTATG-3'; 0.5 µL of 100 mM stocks; Sigma-Genosys). *Mycoplasma* infection was eradicated in the LM-Mel-24 cell line using BM Cyclin (Roche Applied Sciences) according to a modification

(Uphoff and Drexler 2005b) of the manufacturer's instructions. 50 μ L of 0.22 μ m filtered conditioned media from the infected cell line was inoculated into quarantined flasks of other cell lines to cause infection. All replicates were performed in parallel with clean and infected cells at matching passage numbers, the same lot of fetal calf serum, with cells at 70–80 % confluence. Flow cytometry, magnetic cell separation, immunohistochemistry, colony formation assays, proliferation assays and mRNA isolation were performed as described (Aggelis et al. 2009). Expression-level data were generated on Affymetrix Human Genome U133 Plus 2.0 arrays and processed using the Bioconductor framework. Data was RMA-normalized with additional presence/absence calls based on MAS5. Pairwise comparison of individual arrays identified genes with a fold change >1.5 in at least one probeset. Probesets not called present in at least one of the paired arrays were discarded for fold change calculations. Functional changes associated with identified genes were determined by a DAVID analysis (Dennis et al. 2003; Huang et al. 2009) using default parameters restricted to the 'Biological Pathway' category of Gene Ontology. Gene Ontology terms exhibiting a p -value change of more than three orders of magnitude were retained.

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Results

Use of a dedicated treatment and testing facility ensured that no cross-infection occurred. Two weeks after deliberate inoculation *Mycoplasma* was detectable in all infected cultures (Fig. 1 a and b), but morphology and viability were unchanged (Fig. 1 c). Neither assay is quantitative but the burden of infection in LM-Mel-34 appeared lowest. *Mycoplasma* infection had an unpredictable effect on CD133 expression in melanoma cell lines (Fig. 1 d), suppressing (LM-Mel-14), inducing (LM-Mel-24) or having little discernable effect (LM-Mel-34) on CD133 expression. The infected LM-Mel-24 cell line showed isolated cells staining positively for CD133, but complete absence of immunoreactivity after eradication of *Mycoplasma* infection (Fig. 1 e). "CD133+" LM-Mel-24 cells could be enriched by serial positive selection (MACS cell separation followed by re-culturing; Fig. 1 f bottom); however, after *Mycoplasma* eradication, no residual CD133 immunoreactivity could be detected in LM-Mel-24 *Mycoplasma*-negative cultures despite multiple MACS enrichment and re-culture steps (Fig. 1 f, top). *Mycoplasma* infection increased clonogenicity in

melanoma cell lines (Fig. 2a), but this change did not correlate to CD133 expression (Fig. 1d). *Mycoplasma* infection generally increased proliferation (Fig. 2b, c and d), but again this was not proportional to changes induced in CD133 expression.

Fig. 1

a MycoAlert testing showing clean and deliberately infected cell lines with a ratio of >1.2. **b** *Mycoplasma* 16S rRNA DNA sequence PCR. Note a faint positive band of residual DNA following treatment and eradication of *Mycoplasma* infection in the LM-Mel-24 cell line, negative on MycoAlert and subsequently negative on repeat PCR (data not shown). **c** The morphology of clean (top) and *Mycoplasma* infected melanoma cell lines (bottom) is indistinguishable. **d** *Mycoplasma* infection unpredictably alters CD133 expression with decrease of CD133 in one cell line (LM-Mel-14), increase in another cell line (LM-Mel-24) but little effect in LM-Mel-34. **e** CD133 expression was initially detectable in scattered LM-Mel-24 cells by immunohistochemistry, but upon *Mycoplasma* eradication (clean, top) CD133 immunoreactivity was completely lost (magnification 200×, αhE2 antibody). **f** Infected LM-Mel-24 cells could be serially enriched for CD133⁺ (orange) and CD133^{neg} (blue) cells, but after *Mycoplasma* eradication (top panel), and despite multiple attempts to enrich by serial MACS enrichment and re-culture steps, no CD133⁺ cells could be detected in LM-Mel-24 *Mycoplasma*-negative cells

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Fig. 2

Mycoplasma infection variably influences clonogenicity and proliferation in melanoma cell lines. **a** Clonogenic frequencies in soft agar of clean versus infected cells for LM-Mel-14, LM-Mel-24 and LM-Mel-34 (representative of duplicate experiments). **b-d**. *Mycoplasma* infection appeared to generally increase proliferation in melanoma cell lines. Proliferation was measured by formazan production in clean (*squares*) versus infected (*triangles*) cells in the LM-Mel-14, LM-Mel-24 and LM-Mel-34 cell lines. Proliferation was strongly increased in some cell lines (LM-Mel-14), but more modestly influenced in others (LM-Mel-24 and LM-Mel-34)

LM-Mel-14 and LM-Mel-34 seemed most and least perturbed to *Mycoplasma* infection respectively, suggesting that infection may be “discountable” in some lines that are in some way “resistant to infection”. We examined global gene expression in CD133⁺ enriched and CD133^{neg} depleted cell populations from infected LM-Mel-34 cells with comparison to data generated on identical microarray analysis platforms from passage matched clean LM-Mel-34 CD133⁺ and CD133^{neg} cell populations. *Mycoplasma* infection was associated with significant (>3-fold) differential gene expression (Fig. 3 a, c), in Gene Ontology biological programs such as metabolic activity, biogenesis, cellular component organization, cell cycle activity and apoptosis in *Mycoplasma* infected versus clean cell populations (Fig. 3 e). Infection was more pertinent than passage number (Fig. 3 b,d).

Fig. 3

Mycoplasma infection induces substantial differential gene expression in LM-Mel-34 melanoma cells. Affymetrix U133A microarray analysis of gene

expression showed that *Mycoplasma* infection caused significant differential gene expression profile changes in infected CD133^{+/neg} cells compared to matched-passage number CD133^{+/neg} cells (**a** and **c**) in contrast to more stable expression profiles in clean CD133^{+/neg} cells versus alternate passage cells (**b** and **d** respectively). **e** Gene ontology analysis showed evidence of increased metabolic activity, biogenesis, cellular component organization, cell cycle activity and apoptosis in *Mycoplasma* infected versus clean cell populations (y axis shows fold change, only >3-fold differences retained). Thus even in the LM-Mel-34 cell line with the “least” subjective biological distortion, *Mycoplasma* infection still causes wide-ranging, significant and non-discountable differential gene expression

Melanoma cell lines established *de novo* in SCM (Anaka et al. 2012) typically grow as loose spheroids rather than as adherent plaques. These *de novo* lines were easily infected by *Mycoplasma* inoculation (Fig. 4a), which was occult in most lines (Fig. 4b), though one line showed growth arrest and adherent morphology.

Fig. 4

Mycoplasma infects cell lines grown *de novo* in SCM (a), causing little apparent effect in most cell lines (b). However in one cell line (LM-Mel-53) cell growth arrested and cells became adherent and fibroblastic in morphology while in another cell line (LM-Mel-93) clusters became tighter and appeared to increase in volume

Conclusions

Mycoplasma infection has significant, unpredictable, internally inconsistent and non-discountable effects on stem cell like properties in melanoma cell lines, even in apparently “resistant” cell lines. *Mycoplasma* infection causes pleomorphic effects in culture, from apoptosis to enhanced tumorigenicity and

invasiveness (Liu et al. 2006), and can cause considerable waste of resources and confusion in interpretation of experimental results (Callaway 2014). *Mycoplasma* spp. may directly reduce tetrazolium to formazan (Kirchhoff et al. 1992), which may partially account for MTT assay alterations in infected cell cultures. We presume *Mycoplasma* was accidentally historically inoculated from an infected cell line as this is the most common route of contamination (Drexler and Uphoff 2000). All cell lines in our laboratory undergo routine surveillance and remain free of *Mycoplasma* infection. The infecting organism was speciated as *M. hyorhina* (data not shown), which expresses p37, which can induce malignant phenotypes in non-transformed cells (Logunov et al. 2008). *Mycoplasma* may also influence the epidermal growth factor receptor (Gong et al. 2008) and bone morphogenetic protein pathways (Jiang et al. 2008) potentially encouraging host cells survival, and accounting for the generally improved growth observed. This parallels viral infections such as EBV (Kondo et al. 2011), HCV (Ali et al. 2011) and HPV (McLaughlin-Drubin et al. 2011) which can also directly promote self-renewal, survival and “cancer stem cell”-like behavior in mammalian cells. *Mycoplasma* infection has been reported to influence CD133 expression (Mariotti et al. 2010) but only a handful of “cancer stem cell” publications report routine *Mycoplasma* surveillance in cell lines. Our data reinforce the importance of *Mycoplasma* surveillance when utilizing cell lines to study tumor heterogeneity.

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Author Contributions Craig Gedye: Conception and design, collection and/or assembly of data, analysis and interpretation, manuscript writing, final

approval of manuscript.

Tracy Cardwell: Collection and/or assembly of data.

Nektaria Dimopoulos: Collection and/or assembly of data.

Bee Shin Tan: Collection and/or assembly of data.

Heather Jackson: Collection and/or assembly of data.

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Matthew Anaka: Collection and/or assembly of data, data analysis and interpretation.

Andreas Behren: Collection and/or assembly of data, data analysis and interpretation.

Christopher Maher: data analysis and interpretation.

Oliver Hofmann: data analysis and interpretation.

Winston Hide: Conception and design, provision of study materials.

Otavia Caballero: Conception and design, provision of study materials, final approval of manuscript.

Ian D. Davis: Conception and design, provision of study materials, final approval of manuscript.

Jonathan Cebon: Conception and design, financial support, provision of study materials, final approval of manuscript.

Compliance with Ethical Standards

Conflict of Interest We declare that we have no potential conflicts of interest in this work.

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