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Mycoplasma contamination of murine embryonic stem (mES) cells: sensitivity of detection, effects on cytogenetics, germ line transmission and chimeric progeny

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Mykoplasmakontamination von murinen embryonalen Stammzellen: Nachweissensitivität, Einfluss auf Zytogenetik, Keimbahntransmission und Chimärennachkommen

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For ***Georg, Heidi, Chriso*** and ***Andreas***…….

„Με όλη μου την αγάπη, σας ευχαριστώ…“

Table of Contents

1. INTRODUCTION

In this thesis, the sensitivity of four methods for the detection of mycoplasma contamination and the effects of mycoplasmas on the germ line transmission (GLT) of murine embryonic stem (mES) cells and the phenotypes of the chimeras were investigated.

I. Sensitivity of the detection of mycoplasma contamination in cell cultures

Since the first report of the presence of mycoplasmas in cell cultures in the mid 1950's (Robinson 1956), a consolidation of information has been published, concerning new mycoplasma species, their habits, properties and their interaction with cells *in vitro* (Sethi 1972, Barile 1979, McGarrity 1985, Muhlradt 1991, Tsai 1995, Baseman 1997, Razin 1998, Deiters 1999, Rottem 2003, Zhang 2004, Yavlovich 2001, Yavlovich 2006, Zhang 2006). The presence of mycoplasmas can influence the growth and effect morphological, biochemical, immunological and genetically properties of the cells, and therefore endanger the interpretation of the results of biological experiments, as well as the quality of biopharmaceutical products thus resulting in a loss of time, materials and products (Tully 1996). For this reason, European Pharmacopoeia and Federal Drug Administration recommend frequent examination of cell cultures for mycoplasmas, in order to provide quality and safety of products (CBER/FDA 1993, Pheur 2004). In cell cultures, the incidence of single mycoplasma contamination is still high, being 15 to 35% worldwide with extreme incidences of 65 to 80%, whereas multiple mycoplasma infections with 2 or more mycoplasma species are between 7 and 60% (Drexler 2002, Uphoff 2002, Timenetsky 2006). Sources for mycoplasma contaminations in cell cultures are laboratory personnel, contaminated serum, media and already contaminated cell cultures. Among the 20 mycoplasma species that have been isolated from contaminated cell cultures, the species that are found in human are responsible for more than half of all mycoplasma infections (Drexler 2002). These include *M. hominis*, *M. fermentans* and *M. orale*. Mycoplasmas differ from other bacteria found in cell cultures. They could be present without causing any visible changes in cell cultures such as turbidity or change of colour of the media. Due to their lack of a rigid cell wall they are resistant to penicillin and streptomycin, two of the most common

antibiotics used for routine cell culture work. In addition due to their small size conventional microscopy does not detect them.

Over the years, a number of techniques for the detection of mycoplasmas have been developed. These include agar culture, culture of samples on indicator cell lines and DNA fluorochrome staining, biochemical cytotoxicity assay, enzyme-linked immunosorbent assay (ELISA), DNA-RNA hybridization, one step- and nested-PCR, PCR-ELISA, and real-time PCR (McGarrity 1985, Johansson 1990, Uphoff 1992, Uphoff 1992, Hopert 1993, Tully 1996, Uphoff 2002, Uphoff 2002, Garner 2000, Harasawa 2005, Ishikawa 2006). Many of these methods are time-consuming, complex, subjective, detect only a restricted number of mycoplasma species, and some of them appear to be marginally useful (Uphoff 1992, Uphoff 1992).

An ideal detection method should not only be highly sensitive but also simple, rapid, efficient, and cost effective. As such there is still a lack of a suitable method which fulfils all the criteria for routine diagnostic work (Drexler 2000, Drexler 2002, Uphoff, 2002).

In the first part of the thesis, the objective was to determine the sensitivity of two commercial assays, the newly developed MycoAlert™ and MycoSensor™ QPCR assays in detecting *M. hominis, M. fermentans* and *M. orale* and to compare the results with those obtained by agar culture and an in-house established gel-based PCR. Ten-fold serial dilutions of fresh supernatants and cell suspensions of L929 fibroblast cells were analyzed for this purpose. This step was important for reliable detection of mycoplasmas in the ES cell cultures.

II. The influence of mycoplasma species on the germ line transmission of murine embryonic stem cells and phenotypic analysis of chimeras

The use of pluripotent mES cells increased within the last years. In biomedical research, mES cells are used for the production of transgenic and knock-out mice which serve as animal models for human and animal diseases, in studies for investigating embryonic development and for *in vitro* studies of cell systems established after their spontaneous differentiation into well-defined somatic cell types (Rohwedel 1996, Wobus 2005). Additionally, mES cells are used for studies in pharmacology, embryotoxicology, and cell transplantation and replacement therapy (Wobus 2005, Cormier 2006). Due to the high demand of mES cells which results in

extensive and uncontrolled exchange between laboratories worldwide, the risk of contaminations may be high. In contrast to human ES cell lines which are tested for pathogens prior to their use, animal ES cell lines are often used without determination of their microbiological status. As a result, contaminated mES cell lines could lose their pluripotential properties and the ability to contribute to the germ line, becoming worthless for studies, or they could lead to contamination of other laboratories and animal facilities (Nicklas 2000, Nagy 2003).

In the second part of the thesis, the objective was to determine the effects of mycoplasmas on the TBV2 mES cell line (129/SvPas) during *in vitro* culture, the ability to contribute to the germ line, the phenotype of chimeras and their progeny. As such, TBV2 mES cells in the 13th passage (P13) were inoculated with the human species *M. hominis*, *M. fermentans* and *M. orale* to establish a multiple mycoplasma infection. The mES cells were cultured over 20 passages and the growth rate, the viability and the susceptibility of mES cells to mycoplasmas were determined. The karyotype and the differentiation status of mycoplasma-infected and control mES cells were determined at different passages via Spectral Karyotyping (SKY) and Fluorescence Activated Cell Sorting (FACS), respectively. Furthermore, mycoplasma-infected and control mES cells were injected into Crl:CD1 (Icr) blastocysts for the production of chimeras. The phenotype of the resulting chimeras from mycoplasma-infected litters was described based on clinical, morphological, histological, and immunological examinations. The contribution to the germ line was evaluated after mating chimeras produced with C57BL/6 mice and scoring the number of germ line pups.

2. LITERATURE REVIEW

2.1 Mycoplasmas

Mycoplasmas were reported for the first time in 1898 by Nocard and Roux after the successful cultivation of the causative agent of contagious bovine pleuropneumonia (Nocard 1990). The name “mycoplasma” (from the greek words “mykes” which means fungus and “plasma” which means formed) was coined in the 1950s by Edward and Freundt (Edward 1956) replacing the term “pleuropneumonia-like organisms” (PPLO). It was used to describe the only known mycoplasma at that time, the bovine species *M. mycoides*, which has a fungus-like growth pattern, being unique for this mycoplasma species. In 1937, Dienes and Edsall isolated from a gland abscess the first human mycoplasma species, probably the now known *M. hominis* (Dienes 1937, Tully 1996, Waites 2005). The first mycoplasma infection in cell cultures was reported by Robinson et al. in 1956, in which mycoplasmas were detected in negative control cell cultures (Robinson 1956).

2.1.1 Taxonomy of mycoplasmas

Mycoplasmas are one of the eight genera that constitute the class of *Mollicutes*, and with more than 120 recognized species they are the largest group of all (Figure 1) (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi).

Phylogenetic studies involving sequence analysis of the highly conserved 16S mRNA revealed that mycoplasmas are closely related to Gram-positive Clostridia, Bacillus, and Lactobacillus. According to the current molecular biological taxonomy, based on sequence similarities and phenotypic properties, mycoplasmas are reclassified into three groups, the hominis, the pneumoniae and the spiroplasma group (Weisburg 1989, Drexler 2000).

2.1.2 General characteristics and properties of mycoplasmas

Mycoplasmas are 0.3 to 0.8 µm large and are the smallest self-replicating organisms known (Robertson 1975, Razin 1978, Drexler 2000). They lack a rigid cell wall, and are surrounded by a single plasma membrane (Rottem 2003, Razin 1998). As such, mycoplasmas are pleiomorphic, demonstrating a variety of forms, including spherical, pear- and flask-shaped, rod-like and filamentous (Kim 1966, Razin 1998). On solid agar, they build colonies after 3 to 5 days, with a characteristic “fried-egg”

appearance and a diameter of 100 to 400 µm, but in some cases, they grow very slowly and are not recognizable until 1 to 3 weeks or more (Drexler 2002).

Class: Mollicutes
- Order I: Mycoplasmatales
  - Family I: Mycoplasmataceae
    - Genus I: Mycoplasma
    - Genus II: Ureoplasma
- Order II: Entomoplasmatales
  - Family I: Entomoplasmataceae
    - Genus I: Entomoplasma
    - Genus II: Mesoplasma
  - Family II: Spiroplasmataceae
    - Genus I: Spiroplasma
- Order III: Acholeplasmatales
  - Family I: Acholeplasmataceae
    - Genus I: Acholeplasma
- Order IV: Anaeroplasmatales
  - Family I: Anaeroplasmataceae
    - Genus I: Anaeroplasma
    - Genus II: Asteroplasma

Figure 1 Conventional classification of Mollicutes

Mycoplasmas have a double-stranded DNA and divide by binary fusion. The procedures of cytoplasmic division and genome replication are asynchronous, resulting in the formation of filaments and transformation into chains of cocci until division is completed (Razin 1998, Drexler 2000). Their generation time is between 1 to 9 hours and they have a relative long lag phase (Drexler 2002). The mycoplasma genome ranges from 580 (*M. genitalium*) to 2.220 kb (*Spiroplasma*), and it contains 24 to 33 mol% G+C and approximately 500 genes (Dybvig 1996, Pollack 1997, Razin 1998, Drexler 2000, Rottem 2003). This number of genes represents only around

one-fifth of the number of genes found in other bacteria (Drexler 2000). As a result, mycoplasmas lack many enzymatic pathways, such as the pathway for cell wall production and *de novo* biosynthesis of purines, a functional tricarboxylic acid cycle and a cytochrome-mediated electron transport-system, while their energy-yielding pathways are inefficient (Dybvig 1996).

Mycoplasmas are characterized as fermentative and non-fermentative species, based on their ATP-producing abilities. Fermentative mycoplasmas produce ATP by carbohydrate fermentation via the glycolytic pathway, whereas in non-fermentative mycoplasmas, ATP production is achieved by arginine hydrolysis via the arginine-dihydrolase pathway. Some mycoplasma species, such as *M. fermentans* produce ATP, using both metabolic pathways. Furthermore, a mechanism based on ATP production from enzymes commonly found in both groups of mycoplasmas was proposed as a complementary way of ATP production (Razin 1978, Razin 1998, Drexler 2000).

Mycoplasmas are found in humans, animals and in cell cultures, where they live commensally or as pathogens. As parasites, mycoplasmas exhibit host and tissue specificities. As such, they are able to enter the host cells in which they multiply and survive for a long period of time, they develop mechanisms of mimicry against host antigens in order to deal with the host immune system, and they survive within phagocytic and non-phagocytic cells generating phenotypic plasticity (Razin 1998, Rottem 2003).

2.1.3 Mycoplasmas in cell cultures

Mycoplasmas can very easily contaminate *in vitro* cell cultures. They can be present without causing any visible changes, like turbidity or changes of the colour of cell media, and they remain undetectable under conventional microscopy. Their plasticity allows them to pass through commonly used antimicrobiological filters of 0.45 µm in diameter (Drexler 2000). Furthermore, mycoplasmas are gram-negative and show resistance to commonly used antibiotics such as penicillin and streptomycin (Drexler 2002, Uphoff 2005).

Contaminated cell lines, reagents and laboratory equipment often serve as sources for mycoplasma contaminations (McGarrity 1985, Drexler 2002). The ease of droplet generation from mycoplasma contaminated cell lines during cell handling, the high concentration of mycoplasmas in infected cultures and their resistance to

dehydration (up to 7 days on working surfaces) play an important role in the tenacity and transmission of mycoplasmas to cell cultures (McGarrity 1985, Drexler 2000, Drexler 2002). Furthermore, laboratory personnel significantly contribute to spreading of contaminations (Tully 1996, Uphoff 2002, Drexler 2002).

Infection of cells by mycoplasmas depends on the dosage of mycoplasmas and the type of cells involved. Generally, mycoplasmas grow very well in cell cultures. One mycoplasma cell could result in 10^6 CFU/ml in 3 to 5 days in an infected cell culture giving titers of 10^6 to 10^8 organisms/ml. In heavy infections, approximately 100 to 1000 mycoplasmas are attached to each cell (Drexler 2002).

2.1.3.1 Frequency and common mycoplasma contaminants in cell cultures

The frequency of mycoplasma contaminations *in vitro* varies worldwide, depending on the number of cell cultures examined, the detection methods used by the individual laboratories, and the geographic area in which these frequencies were recorded (McGarrity 1985, Tully 1996). Primary cell cultures and cell cultures of early passages are less frequently contaminated with reported incidences of 1 and 5%, respectively. In contrast, continuous human or animal cell lines are the most frequently contaminated with a prevalence of 15 to 35% (Drexler 2002).

Animal mycoplasma species like *M. arginini*, *M. hyorhinis,* and *Acholeplasma laidlawii* used to be the most common mycoplasma species found in cell cultures due to the use of contaminated serum (McGarrity 1985). Currently data, are classifying the human species *M. orale* as the most common contaminant species with a frequency of 20 to 40%, followed by *M. hyorhinis* (10 to 40%), *M. arginini* (20 to 30%), and *M. fermentans* and *M. hominis* with 10 to 20% (Drexler 2002). Multiple infections involving 2 or more mycoplasma species vary between 7% (Uphoff 2002) and 62% (Timenetsky 2006) of mycoplasma contaminations in cell cultures.

2.1.3.2 Effects of mycoplasmas on cell cultures

Mycoplasmas affect several cell parameters *in vitro*, influencing growth, morphology, metabolism, the genome, and the antigenicity of the cells (Stanbridge 1971, Barile 1979, McGarrity 1984, McGarrity 1985, Drexler 2000, Drexler 2002). These effects depend on the infecting mycoplasma species, the infected cell type, the culture conditions, the intensity and duration of the infection (Drexler 2002).

Effects on growth, morphology and metabolism. The requirement of mycoplasmas for nucleic acid precursors and their competitive use of amino acids and other nutrients result in cytopathic effects on the cells. The production of hydrogen peroxide, acidic conditions, depletion of arginine and other nutrients induce increased granularity, cytotoxicity, lysis and cell death (Stanbridge 1971, Barile 1979, McGarrity 1985, Drexler 2000). Mycoplasmas alter the levels of proteins, RNA and DNA synthesis and metabolism of the cells (Drexler 2000, Drexler 2002). Consequently, cells undergo degeneration showing pyknotic nuclei, margination of chromatin, nucleolar segregation and nuclei with condensed chromatin (leopard cells) (Stanbridge 1971, Drexler 2000). The addition of proper nutrients in culture media can reverse some of the above-mentioned effects, but others remain irreversible (Stanbridge 1971, Drexler 2000). Furthermore, mycoplasmas influence the growth rate of infected cells (Stanbridge 1971). Abnormal, stunted growth pattern with rounded degenerated cells and detachment of monolayers could be obtained due to mycoplasma contamination (Barile 1979, Drexler 2000).

Effects on the genome. Mycoplasmas induce chromosomal aberrations in cultured cells, which consist mostly of achromatic gaps, chromatid breaks, translocations, and rearrangements, dicentrics, and pulverization. A further gradual reduction on the number of chromosomes and appearance of new chromosome varieties could be induced by mycoplasmal infections (Stanbridge 1971, Barile 1979, McGarrity 1985, Drexler 2000).

Effects on antigenicity and immunology. Interactions of mycoplasmas with cultured cells result in changes in cell membrane composition thus affecting the expression of surface antigens and receptors (Drexler 2000). In addition, the presence of mycoplasmas influences the propagation of virus in cell cultures. Non-fermentative species decrease the propagation of arginine-dependent DNA virus due to arginine depletion. The mycoplasmal presence could induce or inhibit the production of interferon-γ in cell cultures, resulting in the reduction or increase of virus propagation, respectively (Barile 1979, McGarrity 1985, Drexler 2000). Mycoplasmas affect haematopoietic cells *in vitro*, stimulate or inhibit their activation, proliferation and differentiation. They enhance immunoglobulin secretion of B-cells and induce expression of cytokines and growth factors such as IL-1β, IL-2, IL-6 and TNF-α (McGarrity 1985, Razin 1998, Drexler, 2000).

2.1.3.3 Methods for detection of mycoplasmas in cell cultures

Cell cultures are used extensively in biomedical research and for the production of biological products. Mycoplasmas can affect various cell parameters leading to misinterpretation of experimental results and contamination of biological products. As such, cells should be screened for mycoplasmas prior to their use.

Several techniques that have been developed for the detection of mycoplasmas in cell cultures are summarized in Figure 2 (Drexler 2000).

Mycoplasmologists developed direct and indirect methods for mycoplasma detection in cell cultures. The term “direct” is referred to the classical microbiological colony growth on agar which detects directly viable mycoplasma cells, whereas the term “indirect” refers to procedures that measured gene products of mycoplasmas or they are based on mycoplasmal enzymatic, metabolic and other properties (Drexler 2000).

Most of these methods are complex, difficult to interpret, involve subjective assessments, are lengthy, and expensive. An ideal method should be highly sensitive, specific, simple, rapid, easy to interpret, efficient, and cost effective (Uphoff 1992, Uphoff 1992, Drexler 2000, Drexler 2002, Uphoff 2002). The criteria which methods are based on are sensitivity (detection of true positives), specificity (detection of true negatives), accuracy (detection of true positives and true negatives), predictive value (probability of correct results) and reproducibility (concordance of interpretation of results) (Drexler 2000, Uphoff 2002).

Today, the most reliable methods used for mycoplasma detection in cell cultures are culture in broth and agar, and the PCR method (Drexler 2002). There are several broth and agar media developed for cell-free growth and culture of mycoplasmas including Friis, PH-Hayflick (modified or not), SP4 and DM-1 medium (Hayflick 1965, Uphoff 1992, Uphoff 1992, Tully 1996). For proper and better quality control, results obtained with culture should be confirmed by a second method. For mycoplasma species that cannot be cultured in artificial media, an indicator cell line is proposed as the method of choice (Tully 1996). The PCR method with protocols based on 16S rRNA sequence regions and the 16S-23S intergenic regions of mycoplasma species includes single or nested PCRs, genus or species-specific, gel-based or real-time PCRs (van Kuppeveld 1992, Blanchard 1993, Blanchard 1993, Harasawa 1993, van Kuppeveld 1994, Razin 1994, Dussurget 1994, Rawadi 1995,

Harasawa 1996, Harasawa 1999, Tang 2000, Garner 2000, Kong 2001, Uphoff 2005, Harasawa 2005, Ishikawa 2006, Timenetsky 2006, Sung 2006, Bruchmuller 2006).

Histological staining: Histochemical stains and light microscopy
Electron microscopy: Transmission/Scanning electron microscopy
Microbiological culture: Broth/Colony formation on agar
DNA fluorochrome staining: DAPI/Hoechst 33258 stain
Biochemical methods: Enzyme assays
Gradient electrophoresis separation of labeled RNA
Protein analysis
Immunological procedures: Fluorescence/enzymatic staining with antibodies
ELISA, Autoradiography
RNA hybridization: Filter/Liquid hybridization
Polymerase chain reaction: Species-/genus–specific PCR primers
Universal PCR primers

Figure 2 Methods for detection of mycoplasma in cell cultures (Drexler 2000)

2.1.3.4 Elimination and eradication of mycoplasmas in cell cultures

For routine laboratory work, mycoplasma-positive cell cultures should be discarded and replaced with new mycoplasma-free cell stocks. However, for irreplaceable, valuable cells, attempts to eradicate mycoplasmas and render cells mycoplasma-free can be made (McGarrity 1985, Uphoff 1992, Uphoff 2005).

Over the years, several techniques have been proposed for this purpose including the use of antimycoplasma sera, exposure to detergents selectively toxic for mycoplasmas, induction of chromosomal damage in mycoplasmas using 5-bromouracile and visible light, *in vivo* passage of continuous cell lines through nude mice, co-cultivation of infected cells with macrophages, and antibiotic treatment (Schmidt 1984, McGarrity 1985, Schmitt 1988, Drexler 2000, Drexler 2002, Uphoff 2005). Many of the above techniques are unreliable, ineffective, impracticable, cannot be applied in every situation, are time- and cost-ineffective, and change characteristics and properties of the cells subsequent to treatment (McGarrity 1985, Schmitt 1988, Uphoff 1992, Drexler 2002). Today, the antibiotic treatment is the most

promising, reliable, and effective method for the treatment of mycoplasma-infected cell cultures (Schmidt 1984).

Many antibiotics such as tetracycline, kanamycin, lincomycin, erythromycin, tylosine or spectinomycin have been used in different protocols and combinations for the eradication of mycoplasmas but resistance was induced to several of them (Schmidt 1984, Schmitt 1988). Tiamutin and minocyclin were effective when they were used consecutively for weeks without causing any effects on cell properties followed by cell cloning for the establishment of mycoplasma-free cell line (Schmidt 1984). Optimal results presented ciprofloxacin and other quinolones in respective experiments (Schmitt 1988, Uphoff 1992).

Today, the most prevalent antibiotics with high effectiveness against mycoplasmas *in vivo* (veterinary/human medicine) as well as *in vitro* (cell cultures) are tetracyclines, macrolides, and quinolones. They could be applied in low concentrations, having low or no effects on eukaryotic cells, and the possibility of developing resistance seems to be very unlikely. The antibiotics of choice from these 3 categories are tiamutin, minocyclin, quinolones (ciprofloxacin and enrofloxacin) and Mycoplasmal Removal Agent (MRA) (Uphoff 2005). The macrolide tiamulin (BM-cyclin 1) and the tetracycline minocyclin (BM-cyclin 2), which bind to the 30S and the 50S ribosomal units, inhibit protein synthesis. Quinolones and MRA inhibit the bacterial DNA gyrase, which is essential for DNA replication (Uphoff 2005).

Generally, antibiotic treatment should last between 2 and 4 weeks. However, the treated cells should be monitored for 4 to 6 weeks post treatment (Schmidt 1984, Schmitt 1988, Uphoff 1992, Drexler 2000, Drexler 2002, Uphoff 2005) and should be negative in order to be declared mycoplasma-free. Absence of mycoplasmas should be regularly confirmed by examinations with highly sensitive detection methods (Uphoff 2005).

2.1.4 Mycoplasma species

For the work presented in this thesis, 3 human mycoplasma species were used: *M. hominis*, *M. fermentans*, and *M. orale*. These are described in details below.

2.1.4.1 *Mycoplasma hominis*

M. hominis was the first human mycoplasma species isolated from a gland abscess (Dienes 1937, Tully 1996, Waites 2005). The genome size of different *M. hominis* strains varies between 704 to 825 kb (Ladefoged 1992, Dybvig 1996). Usually, they are round or rod-like (Kim 1966) (Figure 3), but under *in vitro* suboptimal growth conditions produce aberrant forms and show low viability (Robertson 1975). It belongs to the non-fermentative species and uses arginine for the production of ATP (Schimke 1966).

It is located primarily in the human urogenital tract being pathogenic for both males and females (Waites 2005), while it was also found at high prevalence in the urogenital tract of asymptomatic healthy people (Blanchard 1993, Serin 2001, Baczynska 2004, Waites 2005). Clinical studies showed that *M. hominis* could be the causative agent or associated with several inflammatory diseases of the reproductive system of adults and be implicated in conditions directly related to perinatal infections, which could lead to infertility and spontaneous abortions in women. Furthermore, *M. hominis* could show vertical transmission to the foetus or neonate and be implicated with respiratory, neurological, and other systemic infections or septic conditions in newborns (Cassell 1991, Waites 2005). Finally, *M. hominis* could cause extragenital and systemic infections in people suffering from immunodeficiency syndromes (Waites 2005).

Studies in human reproduction and *in vitro* fertilization showed that *M. hominis* adheres to or invades the human sperm cells, showing no apparent damage and significant effects on sperm count, motility and fertilization (Hill 1987, Diaz-Garcia 2006). In addition, *in vitro* washing of infected sperm did not result in eradication of *M. hominis*, whereas this procedure managed to eradicate *Ureoplasma ureolyticum*, another genital mycoplasma (Hill 1987). In experimental animal infections, *M. hominis* persisted for 1 day to 4 months in mice and up to 4 weeks in guinea pigs after subcutaneous inoculation (Kraus 1977). Vaginal colonization of *M. hominis* persisted for more than 200 days in BALB/c mice after direct inoculation, spreading to the uterine horns, ovaries, and oropharynx but not to other organs (Furr 1989).

Under *in vitro* situations, *M. hominis* attaches to the host cell surface or invades the cells (Waites 2005, Taylor-Robinson 1991). A protein called P50 which belongs to Vaa (Variable adherence-associated) antigen was suggested to be involved in adherence of *M. hominis* to the cells (Henrich 1993). Furthermore, P50 undergoes variations and mutations, giving *M. hominis* the chance to evade the host immune system. *In vivo*, the p50 gene of a clinical isolate of *M. hominis* expressed the main form of the respective adhesin protein expressed *in vitro*, suggesting that this is the most stable form of expression. Nevertheless, the p50 gene shows great variability going through point mutations, truncations or duplications. As a result, this protein is highly immunogenic in humans and implicated in arthritis caused by *M. hominis* (Henrich 1998, Razin 1998, Boesen 2001).

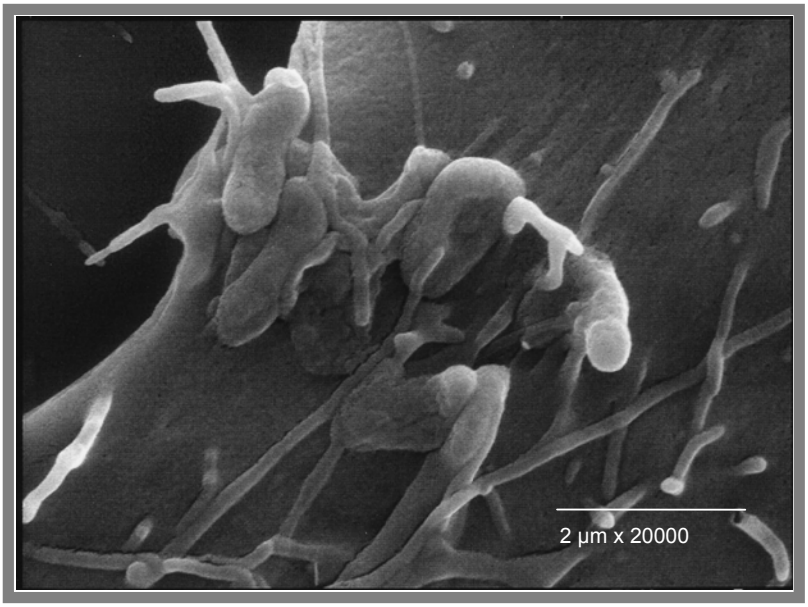


Figure 3 Photo of *M. hominis* in rod-like or round forms attached to a cell, observed in infected cell cultures under an electron microscope at 20000 x magnification. (Kindly provided by Dr. C.C. Uphoff, Department of Human and Animal Cell Cultures, DSMZ, German Collection of Microorganisms and Cell Cultures, D-38124 Braunschweig, Germany)

2.1.4.2 *Mycoplasma fermentans*

M. fermentans was first isolated from the human urogenital tract in the 1950's (Ruiter 1952), has a genome size of 1160 kb (Dybvig 1996), and a spherical or filamentous form (Kim 1966) (Figure 4). It ferments sugars and hydrolyses arginine for the production of ATP, using both metabolic pathways found in mycoplasmas (Özkan 1999).

M. fermentans inhabits the human respiratory and urogenital tract and was considered to be a commensal. The interest in this organism has increased because of its possible role as a pathogen or as an opportunistic co-factor accelerating the progression of human immunodeficiency virus disease (HIV) (Lo 1992, Yavlovich 2001, Waites 2005). Furthermore, *M. fermentans* was possibly involved in the pathogenesis of inflammatory arthritis forms and chronic arthritic conditions (Johnson 2000, Gilroy 2001, Yavlovich 2001, Waites 2005, Yavlovich 2006).

In vitro, *M. fermentans* attaches to or invades the host cells. It possesses a urokinase-type plasminogen activator which binds plasminogen, activating it into plasmin, thus entering the host cell (Yavlovich 2001, Rottem 2003). It has a high reducing antioxidant capacity, serving as a defense mechanism and gives *M. fermentans* the ability to fight against oxidative stress within the host cell, resulting in cell habitation for prolonged period of times (Yavlovich 2006). Furthermore, it is highly fusogenic, capable of fusing with a variety of cells (Franzoso 1992, Rottem 2003), and interacts with haematopoietic cells, inducing production of interleukins and Tumor Necrosis Factor-α (TNF-α) (Muhlradt 1991, Rottem 2003). As previously described, *M. fermentans* induced apoptosis of mouse spleenic T-cells initiated by concanavalin A (Shibata 1997). Induction of malignant transformation and chromosomal abnormalities were obtained in murine embryonic cells after inoculation with *M. fermentans*, correlating to the duration of infection (Tsai 1995).

M. fermentans possess a Macrophage Activating Lipopeptide-2 (MALP-2), which is located in its plasma membrane. This 2 kDa lipopeptide differs from conventional bacterial lipopeptides, carrying only two ester-linked fatty acids, and lacking the N-terminal third fatty acid enhancing a high biological activity (Deiters 1999, Luhrmann 2002). It was capable of liberating chemoattractant chemokines and initiating an inflammatory effect *in vitro* and *in vivo* after intraperitoneal and pulmonary application of MALP-2 lipopeptide in mice, respectively (Deiters 1999, Luhrmann 2002). The mice presented a maximal leukocyte infiltration within 72 hours

returning to normal levels within 10 days, whereas monocytes and macrophages were increased after 3 days (Deiters 1999, Luhrmann 2002). The synthesis of MIP-1α (Macrophage Inflammatory Protein-1α), MCP-1 (Monocyte Chemoattractant Protein-1) increased in the first 6 hours after inoculation, respectively, remaining elevated for 24 to 48 hours, whereas MIP-2 (Macrophage Infiltration Protein-2) reached maximal production within the first 2 hours (Deiters 1999, Luhrmann 2002). Furthermore, MALP-2 induced cross tolerance in mice (Deiters 1999, Luhrmann 2002, Deiters 2003). Additionally, *M. fermentans* suppressed the interferon-γ (INF-γ) expression of major histocompatibility complex (MHC) class II molecules on macrophages and resulted in impaired antigen presentation to helper T-cells in an animal model (Frisch 1996).

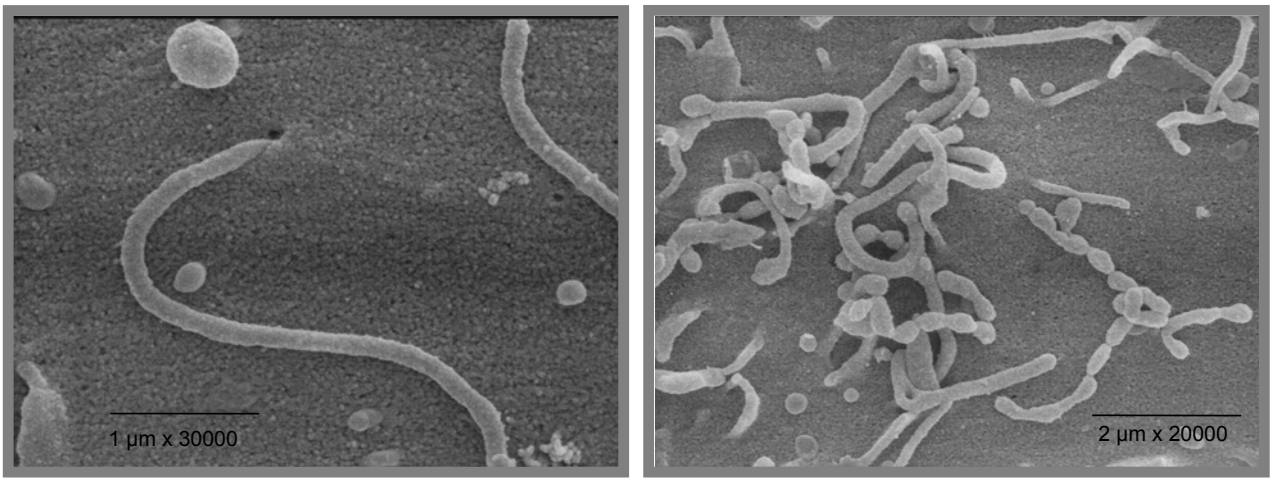


Figure 4 Photos of *M. fermentans* observed in infected cell cultures under an electron microscope at 30000 and 20000 x magnifications, respectively. Left photograph: a filament form of *M. fermentans* in an interaction with the host cell membrane. Right photograph: Forms of *M. fermentans* undergoing binary division and constructing chains of cocci. (Kindly provided by Dr. C.C. Uphoff, Department of Human and Animal Cell Cultures, DSMZ, German Collection of Microorganisms and Cell Cultures, D-38124 Braunschweig, Germany)

2.1.4.3 *Mycoplasma orale*

M. orale is a common inhabitant of the oral and pharyngeal cavity of humans (Taylor-Robinson 1964). It has a characteristic filamentous morphology and uses the arginine-dihydrolase pathway for ATP production (Kim 1966, McGarrity 1985) (Figure 5). *M. orale* is considered to be a non-pathogenic species in immunocompetent persons, but could be responsible for secondary infections in case of immunodeficiency (Paessler 2002, Waites 2005).

In vitro, *M. orale* is mostly found free in medium, between the cells (McGarrity 1984). Whole cells of *M. orale* induced macrophage-mediated cytolysis of fibrosarcoma A9HT (Loewenstein 1983), produced chromosomal aberrations in a human diploid and the WI-38 fibroblast cell lines (McGarrity 1985), induced TNF-α production in a murine tumor cell line; (Gallily 1989) and stimulated lymphocytes for high cell proliferation (Mizushima 1985).

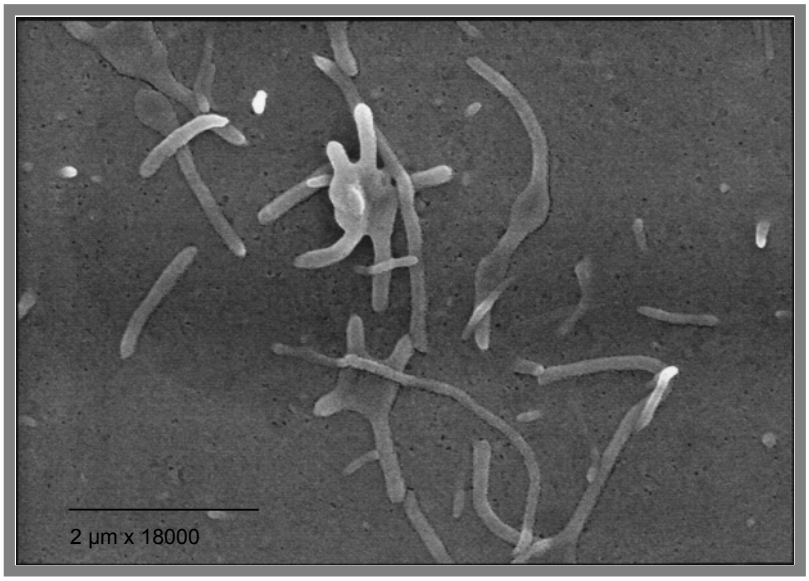


Figure 5 Photo of *M. orale* observed in infected cell cultures under an electron microscope at 18000 x magnification showing filamentous forms. (Kindly provided by Dr. C.C. Uphoff, Department of Human and Animal Cell Cultures, DSMZ, German Collection of Microorganisms and Cell Cultures, D-38124 Braunschweig, Germany)

2.2 Murine embryonic stem (mES) cells

The embryonic development begins with the first entity of life, the zygote, and continues with several cell divisions, the morula and the blastocyst stage, constituting the preimplantation embryonic stages. With respect to embryonic development, a stem cell hierarchy which is described below in Figure 6 characterizes each embryonic stage. mES cells originate from the inner cell mass (ICM) of day 3.5 preimplantation embryos (blastocyst). They are pluripotent which means that they are undifferentiated and capable of forming the 3 primary germ layers (ectoderm, mesoderm, endoderm) and the primordial germ cells (PGCs) (Evans 1981, Martin 1981, Bradley 1990, Smith 2001, Wobus 2005).

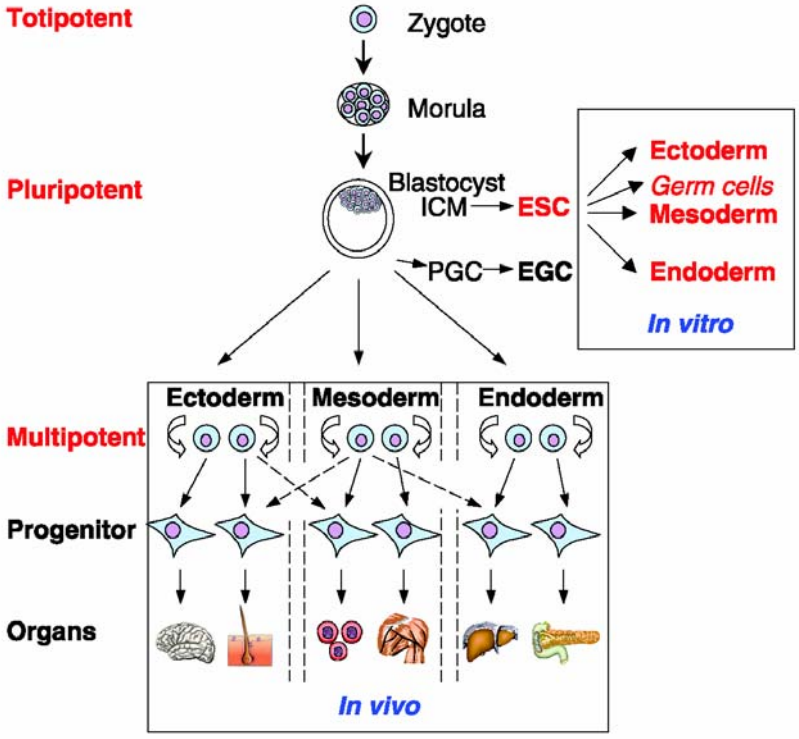


Figure 6 Stem cell hierarchy (Wobus 2005). Zygote and early cell division stages (blastomeres) to the morula stage are defined as totipotent, because they can generate a complex organism. At the blastocyst stage, only the cells of the inner cell mass (ICM) retain the capacity to build up all the three primary germ layers, the endoderm, mesoderm, and ectoderm as well as the primordial germ cells (PGCs), the founder cells of male and female gametes. In adult tissues, multipotent stem and progenitor cells exist in tissues and organs to replace lost or injured cells. At present, it is not known to what extent adult stem cells may also develop (transdifferentiate) into cells of other lineages or what factors could enhance their differentiation capability (dashed lines). Embryonic stem (ES) cells, derived from the ICM, have the developmental capacity to differentiate *in vitro* into cells of all somatic cell lineages as well as into male and female germ cells.

Embryonic stem cell research dates back to the 1970's with the establishment of the first embryonic carcinoma (EC) cell line from tumor stem cells (Jakob 1973, Gearhart 1974). Evans and Kaufman (Evans 1981) and Martin et al. (Martin 1981) isolated and cultured the ICM *in vitro*, establishing the first mES cell lines (Wobus 2005) (Figure 7).

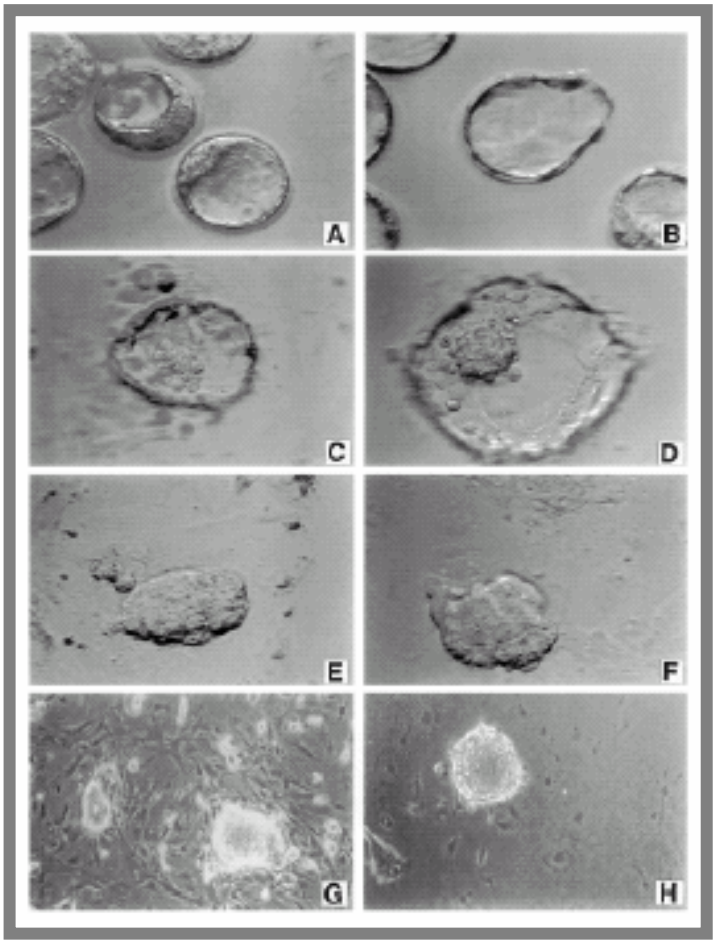


Figure 7 From blastocyst stage embryos to ES cells (Turksen 2002). (A) Blastocyst stage. (B) Blastocyst embryo hatching from the zona pellucida. (C) Blastocyst embryo attached to a PEF feeder layer 2 d after hatching, ICM is apparent inside the blastocyst. (D) Blastocyst embryo attached to tissue culture plastic without a PEF feeder 2 d after hatching, ICM is apparent inside the blastocyst. (E) ICM is distinctive and extends above the flat trophoblasts and PEF feeders. (F) ICM is distinctive and extends above the flat trophoblasts without PEF feeders. (G) ES cell colonies on PEF feeders. (H) ES cell colony on tissue culture plastic without PEF feeders.

mES cells are capable of self-renewal. They show a high proliferative activity *in vitro* remaining undifferentiated (pluripotent) even after a long period of time in culture (Smith 2001, Faherty 2005, Wobus 2005). They possess a large nucleus, little cytoplasm and 1 or more nucleoli (Tielens 2006). They are injected into blastocysts which are transferred to recipients and can contribute to the production of chimeric mice (Gossler 1986, Bradley 1990, Longo 1997, Wobus 2005). Spontaneous differentiation of the mES cells results in a wide range of well-defined differentiated somatic cell lines (Smith 2001, Wobus 2005).

2.2.1 Genetic background of mES cell lines

Mouse strains used for the production of mES cell lines are chosen based on their genetic background. mES cells should be capable of contributing to the germ line. As such, the 129 inbred mouse strain has been used mostly for the establishment of mES cell lines, while mES cell lines were also isolated from the C57BL/6 (B6) mouse strain (Nagy 2003).

The 129 strain is homozygous for the white-bellied agouti allele (A^w/A^w) of the Agouti locus (Nagy 2003). The 129 substrains show great genetic variation, especially in coat and eye colour due to the fact that they were generated by crosses with other inbred lines (Nagy 2003). Currently, there are many established mES cell lines provided by laboratories, commercial sources or research centers. These include R1, AB1 and AB2.1, J1, CCE, W9.5, W4, RW4, E14 derived from 129x1/SvJx129S1/SV-+^{p}+$^{Tyr\text{-}c}$Kitl$^{Sl\text{-}J}$/+, 129S7/SvEvBrd, 129S4/SvJae, 129S6/SvEv, 129X1/SvJ, 129S6/SVEvTac, 129X1/SvJ, 129P2/Ola on a 129 strain background (Nagy 2003, http://www.imgen.bcm.tmc.edu/molgen/labs/bradley/cell.htm, http://www.healthsystem.virginia.edu/internet/transgenic-mouse/aboutESC.cfm).

The C57Bl/6 ES cell lines are considered less stable in maintaining the ability to contribute to the germ line due to possible genetic alterations that occur during gene-targeting procedures (Nagy 2003). The MPI 65-3, WB6, MES-1, 4A4, Bruce4 (Thy1.1 congenic C57BL/6 strain) and B1 (C57BL/6J-Tyrc-2J) are some mES cell lines that originated from C57Bl/6 mice (Auerbach 2000, Liu 2003, http://www.healthsystem.virginia.edu/internet/transgenic-mouse/aboutESC.cfm).

2.2.2 *In vitro* culture of mES cells

The main components of ES cell media are D-MEM medium, foetal calf serum (FCS) pretested for ES-cell culture, β-mercaptoethanol, glutamine, pyruvate, and Leukemia Inhibitory Factor (LIF) (Nagy 2003).

The culture conditions for ES cells are demanding. They often influence the cell properties when they are suboptimal (Nagy 2003). ES cells are sensitive to pH and temperature changes (Turksen 2002); they should be cultured at reasonably high densities and passaged every 2 to 3 days since they have a very short generation time, approximating 12 to 15 hours (Rohwedel 1996, Turksen 2002, Wobus 2005). Culture at low densities, the lack of essential substances, and unstable incubation conditions could lead to production of embryoid bodies, which are mES cell aggregates at the first stage of differentiation, or to chromosomal aberrations and further loss of the germ line competence (Nagy 2003).

Culture of mES cells on feeder cell layers offers an environment for optimal continued proliferation without differentiation allowing and enhancing growth of pluripotent, multicellular, compact, and well-shaped colonies (Nagy 2003, Tielens 2006). The feeder cell layers consist of murine embryonic fibroblast cells (MEFS) which are inactivated prior to their use via irradiation or chemical treatment with Mitomycin C (MMC) (Turksen 2002, Nagy 2003). Culture on feeder cell layers was originally considered a necessity for the *in vitro* culture of mES cells to prevent differentiation (Smith 2001). This consideration was revised after the successful culture and support of mES cells in feeder-free dishes in the present of conditioned media to which LIF (see below) was added (Smith 1987, Smith 1988, Williams 1988). Nevertheless, the culture of mES cells on feeder cells is still widely practiced in stem cell laboratories (Nagy 2003).

mES cells can also be cultured in gelatin-coated dishes. The colonies are flatter than those obtained on feeder layers while their margins appear extensively differentiated (Nagy 2003).

The Leukemia Inhibitory Factor (LIF) or Differentiation Inhibiting Factor (DIA) is essential for the *in vitro* maintenance of mES cell pluripotency. LIF is a cytokine of the interleukin (IL)-6 cytokine family. It supports self-renewal and inhibits differentiation of the mES cells (Smith 1988, Williams 1988) by binding to a 2-part receptor complex, LIF and gp130 receptors, and activating the transcription factor STAT3 (Niwa 1998, Matsuda 1999). LIF is produced by the feeder cells, which are

stimulated by the presence of mES cells (Rathjen 1990, Smith 2001, Nagy 2003). In current protocols for stem cell culture, LIF is added even in co-culture with feeder cells (Nagy 2003).

2.2.3 Markers for determination of pluripotent mES cells

The pluripotent mES cells are characterized by high levels of cellular alkaline phosphatase (AP) (Thomson 1998), high telomerase activity (Tielens 2006), expression of the cell surface glycoprotein Stage-Specific Embryonic Antigen (SSEA)-1 (Knowles 1978), by the presence of the transcription factor Oct 3/4, and other factors (Scholer 1989, Pesce 1999, Wobus 2005, Tielens, 2006).

AP is localized mainly in the plasma membrane (Johnson 1977) of the ES cells and is characteristic but not specific for pluripotent ES cells (Tielens 2006). The AP activity is first expressed between the 8- (Izquierdo 1975) and the 16-cell embryonic stage, and then at the morula stage. Later, at the blastocyst stage, AP is found only in the cells of the ICM (Johnson 1977). It can be detected by cell staining with a dye containing an AP-specific substrate, and depending on the dye used, the cells appear red or dark/blue violet.

Telomerase is a ribonucleoprotein enzyme, which elongates telomeres by adding DNA telomeric sequences onto the 3´ ends of chromosomes (Thomson 1998, Niida 2000), resulting in the maintenance of the telomere length and the replicative life-span of the cells, respectively (Thomson 1998). The telomerase activity of murine and human ES cells is high but not specific for pluripotent cells. It is expressed with lower activity by other somatic cell lines as well (Thomson 1998, Armstrong 2000, Prelle 2000, Wobus 2005).

The expression of the stage-specific embryonic antigen (SSEA)-1 begins at the 8-cell embryo stage, continues at the ICM, and becomes restricted to the embryonic ectoderm and visceral endoderm, respectively (Knowles 1978, Fox 1981, Knowles 1982). Its antigenic determinant is a carbohydrate, whose structure is related to the antigen I of the human blood group (Knowles 1982). It can be detected immunohistochemically using a monoclonal antibody anti-SSEA-1, which reacts with a carbohydrate similar but not identical to antigen I (Nudelman 1980, Gooi 1981, Hakomori 1981). In adult mice, cells of the oviduct, the endometrium, the epididymis, as well as brain areas and the kidney tubules react positively to anti-SSEA-1 antibody (Fox 1981).

The mouse transcription factor OCT-4 (also called OCT-3) is a maternally expressed octamer-binding protein, having 352 amino acids and is encoded by the Oct 4 gene. It belongs to the class V of the POU (Pit-Oct-Unc) family which regulates gene expression by binding to the octamer motif ATGCAAAT (Scholer 1989, Herr 1995, Pesce 1999, Tielens 2006). The OCT-4 factor is expressed in totipotent and pluripotent ES cells, and is downregulated upon differentiation (Pesce 1998, Pesce, 2001), whereas by the PGCs, it is restricted after the 8th day of gestation (Rosner 1990, Scholer 1990, Tielens 2006). This property is retained *in vitro* (Niwa 2000, Tanaka 2002, Tielens 2006). Its unique RNA expression pattern suggests its involvement in the early embryonic events such as the initial formation, self-renewal and maintenance of the pluripotential nature of mES cells (Nichols 1998, Pesce 1999, Niwa 2001, Tielens 2006). The loss of OCT-4 expression has been correlated with the loss of pluripotency, induction of differentiation, and difficulties in delivering pluripotent cells (Buehr 2003, Tielens 2006).

2.2.4 Genetic manipulation and use of embryonic stem cells

The ability of altering the genome of mES cells by homologous recombination and contribution to the germ line (Gossler 1986, Thomas1987, Koller 1989) makes mES cells an attractive tool for studies investigating gene function (Turksen 2002).

mES cells are used for the production of transgenic (insertion of a new gene in mouse genome) and knock-out (inactivation of genes in the mouse genome) mice which serve as animal models for human and other mammalian diseases, in studies of embryonic development, or *in vitro* studies of cell systems, established after their spontaneous differentiation into well-defined somatic cell types (Rohwedel 1996, Wobus 2005). Additionaly, mES cells are used for studies in pharmacology, embryotoxicology, and for experiments concerning cell transplantation and replacement therapy (Wobus 2005, Cormier 2006).

2.2.5 Production and methods for determination of chimeras

The first experimental chimera was created in 1961 (Tarkowski 1961) by placing 2 zona pellucida-free 8-cell stage embryos in contact and transferring the blastocysts afterwards into recipients. The first chimera produced by blastocyst injection was performed in 1968 (Gardner 1968).

Two methods are established for the production of chimeras. The first and the

most common method is the injection method during which mES cells are directly injected into the blastocoel (Gardner 1968) for the production of germ-line chimeras (Bradley 1984) (Figure 8). The aggregate method is the second one where mES cells aggregate with morula stage embryos (Wagner 1985, Nagy 1990, Wood 1993). Both methods have advantages and disadvantages. The injection method requires expensive equipment and time. The aggregate method is simpler, technically less demanding and less expensive than the injection method. A great number of aggregated and injected embryos could be produced daily with both methods but more time is needed for development of aggregated embryos in culture before embryo transfer (Nagy 2003).

Various factors affect the production of germ line chimeras. These include the number and the size of mES cells injected or aggregated, the stage of the aggregated embryos cultured, the genetic background of host blastocysts compared to mES cell line used (Nagy 2003). The number of mES cells injected into a blastocyst is recommended to be 10 to 15 (Bradley 1987, Nagy 2003) depending on the mES cell line used. Too many or too few mES cells could lead to reduction of embryo vitality or failure in producing chimeras (Brown 1992, Nagy 2003). Furthermore, small round mES cells should be chosen for blastocyst injection (Brown 1992). The genetic background of the host blastocysts should be different but compatible with that of the ES cell line in order to obtain chimeras competent for germ line transmission and distinguishable chimeras based on coat colour markers (Nagy 2003).

Chimeras can be distinguished by several ways. The simplest way to evaluate the contribution of mES cells to somatic tissues is the pigmentation markers. It can be scored by simple observation of the coat and eye colour. In this way, distinguishable chimeras can be obtained when 129 agouti mES cells are injected in C57BL/6 or CD-1 Cr host blastocysts or when 129 chinchilla mES cells are injected into C57BL/6 blastocysts (Bradley 1987, Nagy 2003) (Figure 9). Isoenzyme analysis is used to determine chimeric mice in case of undistinguishable coat and eye color. Glucosephosphate Isomerase (GPI) enzyme is detected via electrophoresis and gel analysis (Bradley 1987). Currently, molecular methods like PCR and Southern blot hybridization are used for DNA analysis of chimeras (Nagy 1993, Floss 2002). Finally, the germ line transmission is determined by mouse breeding and simple genetic laws (Bradley 1987) (Figure 9).

2.2.6 Factors influencing germ line transmission

The genetic background significantly influences the germ line competence of mES cell lines. As mentioned above, some mES cell lines are less stable than others and undergo genetic alterations, arising from gene targeting procedures (Nagy 1993). Even though mES cells have the ability for self-renewal and are highly proliferative their long period in culture often leads to development of abnormalities, which influence their germ line competence. These include chromosomal aberrations, abnormal structures in the cytoplasm, changes in metabolism, low replicative efficiency or poor growth, apoptosis or tumorigenicity (Rubin 2002, Kanatsu-Shinohara 2005).

The karyotype of mES cells plays a very important role in their ability to contribute to chimeric tissues and the germ line. Aneuploidy is the main reason for failure of germ line transmission. When a total of 50 to 100% of euploid metaphases was present, mES cells contributed to the germ line, whereas ES cells with metaphases less than 50% euploidy did not (Longo 1997). Other studies revealed that trisomy 8 occurred frequently in mES cell lines during gene targeting, resulting in contribution to somatic tissues but not to the germ line (Liu 1997). Other trisomies like trisomy 11 also occurred in mES cell lines (Sugawara 2006). Therefore, karyotypic analysis of the mES cell lines should be performed prior to their use and lines with 50 or 70% and more of euploid metaphases should be chosen for use only (Turksen 2002, Nagy 2003).

The number of passages influences the germ line competence of the mES cells. Previous studies showed that the contribution to the germ line declined when the number of passages increased correlating to increased aneuploidy which influences the percentage of chimera born and the percentage of chimerism (Nagy 1993, Longo 1997).

The presence of pathogens in mES cell cultures could influence several cell parameters and reduce the germ line potential. The presence of Mouse Hepatitis Virus (MHV) was reported in 2 studies (Okumura 1996, Kyuwa 1997), whereas the E14-1 mES cell line (Hooper 1987, Nagy 2003) reported to be infected with *M. hominis*, showed a relative low frequency (40%) of germ line transmission (http://tikus.gsf.de/ggtc/info/faqs.php). Previous experimental studies using mycoplasma-infected mES cells, showed reductions in the percentage of embryos born, the percentage of chimeric mice and in the contribution to chimeric tissues

compared to results obtained from non-infected mES cells (Bradley 1987). Furthermore, use of mES and feeder cells infected with pathogens could introduce infections into pathogen-free mouse facilities (Nicklas 2000). As a result, screening of mES and feeder cells for pathogens prior to their use is essential, especially when the microbiological status of the cells is unknown (Nicklas 2000, Nagy 2003).

The contribution of mES cells to the germ line can be further influenced by factors such as the number of mES cells injected into a blastocyst, the size and the proliferation of mES cells injected (Brown 1992).

Methods

3.1 Cell culture and preparation of samples for the sensitivity detection

M. hominis, *M. fermentans* and *M. orale* stocks were kindly provided by Dr. C.C. Uphoff, Department of Human and Animal Cell Cultures, DSZM, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany and were stored at -80°C.The number of viable mycoplasmas in stocks was determined before inoculation of the cells by plating on Friis agar and counting the number of CFUs after two weeks of incubation.

The mouse fibroblast L929 cell line (CCL-1 ATCC, Manassas, VA, USA) was grown in antibiotic-free DMEM supplemented with 10% inactivated fetal calf serum in 75 cm^2 cell culture flasks at 37°C in a moisture-saturated atmosphere of 7% CO_2/93% air in an incubator. A total of $6x10^5$ cells was seeded per 75 cm^2 cell culture flask and was inoculated with *M. hominis*, *M. fermentans,* or *M. orale* at a dose of 1 CFU/12 cells ($5x10^4$ CFUs/75 cm^2 flask) (Tsai 1995). Negative control L929 cells were free of mycoplasmas. After 4 passages of the L929 cells over 15 days, supernatants from the mycoplasma-infected and the control cell cultures were collected and the number of cells therein was determined with a haemocytometer FAST-READER 102®. Serial 10-fold dilutions with DMEM up to 10^{-10} from the fresh cell-containing supernatants were made and used for analyses by agar culture, the MycoAlert™ Assay, gel-based PCR, and MycoSensor™ QPCR assays. In addition, for PCR analyses and agar culture, cell suspensions containing 10^4 trypsinized cells from the monolayer/ml DMEM and serial 10-fold dilutions thereof up to 10^{-10} were prepared.

3.2. Agar culture

Friis agar medium contained 128 ml Solution I, composed of 0.82 g Bacto brain heart infusion, 0.87 g Difco™ Bacto PPLO-Broth, 50 ml Hank's BSS (balanced salt solution, see below) and 78 ml dH_2O, 37.3 ml Solution II composed of 33.2 ml heat-inactivated porcine serum, 3.32 ml sterile filtrated yeast extract solution 25%w/v, 0.133 ml sterile filtrated Phenol red solution 1% and 0.343 ml dH_2O, 1.14 g Agar No.1 and 1.6 ml sterile filtrated DEAE-Dextran solution 1%. Hank's BSS composed of 25 ml Solution A, 25 ml Solution B and 450 ml sterile H_2O. Solution A contained 80 g NaCl, 4 g KCl, 1 g $MgSO_4x7H_2O$, and 1 g $MgCl_2x6H_2O$ dissolved in 400 ml dH_2O.

Solution B contained 1.5 g $Na_2HPO_4x2H_2O$ and 0.6 g KH_2PO_4 dissolved in 500 ml dH_2O. Solutions A and B were autoclaved separately and then combined with dH_2O. Solution I was stabilized to pH 7.4 with 1M NaOH and together with agar was autoclaved at 121°C. Before mixing and plating in 6-cm Petri dishes, both Solutions I and II were kept in a water bath at 60°C.

PH-Hayflick Agar medium contained 100 ml Solution I composed of 3.5 g Difco™ Bacto PPLO-Agar, and 100 ml dH_2O and 25 ml Solution II containing 23.36 ml heat-inactivated horse serum, 1.7 ml sterile filtrated yeast extract solution 25%w/v, 0.324 ml DNA-solution, and 0.234 ml dH_2O. Solution I was stabilized to pH 6.5 with 1M HCl and autoclaved at 121°C. Before mixing and plating in 6-cm Petri dishes, both Solutions I and II were kept in a water bath at 60°C.

From the undiluted and diluted cell-containing supernatants and cell suspensions, 100 µl were pipetted directly onto Friis- and PH-Hayflick agar plates, without first culturing them in the respective broths to accommodate quantification (Uphoff 1992, Uphoff 1992, Hopert 1993). Culture was performed at 37°C and 7% CO_2 in a moisture-saturated atmosphere. After 14 days, agar plates were evaluated by counting the number of typical colonies having a fried-egg appearance under the inverted microscope (Zeiss, Göttingen, Germany).

3.3 Biochemical luminescence assay

The MycoAlert™ assay kit was obtained from Cambrex Bio Science, Lonza, Verviers. It is a biochemical luminescence assay based on the presence of mycoplasmal enzymes, and energy pathways that result in ATP generation. A bioluminescent luciferin/luciferase reaction converts the ATP produced into light. The emitted light intensity was measured by a luminometer connected to a computer with the WinGlow program (Berthold Technologies).

In order to assess the sensitivity of the luminometer, dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128 of the MycoAlert™ Assay Control Set were made. From each dilution, 100 µl were pipetted into a 96-well white microplate and 100 µl of the MycoAlert™ Reagent were added to each sample. The emitted light intensity was measured after 5 min (Reading A). Immediately afterwards, 100 µl of the MycoAlert™ Substrate were added. After further 10 min, the second measurement was made (Reading B). The ratio B/A of each sample was calculated. According to the manufacturer's instructions, a luminometer is sufficiently sensitive when it produces a

ratio less than 1 for the negative control and a ratio more than 1 for definite positive results with the MycoAlert™ Assay Control Set at dilutions of at least 1:8.

For detection of mycoplasma, the cell-containing supernatants were centrifuged for 5 min at 1500 rpm according to the manufacturer's instructions. Serial dilutions up to 10^{-10} of the cell-free supernatant were made. From each dilution, 100 µl were analyzed as described above. The ratio B/A of each sample was calculated based on the 2 measurements. According to the manufacturer's instructions, ratios less than 1 indicate mycoplasma-negative samples; ratios greater than 1.2 indicate mycoplasma-positive samples. An aliquot of the MycoAlert™ Assay Control Set was used as the positive control at a dilution of 1:10. For analysis of each mycoplasma species, 5 replications with serial dilution samples were performed.

3.4 Gel-based Polymerase Chain Reaction

Undiluted and diluted cell-containing supernatants and cell suspensions were centrifuged for 6 min at 13.000 rpm. Supernatants were discarded and cell pellets were washed twice with phosphate buffered saline (PBS), resuspended in 100 µl PBS and incubated for 15 min at 95°C. DNA was extracted with the Wizard Clean-Up System according to the manufacturer's instructions and eluted from the column with 50 µl dH_2O at 80°C. Extracted DNA was stored at -20°C and analyzed within 1 week. The degenerated primers Myco-5' and Myco-3' (Uphoff 2002, Uphoff 2005) were used with the following sequences: 5' primers: cgc ctg agt agt acg twc gc, tgc ctg rgt agt aca ttc gc, cgc ctg agt agt atg ctc gc and cgc ctg ggt agt aca ttc gc, 3' primers: gcg gtg tgt aca ara ccc ga, gcg gtg tgt aca aac ccc ga. The PCR with 1 µl DNA was performed in a final volume of 25 µl with 3.5 µl 10xPCR buffer, 1.6 µl 50 mM $MgCl_2$, 1 µl dNTP mix 10 mM each, 1 µl Myco-5' and 1 µl Myco-3' primers (mix of each single primer at 5 µM) (Uphoff 2005), 1.5 U Platinum Taq DNA Polymerase and 15.6 µl dH_2O. The thermal cycler was programmed to perform a hot-start initial cycle carried out at 96°C for 2 min, 65°C for 1 min and 72°C for 1 min. Further 35 cycles were performed with a denaturation time of 94°C for 20 s, annealing time of 65°C for 20 s and amplification time of 72°C for 40 s with 2 s extension time per cycle. Ten microliters of the PCR product were mixed with 2 µl loading buffer, electrophoresed on a 1.4% agarose gel, stained with ethidium bromide, visualized under UV light, and photographed. Internal and positive controls for the PCR were provided as described (Uphoff 2005). *M. orale* stocks were used as the positive control. Mycoplasma-

positive samples show a band at 502-520 bp, depending on the mycoplasma species present. For analysis of each mycoplasma species, 5 replications with supernatant samples and 3 replications with cell suspensions were performed. The mycoplasma species were identified by digestion of the PCR products with the restriction enzymes Xba I, Hpa II, and Hae III using React® 2 buffer, React® 8 buffer and React® 2 buffer, respectively. One microliter of the diluted enzyme (1:10 in 1x diluted buffer) and 1 µl of the corresponding 10x buffer were added to 8 µl of the PCR reaction mix containing the PCR products and incubated at 37°C for 1 h. The restriction fragment patterns were determined by electrophoresis on a 1.4% agarose gel, staining with ethidium bromide, and then visualized under the UV light and photographed. The DNA from *M. hominis* was digested by Xba I and Hae III enzymes, that of *M. fermentans by* Hpa II and Hae III and the DNA from *M. orale* was digested by Xba I and Hpa II enzymes. Xba I produced fragments of 253 bp or 265 bp for *M. hominis* and *M. orale*, respectively, Hpa II produced fragments of 288 bp and 230 bp for *M. orale* and 357 bp, 111 bp and 48 bp for *M. fermentans*. Hae III produced fragments of 336 bp or 180 bp and 356 or 160 bp for *M. hominis* and *M. fermentans,* respectively (Uphoff 2002, Uphoff 2005). For determination of each mycoplasma species, PCR products from 2 replications were analyzed.

3.5 Real-time Polymerase Chain Reaction

The MycoSensor™ QPCR Assay kit was obtained from Stratagene. In order to directly compare the results from the gel-based PCR and real-time PCR methods, extracted DNA from the same aliquots was used for both analyses. The MycoSensor™ QPCR Assay was performed by analyzing 5 µl DNA in a final volume of 50 µl with 2xMycoSensor™ QPCR Master Mix containing SureStart® *Taq* DNA polymerase, dNTPs and optimized buffer components, mycoplasma primer mix provided with the kit, SYBR® green reference dye, diluted 1:50, and PCR-grade H_2O. Amplification and detection were performed with the Applied Biosystems 7500 Real-time PCR System using the following settings: initial cycle at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min and 79°C for 30 s. Subsequently, a melting curve analysis was performed at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. Fluorescence was emitted at 520 nm. According to the manufacturer's instructions, negative controls give no peaks or have amplification curves with a melting temperature (T_m) of ~ 74°C due to a primer dimer formation,

whereas positive controls give amplification curves with a melting temperature (T_m) of ~82°C.

Standard curves for each real-time PCR run were constructed using dilutions containing 50, 100 and 1000 copies of *M. orale*-positive control provided with the kit at a concentration of 200 copies/µl. For real-time PCR with each mycoplasma species, 3 to 5 replications with supernatant samples and 3 replications with cell suspensions were performed.

3.6 Preparation of primary murine embryonic fibroblast feeder cells

For the production of primary murine embryonic fibroblast feeder cells (EMFI), pregnant C57BL/6J-Tg(pPGKneobpA)3Ems/J inbred mice were sacrificed 13.5 days post coitus (dpc) by cervical dislocation. The uteri were removed with sterile surgical instruments, placed in 10 cm Petri dishes containing phosphate buffered saline (PBS) without Ca^{+2} and Mg^{+2}, and the foetuses were collected. Their membranes, heads, and the internal organs were removed, and discarded. The carcasses of 10 embryos were collected in a 50 ml Falcon tube containing 35 ml PBS and washed in order to remove as much blood as possible. The washed carcasses were minced with a scalpel in a Petri dish placed on ice for about 5 min. Minced tissues were collected in 10 ml 0.05% Trypsin/0.02% EDTA solution (w/v) in PBS without Ca^{+2} and Mg^{+2}, 200 µl DNase I (100 mg) were added and the tissues were incubated for 15 min at 37°C. With the use of a syringe plunger, the tissues were pressed through a sterilized mesh into an Erlenmeyer flask. A total of 50 ml Trypsin/EDTA solution was added to flush the remaining tissue clumps on the mesh. In case of a viscous liquid, 200 to 600 µl DNase I were added directly onto the mesh. The liquid was incubated thereafter for 45 min at 37°C by shaking. The cell suspension was distributed evenly into two 50 ml Falcon tubes, filled up with 1xDMEM medium enriched with 4.5 g/L glucose, L-glutamine and pyruvate and centrifuged for 5 min at 1500 rpm. Cell pellets were washed twice with DMEM (1x), resuspended in a volume of 10 ml DMEM (1x) and the number of viable cells was determined using 1:10 trypan blue dye (100 µl trypan blue, 100 µl cell suspension and 800 µl PBS) and a haemocytometer (FAST-READER 102®). A total of $5x10^6$ cells was seeded into 15 cm Petri dishes containing 25 ml DMEM medium (1x) and 200 µl of Penicillin-Streptomycin, and cultured overnight. The next day, the medium was replaced with antibiotic-free feeder cell medium. Feeder cell medium contained

100 ml DMEM (1x) medium enriched with 4.5 g/L glucose, L-glutamine and pyruvate, 11.4 ml inactivated fetal calf serum (FCS), 1.14 ml 200 mM glutamine and 1.14 ml non-essential amino acids (100x). Cells were cultured for 3 days at 37°C in a moisture-saturated atmosphere of 7% CO_2/93% air in an incubator (Binder), splitted 1:5, and cultured for 3 more days until they were confluent. They were frozen in vials (Nunc^{TM} Cryo tube vials) containing 5x10^6 cells/ml freezing medium consisting of 70% DMEM medium (1x), 20% FCS and 10% Dimethyl Sulfoxide (DMSO). After 1 day storage at -80°C, vials were stored at -160°C.

For the production of inactivated EMFI, cells were quickly thawed at 37°C in a water bath, placed in a 15 ml Falcon tube, filled up with 5 ml feeder cell medium and centrifuged (Hettich Universal 30 F) at 1200 rpm for 5 min. The cell pellet was resuspended in 5 ml feeder cell medium, distributed in five 15 cm Petri dishes, each containing 19 ml feeder cell medium, and incubated at 37°C in a moisture-saturated atmosphere of 7% CO_2/93% air in an incubator for 3 days, followed by a 1:3 split and further culture until confluence was observed. A total of 20 ml fresh feeder cell medium containing 200 µl Mitomycin C (1 mg/ml) was given to the confluent cells followed by incubation at 37°C in a moisture-saturated atmosphere of 7% CO_2/93% for 2 to 2.5 hours. Then they were washed twice with 5 ml PBS, 5 ml of Trypsin/EDTA solution were added, and cells were incubated for a few minutes at 37°C until they detached from the bottom of the plates. A cell suspension was made by repeated pipetting and the cells were transferred into a 15 ml Falcon tube. The plate was washed with 5 ml DMEM, and then the medium containing cells was added to the Falcon tube to stop enzymatic activity. The number of cells was determined using a haemocytometer. Cells were frozen in vials containing 3 to 5x10^6 cells/ml freezing medium. After 1 day storage at -80°C, vials were stored at -160°C.

For preparation of plates for ES cell culture frozen inactivated EMFI cells were thawed quickly at 37°C in a water bath, placed in a 15 ml Falcon tube, filled up with 5 ml of feeder cell medium and centrifuged at 1200 rpm for 5 min. The cell pellet was resuspended in 5 ml fresh feeder cell medium and the number of cells was determined using a haemocytometer. A total of 2.5x10^5 and 7 to 7.5x10^5 inactivated feeder cells was seeded with 4 ml in 6 cm and with 8 ml in 10 cm feeder cell medium in Petri dishes, respectively.

3.7 Mycoplasma species

The number of viable mycoplasmas in stocks was determined before inoculation of the mES cells by plating on Friis agar and counting the number of CFUs after 2 weeks of incubation.

3.8 Murine embryonic stem cell culture

In this study, the murine embryonic stem cell line ES TBV2 which was produced from the 129/SvPas strain of mouse (Institute of Developmental Genetics, Helmholtz Zentrum München, HMGU), was used in the 13th passage (P13). They were free of mycoplasmas and FELASA (Federation of Laboratory Animal Science Associations)-relevant pathogens and contributed to the germ line transmission at P13+1. At P13+2, the karyotype of the ES cells was normal (40, XY).

TBV2 mES cells (P13) were thawed quickly at 37°C in a water bath, placed in a 15 ml Falcon tube, filled up with 5 ml of Embryonic Stem cell (ESC) medium and centrifuged (Eppendorf 5804) at 1200 rpm for 5 min. The ESC medium consisted of 100 ml DMEM (1x) enriched with 4.5 g/L glucose, L-glutamine and pyruvate, 15 ml fetal calf serum pre-tested for ES cells, 0.2 ml β-Mercaptoethanol (50 mM), 1 ml glutamine (200 mM) and 18 µl LIF (10^7 I.U/ml). The cell pellet was resuspended in 5 ml fresh ESC medium and the number of cells was determined using a haemocytometer. A total of $4.14x10^5$ ES cells ($2x10^4$ ES cells/cm^2) was seeded in 6 cm Petri dishes on inactivated feeder cells together with 4 ml ESC medium.

For the establishment of a multiple mycoplasma infection, frozen stocks of *M. hominis*, *M. fermentans* and *M. orale* were thawed quickly at 37°C in a water bath and added to the freshly seeded ES cells at a dose of 1 CFU/12 cells (Tsai 1995). A total of $1x10^4$ CFUs of each mycoplasma species was added to $4.14x10^5$ freshly seeded ES cells and were cultured for 2 days. Control cells were not inoculated with mycoplasmas.

For the following passages, a total of $1.134x10^6$ mycoplasma-infected and control mES cells ($2x10^4$ ES cells/cm^2) was seeded in separate 10 cm Petri dishes with 10 ml ESC medium, cultured over 20 passages on inactivated EMFI cells at 37°C and 7% CO_2/93% air in an incubator (Queue Systems), and passaged every 2 days. At each passage, mES cells from each experimental group were cultured in four to eight 10 cm Petri dishes. The supernatants were collected and the mES cells were washed twice with 3 ml PBS and once with Trypsin/EDTA solution and

incubated with 3 ml Trypsin/EDTA solution at 37°C for a few minutes until they detached from the bottom of the plates. A cell suspension was made by repeated pipetting and the ES cells were transferred into a Falcon tube. Each plate was washed with 3 ml ESC medium and the medium containing cells was added to the Falcon tube with the trypsinized cell suspension to stop enzymatic activity. For each experimental group, the supernatants, the washing solutions containing detached cells and the cell suspensions were pooled together, respectively.

The morphology, the number and the viability of mycoplasma-infected and control mES cells were determined at all 20 passages. The morphology of the ES cells was determined by observing the confluence, the size of the mES cell colonies, and the development of embryoid bodies. The total number of mES cells at each passage was determined by calculating the number of cells in the supernatant, the washing solution and the cell suspension using a haemocytometer. The viability of mES cells in the cell suspension was determined using 1:10 Trypan blue dye (100 µl cell suspension, 100 µl Trypan blue, 800 µl PBS).

The remaining mES cells were frozen in 1.8 ml vials with ESC freezing medium consisting of 65% DMEM (1x), 25% FCS and 10% DMSO, stored 1 day at -80°C and then at -160°C.

3.9 Detection of mycoplasmas in stem cell cultures

The ability of *M. hominis*, *M. fermentans* and *M. orale* to grow in ES TBV2 cell cultures was determined at all 20 passages by agar culture, the MycoAlert™ assay, the in-house established PCR, and the Mycosensor™ QPCR assay.

For agar culture, aliquots of 100 µl fresh supernatant from mycoplasma-infected and control ES cells were inoculated on Friis and PH-Hayflick agar plates at passages P13+5, P13+10, P13+15 and P13+20. Results were evaluated after 2 weeks of incubation in a moisture-saturated atmosphere of 7% CO_2/93% air at 37°C. Three aliquots of 1 ml supernatant from the 20 passages of mycoplasma-infected and control mES cell lines were collected in 1.5 ml Eppendorf tubes stored at -80°C and examined subsequently. Supernatants from all passages were tested with the MycoAlert™ assay and the gel-based PCR, while only mycoplasma-infected samples from the passages P13+5, P13+10, P13+15 and P13+20 were tested with Mycosensor™ QPCR assay. Additionally, a total of $1x10^4$ mycoplasma-infected mES cells prepared for blastocyst injection was tested with the Mycosensor™ QPCR

assay in order to determine the number of mycoplasmal genomes injected into a blastocyst. The identification of mycoplasma species found in each passage was determined by digestion of the PCR products with restriction enzymes (Restriction Fragment Length Polymorphism, RFLP).

3.10 Spectral Karyotyping (SKY)

The karyotype of mycoplasma-infected and control mES cells was determined by Spectral Karyotyping at passages P13+5, P13+10, P13+15 and P13+20. The analyses were performed in the Institute of Molecular Radiation Biology, HMGU.

A total of 100 µl Colcemid (Colcemid stock solution 10 µg/ml) was added to mES cells in a 10 cm Petri dish (10 ml ESC medium), in order to capture metaphases. The ES cells were incubated at 37°C in an incubator (Queue Systems) for 2 to 2.5 hours. Then the cells were washed twice with 3 ml PBS and trypsinized with 3 ml EDTA/Trypsin solution. The supernatant, the washing solution and the cells were collected in a 50 ml Falcon tube and centrifuged at 1100 rpm for 5 min. The supernatant was discarded and the tube was flicked with the finger to distinguish the cell pellet. A total of 10 ml KCl (75 mM) hypotonic solution (0.56% in dH_2O) (KCl M=74.56 g/mol) was added dropwise while the tube was gently flicked with the finger. The cells were left for 10 min at room temperature and centrifuged afterwards at 1100 rpm for 10 min. The supernatant was discarded and the tube was flicked with the finger to distinguish the cell pellet. A total of 10 ml fresh cold methanol/acetic acid fixative (3:1 vol/vol) was added dropwise to the cells, stored for 30 min at -20°C and centrifuged at 1500 rpm for 10 min. The same procedure was repeated 3 times. The cells were finally resuspended in a final volume of 2 ml fixative and stored overnight at 4°C. The next day, 10 to 20 µl of fixed metaphases were dropped onto super frost object slides and aged for a week at room temperature. The slides were stored under a nitrogen atmosphere at -20°C until used.

Metaphase preparations were thawed in a humid chamber at 37°C for 20 min and treated thereafter with 100 µl RNase solution (0.05 mg RNase A stock solution/1 ml 2xSSC), covered with plastic foil, and incubated in a humid chamber for 20 min at 37°C in an incubator (Memmert). They were washed twice in 2xSSC (100 ml of 20xSSC in 1 liter dH_2O) for 3 min, placed in denaturating solution (70 ml formamide, 10 ml 20xSSC, and 20 ml dH_2O) for 2 min at 72°C and dehydrated in a 70% (kept at -20°C), 90% (kept at 4°C) and 100% (kept at 4°C) ethanol series for 2 min, each. The

20xSSC solution consisted of 175.3 g NaCl, 88.2 g sodium citratex2H_2O in 1 liter dH_2O (autoclaved).They were subsequently hybridized with 5 µl/slide probe mixture (Mouse Spectral Karyotyping Reagent Lot Nr. 0666, Applied Spectral Imaging), which was denaturated before for 7 min at 80°C and incubated for an hour in the dark at 37°C on a heated block. The denaturated metaphases with the hybridization solution were covered with 18x18 mm coverslips, sealed with rubber cement (Fixogum, Marabu) and incubated for 46 to 48 hours in a humid chamber at 37°C. Post hybridization washes were performed twice in 2xSSC at 37°C for 5 min, once in 0.5xSSC at 75°C for 5 min and once in 4xSSC/0.1% Tween (80 ml dH_2O, 20 ml 20xSSC and 0.1 ml Tween® 20) for 2 min at room temperature. After a short wash with dH_2O, 100 µl of PNM blocking reagent were added onto the slides, covered with plastic foil and incubated in a humid chamber at 37°C for 30 min. PMN consisted of 500 ml PN buffer, 25 g of Blotting Grade Blocker Non-Fat Dry Milk 5% and 0.1 g sodium azide. PN solution contained 1 liter solution A (17.79 g Na_2HPO_4 M = 177.99 g/mol, in 1 liter dH_2O), 62.5 ml solution B (0.86 g NaH_2PO_4 M = 137.99 g/mol, in 62.5 ml dH_2O) and 0.1% Nonidet P40 which was added after the stabilization of the final volume at pH 8.0. The detection of digoxigenin-labelled and biotinylated probes was carried out by the addition to the slides of 50 µl of Anti-digoxigenin solution diluted in PMN (5 µl Anti-digoxigenin solution in 1245 ml PMN) followed by 50 µl mixture solution of a goat anti-mouse antibody conjugated to Cy-5 and Cy-5.5, respectively (5 µl of each antibody+245 µl PMN, mixed together 1:1). The slides with the antibody solutions were incubated at 37°C for 45 min and washed 3 times afterwards in 4xSSC/0.1% Tween at 45°C for 5 min. The chromosomes were then stained with 30 µl DAPI-antifade solution (5 µl DAPI 1 mg/ml, 30 µl Vectashield mounting medium, 100 ml 2xSSC) and covered with 24x50 mm coverslips.

Metaphases were observed under an epifluorescence microscope (Axioplan 2, Carl Zeiss GmbH) connected to a interferometer-CCD Camera Combination (SpectraCube, Applied Spectral Imaging) and analyzed with the SKY View imaging software. A total of 15 to 20 metaphases from each cell passage was evaluated for chromosomal aberrations such as translocations, deletions, numeric aberrations, insertions and chromosome breaks using a mouse cytogenetic nomenclature. An alteration was characterized “clonal” when it appeared 2 or more times in 15 metaphases and “non clonal” when it appeared only once. Numeric aberrations were only of importance when they appeared 3 or more times in 15 metaphases.

3.11 Fluorescent Activated Cell Sorting (FACS) of the ES cells

The differentiation status of mycoplasma-infected and control ES TBV2 cells was determined via flow cytometric analysis (FACS) by evaluation of OCT-4 and SSEA-1 markers. mES cells at passages P13+6, P13+11, P13+16 and P13+21 were analysed at the Institute of Molecular Immunology, HMGU.

Frozen mycoplasma-infected and control mES cells at passages P13+5, P13+10, P13+15 and P13+20, were thawed and cultured for 2 days. The mES cells were harvested and collected in 4 ml ESC medium after 2 and 1 washing steps with PBS and Trypsin/EDTA solution, respectively, and short incubation with Trypsin/EDTA solution. They were centrifuged at 1700 rpm for 7 min (Heraeus Megafuge 2.0) and the cell pellets were washed with 1 ml Hank's Balanced Salt Solution (HBSS). In order to discriminate live from dead cells, the cell pellets were resuspended in 2 µl ethidium monoazide (EMA 500 mg/ml), transferred in 2.5 ml tubes, incubated in the dark for 10 min and then illuminated for further 15 min. Then, they were washed with 1 ml HBSS, centrifuged at 1700 rpm for 5 min and resuspended in 100 µl mouse buffer. The mouse buffer consisted of 1 ml HBSS, 50 µl mouse serum and 10 µl NaN_3 (NaN_3 0.1%). Aliquots of 50 µl cell suspension were prepared for double staining, isotype controls, and single staining controls, respectively. A total of 3 µl Anti-SSEA-1 and Mouse IgM coupled with APC was added to the samples prepared for double and single staining, and isotype control, respectively, and incubated by shaking on ice. After 1 hour incubation time, the samples were washed twice with 1 ml HBSS, centrifuged at 1900 rpm for 5 min, resuspended in 0.5 ml 1:3 Fixation/Permeabilization solution (Foxp3 Staining Buffer set), incubated in the dark at 4°C for 40 min to allow fixation of the cells, and washed twice with 1 ml permeabilization buffer solution (1 ml 10xPermeabilization buffer/9 ml dH_2O). Furthermore, 2 µl of Fc block solution (2 mg/ml FCR G Rat 2b in 0.1% NaN_3) were added to the samples. A total of 3 µl of Anti-OCT-3/4 and Anti-mouse CD4 coupled with PE (L3T4) was given to samples prepared for double and single staining, and isotype control, respectively, and incubated by shaking at room temperature for 45 min. They were washed twice with 1 ml permeabilization buffer solution and resuspended in 250 µl HBSS. Measurements were performed with a FACS LSR II and results were evaluated with the FloJo software (TreeStar, Inc. USA).

3.12 Mice and husbandry

Outbred Crl:CD1 (Icr) and inbred C57BL/6J-Tg(pPGKneobpA)3Ems/J mice used for the production of blastocysts and embryo transfer recipients, and the production of murine embryonic fibroblasts (feeder cells), respectively, were bred in a full barrier unit at the HMGU animal facilities and kept in filter-topped Type II Makrolon cages at a temperature of 20 to 24^{o}C, humidity of 50 to 60%, 20 air exchanges per hour and a 12/12-hour light/dark cycle. They were free of FELASA-relevant infectious agents. Wood shavings were provided as bedding. Mice were fed a standardized mouse diet (1314, Altromin) and provided sterilized drinking water *ad libitum*.

Animal manipulations were performed in a mouse room. Before entering the area, staff were clothed in a clean garment and wore disposable gloves, bonnets and face masks. During routine weekly changes of cages including wire bar lids and water bottles in class II laminar flow changing stations, mice were transferred to new cages with forceps padded with silicone tubing. Forceps were disinfected after each cage change with 70% ethanol. All materials, including individually ventilated cages (IVCs, VentiRacks™), Makrolon® cages, lids, feeders, bottles, bedding and water, were autoclaved before use.

Experimental mice were kept in IVCs under positive pressure and the conditions stated above. All animal studies were approved by the HMGU Institutional Animal Care and Use Committee and the Government of Upper Bavaria, Germany (211-2531-8/02).

3.13 Production of blastocysts and pseudopregnant recipients

Female outbred Crl:CD1 (Icr) mice aging 6 to 8 weeks were used as blastocysts donors. They were induced to ovulate by intraperitoneal injections of 5 IU equine Chorionic Gonadotropin (eCG) (Intergonan® 1000 I.U) followed 48h later by 5 IU human Chorionic Gonadotropin (hCG) (Ovogest® 1500 I.U). They were mated immediately thereafter with males of proven fertility. The presence of vaginal plugs indicating successful mating was determined the following morning.

For the collection of blastocysts, pregnant mice were sacrificed with CO_2 3.5 days post coitus (3.5 days after plug checking), the uteri were removed with sterile surgical instruments and they were placed in 8-cm Petri dishes (Nunc IVF Product line) containing filtered M2 medium pre-tested for embryos. Then, they were flushed

with the respective medium and the blastocysts were collected thereafter under a stereoscopic microscope (Leica MZ75). They were placed in 3.5 cm sterile tissue culture dishes, and washed through 4 drops of 100 µl M2 medium each. The washed blastocysts were kept in a 3.5 cm Petri dish in a 100 µl drop of M2 medium, covered with mineral oil on a heated block at 37°C until used.

Female outbred Crl:CD1 (Icr) mice aging 8 to 12 weeks were used as pseudopregnant recipients. They were mated with vasectomized males and used for embryo transfers 2.5 days after the vaginal plug was detected.

3.14 Blastocyst injection, embryo transfer and production of chimeras

Mycoplasma-infected and control TVB2 mES cells at passages P13+5, P13+10, P13+15 and P13+20 were used for blastocyst injection and embryo transfers. The ES cells were prepared as previously described. However, incubation of the cells with Trypsin/EDTA solution was shorter in order to obtain feeder-free mES cells. The cell suspension was then centrifuged at 1200 rpm for 5 min, and the cell pellet was resuspended in a final volume of 0.5 ml to 1 ml ESC medium and kept on ice until used.

Blastocyst injection and embryo transfer were performed as described (Nagy 2003). Blastocyst injection was performed using a microinjection manipulator (Leitz DM IRB) on a super frost object slide covered with filtered M2 medium using injection pipettes GC100T-15, size 38, straight with spike (BioMedical Instruments) and hand-made blastocyst-holding pipettes. After uptake of the mES cells into the injection pipette, the blastocysts were held in the correct position and a total of 15 to 20 mES cells was injected in each blastocoel (Figure 8). The injected blastocysts were collected afterwards into a drop of M2 medium covered with mineral oil and left on a heated block to recover and re-expanded until used. Injection of blastocysts with control mES cells was performed first, followed by the injection with mycoplasma-infected mES cells.

The pseudopregnant recipients were anaesthetized with 0.15 ml of Ketamin (0.39 ml Ketavet® 100 mg/ml), Xylazine (0.39 ml Sedaxylan 20 mg/ml), and Acepromazinemaleat and chlorobutanol (0.12 ml Vetranquil 1%) mixture solution diluted in 2.1 ml Sodium Chloride (0.9%). The mice were dissected laterally in the abdominal area with sterile surgical instruments and the uteri horns were taken out of the cavity gently and held with a clamp. A 27 gauge injection needle was inserted

near the utero-tubal junction into the endometrial stroma under a stereoscopic microscope, and immediately after the removal of the needle, a glass micropipette containing the injected blastocysts and a very small amount of M2 medium was inserted and embryos were deposited into the uterus. A total of 7 injected blastocysts was transferred to each uterus horn. The abdominal muscles were sutured with sterile absorbable Vicryl 4-0 and the skin was closed with 9 mm auto clips. Embryos injected with control mES cells were transferred first, followed by the transfer of blastocysts injected with mycoplasma-infected ES cells.

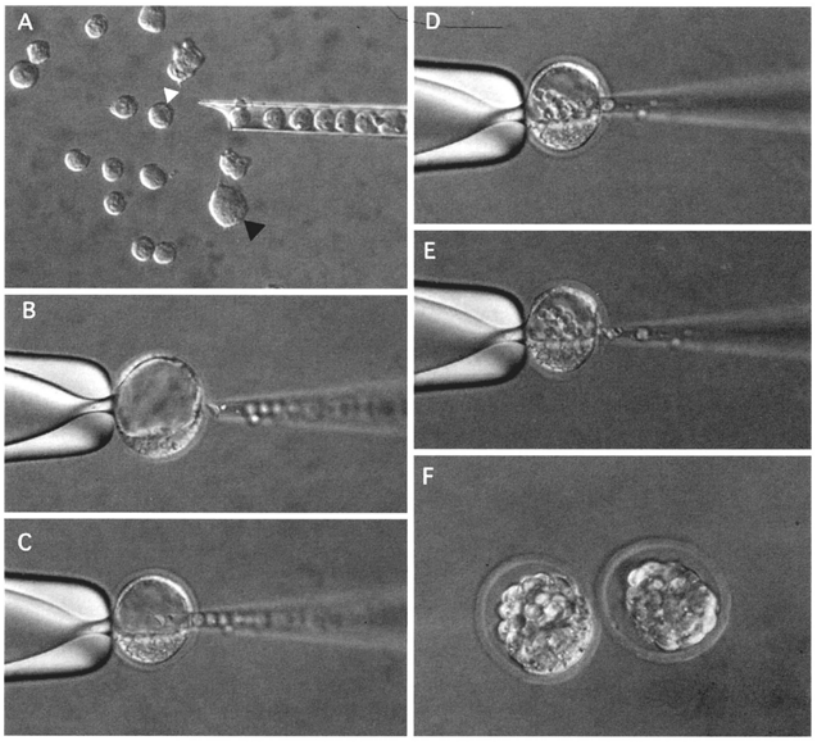


Figure 8 Procedure of blastocyst injection, Photograph from Nagy et al., Manipulating the mouse embryo, 2003. A: Collection of ES cells, White arrow: ES cell, Black arrow: Fibroblast cell, B: Immobilization of blastocyst by holding pipette in the right position (inner cell mass positioned at 6 or 12 o´ clock), C: Insertion of injection pipette into the blastocoel, D: Release of ES cells into blastocoel, E: Slowly withdrawal of the injection pipette, F: Collapse of blastocysts, resulting in close contact of the ES cells with the surface of the inner cell mass. Blastocysts are re-expanded after 1 to 3 hours of culture.

After the operation, pseudopregnant recipients received one drop of analgesic (Vetalgin® 500 mg/ml) orally. They were kept singly in IVCs.

A total of 8, 14, 14 and 16 embryo transfers was performed with mycoplasma-infected mES cells from the passages P13+5, P13+10, P13+15, and P13+20, respectively, whereas 6, 8, 11, and 7 embryo transfers were performed from the respective passages with control TBV2 mES cells.

The number of litters, pups, and chimeric pups of each respective passage, as well as clinical signs of the recipients and pups were protocolled. Furthermore, the weight and length of a total of 108 and 69 pups coming from litters produced with mycoplasma-infected and control mES cells, respectively, were scored weekly until the 13th week of age.

3.15 Determination of chimeras and germ line transmission (GLT)

For determination of mES cell contribution to the germ line, chimeras obtained were mated with C57BL/6 mice (Charles River Laboratories, Inc., Germany). The litter size, color and gender of the mice were recorded. Progeny were either agouti with a light-coloured belly (GLT, originating from the 129/SvPas strain) or black in colour (wild type, originating from the C57BL/6 strain) (Figure 9).

3.16 Histological examination and stains

A total of 6 and 3 clinically affected and healthy male chimeras, respectively, was sacrificed at 2 (n=2 and 1), 7 (n=1 and 1) and 16 weeks of age (n=3 and 1). The heads, the internal organs, the femurs and the humerus were investigated histologically. A total of 2 male chimeras, 2 and 7 weeks old, respectively, from control litters and 3 male outbred wild type Crl:CD1 mice (pathogen-free from the core breeding unit of the HMGU), 7 week old, were sacrificed and served as control mice (additionally to the clinically healthy chimeras obtained after blastocyst injection with mycoplasma-infected mES cells). The weight and length of mice, as well as the size of the internal organs of the 7- and 16- week old mice were scored whereas their carcasses were x-rayed in order to determine the structure of the skeleton. Histological examinations were performed at the Institute of Pathology, HMGU.

Internal organs were fixed in 10% buffered formalin for 1 to 2 days and embedded in paraffin. For histological examination, 2-5 µm thick sections were prepared (Microm 355), mounted on super frost plus slides and dewaxed in xylol for

5 min. This was followed by dehydration in a 70%, 96% and 100% ethanol series for 3 min, respectively, and washing with dH_2O for 30 sec. The slides were then stained for 2 min with Mayer`s hematoxylin solution, and washed with running water for 5 to 10 min until the water appeared clear. Hematoxylin solution consisted of 1 g hematoxylin, 0.2 g $NaJO_3$, 50 g $KAl(SO_4)12H_2O$, 50 g chloral hydrate and 1 g of citric acid, diluted and filtered in 1 liter dH_2O. The samples were dipped for 1 second in a 1% alcoholic eosin solution (1g Eosin Y/100 ml 100% ethanol), dehydrated through a 70%, 96% and 100% ethanol series and placed in xylol for about 5 min. The slides were then covered with Eukitt mounting medium and then with 24x50 mm coverslips. Hematoxylin-Eosin stains the cytoplasm of the cells red, and the nuclei blue.

After 7 days of fixation in formalin, the head, the femurs and the humerus of the respective mice were decalcified in EDTA for 6-8 weeks at room temperature or 2 weeks at 58°C, washed, dehydrated, prepared in Tissue-Tek® VIP™ 5 (Vacuum Infiltrations Processsor) and embedded afterwards in liquid paraffin. For histological examination, 4 µm thick sections were prepared, mounted on super frost plus slides, dewaxed in xylol, dehydrated through a 70%, 96% and 100% ethanol series for 3 min and washed with dH_2O for 30 sec. For examination of the bone structure, the samples were stained for 10 min with Weigert´s Fe-Hematoxylin stain. The Fe-Heamatoxylin consisted of 1 part solution A (10 g Hematoxylin in 1 liter ethanol) and 1 part solution B (11.6 g $FeCl_3$, 7.8 ml 32% HCL in 992 ml solution A). The sections were washed for 10 min in dH_2O and then they were stained for 5 min in Van Gieson solution saturated with picric acid, rinsed in dH_2O and ethanol, respectively, and mounted in Eukitt mounting medium. The Van Gieson´s stain consisted of 400 ml picric acid and 10 ml 1% acid violet. Van Gieson stains mineralised bones black, ostoid and collagen red, and bone marrow and muscles yellow.

3.17 Flouresence Activated Cell Sorting of peripheral white blood cells

The immunological status of chimeras obtained after blastocyst injection with mycoplasma-infected and control mES cells, was determined. Immunoglobulin levels, Anti-DNA Abs, rheumatoid factor and peripheral blood leucocytes were investigated via multiplex bead array based assay, ELISA, and flow cytometric analysis (FACS), respectively. Analyses were performed in the Immunological Screening Module of the German Mouse Clinic, HMGU.

A total of 200 to 300 µl blood was collected with a 24-gauge needle from the

lateral veins of the mouse tail in 1.5 ml Eppendorf tubes containing 10 µl Heparin (25.000 I.U). The tubes were inverted several times to distribute the blood to anticoagulant and kept at room temperature for maximal 3 hours. The blood samples were centrifuged at $7.5x10^3$ rpm for 3 min at 10°C, and 50 to 100 µl of plasma were taken for determination of the IgG1, IgG2a, IgM, IgG3, IgA levels, anti-DNA antibodies (abs) and rheumatoid factor.

A total of 25 µl/well of plasma diluted 1:1000 in FACS buffer, and 25 µl of 7-fold serial dilutions of unlabelled mouse IgG1, IgG2a, IgM, IgG3 and IgA standard mixture (10^0=2.5 µl/ml) were added in duplicate in 96-well plate. The FACS buffer consisted of 1 liter filtered PBS, 5 g BSA, and 660 µl NaN_3 30%, stabilized at pH=7.5. A total of 5 µl/well beads mixture was added and the plate was covered with aluminium foil and incubated at room temperature on a shaker for 10 min at 300 rpm. The bead mixture contained luminex beads of five different regions, coupled with antibodies specific for mouse IgG1, IgG2a, IgM, IgG3, and IgA. A mixture of biotinylated IgM, IgG1, IgG2a, IgG3 and IgA standards (200 ng/ml in FACS buffer) was prepared and 25 µl/well were added to the samples, covered with aluminium foil and incubated for further 20 min at room temperature on a shaker at 300 rpm. Afterwards, the samples were washed three times with 100 µl/well FACS buffer using a vacuum manifold and then 50 µl/well of streptavidin-PE (1 µl/ml) were added and incubated in the dark on a shaker for 10 min at 300 rpm. The washing step with the FACS buffer was repeated 3 more times and the samples were resuspended in 100 µl/well FACS buffer/0.05% Tween 20 and shaken for 30 seconds at 1000 rpm. Results were obtained on a Bio-Plex reader (100 beads/region/analyte).

The DNA plate was incubated for an hour at room temperature with 50 µl/well Poly L-Lysine (50 µg/ml) and washed with the TECAN washer program, dried upside down on tissue paper to remove remaining liquid and 50 µl of single- and double-stranded DNA (1:1) mixture were added per well. The plate was covered with aluminium foil and stored at 4°C overnight. The wash buffer consisted of 2.5 l PBS, 12.5 ml Tween® 20, and 5 ml 10% NaN_3. For the rheumatoid factor, the plate was incubated with 50 µl/well rabbit IgG, covered with aluminium foil and stored at 4°C overnight. A total of 60 µl of plasma diluted 1:5 in PBS was added per well in the respective plates in duplicate. Coated-blocked plates were washed with the TOMTEC-Robot Quadra 3 system after 30 min of incubation at room temperature, and dried on tissue paper until there was no liquid in the wells. The washing solution

of Quadra 3 constisted of 10 l PBS and 0.01% Tween® 20. A total of 200 µl of DNA (diluted 1:100 in PBS) and RH (diluted 1:200 in PBS) dilutions was added per well in the respective plates, whereas serial 7-fold dilutions of control DNA (10^0 = 1 µl in 1250 µl PBS) and RH standard controls (10^0 = 3.2 µl in 800 µl PBS) were made and 200 µl of each serial dilution were added per well in the respective plates. The plates were incubated for 1 hour in a moist chamber at room temperature, washed and dried as previously described. A total of 100 µl of the appropriate detection antibody (Anti-mouse Polyvalent Immunoglobulins-AP diluted 1:3000 in PBS) was given in each well and the plates were incubated for more 1.5 hours in a moist chamber at room temperature. Wash and dry steps were repeated as previously described. Furthemore, 100 µl of p-Nitrophenyl Phosphat tabs/substrate buffer dilution (20 mg in 20 ml) were added per well and incubated for exactly 17 min at room temperature and washed thereafter with the TECAN washer program. The substrate buffer consisted of 1 l dH_2O, 97 ml Diethanolamine, 2 ml 10% NaN_3, 200 mg $MgCl_26H_2O$, stabilized of pH 9.5. Measurements were performed on a TECAN SUNRISE^{TM} reader (TECAN GmbH, Crailsheim, Germany).

Peripheral blood leucocytes were collected after resuspension of the remaining cell pellet in 500 µl NH_4Cl-Tris solution (lysis buffer) at room temperature which led to lysis of the red blood cells, followed by filtration of the cell suspension through a nylon net into a 1.5 ml Eppendorf tube and shaking for 10 min at room temperature. The Lysis buffer consisted of 45 ml solution A (1 liter dH_2O and 9.1 g NH_4Cl, filtered) and 5 ml solution B (1 liter dH_2O and 28.8 g Tris-HCl, pH=7.5, filtered). A total of 200 µl/well of the cell suspension was added to 2 wells of a 96-well plate with a U-bottom and the plate was centrifuged at 10°C for 3 min at $7.5x10^3$ rpm. The cells were washed twice in 200 µl NH_4Cl-Tris/well and 200 µl FACS buffer/well, respectively, and centrifuged at 10°C for 3 min at $7.5x10^3$ rpm. Peripheral blood leucocytes were then incubated with 20 µl Fc block solution (anti-mouse CD16/CD32) (1µl Fc block stock solution 2 mg/ml, in 499 µl FACS buffer) for 20 min on ice, 150 µl FACS buffer were added to the samples and centrifuged thereafter at 10°C for 3 min at $7.5x10^3$ rpm. The cells were resuspended in 50 µl Antibody mix (Panel 1 and Panel 2), incubated in the dark on ice for 15 min, then 50 µl Propidium Iodide solution/well (1 µl PI stock solution 2 mg/ml, in 99 µl PBS, stored at -20°C) (PI 2 mg/ml) were added therein, and the cells were incubated for further 5 min in the dark on ice. They were washed and centrifuged repeatedly in 100 and 200 µl FACS

buffer 5 times, respectively. The Panel 1 mixture consisted of the following antibodies: 1:400 of 0.5 mg/ml FITC-conjugated rat anti-mouse CD44, 1:100 of 0.2 mg/ml PE-conjugated armenian hamster anti-mouse γδTCR and APC-conjugated rat anti–mouse CD25, respectively, 1:1000 of 0.2 mg/ml PerCpCy55-conjugated rat anti-mouse CD4, 1:100 of 0.2 mg/ml PE-Cy7 conjugated rat anti-mouse CD62L and 0.2 mg/ml PB-conjugated rat anti-mouse CD3, 1:400 of 0.1 mg/ml APC-Alexa 750-conjugated rat anti-mouse CD8a and 1:200 of 0.5 mg/ml Alexa Fluor 700-conjugated rat anti-mouse CD45, whereas the Panel 2 antibody mixture contains 1:1000 of 0.5 µl/ml FITC-conjugated rat anti-mouse IgD, 1:200 of 0.2 mg/ml PE-conjugated Rat anti-mouse NK-T/NK, 1:800 of 0.2 mg/ml PerCPCy55-conjugated rat anti-mouse Gr1, 1:1000 of PECy7-conjugated rat anti-mouse CD19, 1:2000 of 1 mg/ml APC-conjugated rat anti-CD5, 1:800 of 0.1 mg/ml PB-conjugated rat anti-mouse CD11b, 0.1 mg/ml APC-Alexa 750-conjugated rat anti-mouse B220 and 1:2000 of 0.5 mg/ml Alexa fluor 610-conjugated rat anti-mouse CD45. On the same plate, single color controls (SCC) containing single antibodies and PI were run parallel using the rest of the blood samples and 1:100 of 0.1 mg/ml PE-Alexa Fluor 610-conjugated rat anti-mouse CD4, as well as an unstained sample containing only PI.

Data were obtained with a FACS LSR II and were analyzed using the FlowJo software (TreeStar, Inc. USA). Discrimination between live and dead cells was performed using the PI versus PE ratio. The minimum number of living cells was expected to be ≥ 30.000. The CD45+ cells were then defined versus the single colour controls and were taken for further analysis. T-cells were defined with the Panel 1 antibodies, whereas Panel 2 detected B- and NK- cells. Subtypes of these cells were defined after subsequent subgatings of the respective cell population.

Blood samples were collected and tested from 56 male mice, aging 13 to 18 weeks, and were divided into 5 groups. Group A constituted 9 chimeric mice obtained after the blastocyst injection with control mES cells, group B constituted 13 clinically healthy chimeric mice obtained after the blastocyst injection with mycoplasma-infected mES cells, group C constituted 13 affected chimeric mice with clinical signs obtained after the blastocyst injection with mycoplasma-infected mES cells, group D constituted 10 control CD-1 wild type mice from the HMGU core breeding unit, and group E constituted 11 non-chimeric mice obtained after the blastocyst injection with mycoplasma-infected mES cells. All mice were housed under the same conditions in IVCs.

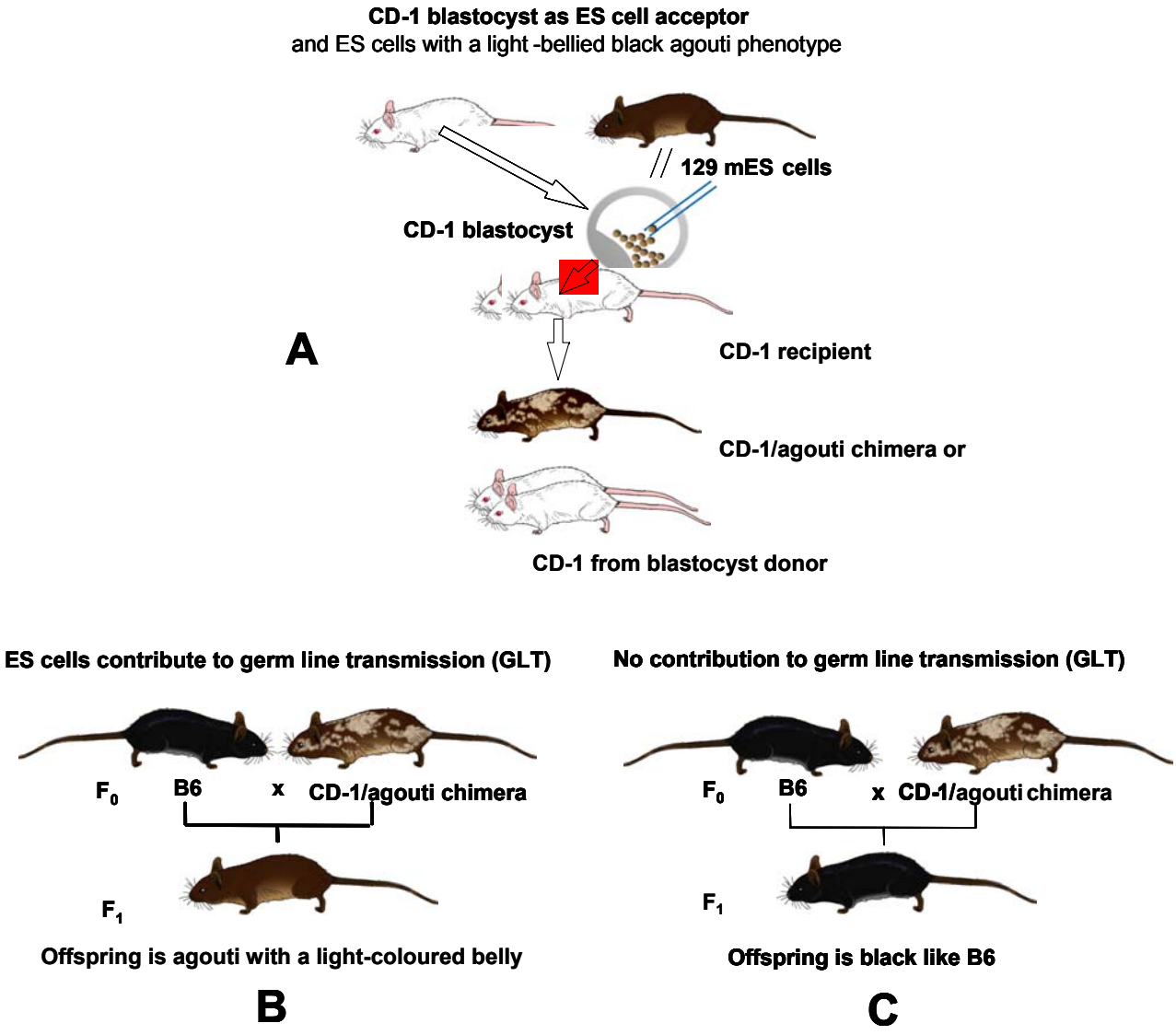


Figure 9 Determination of the germ line transmission based on the coat colour of the germ line pups. A. Offspring chimeras coming from CD-1 blastocysts injected with ES TBV2 cells. B. When ES cells show germ line transmission (GLT), the offspring appear agouti with light-coloured belly. C. When ES cells do not show GLT, the offspring appear black. (Kindly provided by Dr. Michael van der Waal)

4. RESULTS

Detection of mycoplasma species

The presence of mycoplasmas in serial 10-fold dilutions of cell culture supernatants and cell suspensions was determined using agar culture, MycoAlert™, gel-based PCR, and MycoSensor™ QPCR assays. The number of cells contained in the supernatant was $1x10^4$/ml for *M. hominis*, $1.2x10^4$/ml for *M. fermentans,* and $2x10^4$/ml for *M. orale*, whereas $1x10^4$ trypsinized cells/ml DMEM were in the undiluted cell suspensions. The results are shown in Tables 1 to 3.

Detection of *M. hominis*

The results for the comparison of agar culture, MycoAlert™, gel-based PCR and MycoSensor™ for the detection of *M. hominis* are shown in Table 1. Plating of *M. hominis* from the undiluted cell-containing supernatant led to growth on both Friis and PH-Hayflick agar plates. Since the colonies were too dense they were not counted. With respect to the dilutions of the cell-containing supernatants, *M. hominis* colonies were detected up to the 10^{-5} dilution on Friis agar plates with $2x10^1$ CFUs/ml and up to the 10^{-4} dilution on PH-Hayflick agar plates with $7x10^1$ CFUs/ml. The limit of detection (LOD) of *M. hominis* in the cell suspensions was the 10^{-2} dilution with $6x10^2$ CFUs/ml and the 10^{-3} dilution with $4x10^1$ CFUs/ml on Friis and on PH-Hayflick agar plates, respectively. The MycoAlert™ assay produced positive results with the undiluted *M. hominis* aliquots (5/5) and with the 10^{-1} dilution (5/5). All gel-based PCR runs (5/5) from the undiluted cell-containing supernatants were positive. Positive runs were obtained up to the 10^{-2} dilutions (3/5) (Figure 10). The gel-based PCR gave positive results for *M. hominis* only with the undiluted cell suspension in 2 out of 3 runs. The MycoSensor™ QPCR detected *M. hominis* in the undiluted cell-containing supernatant ($8x10^7$ copies/ml) and up to a dilution of 10^{-3} ($1.3x10^5$ copies/ml) in all runs. Positive runs were obtained up to the 10^{-6} dilution (1/5), corresponding to $8x10^1$ copies/ml and 8 copies per reaction mixture (Figure 11). The MycoSensor™ QPCR detected *M. hominis* in the undiluted cell suspension ($1.6x10^6$ copies/ml) and up to a dilution of 10^{-2} ($1.9x10^4$ copies/ml) in all runs. Positive runs were obtained up to the 10^{-4} dilution (1/3), corresponding to $6x10^1$ copies/ml and 6 copies per reaction mixture. The cycle threshold (C_t) value for the standard sample of 50 copies was an average of 32 cycles for the *M. hominis* runs (data not shown), and the melting

temperature (T_m) was between 80.1°C and 82°C. The negative control gave no amplification curves and the T_m was between 65°C and 73°C. As such, it was judged as negative. The standard curves for run 1 to run 5 with *M. hominis* cell-containing supernatants and dilutions were linear with an average correlation coefficient (r^2) of 0.99 and an average slope of -3.29, corresponding to a PCR efficiency of 2 (100%) (Figure 11). Likewise, the standard curves for run 1 to run 3 with *M. hominis* cell suspensions and dilutions were linear with an average correlation coefficient (r^2) of 0.99 and an average slope of -3.35, corresponding to a PCR efficiency of 1.99 (99%).

Detection of *M. fermentans*

The results for the comparison of agar culture, MycoAlert™, gel-based PCR and MycoSensor™ for the detection of *M. fermentans* are shown in Table 2. Plating of *M. fermentans* from the undiluted cell-containing supernatant led to the growth of $4.5x10^4$ CFUs/ml on Friis agar plates but no colonies were observed on PH-Hayflick agar plates. With respect to the dilutions of the cell-containing supernatants, *M. fermentans* colonies were detected up to the 10^{-4} dilution on Friis agar plates with $1x10^1$ CFUs/ml. Plating of cell suspensions led to the growth of colonies up to the 10^{-4} dilution with $5x10^1$ CFUs/ml on Friis agar plates while growth on PH-Hayflick plates with $3x10^1$ CFUs/ml was observed up to the 10^{-2} dilution. The MycoAlert™ assay produced positive results for the undiluted aliquots (5/5). All gel-based PCR runs (5/5) with the undiluted *M. fermentans* cell-containing supernatants were positive. Positive runs were also obtained with the 10^{-1} (3/5) dilutions (Figure 10). *M. fermentans* was detected in the undiluted cell suspension in all 3 runs and up to the 10^{-1} dilution (2/3) with the gel-based PCR. The MycoSensor™ QPCR detected *M. fermentans* in the undiluted cell-containing supernatant ($3x10^5$ copies/ml) and up to the 10^{-1} dilution ($2.9x10^4$ copies/ml) in all runs. Positive runs were obtained up to the 10^{-3} dilution (1/5), corresponding to $6.8x10^1$ copies/ml and 7 copies per reaction mixture (Figure 12). The MycoSensor™ QPCR detected *M. fermentans* in the undiluted cell suspension ($1.6x10^5$ copies/ml) and up to a dilution of 10^{-2} ($2.4x10^3$ copies/ml) in all runs. Positive runs were obtained up to the 10^{-3} dilution (2/3), corresponding to $2.4x10^2$ copies/ml and 24 copies per reaction mixture. The cycle threshold (C_t) value for the standard sample of 50 copies was an average of 32 cycles (data not shown) and the melting temperature (T_m) was between 80.1°C and

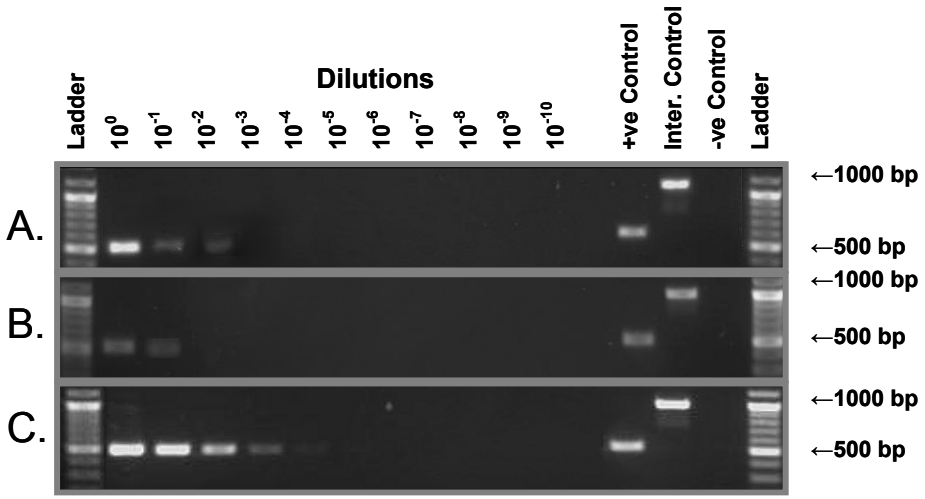


Figure 10 Gel-based PCR analyses of supernatant and 10-fold serial dilutions for the three human mycoplasma species. The PCR products of the mycoplasma-positive samples gave bands of about 510 bp. The internal control gave a 986 bp product. A.: *M. hominis* showed detectable bands up to the 10^{-2} dilution, B.: *M. fermentans* showed detectable bands up to the 10^{-1} dilution, C.: *M. orale* showed detectable bands up to the 10^{-4} dilution.

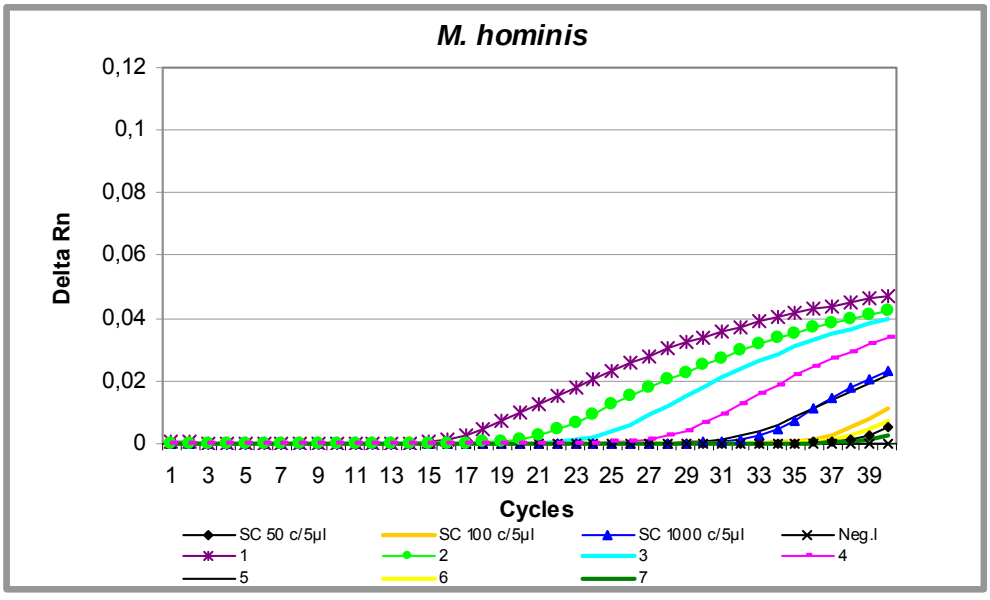


Figure 11 Amplification curves of the standard sample and serial dilutions of supernatant up to the respective dilution limit for *M. hominis* samples. SC: Standard curve. The standard samples gave Ct values of 31.75, 30.9, and 27.37 for 50, 100, and 1000 copies per 5 µl reaction volumes, respectively. 1-7: 10^{0}-10^{-6} dilution. *M. hominis* gave Ct values of 13.5 (1), 15.25 (2), 17.6 (3), 22.05 (4), 25.2 (5), 30.66 (6) and 34.35 (7) with $1.64x10^{7}$, $4.75x10^{6}$, $9.3x10^{5}$, $4.1x10^{4}$, $4.5x10^{3}$, $1x10^{2}$ and 8 copies per reaction, respectively.

Table 1 Comparison of agar culture, MycoAlert™, gel-based PCR and MycoSensor™ QPCR assays for detection of *M. hominis*

Dilutions	Agar culture (CFUs/ml)				MycoAlert™ Positives/ total runs	Gel-based PCR Positives/total runs		MycoSensor™ QPCR Positives/total runs (copies/ml)	
	Friis		PH-Hayflick						
	Supernatant ($1x10^4$ cells/ml)	Cell suspension ($1x10^4$ cells/ml)	Supernatant ($1x10^4$ cells/ml)	Cell suspension ($1x10^4$ cells/ml)	Cell-free supernatant	Supernatant ($1x10^4$ cells/ml)	Cell suspension ($1x10^4$ cells/ml)	Supernatant ($1x10^4$) cells/ml	Cell suspension ($1x10^4$) cells/ml
10^0	n.c.	n.c.	n.c.	n.c.	5/5	5/5	2/3	3/3 ($8x10^7$)	3/3 ($1.6x10^6$)
10^{-1}	n.c.	$4x10^3$	n.c.	$5x10^3$	5/5	3/5	0/3	3/3 ($1.7x10^7$)	3/3 ($9x10^4$)
10^{-2}	$8.7x10^3$	$6x10^2$	$6x10^3$	$3.6x10^2$	0/5	3/5	0/3	3/3 ($3.4x10^6$)	3/3 ($1.9x10^4$)
10^{-3}	$7x10^2$	0	$5x10^2$	$4x10^1$	0/5	0/5	0/3	5/5 ($1.3x10^5$)	2/3 ($4.5x10^2$)
10^{-4}	$8x10^1$	0	$7x10^1$	0	0/5	0/5	0/3	4/5/ ($1.6x10^4$)	1/3 ($6x10^1$)
10^{-5}	$2x10^1$	0	0	0	0/5	0/5	0/3	2/5 ($1.8x10^3$)	0/3
10^{-6}	0	0	0	0	0/5	0/5	0/3	1/5 ($7.6x10^1$)	0/3
10^{-7*}	0	0	0	0	0/5	0/5	0/3	0/5	0/3

n.c.: not counted because the colonies were too dense

*dilutions from 10^{-7} to 10^{-10} gave negative results with all methods

82°C. The negative control gave no amplification curves and the T_m was between 65°C and 73°C. As such, it was judged as negative. The standard curves for run 1 to run 5 with *M. fermentans* cell-containing supernatants and dilutions were linear with an average correlation coefficient (r^2) of 0.99 and a slope of -3.38, corresponding to a PCR efficiency of 1.98 (99%) (Figure 12). Likewise, the standard curves for run 1 to run 3 with *M. fermentans* cell suspensions and dilutions were linear with an average correlation coefficient (r^2) of 0.99 and an average slope of -3.35, corresponding to a PCR efficiency of 1.99 (99%).

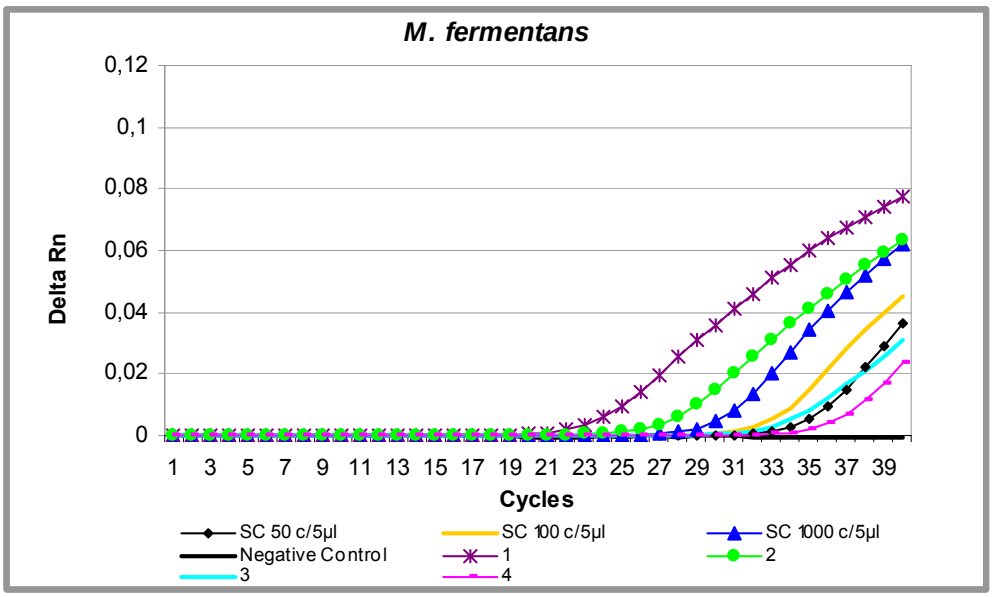


Figure 12 Amplification curves of the standard sample and serial dilutions of supernatant up to the respective dilution limit for *M. fermentans* samples. SC: Standard curve. The standard samples gave Ct values of 31.75, 30.9, and 27.37 for 50, 100, and 1000 copies per 5 µl reaction volumes, respectively. 1-4: 10^0-10^{-3} dilution. *M. fermentans* gave Ct values of 20.57 (1), 24.3 (2), 30.77 (3) and 33.4 (4) with $8x10^4$, $6.8x10^3$, $9.3x10^1$ and 7 copies per reaction, respectively.

Table 2 Comparison of agar culture, MycoAlert™, gel-based PCR and MycoSensor™ QPCR assays for detection of *M. fermentans*

Dilutions	Agar culture (CFUs/ml)				MycoAlert™ Positives/ total runs	Gel-based PCR Positives/total runs		MycoSensor™ QPCR Positives/total runs (copies/ml)	
	Friis		PH-Hayflick						
	Supernatant ($1.2x10^4$ cells/ml)	Cell suspension ($1x10^4$ cells/ml)	Supernatant ($1.2x10^4$ cells/ml)	Cell suspension ($1x10^4$ cells/ml)	Cell-free supernatant	Supernatant ($1.2x10^4$ cells/ml)	Cell suspension ($1x10^4$ cells/ml)	Supernatant ($1.2x10^4$) cells/ml	Cell suspension ($1x10^4$) cells/ml
10^0	$4.5x10^4$	n.c.	0	$3x10^3$	5/5	5/5	3/3	3/3 ($3x10^5$)	3/3 ($1.6x10^5$)
10^{-1}	$4.4x10^3$	$2x10^4$	0	$1.5x10^2$	0/5	3/5	2/3	3/3 ($2.9x10^4$)	3/3 ($1.6x10^4$)
10^{-2}	$3.8x10^2$	$5x10^3$	0	$3x10^1$	0/5	0/5	0/3	2/3 ($2.2x10^3$)	3/3 ($2.4x10^3$)
10^{-3}	$2.5x10^1$	$7x10^2$	0	0	0/5	0/5	0/3	1/5 ($6.8x10^1$)	2/3 ($2.4x10^2$)
10^{-4}	$1x10^1$	$5x10^1$	0	0	0/5	0/5	0/3	0/5	0/3
10^{-5}	0	0	0	0	0/5	0/5	0/3	0/5	0/3
10^{-6}	0	0	0	0	0/5	0/5	0/3	0/5	0/3
10^{-7*}	0	0	0	0	0/5	0/5	0/3	0/5	0/3

n.c.: not counted because the colonies were too dense

*dilutions from 10^{-5} to 10^{-10} gave negative results with all methods

Detection of *M. orale*

The results for the comparison of agar culture, MycoAlert™, gel-based PCR and MycoSensor™ for the detection of *M. orale* are shown in Table 3. Plating of *M. orale* from the undiluted cell-containing supernatant led to growth on both Friis and PH-Hayflick agar plates. With respect to the dilutions of the cell-containing supernatants, *M. orale* colonies were detected up to a dilution of 10^{-6} on Friis agar plates with 10 CFUs/ml and up to a dilution of 10^{-6} on PH-Hayflick agar plates with 5 CFUs/ml. Plating of the cell suspension led to growth on both Friis and PH-Hayflick agar plates. *M. orale* colonies were detected up to a dilution of 10^{-4} with 60 CFUs/ml on Friis agar plates and up to a dilution of 10^{-4} with $1.3x10^3$ CFUs/ml on PH-Hayflick agar plates (Figure 13). The MycoAlert™ assay produced positive results for the undiluted supernatants (5/5) and up to a dilution of 10^{-2} (5/5) in all runs. All gel-based PCR runs (5/5) with the undiluted *M. orale* supernatant and up to the 10^{-3} dilution were positive. Positive runs were also obtained with the 10^{-4} (1/5) dilution (Figure 10). *M. orale* was detected in the undiluted cell suspension in all 3 runs. Positive runs were also obtained up to the 10^{-2} dilution (1/3) with the gel-based PCR. The MycoSensor™ QPCR detected *M. orale* in the cell-containing supernatant ($1x10^8$ copies/ml) and up to a dilution of 10^{-3} ($1.2x10^4$ copies/ml) in all runs. Positive runs (1/5) were also obtained up to the 10^{-5} dilution, corresponding to $8x10^2$ copies/ml and 80 copies/reaction mixture (Figure 14). The MycoSensor™ QPCR detected *M. orale* in the undiluted cell suspension ($4.6x10^5$ copies/ml) and up to a dilution of 10^{-2} in all 3 runs, corresponding to $3.2x10^3$ copies/ml and 320 copies per reaction mixture. The cycle threshold (C_t) value for the standard sample of 50 copies was an average of 32 cycles (data not shown) and the melting temperature (T_m) was between 80.1°C and 82°C. The negative control gave no amplification curves and the T_m was between 65°C and 73°C. As such, it was judged as negative. The standard curves of *M. orale* cell-containing supernatants and dilutions for run 1 to run 5 were linear with an average correlation coefficient (r^2) of 0.99, and an average slope of -3.24, corresponding to a PCR efficiency of 2 (100%) (Figure 14). Likewise, the standard curves for run 1 to run 3 with the *M. orale* cell suspensions and dilutions were linear with an average correlation coefficient (r^2) of 0.99 and an average slope of -3.35, corresponding to a PCR efficiency of 1.99 (99%).

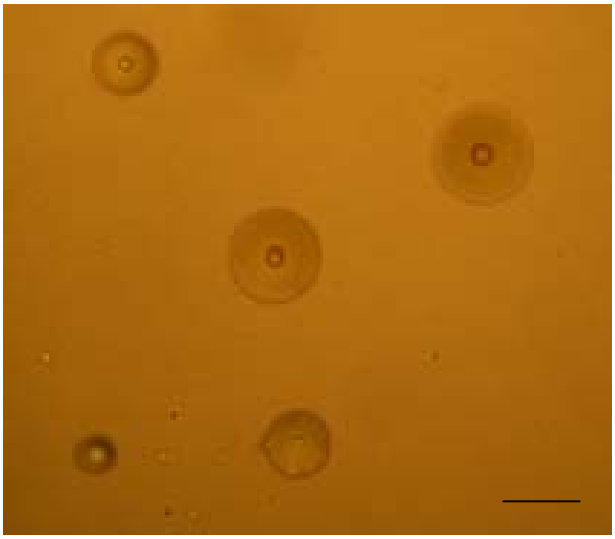


Figure 13 Typical colonies of *M. orale* with "fried egg appearance" on Friis agar, observed under a Zeiss inverted microscope. Scale bar = 10mm

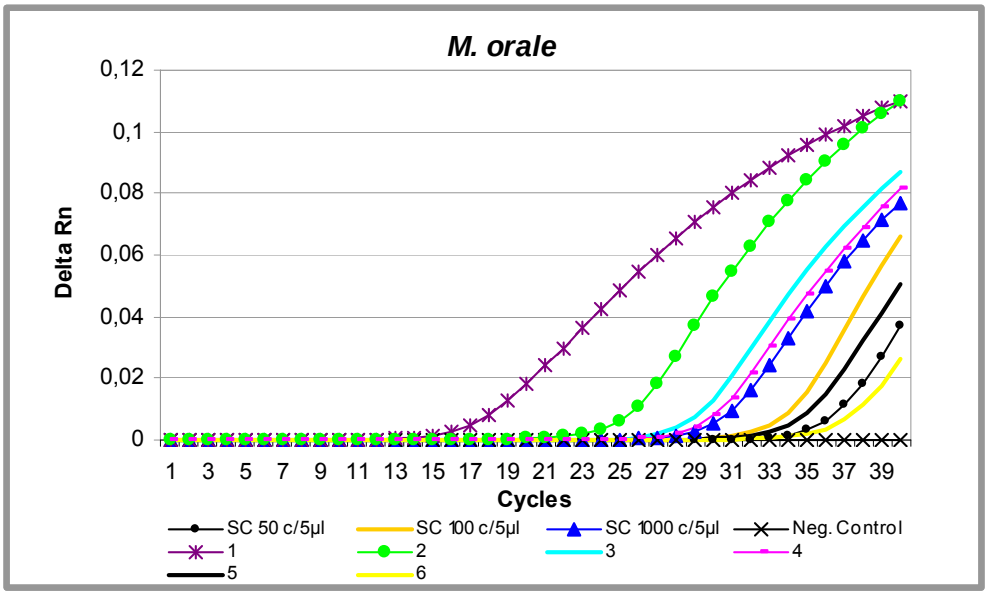


Figure 14 Amplification curves of the standard sample and serial dilutions up to the respective dilution limit for *M. orale* samples. SC: Standard curve. The standard samples gave Ct values of 31.75, 30.9, and 27.37 for 50, 100, and 1000 copies per 5 µl reaction volumes, respectively. 1-6: 10^{0}-10^{-5} dilution. *M. orale* gave Ct values of 12.86 (1), 19.20 (2), 25.12 (3), 25.86 (4), 29.35 (5) and 31.29 (6) with $1.98x10^{7}$, $3.13x10^{5}$, $5.2x10^{3}$, $3.1x10^{3}$, $3x10^{2}$ and $8x10^{1}$ copies per reaction, respectively.

Table 3 Comparison of agar culture, MycoAlert™, gel-based PCR and MycoSensor™ QPCR assays for detection of *M. orale*

Dilutions	Agar culture (CFUs/ml)				MycoAlert™ Positives/ total runs	Gel-based PCR Positives/total runs		MycoSensor™ QPCR Positives/total runs (copies/ml)	
	Friis		PH-Hayflick						
	Supernatant ($2x10^4$ cells/ml)	Cell suspension ($1x10^4$ cells/ml)	Supernatant ($2x10^4$ cells/ml)	Cell suspension ($1x10^4$ cells/ml)	Cell-free supernatant	Supernatant ($2x10^4$ cells/ml)	Cell suspension ($1x10^4$ cells/ml)	Supernatant ($2x10^4$) cells/ml	Cell suspension ($1x10^4$) cells/ml
10^0	n.c.	n.c.	n.c.	n.c.	5/5	5/5	3/3	3/3 ($1x10^8$)	3/3 ($4.6x10^5$)
10^{-1}	n.c.	$9x10^3$	n.c.	n.c.	5/5	5/5	2/3	3/3 ($1.9x10^6$)	3/3 ($6x10^4$)
10^{-2}	n.c.	$1x10^3$	n.c.	$9x10^3$	5/5	5/5	1/3	3/3 ($2.4x10^4$)	3/3 ($3.2x10^3$)
10^{-3}	$7.2x10^3$	$6.8x10^2$	$9x10^3$	n.e.	0/5	5/5	0/3	5/5 ($1.2x10^4$)	0/3
10^{-4}	$6.7x10^2$	$6x10^1$	$1x10^3$	$1.3x10^3$	0/5	1/5	0/3	2/5/ ($1.5x10^3$)	0/3
10^{-5}	$1x10^2$	0	$1.2x10^2$	0	0/5	0/5	0/3	1/5 ($8x10^2$)	0/3
10^{-6}	$1x10^1$	0	$5x10^0$	0	0/5	0/5	0/3	0/5	0/3
10^{-7*}	0	0	0	0	0/5	0/5	0/3	0/5	0/3

n.c.: not counted because the colonies were too dense, n.e.: could not be evaluated

*dilutions from 10^{-7} to 10^{-10} gave negative results with all methods

mES cell culture

To determine if mycoplasmas have an effect on mES cell culture, the morphology, the growth rate and the viability of mycoplasma-infected and control TBV2 mES cells were investigated over 20 passages by use of murine feeder cells. The morphology was determined by observing the size of ES cell colonies and the development of embryoid bodies (cell aggregates which implicates *in vitro* differentiation of the mES cells). The growth rate was determined by calculating the total number of mES cells which included dead, apoptotic and live cells in the supernatant, in the washing solution and in trypsinized cell suspensions. The total number of viable mES cells in trypsinized cell suspensions was determined using trypan blue staining. The numbers of mES cells were extrapolated at each passage.

Morphology of TBV2 mES cells

The results obtained for morphology are shown in Figure 15. Control mES cells led to the growth of medium to large, multicellular, and well-shaped ES cell colonies up to the passage P13+15 (Figure 15, A, B, C). Up to passage P13+20, control mES cells produced very large colonies (Figure 15, D). Mycoplasma-infected mES cells led to the growth of very small, small and medium colonies up to the passage P13+20 (Figure 15, E, black arrow, F, G, H) which appeared flatter than the colonies obtained from the control group. No embryoid bodies were observed for the two experimental groups over the 20 passages of culture.

Growth and viability of TBV2 mES cells

The results obtained for the growth and viability of mycoplasma-infected and control mES cells are shown in Table 4 and Figure 16. A total of 4.16×10^5 control TBV2 mES cells was seeded at P13 and increased to 1.2×10^{22} at passage P13+20 (Table 4). The number of dead and apoptotic cells at each passage was calculated by the difference of the total number to the number of trypsinized cells and was on average 18%. The viability of the trypsinized cells was 89% to 100% over the 20 passages of culture. With respect to the mycoplasma-infected cultures, 4.16×10^5 of TBV2 mES cells were seeded at passage P13, inoculated with *M. hominis*, *M. fermentans* and *M. orale* and the cell number was 3.9×10^{15} at passage P13+20.

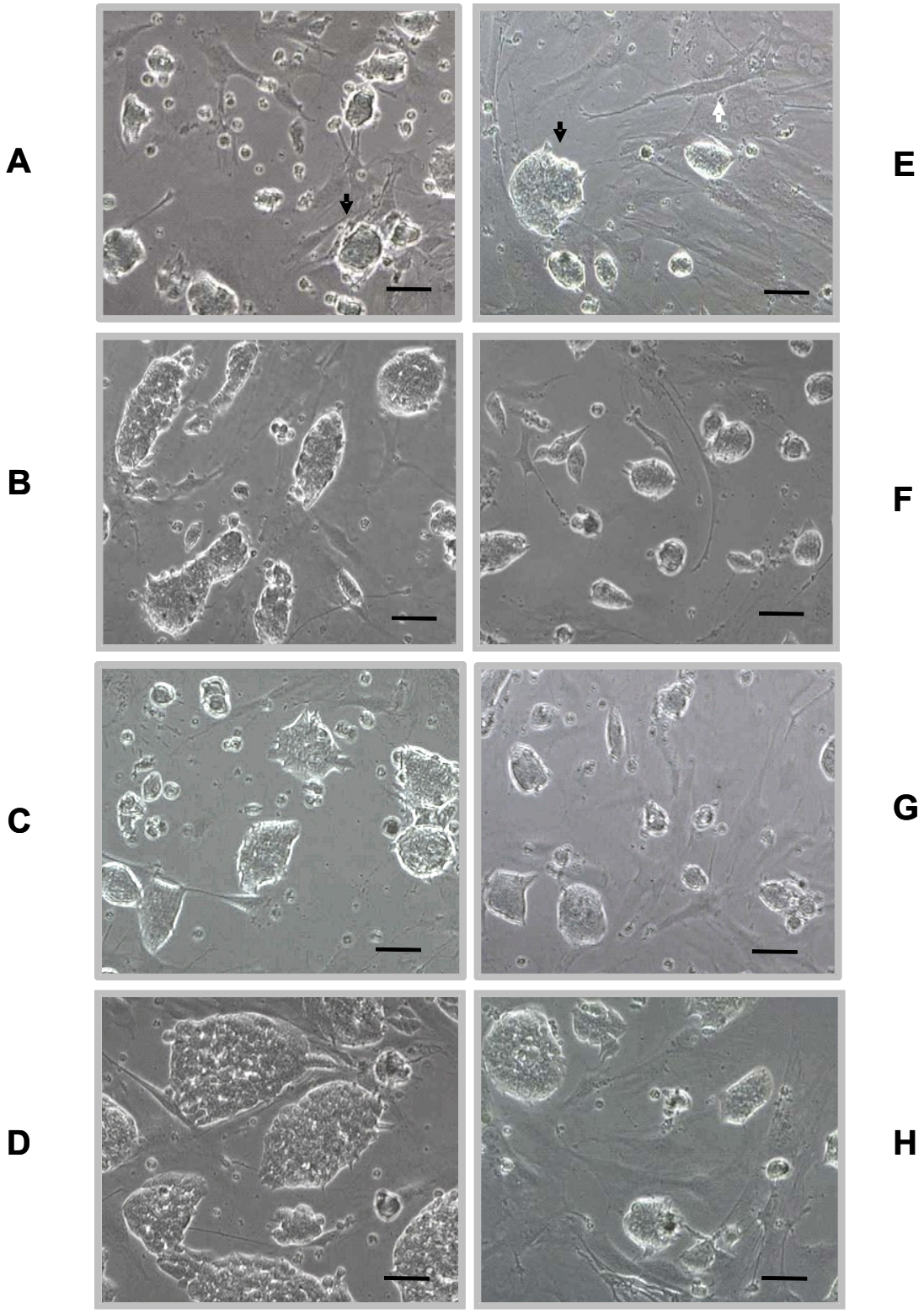


Figure 15 Morphology of control (A, B, C, D) and mycoplasma-infected (E, F, G, H) TBV2 murine ES cell colonies at passages P13+5 (A, E), P13+10 (B, F), P13+15 (C, G), and P13+20 (D, H), respectively. Black arrow: colonies of TBV2 murine ES cells, white arrow: murine embryonic feeder cells. Scale bar = 100 µm.

The average number of dead and apoptotic cells was 27% whereas the viability obtained in trypsinized cell suspensions was 74% to 95%.

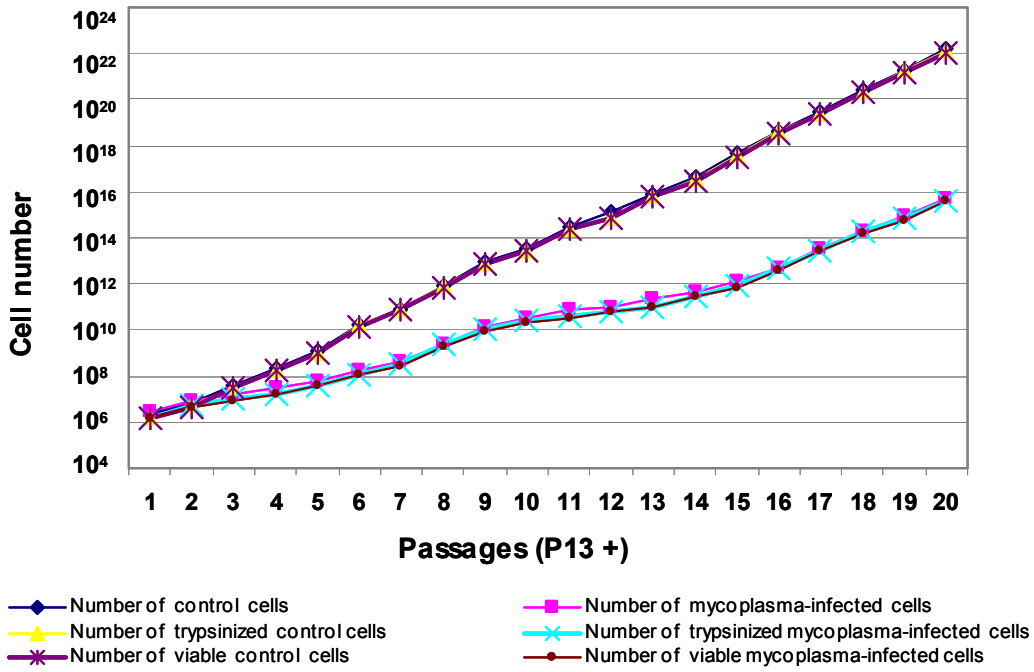


Figure 16 Growth rates and viability of control and mycoplasma-infected TBV2 mES cells over 20 passages (P13+1 to P13+20).

Detection of mycoplasmas in TBV2 mES cell cultures

The presence of mycoplasmas in TBV2 mES cell cultures was determined by agar culture, the MycoAlert™ assay, the gel-based PCR, and the MycoSensor™ QPCR assay. The results are shown in Table 5. The number of cells found in the mycoplasma-infected supernatants ranged from 2.6×10^4 to 1×10^5 mES cells per ml.

Plating of cell-containing control supernatant from passages P13+5, P13+10, P13+15, and P13+20 on Friis and PH-Hayflick agar plates did not lead to growth of mycoplasmas. Plating of mycoplasma-infected cell-containing supernatants from the respective passages led to growth of mycoplasmas on both Friis and PH-Hayflick agar plates. The colony forming units (CFUs) on agar plates were too dense to allow counting.

Table 4 Growth rates and viability of control and mycoplasma-infected TBV2 murine ES cells

Passages	Total number of TBV2 mES cells*£		Number of TBV2 ES cells* in trypsinized cell suspensions		Viability of TBV2 mES cells* in trypsinized cell suspension (%)	
P13 +	Control	Mycoplasma-infected	Control	Mycoplasma-infected	Control	Mycoplasma-infected
1	$2.4x10^6$	$2.7x10^6$	$1.6x10^6$	$1.6x10^6$	$1.6x10^6$ (100)	$1.5x10^6$ (95)
2	$7.4x10^6$	$7.9x10^6$	$4.9x10^6$	$5.4x10^6$	$4.7x10^6$ (95)	$4.7x10^6$ (86)
3	$3.8x10^7$	$1.6x10^7$	$3.4x10^7$	$1.0x10^7$	$3.3x10^7$ (95)	$9.2x10^6$ (85)
4	$2.0x10^8$	$2.8x10^7$	$1.7x10^8$	$1.8x10^7$	$1.6x10^8$ (97)	$1.5x10^7$ (87)
5	$1.3x10^9$	$5.6x10^7$	$1.0x10^9$	$4.2x10^7$	$9.5x10^8$ (95)	$3.7x10^7$ (89)
6	$1.8x10^{10}$	$1.7x10^8$	$1.7x10^{10}$	$1.3x10^8$	$1.5x10^{10}$ (89)	$1.2x10^8$ (94)
7	$8.5x10^{10}$	$4.2x10^8$	$7.3x10^{10}$	$3.6x10^8$	$7.3x10^{10}$ (100)	$2.7x10^8$ (74)
8	$8.9x10^{11}$	$2.6x10^9$	$7.8x10^{11}$	$2.3x10^9$	$6.9x10^{11}$ (89)	$2.0x10^9$ (89)
9	$8.4x10^{12}$	$1.3x10^{10}$	$7.7x10^{12}$	$1.1x10^{10}$	$7.0x10^{12}$ (91)	$9.6x10^9$ (89)
10	$3.4x10^{13}$	$3.1x10^{10}$	$2.9x10^{13}$	$2.5x10^{10}$	$2.7x10^{13}$ (91)	$2.0x10^{10}$ (80)
11	$2.9x10^{14}$	$7.5x10^{10}$	$2.5x10^{14}$	$3.7x10^{10}$	$2.4x10^{14}$ (96)	$3.2x10^{10}$ (86)
12	$1.3x10^{15}$	$1.0x10^{11}$	$7.9x10^{14}$	$6.3x10^{10}$	$7.6x10^{14}$ (97)	$5.7x10^{10}$ (90)
13	$8.0x10^{15}$	$2.2x10^{11}$	$6.0x10^{15}$	$1.0x10^{11}$	$5.8x10^{15}$ (96)	$8.7x10^{10}$ (85)
14	$4.5x10^{16}$	$4.5x10^{11}$	$3.2x10^{16}$	$3.1x10^{11}$	$3.0x10^{16}$ (93)	$2.9x10^{11}$ (93)
15	$4.3x10^{17}$	$1.3x10^{12}$	$3.6x10^{17}$	$7.8x10^{11}$	$3.4x10^{17}$ (95)	$6.8x10^{11}$ (88)
16	$4.2x10^{18}$	$4.8x10^{12}$	$3.8x10^{18}$	$4.4x10^{12}$	$3.6x10^{18}$ (95)	$3.7x10^{12}$ (84)
17	$2.8x10^{19}$	$3.5x10^{13}$	$2.4x10^{19}$	$2.8x10^{13}$	$2.3x10^{19}$ (95)	$2.7x10^{13}$ (95)
18	$2.5x10^{20}$	$2.0x10^{14}$	$2.2x10^{20}$	$1.8x10^{14}$	$2.0x10^{20}$ (90)	$1.6x10^{14}$ (94)
19	$1.9x10^{21}$	$8.3x10^{14}$	$1.7x10^{21}$	$6.5x10^{14}$	$1.6x10^{21}$ (96)	$5.8x10^{14}$ (89)
20	$1.5x10^{22}$	$4.5x10^{15}$	$1.2x10^{22}$	$3.9x10^{15}$	$1.1x10^{22}$ (96)	$3.6x10^{15}$ (93)

*Numbers of cells were extrapolated, £: the total number of cells was enumerated from the supernatants, washes and trypsinized cell suspensions

The MycoAlert™ detection assay was performed for all 20 passages of the control and mycoplasma-infected mES cells. Control supernatants were all negative and the ratio B/A was between 0.4 and 0.9. The MycoAlert™ assay gave positive results for mycoplasma-infected samples for all 20 passages. The ratio B/A at passage P13+1 was 2.6 whereas for the next passages and up to passage P13+19, the ratio B/A ranged between 10.2 and 51.7 (Table 5). At passage P13+20, the ratio B/A was 5.7.

The gel-based PCR was performed for all 20 passages of the 2 experimental groups. Control samples gave no PCR products and no bands were observed on agarose gel while PCR products and bands on agarose gel were observed from all 20 passages of mycoplasma-infected samples. Furthermore, positive PCR products were used for mycoplasma-species identification with Restriction Fragment Length Polymorphism (RFLP). At passage P13+1, only *M. hominis* and *M. orale* were detected whereas from passage P13+2 to P13+20 *M. hominis*, *M. fermentans* and *M. orale* were detected.

The MycoSensor™ QPCR assay was performed with mycoplasma positive cell-containing supernatants and cell suspension samples from passages P13+5, P13+10, P13+15 and P13+20. The supernatants from the respective passages contained $2.9x10^4$, $3.7x10^4$, $9.2x10^4$ and $7x10^4$ mES cells, respectively, whereas the cell suspensions contained $1x10^4$ mES cells per ml. The MycoSensor™ QPCR assay detected $5.5x10^7$ and $5.8x10^6$, $1.7x10^8$ and $4.9x10^5$, $3.2x10^7$ and $1x10^5$, and $6x10^6$ and $6.5x10^3$ mycoplasma genome copies in cell-containing supernatants and in cell suspensions at passages P13+5, P13+10, P13+15 and P13+20, respectively. Consequently, a total of $5.8x10^2$, 49, 10 and 1 mycoplasmas was adhered to or invaded 1 mES cell at the respective passages.

Karyotype of TBV2 mES cells

The karyotype of mycoplasma-free TBV2 mES cells was examined at passage P13+2. Furthermore, control and mycoplasma-infected TBV2 mES cells at passages P13+5, P13+10, P13+15 and P13+20 were examined using Spectral Karyotyping (SKY). The results are shown in Table 6. At passage P13+2, 15 metaphases were studied. A total of 9 metaphases showed a normal karyotype (60%) having {40, XY} chromosomes and 6 of them showed numeric aberrations including 2 tetraploid metaphases. Absence of chromosome X appeared twice (13%), whereas absence of

Table 5 Detection of mycoplasmas in supernatants and cell suspensions of TBV2 mES cell cultures by four methods

Passages	Number of TBV2 mES cells in supernatant/ ml	Agar culture (CFUs/ml)		MycoAlert™ detection assay		Gel-based PCR		MycoSensor™ QPCR assay	
P13 +		Friis/PH-Hayflick	Friis/PH-Hayflick					Cell-containing supernatant (genome copies/ml)	Cell suspension ($1x10^4$ cells/ml)
	Mycoplasma-infected	Control	Mycoplasma -infected	Control	Mycoplasma -infected	Control	Mycoplasma -infected	Mycoplasma -infected	Mycoplasma -infected
1	$1.0x10^5$	n.d	n.d	0.74 (-)	2.6 (+)	-	+*	n.d	n.d
2	$6.3x10^4$	n.d	n.d	0.68 (-)	36.5 (+)	-	+**	n.d	n.d
3	$2.6x10^4$	n.d	n.d	0.71 (-)	51.7 (+)	-	+	n.d	n.d
4	$4.0x10^4$	n.d	n.d	0.78 (-)	34.9 (+)	-	+	n.d	n.d
5	$2.9x10^4$	-/-	+/+†	0.66 (-)	37.9 (+)	-	+	$5.5x10^7$	$5.8x10^6$
6	$2.6x10^4$	n.d	n.d	0.65 (-)	18.2 (+)	-	+	n.d	n.d
7	$3.0x10^4$	n.d	n.d	0.76 (-)	23.0 (+)	-	+	n.d	n.d
8	$2.9x10^4$	n.d	n.d	0.63 (-)	34.0 (+)	-	+	n.d	n.d
9	$3.0x10^4$	n.d	n.d	0.68 (-)	28.7 (+)	-	+	n.d	n.d
10	$3.7x10^4$	-/-	+/+†	0.62 (-)	42.3 (+)	-	+	$1.7x10^8$	$4.9x10^5$
11	$4.0x10^4$	n.d	n.d	0.64 (-)	18.6 (+)	-	+	n.d	n.d
12	$7.6x10^4$	n.d	n.d	0.63 (-)	10.8 (+)	-	+	n.d	n.d
13	$9.3x10^4$	n.d	n.d	0.41 (-)	17.9 (+)	-	+	n.d	n.d
14	$8.0x10^4$	n.d	n.d	0.49 (-)	16.2 (+)	-	+	n.d	n.d
15	$9.2x10^4$	-/-	+/+†	0.61 (-)	12.3 (+)	-	+	$3.2x10^7$	$1.0x10^5$
16	$3.3x10^4$	n.d	n.d	0.88 (-)	10.2 (+)	-	+	n.d	n.d
17	$5.3x10^4$	n.d	n.d	0.90 (-)	17.6 (+)	-	+	n.d	n.d
18	$5.0x10^4$	n.d	n.d	0.86 (-)	14.8 (+)	-	+	n.d	n.d
19	$4.0x10^4$	n.d	n.d	0.72 (-)	16.6 (+)	-	+	n.d	n.d
20	$7.0x10^4$	-/-	+/+†	0.50 (-)	5.7 (+)	-	+	$6.0x10^6$	$6.5x10^3$

n.d: not done, +: positive, -: negative, †: Colony forming units (CFUs) were not counted, *:P13+1: Restriction Fragment Length Polymorphism (RFLP) assay detected only *M. hominis* and *M. orale*, **P13+2 to P13+20: Restriction Fragment Length Polymorphism assay detected *M. hominis*, *M. fermentans*, *M. orale.*

chromosome 2, 3, 6 and 17 appeared once. A trisomy 10 was observed in 1 metaphase.

From the control TBV2 mES cells at passage P13+5, 18 metaphases were studied, 11 of which were normal (60%) and 7 showed an abnormal karyotype with numeric aberrations. A total of 1 tetraploid metaphase was observed. Absence of chromosome Y appeared twice while 2 metaphases had only 31 chromosomes in which both chromosome 8 were absent. Trisomy 11 and 6 was observed once.

From the mycoplasma-infected TBV2 mES cells at passage P13+5, 16 metaphases were studied. A total of 9 metaphases was normal (56%) and 7 were abnormal with numeric aberrations. In 1 metaphase, both X and Y chromosomes were absent whereas absence of chromosome Y, 4, 6, and 11 was observed once. Trisomy 1 appeared twice and trisomy 16 and 18 once.

From the control TBV2 mES cells at passage P13+10, 15 metaphases were studied. A total of 3 metaphases was normal (20%) and 12 showed an abnormal karyotype. The Y chromosome was absent in 2 metaphases (13%), and trisomy 1, 8, 11 and 14 appeared in 5 (33%), 1 (7%), 3 (20%) and 2 (13%) metaphases, respectively. Absence of chromosome 15 and 18 as well as an acentric fragment of the chromosome 17 {41, XY; ace(17)} appeared once. From mycoplasma-infected TBV2 mES cells of the respective passage 15 metaphases were studied. A total of 10 metaphases showed a normal karyotype (67%) and 5 were abnormal. Absence of chromosome Y and trisomy 1 was observed once (7%). A balanced translocation between the chromosomes 10 and 18, which were attached to each other at the centromeres {t(10;8)(cen-cen)} was observed in 1 metaphase. Deletions of chromosome 7 and 10 segregated at the A2 and B3 bands up to the terminal end of the chromatids, respectively, were observed once (7%).

At passage P13+15, 15 control metaphases were studied. A total of 5 metaphases was normal (33%) and 10 showed numeric aberrations. Absence of chromosomes Y and 18 was observed once (7%), whereas trisomy 1, 6, 8, 11, 14, and 16 appeared in a total of 3 (20%), 1 (7%), 2 (13%), 3 (20%), 2 (13%) and 1 (7%) metaphases, respectively. A total of 18 metaphases was studied from mycoplasma-infected TBV2 mES cells of the respective passage. A normal karyotype appeared in 6 metaphases (33%). The 12 abnormal metaphases showed trisomy 1, 6, and 13 in 4 (22%), 3 (17%) and 1 (6%) cases, respectively, whereas absence of chromosome 7, 9, 13, 14, 17, and 19 appeared once (6%). Both chromosomes 17 were absent from 1 metaphase. Deletions of chromosome 4, 11 and 15 segregated at the C5, D3 and

C bands and up to the terminal end of the chromatids, respectively, were observed once. A translocation between chromosome 3 and 7 and 5 and 9 was present in 1 metaphase at which the derivative fragment of chromosome 7 from the C3 band up to the terminal end of the chromatids was connected to the segregated chromosome 3 at the G3 bands, whereas 5 and 9 chromosomes were connected to each other at the centromeres.

For control TBV2 mES cells at passage P13+20, 18 metaphases were studied. A total of 6 metaphases was normal (33%) and 12 were abnormal. Absence of chromosome 3, 5, 10 was observed in 1 (6%), 2 (11%) and 1 (6%) metaphase, respectively, whereas trisomy 1 and 11 appeared in 5 (28%) and 3 (17%) metaphases, respectively. In a case of trisomy 11, a balanced translocation was observed between the chromosomes 11 which were joined to each other at the centromeres. A deletion of chromosome 15 segregated at the C2 band and up to the terminal end of the chromatids, and a translocation between chromosome 3 and 4 at which the derivative fragment of chromosome 4, segregated at the E1 band and up to the terminal end of the chromatids, was connected to the segregated chromosome 3 at the H4 bands, were also observed. Twenty metaphases were studied from mycoplasma-infected TBV2 mES cells of the respective passage. A total of 4 metaphases was normal (20%) (Figure 17, A) and 16 showed abnormal karyotype. Absence of chromosome Y, 11 and 7 was observed in 3 (15%), 1 (5%) and 1 (5%) metaphases, respectively, whereas trisomy 1 (Figure 17, B), 6, and 11 was observed in 5 (25%), 2 (10%) and 1 (5%) metaphases, respectively. An acentric fragment of chromosome 1 {41,XY;ace(1)} was observed once. Balanced translocations were observed between chromosomes 1, and between the chromosomes 5 and 14, which were connected to each other at the centromeres, respectively. Unbalanced translocations were observed between the chromosomes 14 and 4 at which the derivative fragment from chromosome 4, segregated at the E1 band up to the terminal end of the chromatids, was connected to the chromosome 14 at the E4 band. An insertion between chromosome 11 and 19 {41,XY;der11(B5-ter) ;ins(11;19)(B5-ter;cen-A9} was observed in 1 metaphase (5%) (Figure 17, B). Deletions of chromosome 1, 6, 10, 11, and 12, segregated at the E4, C3, B3, A2 and D1 bands up to the terminal end of the chromatids, respectively, were observed once (Figure 17, C).

Table 6 Spectral karyotypic analysis of control and mycoplasma-infected TBV2 mES cells

TBV2 mES cells	Number of metaphases studied		Aberrant karyotype
Passage	Total	Abnormal	
P13+2 mycoplasma-free	15	6	{39,Y}, {38,Y;-17}, {39,XY;-2}, {41,XY;+10}, {79,XXYY;-3}, {79,XXYY;-6}
P13+5 control	18	7	{41,XY;+6;}, {39,XY;-3}, {38,X;-14;}, {39,XY;-17;}, {31,XY;-3;-6;-7;-8;-8;-9;-11;-17;-19}, {31,X;-5;-8;-8;-9;-15;-16;-17;-19}, {78,YY;+11;-12}
P13+5 mycoplasma-infected	16	7	{2x41,XY+1;}, {38;}, {39,X;}, {39,XY;-11;}, {38,XY;-4;-6;}, {42,XY;+16;+18;}
P13+10 control	15	12	{2x39,X}, {3x41,XY;+1}, {41,XY;+8}, {2x41,XY;+11}, {40,XY;+1;-18}, {41,XY;+6; +14;-15}, {41,XY;ace(17)}, {41,XY;+1;+11;+14}
P13+10 mycoplasma-infected	15	5	{39,X}, {41,XY;+1}, {39,XY;t(10;18)(cen;cen}, {40,XY;d7(A2-ter)}, {40,XY;d10(B3-ter)}
P13+15 control	15	10	{39,X}, {2x41,XY;+11}, {40,XXY;-18}, {41,XY;+1}, {42,XY;+1;+14}, {42,XY;+8;+12}, {41,XY;+8}, {41,XY;+6}, {43,XY;+1;+11;+14,+16}
P13+15 mycoplasma-infected	18	12	{2x41,XY;+1}, {41,XY;+6}, {41,XY;+13}, {39, XY; -3}, {40,XY;+1;-14}, {41,XY;d4(C5-ter);+6}, {39,XY;d15(D3-ter);-19}, {40,XY;d11(D-ter)}, {35,Y;+1;-7;-9;-13;-17;-17}, {40,XY;der(3;7);t(3;7)(cen-G3;C-ter);-17;+6}, {39, XY; t(5;9)(cen-cen)}
P13+20 control	18	12	{2x41,XY;+1}, {2x41,XY;+11}, {39,XY;-5}, {39,XY;-10}, {40,XY;+1,-5}, {40,XY;+11;d15(D2-ter)}, {40,XY;+1;-3;amp 16}, {40,XY;amp14}, {41,XY;t(11;11)(cen;cen)}, {41,XY;+1;t(3;4)(H4;E1-ter)}
P13+20 mycoplasma-infected	20	16	{2x39,X}, {41,XY;+1}, {40,X;+1}, {39,XY;-11}, {41,XY;ace(1)}, {40,XY;+1;-7}, {41,XY;t(1;1)(cen;cen)}, {39,XY;t(5;14)(cen-cen)}, {40,XY;d12(D1-ter)}, {41,XY;+6;d10(B3-ter)}, {41,XY;+6;d6(C3-ter)}, {40,XY;d1(E4-ter)}, {40,XY;t(14;4)(E5;E1-ter)}, {41,XY;der 11(B5-ter) ;ins(11;19)(B5-ter;cen-A)}, {42,XY;+1;d11(A2-ter); (11;11)(cen;cen)}

-, +: numeric aberrations, ace: acentric fragment, cen: centromere, t: translocation, d: deletion, ter: terminal end, der: derivative, ins: insertion, amp: amplification, A2, B3, C5, D3, D, G3, C, D1, C3, E4, E5, E1, A, B5: regions of chromosome bands based on the mouse ideogram (http://www.pathology.washington.edu/research/cytopages/idiograms/mouse/ #374).

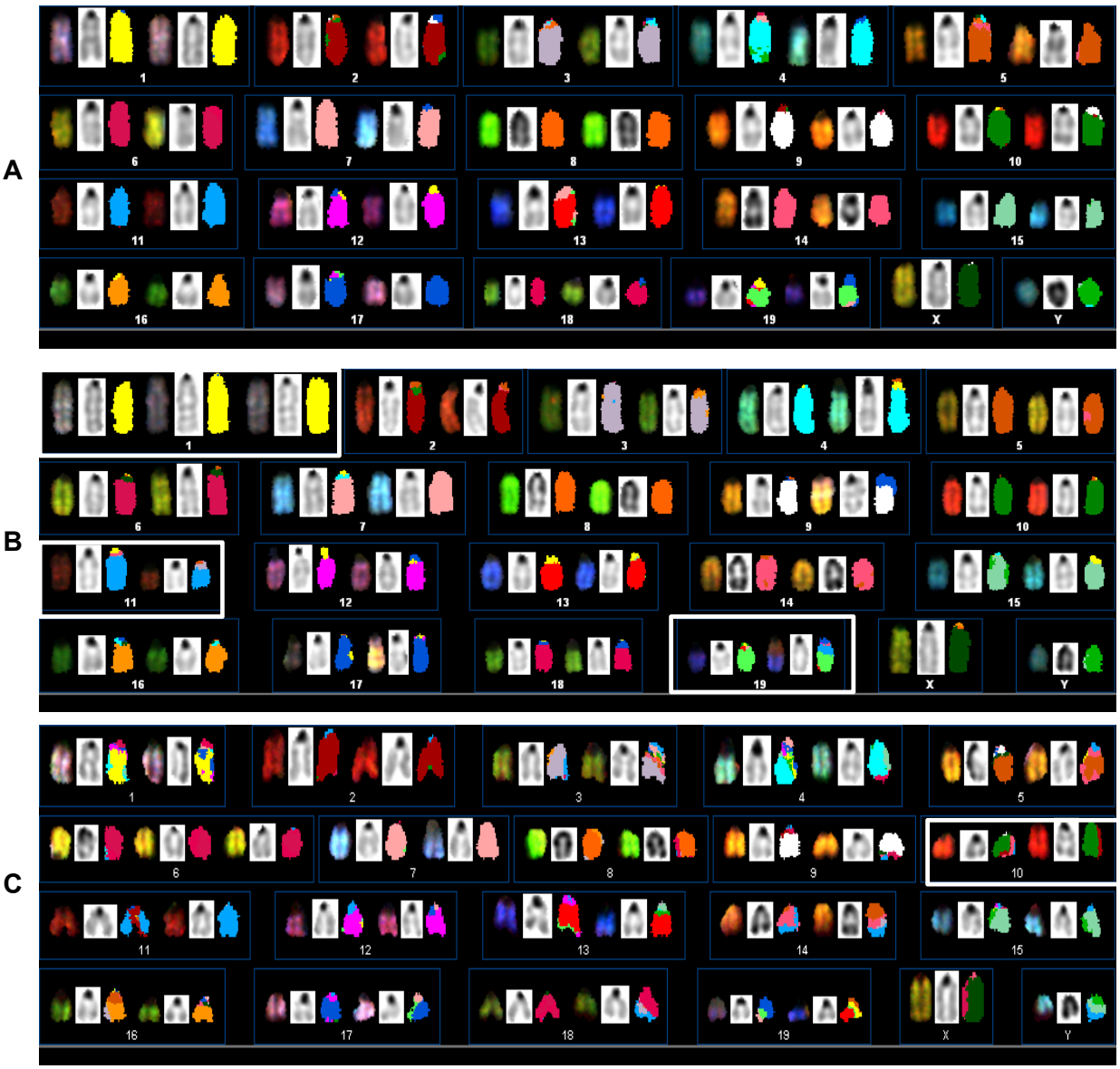


Figure 17 Spectral karyotyping of mycoplasma-infected TBV2 mES cells at passage P13+20 (A, B, C). A: normal karyotype {40, XY}. B: Trisomy 1 {41,XY;+1}, ins {41,XY;der 11(B5-ter);ins(11;19)(B5-ter;cen-A)}, the segregated fragment of chromosome 11 at the B5 band and up to the terminal end of the chromatids was inserted between the A and B bands of the chromosome 19 (aberrations are shown in white frames). C: d10 (B3-ter), chromosome 10 was deleted in both chromatids at point B3 (white frame).

Differentiation status of TBV2 mES cells

The differentiation status of the control and mycoplasma-infected TBV2 mES cells was determined at passages P13+6, P13+11, P13+16 and P13+21, respectively, using flow cytometric analysis (FACS) and double labeling with antibodies against the markers OCT-4 and SSEA-1. The results are shown in Figure 18.

A total of 84.8% of the control TBV2 mES cells at passage P13+6 was positive for both OCT-4 and SSEA-1 markers, an additional 5.06% and 4.66% was positive only for OCT-4, and only for SSEA-1, respectively, and 5.49% was negative for both markers (Figure 18, A). Mycoplasma-infected TBV2 mES cells at the same passage were positive for OCT-4 and SSEA-1 markers at a rate of 46.3%, whereas 10% was positive only for OCT-4, 24.4% was positive only for SSEA-1, and 19.2% was negative for both markers (Figure 18, E).

At passage P13+11, 86.7% of the control TBV2 mES cells were positive for both OCT-4 and SSEA-1 markers, 10.3% were positive only for OCT-4, 1.13% only for SSEA-1 and 1.94% were negative for both markers (Figure 18, B). Mycoplasma-infected TBV2 mES cells at the same passage were positive for OCT-4 ad SSEA-1 markers at a rate of 83%, whereas 11.3% were positive only for OCT-4, 1.92% were positive only for SSEA-1, and 3.81% were negative for both markers (Figure 18, F).

A total of 87.8% of the control TBV2 mES cells at passage P13+16 was positive for both OCT-4 and SSEA-1 markers, 7.86% was positive only for OCT-4, 2.05% only for SSEA-1 and 2.3% was negative for both markers (Figure 18, C). Mycoplasma-infected TBV2 mES cells at the same passage were positive for both OCT-4 and SSEA-1 markers at a rate of 78%, whereas 14.3% were positive only for OCT-4, 2.2% were positive only for SSEA-1 and 5.5% were negative for both markers (Figure 18, G).

At passage P13+21, 78% of the control TBV2 mES cells were positive for both OCT-4 and SSEA-1 markers, 18.4% were positive only for OCT-4, 1.82% were positive only for SSEA-1 and 1.79% was negative for both markers (Figure 18, D). Mycoplasma-infected TBV2 mES cells at the same passage were positive for both OCT-4 and SSEA-1 markers at a rate of 69.4%, whereas 20.1% were positive only for OCT-4, 3.63% were positive only for SSEA-1 and 6.89% were negative for both markers (Figure 18, H).

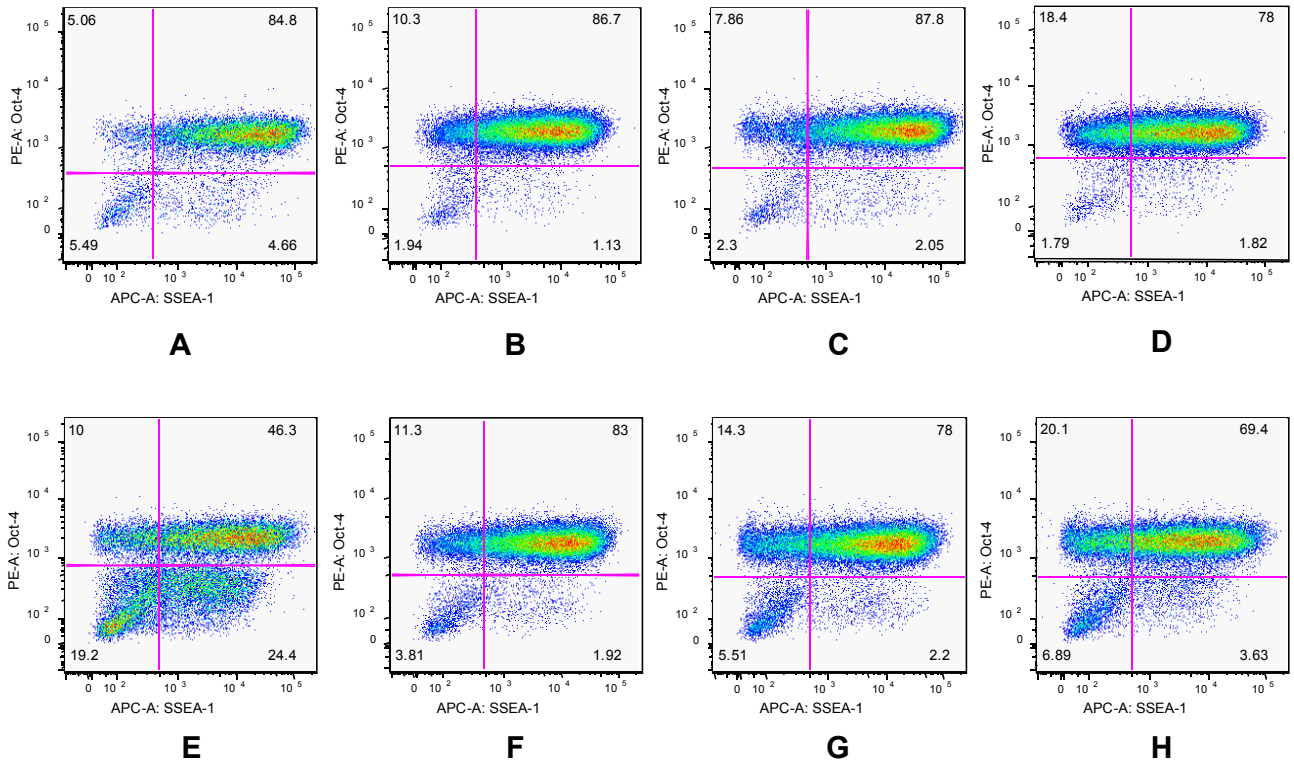


Figure 18 Determination of pluripotency of control (A, B, C, D) and mycoplasma-infected (E, F, G, H) TBV2 mES cells at passages P13+6, P13+11, P13+16 and P13+21 using flow cytometric analysis (FACS) and antibodies against the differentiation markers OCT-4 and anti-SSEA-1.

Results of embryo transfers, chimera production and germ line transmission

Mycoplasma-free TBV2 mES cells at passage P13+2 and control and mycoplasma-infected TBV2 mES cells at passages P13+5, P13+10, P13+15 and P13+20 were injected into CD-1 blastocysts, and the embryos were transferred in CD-1 pseudopregnant recipients. The number of litters, the percentage of chimeric pups and the contribution to the germ line were determined. The contribution of TBV2 mES cells to somatic tissues and to the germ line of chimeras was determined by coat and eye colour, and breeding of chimeric progeny with C57/BL6 (B6) mice, respectively. Each chimera was mated twice with B6 mice. Reproductive and germ line transmission (GLT) results are shown in Table 7.

At passage P13+2, 6 out of 9 recipients littered and 32% of the embryos injected resulted in the birth of 40 live pups. A total of 13 chimeras (11 males and 2 females) was obtained from 5 out of 6 litters, 12 (10 males and 2 females) of which showed GLT

Table 7 Results obtained from embryo transfers after injection of control and mycoplasma-infected TBV2 mES cells into blastocysts

Parameter	P13+1	P13+5		P13+10		P13+15		P13+20	
	Mycoplasma-free	Control	Mycoplasma-infected	Control	Mycoplasma-infected	Control	Mycoplasma-infected	Control	Mycoplasma-infected
Pups born /embryos transferred (%)	40/126 (32)	38/84 (45)	25/112 (22)	31/112 (27)	67/196 (34)	34/154 (22)	48/196 (24)	30/98 (31)	59/224 (26)
Litters/No. of transfers performed	6/9	6/6	4/8	5/8	10/14	6/11	9/14	5/7	10/16
Litters with chimeras (No. of mice)	5/6 (13)	6/6 (10)	3/4 (10)	4/5 (10)	10/10 (28)	5/6 (11)	8/9 (22)	4/5 (9)	8/10 (23)
♂/♀ chimeras	11/2	8/2	9/1	10/0	24/4	9/2	17/5	4/5	19/4
Litters with GLT*	5/5	6/6	3/3	4/4	7/10	3/5$^{\pm}$	8/8	5/5	8/8
GL chimeras/total no. of chimeras (%)	12/13$^{\pm}$ (92)	9/10 (90)	5/10 (50)	7/9† (78)	15/24† (62)	6/11$^{\pm}$ (55)	12/20† (60)	7/9 (78)	18/22† (81)
♂ GL chimeras/ ♂ chimeras	10/11†	7/8	5/9	7/9	15/21	6/9$^{\pm}$	9/15	2/4	15/18
♀ GL chimeras/ ♀ chimeras	2/2	2/2	0/1	0/0	0/3	0/2$^{\pm}$	3/5	5/5	3/4
GL pups/pups born (%)	103/139 (74)	94/146 (64)	45/108 (42)	91/92 (98)	154/258 (60)	67/89 (75)	161/192 (83)	89/115 (77)	172/239 (72)
GL pups/pups born from ♂ chimeras	85/116	68/99	45/108	91/92	134/212	67/89	110/134	36/52	128/183
GL pups/pups born from ♀ chimeras	18/23	26/47	0/0	0/0	0/46	0/0	51/58	53/63	44/56

*GLT: Germ line transmission, GL: germ line, ♂: male, ♀: female, †: pups were sacrificed or died before weaning, $^{\pm}$: incomplete data

(92%). Breeding of chimeric progeny with B6 mice resulted until now in 139 live pups, 103 (74%) of which were germ line pups. This experiment is still in progress.

At passage P13+5, all 6 recipients littered and 45% of the embryos injected with control mES cells resulted in 38 live pups. A total of 10 chimeras (8 males and 2 females) was obtained from all 6 litters and 9 out of 10 chimeras (7 males and 2 females) showed GLT (90%). The number of pups born from chimera breeding with B6 mice was 146, 94 of which were germ line pups (64%). With respect to the embryos injected with mycoplasma-infected mES cells of the respective passage, 4 out 8 recipients littered and 22% resulted in 25 live pups. A total of 10 chimeras (9 males and 1 female) was obtained from 3 out of 4 litters and 5 males showed GLT (50%). The number of pups born from chimera breeding with B6 mice was 108, 45 of which were germ line pups (42%). Statistical analysis with the Fischer's test showed significant differences between the 2 experimental groups for the number of litters born ($p<0.05$), the number of pups born ($p<0.001$), the GLT ($p<0.07$) and the number of germ line pups ($p<0.001$). The ($p<0.07$) value is borderline due to the small number of chimeras in the 2 groups.

At passage P13+10, 5 out of 8 recipients littered and 27% of the embryos injected with mES cells resulted in 31 live pups. A total of 10 male chimeras was obtained from 4 out of 5 litters and 7 out of 9 remaining chimeras showed GLT (78%). The number of pups born from chimera breeding with B6 mice was 92, 91 of which were germ line pups (98%). With respect to the embryos injected with mycoplasma-infected mES cells of the respective passage, 10 out of 14 recipients littered and 34% resulted in 67 live pups. A total of 28 chimeras (24 males and 4 females) was obtained from all 10 litters and 15 males out of the 24 remaining chimeras contributed to the germ line (62%). The number of pups born from chimera breeding with B6 mice was 258, 154 of which were germ line pups (60%).

At passage P13+15, 6 out of 11 recipients littered and 22% of the embryos injected with control mES cells resulted in 34 live pups. A total of 11 chimeras (9 males and 2 females) was obtained from 5 out of 6 litters and 6 males out of the 11 chimeras showed until now GLT (55%). The number of pups born from chimera breeding with B6 mice was 89, 67 of which were germ line pups (75%). With respect to the embryos injected with mycoplasma-infected mES cells of the respective passage, 9 out of 14 recipients littered and 24% resulted in 48 live pups. A total of 22 chimeras (17 males and 5 females) was obtained from 8 and out of 9 litters and 12 (9 males and 3 females)

out of the 20 remaining chimeras showed GLT (60%). The number of pups born from chimera breeding with B6 mice was 192, 161 of which were germ line pups (83%). This experiment is still in progress.

At passage P13+20, 5 out of 7 recipients littered and 31% of the embryos injected with control mES cells resulted in 30 live pups. A total of 9 chimeras (4 males and 5 females) was obtained from 4 out of 5 litters and 7 out of 9 chimeras (2 males and 5 females) showed GLT (78%). The number of pups born from chimera breeding with B6 mice was 115, 89 of which were germ line pups (77%). With respect to the embryos injected with mycoplasma-infected mES cells of the respective passage, 10 out of 16 recipients littered and 26% resulted in 59 live pups. A total of 23 chimeras (19 males and 4 females) was obtained from 8 out of 10 litters and 18 out of the 22 remaining chimeras showed GLT (81%). The numbers of pups born from chimera breeding with B6 mice was 239, 172 of which were germ line pups (72%).

Appearance and clinical signs of chimeras

A number of chimeras resulting from blastocysts that were injected with mycoplasma-infected mES cells showed morphological and clinical signs which are summarized in Table 8 and are shown in Figure 19 (A, B). A total of 10 (36%) (8 males and 2 females), 5 (23%) (males) and 5 (22%) (4 males and 1 female) chimeras produced from mycoplasma-infected TBV2 mES cells at the P13+10, P13+15 and P13+20 passages, respectively, was smaller in size with deformed extremities and had difficulties in moving (Figure 19, A). They showed retarded development and were underweight.

The weekly weight of 22, 35, 32, and 20 male mice and 2, 9, 12, and 10 female mice, including chimeric and wild type CD-1 mice produced from control and mycoplasma-infected TBV2 mES cells at passages P13+1, P13+10, P13+15 and P13+20 was determined, respectively. The weight of the male mice is presented in Figure 20 (A-D). The male chimeras that showed retarded development were constantly lighter and, at the age of 13 weeks, weighed 15 to 20 g less than the healthy chimeras and wild type mice. Statistical analysis which obtained using the t-test showed significant differences between the group of the diseased mice compared to the other groups of mice ($p<0.0001$). Similarly, reduced weight was observed for diseased female chimeras (n=3). Furthermore, 11 (39%) (10 males and 1 female), 7 (22%) (males) and 12 (52%) (10 males and 2 females) chimeras produced from mycoplasma-infected

Table 8 Chimeras with morphological and clinical signs of disease

Parameter	P13+1	P13+5		P13+10		P13+15		P13+20	
	Mycoplasma-free	Control	Mycoplasma-infected	Control	Mycoplasma-infected	Control	Mycoplasma-infected	Control	Mycoplasma-infected
Litters with chimeras having clinical and morphological signs/No. of litters	0/6	0/6	0/4	0/5	8/10	0/6	5/9	0/5	7/10
Small chimeras/ chimeras born (%)	0/14	0/10	0/10	0/10	10/28 (36)	0/12	5/22 (23)	0/9	5/23 (22)
♂/♀small chimeras	0/0	0/0	0/0	0/0	8/2	0/0	5/0	0/0	4/1
Chimeras with nasal discharge /chimeras born (%)	0/14	0/10	0/10	0/10	11/28 (39)	0/12	7/22 (32)	0/9	12/23 (52)
♂/♀chimeras with nasal discharge	0/0	0/0	0/0	0/0	10/1	0/0	7/0	0/0	10/2
Small chimeras with nasal discharge /chimeras born (%)	0/14	0/10	0/10	0/10	8/28 (29)	0/12	5/22 (23)	0/9	4/23 (17)
♂/♀ small chimeras with nasal discharge	0/0	0/0	0/0	0/0	7/1	0/0	5/0	0/0	4/0

♂: male, ♀: female

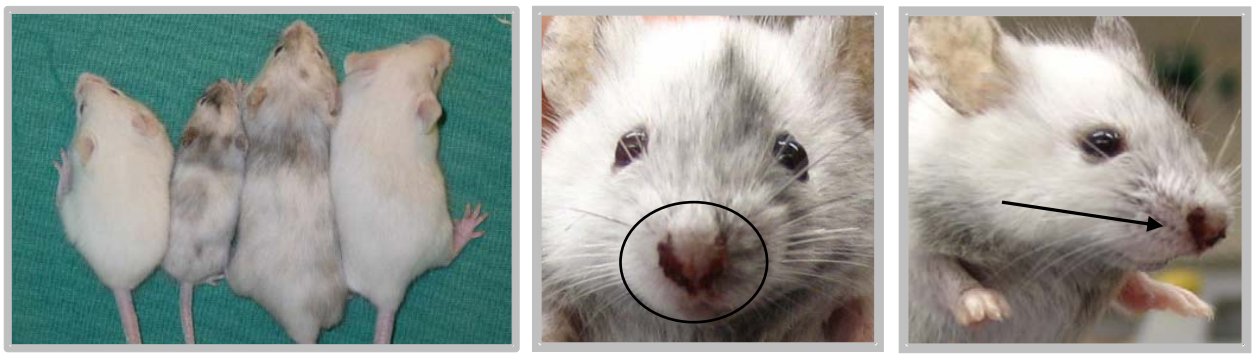


Figure 19 Appearance and clinical signs of disease of 4 week-old chimeric pups. A: Chimeric pups produced after blastocyst injection with mycoplasma-infected TBV2 cells at the passage P13+15. Some chimeras showed a significantly delayed development; they were smaller with shorter extremities and were underweight compared to chimeric pups of the same age (mouse no. 1 and 2). Clinical signs of disease included nasal discharge (B, mouse no. 2) starting at the third week of age.

TBV2 mES cells at passages P13+10, P13+15 and P13+20, respectively, developed hemorrhagic disorders and showed chronic nasal discharge which started at the age of 3 weeks. These symptoms were observed during the study and alternated between strong hemorrhages and mild serum secretion (Figure 19, B). A total of 8 (29%) (7 males and 1 female), 5 (23%) (males), and 4 (17%) (males) chimeras produced from mycoplasma-infected TBV2 mES cells at passages P13+10, P13+15 and P13+20, respectively, was smaller, showed retarded development and had nasal discharge (Table 8).

Pathological and histological analysis of chimeras

A total 2, 1, and 3 male chimeric mice with clinical signs and 3 (1 chimeric and 2 wild type mice), 5 (2 chimeric and 3 wild type mice), and 1 control chimeric and wild type mice were X-rayed and examined histologically at the age of 2, 7 and 16 weeks, respectively. The sections of internal organs were stained with hematoxylin-eosin while sections of the nasal cavity obtained from 3 different levels as well as sections of the humerus and the femur were stained with hematoxylin-eosin and Van Gieson stainings. The histological results and the X-Rays are shown in Figures 21-27.

At the age of 2 weeks, control chimeric and wild type mice weighed 7, 8.1 and 10.8 g and were 6 cm long, respectively, whereas chimeric mice with clinical signs

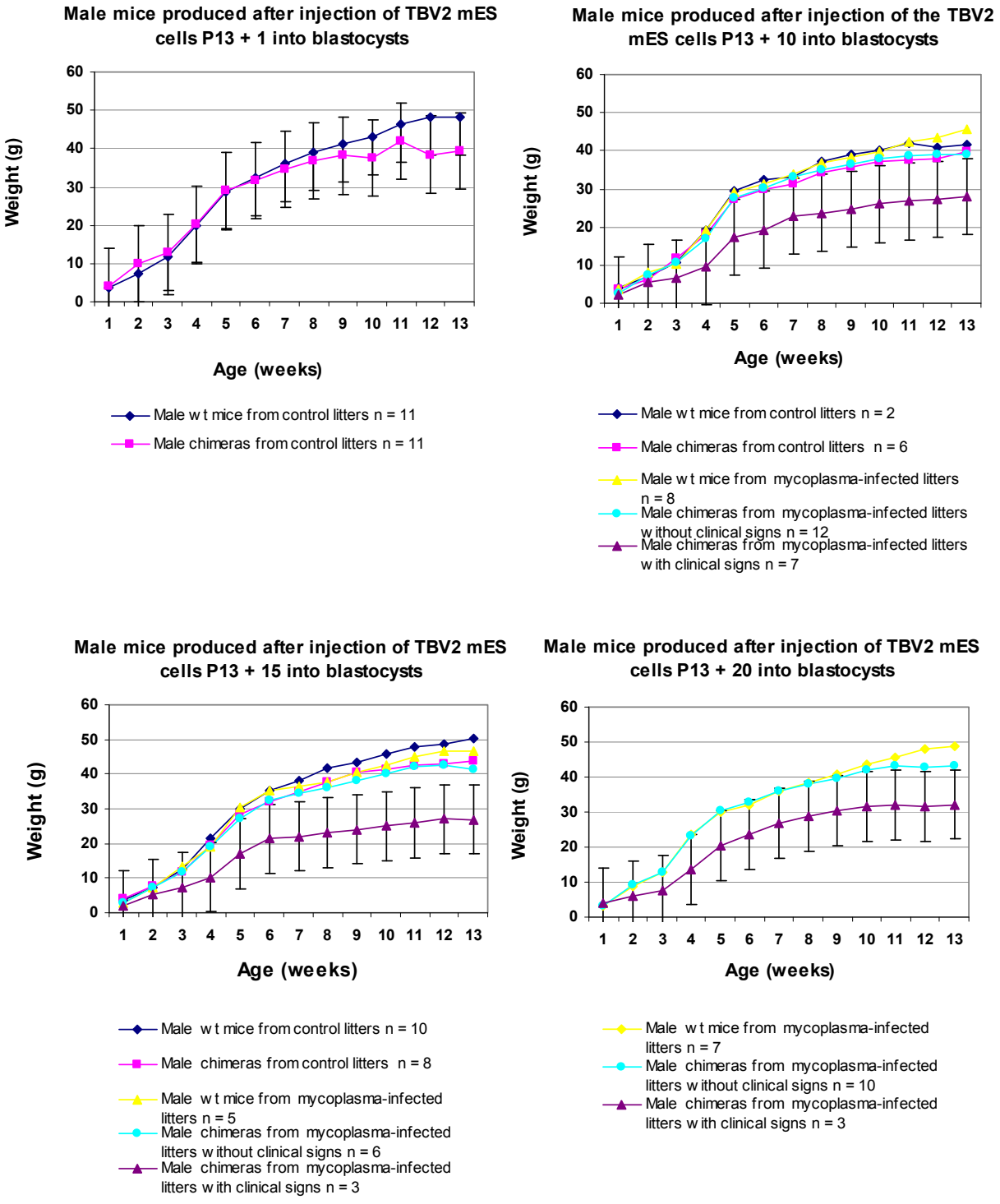


Figure 20 Development of male mice and weekly weight data up to the 13th week of age. Mice were produced by injecting control and mycoplasma-infected TBV2 mES cells at the P13+1, P13+10, P13+15 and P13+20 passages into blastocysts and performing embryo transfer. Clinically affected chimeras at passages P13+10, P13+15 and P13+20 showed statistically significant differences ($p<0.0001$).

weighed 3.7 and 4.8 g, were 4 and 5 cm long and had yellow nasal secretion, respectively. The histological examination showed a mild pneumonia in 1 chimeric mouse.

At the age of 7 weeks, control mice weighed 32.4 ± 2.6 g , and were 20.5 ± 0.2 cm long, respectively, while the chimeric mouse with clinical signs weighed 17.1 g, was 15 cm long and had strong hemorrhagic nasal discharge (Figure 19, A and B). Histological examination detected a mild pneumonia in a wild type mouse and a tubular atrophy of the testicles (Figure 23) in a clinically healthy chimeric mouse obtained after blastocyst injection with mycoplasma-infected mES cells. For the clinically affected chimeric mouse with clinical signs, histological examination revealed a lymphocytic cellular smear at the epithelial surface of the nasal septum (Figure 21, E and 22), mild pneumonia (Figure 24), monorchidy and tubular atrophy of the testicles (Figure 23), follicles and increased erythropoiesis of the spleen, and arthritis at the knee and shoulder joint which resulted in osteoarhtropathia deformans and destruction of the normal articular structures, including metaphyseal joint surfaces, the joint space and the joint capsule, respectively (Figure 27, C-H). The chondroblasts of the growth plate showed hyperproliferation and lacked column-like orientation and organization (Figure 27, D). The results obtained from the X-Ray analysis were concordant with the results obtained for the histological examination. Control mice showed no abnormalities (Figure 25, A and 26, E), whereas severe deformation of the limbs was observed for the chimeric mouse with clinical signs. The alterations affected predominantly the humerus and the femur but also other long bones which were shortened, irregularly formed and distorted with the diaphyseal bone density being reduced, having a thickened bony cortex and exostotic deformations (Figure 25, B and 26, F). Metaphysis of the proximal humerus and distal femur were severely deformed, including metaphyseal flaring, patchy lyrics changes and osteogartilaginous loose bodies.

At the age of 16 weeks, control chimeric mice (clinically healthy chimeric mouse obtained after blastocyst injection with mycoplasma-infected mES cells) weighed 36.9 g and was 20.8 cm long, while chimeric mice with clinical signs weighed 28.7 ± 1.6 g, were 18.1 ± 0.5 cm long, and had hemorrhagic nasal discharge, respectively. Histological examination of control chimeric mouse revealed a mild pneumonia, follicular hyperplasia of the spleen, and increased hematopoiesis of the liver, whereas for chimeric mice with clinical signs a mild pneumonia and

kidney hydronefrosis was present in one mouse, and follicular hyperplasia of the spleen and osteoarthropathia were present in all 3 mice, respectively. X-Ray analysis of the 16 week-old control mouse showed no alterations (Figure 25, C and 26, G). Clinical affected chimeric mice showed similar appearance as that obtained in the 7-week old chimeric mouse with clinical signs which were characterized by irregular and bending deformation of the humeral diaphysis and the olecranon ulnae but no loosen bodies (reossification). Deformation of the femur was less pronounced as compared to the humerus and the shoulder joint of the 7 week old mouse with clinical signs, which presented an incongruent articular neoformation of the shoulder, elbow, and knee with metaphyseal flaring (Figure 25, D and 26, H).

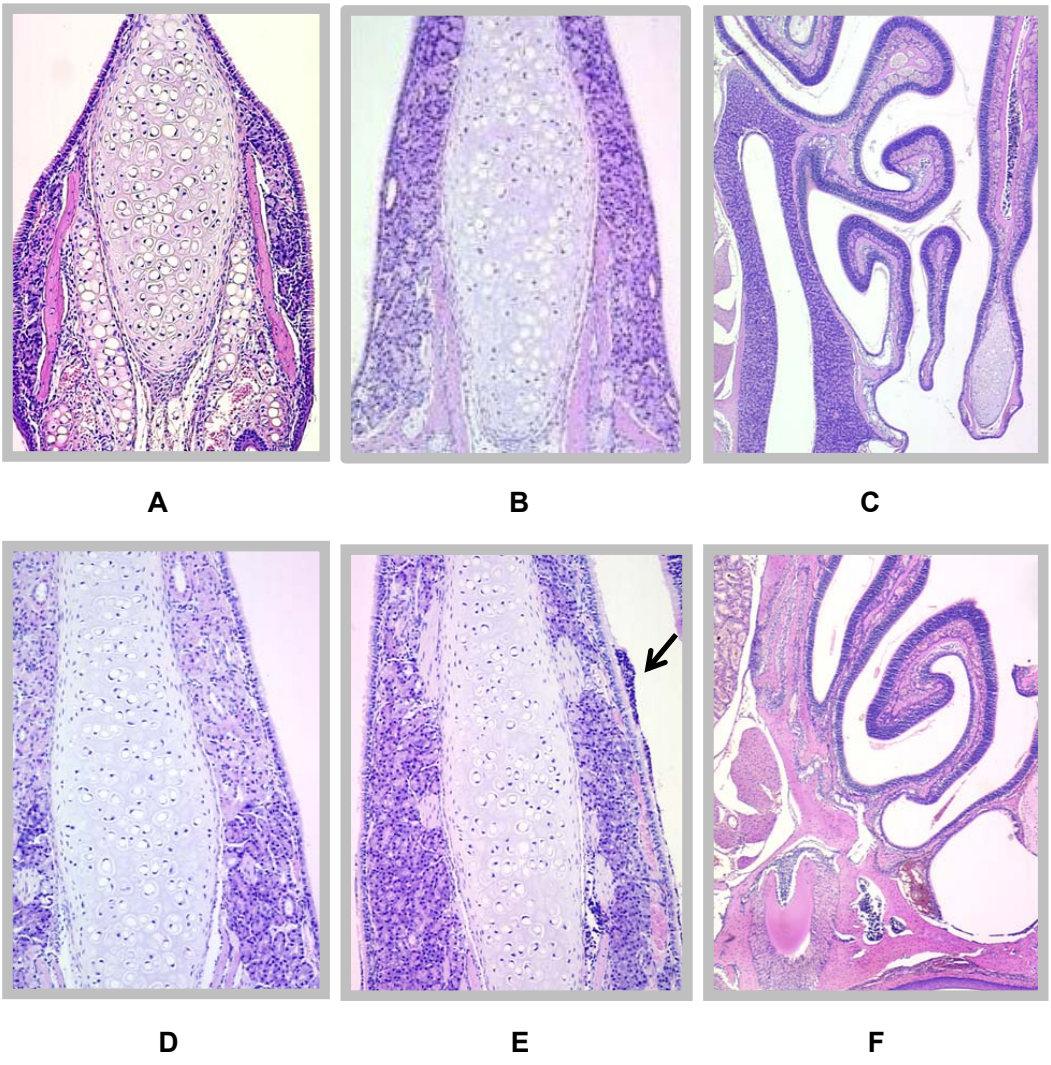
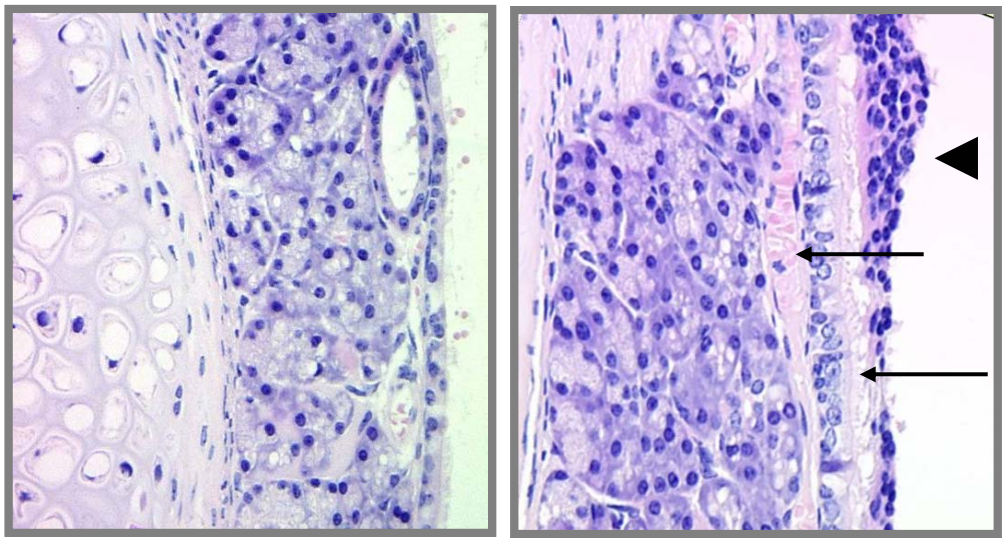
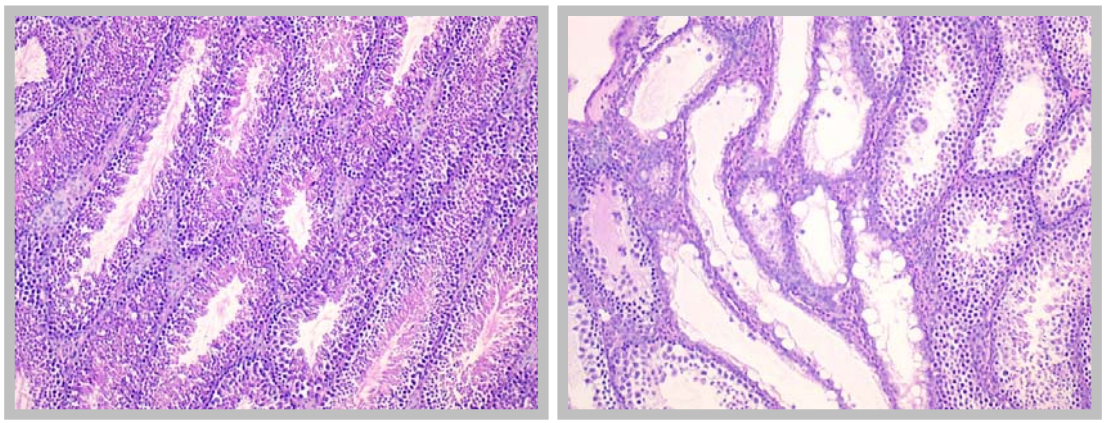


Figure 21 Section of the nasal septum from 7 week-old clinically healthy (A, B, C) and clinically affected chimeric mouse (D, E, F) obtained after blastocyst injection with mycoplasma-infected mES cells, respectively (rostral 125x; middle 125x; and caudal 160x; hematoxylin-eosin). Cellular smear was detected at sections of clinically affected chimeric mouse (E, black arrow).



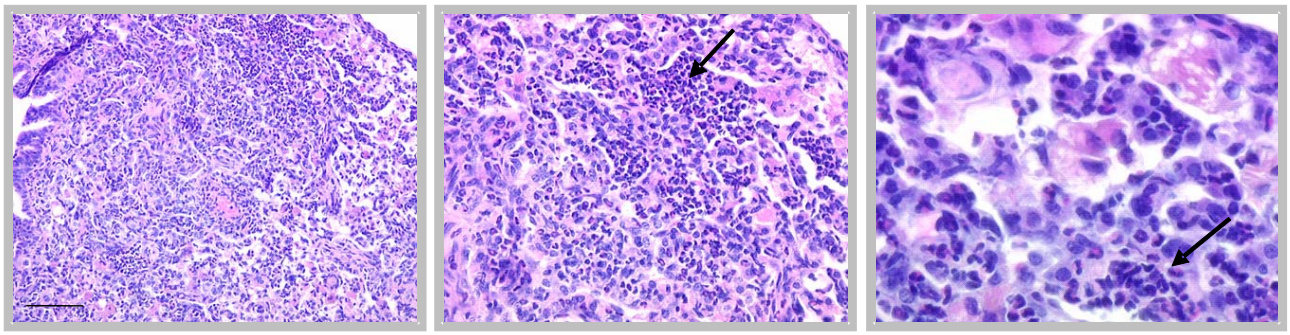
A B

Figure 22 A, B: Middle sections of the nasal septum of 7 week-old clinically healthy (A) and affected (B) chimeric mice. Larger magnifications of Figure 21, B, and E (320x, hematoxylin-eosin). Clinically affected chimeric mouse (B) showed cellular alterations of the mucosa and the submucosal layers (arrows). Deposits of cellular smear with numerous lymphocytes covering the mucosa (arrowhead).



A B

Figure 23 Histological appearances of the testicles. A: Normal spermatogenesis of a 7 week-old mouse generated with control TBV2 mES cells (100x, hematoxylin-eosin staining). B: Testicles atrophy with arrest of the germ cell development and abundant fatty cells of a 7 week-old chimeric mouse generated with mycoplasma-infected TBV2 mES cells (100x, hematoxylin-eosin).



A B C

Figure 24 Histologically appearance of lung tissue of clinically affected chimeric mice. Acute pneumonia with granulocytes and macrophages from a 16 week-old chimeric mouse with clinical signs at different magnifications. (A: 160x, B: 320x, C: 640x, hematoxylin-eosin) Scale bar = 10 mm.

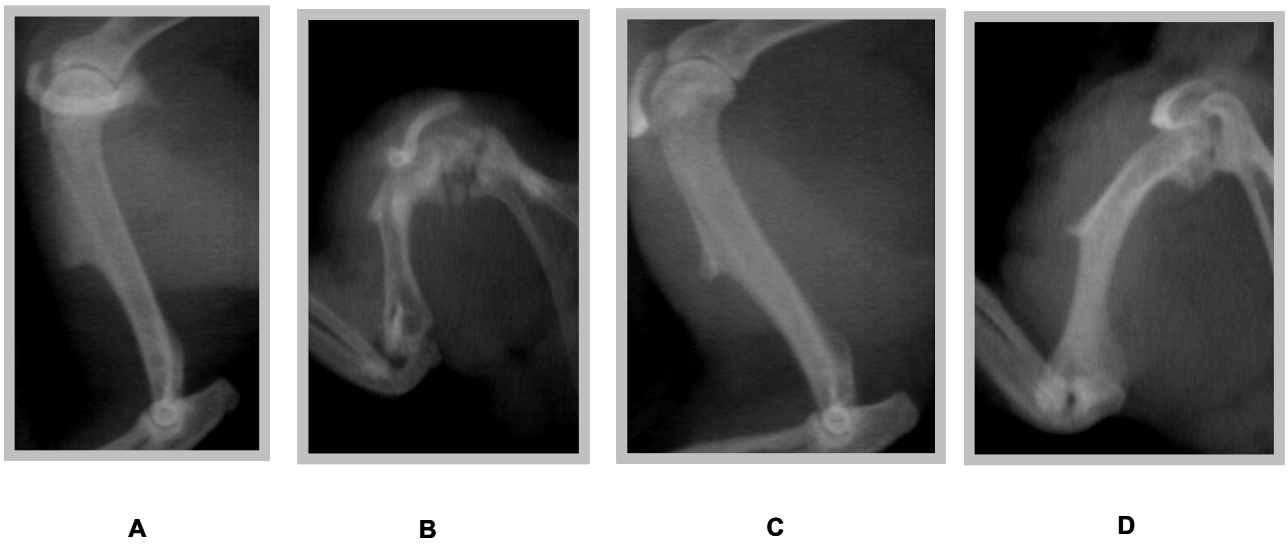


Figure 25 X-Rays of the humerus (A, B, C, D). A, C: Appearance of the humerus from 7 and 16 week-old control chimeric mice. B, D: humeral diaphysis of 7 and 16 week-old clinically affected chimeric mice.

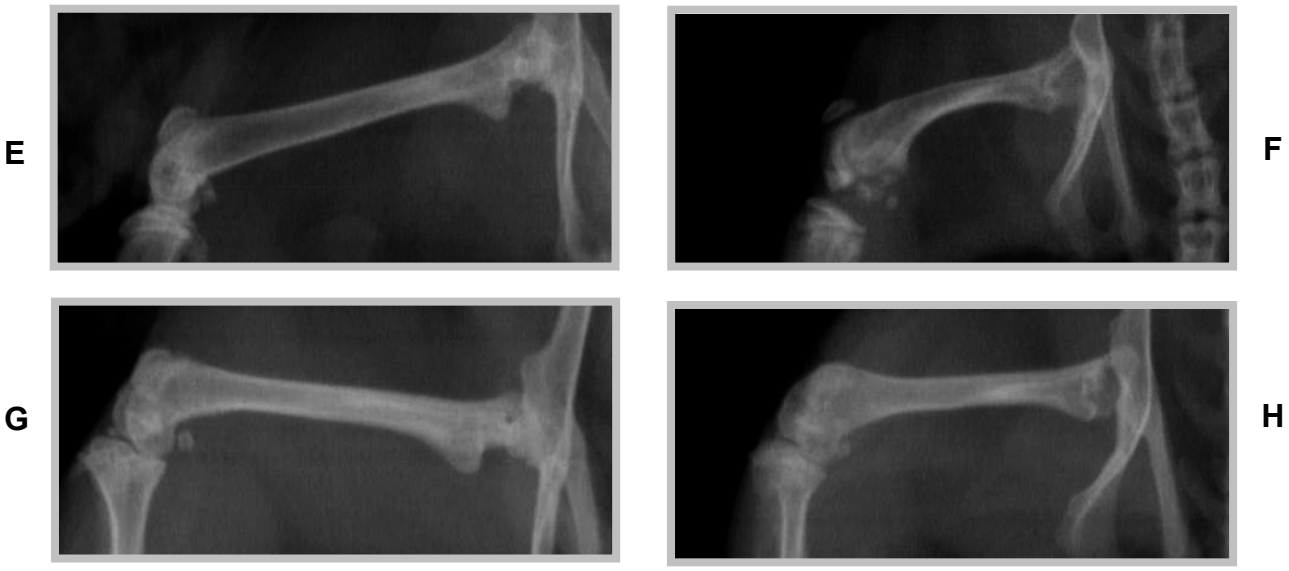


Figure 26 X-Rays of the femur (E, F, G, H). E, G: Appearance of the femur from 7 and 16 week-old control chimeric mice. F, H: femoral diaphysis of 7 and 16 week-old clinically affected chimeric mice.

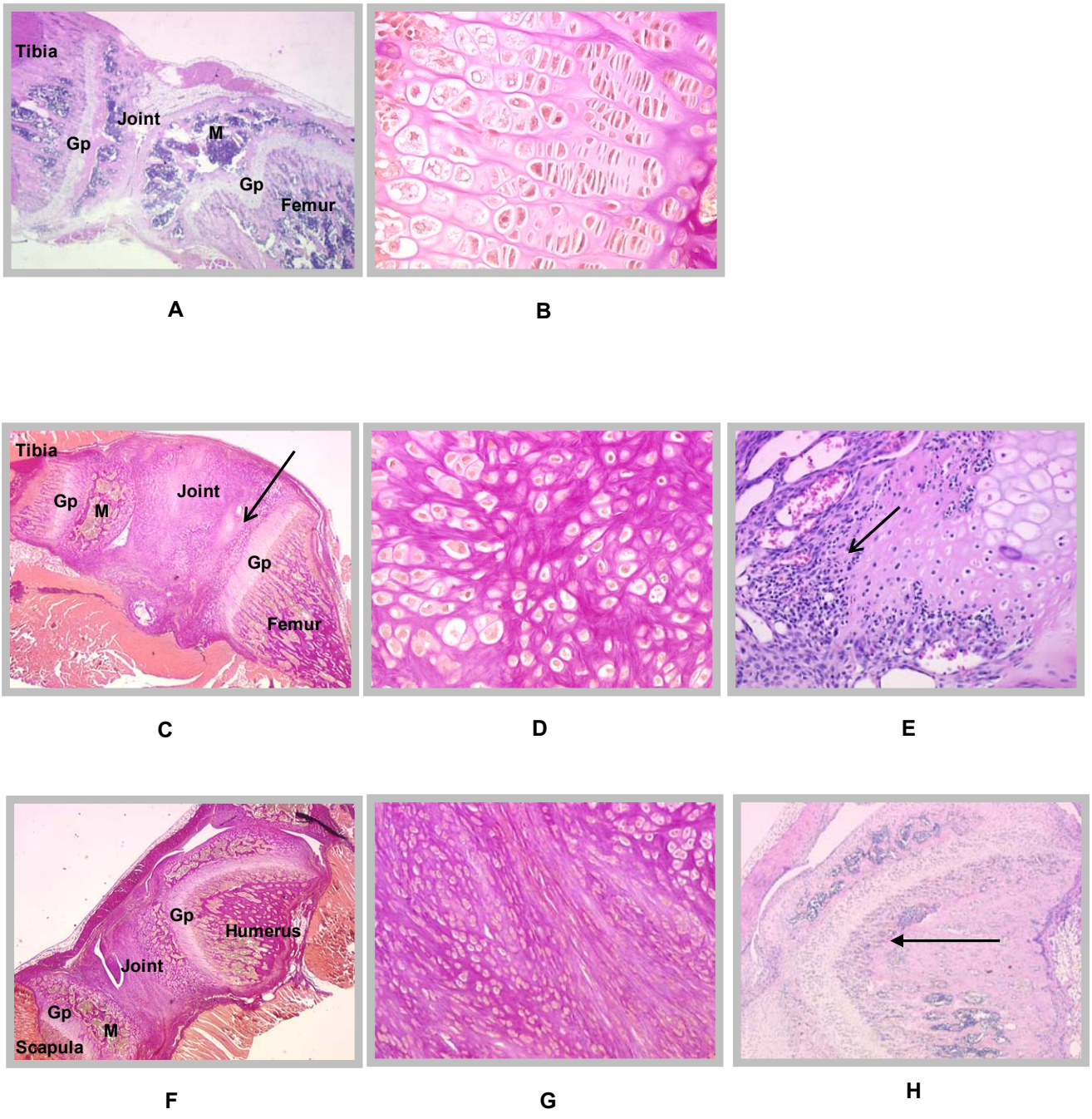


Figure 27 A: Histological appearance of the knee of a 7 week-old control chimeric mice (20x, hematoxylin-eosin), Gp: Growth plate, M: Metaphysis, B: Chondroblasts in the tibioproximal growth plate, presenting a well-organized column-like appearance (320x, hematoxylin-eosin). Deformed knee (C, D, E) and shoulder joint (F, G, H) obtained from the 7 week-old chimeric mouse with clinical signs, respectively. C, F: Metaphyseal structures are completely disorganized and a part of the metaphysis is absent (black arrow, 20x, Van Giesson), Gp: Growth plate, M: Metaphysis. C, D, F, G: Fibrocartilaginous masses are proliferating within the joint, lacking orientation and organization (D: 320x, G: 160x, Van Giesson). E: arthritis in knee resulted in osteoarthropathia deformans appeared in sections C and D (200x, hematoxylin-eosin). H: irregular line of the humeral growth plate (40x, hematoxylin-eosin).

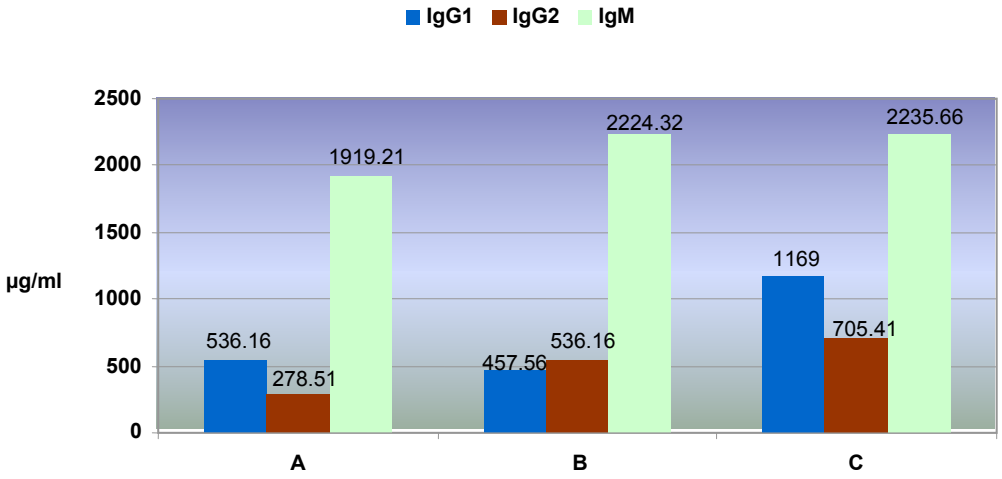
Immunological analyses

The levels of IgG1, IgG2a, IgG3, IgM, and IgA immunoglobulins, anti-DNA antibodies (Abs), rheumatoid factor (Abs), and the number of T-, B- and NK-T cells were determined from the plasma of 56 mice aging 13 to 18 weeks, by use of a bead array based system (bioplex assay), ELISA, and flow cytometric analysis (FACS). The mice were divided into 5 groups: group A constituted 9 chimeric mice obtained after blastocyst injection with control mES cells, group B constituted 13 clinically healthy chimeric mice obtained after blastocyst injection with mycoplasma-infected mES cells, group C constituted 13 chimeric mice with clinical signs of disease, obtained after blastocyst injection with mycoplasma-infected mES cells, group D constituted 10 control CD-1 wild type mice from our core breeding unit, and group E constituted 11 non-chimeric mice obtained after blastocyst injection with mycoplasma-infected mES cells. The results obtained from the respective groups were analyzed statistically using the t-test and significant differences were observed which are shown in Figure 28 and Table 9.

The plasma levels of IgG1, IgG2a, and IgM were $5.4x10^2$, $2.8x10^2$, and $1.9x10^3$ µg/ml for group A. For group B the levels were $4.6x10^2$, $5.4x10^2$, and $2.2x10^3$ µg/ml, and for group C values were $1.2x10^3$, $7.1x10^2$, $2.2x10^3$ µg/ml, respectively. The levels of anti-DNA antibodies and rheumatoid factor were 0.6 and 0.3 OD for group A, 0.7 and 0.4 OD for group B, and 0.8 and 0.4 OD for group C, respectively. Comparison of group A (control group) to group B (clinically healthy chimeras produced with mycoplasma-infected mES cells) showed significant differences for IgM ($p<0.05$) and rheumatoid factor ($p<0.01$). Comparison of group A to group C (clinically affected chimeras produced with mycoplasma-infected mES cells) showed significant differences for IgG1 ($p<0.001$), IgG2a ($p<0.05$), IgM ($p<0.05$), anti-DNA antibodies ($p<0.05$), and rheumatoid factor ($p<0.01$). The IgG1 levels were significantly different between group B and group C ($p<0.001$) (Figure 28). There were no significant differences in the levels of IgG3 and IgA obtained between groups A, B and C ($p>0.05$). Between the group D (control CD-1 wild type mice) and group E (non-chimeric mice produced after the injection of mycoplasma-infected mES cells into blastocysts), no statistically significant differences could be revealed for the above-mentioned immunoglobulins ($p>0.05$).

The results obtained for the white blood cells are shown in Table 9. There were significant differences between group A (control chimeric mice) and group B

(clinically healthy chimeric mice produced after the injection of mycoplasma-infected mES cells into blastocysts), group A and group C (clinically affected chimeric mice produced after the injection of mycoplasma-infected mES cells into blastocysts), but not between group B and group C. The proportions of T-regulatory cells (CD4+>CD3+>CD25+), NK-T cells (NK-U52A>CD3+), and gdT-cells (CD3+>gdTCR+) in group B and group C were decreased compared to group A, whereas the proportions of a subset of cytotoxic T-cells (CD8a+CD4->CD3+CD44-CD62l-) and cells expressing Gr1 marker (CD19-/Gr1+CD11b-) were increased. Furthermore, several non T-cell leukocyte subsets were altered between the groups. In the non T-cell compartment (B-cells or granulocytes) bivariate gating revealed differences within the CD44/CD62L subsets. Similar results were observed between group E (non-chimeric mice produced after the injection of mycoplasma-infected mES cells into blastocysts) and group D (CD-1 wild type mice).



A vs. B: IgM (p<0.05)

A vs. C: IgG1 (p<0.001), IgG2 (p<0.05), IgM (p<0.05)

B vs. C: IgG1 (p<0.001)

Figure 28 Immunoglobulin levels obtained from the examination of plasma. A, B, C: group A (control chimeric mice n =9), group B (chimeric mice without clinical signs n = 13), group C (chimeric mice with clinical signs n = 13)

Table 9 Immunological screening of chimeric mice

Parameters (**>**, **/**) [#]	Group A* (n = 9)	Group B[£] (n = 13)	Group C[‡] (n = 13)	Group A vs. Group B	Group A vs. Group C	Group B vs. Group C
				p-value	p-value	p-value
Non duplets**>**living cells**>**CD45+**>**CD19-**/**Gr1+CD11b- (%)[†]	0.4 ± 0.1	0.8 ± 0.2	1.1 ± 0.2	p<0.05	p<0.01	n.s
Non duplets**>**living cells**>**CD45+**>**CD19-**>** non granulocytes**/**NK-U5A2, freq. of 45+ (%)[†]	4.7 ± 0.3	3.3 ± 0.2	3.4 ± 0.2	p<0.01	p<0.01	n.s
Non duplets**>**living cells**>**CD45+**/**CD19-**>**non granulocytes**/**NK-U5A2,CD3+, freq. of 45+ (%)	1.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	p<0.001	p<0.001	n.s
Non duplets**>**living cells**>**CD45+**>**CD3+**/**gdTCR+, freq. of 45+ (%)[†]	0.7 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	p<0.001	p<0.01	n.s
Non duplets**>**living cells**>**CD45+**>**CD4+, CD8-**/**CD3+, CD25+ (%)[†]	4.9 ± 0.3	2.8 ± 0.3	3.2 ± 0.3	p<0.001	p<0.01	n.s
Non duplets**>**living cells**>**CD45+**>**CD8a+, CD4-**/**CD3+, CD44-CD62l-(%)[†]	18.7 ± 2.6	30.2 ± 1.7	28.2 ± 3	p<0.05	p<0.01	n.s
Non duplets**>**living cells**>**CD45+**/**CD4-, CD8a-, CD44+, CD62L- (%)[†]	5.7 ± 0.3	8 ± 0.5	9.3 ± 1	p<0.001	p<0.01	n.s
Non duplets**>**living cells**>**CD45+**/**CD4-, CD8a-, CD44-, CD62L+ (%)[†]	12 ± 1.8	10 ± 1.2	6 ± 1	n.s	p<0.05	n.s

Group A*: control chimeric mice, Group B[£]: clinically healthy chimeric mice produced with mycoplasma-infected mES cells, Group C[‡]: clinically affected chimeric mice produced with mycoplasma-infected mES cells, n.s: not significant, [#] (**>**): parent gate. Parameters of the parent gate are presented in the order of gating hierarchy, (**/**): parameters of leukocyte clusters into cell population, (%)[†]: values present percentages of parent gate, and/or CD45+ cells if indicated

5. DISCUSSION

The aim of this thesis was to investigate the effects of mycoplasma contamination on mES cell cultures, the germ line transmission, and the resulting chimeric progeny.

Since mES cells were experimentally infected, appropriate methods were established for reliable detection of mycoplasmas in the ES cell cultures. Therefore, in a first step of this thesis, the agar culture, the MycoAlert™ assay, the gel-based PCR, and the MycoSensor™ QPCR were evaluated concurrently with regards to the limit of detection (LOD) of *M. hominis, M. fermentans* and *M. orale* in cell culture supernatants and in cells. Due to the diversity of mycoplasma species with respect to their properties and growth, samples of supernatants containing apoptotic and dead cells and cell suspensions containing viable cells were used for agar culture and PCRs. The present data show that each method was able to detect mycoplasmas in the undiluted samples while differences were obvious with respect to analysis of the dilutions where agar culture and the MycoSensor™ QPCR were the most sensitive methods.

For detection of *M. hominis* in cell-containing supernatants and dilutions, the MycoSensor™ QPCR assay was the most sensitive method detecting mycoplasmas up to the 10^{-6} dilution, followed by Friis agar culture, PH-Hayflick agar culture, the gel-based PCR, and the MycoAlert™ assay being 10^1, 10^2, 10^4 and 10^5 times more sensitive, respectively. With respect to reproducibility, where all 3 to 5 runs were positive, the MycoSensor™ QPCR assay was 10^3 and 10^2 times more sensitive than the gel-based PCR and the MycoAlert™ assay, respectively. Analysis of cell suspensions and dilutions for *M. hominis* showed that the MycoSensor™ QPCR assay was again the most sensitive method detecting mycoplasmas up to the 10^{-4} dilution, followed by PH-Hayflick agar culture, Friis agar culture and the gel-based PCR being 10^1, 10^2 and 10^4 times more sensitive, respectively. Where all 3 runs were positive, in contrast to the gel-based PCR, the MycoSensor™ QPCR assay gave reproducible results.

For detection of *M. fermentans* in cell-containing supernatants and dilutions, Friis agar culture was the most sensitive method detecting mycoplasmas up to the 10^{-4} dilution, followed by the MycoSensor™ QPCR assay, the gel-based PCR, and the MycoAlert™ assay being 10^1, 10^3, and 10^4 times more sensitive, respectively. Where all 3 to 5 runs were positive, the MycoSensor™ QPCR assay was 10 times

more sensitive than both the gel-based PCR and the MycoAlert™ assay. Analysis of cell suspensions and dilutions for *M. fermentans* revealed that Friis agar culture was again the most sensitive method detecting mycoplasmas up to the 10^{-4} dilution, followed by the MycoSensor™ QPCR assay, PH-Hayflick agar culture, and the gel-based PCR being 10^1, 10^2 and 10^3 times more sensitive, respectively. Where all 3 runs were positive, the MycoSensor™ QPCR assay was 10^2 times more sensitive than the gel-based PCR assay.

For detection of *M. orale* in cell-containing supernatants and dilutions, Friis and PH-Hayflick agar culture were the most sensitive methods detecting mycoplasmas up to the 10^{-6} dilution, followed by the MycoSensor™ QPCR assay, the gel-based PCR, and the MycoAlert™ assay being 10^1, 10^2 and 10^4 times more sensitive, respectively. Where all 3 to 5 runs were positive, the MycoSensor™ QPCR assay was equally sensitive to the gel-based PCR and 10 times more sensitive than the MycoAlert™ assay. Analysis of cell suspensions and dilutions for *M. orale* showed that Friis and PH-Hayflick agar culture were the most sensitive methods detecting mycoplasmas up to the 10^{-4} dilution, followed by the MycoSensor™ QPCR assay and the gel-based PCR being 10^2 times more sensitive. Where all 3 runs were positive, the MycoSensor™ QPCR assay was 10^2 times more sensitive than the gel-based PCR assay.

In this study, due to quantification of the mycoplasmas present, samples were inoculated directly on agar plates omitting culture in broth (Uphoff 1992, Uphoff, 1992). With the exception of PH-Hayflick agar plates inoculated with cell-containing supernatants and dilutions on which *M. fermentans* failed to grow, agar culture was the most sensitive method for detection of *M. fermentans* and *M. orale*. Failure of *M. fermentans* to grow on PH-Hayflick agar in this study confirms previous reports (Blanchard 1993, Serin 2001) and may be due to the lack of appropriate growth conditions (Drexler 2000). Mycoplasmas often show an elongated lag phase and slower or no growth as a compensatory survival mechanism. Incubation in broth is essential for counteracting the long lag phase, allowing replication of mycoplasmas. In this study, Friis agar culture was conducive to the growth of *M. fermentans* despite omission of the broth phase. However, for PH-Hayflick culture, incubation in broth seems to be mandatory for growth of *M. fermentans*. Therefore, it is recommended to use both types of culture media for more reliable results with supernatants (Uphoff, 1992). The viable cells in the cell suspension provided the cell-associated *M.*

fermentans with the necessary nutrients, allowing growth on PH-Hayflick agar. In contrast, the cells that were found in the supernatants failed to support growth of *M. fermentans* most likely since they are apoptotic or dead.

Using both agar culture and PCRs, more mycoplasmas were detected in the cell-containing supernatants than in the corresponding dilution of the cell suspensions confirming that *M. hominis* and *M. orale* are present mostly in cell culture supernatants. Friis agar culture and the PCRs detected *M. fermentans* equally in cell-containing supernatants and in cell suspensions. However, for PH-Hayflick agar culture, only cell suspensions led to growth of colonies. These observations confirm that *M. fermentans* is mainly a cell-associated species found mostly attached to or in the cells. The present data also show that detection of mycoplasmas in cell cultures is more reliable when both cell-containing supernatants and viable cells are analyzed.

In the present study, the in-house established gel-based PCR used is based on previous reports (Uphoff 2002, Uphoff 2002) with modified annealing and amplification times, number of cycles and master mix. The primers used anneal to gene sequences coding for conserved 16S rRNA of several different mycoplasma species, allowing specific detection of mycoplasmal DNA (Uphoff 2005). In addition, the mycoplasma species present was identified by restriction fragment length polymorphism. The present data reveal that the gel-based PCR showed the highest sensitivity for *M. orale*, followed by *M. fermentans* and *M. hominis*. Based on the quantification data generated by the MycoSensor^{TM} QPCR, the LOD per reaction for the cell-containing supernatant and the cell suspension, respectively, was 30 and 60 genomic copies of *M. orale,* $5.8x10^2$ and $3.2x10^2$ genomic copies of *M. fermentans*, and $6.8x10^4$ and $3.2x10^4$ genomic copies of *M. hominis*. Where all 3 to 5 runs were positive for the supernatant dilutions, the gel-based PCR detected $1.6x10^6$, $6x10^3$ and $4.8x10^2$ copies of *M. hominis, M. fermentans,* and *M. orale* per reaction, respectively. In contrast, for cell suspension dilutions, the gel-based PCR gave reproducible results only for *M. fermentans* and *M. orale* with $3.2x10^3$ and $9.2x10^3$ copies per reaction, respectively.

Published LODs of gel-based PCRs are 1 to 2 copies of the target DNA from 13 mycoplasma species (Sung 2006), 1 organism of *M. pneumoniae* (Vojdani 1998), 12 to 18 organisms of *M. hominis* (Blanchard 1993), 1 CFU of 4 mycoplasma species including *M. fermentans* and *M. orale* (Harasawa 1993), 8.8 CFUs of *M. hominis*

(Stellrecht 2004), 10^2 CFUs (Harasawa 2005), and 10 to 90 CFUs of *M. hominis*, *M. fermentans,* and *M. orale* (Tang 2000). In the present study, the sensitivity of the gel-based PCR for detection of *M. orale* and *M. fermentans* was similar to that of previous works (Harasawa 1993, Tang 2000, Harasawa 2005) but lower than that reported for detection of *M. hominis* (Blanchard 1993, Stellrecht 2004, Sung 2006).

The MycoSensor^{TM} QPCR showed the highest sensitivity of all methods for analysis of *M. hominis*, detecting as little as 8 copies per reaction. For *M. fermentans* and *M. orale*, the MycoSensor^{TM} QPCR was the second most sensitive method after agar culture, detecting 7 and 80 copies per reaction, respectively. Where all 3 to 5 runs were positive, the MycoSensor^{TM} QPCR detected in supernatant dilutions $1.3x10^4$, $2.9x10^3$ and $1.2x10^3$ copies of *M. hominis, M. fermentans*, and *M. orale* per reaction, respectively. For cell suspensions, the MycoSensor^{TM} QPCR detected $1.9x10^3$, $2.4x10^2$, and $3.2x10^2$ copies of *M. hominis, M. fermentans*, and *M. orale* per reaction, respectively.

The melting temperatures were similar for all three mycoplasma species, lying between 80.1°C and 82°C, thus hindering differentiation between species, as previously reported (Harasawa 2005). The efficiency of the real-time PCR was 1.98 to 2 showing that the quantity of DNA doubled in each cycle. Published LODs of the real-time PCR are 10, 5, and 5 genome copies of *M. genitalium* per reaction (Svenstrup 2005, Yoshida 2002, Dupin 2003), respectively, 10 genomic copies of *M. hominis* (Baczynska 2004), and 10 CFUs per assay (Harasawa 2005). The SYBR green-based real-time PCR detected 100 fg of mycoplasmal genomic DNA from *M. hominis*, *M. fermentans* and *M. orale* (Ishikawa 2006). As such, the LODs obtained in this study are comparable to those published by the above-mentioned authors.

As shown in the present study, each detection method has its own advantages and disadvantages which should be considered. Agar culture is highly sensitive; it detects viable mycoplasmas, colonies can generally be readily recognized, it is easy to perform, and is cost effective. The disadvantages of this method are the long incubation time of 2 to 3 weeks and the artifacts that are occasionally observed on agar which makes it difficult for the inexperienced observer to distinguish true mycoplasma colonies from cell clumps or pseudocolonies arising from crystals and air bubbles. In addition, instead of the classical fried-egg colonies certain mycoplasmas produce a more diffuse, granular pattern, which makes evaluation difficult. Finally, not all mycoplasma species are cultivatable in artificial media

(McGarrity 1985, Uphoff 1992, Uphoff 1992, Tully 1996, Drexler 2000, Drexler 2002).

The MycoAlert™ assay is easy to perform, simple to interpret and results are obtained within 20 minutes. However, the absence of cells for analysis makes the MycoAlert™ assay less sensitive for mycoplasmas that are mainly cell-associated.

The results of the gel-based PCR can be obtained within hours. The gel-based PCR is susceptible to technical artifacts if not performed with extraordinary care including strict quality control of reagents, aseptic procedures and appropriate laboratory environment (Tully 1996). In general, PCR results should be verified by sequencing, restriction analysis or hybridization. The MycoSensor™ QPCR is a quantitative assay and is highly sensitive. It is easy to use and interpret, and results can be obtained within hours. In addition, it eliminates post-PCR processing and carry-over contamination since it employs a closed-tube detection system.

European Pharmacopoeia and the FDA still recommend culture in broth and agar media as the method of choice for detection of mycoplasmas in cell cultures and biological products produced in cell substrates. For mycoplasma species that cannot be cultured in artificial media, use of an indicator cell line is proposed as the method of choice. The PCR method was also added to this list (CBER/FDA 1993, Pheur, 2004).

For routine diagnostic work with cell cultures, the presence of mycoplasmas *per se* and not necessarily the species is of interest. Although sensitive methods bring some advantages, acute and chronic cell culture infections involve at least 10^6 to 10^8 organisms per ml and 100 to 1000 mycoplasmas can be attached to each infected cell (Drexler 2002). In the present study, all methods reliably detected the presence of mycoplasmas in the undiluted supernatants and cell suspensions. Highly sensitive methods are necessary where minute amounts of mycoplasmas are present such as in cells that are routinely cultured with antibiotics, which could lead to covered contaminations, after antibiotic treatment or screening of imported cells. Based on the present data, agar culture and the MycoSensor™ QPCR can be used for routine diagnostic work. In addition to the use of supernatants, analysis of viable cells would aid in increasing the probability of detecting cell-associated mycoplasmas.

As infections of cell lines with more than one mycoplasma strain are frequently observed (Timenetsky 2006), in the second part of this thesis, the susceptibility of the TBV2 mES cells to infection with a combination of *M. hominis*, *M. fermentans,* and *M.*

orale and the resulting effects on morphology, growth, viability, karyotype, and differentiation status were determined over 20 passages. The influence on Germ Line Transmission (GLT) of the infected TBV2 mES cells was evaluated after injection of the mES cells into blastocysts, transfer of the injected embryos into pseudopregnant recipients, production of chimeras, and breeding of the chimeric progeny with the B6 mouse strain.

The human species *M. hominis*, *M. fermentans* and *M. orale* were chosen since they are the most common mycoplasma contaminants of cell cultures (Drexler 2002). In addition, a recent study including 98 cell lines showed 60% prevalence of multiple mycoplasma infections with 2 or more mycoplasma species including those used in the present study (Timenetsky 2006). Continuous culture of the inoculated cell line indicates that TBV2 mES cells are susceptible to infection with the combination of *M. hominis*, *M. fermentans,* and *M. orale* and that mycoplasma-infected mES cells exhibit a number of severe effects both *in vitro* and *in vivo*.

Control mES cells led to the growth of large to very large mES cell colonies, showed an average growth rate of 1:7 per passage and reached a hypothetical number of $1.2x10^{22}$ trypsinized mES cells at passage P13+20. Subsequent to inoculation with *M. hominis*, *M. fermentans,* and *M. orale* at passage P13, all 3 species were present from passage P13+2 to P13+20, as determined by restriction fragment length polymorphism (RFLP) indicating productive infection of the mES cells with all 3 mycoplasma strains. The MycoSensor^{TM} QPCR assay detected between $1.7x10^{8}$ (at P13+10) and $6.5x10^{3}$ (at P13+20) mycoplasma genome copies per ml in cell-containing supernatants and in cell suspensions. Thus, an average of 1 to 580 mycoplasmas adhered to or was present in each mES cell.

The mycoplasma infection affected the morphology, the growth rate, and the viability of the mES cells and led to very small to medium cell colonies. The average growth rate was 1:3 per passage up to passage P13+14, increased to 1:6 for passages P13+15 to P13+20, and resulted in $3.9x10^{15}$ trypsinized mES cells at passage P13+20. As such, the number of cells was approximately 7 logs lower than that of the control cell line at this passage level. The average viability of the mycoplasma-infected mES cells was 85% and thus 10% lower than that of the control cell line (95%). Earlier studies showed that *M. fermentans* increased the population doubling time of FL amnion cells from 17.5 to 30 hours (Fogh 1971), whereas *M. hominis* reduced the growth rate of the HAIN-55 human diploid

fibroblasts (Sasaki 1981). Other studies demonstrated similar results (Kenny 1963, Stanbridge 1969, Stanbridge 1971, McGarrity 1985).

The GLT rate of the control mES cells at passage P13+1 was 92%; it decreased slightly to 90% at passage P13+5 and further to 78% at passage P13+20, showing that extended periods of culture reduced the GLT of the TBV2 mES cells. Similar results have been reported for the R1 mES cell line with a 129 genetic background (Nagy 1993). With respect to control TBV2 mES cells at passage levels P13+6, P13+11, P13+16, and P13+21, 84% expressed both differentiation markers OCT-4 and SSEA-1, as determined by flow cytometric analysis (FACS). These data agree with those of previous reports, where low OCT-4 expression resulted in a low GLT (Buehr 2003, Tielens 2006). Control mES cells showed low euploidy of 20% at passage P13+10 and trisomy 1, 6 and 11, indicating that these features did not affect the GLT of mES cells or the chimeric progeny. In contrast, previous studies reported that various mES cell lines failed to contribute to the germ line, when euploidy was less than 50%. Moreover, sterility, polydactylia, and eye anomalies were detected in the progeny (Longo 1997, Guo 2005). Two more studies showed that trisomy 8 had no effects on the differentiation potential of the mES cells but influenced the GLT (Liu 1997, Park 1998). The reason for the high GLT of the control TBV2 mES cells observed even at late passages in the present study could be due to the fact that 15 to 20 mES cells were injected into each blastocyst, indicating that there were sufficient ES cells capable of contributing to the germ line. The number of mES cells injected has been shown to markedly influence GLT as previously reported (Brown 1992) where 8 to 12 mES cells with a diameter of 7 to 13 µm resulted in 18.5% of chimeric mice, whereas blastocyst injections with 3 to 5 and 5 to 8 mES cells resulted in 0% and 7.4% chimeric mice, respectively.

Germ line transmission of mycoplasma-infected mES cells at passage P13+5, which was the strongest infected passage, was only 50%. This effect was less pronounced at P13+10 and P13+15, where GLT was 62% and 60%, respectively. At passage level P13+20, at which approximately 1 mycoplasma attached to each mES cell, GLT was 81% and similar to that obtained for control mES cells at the same passage. The low GLT rates were most likely due to the effects of mycoplasmas on various cell parameters. Based on the MycoSensor^{TM} QPCR assay, approximately $1.2x10^4$ to 15 mycoplasmas were injected together with the mES cells into each blastocyst between passages P13+5 and P13+20, respectively. Since each recipient

received 14 blastocysts, approximately $1.6x10^5$ to 210 mycoplasmas were transferred to the recipients via the blastocysts at the four respective passages.

Karyotypic analysis of mycoplasma-infected and control mES cell metaphases revealed numeric aberrations and a number of non-clonal chromosomal aberrations, which were first observed at passage P13+10 and increased with further passages. Previous studies in a human amnion cell line showed that chronic mycoplasma contamination led to gradual reduction of the chromosome number (Fogh 1965), whereas other studies reported that *M. hominis*, *M. fermentans*, *M. orale*, *A. laidlawii* and Ureoplasmas resulted in chromosome breaks, rearrangements, and translocations (Paton 1965, Stanbridge 1969, Kundsin 1971, Tsai, 1995). The duration of an infection with mycoplasmas plays an important role in the establishment of reversible or irreversible effects with clonal or non-clonal chromosomal aberrations. Reversible chromosomal aberrations caused by *M. fermentans* were described in the mouse embryo cell line C3H in the first 7 to 11 weeks of infection; they became irreversible 5 weeks later (Tsai 1995). Even though no influence on GLT or on the resulting chimeras was observed in the present study after 6 weeks of infection, it is subject of speculation whether irreversible clonal chromosomal aberrations occur in mES cells after longer periods of mycoplasma infection and whether consequences on GLT of the mES cells and the genome of the resulting chimeric progeny are brought about at later time points. This, however, is rather irrelevant, since mES cells with high passage numbers are generally no longer used for transgenesis.

Mycoplasma-infected mES cells at passage P13+6 showed expression of both OCT-4 and SSEA-1 at a rate of 46%, 10% expressed only OCT-4 and 24% expressed only SSEA-1; 20% expressed neither OCT-4 nor SSEA-1. At further passages, OCT-4 and SSEA-1 levels were comparable to those of control mES cells. The low OCT-4 expression rate observed at passage P13+6 correlated with the low GLT of mycoplasma-infected mES cells. A previous study reported that changes in culture conditions led to reduced OCT-4 expression of the IOUD2 murine ES cell line (Faherty 2005). In this study, suboptimal culture conditions were very likely generated due to mycoplasma consumption of essential nutrients and other components of the media, as well as by changes of the pH due to high metabolic activity, especially in the early stages of the infection.

At passage P13+5, a smaller numbers of pups were born and low GLTs were observed, probably due to the heavy mycoplasma infection. A previous study showed that mycoplasma contamination led to reductions in the number of chimeras born, percentage of male progeny and average contribution of ES cells to tissues (Bradley 1987). It was further reported that infection could be transmitted from mES cells to the embryo where mycoplasmas replicate uncontrollably, resulting in embryo death and that the level of cell contamination could affect the number and quality of the chimeras born (Bradley 1987).

Chimeric progeny from mycoplasma-infected mES cells at passages P13+10 to P13+20 showed abnormal morphology and signs of disease. In contrast, no effects were observed in chimeric progeny produced with mycoplasma-infected mES cells at passage P13+5 even though GLT was lowest at this passage. This may be due to a strong immunological reaction of the recipients to the large amount of mycoplasma antigens at passage P13+5. At later passage levels, when less antigen was transferred to the blastocysts immunological interactions between the host and mycoplasma-containing mES cells could be the reason for the development of disease. The development of morphological and clinical effects in chimeric progeny may depend on the mycoplasma species, since *M. hominis* and *M. fermentans* are highly pathogenic, whereas *M. orale* act as a pathogen only under certain conditions. Moreover, it appears to be individually controlled by the recipients' defense mechanisms, since healthy and affected chimeras with morphological and clinical signs were observed in the same litters.

Morphological alterations and diseased mice were present in the chimeric progeny but not in the F1 generation after breeding of chimeras with B6 mice, indicating that the disease developed as a consequence of infection rather than due to genetic reasons. Histological analysis and the X-rays of affected progeny suggested that dwarfism and locomotory problems are due to alterations in the growth plate, arthritis and generalized osteoarthropathia as a consequence of mycoplasma infection of the joints.

Generalized mycoplasma infection in the chimeras resulted in retarded development and affected chimeras were significantly lighter than their healthy mates weighing 30 g or less until 13 weeks of age. The contribution of mES cells to the somatic tissues of the affected chimeras was 5 to 95%, excluding the possibility that the 129 mouse genetic background of the mES cells is the reason for this condition.

Furthermore, generalized mycoplasma infection induced subtle changes in the proportions of leukocyte subsets in peripheral blood of the adult chimeras produced with mycoplasma-infected mES cells, a decrease in T-regulatory cells, gdTCR cells, NK-T cells, and changes in the levels of IgG1, IgG2a, and IgM. Furthermore, the levels of DNA and rheumatoid antibodies were elevated in some of the affected chimeric mice.

Nasal hemorrhagic discharge was observed in 30 chimeras from 3 weeks of age during the whole experimental period. The severity of the clinical signs, the quality (hemorrhagic, serum) and the quantity (strong or mild) of the secretion varied and the condition was not contagious or life-threatening. The nasal epithileum was also affected and the clinical smear observed was infiltrated with lymphocytes, indicating a chronic situation. Acute pneumonia with granulocyte infiltration of the lungs as well as hydronephrosis and follicular hyperplasia of the spleen with increased erythropoiesis were observed probably due to nasal discharge as a result of aspiration and bronchiolar obstruction and hemorrhagic compensatory and immunological mechanisms, respectively.

There are many studies which show the interactions of mycoplasmas with various cell types *in vitro* and *in vivo* and their implication as causative factors, co factors or opportunistic agents in human diseases (Baseman 1997, Nicolson 1999). Mycoplasmas exhibit host and tissue specificities. As such, they are able to enter the host cells, multiply, and survive within phagocytic and non-phagocytic cells for a long period of time showing slow growth rates. They develop mechanisms of mimicry showing antigenic variability and the ability to suppress the host's immune responses (Razin 1998, Rottem 2003, Nicolson 1999). As a result, mycoplasmal infections have a chronic and persisting nature and the host is unable to suppress these infections through immune and non-immune responses (Nicolson 1999).

The presence of *M. hominis* and *M. fermentans* in synovial fluids of the human joints was reported previously, implicating the involvement of these 2 mycoplasma species in the development of human arthritis and chronic arthritic conditions including rheumatoid arthritis (Schaeverbeke 1997, Haier 1999, Nicolson 1999, Henrich 1998, Razin1998, Johnson 2000, Boesen 2001, Gilroy 2001, Yavlovich 2001, Waites 2005, Yavlovich 2006). It was reported that in cases of chronic active arthritis the lipoprotein surface of *M. hominis* Vaa antigen changes in structure and its expression, thus affecting the adhering properties of *M. hominis* and enhancing

evasion of host-mediated immunity (Henrich 1993, Razin 1998, Nicolson 1999, Boesen 2001). In animal models, experimental arthritis was induced in rabbits by *M. fermentans* after intratracheal and intrasynovial application (Rivera 2002). Furthermore, *M. fermentans* was found alone or in combination with other mycoplasma species in synovial fluids and the blood of patients with rheumatoid arthritis (Schaeverbeke 1997, Haier 1999, Nicolson 1999), suggesting that even though the underlying causes of rheumatoid arthritis remain unknown, mycoplasmas could play a role as co-factor in this desease. In experimental rat and mice models of rheumatoid arthritis, *M. arthritidis*-related superantigens compromised T-cells and resulted in autoimmune arthritis, while release of substances from the same species of the same animal model including oxygen radicals, chemotactic and aggregating substances acted on polymorphonuclear granulocytes (Kirchhoff 1989, Nicolson 1999).

Mycoplasmas activate or suppress the host's immune system and induce the production of interleukins, TNF-α, interferon-γ (INF-γ) and other inflammatory mediators (McGarrity 1985, Muhlradt 1991, Drexler 2000, Rottem 2003). Earlier studies showed that they affect macrophages, lymphocytes and the balance between the Th1 and Th2 populations of CD4+ T-cells, thus influencing the direction of the subsequent effector phases of the immune response, as well as increasing natural killer cell activity (Rottem 1998, Razin 1998, Rottem 2003). Mycoplasma-infected fibroblast cells were also shown to produce IL-13. IL-13 was shown to be produced by CD4+, CD8+ cells, neutrophils, and other non-immune cells, and has a down-regulating activity on macrophages. Moreover, IL-13 stimulates B-cells for IgG1 and IgE production. As a result, down-regulating cytokines influence T-cell proliferation and the balance between CD4+ Th1 and Th2 phenotypes (Zurita-Salinas 1996, Razin, 1998). As previously described, *M. fermentans* suppressed the interferon-γ (INF-γ) expression of major histocompatibility complex (MHC) class II molecules on macrophages and resulted in impaired antigen presentation to helper T-cells in an animal model (Frisch 1996). Additionally, *M. fermentans* induced apoptosis of T-cells initiated by concanavalin A (Shibata 1997). Furthermore, the mycoplasmal lipoprotein spiralin stimulated *in vitro* proliferation of human peripheral blood mononuclear cells and murine splenocytes, resulting in the production of proinflammatory cytokines (Brenner 1997), whereas the MALP-2 lipoprotein of *M. fermentans* stimulated *in vitro* and *in vivo* the production of chemokines and induced inflammatory effect and

endotoxin cross tolerance in mouse models (Urbaschek 1987, Deiters 1999, Luhrmann 2002, Deiters 2003). As a result of mycoplasmal properties and their ability to invade the cells, infections showed persistence and mycoplasmas are found in high incidences in the blood and tissues of patients with chronic illnesses, resulting in various clinical conditions (Nicolson 1999).

In our study, the clinical signs of the affected chimeras indicate a chronic inflammatory situation. The immunological analyses revealed differences in the proportion of certain T-cells subsets especially regulatory T-cells, NK-T and gdTCR cells, differences in the subsets of CD8a+ cells, increased CD44+ expression in the non T-cell compartment and increased immunoglobulin levels, which are in agreement with several of the above-mentioned studies. The increased levels of anti-DNA Abs and rheumatoid factor in chimeras produced of mycoplasma-infected mES cells suggest activated, however, not as yet identified autoimmune mechanisms. Interestingly, the immunological data of the clinically healthy and affected chimeras produced with mycoplasma-infected mES cells revealed no significant differences in the clinically-affected chimeras, except for an increased IgG1 level. Different levels of Ig classes and IgG isotypes could be due to genetic variation of various inbred mouse strains or due to different activated status of the immune system (Sant'Anna 1985). The profile of the subclasses reflects the general direction of T helper cell differentiation. Th2-directed responses correlate with higher IgG1 levels, whereas Th1 bias towards IgG2a (Stevens 1988, Adler 2007). The levels of immunoglobulins of the clinically affected chimeras were generally increased compared to the other groups, suggesting a higher infection status, which could be responsible for the development of the clinical signs. No mycoplasmas were detected in nasal secretion and plasma samples of 8 week-old affected chimeras using the agar culture and the Mycosensor^{TM} QPCR. However, further efforts to detect mycoplasmas in the chimeric mice are continuing. Based on the present data, osteoarthropathia, nasal discharge and cachexia of the affected chimeras could be due either to the presence of mycoplasmas in the organism or to autoimmune reactions. Further investigations into the etiopathological cause of the nasal discharge are part of the current work.

Since mES cells were infected with 3 different mycoplasma species the present results cannot be ascertained to a particular species. To elucidate these effects, mES cells should be infected with a single species. At our laboratory, attempts to work with the E14 ES cell line, which is permanently infected with *M.*

hominis, resulted in a lower number of chimeras with a GLT of 40% in comparison to E14 Tg2a mycoplasma-free ES cells which show a GLT of 80%. However, the chimeras obtained were normal, in contrast to some obtained in the present study. This observation, the knowledge based on the effects of a *M. fermentans* infection on humans and the fact that *M. orale* is pathogenic only under certain conditions implicate that *M. fermentans* plays a major role in the phenotype of the chimeras obtained in the present study.

In view of the increasing use of mES cell lines in biomedical research for the production of transgenic und knock-out mice and the present data, it is of paramount importance to screen mES cells for mycoplasmas prior to their use. In this way, one can contribute to animal welfare since the number of mice used would be reduced and misinterpretation of experimental results and phenotypic characteristics is avoided.

6. Summary

In biomedical research, murine ES (mES) cells are used for the production of transgenic and knock-out mice which serve as animal models for human and animal diseases. The extensive exchange of mES cells between laboratories increases the risk of contaminations since mES cell lines are often used without determination of their microbiological status. Mycoplasmas can influence various cell parameters and ultimately, the interpretation of experimental data. In cell cultures, mycoplasma contaminations are 15 to 35% worldwide with extreme incidences of 65 to 80%. In this study, the objective was to determine the effects of mycoplasmas on mES cells during *in vitro* culture, the germ line transmission (GLT) of mES cells, and the resulting chimeric progeny. For reliable detection of mycoplasmas in mES cell cultures, 4 detections methods were evaluated concurrently with regards to the limit of detection of the human species *M. hominis, M. fermentans,* and *M. orale* in cell culture supernatants and in cells. The mES cell line TBV2 (129/SvPas) was inoculated at passage P13 with the above-mentioned mycoplasma species and cultured over 20 passages, whereby the growth rate, viability, the karyotype, and the differentiation status of the mES cells were determined. Furthermore, the GLT was examined after injection of mES cells at passages P13+5, P13+10, P13+15, and P13+20 into Crl:CD1 (Icr) blastocysts, transfer of the embryos in Crl:CD1 (Icr) pseudopregnant recipients and breeding of the resulting chimeras with C57BL/6 mice.

The data show that each method was able to detect mycoplasmas while agar culture and the MycoSensor™ QPCR were the most sensitive methods compared to the in-house established PCR and the MycoAlert™ assay. mES cells became infected with all three mycoplasma species, showing reduced growth rate and viability compared to control mES cells. Spectral karyotypic analysis of both control and infected mES cells revealed numeric aberrations with trisomies 1, 6, and 11. In addition, mycoplasma-infected mES cells showed a number of non-clonal chromosomal aberrations, which increased with the duration of infection. The pluripotential status of the control mES cells, determined by double staining with the differentiation markers anti-OCT-4 and anti-SSEA-1 and flow cytometric analysis (FACS), showed that control mES cells were highly pluripotent, expressing both markers at passages P13+6 (84.8%), P13+11 (86.7%), P13+16 (87.8%), and P13+21 (78%). Mycoplasma-infected mES cells were less pluripotent at passage P13+6 (46.3%), where the infection was strongest, and

pluripotency at passages P13+11, P13+16, and P13+21 was 83%, 78%, and 69.4%, respectively. As a result, the GLT of mycoplasma-infected mES cells at passage P13+5 was most affected (50%) with reductions in the number of litters obtained (50%) and in the numbers of pups born (22%) compared to the number of embryo transfers performed. In comparison, the control group showed 90% GLT at passage P13+5, with 100% litters and 45% pups born. Furthermore, 30 out of 73 chimeras obtained from blastocyst injection with mycoplasma-infected mES cells at passages P13+10 to P13+20 showed a phenotype with nasal discharge, osteoarthropathia, and cachexia. The same phenotype was not observed in the F1 generation after breeding of chimeras with C57BL/6 mice. For chimeras generated with mycoplasma-infected mES cells, immunological analysis of murine plasma by the bead array based assay (bioplex), ELISA and flow cytometric analysis (FACS) showed reduced proportions of T-cells and increased levels of IgG1, IgG2a and IgM as well as increased levels of anti-DNA and rheumatoid antibodies, suggesting the presence of infection and/or an autoimmune situation.

In view of the increasing use of mES cell lines in biomedical research for the production of transgenic und knock-out mice and the present data, it is of paramount importance to screen mES cells for mycoplasmas prior to their use. In this way, one can contribute to animal welfare since the number of mice used would be reduced and misinterpretation of experimental results and phenotypic characteristics are avoided.

7. Zusammenfassung

Mykoplasmakontamination von murinen embryonalen Stammzellen: Nachweissensitivität, Einfluss auf Zytogenetik, Keimbahntransmission und Chimärennachkommen

Murine embryonale Stammzellen (mES Zellen) werden in der biomedizinischen Forschung zur Herstellung von transgenen und knock-out Mäusen, die als Tiermodelle für Pathogenesestudien dienen, eingesetzt. Mit zunehmendem Austausch von mES Zellen zwischen Laboratorien steigt auch die Gefahr ihrer mikrobiellen Kontamination, insbesondere mit Mykoplasmen. Dadurch werden verschiedene Zellparameter verändert und die Interpretation von experimentellen Daten beeinflusst. Weltweit kommen Mykoplasmen in 15 bis 35% der Zellkulturen vor und erreichen eine Prävalenz von mitunter bis zu 80%.

Ziel der vorliegenden Arbeit war es, die Einflüsse von Mykoplasmen auf mES Zellen bezüglich zellbiologischer Parameter, auf die Keimbahntransmission und die daraus resultierenden Chimären zu untersuchen. Dazu wurden mES Zellen der Linie TBV2 (129/SvPas) bei Passage P13 mit den bei Zellkulturen prävalenten Stämmen *M. hominis, M. fermentans* und *M. orale* inokuliert und über 20 Passagen kultiviert.

Von den untersuchten Nachweismethoden waren die Agarkultur und die MycoSensor™ QPCR die sensitivsten, gefolgt von einer selbst dafür etablierten PCR und dem MycoAlert™ Assay. Mycoplasma-infizierte mES Zellen zeigten eine niedrigere Wachstumsrate und geringere Vitalität im Vergleich zu Kontrollzellen. Die Karyotypisierung von nicht-infizierten und infizierten mES Zellen zeigte eine Reihe von chromosomalen Aberrationen einschließlich Trisomie 1, 6 und 11. Bei den infizierten mES Zellen wurden außerdem mehrere non-klonale chromosomale Aberrationen festgestellt, die mit der Infektionsdauer zunahmen. Die durch eine Doppelmarkierung der Differenzierungsmarker OCT-4 und SSEA-1 und mittels FACS ermittelte Pluripotenz nicht-infizierter mES Zellen lag zwischen Passage P13+6 und P13+21 bei 84.8 - 78%. Bei Mykoplasma-infizierten mES Zellen lag sie bei Passage P13+6 mit der stärksten Infektion bei 46.3%, während sie bei Passage P13+11, P13+16 bzw. P13+21 jeweils 83%, 78% bzw. 69.4% betrug. Die Keimbahntransmission Mykoplasma-infizierter mES Zellen lag bei Passage P13+5 bei 50% mit 22% Nachkommen, bei den Kontrollen lag sie bei 90%, mit 100% Würfen und 45% Nachkommen. In 30 von 73 Chimären der F1

Generation, die durch die Blastozysteninjektion mit Mykoplasma-infizierten mES Zellen von Passage P13+10 bis P13+20 entstanden, wurden Nasenausfluss, Osteoarthropathie, und Kachexie festgestellt. Bei Rückverpaarungen der Chimäre mit C57BL/6 Mäusen wurden keine Veränderungen festgestellt. F1 Chimären, die aus Mykoplasma-infizierten mES Zellen entstanden, zeigten eine Reduktion der Proportion von T-Zellen und eine Erhöhung der IgG1-, IgG2a-, IgM-, anti-DNA- und rheumatypischen Antikörper-Spiegel. Diese Ergebnisse deuten auf die Anwesenheit einer Infektion und/oder einen autoimmunen Zustand hin.

Ausgehend vom zunehmenden Einsatz von mES Zellen in der biomedizinischen Forschung bei der Herstellung von transgenen und knock-out Mäusen und den vorliegenden Daten ist es unabdingbar, mES Zellen vor ihrer Verwendung auf eine Kontamination mit Mykoplasmen zu überprüfen und kontaminierte Zellen zu verwerfen. Dadurch kann der Bedarf an Versuchstieren reduziert und langfristig ein Beitrag zum Tierschutz geleistet werden. Schließlich vermeiden derartige Kontrollmaßnahmen falsche Interpretationen von experimentellen und Phänotypisierungsergebnissen.

8. MATERIALS

The following antibodies, chemicals, equipment, enzymes, kits, materials, hormones, anaesthetics, and reagents were used for this project.

Antibodies

Alexa Fluor 700/ rat anti-mouse CD45	BioLegend, San Diego, CA, USA
Alexa fluor 610/ rat anti-mouse CD45	BioLegend, San Diego, CA, USA
Anti-BiotinCy-5	Rockland, Biomol, Hamburg, Germany
Anti-Biotin Cy-5.5	Rockland, Biomol, Hamburg, Germany
Anti-digoxigenin	Roche Diagnostics, Mannheim, Germany
Anti-DNA Abs (LPR control)	Jackson Labs, Maine, USA
Anti-mouse CD16/CD32	Pharmingen, BD, Heidelberg, Germany
Anti-mouse Polyvalent Immunoglobulins-AP	Biozol Diagnostica GmbH, Eching, Germany
Anti-Oct 3/4	R and D Systems Inc., Minneapolis, USA
Anti-SSEA-1	R and D Systems Inc., Minneapolis, USA
APC-Alexa 750/ rat anti-mouse B220	Caltag Laboratories GmbH, Hamburg, German
APC-Alexa 750/ rat anti-mouse CD8a	Caltag Laboratories GmbH, Hamburg, Germany
APC rat anti-mouse CD5	Pharmingen, BD, Heidelberg, Germany
DNA (LPR-control)	Jackson Labs, Maine, USA
FITC rat anti-mouse CD44	Pharmingen, BD, Heidelberg, Germany
FITC rat anti-mouse IgD	Pharmingen, BD, Heidelberg, Germany
Mouse IgM APC	Pharmingen, BD, Heidelberg, Germany
PE anti-mouse CD4 (L3T4)	Pharmingen, BD, Heidelberg, Germany
PB rat anti-mouse CD3	eBioscience, San Diego, CA, USA
PB rat anti-mouse CD11b	Caltag Laboratories GmbH, Hamburg, German
PE-Alexa Fluor 610/ rat anti-mouse CD4	Caltag Laboratories GmbH, Hamburg, German
PE armenian hamster/	Pharmingen, BD, Heidelberg, Germany

anti-mouse γδTCR	
PerCpCy55 rat anti-mouse CD4	Pharmingen, BD, Heidelberg, Germany
PE-Cy7 rat anti-mouse CD62L	eBioscience, San Diego, CA, USA
PE-Cy7 rat anti-mouse CD19	Pharmingen, BD, Heidelberg, Germany
PE rat anti-mouse NK-T/NK	Pharmingen, BD, Heidelberg, Germany
RH factor (LPR control)	Dr Bruno Lukow, LMU, Germany

Chemicals

Acetic acid CH_3COOH	Merck, Darmstadt, Germany
Acid violet	Merck, Darmstadt, Germany
Chloral hydrate	Merck, Darmstadt, Germany
Citric acid	Merck, Darmstadt, Germany
Colcemid 10µg/ml	Roche Diagnostic GmbH, Mannheim, Germany
DAPI 1mg/ml	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
EMA (500 mg/ml)	Mol. Bioprobes, Invitrogen™, Karlsruhe, Germany
Eosin	Medite, Burgdorf, Germany
Ethanol	Merck, Darmstadt, Germany
Ethidium bromide	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
$FeCl_3$	Merck, Darmstadt, Germany
Formamide	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Formalin	Sigma-Aldrich Chemie GmbH, Steinhein, Germany
HCl	Merck, Darmstadt, Germany
$KAl(SO_4)\ 12H_2O$	Merck, Darmstadt, Germany
KCl	Neolab GmbH, Heidelberg, Germany
KCl	Merck, Darmstadt, Germany
KH_2PO_4	Merck, Darmstadt, Germany
Methanol CH_3OH	Merck, Darmstadt, Germany
$MgCl_2$	Merck, Darmstadt, Germany
$MgCl_2$ (50 mM)	Invitrogen™, Carlsbad, CA, USA
$MgCl_26H_2O$	Sigma-Aldrich Chemie GmbH, Steinhein, Germany
$MgSO_4$	Merck, Darmstadt, Germany
NaCl	Merck, Darmstadt, Germany
NaH_2PO_4	Merck, Darmstadt, Germany
Na_2HPO_4	Merck, Darmstadt, Germany

$NaJO_3$	Merck, Darmstadt, Germany
NaN_3	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
NaOH	Merck, Darmstadt, Germany
NH_4Cl	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Nitric acid	Merck, Darmstadt Germany
Nonidet P40	Fluka, Sigma-Aldrich Chemie GmbH Germany
Paraffin w/o DMSO	Merck, Darmstadt, Germany
PI (2 mg/ml)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Picric acid	Merck, Darmstadt Germany
Phenol Red 1%	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Sodium azide	Merck, Darmstadt, Germany
Sodium citrate	Merck, Darmstadt, Germany
Tris-HCl	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Trypan blue dye	Biochrom AG, Berlin, Germany
Xylol	Merck, Darmstadt, Germany

Equipment

Applied Biosystem 7500	
Real-time PCR System	Applied Biosystem, Singapore
Biometra Thermocycler	Biometra, Germany
Bio-Plex reader	Biorad, Hercules, CA, USA
Centrifuge	Eppendorf, Hamburg, Germany
Centrifuge	Hettich Universal 30 F, Tuttingen, Germany
Centrifuge 5415 D	Eppendorf, Hamburg, Germany
Eppendorf centrifuge 5804	Eppendorf, Hamburg, Germany
FACS LSR II	BD, San Diego, USA
Heated block HLC	HBT 130, Bovenden, Germany
Heraeus Megafuge 2.0	Hereaus GmbH, Hanau, Germany
Incubator	Queue Systems Inc., Ashville, USA
Incubator	Binder, Tuttingen, Germany
Luminometer Microlumat LB 96P	Berthold Technologies, Bad Wildbad, Germany
Microinjection manipulator	Leitz DM IRB, Leica, Bensheim, Germany
Microm 355	Walldorf, Germany
Plate shaker	Heidolph, Schwabach, Germany

Shaker, Multifuge 3 S-R	Hereaus, Hanau, Germany
Stereoscopic microscope	Leica, Bensheim, Germany
Thermomixer compact	Eppendorf, Hamburg, Germany
Vacuum Infiltrations Processsor	Sakura Finetek GmbH, Heppenheim, Germany
Vacuum manifold	Biorad, Hercules, CA, Germany
TECAN SUNRISE™ reader	TECAN GmbH, Crailsheim, Germany
TECAN washer	TECAN GmbH, Crailsheim, Germany
Tomtec-Robot Quadra 3	Biozym Scientific GmbH, Oldendorf, Germany

Enzymes

Platinum Taq DNA Polymerase	Invitrogen™, Carlsbad, CA, USA
Poly-L-Lysine	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
RNase A	Fermentas, St. Leon-Rot, Germany
Trypsin/EDTA	Biochrom AG, Berlin, Germany
Xba I, Hpa II, and Hae III	Invitrogen™, Carlsbad, CA, USA

Kits

Bio Plex assay kit	Biorad, Hercules, CA, USA
Foxp3 Staining Buffer set	eBioscience, San Diego, CA, USA
MycoSensor™ QPCR Assay kit	Stratagene, La Jolla, CA, USA
MycoAlert™ Assay Control Set	Lonza Verviers, Liege, Belgium
MycoAlert™ assay kit	Lonza Verviers, Liege, Belgium
MycoSensor™ QPCR Assay kit	Stratagene, La Jolla, CA, USA
Wizard Clean-Up System	Promega, Mannheim, Germany

Materials

Auto clips 9 mm	Clay Adams, BD Company, NJ., USA
Cryo tube vials	Nunc™, Roskilde, Denmark
Coverslips	Menzel, Braunschweig, Germany
Coverslips	IDL GmbH, Nidderau, Germany
Cryo tube vials	Nunc™, Roskilde, Denmark
Culture dishes CELLSTAR®	Greiner Bio-One GmbH, Frickenhausen, Germany
Dilution plates	Greiner Bio-One GmbH, Frickenhausen, Germany
ELISA plates	Nunc™, Roskilde, Denmark

Eppendorf tubes	Eppendorf, Hamburg, Germany
Falcon tubes	Becton Dickinson and Company, N.J., USA
Falcon Petri dishes	BD and Company, Sparks, USA
Injection pipettes	BioMedical Instruments, Zöllnitz, Germany
Haemocytometer	Hycor Biomedical Inc., Kassel, Germany
IVCs	VentiRacks™; BioZone, Margate, UK
Makrolon® cages	
Millex™ Filter 0.22 µm	Millipore, Carrigtwohill, Co. Cork, Ireland
Mineral oil	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Needle, 27-gauge	Braun, Melsungen, Germany
Petri dishes	Nunc™, Roskilde, Denmark
Petri dishes	Nunclon™ Surface, Nunc™, Roskilde, Denmark
Rubber cement	Fixogum, Marabu, Tamm, Germany
Standardized mouse diet1314	Altromin, Lage, Germany
Super frost object slide	Neolab Migge GmbH, Munich, Germany
Super Frost plus slides	Roth, Karlsruhe, Germany
Tubes	Sarstedt, Mumbrecht, Germany
Vectashield mounting medium	Vector Laboratories, Burlingame, CA, USA
Vicryl 4-0	Johnson and Johnson Intl., Woluwe, Belgium
Wood shavings	Altromin, Lage, Germany
96-well white microplate	Nunc™, Roskilde, Denmark
96-well white microplate	Applied Biosystems, Singapore

Hormones and anaesthetics

Equine chorionic gonadotropin	Intergonan® 1000 I.U, Intervet, Germany
Human chorionic gonadotropin	Ovogest® 1500 I.U, Intervet, Germany
Ketavet® 100 mg/ml	Pfizer, Karlsruhe, Germany
Sedaxylan 20 mg/ml	WDT, Garbsen, Germany
Vetranquil 1%	Ceva GmbH, Düsseldorf, Germany
Vetalgin® 500 mg/ml	Intervet GmbH, Unterschleißheim, Germany

Reagents

Agar No.1	Oxoid, Hampshire, England

Agarose	Biozym Scientific GmbH, Oldendorf, Germany
Bacto brain heart infusion	Oxoid, Hampshire, England
Blotting Blocker Dry Milk	Biorad, Hercules, CA, USA
BSA	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Calf thymus DNA	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
DEAE-Dextran 1%	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Difco™ Bacto PPLO-Agar	BD and Company, Sparks, USA
Difco™ Bacto PPLO-Broth	BD and Company, Sparks, USA
DMEM (1x)	Gibco, Invitrogen™, Paisley, UK
DMEM	Biochrom, Berlin, Germany
DMSO 99.5% GC	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
DNase I (100 mg)	Roche Diagnostics, Mannheim, Germany
DNA solution	Takara BIO Inc., Japan
dNTP	Fermentas, St- Leon-Rot, Germany
EDTA	Roth, Karsruhe, Germany
Ethanol	Merck, Darmstadt, Germany
Eukitt mounting medium	Kindler, Freiburg, Germany
Fetal calf serum	Seromed, Biochrom, Berlin, Germany
Fetal calf serum for ES cells	PAN™, BIOTECH GmbH, Aidenbach, Germany
Glutamine 200 mM	Biochrom, Berlin, Germany
HBSS	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Heat-inactivated horse serum	Gibco, Invitrogen™, Auckland, New Zealand
Heat-inactivated porcine serum	Gibco, Invitrogen™, Auckland, New Zealand
Heparin (25.000 I.U)	Roche, Mannheim, Germany
Inactivated fetal calf serum	Seromed, Biochrom, Berlin, Germany
LIF	Chemicon International Inc., CA, USA
Loading buffer	Fermentas, St. Leon-Rot, Germany
Mitomycin C (1 mg/ml)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Mouse Spectral Karyotyping	Applied Spectral Imaging, Edingen, Germany
M2 Medium	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Non-essential amino acids	Biochrom, Berlin, Germany
PBS	Biochrom, Berlin, Germany
PBS	Gibco, Invitrogen™, Auckland, New Zeeland
PCR buffer (10 x)	Invitrogen™, Carlsbad, CA, USA

Penicillin-Streptomycin	Gibco, Invitrogen™, Paisley, UK
React® 2 and 8 buffer	Invitrogen™, Carlsbad, CA, USA
p-Nitrophenyl Phosphate tabs	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Tween® 20	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Sodium Chloride 0.9%	Fresenius Kabi GmbH, Homburg v. d. H., Germany
Yeast extracts 25%w/v	Fluka, Sigma-Aldrich Chemie GmbH, Germany
β-Mercaptoethanol	Gibco, Invitrogen™, Paisley, UK

9. List of abbreviations

Abs	Antibodies
amp	amplification
AP	alkaline phosphatase
APC	allophycocyanin
ATP	adenosine triphosphate
BSA	bovine serum albumin
CFU	colony forming unit
Cy	cyanine
d	day
d	deletion
DAPI	diamidinophenylindole
der	derivation
DEAE	diethylaminoethyldextran
DIA	differentiation inhibitoring factor
DNA	deoxyribonucleic acid
dNTP	deoxynucleotides
D-MEM	Dulbeccos's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
dpc	days post coitus
eCG	equine Chorionic Gonadotropin
EC	embryonic carcinoma
EDTA	ethylene diamine tetraacetate
ELISA	Enzyme Linked Immunobsorbent Assay
EMA	ethidium monoazide
EMFI	embryonic murine fibroblasts
ESM	embryonic stem cell medium
FACS	fluoresence activated cell sorting
FCS	fetal calf serum
$FeCl_3$	iron chloride
FITC	fluorescein isothiocyanate
g	gram
G	gauge
G+C	guanine+cytosine

GLT	germ line transmission
GPI	glucosephosphate isomerase
h	hour
HBSS	hank's balanced salt solution
hCG	human chorionic gonadotropin
HIV	Human immunodeficiency virus
ICM	inner cell mass
IL	Interleukin
ins	insertion
IU	international unit
i.p	intraperitoneal
$KAl(SO_4)$	potassium aluminium sulfate
kb	kilo base
KH_2PO_4	mono potassium phosphate
KCl	potassium chloride
l	liter
LIF	leukemia inhibitory factor
LOD	limit of detection
MALP	macrophage activating lpoprotein
µg	microgram
µl	microliter
ml	mililiter
mm	milimeter
mM	milimole
M2	wash medium for blastocysts
MCP	macrophage chemoattractant lipoprotein
MEFS	murine embryonic fibroblasts
mES	murine embryonic stem cells
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
MIP	macrophage inflammatory protein
MMC	mitomycin C
MRA	mycoplasmal removal agent
mRNA	messenger ribonucleic acid

NaCl	sodium chloride
Na_2HPO_4	disodium hydrogen phosphate
NaH_2PO4	monosodium phosphate
$NaJO_3$	sodium iodate
NaN_3	sodium azide
NaOH	sodium hydroxide
n.d	not done
NH_4CH	ammonium chloride
Nr.	number
Oct-4	Octamer-binding protein-4
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PerCp	peridinin chlorophyll protein
PGCs	primordial germ cells
PE	phycoerythrine
PI	propidium iodide
PN	phosphate nonidet
PNM	phosphate nonidet with dry milk
POU	pit (pituitary), oct (octamer), unc
PPLO	pleuropneumonia like organisms
QPCR	quantitative polymerase chain reaction
RH	rheumatoid
RNA	ribonucleic acid
s.c	subcutaneously
SSC	standard saline citrate
SSEA	stage specific embryonic antigen
SKY	spectral karyotyping
t	translocation
ter	terminal end of chromatid
TNF-α	tumor necrosis factor-α
T_m	melting temperature
Tris-HCl	hydroxymethyl aminomethane hydrochloride
Vaa	variable adherence associated

10. References

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11. List of Figures

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