Immortalization and proliferation of adult canine Schwann cells and olfactory ensheathing cells and their infection with canine distemper virus

Somporn Techangamsuwan

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Immortalization and proliferation of adult canine Schwann cells and olfactory ensheathing cells and their infection with canine distemper virus

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Hannover, Germany 2009
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To my family
“For whatever I am and will become,
I owe it to my mother.”

...Abraham Lincoln...
Parts of the thesis have already been published or communicated:

Publications:


Oral presentations:


Poster presentation:

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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2’-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy deoxyribonucleic acid</td>
</tr>
<tr>
<td>CDV</td>
<td>canine distemper virus</td>
</tr>
<tr>
<td>CDV-2544</td>
<td>canine distemper virus strain 2544</td>
</tr>
<tr>
<td>CDV-5804PeGFP</td>
<td>canine distemper virus strain 5804P expressing green fluorescent protein</td>
</tr>
<tr>
<td>CDV-A75-17</td>
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<td>canine distemper virus strain Ondersteoport</td>
</tr>
<tr>
<td>CDV-OndeGFP</td>
<td>canine distemper virus strain Ondersteoport expressing green fluorescent protein</td>
</tr>
<tr>
<td>CDV-R252</td>
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</tr>
<tr>
<td>CDV-RO</td>
<td>canine distemper virus strain Rockborn</td>
</tr>
<tr>
<td>CDV-SH</td>
<td>canine distemper virus strain Snyder Hill</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CPD</td>
<td>cumulative population doubling</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>dibutyryl cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>DHH</td>
<td>desert hedgehog</td>
</tr>
<tr>
<td>DL</td>
<td>demyelinating leukoencephalitis</td>
</tr>
<tr>
<td>DME</td>
<td>Dulbecco’s modified Eagle</td>
</tr>
<tr>
<td>DMV</td>
<td>dolphin morbillivirus</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpi</td>
<td>day post infection</td>
</tr>
<tr>
<td>DT</td>
<td>doubling time</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental allergic (autoimmune) encephalomyelitis</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunoadsorbent assay</td>
</tr>
<tr>
<td>EpR</td>
<td>epithelial cell receptor</td>
</tr>
<tr>
<td>F</td>
<td>fusion (protein)</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FGF-2</td>
<td>fibroblast growth factor-2</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate-dehydrogenase</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>H</td>
<td>hemagglutinin (protein)</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HRG-1β</td>
<td>heregulin-1β</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>L</td>
<td>large (protein)</td>
</tr>
<tr>
<td>M</td>
<td>matrix (protein)</td>
</tr>
<tr>
<td>MACS</td>
<td>magnet-activated cell separation</td>
</tr>
<tr>
<td>MALT</td>
<td>mucosa-associated lymphatic tissues</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>MCP</td>
<td>membrane cofactor protein</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>MV</td>
<td>measles virus</td>
</tr>
<tr>
<td>N</td>
<td>nucleocapsid (protein)</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>NRG1-III</td>
<td>neuregulin-1 type III</td>
</tr>
<tr>
<td>NT3</td>
<td>neurotrophin 3</td>
</tr>
<tr>
<td>OEC</td>
<td>olfactory ensheathing cell</td>
</tr>
<tr>
<td>ORN</td>
<td>olfactory receptor neuron</td>
</tr>
<tr>
<td>P</td>
<td>phospho-(protein)</td>
</tr>
<tr>
<td>P₀</td>
<td>protein zero</td>
</tr>
<tr>
<td>p75&lt;sup&gt;NTR&lt;/sup&gt;</td>
<td>p75 neurotrophin receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>PBS-triton-x100</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDV</td>
<td>phocine distemper virus</td>
</tr>
<tr>
<td>PLL</td>
<td>poly-L-lysine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PLP</td>
<td>proteolipid protein</td>
</tr>
<tr>
<td>PMP-22</td>
<td>peripheral myelin protein-22</td>
</tr>
<tr>
<td>PMV</td>
<td>porpoise morbillivirus</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PPRV</td>
<td>peste des petits ruminants virus</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPV</td>
<td>rinderpest virus</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SCBG</td>
<td>Schwann cell-like brain glia</td>
</tr>
<tr>
<td>SLAM</td>
<td>signaling lymphocyte activation molecule</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single strand ribonucleic acid</td>
</tr>
<tr>
<td>TCID50</td>
<td>tissue culture infectious dose 50</td>
</tr>
<tr>
<td>TERT</td>
<td>telomerase reverse transcriptase</td>
</tr>
<tr>
<td>TMEV</td>
<td>Theiler’s murine encephalomyelitis virus</td>
</tr>
<tr>
<td>TR</td>
<td>telomeric ribonucleic acid</td>
</tr>
<tr>
<td>TRAP</td>
<td>telomeric repeat amplification protocol</td>
</tr>
<tr>
<td>TrkC</td>
<td>tyrosine kinase receptor C</td>
</tr>
</tbody>
</table>
Chapter 1

General introduction
1.1 Neuroglia

The nervous system is the most complicated and important part of the body and controls all communications within the body. It is the center of mental activity, including thought, learning and memory. The nervous system consists of two divisions designated the central nervous system (CNS) and the peripheral nervous system (PNS). The brain and the spinal cord make up the CNS, while the cranial and spinal nerves along with their associated ganglia form the PNS. The CNS consists of a variety of cell populations, including neurons, glia, ependymal cells, endothelial cells and pericytes of blood vessels and meningeal cells. The PNS is divided into the sensorimotor, autonomic and enteric division (Zachary, 2007).

Besides neurons, neuroglia or glia as the major cell component in the mammalian CNS form the brain and outnumber the neurons by about 10 to 1 (Liu and Rao, 2004). Histologically, glial cells are classified into two categories; macroglia and microglia. Macroglia which is derived from the neuroectoderm consists of astrocytes, oligodendrocytes, ependymal cells and radial glia, while microglia as part of the monocyte-macrophage system emanates from the mesoderm (hematopoietic precursors from bone marrow) and makes up approximately 20% of the total glial cell population of the brain (Lawson et al., 1990). Schwann cells, satellite cells and enteric glial cells which are derived from the neural crest comprise the PNS glia and serve a multitude of functions (see table 1; Jessen, 2004; Zachary, 2007).
Table 1: Localization, function and properties of central nervous system (CNS) and peripheral nervous system (PNS) glia (modified after Zachary, 2007)

<table>
<thead>
<tr>
<th>Localization</th>
<th>Glia</th>
<th>Functions / Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS</td>
<td>Astrocyte</td>
<td>• Support functionally-related axons of the CNS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Formation of protective barriers (glia limitans, blood-brain barrier)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Proliferation in response to injury (glial scar formation)</td>
</tr>
<tr>
<td>CNS</td>
<td>Oligodendrocyte</td>
<td>• Myelination of CNS axons</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Maintenance of CNS homeostasis</td>
</tr>
<tr>
<td>CNS</td>
<td>Ependymal cell</td>
<td>• Secretion and circulation of cerebrospinal fluid</td>
</tr>
<tr>
<td>CNS</td>
<td>Radial glia</td>
<td>• Generation of neural progenitors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Scaffolding of migrating neurons during neural development</td>
</tr>
<tr>
<td>PNS</td>
<td>Schwann cell</td>
<td>• Myelination and ensheathment of PNS axons</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Phagocytosis of myelin debris following nervous system injury</td>
</tr>
<tr>
<td>PNS</td>
<td>Satellite cell</td>
<td>• Lining of the exterior surface of PNS neurons and regulation of the external chemical environment</td>
</tr>
<tr>
<td>PNS</td>
<td>Enteric glia</td>
<td>• Ensheathment of axons in the gut</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Regulation of barrier and neuronal functions in the gut</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Neunlist et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Putative roles during gastrointestinal tract inflammation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Sharkey et al., 2004)</td>
</tr>
</tbody>
</table>

The myelin sheath provided by Schwann cells and oligodendrocytes of the PNS and CNS, respectively, is crucial for the rapid conduction of action potentials along the axon. Although the principal organization of the central and peripheral myelin sheath is quite similar there are a number of specific characteristics. At the molecular level, there is specific expression of myelin proteins, such as protein zero (P₀) and proteolipid protein (PLP), which are restricted to the PNS and CNS, respectively. At the morphological level, there are differences regarding the quantitative ratio between axons and glial cells. Schwann cells in the periphery are associated with single axons, whereas oligodendrocytes of the CNS myelinate multiple axons (Quarles, 2005).
1.1.1 Schwann cells

Schwann cells – named after Theodore Schwann, a German physiologist (1810-1882) – are the major glial cell type in the PNS (Zujovic et al., 2007). Embryonic and postnatal development of Schwann cells occur through a series of stages defined by specific gene expression profiles. Moreover, the distinct developmental stages are characterized by differential trophic requirements allowing survival and expansion (Jessen and Mirsky, 2005). During embryogenesis, neural crest cells give rise to Schwann cell precursors, which represent the first transitional state in the Schwann cell lineage. Schwann cell precursors associate with axons and initiate expression of a specific set of markers, including P0, desert hedgehog (DHH), CD9 (a tetraspan transmembrane protein) and peripheral myelin protein-22 (PMP-22). Subsequently, Schwann cell precursors develop into immature Schwann cells that acquire expression of further molecules, including glial fibrillary acidic protein (GFAP), S100 (calcium-binding protein), and O4 (lipid antigen). Immature Schwann cells do not only develop autocrine survival loops but also start formation of a basal lamina (Jessen and Mirsky, 2005). At birth, immature Schwann cells upon contact with axons differentiate into either the myelinating or non-myelinating phenotype, which are the final transitional stages and form the mature nerve trunk (Bhatheja and Field, 2006; Zujovic et al., 2007). Contrary to myelinating Schwann cells that dramatically change their gene expression and initiate synthesis of myelin constituents, the phenotypic changes involved in the establishment of non-myelinating Schwann cells are comparatively small. The majority of markers specific for immature Schwann cells are maintained during development of non-myelinating Schwann cells. However, there are several lines of evidence indicating that the development of the non-myelinating Schwann cell phenotype is also controlled by axon-derived signals (Jessen and Mirsky, 2005).

Considering the morphology and anatomical localization, Schwann cells are divided into several categories: myelinating Schwann cells, non-myelinating Schwann cells, peri-synaptic Schwann cells of the neuromuscular junction and satellite cells that cover the cell body of sensory neurons (Corfas et al., 2004; Hanani, 2005). Myelinating Schwann cells as the best characterized population gained wide attention because of the severe clinical deficits associated with their malfunction (Suter et al., 1993). Schwann cells are not only necessary for myelination but also for giving trophic support to axons helping them to maintain certain parameters, such as axon diameter. Unlike olfactory ensheathing cells (OECs, see below), Schwann cells do not appear to guide axons during the formation of the PNS. Moreover, non-myelinating Schwann cells play an important role in mediating pain sensations (Chen et al., 2003; Taveggia et al., 2005).
The composition of the peripheral myelin established by Schwann cell membranes, including lipids and proteins is different from the CNS myelin formed by oligodendrocytes. The plasma membrane of a single Schwann cell wraps around a single axon while one oligodendrocyte is associated with multiple axons. This fact is the reason why cell death of myelinating cells in the CNS is more devastating than in the PNS (Rosenbluth, 1999). The formation of the PNS myelin is a complex and dynamic process involving a series of mutual interactions between Schwann cells and neurons (Bunge et al., 1982; Bunge, 1993). After proliferation and migration along axons, Schwann cells ensheath individual axons leading to myelination which is critically determined by axonal caliber. Myelination will not be initiated when axons display a diameter of less than 1µm. The molecular signal crucial for myelination was identified recently. The amount of neuregulin-1 type III (NRG1-III) provided by the axon is proportional to the axon diameter. While large caliber axons induce wrapping of the Schwann cells around the axon, smaller axons produce less NRG1-III resulting in ensheathment but not myelination of multiple axons, which are called Remak bundles (Taveggia et al., 2005).

The family of neurotrophins and their receptors are known to play an important role during myelination. Whereas interaction of the brain-derived neurotrophic factor (BDNF) with the p75 neurotrophin receptor (p75NTR) has been shown to promote myelination, neurotrophin 3 (NT3) signaling mediated by the tyrosine kinase receptor C (TrkC) is blocking this process (Chan et al., 2001; Cosgaya et al., 2002). These data are evidence for opposite actions of neurotrophin receptors on PNS myelination.

During isolation and dissociation of mature peripheral nerves containing non-myelinating and myelinating Schwann cells, both phenotypes develop into a single homogeneous cell population displaying the typically elongated, bi- to tripolar phenotype and expressing the molecular markers typical for non-myelinating Schwann cells in vivo (Jessen et al., 1990). It is well established that purified rodent Schwann cells are mitogenically quiescent in vitro and require growth factors, such as fibroblast growth factor-2 (FGF-2) or heregulin-1β (HRG-1β) in addition to reagents elevating the intracellular cAMP level, such as forskolin and dibutyryl cAMP (dbcAMP) for in vitro expansion (Levi et al., 1995; Zhang et al., 1995). Moreover, growth factor effects are modulated by addition of serum (Dong et al., 1997).

### 1.1.2 Olfactory ensheathing cells

Olfactory ensheathing cells (OECs) of the olfactory nerves and bulb are non-myelin-forming glial cells that generate fine processes communally ensheathing 50-100 olfactory neuron axons (Field et al., 2003; Raisman, 1985). In the olfactory fascicles, the OEC somata are localized to the periphery, where they are separated from overlaying olfactory nerve fibroblasts by a basal lamina (Field et al., 2003; Raisman, 1985). In addition, OECs are
involved in the formation of the glia limitans (Doucette, 1984, 1993). These properties discriminate OECs from both non-myelinating and myelinating adult Schwann cells. OECs that emerge from the olfactory placode during development are specialized glial cells that guide axonal growth of non-myelinated olfactory neurons from the olfactory mucosa via the cribriform plate to the olfactory bulb (Au and Roskams, 2003). OECs as part of the PNS and CNS are considered an intermediate glial cell type sharing expression of Schwann cell- and astrocyte-specific marker molecules (see table 2; Barber and Lindsay, 1982; Doucette, 1984; Wewetzer et al., 1997, 2005). Compared to Schwann cells, whose lineage has been characterized in detail (see above), very little is known about gene expression of developing OECs. Expression of p75NTR in neonatal OECs is restricted to a certain subpopulation (Franceschini and Barnett, 1996; Wewetzer et al., 2005). This is in striking contrast to non-myelin-forming Schwann cells at the same developmental age that express p75NTR without exception. Moreover, OECs down-regulate p75NTR during postnatal development, whereas non-myelinating Schwann cells maintain its expression (Jessen and Mirsky, 1991; Jessen et al., 1990).

Contrary to the in situ situation, where OECs and Schwann cells display distinct morphologies and gene expression, cultured OECs and Schwann cells have the same morphological phenotype and behave similarly. This is true for both the antigenic expression and the growth factor requirements. Both neonatal and mature OECs in addition to Schwann cells stably express p75NTR. Reports that identified OEC subpopulations in vitro (Franceschini and Barnett, 1996) await further confirmation. Rodent OECs and Schwann cells respond to the same growth factors, including HRG-1ß, FGF-2 and hepatocyte growth factor (HGF) (Chuah et al., 2000; Pollock et al., 1999; Ramón-Cueto et al., 1998; Wewetzer et al., 2001; Yan et al., 2001). Proliferation is also potentiated by intracellular cAMP concentration elevating substances, such as forskolin. Moreover, prolonged growth factor stimulation has been shown to induce spontaneous immortalization in both cell types (Bolin et al., 1992; Eccleston et al., 1991; Sonigra et al., 1996).
Table 2: Antigenic phenotype of rodent myelinating and non-myelinating Schwann cells, olfactory ensheathing cells and astrocytes in situ and in vitro

<table>
<thead>
<tr>
<th>Marker *</th>
<th>Myelinating Schwann cells</th>
<th>Non-myelinating Schwann cells</th>
<th>Olfactory ensheathing cells</th>
<th>Astrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in situ</td>
<td>in vitro</td>
<td>in situ</td>
<td>in vitro</td>
</tr>
<tr>
<td>P0</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MBP</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>p75NTR</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GFAP</td>
<td>-</td>
<td>+</td>
<td>+</td>
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</tr>
</tbody>
</table>

1 Myelinating Schwann cells in the presence of neurons in vitro display antigenic phenotype similar to the in situ, while cells in the absence of neurons exhibit different phenotype as shown in table.
2 p75NTR is expressed by a certain subpopulation of neonatal OECs but not by adult OECs (Wewetzer et al., 2005)
3 O4 has been shown to be expressed by neonatal olfactory neurons in situ and is associated with axonal fragments adhered to the cell surface of neonatal OECs in vitro (Wewetzer et al., 2005)

* Abbreviations: P0 = protein zero, MBP = myelin basic protein, p75NTR = p75 neurotrophin receptor, GFAP = glial fibrillary acidic protein, (-) = immunonegative, (+) = immunopositive

During the last decade, transplantation of Schwann cells and OECs has been used to foster CNS regeneration. A variety of studies has shown that both cell types are capable of promoting axonal regrowth and remyelination of CNS axons. However, since there are still only a limited number of studies available that applied both cell types in the same experimental paradigm (Wewetzer et al., 2002), it is still not clear, which cell type is more suitable for gaining optimal functional recovery. Another dilemma in Schwann cell and OEC research is the lack of cell type-specific marker molecules. So far no markers have been identified that allow selective visualization of both cell types in vitro. Thus, it is still not clear in how far OEC preparations from the olfactory bulb and olfactory mucosa are contaminated by Schwann cells, emanating from the meninges and trigeminal afferents, respectively. This fact is the reason for the ongoing controversy of whether remyelination following transplantation of OECs is in fact due to the OECs themselves or to Schwann cells contaminating the preparations (Harvey and Plant, 2006; Wewetzer and Brandes, 2006, 2007). Finally, it has to be pointed out, that the vast majority of experimental observations still refer to rodent models. Due to the lack of studies on large mammals (Jeffery et al., 2005, 2006; Radtke et al., 2004) transplantation of Schwann cells and OECs in humans are still mainly based on evidence from laboratory animals. This might be misleading, because several reports have provided evidence for pronounced species-specific properties of OECs (Krudewig et al.,
2006; Rubio et al., 2008). For that reason, Schwann cells and OECs from large mammals, such as dogs and pigs, are becoming increasingly important as alternative models to study their distinct properties.

### 1.2 Animal models for demyelinating diseases

Demyelination results from destruction of the myelin sheath in the CNS and PNS, and may be caused by genetic, autoimmune and other unknown factors as well as by infectious agents (Herrmann, 2008; Monahan et al., 2008). In humans, multiple sclerosis (MS) represents a devastating inflammatory demyelinating and neurodegenerative disease of the CNS which commonly affects young adults (Franklin and Ffrench-Constant, 2008). Although the pathomechanism of demyelination in MS has been studied for decades, the underlying processes are still poorly understood. Experimentally-induced or naturally-occurring demyelinating animal models are considered powerful tools to study the pathogenesis of MS. The prototype of an immune-mediated demyelinating model is the experimental allergic (autoimmune) encephalomyelitis (EAE), which includes the injection of spinal cord homogenates or isolated myelin proteins and peptides into experimental animals, including mice (Olitsky and Yager, 1949), rats (Paterson et al., 1970), pigs (Singer et al., 2000) and non-human primates (Rauch and Einstein, 1974). Unfortunately, this model does not reproduce all of the pathomechanisms occurring in human MS (Friese et al., 2006). To overcome this problem, virus-induced animal models mimicking the triggered pathomechanism of MS have been introduced, including coronavirus (mouse hepatitis virus; Shindler et al., 2008) and enterovirus (Theiler’s virus) infection in mice (Kumnok et al., 2008; Ulrich et al., 2006) as well as visna infection in sheep (Murrell et al., 1986) and distemper infection in dogs (Sips et al., 2007).

### 1.3 Canine distemper virus

Canine distemper is a highly contagious and immunosuppressive viral disease caused by canine distemper virus (CDV), an enveloped RNA virus of the family *Paramyxoviridae*. The susceptible host spectrum of CDV is broad and includes all families of the order Carnivora; the *Canidae* (dogs, foxes, dingos), *Felidae* (cats), *Mustelidae* (ferrets, minks, badgers, weasles) and *Proconidae* (raccoons) (Baumgärtner et al., 2003; Beineke et al., 2009; Deem et al., 2000; van Moll et al., 1995; Wohlsein et al., 2007). The most vulnerable age of dogs
for CDV infection is 3-6 months. As a result of the epitheliotropic properties of CDV, organs containing epithelium such as skin, conjunctiva, urogenital and gastrointestinal tract are frequently affected. The infected animal shows several clinical manifestations ranging from the rare peracute to the most common acute and subacute form with and without nervous signs. In addition, other manifestations include old dog encephalitis and hard pad disease (Gröne et al., 2003; Koutinas et al., 2004).

1.3.1 Viral properties

CDV is an RNA virus belonging to the genus *Morbillivirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, order *Mononegavirales* (Murphy et al., 1999). CDV is closely related to other morbilliviruses, such as rinderpest virus (RPV), peste des petits ruminants virus (PPRV) and measles virus (MV). Other morbilliviruses that particularly infect aquatic mammals have been termed phocine distemper virus (PDV), dolphin morbillivirus (DMV), and porpoise morbillivirus (PMV) (Barrett et al., 1995; Hall, 1995; Kennedy, 1998; Kreutzer et al., 2008; Müller et al., 2008). Among the members of the morbilliviruses, CDV and MV are well known for their ability to cause a chronic demyelinating disease of the CNS in their natural hosts, dogs and humans, respectively (Sips et al., 2007). Therefore, dogs infected with CDV have considered a naturally-occurring translational model to investigate the pathogenesis of virus-triggered and immune-mediated demyelination in human diseases, including multiple sclerosis (MS) (Baumgärtner and Alldinger, 2005). Apart from that, there is some speculation upon the participation of CDV and MV in the pathogenesis of human Paget’s disease of bone and MS (Mee et al., 1998; Reddy et al., 1996; Selby et al., 2006).

The CDV virion is a spherical structure of about 150-300 nm and it contains a 15,600-kb genome surrounded by a lipid envelope. CDV possesses six structural proteins; the hemagglutinin (H), the fusion (F), the nucleocapsid (N), the phospho- (P), the large (L) and the matrix (M) protein, and two other non-structural proteins termed C and V protein (Diallo, 1990; Hall et al., 1980; Lamb and Kolakofsky, 2001; Örvell, 1980). The H and F proteins are the two principle membrane-anchored glycoproteins projecting from the viral membrane. The H glycoprotein is a type II membrane protein where the amino (N)-terminus is oriented towards the cytoplasm and the carboxy (C)-terminus is extracellularly. The F protein is a type I integral membrane glycoprotein with an extracellular N-terminus. The H protein is responsible for viral attachment to the specific receptor of the target cell (virus-cell interaction) indicating that this protein represents a key determinant of CDV cell tropism and cytopathogenicity (von Messling et al., 2001). The F protein mediates membrane fusion between the viral envelope and the host cell plasma membrane (Takeda, 2008). Both H and F proteins function concomitantly to mediate membrane fusion leading to the entry and exit
of viral particles from the susceptible host cells. In a related process, the infected cells expressing attachment (H) and fusion (F) proteins on their surface can fuse with the receptor-containing neighbour cells (cell-cell spread) displaying a characteristic feature of morbillivirus infection; the presence of multinucleated syncytial cells (Baumgärtner et al., 1989; Murphy et al., 1999). Similar to the in vivo situation, different CDV strains grow efficiently in cell cultures from many species, including African green monkey kidney cells (Vero cells), marmoset lymphoid cells (B95a), Madin-Darby canine kidney (MDCK) cells, canine macrophages (DH82), primary canine macrophages and lymphocytes (Appel and Jones, 1967; Baumgärtner et al., 1987; Gröne et al., 2002; Kai et al., 1993; Puff et al., 2008; Suter et al., 2005) suggesting that CDV has a broad cell tropism in vitro as well. However, the mechanisms underlying the functional interaction of the H and F proteins during infection have remained elusive.

Following fusion of the viral and host cell membrane, the nucleocapsid associated with the N, P and L protein is delivered into the host cells and initiates intracellular replication. The core of the CDV nucleocapsid consists of a single stranded RNA (ssRNA) in antisense (negative) orientation encapsidated by the N protein which protects RNA from degradation. Associated with the N protein is the polymerase complex composed of the L protein (a RNA-dependent RNA-polymerase) and the P protein (a cofactor of L protein). This viral nucleocapsid has the capacity to transcribe mRNAs and to replicate the viral genome in infected cells without being disassembled during the infectious cycle (Lamb and Kolakofsky, 2001). The M protein is underlying the cellular or viral lipid envelope and interacts with the cytoplasmic tails of the envelope glycoproteins as well as with the N protein revealing its important role in virus assembly and budding (Takimoto and Portner, 2004). All of the aforementioned proteins except the P protein are derived from a mRNA transcription of a single corresponding gene. The P gene does not only give rise to the P protein but also to the two non-structural proteins, C and V, which are facilitating the establishment of CDV infection by interacting with the host innate immune system (von Messling et al., 2006; Wang et al., 1998). The replication site is localized in the cytoplasm of the host cells. After replication, the progeny viruses are formed and released from the host plasma membrane by budding with an inactive precursor form of the fusion protein (F₀). To become active, the F₀ has to be activated by a host cellular protease and proteolytic cleavage resulting in the generation of the two subunits F₁ and F₂. When a host cell does not contain appropriate proteases the formed virus is not infectious. The cleavage of F₀ is postulated to be a key factor that influences both infectivity and pathogenicity of paramyxoviruses causing direct cell-cell spread via fusion or persistent infection (Murphy et al., 1999; Plattet et al., 2005). Recently, it was shown that the transcriptional control of the F gene expression occurring in the region
between the CDV M and F protein is able to modulate viral virulence (Anderson and von Messling, 2008). In addition, the restricted cell-cell fusion favoring persistent infection in the CNS is mediated by complex interactions between all viral structural proteins (Wiener et al., 2007).

1.3.2 Clinical and pathological manifestations

Early clinical manifestation of CDV infected dogs includes a biphasic fever (103°F to 106°F), anorexia, depression, vomiting, diarrhea and conjunctivitis. When the disease progresses, symptoms become more serious and vary considerably depending on the virus strain and the immune status of the dog. Secondary bacterial infections always aggravate the respiratory and gastrointestinal tract manifestations. The neurological complications of a CDV infection range from ataxia (muscle incoordination), hyperesthesia (increased sensitivity to sensory stimuli such as pain or touch), myoclonus (muscle twitching or spasm), paralysis, paresis (partial paralysis), progressive mental and motor disability, seizure and optic neuritis which can lead to blindness. In addition, enamel hypoplasia and hyperkeratosis of the nose and footpads may occur (Beineke et al., 2009; Koutinas et al., 2002; Martella et al., 2008; Raw et al., 1992; Schwab et al., 2007). Some affected animals may recover due to upregulation of virus-specific neutralizing antibodies, in others the disease will progress. CDV persistence in certain tissues, such as uvea, CNS, lymphoid organs and footpads, can be observed (Appel, 1970, 1987; Gröne et al., 2003; Schobesberger et al., 2005; Zurbriggen et al., 1995a, 1995b).

Pathological findings of CDV infection can be found both in non-nervous and nervous tissues, frequently associated with characteristic cytoplasmic and intranuclear inclusion bodies (Kubo et al., 2007; Palmer et al., 1990). Respiratory lesions include serous to mucopurulent rhinitis, interstitial pneumonia, necrotizing bronchiolitis which is often complicated by secondary bacterial pneumonia (Caswell and Williams, 2007; Pandher et al., 2006). Gastrointestinal manifestations result in gastroenteritis associated with the depletion of Peyer’s patches (Decaro et al., 2004; Krakowka et al., 1985; Okita et al., 1997). A generalized lymphocytic depletion of lymphoid organs is commonly found and is associated with an impairment of the immune response (Krakowka et al., 1975; Krakowka, 1982). Skin infections display variable features, including pustular dermatitis of the thighs and ventral abdomen, and hyperkeratosis of the footpads and nasal planum (Gröne et al., 2003; Koutinas et al., 2004). In addition, CDV can cause enamel hypoplasia due to the infection of tooth buds and ameloblasts during permanent tooth development (Bittegeko et al., 1995; Dubielzig et al., 1981). Furthermore, CDV-associated bone lesions have been shown in young dogs with systemic distemper infection. Metaphyseal osteosclerosis develops due to
persistence of the primary spongiosa and atrophy as well as necrosis of osteoclasts and bone marrow cells (Baumgärtnert et al., 1995a, 1995b). Due to the similarities with morphological alterations found in human Paget’s disease of bone, paramyxovirus infection, including CDV or measles virus have been discussed as a possible etiology (Hoyland et al., 2003, Selby et al., 2006). The risk of developing human Paget’s disease of bone may be increased after previous exposure to dogs unvaccinated for canine distemper virus (Khan et al., 1996).

In canine distemper encephalitis two major forms, polioencephalitis and leukoencephalitis, can be distinguished (Krakowka and Koestner, 1976; Pearce-Kelling et al., 1990; Raw et al., 1992; Summers et al., 1984). **Polioencephalitis**, including old dog encephalitis, inclusion body encephalitis and postvaccinal encephalitis is a rare finding of CDV infection and is predominantly associated with lesions of the cortex and brain stem nuclei. The restricted viral infection found in neurons and protoplasmic astrocytes is characterized by neuronal necrosis and neuronophagia (Baumgärtnert et al., 1989; Nesseler et al., 1997, 1999). In contrary, distemper **leukoencephalitis** (DL) represents the most common CNS manifestation frequently associated with demyelination. Lesions of DL are frequently observed in the cerebellum and less frequently in the cerebral white matter and spinal cord (Bathen-Nöthen et al., 2008; Baumgärtnert et al., 1989). Additionally, demyelination is consistently found in fibre tracts adjacent the ventricles and within the cerebellar velum, cerebellar peduncles and optic tracts (Summers and Apple, 1994). DL can be categorized as acute, subacute non-inflammatory, subacute inflammatory, chronic, and sclerotic plaques (see table 3; Baumgärtnert and Alldinger, 2005; Kipar et al., 1998; Krakowka et al., 1985; Wünschmann et al., 1999).
Table 3: The histopathological classification of lesions found in demyelinating distemper leukoencephalitis (DL) (Baumgärtner and Alldinger, 2005)

<table>
<thead>
<tr>
<th>Classification</th>
<th>Lesions</th>
</tr>
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<tbody>
<tr>
<td>Acute</td>
<td>• Focal vacuolation</td>
</tr>
<tr>
<td></td>
<td>• Mild astrogliosis and microgliosis</td>
</tr>
<tr>
<td>Subacute non-inflammatory</td>
<td>• Demyelination</td>
</tr>
<tr>
<td></td>
<td>• Astrogliosis accompanied by gemistocytes and syncytial cell formation</td>
</tr>
<tr>
<td></td>
<td>• Microgliosis maybe associated with malacia and gitter cells</td>
</tr>
<tr>
<td></td>
<td>• Axonal injury (spheroid formation)</td>
</tr>
<tr>
<td>Subacute inflammatory</td>
<td>• Similar to subacute non-inflammatory lesions</td>
</tr>
<tr>
<td></td>
<td>• Mild to moderate non-suppurative perivascular cuffing and encephalitis</td>
</tr>
<tr>
<td>Chronic</td>
<td>• Similar to subacute non-inflammatory lesions</td>
</tr>
<tr>
<td></td>
<td>• Severe non-suppurative perivascular cuffing and encephalitis</td>
</tr>
<tr>
<td>Sclerotic</td>
<td>• Demyelinating areas are replaced by astrocytic scar tissue</td>
</tr>
</tbody>
</table>

1.3.3 **Pathogenesis**

CDV is generally transmitted by aerosol infection of the upper respiratory tract. The primary viral replication occurs in lymphoid tissues leading to severe continuing immunosuppression (Krakowka et al., 1980; Krakowka, 1982). Tonsils and local tissue macrophages of the respiratory epithelium represent the first line of defense and are the primary viral replication site. Subsequently, CDV spreads from the primary site via lymphatics and hematogenous routes to distant hematopoietic tissues either cell-free or cell (leukocyte/platelet)-associated during this first viremic phase (Axthelm and Krakowka, 1987; Higgins et al., 1982a, 1982b; Summers and Appel, 1987). After 3-6 days post infection (dpi) accompanied by a transient fever and lymphopenia, a generalized infection of all lymphoid tissues, including spleen, thymus, lymph nodes, bone marrow, mucosa-associated lymphatic tissues (MALT), hepatic Kupffer cells and macrophages in the lamina propria of the gut is observed (Appel, 1970, 1987; Wright et al., 1974). Around 10dpi, the second viremic phase occurs associated with a high fever and further virus dissemination to various epithelial tissues and the CNS.
This model of morbillivirus pathogenesis postulating an apical infection of the respiratory epithelium and the systemic spread depending on infection of signaling lymphocyte activation molecule (SLAM, CD150)-expressing lymphocytes has been widely accepted (de Swart et al., 2007; von Messling et al., 2006; Yanagi et al., 2006). Recently, Leonard et al. (2008) provided an alternative hypothesis for the systemic dissemination of morbillivirus by demonstrating an interaction between the virus and an unidentified epithelial cell receptor (EpR) which is located on the basolateral side of the epithelium (Leonard et al., 2008). This finding implies that the initial viral infection of the respiratory epithelium is not always a prerequisite for virus spread.

CDV gains access to the CNS by different pathways. The hematogenous route is a typical transmitting pathway carrying infected peripheral blood mononuclear cells which penetrate the blood brain barrier into the CNS. In addition, cell-free viruses are circulating in the cerebrospinal fluid and fuse with the ependymal lining of the ventricles (Frisk et al., 1999a, 1999b; Higgins et al., 1982a). The olfactory route represents an alternative pathway allowing CDV transmission and transneuronal spreading along the olfactory axons into the nervous system (Rudd et al., 2006). The latter pathway is also used by neurotropic viruses, including Herpes simplex virus, Borna disease virus, Influenza A virus and rabies virus (Aronsson et al., 2003; Esiri and Tomlinson, 1984; Lafay et al., 1991; Morales et al., 1988). So far, the investigations have mentioned the viral infection only of olfactory receptor neurons (ORNs) which are directly exposed to the pathogens in their apical dendritic nerve terminals followed by a trans-synaptical transportation of the agents to the CNS (Yoshihara, 2002). Whether olfactory axonal ensheathing cells (OECs) are also infected by CDV or how they respond to the virus has not been demonstrated. Recent evidence suggested that OECs might play a protective role against bacterial pathogens (Chuah et al., 2004-05; Leung et al., 2008). Whether they have a similar function during viral infection remains underdetermined. In addition, Schwann cells from sciatic nerves that share a number of similarities with and are closely related to OECs in vitro have not been investigated regarding CDV infection in vitro and in vivo.

1.3.4 Cell tropism and viral receptors

The tropism of morbillivirus-infectable cells has been correlated to the presence of both well known and unidentified cellular receptors. SLAM, a membrane glycoprotein, is a well known specific morbillivirus receptor expressed on a variety of different lymphoid cell subpopulations, including immature thymocytes, primary B cells, activated T cells, memory T cells, macrophages and mature dendritic cells (Kruse et al., 2001; Ostrakhovitch and Li, 2006). The distribution of SLAM-presenting cells is in accordance with the lymphotropism
and immunosuppression following morbillivirus infection (Schneider-Schaulies and Schneider-Schaulies, 2008; Tatsuo and Yanagi, 2002; Tatsuo et al., 2001; Wenzlow et al., 2007). SLAM acts as an efficient receptor for vaccine and field strains/clinical isolates of morbillivirus (Tatsuo et al., 2000). However, laboratory virus strains infect well known target cells, such as epithelial, endothelial and neuronal cells via a SLAM-independent mechanism as shown by the lack of cellular SLAM receptor expression (Andres et al., 2003; Wenzlow et al., 2007). **CD46**, a membrane cofactor protein (MCP) or a complement regulatory molecule, represents a primate-specific receptor for the laboratory-adapted Edmonston strain of MV which is expressed on all human cells except erythrocytes (Dörig et al., 1993, 1994). **CD9**, a tetraspan transmembrane protein, plays a role in a variety of physiological conditions; such as cell adhesion, motility, activation and proliferation, as well as during pathological conditions, including tumor metastases or viral infection. CD9 was found in the myelin and in exosomes of dendritic cells (Bronstein, 2000; Charrin et al., 2001). CD9 is considered an essential factor for CDV uptake by target cells, cell-cell (but not virus-cell) syncytial cell formation and the production of progeny virus (Löffler et al., 1997; Schmid et al., 2000). In addition, an unidentified epithelial cell receptor (**EpR**) located on the basolateral side of epithelial cell has been reported as a tight junction-related molecule to facilitate viral transmission directly to the airway lumen and to disseminate the pathogen without initial infection of the respiratory epithelium (Leonard et al., 2008; Tahara et al., 2008).

Within the CNS, astrocytes, microglia, oligodendrocytes, neurons, ependymal cells and choroid plexus epithelial cells have been documented as CDV target cells (Alldinger et al., 1993; Seehusen et al., 2007; Stein et al., 2006). Studies of in vivo and in vitro infection revealed differential susceptibility of various glial cell types to specific CDV strains. Astrocytes represent the first cell target of CDV following CNS infection and play a role for virus persistence and replication leading to chronic demyelinating lesions in the CNS (Headley et al., 2001; Mutinelli et al., 1989; Pearce-Kelling et al., 1990). Recently, Seehusen et al. (2007) demonstrated CDV infection in situ of GFAP+ astrocytes in acute distemper lesions, while vimentin+ astrocyte-like cells were targeted in chronic demyelinating disease. This finding suggests a change of cell tropism and/or susceptibility of glial cells during disease progression in DL. Subsequently, in vitro experiments using mixed adult canine brain cell cultures demonstrated the differential susceptibility of glial cells following infection with the CDV-R252 strain. The dominant cytopathic effect (CPE) noted in infected GFAP+ astrocytes, especially in the multinucleated syncytial cells, consisted of a ruptured cytoskeleton, whereas vimentin+ cells displayed no change in the filament network. The in vitro findings support a role of immature astrocytes for virus persistence and spread in advanced DL lesions (Seehusen et al., 2007).
Focusing on CDV strain-specific CNS lesions, the virulent CDV Snyder Hill (CDV-SH) strain initiated an acute encephalitis with predominantly induced gray matter lesions, while the demyelinating CDV-A75-17 and CDV-R252 strains resulted in a chronic encephalitis that more strikingly affected the white matter (Summers et al., 1984). In cultures of neonatal dog brains, the attenuated CDV Rockborn (CDV-RO) and the virulent CDV-SH strains rapidly replicated in various cell types, including neurons, astrocytes, bipolar oligodendrocytes and macrophages by 14dpi. Contrary to this, infection with the CDV-A75-17 strain did not affect neurons and replication was delayed until after 28-35dpi. In addition, multipolar oligodendrocytes were rarely infected by any of the virus strains (Pearce-Kelling et al., 1990, 1991). Similarly, the CPE characteristics also differed between different CDV strains. Both CDV-SH and CDV-A75-17 strains induced a non-cytolytic infection whereas CDV-RO caused a cytolysis of infected astrocytes (Pearce-Kelling et al., 1990).

Previously, CDV-mediated demyelination was thought to be caused by a selective infection of myelin-forming oligodendrocytes that leads to morphological changes, metabolic impairment and complete disappearance of oligodendrocytes in demyelinating lesions (Blakemore et al., 1989; Glaus et al., 1990; Summers and Appel, 1987). Although a down-regulation of myelin gene transcription was observed before demyelination occurred; the number of oligodendrocytes was not decreased until demyelination became evident and they remained presented in a significant amount even in chronic, completely demyelinated distemper lesions. These findings suggest that demyelination precedes oligodendrocyte loss (Schobesberger et al., 1999, 2002).

Besides astrocytes and oligodendrocytes that represent pivotal factors in the pathogenesis of distemper demyelination, microglia and peripheral macrophages invading the CNS during the course of the inflammatory response also play an important role in the demyelinating process (Stein et al., 2004; Vandevelde and Zurbriggen, 2005). In vivo and in vitro studies showed a significant upregulation of certain surface molecules, such as CD1c, B7-1, B7-2, MHC I and CD11b, in microglia and peripheral blood monocytes following CDV infection (Stein et al., 2004, 2008). All these molecules play a key role in the host’s immune response, notably antigen presentation and cell adhesion. Therefore, these findings suggest an enhancement of macrophage functions which may facilitate the entry of peripheral monocytes in the CNS leading to effective clearance of the virus but may also increase demyelination via a bystander effect (Stein et al., 2008).

Recently, the infection of the attenuated CDV-Ondersteapoort strain expressing the green fluorescent protein (CDV-OndeGFP) and CDV-R252 strain has been investigated in mixed adult canine brain cultures. Infection with the CDV-R252 strain resulted in a preferential affect in microglia and astrocytes compared to CDV-OndeGFP strain. Following infection, a
bipolar spindle-shaped Schwann cell-like population designated presently “Schwann cell-like brain glia (SCBG)” was observed. These cells displayed a high susceptibility to attenuated CDV strains as early as 3dpi (Orlando et al., 2008). So far there are no data available on CDV infection of p75^{NTR}-expressing SCBG in vivo.
References


1.7 Aim and hypothesis of the study

The aim of the study is the comparative analysis of two closely related glial cell types, Schwann cells and olfactory ensheathing cells (OECs), that have been shown to promote regeneration following myelin loss in the central nervous system. The following hypotheses were challenged in the different in vitro experiments:

- Transfection with human telomerase reverse transcriptase (hTERT) is an efficient approach to generate sufficient numbers of physiologically-behaving cells for transplantation purposes because it induces immortalization without substantial alterations of the cellular phenotype.

- Adult canine Schwann cells and OECs as closely related cell types share antigenic expression, growth factor requirements and proliferation behavior but display species-specific properties distinct from the well characterized rodent counterpart.

- Adult canine Schwann cells and OECs as closely related cell types do not differ in their susceptibility to infection with different attenuated and virulent CDV strains in vitro.
Chapter 2

Transfection of adult canine Schwann cells and olfactory ensheathing cells at early and late passage with human TERT differentially affects growth factor responsiveness and in vitro growth

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ABSTRACT

Adult canine Schwann cells and olfactory ensheathing cells (OECs) are closely related cell types that are considered attractive candidates for translational studies of neural repair. To establish a reliable cell source by comparing the in vitro properties of immortalized Schwann cells and OECs for transplantation purposes, we transfected both cell types with human telomerase reverse transcriptase (hTERT). Ectopic hTERT expression has been shown to induce immortalization of various cell types without substantial alterations of their phenotypes. Schwann cells and OECs were isolated from adult dogs, transfected with hTERT at early (P4) and late passage (P26), characterized regarding in vitro proliferation, antigenic expression and senescence-associated genes in the presence and absence of fibroblast growth factor-2 (FGF-2). Ectopic hTERT expression in late passage glia treated with but not without FGF-2 prevented the decline in proliferation observed in non-transfected cells. Immortalization did not alter p75NTR and GFAP but O4 and A2B5 expression. Contrary to this, early passage hTERT transfection significantly reduced proliferation independent of FGF-2 and lowered expression of O4 and GFAP in both cell types. Transfection did not alter mRNA expression of senescence-associated genes such as p53 and p16. No substantial differences were found between Schwann cells and OECs underscoring the close relationship of both cell types. Taken together, we established a stable source of adult canine Schwann cells and OECs and demonstrated that the effects of hTERT expression on in vitro growth and growth factor responsiveness depend on the replicative age.

KEYWORDS Schwann cell; olfactory ensheathing cell; telomerase; proliferation; replicative age

INTRODUCTION

Schwann cells and olfactory ensheathing cells (OECs) have been shown to promote axonal regeneration and remyelination following transplantation into the lesioned nervous system (Baron-Van Evercooren et al., 1997; Wewetzer et al., 2002; Barnett and Riddell, 2007). Based on these properties both cell types are considered promising candidates for transplantation-based therapies of CNS injury and disease (Moreno-Flores and Avila, 2006; Franssen et al., 2007). So far, the vast majority of studies have been performed in the rodent model. However, there is evidence for species-specific differences in the proliferation control of glial cells that may be relevant for the therapeutic application in humans (Barnett et al., 2000; Krudewig et al., 2006; Bock et al., 2007; Rubio et al., 2008). Previously, adult dogs were introduced as a translational model for the study of spinal cord repair (Smith et al.,
2002; Jeffery et al., 2005, 2006). Recently, we showed that adult canine OECs are different from rodent cells with regard to proliferation and antigenic expression (Krudewig et al., 2006). Several methods have been described for immortalization of mammalian cells in vitro, including application of chemical agents, radiation, infection with oncogenic virus or transfection with telomerase reverse transcriptase (TERT) (Cannell et al., 1996; Ouyang et al., 2000; Lee et al., 2002; Olson et al., 2003; Akimov et al., 2005; Uebing-Czipura et al., 2008). The advantages of TERT transfection compared to alternative methods are maintenance of a stable genotype and preservation of the differentiated and functional phenotype in the immortalized cells whereas abnormal growth patterns, cell transformation and genomic instability were rarely described. These characteristics are essential for providing cells that display physiological responses suitable for cell transplantation. To gain full telomerase activity, both components of the enzymatic complex, the TERT and the RNA subunit (TR), have to be expressed as a minimum requirement to keep cells in a dividing state by extending the telomeric DNA back (Blackburn, 1992; McEachern et al., 2000; Shay and Wright, 2007; Cairney and Keith, 2008).

Telomeres are the repetitive DNA elements which protect the chromosome ends and shorten with each round of cell division (Holt and Shay, 1999; McEachern et al., 2000). When the telomere length reaches a critical status for the chromosomal stability, a DNA damage response mediated by p53 signaling is initiated (Mu and Wei, 2002). As a result, the cell enters into an irreversible non-dividing state known as replicative senescence or G1 cell cycle arrest (Schafer, 1998; Shay and Wright, 2007). In addition, a telomere-independent senescence mechanism executed through a p16-mediated pathway was described (Herbig and Sedivy, 2006). Based on these properties, telomerase is considered a key molecule for the deceleration of senescence and cellular immortalization (Forsyth et al., 2002; Shay and Wright, 2005; Stewart and Weinberg, 2006). In particular cell types such as human mammary epithelial cells, telomerase-induced immortalization is mediated in part by Akt activity and is growth factor-dependent (DiRenzo et al., 2002; Kurz and Erusalimsky, 2003). Moreover, hTERT induces expression of growth-promoting genes, such as epidermal growth factor receptor (EGFR) and fibroblast growth factor-2 (FGF-2) (Smith et al., 2003).

In the present study, the catalytic subunit of human TERT (hTERT) was introduced into adult canine Schwann cells and OECs to establish a homogenous cell population for transplantation purposes. We show here that ectopic hTERT expression in late passage cells with FGF-2-treated but not in untreated Schwann cells and OECs induced immortalization while only inducing subtle alterations of their antigenic phenotype. Introduction of hTERT into early passage Schwann cells and OECs unexpectedly reduced proliferation of both cell types independent of the presence of FGF-2 and changed their antigenic profile. This is the first
demonstration that hTERT alters growth factor responsiveness and in vitro growth in dependence on the replicative age of cultured cells. The observation that hTERT in early passage glia inhibits proliferation without changing the responsiveness of Schwann cells and OECs to FGF-2 may help to identify novel functions of hTERT.

MATERIALS AND METHODS

**Primary culture of adult canine Schwann cells and olfactory ensheathing cells (OECs)**

Schwann cells and OECs were isolated from sciatic nerve and olfactory bulb from dogs (4 beagles, 5-17 month-old) as previously described with modifications (Krudewig et al., 2006; Bock et al., 2007). For isolation of Schwann cells, the sciatic nerve was cut into pieces (1 mm³) following removal of the epineurium, and plated on collagen type-I (5µg/cm², BD Bioscience, Germany) coated 24-well plates containing Dulbecco’s modified Eagle (DME) medium (100µl, Gibco Life Technologies, Eggenstein, Germany) supplemented with fetal calf serum (10%, PAA, Marburg, Germany), penicillin/streptomycin (1%, PAA, Marburg, Germany), sodium pyruvate (1%, PAA, Marburg, Germany). Cultures were maintained under standard conditions (5% CO₂, 37°C) for 1 h. After nerve fragments adhered to the plate, DME/10%FCS medium (1ml) was added and fragments were maintained under standard conditions for 7 days. Thereafter, nerve fragments were treated with a mixture of trypsin type I (1.5%), collagenase (0.5%) and hyaluronidase (0.5%, Sigma-Aldrich, Taufkirchen, Germany) in DME medium for 30 min at 37°C under gentle agitation. For OEC cultures, the meninges and blood vessels were carefully removed under microscopic control and bulb grey matter was isolated, chopped into pieces (1 mm³), and treated with trypsin/EDTA (0.25%, PAA, Marburg, Germany) in DME medium for 30 min at 37°C. After centrifugation (450xg, 4°C, 5 min), tissue was dissociated into single cells by trituration (Wewetzer et al., 1997) in the presence of DNase I (0.05%, Roche Diagnostics, Mannheim, Germany). Both cell suspensions were then passed through pre-separation filters (30µm mesh, Miltenyi Biotec, Bergisch-Gladbach, Germany) to remove cell aggregates. Cells were seeded on poly-L-lysine (PLL; 0.1 mg/ml, Sigma-Aldrich, Taufkirchen, Germany) coated culture flasks (Nunclon, Nunc, Germany) and maintained in DME/10%FCS medium under standard conditions.

**Antibody-based purification of Schwann cells and OECs**

Purification of Schwann cells and OECs was done from confluent cultures using magnet-activated cell sorting (MACS, Miltenyi Biotec, Bergisch-Gladbach, Germany) essentially as
described previously (Wewetzer et al., 2005; Krudewig et al., 2006; Bock et al., 2007). The anti-human-p75\textsuperscript{NTR} antibody (supernatant hybridoma, dilution 1:5, American Tissue Culture Collection, USA) and goat-anti-mouse IgG MicroBeads (dilution 1:20, Miltenyi Biotec) were used as primary and secondary antibodies, respectively. Following labeling with anti-p75\textsuperscript{NTR} antibodies and adsorption to antibody-coated magnetic beads, cells were seeded in PLL-coated culture flasks and expanded in DME/10\%FCS medium.

Transfection
Schwann cells and OECs were transfected at early (P4) and late passage (P26) with pBabe puro-hTERT plasmid (Addgene Inc., MA, USA) using Lipofectamine\textsuperscript{TM} 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions. After selection with puromycin (0.4 \( \mu \)g/ml, Sigma-Aldrich, Taufkirchen, Germany), the resistant clones were picked using Corning\textsuperscript{®} cloning cylinders (Fisher Scientific, Germany) and expanded under standard conditions (see above). Schwann cells and OECs 4 and 5 passages after transfection were used for RT-PCR, telomerase activity and proliferation assay.

Reverse transcriptase polymerase chain reaction (RT-PCR)
For RNA isolation, the RNeasy Mini Kit (Qiagen, Hilden, Germany) followed by a DNase treatment using RNase-Free DNase set (Qiagen, Hilden, Germany) according to the manufacturer's instructions was used. The reverse transcriptase (RT) reaction was carried out using 0.5 \( \mu \)g RNA, random primers (Promega, Mannheim, Germany), and the Omniscript RT-PCR kit (Qiagen, Hilden, Germany). The PCR conditions are depicted in table 1. Human cervical carcinoma cells (HeLa, ATCC: CCL-2) and Madin Darby canine kidney (MDCK, ATCC: CCL-34) were used as positive controls and the products were visualized on 2% agarose gels.

Table 1: List of used primers: sequences, annealing temperatures and the length of the obtained PCR products.

<table>
<thead>
<tr>
<th></th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Ta (( ^\circ )C)</th>
<th>Amplicon length</th>
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<tr>
<td>hTERT</td>
<td>5'-TGACTTTTGCAAGGTTGATGG-3'</td>
<td>5'-GTACGGCTGGAGGCTGATG-3'</td>
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<td>200</td>
</tr>
<tr>
<td>dTERT</td>
<td>5'-CAGCATCTCACTTCACA-3'</td>
<td>5'-CCCCGATCCTTACGA-3'</td>
<td>50</td>
<td>515</td>
</tr>
<tr>
<td>dTR</td>
<td>5'-CTAAACCTAACTGAGCAGG-3'</td>
<td>5'-CCAGCAGTGTACATTTTGT-3'</td>
<td>56</td>
<td>149</td>
</tr>
<tr>
<td>p53</td>
<td>5'-GAGTTCATGAGCCGGGATGT-3'</td>
<td>5'-CTCCCCAATGACAGACACGGA-3'</td>
<td>55</td>
<td>238</td>
</tr>
<tr>
<td>p16</td>
<td>5'-GGTGAGGGTCACAGCAGCCTGT-3'</td>
<td>5'-CGAAGGTACCAGTGCGCAT-3'</td>
<td>50</td>
<td>125</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-AAGGTCAAGGAGTCAACGGATT-3'</td>
<td>5'-GCAGAAGGAGACAGAGATGAG-3'</td>
<td>50</td>
<td>365</td>
</tr>
</tbody>
</table>
**Telomerase activity assay**

Cell extracts corresponding to 10⁴ cells were assayed for telomerase activity using the telomeric repeat amplification protocol (Kim et al., 1994) and the TRAPEZE® telomerase detection kit (Intergen, Manhattanville, NY, USA). HeLa and DH82 (ATCC: CRL-10389) cells were used as positive controls (Frisk et al., 1999). The products were run on a 12.5% non-denaturing polyacrylamide gel (Bio-Rad, Munich, Germany) and visualized by ethidium bromide (0.01% v/v).

**Proliferation assays**

To monitor the proliferation kinetics of Schwann cells and OECs in long term culture, the cumulative population doublings (CPDs) were determined by manually counting of the trypsinized cells upon passaging. For this, cells were grown in the presence and absence of FGF-2, dislodged when they reached confluency and counted in a hemocytometer. PDs were calculated at every passage using the formula log(cellsharvested/cellsseeded)/log(2). Cumulative PDs (CPDs) were determined by summing the log until the last passage as described previously (Rheinwald et al., 2002).

**Antibodies**

The used mouse monoclonal antibodies included anti-sulfatide (O4) and anti-ganglioside Q (A2B5, gift of J. Trotter, Mainz, Germany) and anti-p75<sup>NTR</sup>-antibodies (clone HB8737, American Type Culture Collection, ATCC, Rockville, USA). Antibodies were used as hybridoma supernatants at a dilution of 1:2 (O4, A2B5) and 1:5 (p75<sup>NTR</sup>). Monoclonal anti-GFAP antibodies were from Sigma-Aldrich, Taufkirchen, Germany) and were used at a 1:400 dilution.

**Immunofluorescence**

For immunostaining of vital and fixed cells, Schwann cells and OECs were seeded in 96-well PLL-coated plates and maintained for 3 days under standard conditions. Sulfatide (O4), ganglioside Q (A2B5), and the neurotrophin receptor p75 (p75<sup>NTR</sup>) were visualized on vital cells, while GFAP expression was studied in fixed cells. For vital staining, the monoclonal antibodies were applied for 20 min at 37°C followed by the secondary antibodies under identical conditions (1:200, Cy3-coupled goat anti-mouse antibodies, Jackson Immunoresearch, Dianova, Hamburg, Germany). Cells were fixed using paraformaldehyde (4% in PBS, pH 7.4) for 15 min and nuclei were counterstained using bisbenzimide Hoechst 33258 (0.01% in methanol) for 10 min at room temperature. Intracellular staining included
fixation with paraformaldehyde (4%), pre-treatment with PBS-Triton X-100 (PBST; 0.25%, 5min) blocking of non-specific binding with BSA (1% in PBST) for 15 min. Cells were labelled with primary antibodies in the same solution for 2 h at room temperature and secondary antibodies (1:200, Cy3-coupled goat anti-mouse antibodies) in blocking solution for 1 h at room temperature. Nuclei were counterstained as described above. The percentage of positive cells was determined using an inverted fluorescence microscope (Olympus IX-70, Hamburg, Germany) by manually counting Cy3-labelled cells versus Hoechst-labelled cells. Experiments were performed in triplicates and five fields per well were counted.

**Statistical analysis**

The data of the immunofluorescence assays were analyzed by Friedman-rank-sum-test for comparison between cells without and with FGF-2 treatment and Wilcoxon's rank-sum-test for comparison between non-transfected and transfected cells. For long-term proliferation assays, the slopes of linear regression function of CPD were compared between cells without and with FGF-2 treatment and between non-transfected and transfected cells over time by calculating analysis of covariance. All statistical tests were carried out using the Statistical Analysis System, version 9.1 (SAS Institute, Cary, North Carolina, USA). P values of less than 0.01 were considered to be statistically significant.

**RESULTS**

*Expression of dog telomerase (dTERT), telomerase RNA (dTR), p53 and p16 in Schwann cells and OECs*

Adult canine Schwann cells and OECs derived from the sciatic nerve and olfactory bulb, respectively, were immunopurified by anti-p75NTR antibody-based MACS and expanded in the presence of FGF-2. Expression of endogenous dog TERT (dTERT) and its RNA subunit (dTR) were monitored using RT-PCR (Fig.1A). Adult canine Schwann cells and OECs at early passage (P4) lacked dTERT expression but were positive for dTR compared to MDCK used as a positive control. Identical expression profiles were found in Schwann cells and OECs at late passage (P28) (Fig.1A). Schwann cells and OECs expressed p53 and lacked p16 as demonstrated by using RT-PCR (Fig.1B). The same expression profile was observed in early and late passage cells (Fig.1B; SC_E -, SC_L -, OEC_E -, OEC_L -).
Fig. 1: RT-PCR analysis of dTERT, dTR expression (A) and p53, p16 expression (B) in adult canine Schwann cells (SC) and olfactory ensheathing cells (OEC). Both cell types expressed telomere RNA (dTR) and lacked telomerase reverse transcriptase (dTERT) at early (A, SC_E, OEC_E, passage 4) and late passage (A, SC_L, OEC_L, passage 28). In addition, both cell types expressed the senescence-associated molecule p53 but not p16 at early (B, SC_E, OEC_E) and late passage (B, SC_L, OEC_L) independent of hTERT transfection. MDCK and HeLa cells served as positive controls for dTERT/dTR and p53/p16, respectively. GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) was used as a cDNA qualitative control.

Transfection of primary adult canine Schwann cells and OECs with human TERT
Cells at early (P4) and late passage (P26) were transfected with the pBabe puro-hTERT construct expressing the catalytic subunit of the hTERT and selection of positive clones was done in puromycin-containing medium. All the transfected Schwann cell and OEC clones but not the non-transfected cells expressed hTERT mRNA (Fig.2A). The hTERT mRNA expression was detected both in early and late passage-transfected Schwann cells and OECs (Fig.2A). Subsequently, the early and late passage transected Schwann cells and OECs were monitored for telomerase activity using the TRAPEZE® Kit and compared to non-transfected counterparts (Fig.2B). Early and late passage transfected but not non-transfected Schwann cells and OECs displayed high levels of telomerase activity as indicated by the presence of a 6-base pair interval ladder starting from 50 bp (Fig.2B). In addition, no alteration of p53 and p16 expression was observed in early and late passage transfected
Schwann cells and OECs compared to their non-transfected counterparts as demonstrated by using RT-PCR (Fig. 1B; SC_E+, SC_L+, OEC_E+, OEC_L+).

**Fig. 2:** RT-PCR analysis of hTERT expression (A) and telomerase activity (B) in adult canine Schwann cells (SC) and olfactory ensheathing cells (OEC) before (-) and after transfection (+) at early (SC_E, OEC_E, passage 4) and late passage (SC_L, OEC_L, passage 26). All transfected cells were positive for hTERT independent of passage level (A, SC_E+, SC_L+, OEC_E+, OEC_L+). The pBabe puro-hTERT plasmid and HeLa cells served as positive controls, while MDCK cells were used as a negative control for hTERT analysis (A). Early and late passage transfected (B, SC_E+, SC_L+, OEC_E+, OEC_L+) but not non-transfected Schwann cells and OECs (B, SC_E-, SC_L-, OEC_E-, OEC_L-) displayed high levels of telomerase activity. HeLa and DH82 cell lines served as positive controls for telomerase activity (B).
**Morphology and antigenic phenotype of hTERT-transfected Schwann cells and OECs**

In the presence of FGF-2, both non-transfected Schwann cells and OECs at early passage displayed the same typical spindle-shaped, bi- to tripolar morphology (Fig.3A, E). This morphological phenotype was not altered upon hTERT transfection showing that both cell types maintained the same spindle-shaped morphology (Fig.3C, G). At late passage, Schwann cells and OECs acquired a more flattened morphology (Fig.3B, F) but no substantial alterations in the cell morphology were observed after hTERT transfection (Fig.3D, H).

![Morphology of non-transfected and hTERT-transfected cells](image)

*Fig. 3: Morphology of non-transfected (A,B; E,F) and hTERT-transfected cells (C,D; G,H) adult canine Schwann cells (A-D) and olfactory ensheathing cells (OECs, E-H) at early (passage 4; A, C, E, G) and late passage (passage 42; B, D, F, H). Both cell types displayed the typical spindle-shaped, bi- to tripolar morphology and no substantial differences in the morphological phenotype were observed. (Scale bar: 100 µm)*
To study whether hTERT transfection induced alterations of the antigenic phenotype, early and late passage transfected Schwann cells and OECs were maintained in the presence and absence of FGF-2. The cells were then immunostained with antibodies against sulfatide (O4), ganglioside Q (A2B5), neurotrophin receptor p75 (p75\textsuperscript{NTR}), GFAP and the expression of these antigens was compared between non-transfected and transfected cells (Table 2, Fig.4). The expression of O4 in early passage Schwann cells and OECs was significantly reduced upon transfection ($d$, $p<0.01$, Fig.4A, C: Schwann cells; Fig.4E, G: OECs) and FGF-2 treatment ($c$, $p<0.01$). For the late passage cells, the transfection significantly lowered the expression of O4 in FGF-2 untreated Schwann cells, FGF-2 untreated and treated OECs ($d$, $p<0.01$, Fig.4B, D: Schwann cells; Fig.4F, H: OECs) while the addition of FGF-2 only reduced the expression in non-transfected Schwann cells ($c$, $p<0.01$). The significant reduction of A2B5 expression was limited to late passage transfected Schwann cells independent of FGF-2 addition ($d$, $p<0.01$). Interestingly, the expression of the typical Schwann cell and OEC marker p75\textsuperscript{NTR} was confined to all Schwann cells and OECs (Table 2). Expression of GFAP was found to be reduced after early passage transfection in FGF-2-untreated cultures of both cell types ($d$, $p<0.01$). Addition of FGF-2 resulted in the significant down-regulation of GFAP in early passage non-transfected Schwann cells and up-regulation of GFAP in late passage Schwann cells independent of transfection ($c$, $p<0.01$).
Fig. 4: Micrographs illustrating O4 immunoreactivity of non-transfected (A,B; E,F) and transfected (C,D; G,H) adult canine Schwann cells (A-D) and olfactory ensheathing cells (OECs, E-H) at early (passage 4) and late passage (passage 26) in the absence of FGF-2. O4 expression was significantly reduced in both early and late passage Schwann cells and OECs after hTERT transfection. (Scale bar: 100 µm)
Table 2: Comparison of the mean percentage of O4, A2B5, p75NTR and GFAP-expressing hTERT-transfected Schwann cells and OECs with their normal counterparts at early and late passage.

<table>
<thead>
<tr>
<th></th>
<th>Early passage</th>
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<th>Late passage</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Schwann cells</td>
<td>OECs</td>
<td>Schwann cells</td>
<td>OECs</td>
</tr>
<tr>
<td></td>
<td>Non-transfected</td>
<td>hTERT-transfected</td>
<td>Non-transfected</td>
<td>hTERT-transfected</td>
</tr>
<tr>
<td>O4a</td>
<td>46.55&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.60&lt;sup&gt;c, d&lt;/sup&gt;</td>
<td>51.97&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.09&lt;sup&gt;c, d&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>8.63</td>
<td>4.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14.81</td>
<td>0.00&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>A2B5</td>
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<td>0.34</td>
<td>3.41</td>
<td>1.07</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.93</td>
<td>2.18</td>
</tr>
<tr>
<td>p75&lt;sup&gt;NTR&lt;/sup&gt;</td>
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<td>100.00</td>
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<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
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<tr>
<td>GFAP</td>
<td>11.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.55&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.03</td>
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<tr>
<td>GFAP</td>
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<td>8.21</td>
<td>9.75</td>
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<sup>a</sup> O4 (sulfatide); A2B5 (ganglioside Q); p75<sup>NTR</sup> (neurotrophin receptor p75); GFAP (glial fibrillary acidic protein)

<sup>b</sup> ∅ FGF-2 (fibroblast growth factor-2 was omitted from the culture); + FGF-2 (FGF-2 was added to the culture)

<sup>c</sup> Significant differences between cells without and with FGF-2 treatment in each non-transfected and transfected cells.

<sup>d</sup> Significant differences between non-transfected and transfected cells in the same culture conditions.
Long-term in vitro growth of hTERT-transfected Schwann cells and OECs

To study the effects of ectopic hTERT expression on the long-term proliferation of adult canine glia, transfected Schwann cells and OECs were maintained in the presence and absence of FGF-2 and the cumulative population doublings (CPDs) of early and late passage cells were determined (Fig.5). By comparing the CPDs from transfected and non-transfected cells, it was observed that hTERT expression exhibited differential effects in early and late passage cells. Ectopic hTERT expression in late passage cells significantly restored proliferation levels up to early passage levels (◊, p<0.01, Fig.5B, D). The differences of CPDs in hTERT-transfected cells were at least 7-fold (Schwann cells) and 10-fold (OECs) increases compared to their non-transfected controls. However, this effect was dependent on FGF-2 treatment (*, p<0.01, Fig.5B, D). In the absence of FGF-2, both Schwann cells and OECs stopped proliferation like their non-transfected counterpart (Fig.5B, D). Contrary to this, hTERT expression slowed down proliferation of both Schwann cells and OECs in early passage cells independent of the presence of FGF-2 (◊, p<0.01, Fig.5A, C). Both early passage cell types, either non-transfected or transfected, similarly increased proliferation upon FGF-2 treatment as compared to their FGF-2 untreated counterparts (*, p<0.01, Fig.5A, C).
Fig. 5: Effects of hTERT transfection on the proliferation of Schwann cells (SCs) and olfactory ensheathing cells (OECs) at early passage (T/SCe, A; T/OECe, C, starting from passage 4) and late passage (T/SCl, B; T/OECl, D, starting from passage 26) compared to non-transfected cells (SCe, SCl, OECe, OECl) in the presence (+FGF) and absence of FGF-2 (ØFGF). At early passage, the proliferation was significantly lowered after introduction of hTERT independent of FGF-2 treatment (A, C, *), while hTERT expression in late passage cells increased proliferation only in the presence of FGF-2 (B, D, *) and cells ceased proliferation in its absence (B, D). Significant differences between cells cultured without and with FGF-2 (*) and between non-transfected and transfected cells maintained under the same culture conditions (•) as revealed by analysis of covariance (P values < 0.01).
DISCUSSION

The adult dog is an interesting translational model to study Schwann cell- and OEC-mediated neural repair (Smith et al., 2003; Jeffery et al., 2005, 2006). In the present study, we used human telomerase reverse transcriptase (hTERT) transfection of adult canine Schwann cells and OECs derived from the sciatic nerve and olfactory bulb, respectively, to establish a stable source for cell transplantation. Several studies described immortalization of both Schwann cells (Langford et al., 1988; Eccleston et al., 1991; Thi et al., 1998; Watabe et al., 2003) and OECs (Moreno-Flores et al., 2003, 2006). It was demonstrated that forskolin-driven immortalized Schwann cells were able to myelinate peripheral neurons but formed tumours in vivo following transplantation (Langford et al., 1988). OECs immortalized by SV 40 large T antigen (TEG3) stimulated axonal outgrowth of retinal ganglionic neurons in vitro and promoted the functional recovery of the injured spinal cord in vivo (Moreno-Flores et al., 2003, 2006). However, so far the majority of studies were carried out in rodents that have recently been reported to display species-specific properties, different from primates (Rubio et al., 2008).

Normal adult canine Schwann cells and OECs constitutively expressed dTR but were negative for dTERT. This is in contrast to rodent glia, that was shown to express both components of the enzymatic complex and which lack replicative senescence (Prowse and Greider, 1995; Mathon et al., 2001; Tang et al., 2001; Hahn and Weinberg, 2002). Rodent glia in the presence of mitogens grows throughout multiple passages before spontaneously immortalizing while the adult canine glia used in the present study grew well at early passage but ceased proliferation from passage 26 on. Prior to hTERT transfection, the expression of the senescence key mediators p53 and p16 was analyzed in Schwann cells and OECs. There are two main pathways by which cells enter cellular senescence. The one is dependent on the telomere erosion and is supposed to be initiated by a DNA damage response mediated through p53/p21 signalling while the other one is telomere-independent and is executed either by p16/Rb and/or p14/19ARF/p53 (Shamanin and Androphy, 2004). Normal canine Schwann cells and OECs at early and late passage consistently expressed p53 but lacked p16. This may indicate that canine Schwann cells and OECs undergo senescence through a telomere-dependent pathway similar to human cells as previously suggested (Argyle and Nasir, 2003; Nasir, 2008). It was observed that in the majority of human adult somatic cells telomerase expression is repressed and the cells undergo replicative senescence not until the telomere length becomes critical for chromosomal stability (Blasco, 2005).

Transfection of adult canine glia was done at early and late passage and the morphological and antigenic phenotype as well as the proliferation of Schwann cells and OECs was
studied. Assuming that hTERT expression alone is sufficient to escape from the growth arrest, hTERT transfection should permit indefinite *in vitro* growth of Schwann cells and OECs without altering their phenotype. All of the studied Schwann cell and OEC clones were successfully transfected and gained full telomerase activity. Following hTERT transfection at late passage, Schwann cells and OECs escaped from the growth arrest observed in their non-transfected counterparts and underwent cellular immortalization in the presence of FGF-2. No alterations in the expression of senescence-associated molecules such as p53 and p16 and the morphological phenotypes of transfected glial cells were observed. The expression of p75NTR and GFAP but not of O4 and A2B5 remained unchanged upon transfection.

Contrary to this were the effects of ectopic hTERT expression in early passage glia. Here, hTERT did not only induce a significant decrease in the proliferation of Schwann cells and OECs but also lowered the expression of O4 and GFAP both in the presence and absence of FGF-2. This is the first demonstration that ectopic hTERT expression slows down proliferation. So far, TERT expression has always been shown to increase cellular proliferation (Xiang et al., 2002; Gorbunova and Seluanov, 2003; Smith et al., 2003; Young et al., 2003; Jin et al., 2006). However, the majority of studies introduced ectopic TERT at a time point when telomeres had already reached a critical length. Thus, the unexpected finding of the present study may be explained by the fact that ectopic hTERT was introduced at early passage a long time before cells slowed down proliferation and suggest that hTERT has additional functions in the control of proliferation.

Schwann cells and OECs are considered closely related cell types that promote CNS regeneration following transplantation (Wewetzer et al., 2002; Wewetzer and Brandes, 2006). Based on a number of studies, it was concluded that OECs display a superior regenerative potential compared to Schwann cells (Franklin and Barnett, 1997). In the present study, both Schwann cells and OECs were successfully transfected and displayed full telomerase activity. No differences in the expression of p53 and p16 prior to and after transfection were detected between both cell types. Moreover, hTERT transfection of early passage glia induced identical reductions of *in vitro* proliferation on both Schwann cells and OECs, while transfection of late passage glia increased FGF-2-dependent proliferation to the same extent in both cell types. Taken together, adult canine Schwann cells and OECs at late passage were successfully immortalized by ectopic telomerase transfection in combination with mitogen stimulation thereby providing a stable and homogeneous cell population for further cell-based transplantation studies.
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Chapter 3

Similar behaviour and primate-like properties of adult canine Schwann cells and olfactory ensheathing cells in long-term culture

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ABSTRACT
Adult canine Schwann cells and olfactory ensheathing cells (OECs) have been shown to promote neural regeneration in vivo. Since the majority of studies has been performed in rodents, it is not yet clear in how far OECs from large animals and humans share the reported properties. Moreover, due to the lack of comparative studies, it remains to be established whether Schwann cells and OECs display cell type-specific characteristics. In the present study, adult canine Schwann cells and OECs were comparatively analyzed regarding long-term growth, morphology, growth factor responsiveness, and antigenic expression. Adult canine Schwann cells and OECs displayed the same typical spindle-shaped morphology and expressed the cell type-specific marker p75NTR. Moreover, the proliferation of both cell types was promoted by the same mitogens, including fibroblast growth factor-2 (FGF-2) and heregulin-1β (HRG-1β). Several observations indicate that canine OECs differ from the well characterized rodent OECs and display properties reminiscent on primate cells. Both cell types (i) proliferated through multiple passages in the absence of growth factors and did not enter a senescent state until 3 months in culture, (ii) were not responsive to the cAMP-elevating agent forskolin, and stably expressed p75NTR in long-term culture. Taken together, this is the first report demonstrating that adult canine Schwann cells and OECs in long-term culture share the same in vitro characteristics and display primate-like properties. This underscores the relevance of the dog as a translational species between rodents and humans.

Classification terms:

Keywords Schwann cell, OEC, canine, long-term culture, primate-like, growth factor responsiveness

Section Nervous System Development, Regeneration and Aging

ABBREVIATIONS
BrdU 5-Bromo-2′-deoxy-uridine
CNTF Ciliary neurotrophic factor
dbcAMP dibutyryl cyclic adenosine monophosphate
DME Dulbecco’s modified Eagle
EGF Epidermal growth factor
ELISA Enzyme-linked immunosorbent assay
FCS Fetal calf serum
FGF-2 Recombinant human fibroblast growth factor-2
CHAPTER 3: LONG-TERM IN VITRO GROWTH OF CANINE GLIA

GFAP  Glial fibrillary acidic protein
HCl   Hydrogen chloride
HRG-1ß Heregulin-1ß
MACS  Magnet-activated cell separation
OECs  Olfactory ensheathing cells
p75NTR p75 neurotrophin receptor
PBS   Phosphate buffer saline
PBST  PBS-Triton-X100
PLL   Poly-L-lysine

1. INTRODUCTION

Schwann cells and olfactory ensheathing cells (OECs) are closely related glial cells that have been shown to promote neural repair following transplantation into the lesioned nervous system (Honmou et al., 1996; Ramón-Cueto et al., 1998; Barnett et al., 2000; Kohama et al., 2001; Wewetzer et al., 2002; Akiyama et al., 2004). Based on these properties, both cell types are considered promising candidates for cell transplantation-based therapies of human CNS injury and disease (Santos-Benito and Ramón-Cueto, 2003; Barnett and Riddell, 2007; Lavdas et al., 2008). However, the vast majority of the studies has been performed in the rodent model and it is not clear in how far the obtained data can be extrapolated to humans (Wewetzer and Brandes, 2006). Although the adult dog has been recently introduced as a translational model for the study of spinal cord repair and in vivo studies reported beneficial effects of adult canine OECs (Smith et al., 2001; Jeffery et al., 2005, 2006; Ito et al., 2006; Krudewig et al., 2006), little is known about the in vitro properties of OECs and their characterization has remained fragmentary. Even less is known about the biology of canine Schwann cells, whose purification has been described in a single report (Pauls et al., 2004). Thus, the question of which cell type is most suitable for CNS repair, which is discussed controversially in the rodent model, is also open for the canine system. The assumption of species-specific properties of Schwann cells and OECs is underscored by recent studies. In contrast to rat OECs, adult human and canine OECs do not respond to forskolin with proliferation and transition of their morphology (Barnett et al., 2000; Krudewig et al., 2006). Moreover, primate OECs but not rodent OECs display a prolonged life span in vitro and a stable expression of the neurotrophin receptor p75 (Rubio et al., 2008).

In the present study, we focused on the comparative analysis of adult canine Schwann cells and OECs in order to lay the groundwork for the transplantation of both cell types into the lesioned spinal cord. We show here that both normal adult canine Schwann cells and OECs
displayed long-term proliferation even in the absence of mitogens and did not enter senescence until 3 months \textit{in vitro}. Moreover, both Schwann cells and OECs maintained stable expression of p75\textsuperscript{NTR} and did not respond to forskolin.

2. EXPERIMENTAL PROCEDURES

2.1 Primary culture of adult canine Schwann cells and olfactory ensheathing cells (OECs)

Schwann cells and OECs were isolated from sciatic nerve and olfactory bulb from 5 six-month-old beagle dogs as previously described (Wewetzer et al., 1996; Krudewig et al., 2006; Bock et al., 2007). Both cell suspensions were passed through pre-separation filters (30\textmu m mesh, Miltenyi Biotec, Bergisch-Gladbach, Germany) to remove cell aggregates. Cells were seeded on poly-L-lysine (PLL; 0.1 mg/ml, Sigma-Aldrich, Taufkirchen, Germany) coated culture flasks (Nunclon, Nunc, Germany) and maintained in DME/10\%FCS medium under standard conditions (37\textdegree C, 5\% CO\textsubscript{2} incubator).

2.2 Antibody-based purification of Schwann cells and OECs

Purification of Schwann cells and OECs was performed from confluent cultures using magnet-activated cell sorting (MACS, Miltenyi Biotec, Bergisch-Gladbach, Germany) essentially as described previously (Wewetzer et al., 2005; Krudewig et al., 2006; Bock et al., 2007). Anti-human-p75\textsuperscript{NTR} antibodies (supernatant hybridoma, dilution 1:5, American Tissue Culture Collection, USA) and goat-anti-mouse IgG MicroBeads (dilution 1:20, Miltenyi Biotec) were used as primary and secondary antibodies, respectively. The magnetically p75\textsuperscript{NTR}-labelled cells were seeded in PLL-coated culture flasks and expanded in DME/10\%FCS medium.

2.3 Proliferation assays

Growth of Schwann cells and OECs in culture was monitored either by BrdU short-term assays (5-Bromo-2’-deoxy-uridine labeling and detection kit III, Roche, USA) or neutral red (Wewetzer et al., 2001; Orlando et al., 2008). For BrdU uptake, Schwann cells (P4, P26) and OECs (P4, P28) were seeded at a density of 5000 cells/well in PLL-coated 96-well microtiter plates (Nunclon) and received FGF-2 or not (20 ng/ml; recombinant human, Peprotech, USA) 24 h later. BrdU (100\mu M) was applied for 12 h and cells were washed with DME medium without serum. Then, cells were fixed with chilled ethanol (70\% ) / HCl (25\%) for 30 min at -20\textdegree C and immunostained according to the manufacturer’s instructions. Proliferating cells
were labelled with secondary antibodies (1:100, Cy3-coupled goat anti-mouse antibodies, Jackson Immunoresearch, Dianova, Hamburg, Germany) while nuclei were counterstained with bisbenzimide Hoechst 33258 (0.01% in methanol, Sigma-Aldrich, Taufkirchen, Germany) for 10 min at room temperature. The percentage of BrdU-positive cells was determined using an inverted fluorescence microscope (Olympus IX-70, Hamburg, Germany) by manually counting of Cy3-labelled cells versus Hoechst-labelled cells. Experiments were performed in triplicates and five high-power fields per well were counted.

For neutral red assays, Schwann cells and OECs (P6-10 and P20-22) were seeded in quadruplicate at a density of 6000 cells/well in PLL-coated 96-well microtiter plates and maintained in the absence of growth factors for at least 48 h in either serum-containing medium (DME/10%FCS) or serum-free medium (DME/F12 medium, Gibco Life Technologies) supplemented by additives as described previously (Giulian and Baker, 1986), a concentration of 40 ng/ml of FGF-2, epidermal growth factor (EGF), ciliary neurotrophic factor (CNTF; all from Peprotec, Tebu, Frankfurt, Germany), heregulin-1β (HRG-1β, EGF domain, R & D Systems, Wiesbaden, Germany) and 4µM of forskolin (Calbiochem/Novabiochem, Schwalbach, Germany) were applied for 3 days. Cultures were maintained under standard condition (see above). Cells were fixed and processed for enzyme-linked immunoadsorbent assay (ELISA) as described previously (Wewetzer et al., 2001). Plates were read at 570 nm using a Dynex ELISA reader (Dynex Revelation 4.24). Cell titration experiments and standard curves for correlating OD values to absolute cell numbers can be obtained from Krudewig et al. (2006).

To monitor the proliferation kinetics of Schwann cells and OECs in long-term culture, the doubling times (DTs) of single clones were determined. For this, cells were seeded at a density of 5 x 10^5 cells in PLL-coated 25-cm² flasks and grown in the presence and absence of FGF-2. Cells were dislodged at confluency, and counted in a hemocytometer. DTs were calculated at every passage using the formula times in vitro (days) multiplied by the log(cellsharvested/cellsseeded)/log(2).

2.4 Antibodies

The used mouse monoclonal primary antibodies included anti-sulfatide (O4) and anti-ganglioside Q (A2B5, gift of J. Trotter, Mainz, Germany) and anti-p75NTR-antibodies (clone HB8737, American Type Culture Collection, ATCC, Rockville, USA). Antibodies were used as hybridoma supernatants at a dilution of 1:2 (O4, A2B5) and 1:5 (p75NTR). Monoclonal anti-
GFAP antibodies (Sigma-Aldrich, Taukirchen, Germany) were used at a 1:400 dilution. The second antibodies was Cy3-coupled goat anti-mouse antibodies and used at a 1:200 dilution.

2.5 Immunofluorescence

Schwann cells and OECs were seeded in 96-well PLL-coated microtiter plates and maintained for 3 days in the presence and absence of FGF-2 under standard conditions. Sulfatide (O4), ganglioside Q (A2B5), and the neurotrophin receptor p75NTR were visualized on vital cells, while GFAP expression was studied in fixed cells. For vital staining, the monoclonal antibodies were applied for 20 min at 37°C followed by the secondary antibodies under identical conditions. Cells were fixed using paraformaldehyde (4% in PBS, pH 7.4) for 15 min and nuclei were counterstained using bisbenzimide Hoechst 33258 (0.01% in methanol) (see above). For fixed cell staining, cells were immersed with paraformaldehyde (4%), pre-treated with PBS-Triton X-100 (PBST; 0.25%, 5min) and the non-specific binding was blocked with BSA (1% in PBST) for 15 min. Cells were labelled with primary antibodies in the same solution for 2 h at room temperature and secondary antibodies for 1 h at room temperature in blocking solution. Nuclei were counterstained (see above). Triplicate experiments were performed and the percentage of positive cells was calculated as described above.

2.6 Statistical analysis

The data of the BrdU and immunofluorescence assays were analyzed by Friedman-rank-sum-test for comparison between cells without and with FGF-2 treatment. Wilcoxon’s rank-sum-test was employed to compare cells at low and high passages. For the neutral red assays, the data were analyzed by Friedman’s Signed Rank test. All analysis was done using SAS Online Doc® Software, version 8 (SAS Institute Inc., 1999). P-values of less than 0.01 were considered to be statistically significant. All data were expressed as mean, maximal and minimal.

3. RESULTS

3.1 In vitro long-term characteristics and morphology of adult canine Schwann cells and OECs

For the analysis of the long-term growth of Schwann cells and OECs, the doubling times (DTs) were determined at every passage (Fig.1A,B). Cells that reached confluency were dislodged, counted, and the DTs were calculated. In the presence of FGF-2, Schwann cells
passed through 14 passages whereas FGF-2 untreated control cells reached only 6 passages within 3 months. The mean average of DTs was about 2 days (range 1.46-2.97 days) for FGF-2-treated Schwann cells and about 7 days (range 4.32-18.32 days) for untreated controls (Fig.1A). OECs displayed similar long-term proliferation properties (Fig.1B). In the absence of FGF-2, OECs went through 10 passages while addition of FGF-2 increased the number of passages up to 19 in the same observation period. The mean average of DTs was about 2 (range 1.31-3.07 days) for FGF-2-treated OECs compared to 6 days (range 2.66-14.5 days) for the untreated controls (Fig.1B).

The peak in DTs at around day 40 in both Schwann cells and OECs grown in the absence of FGF-2 revealed low proliferation of the cells following thawing of the cryopreserved stocks. Both cell types recovered within a single passage and the DTs were lowered again, with about 6.5 days (range 4.32-8.41 days) for Schwann cells and 6 days (range 2.66-7.76 days) for OECs (Fig.1A,B).

Both Schwann cells and OECs displayed the typical bi- to tripolar morphology that was not changed up to passage 20 (Fig.1C-F). At higher passages (>25), Schwann cells and OECs changed their morphology to a more flattened phenotype (Fig.4C,D).
Fig. 1: Long-term in vitro growth and morphology of adult canine Schwann cells (SC, A, C, E) and olfactory ensheathing cells (OEC, B, D, F) in the presence (+ FGF-2) and absence of FGF-2 (∅ FGF-2). During the 3-month-culturing period, single clones of FGF-2-treated Schwann cells and OECs displayed shorter population doubling times than untreated cells throughout multiple passages (P) (A, B). Morphology of Schwann cells (P4: C and P16: E) and OECs (P4: D and 21: F) in the absence of FGF-2. Both glial cell types displayed the typical bi- to tripolar shape and did not change upon FGF-2 treatment up to P25. Scale bar ~ 200 µm.
3.2 Proliferation rate and growth factor responsiveness of adult canine Schwann cells and OECs

Schwann cells and OECs from passages 4-10 (Fig.2C,D; Fig.3A,B), 20-22 (Fig.2A,B), and 26-28 (Fig.3A,B) were further analyzed by short-term neutral red (Fig.2A-D) and BrdU assays (Fig.3A,B). Both cell types increased proliferation after treatment with established Schwann cell mitogens, such as FGF-2 and HRG-1ß but not with ciliary neurotrophic factor (CNTF) and epidermal growth factor (EGF) both under serum-containing and serum-free culture conditions up to passage 20 (⁎, Fig.2, Fig.3A,B). Addition of FGF-2 to Schwann cell and OEC cultures in serum-containing but not in serum-free media significantly increased proliferation compared to HRG-1ß (♦, Fig.2A,B). Furthermore, the intracellular cAMP level-elevating agent forskolin did not increase proliferation, neither in combination nor applied alone (Fig.2C,D). The only exception to this was the serum-free culture of OECs where the combined treatment with FGF-2/forskolin increased proliferation compared to addition of FGF-2 alone (♦, Fig.2D). In addition, forskolin under serum-free conditions significantly reduced proliferation compared to untreated controls in both Schwann cells and OECs (♦, Fig.2C,D). At the highest passages analyzed (P26-28, Fig.3A,B), FGF-2 did not increase BrdU uptake in both Schwann cells and OECs suggesting that the cells at these passages had lost the ability to respond to FGF-2 treatment (Fig.3A,B).
Fig. 2: Growth factor effects on proliferation (A, B) and forskolin responsiveness (C, D) were analyzed by neutral red incorporation assays in Schwann cells (SC, A, C) and olfactory ensheathing cells (OEC, B, D) in serum-containing (FCS) and serum-free culture. FGF-2 and HRG-1β but not CNTF and EGF significantly increased proliferation (*, p<0.01) independent of FCS (A, B). Addition of FGF-2 in FCS-containing but not serum-free culture significantly increased proliferation rate when compared to HRG-1β (●, p<0.01) (A, B). FGF-2 and in combination with forskolin (FGF-2/Forskolin) but not forskolin alone increased the proliferation in both FCS- and serum-free cultures (*, p<0.01) (C, D). FGF-2/forskolin stimulation in OEC slightly increased proliferation when compared to stimulated with FGF-2 alone (●, p<0.01) (D). In addition, forskolin alone in serum-free culture condition decreased growth rates in both cell types when compared to control (●, p<0.01) (C, D).
3.3 Antigenic expression of adult canine Schwann cells and OECs
To investigate whether the morphological transition at higher passages (>25) and the loss of growth factor responsiveness were paralleled by an alteration in the antigenic phenotype, Schwann cells and OECs from low (P4) and high (P26) passages were stained with a panel of monoclonal antibodies (Fig.3C,D, Fig.4). There was no significant alteration in the percentage of p75NTR-positive cells during culturing and the expression of this receptor was maintained up to the highest passage analyzed in Schwann cells (Fig.3C, Fig.4A,C). The reduced percentage of p75NTR-positive OECs at low passage was due to an increased proportion of p75NTR-negative fibroblast-like cells (♦, Fig.3D, Fig.4B) and not to an incomplete staining of OECs as revealed by microscopic analysis of OEC cultures at passage 26 (Fig.4D). Here, all OECs displayed positive staining for p75NTR (Fig.4D). In addition, p75NTR expression did not change upon FGF-2 treatment. Contrary to this was expression of O4, A2B5 and GFAP. Whereas the expression of O4 and GFAP showed significant alterations between low and high passages of non-FGF-2-treated Schwann cells and OECs (♦, Fig.3C,D), the change in A2B5 expression was confined to Schwann cells only (♦, Fig.3C). Moreover, addition of FGF-2 significantly decreased O4 expression at low passage in Schwann cells and OECs (∗, Fig.3C,D).
Fig. 3: Short-term growth analysis using BrdU incorporation assays (A, B) and antigenic profile (C, D) of Schwann cells (SC, A, C) and olfactory ensheathing cells (OEC, B, D) in the absence (∅ FGF-2) and presence of FGF-2 (+ FGF-2). For BrdU assays, the significances were observed in FGF-2-treated cultures of passage 4 compared to untreated control cells (*, p<0.01) and compared to FGF-2-treated high passage cells (P26; †, p<0.01). Identical results were obtained for both cell types. p75NTR expression was stable throughout the culturing period, whereas O4 and GFAP expression were changed either at higher passages (P26) or after FGF-2 treatment. Both cell types showed similar antigenic expression profiles with exception of A2B5 that was more stably expressed in OECs.
Fig. 4: Micrographs illustrating p75<sup>NTR</sup> expression in Schwann cells (SC, A, C) and olfactory ensheathing cells (OEC, B, D) at low (P4) and high passage (P26). Schwann cells and OECs at passage 26 displayed a more flattened morphology (C, D) compared to both cells from passage 4 (A, B). Scale bar ~ 200 µm.

4. DISCUSSION
The adult dog is an important translational model system to study Schwann cell- and olfactory ensheathing cell (OEC)-mediated neural repair (Smith et al., 2002; Jeffery et al., 2006). It was shown that cryopreserved adult canine OECs can remyelinate the rat spinal cord (Smith et al., 2002) and that the autologous transplantation of adult canine OECs is a clinically safe procedure (Jeffery et al., 2005). However, so far only a few studies reported on their in vitro properties (Krudewig et al., 2006; Ito et al., 2006, 2008; Bock et al., 2007), and the relationship of canine OECs to rodent and human cells has remained elusive. What has also remained unclear is whether OECs and Schwann cells display differential in vitro properties that may be relevant to their therapeutic transplantation into the lesioned nervous system (Franklin and Barnett, 1997). This important question is still discussed controversially in the well studied rodent model and is completely open for the canine system.
In the present study, we, therefore, purified Schwann cells and OECs from the adult canine sciatic nerve and olfactory bulb, respectively, and further comparatively analyzed their in vitro properties. Main focus was on the analysis of long-term proliferation and antigenic expression since significant species-specific differences between rodent and primate OECs were recently reported (Rubio et al., 2008). The understanding of glial cell proliferation is not only relevant for transplantation purposes, e.g. for generating sufficient cell numbers or for estimating the tumor-forming capacity of transplanted cells, but may also help to devise therapeutic strategies counteracting malignant transformation (Emery et al., 1999).

The main findings of the present study are that adult canine glia displays long-term growth in the absence of mitogens and that it does not enter senescence until 3 months in vitro. This is in striking contrast to rodent glia that requires mitogenic stimulation during expansion in vitro (Sonigra et al., 1996; Mathon and Lloyd, 2001). Moreover, no significant differences were found in the growth behaviour and antigenic expression between Schwann cells and OECs underscoring the assumption of a close relationship of both cell types (Wewetzer et al., 2002; Wewetzer and Brandes, 2006).

Only a few studies so far have focused on the long-term growth characteristics of rodent glia. It is well established that rodent Schwann cells and OECs are mitotically quiescent upon dissociation and culturing (Davis and Stroobant, 1990; Wewetzer et al., 2001; Yan et al., 2001). Proliferation is routinely promoted by a combination of growth factors, such as neuregulins or FGF-2, and agents elevating the intracellular cAMP level, such as forskolin or dbcAMP (Sobue et al., 1986; Jessen et al., 1991; Wewetzer et al., 2001; Yan et al., 2001; Alexander et al., 2002). It was shown that prolonged mitogenic stimulation of both Schwann cells and OECs can result in spontaneous immortalization (Eccleston et al., 1991; Bolin et al., 1995; Sonigra et al., 1996; Funk et al., 2007). Recently, Rubio et al. (2008) comparatively analyzed the long-term proliferation of rat and primate OECs and showed that rat OECs entered a senescent state early in culture, whereas primate OECs exhibited an extended life span in vitro and maintained proliferation in the absence of growth factors for more than 3 months (Rubio et al., 2008). Moreover, primate but not rodent OECs maintained expression of p75NTR and did not increase proliferation in response to forskolin (Rubio et al., 2008). As observed in the present study, adult canine Schwann cells and OECs did not only display long-term proliferation in the absence of growth factors but also stably expressed p75NTR throughout the entire observation period. In agreement with previous findings, we confirmed that canine OECs do not respond to forskolin (Krudewig et al., 2006) and extended this observation to the closely related Schwann cells. This suggests that canine glia shares characteristics with primate cells and may, therefore, serve as a good translational model for humans. Further studies have to demonstrate in how far the data on canine and primate
OECs can be extrapolated to humans. The growth factor responsiveness of human glia so far has not been comprehensively studied. While human OECs like canine OECs did not respond to forskolin (Barnett et al., 2000), controversial results have been reported for human and monkey Schwann cells (Levi et al., 1995; Avellana-Adalid et al., 1998; Monje et al., 2006).

Another difference between the rodent and the canine system is the susceptibility to spontaneous immortalization. This is a common phenomenon observed in the rodent system (Eccleston et al., 1991; Sonigra et al., 1996; Pringproa et al., 2008). Interestingly, at no time point and under no culture condition immortalization of canine glia was observed in this study. Independent of the presence of growth factors, both canine Schwann cells and OECs up to passage 20 retained the ability to respond to added FGF-2 with proliferation. At higher passages, both cell types did not undergo immortalization but lost the ability to respond to FGF-2. Whether the reduced probability of adult canine glia to undergo immortalization is related to its strong inherent proliferation capacity has to be clarified by further studies. This property of canine glia might be relevant for their transplantation since it may correspond to a reduced potential for malignant transformation that is reminiscent of the human system. It was shown that mitogen-expanded rodent but not human Schwann cells induced tumor growth following transplantation into the sciatic nerve (Langford et al., 1988; Emery et al., 1999).

With regard to their regenerative effects following transplantation it is important to compare the morphological and molecular phenotype as well as the in vitro growth characteristics of both OECs and Schwann cells. Since isolation of canine Schwann cells has so far only been described in a single report (Pauls et al., 2004), it was not clear in how far canine Schwann cells differ from OECs. What is well known from the rodent model is that OECs and Schwann cells display a similar morphological phenotype, share the expression of marker molecules, such as p75NTR, and proliferate in response to the same mitogens, e.g. FGF-2 and heregulin (Davis and Stroobant, 1990; Wewetzer et al., 2001; Yan et al., 2001). This is in agreement with the present findings. Adult canine Schwann cells and OECs displayed the typical spindle-shaped morphology and maintained p75NTR expression even at extended culture periods. Alterations in O4, A2B5, and GFAP expression were identical in both cell types. FGF-2 in OECs and Schwann cells decreased A2B5 and GFAP expression at early passage while increasing their expression at late passage. The FGF-2 induced down-regulation of O4 observed in both OECs and Schwann cells is in agreement with previous findings in adult canine OECs (Krudewig et al., 2006). Taken together, adult canine Schwann cells and OECs displayed the same expression and regulation of the studied antigens implying a close relationship of both cell types. The assumption that OECs and Schwann cells which arise
during development from the olfactory placode and the neural crest, respectively, are not identical at the molecular level is underscored by a recent study. Franssen et al. (2008) used DNA arrays of cultured rat OECs and Schwann cells to reveal differential gene expression in both cell types in vitro.

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Chapter 4

Distinct cell tropism of canine distemper virus strains to adult olfactory ensheathing cells and Schwann cells in vitro

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(Submitted)
ABSTRACT

Canine distemper virus (CDV) can enter the brain via infection of olfactory neurons. Whether olfactory ensheathing cells (OECs) are also infected by CDV, and if yes, how they respond to the virus has remained enigmatic. Here, we exposed adult canine OECs in vitro to several attenuated (CDV-2544, CDV-R252, CDV-Ond, CDV-OndeGFP) and one virulent CDV strain (CDV-5804PeGFP) and studied their susceptibility compared to Schwann cells, a closely related cell type sharing the phagocytizing activity. We show that OECs and Schwann cells were infected by CDV strains albeit to different levels. Ten days post infection (dpi), a mild to severe cytopathic effect ranging from single cell necrosis to layer detachment was noted. The percentage of infection increased during 10dpi and viral progenies were detected in each culture using virus titration. Interestingly, CDV-2544, CDV-OndeGFP, and CDV-5804PeGFP predominantly infected OECs, while CDV-Ond targeted Schwann cells. No significant differences were found between the virulent and attenuated CDV strains. The observation of a CDV strain-specific cell tropism is evidence for significant molecular differences between OECs and Schwann cells. Whether these differences are either related to strain-specific distemper pathogenesis or support a role of OECs during CDV infection and virus spread needs to be addressed in future studies.

KEYWORDS Canine distemper virus, Olfactory ensheathing cell, Schwann cell, Cell tropism

1. INTRODUCTION

Demyelinating distemper leukoencephalitis (DL) is caused by canine distemper virus (CDV), a morbillivirus of the family Paramyxoviridae. Naturally CDV-affected dogs besides rodents experimentally infected by Theiler’s murine encephalomyelitis virus (TMEV) serve as an alternative animal model to study the pathogenesis of demyelination in numerous diseases, including multiple sclerosis (MS) (Baumgärtner and Alldinger, 2005; Kumnok et al., 2008; Ulrich et al., 2006; Vandevelde and Zurbriggen, 2005). CDV-induced lesions frequently found in the central nervous system (CNS), are characterized by variable pathological changes, including acute, subacute non-inflammatory, subacute inflammatory, chronic and sclerotic alterations (Alldinger et al., 2000; Beineke et al., 2009). Outside the CNS, non-nervous tissues such as skin, respiratory and urinary tract are also infected with CDV and display a variety of inflammatory and degenerative lesions (Baumgärtner and Alldinger, 2005; Gröne et al., 2003; Koutinas et al., 2004). However, CDV-induced changes of the peripheral nervous system (PNS) have not been addressed so far.
The broad range of affected species and the wide spread of CDV to various organs and tissues can be recapitulated in vitro (Baumgärtner et al., 2003; Seehusen et al., 2007; van Moll et al., 1995; Wohlsen et al., 2007). Several cell lines and primary cells from different species and tissues have been used for CDV isolation and infection, including African green monkey kidney cells (Vero cells), marmoset lymphoid cells (B95a), Madin-Darby canine kidney (MDCK) cells, canine macrophages (DH82), primary canine macrophages and lymphocytes (Appel and Jones, 1967; Baumgärtner et al., 1987; Frisk et al., 1999a; Gröne et al., 2002; Kai et al., 1993; Puff et al., 2008; Suter et al., 2005; von Messling et al., 2004). The envelope proteins consisting of the hemagglutinin and fusion protein play a key role for cellular attachment and membrane fusion leading to virus entry into the cells (Stern et al., 1995; Takeda, 2008; von Messling et al., 2001) In addition, the restricted cell-cell fusion favoring persistent infection in the CNS is mediated by complex interactions between all viral structural proteins (Wiener et al., 2007).

CDV can gain access to the CNS by different pathways. The hematogenous route transmits infected peripheral blood mononuclear cells which penetrate the blood brain barrier and invade the CNS. Additionally, cell-free viruses are circulating in the cerebrospinal fluid and fuse with the ependymal lining of the ventricles (Frisk et al., 1999a, 1999b; Higgins et al., 1982). Recently, it was shown that CDV enters the brain via the olfactory system in ferrets where transneuronal transmission along the olfactory axons occurs (Rudd et al., 2006). The latter pathway is also used by neurotropic viruses, including Herpes simplex virus, Bornedisease virus, Influenza A virus and rabies virus (Aronsson et al., 2003; Esiri and Tomlinson, 1984; Lafay et al., 1991; Morales et al., 1988). So far, investigations have focussed on olfactory receptor neurons and their role as vehicles for virus infection, while very little is known about the infection and the role of associated glial cells.

Olfactory ensheathing cells (OECs) encircle bundles of olfactory neuron axons spanning the region between the olfactory mucosa and bulb. Besides their role in axonal guidance during prenatal and postnatal development (Doucette, 1990), OECs have been shown to display active phagocytosis (Chuah et al., 1995; Wewetzer et al., 2005) and to secrete cytokines and bacteriocidal substances (Chuah et al., 2004-05; Getchell et al., 2002; Li et al., 2005; Wewetzer et al., 2005). These findings suggest that OECs may play an important role as an immunoregulatory and/or antigen-presenting cell protecting the CNS from invading pathogens. However, whether OECs are infected by neurotropic viruses in vivo remains to be established. To further elucidate the role of these unique glial cell populations for virus spread and distemper pathogenesis, we investigated purified cultures of adult canine OECs and Schwann cells infected with different attenuated and one mustelid virulent CDV strain for a 10-day observation period. This is the first report revealing that OECs and Schwann cells
are infected differently by CDV strains in vitro indicating a strain-specific cytotropic replication of CDV.

2. MATERIALS AND METHODS

2.1 Purification and cultivation of olfactory ensheathing cells (OECs) and Schwann cells

Cells were obtained from Beagle dogs (6-month-old) not suffering from a nervous system disease, as determined by clinic and histopathologic examination. Primary OEC and Schwann cell cultures were isolated from the olfactory bulb and sciatic nerve, respectively, as previously described (Bock et al., 2007; Krudewig et al., 2006; Techangamsuwan et al., 2009). Both single cell suspensions were passed through pre-separation filters (30µm mesh, Miltenyi Biotec, Bergisch-Gladbach, Germany) to remove cell aggregates. Cells were seeded on poly-L-lysine (PLL, 100µg/ml) -coated culture flasks (Nunc™, Nunc GmbH & Co KG, Wiesbaden, Germany) and maintained in DME/10%FCS medium under standard conditions (37°C, 5% CO₂ incubator, water-saturated atmosphere).

Antibody-based purification was done from confluent cultures using magnet-activated cell sorting (MACS, Miltenyi Biotec) as previously described (Krudewig et al., 2006; Techangamsuwan et al., 2008; Wewetzer et al., 2005). Anti-human-p75NTR antibodies (supernatant hybridoma, dilution 1:5, American Tissue Culture Collection, USA) and goat-anti-mouse IgG MicroBeads (dilution 1:20, Miltenyi Biotec) were used as primary and secondary antibodies, respectively. Following labeling with anti-p75NTR antibodies and adsorption to antibody-coated magnetic beads, cells were seeded in PLL-coated culture flasks and expanded in the same medium under standard conditions (see above). Cells from passages 5-7 were seeded at a density of 8 x 10³ cells/well in 96-well PLL-coated microtiter plates (Nunc™) for immunofluorescence assays and of 1.6 x 10⁵ cells/well in 6-well PLL-coated plates (Nunc™) for virus titration assays.

2.2 Canine distemper virus (CDV) infection of OECs and Schwann cells in vitro

Virus infection was done 3 days post seeding. Cultures were inoculated with four attenuated and a virulent CDV strains separately at a multiplicity of infection (MOI) of 0.1 (detailed shown in table 1). The attenuated and mustelid virulent CDV strains were propagated in normal Vero cells and Vero cells expressing canine signaling lymphocyte activation molecule (Vero.DogSLAM; kindly provided by Dr. von Messling, Institute Armand-Frappier, University of Quebec, Canada), respectively. Infection was done as previously described (Orlando et
Briefly, cultures were washed twice with FCS-free DME medium and incubated with 100μl (96-well plate) and 2ml (6-well plate) FCS-free DME medium (mock-infection) or the inoculum (CDV-infection) for 2 h under standard conditions. After removal of the supernatant, cells were washed twice with FCS-free medium and maintained in DME medium supplemented with FCS (10%). Media were replaced every 3 days and the cytopathic effect (CPE) was monitored daily for 10 days.

Table 1 Titers, virulence and cell lines used for canine distemper virus (CDV) strain propagation

<table>
<thead>
<tr>
<th>CDV strains</th>
<th>TCID&lt;sub&gt;50&lt;/sub&gt;/ml</th>
<th>Virulence</th>
<th>Viral-propagated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDV-2544</td>
<td>10&lt;sup&gt;6.5&lt;/sup&gt;</td>
<td>Attenuated</td>
<td>Vero</td>
</tr>
<tr>
<td>CDV-R252</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Attenuated</td>
<td>Vero</td>
</tr>
<tr>
<td>CDV-Ond</td>
<td>10&lt;sup&gt;6.25&lt;/sup&gt;</td>
<td>Attenuated</td>
<td>Vero</td>
</tr>
<tr>
<td>CDV-OndeGFP</td>
<td>10&lt;sup&gt;4.5&lt;/sup&gt;</td>
<td>Attenuated</td>
<td>Vero</td>
</tr>
<tr>
<td>CDV-5804PeGFP</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Virulent</td>
<td>Vero.DogSLAM</td>
</tr>
</tbody>
</table>

1 The CDV-2544 strain was kindly provided by Dr. Haas, Institute of Virology, University of Veterinary Medicine Hannover, Germany. The CDV-R252 strain was gently provided by Dr. Krakowka, Ohio State University, USA. The CDV-Ond strain was a kind gift from Dr. Metzler, Institute of Virology, Veterinary Medical Faculty, University of Zürich, Switzerland. The CDV-OndeGFP and the recombinant mustelid virulent CDV-5804PeGFP strain were kindly provided by Dr. von Messling, Institute Armand-Frappier, University of Quebec, Canada.

2 Tissue Culture Infectious Dose 50 per milliliter (TCID<sub>50</sub>/ml)

3 African green monkey kidney cell line (Vero), Vero.DogSLAM (Vero cells constitutively expressing the canine signaling lymphocyte activation molecule; SLAM; kindly provided by Dr. von Messling, Institute Armand-Frappier, University of Quebec, Canada)

2.3 Virus titration

After 2 h (hpi), 3 and 10 days post infection (dpi), the supernatant (SNT) and the cell sediment (SED) were harvested to calculate the 50% log<sub>10</sub> tissue culture infectious dose/milliliter (TCID<sub>50</sub>/ml) of cell-free (SNT) and cell-associated progeny virus (SED) of CDV-infected cultures. Briefly, the SNT was centrifuged (700x g, 10 min, 4°C), aliquoted and stored at -80°C until used, while the SED was obtained from scraped cells collected into FCS-free DME medium followed by three cycles of freezing/thawing to release cell-associated viral particles prior to centrifugation. Both SNT and SED were diluted logarithmically from 10<sup>0</sup> to 10<sup>-8</sup> in DME medium containing FCS (10%) and zeocin (0.5%) (InvivoGen, CAYLA, Toulouse, France) and titrated in quadruplicates in 96-well microtiter plates (Nunc™) containing Vero.DogSLAM cells (1.5 x 10<sup>4</sup>/well). After a 5-day incubation period under standard conditions, the cells were examined for CPE and the TCID<sub>50</sub> was
calculated according to the Reed and Muench method (Frisk et al., 1999a). Analysis was done by using three independent samples.

2.4 Immunofluorescence

For characterization of cultures and for the numeric determination of virus infection, OECs and Schwann cells were incubated with antibodies against p75 neurotrophin receptor (p75NTR, dilution 1:5; ATCC) and CDV nucleoprotein (CDV-NP, batch #25, dilution 1:2000, kindly provided by Dr. C. Örvell, Central Microbiological Laboratory of Stockholm, Sweden). CDV antigen and p75NTR were identified on fixed and viable cells, respectively. The used secondary antibodies were goat-anti-mouse Cy3 (for p75NTR) and goat-anti-rabbit Cy2 (for CDV-NP) antibodies, each used at a dilution of 1:200 (Jackson Immunoresearch, Dianova, Hamburg, Germany). Nuclei were counter-stained with bisbenzimide (Hoechst 33258, 0.01% in methanol, Sigma-Aldrich, Taufkirchen, Germany) and the experiments were done in triplicates. The numeric evaluation of infected cells was done by determining the number of CDV-positive cells in relation to the total number of cells, visualized by bisbenzimide staining. Five high-power fields per 96-well were counted using an inverted fluorescence microscope (Olympus IX-70, Hamburg, Germany).

2.5 Statistical analysis

Data analysis was done using the statistical software SAS, version 9.1 (SAS Institute, Cary, NC). The infection data were logarithmically transformed to achieve an approximately normal distribution; however geometric mean and geometric standard deviation were calculated using the original scale. The data between CDV-infected OECs and Schwann cells were compared using one-way analysis of variance (ANOVA) with “virus strain” as independent effect and “time post infection” as repeated measures applying the Tukey’s post-hoc test for multiple pairwise comparisons. P-values of less than 0.05 were considered to indicate statistically significant differences. All data were expressed as mean, maximum and minimum.

3. RESULTS

3.1 In vitro infection of adult canine OECs and Schwann cells

After inoculation of OECs and Schwann cells with CDV, cultures were monitored daily for CPE during 10 days. The CPE characteristics defined by single cell necrosis and cellular detachment were classified as minimal (less than 5%), mild (6-20%), moderate (21-60%) and
severe (greater than 60%) compared to mock-infected controls at the same time points (Table 2).

Initially, the CPE in both OEC and Schwann cell cultures was characterized by single cell necrosis of a low percentage (minimal-mild) and increased in severity to monolayer detachment (moderate-severe) at 10dpi (Fig. 1). No multinucleated syncytial cell formation was found in both infected cultures at any time point. At 3dpi, a mild CPE was seen in the infected OEC cultures independent of the virus strain used. A moderate CPE was observed in CDV-Ond-infected Schwann cells while a minimal to mild CPE was found after infection with other strains (Table 2). The severity of the CPE increased in OEC but not in Schwann cell cultures during the observation period. At 10dpi, the CPE was dependent on the cell type and CDV strain (Table 2). Both cultures infected with CDV-Ond displayed a severe CPE. Whereas OECs infected with CDV-2544, CDV-R252, CDV-OndeGFP and CDV-5804PeGFP demonstrated a moderate CPE, affected Schwann cells showed only a minimal to mild CPE (Table 2).

The use of an enhanced GFP-expressing virus allowed the direct observation of infected cells in living cultures. Single CDV-OndeGFP-expressing OECs and Schwann cells were seen as early as 1dpi, while CDV-5804PeGFP-expressing cells were observed later at 3dpi. Interestingly, the virus spread differed between the GFP-expressing viruses but not between the cell types. While the CDV-OndeGFP displayed a random single cell distribution, CDV-5804PeGFP expressing cells were found in patches consisting of multiple cells. The number of infected cells and the size of the patches increased continuously from 3 to 10dpi.

Table 2  Cytopathic effect (CPE) induced by various canine distemper virus (CDV) strains in olfactory ensheathing cells and Schwann cells

<table>
<thead>
<tr>
<th>CDV strains</th>
<th>Olfactory ensheathing cells</th>
<th>Schwann cells</th>
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<tbody>
<tr>
<td></td>
<td>3 dpi ²</td>
<td>7 dpi</td>
</tr>
<tr>
<td>CDV-2544</td>
<td>Mild</td>
<td>Moderate</td>
</tr>
<tr>
<td>CDV-R252</td>
<td>Mild</td>
<td>Moderate</td>
</tr>
<tr>
<td>CDV-Ond</td>
<td>Mild</td>
<td>Moderate</td>
</tr>
<tr>
<td>CDV-OndeGFP</td>
<td>Mild</td>
<td>Moderate</td>
</tr>
<tr>
<td>CDV-5804PeGFP</td>
<td>Mild</td>
<td>Mild</td>
</tr>
</tbody>
</table>

¹ CDV (canine distemper), ² dpi (days post infection)
Fig. 1 Adult canine olfactory ensheathing cells (OECs) mock-infected (A) and infected with CDV-R252 (B) and CDV-Ond (C) at 10 days post infection. CDV-R252 and CDV-Ond induced a moderate and severe cytopathic effect (CPE) consisting of single cell necrosis and cellular loss compared to mock-infected control. Scale bar: 100 μm.

3.2 Differential CDV infection of OECs and Schwann cells but stable expression of p75NTR

The numeric evaluation of mock-infected and CDV-infected cells at different time points with anti-p75NTR and/or anti-CDV-NP antibodies for non-GFP-expressing viruses, revealed a strain-specific and cell type-specific tropism of CDV infected OECs and Schwann cells. The purity of cultures was determined by immunostaining with p75NTR antibodies revealing the presence of more than 95% OECs and Schwann cells (data not shown). Following CDV infection, no substantial alteration of p75NTR expression was found in any culture compared to the mock-infected controls, regardless of the virus strain used (Fig. 3).

To demonstrate CDV infection of OECs and Schwann cells, co-localization of p75NTR and CDV-NP, was numerically determined (Fig. 2, 3). Virus antigen was found in the cytoplasm of infected cells in the soma and along cell processes (Fig. 3). The percentage of infected cells at 10dpi was significantly increased compared to 3dpi for all virus strains used (Fig. 2, p<0.01). At 3dpi, p75NTR+ OECs were infected by CDV-2544, CDV-OndeGFP and CDV-5804PeGFP at a percentage of 24%, 2% and 2%, respectively, compared to about 0.3% of Schwann cells infected with the same strains (Fig.2, p<0.01). Similar differences of the percentage of infected cells were found for the same strains at 10dpi and 100%, 17% and 34% of OECs and 46%, 5%, and 5% of Schwann cells were infected (Fig. 2, p<0.05, Fig. 3). Contrary to this, infection with CDV-Ond was more prominent in Schwann cells. At 3 and 10dpi, 13% and 70% of Schwann cells were infected compared to about 5% and 27% of OECs, respectively (Fig. 2, p<0.01, Fig. 3). The percentage of CDV-R252 infection was not different between both cell types (Fig. 2).
Fig. 2 Percentage of adult canine olfactory ensheathing cells (OECs) and Schwann cells (SCs) expressing CDV antigens at different time points post infection. The number of infected OECs and SCs increased at least three-fold between day 3 and 10, independent of the virus strain used (φ, p<0.01). At 3 and 10 days post infection (dpi), the percentage of OECs infected by CDV-2544, CDV-OndeGFP, and CDV-5804PeGFP was higher compared to SCs (*, p<0.05), while infection with CDV-Ond was found predominantly in SCs at the same time points (♦, p<0.01). No differences were detected following CDV-R252 infection. The data are displayed as median, maximum and minimum.
Fig. 3 Immunostaining for p75NTR (A, D; red) and CDV antigens (B, E, green) of CDV-2544-infected adult canine olfactory ensheathing cells (OECs, A-C) and CDV-Ond-infected Schwann cells (SCs, D-F) and their co-localization (merged, C, F) at 10 days post infection. CDV antigen is localized in the cytoplasm and along cell processes (B, E) of both p75NTR-positive OECs and SCs. In infected SCs, some CDV-negative cells could be detected (D-F; arrows), while infection of OECs by CDV-2544 was found in 100% of all cells. Scale bar: 50 μm.

3.3 Detection of progeny virus in OEC and Schwann cell cultures infected with different CDV strains

To examine the infected cultures for progeny virions, both cell-free virus in the SNT and cell-associated virus in the SED was determined. All samples were titrated in quadruplicates using the Vero.DogSLAM cells and the TCID<sub>50</sub>/ml was calculated after 5 days (Fig. 4). Replicating virus could be detected throughout the observation period in all cultures and virus strains used. The cell-associated virus in the SED was detected as early as 2hpi after infection with the highest titers in CDV-R252-infected cultures. However, there were no substantial differences of the SED titers between virus strains at this time point. Cultures infected with CDV-R252 displayed the highest titers in both SNT and SED at 3 and 10dpi (Fig. 4).
Progeny virions of non-GFP-expressing CDV strains (CDV-2544, CDV-R252, CDV-Ond) were more prominent in the SNT compared to the SED. Contrary to this, the CDV-OndeGFP and CDV-5804PeGFP strain displayed titers higher in the SED than in the SNT (Fig. 4).

**Fig. 4** The cell-free and cell-associated virus titers of CDV-infected adult canine olfactory ensheathing cells (OECs) and Schwann cells (SCs) expressed as 50% log$_{10}$ tissue culture infectious dose/milliliter (log$_{10}$ TCID$_{50}$/ml). For cultures infected with CDV-2544, CDV-R252 and CDV-Ond, the amount of cell-free virus was higher compared to of cell-associated progeny virus, whereas in cultures infected with CDV-OndeGFP and CDV-5804PeGFP the higher titers
4. DISCUSSION

It is well established that CDV infection of the CNS primarily affects central glial cells, such as microglia, oligodendrocytes and astrocytes, and that the virus enters neurons via transmission from infected glial cells (Baumgärtner and Alldinger, 2005; Beineke et al., 2009; Vandevelde and Zurbriggen, 2005). However, very little is known about infection of peripheral glia, such as Schwann cells. CDV has not been detected in Schwann cells so far, and although infected dogs display neurological symptoms, such as paresis, there is no evidence for a CDV-induced peripheral neuropathy during naturally occurring CDV infection.

Olfactory ensheathing cells (OECs) are the specialized glial cells of the olfactory system and are closely related to Schwann cells (Wewetzer and Radtke, 2009; Wewetzer et al., 2002). OECs that are associated with olfactory neurons within the olfactory nerves and olfactory bulb are considered an intermediate glial cell type sharing properties with astrocytes and Schwann cells (Barber and Lindsay, 1982; Doucette, 1984). The life-long production of olfactory neurons and axonal growth, guided to the olfactory bulb by OECs has been interpreted as a protective mechanism preventing the impairment of the olfactory senses (Loseva et al., 2008). However, recently, it was shown that CDV uses the olfactory pathway in addition to the well established hematogenous route for neuroinvasion (Rudd et al., 2006).

Infection of ferrets with the CDV strain A75/17 engineered to express eGFP resulted in infection of olfactory neurons at 14 days post infection and subsequent transsynaptic transmission of CDV to the olfactory bulb (Rudd et al., 2006). Since OECs display very thin processes encircling bundles of olfactory axons (Field et al., 2003; Raisman, 1985) and no reliable markers for adult OECs have become available so far (Wewetzer et al., 2002), an association or interaction of CDV with OECs is difficult to demonstrate in situ.

In the present study, we investigated whether OECs and Schwann cells can be infected in vitro with different attenuated or virulent CDV strains, and whether there are significant differences regarding susceptibility to the different viruses used. OECs, like Schwann cells, are known to display active phagocytosis (Chuah et al., 1995; Wewetzer et al., 2005) and were proposed to act as immunoregulatory and/or antigen-presenting cells (Chuah et al., 2004-05; Getchell et al., 2002; Li et al., 2005).

We show here that both OECs and Schwann cells can be infected by various CDV strains, albeit to a different extent. The CDV-2544, CDV-OndeGFP, and CDV-5804PeGFP strains infected significantly more OECs than Schwann cells, whereas the CDV-Ond strain mainly targeted Schwann cells. No significant differences were found between OECs and Schwann cells following infection with CDV-R252. Moreover, the mustelid virulent CDV-5804PeGFP and the attenuated CDV-OndeGFP strain did not differ in their ability to infect OECs and Schwann cells. Both strains infected OECs to a higher level than Schwann cells.
Although it remains to be clarified, whether OECs and Schwann cells are targets for CDV *in vivo*, putative infection is apparently not prominent (Rudd et al., 2006). Successful and differential infection of OECs and Schwann cells as observed in the present study, therefore, could have two main implications. Firstly, the susceptibility of the cells would be apparently increased upon isolation and culturing. As discussed below, this might point to the underlying mechanism of CDV infection and associated cellular receptor expression. Secondly, the differential infection of both closely related cell types could be interpreted as evidence for subtle differences in the molecular setup between both cell types. However, this needs to be substantiated by detailed molecular *in vitro* and *in vivo* studies.

Increased susceptibility of OECs and Schwann cells *in vitro* may be due to alterations in gene expression. It was shown that the p75 neurotrophin receptor (p75NTR) is a functional but not essential receptor for the neurotropic rabies virus (Tuffereau et al., 1998, 2007). Previously, we demonstrated that p75NTR-positive Schwann cell-like glial cells of the adult canine brain are preferentially infected *in vitro* with attenuated CDV strains compared with other p75NTR-negative cell populations, such as astrocytes, microglia and fibroblasts (Orlando et al., 2008). The up-regulation of p75NTR expression observed in Schwann cells and OECs (Bock et al., 2007; Wewetzer et al., 2005) following cultivation would help to explain the discrepancy between *in situ* and *in vitro* CDV infection.

The observation that OECs and Schwann cells display differential susceptibility to the different CDV strains may have important cell biological implications regarding their molecular identity. OECs and Schwann cells differ substantially in morphology and molecular expression *in situ* but not *in vitro* (Jessen and Mirsky, 1991; Jessen et al., 1990; Wewetzer and Brandes, 2006; Wewetzer et al., 2002). This discrepancy has been attributed to specific neuron-glia interactions determining their phenotype *in situ* (Wewetzer and Brandes, 2006). The degree of homology between both cell types is reflected by the fact that no cell type-specific markers for cultured OECs and Schwann cells have been identified so far (Bock et al., 2007; Harvey and Plant, 2006; Wewetzer et al., 2005). The differential susceptibility of both cell types can only be explained on the basis of molecular differences. Recently, Franssen et al. (2008) performed microarray analysis of cultured adult rat OECs and Schwann cells and demonstrated significant differences in the gene expression profile between OECs and Schwann cells (Franssen et al., 2008). This is the first convincing evidence for differences between OECs and Schwann cells at a molecular level and is in agreement with the present study. Assuming an involvement of p75NTR in CDV infection, cell type-specific differences in the expression level of this receptor could be expected. Thus, future studies have to analyze quantitatively p75NTR expression and to investigate possible correlations between p75NTR expression level and susceptibility to CDV infection.
Taken together, our data show that non-virulent and virulent strains of CDV differentially infect OECs and Schwann cells in vitro. Future studies have to investigate the role of p75\textsuperscript{NTR} expression during CDV entry into the cells and to determine whether OECs represent a crucial cell population during in vivo CDV infection and distemper pathogenesis.

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Chapter 5

General discussion
5.1 Transfection of adult canine Schwann cells and olfactory ensheathing cells at late passage with human TERT differentially affects growth factor responsiveness and in vitro growth

Schwann cells and olfactory ensheathing cells (OECs) have been shown to promote axonal regeneration and remyelination following transplantation into the lesioned nervous system (Barnett and Riddell, 2007; Baron-Van Evercooren et al., 1997; Wewetzer et al., 2002). However, most of the research has been done in the rodent model and it is not clear in how far data can be extrapolated and used for clinical application. Large animal models offer the opportunity of being more closely related to humans. However, there are limitations in supply with tissues and cells that hinder a systematic characterization. One possibility to overcome these limitations is the generation of stable cell lines by immortalization that display infinite growth in vitro without substantial alterations of their morphological and molecular phenotype. Immortalization of cultured mammalian cells has been achieved using different methods, including application of chemical agents, radiation, infection with oncogenic viruses or transfection with telomerase reverse transcriptase (TERT) (Akimov et al., 2005; Cannell et al., 1996; Lee et al., 2002; Olson et al., 2003; Ouyang et al., 2000; Uebing-Czipura et al., 2008). TERT transfection offers the advantage of maintaining a stable genotype and preserving the differentiated and functional phenotype of the immortalized cells, while abnormal growth patterns, cell transformation and genomic instability are rarely described (Chen and Thibeault, 2008; Lee et al., 2004). These characteristics are essential for cells that will be used for cell transplantation.

Adult canine Schwann cells and OECs at early (passage 4) and late passage (passage 26) were transfected using ectopic human TERT (hTERT). Control experiments with non-transfected cells showed that cellular senescence of both cell types occurred after passage 26. Selection of the clones was done using cloning rings and cultivation in puromycin-containing medium. All transfected Schwann cell and OEC clones expressed hTERT mRNA and gained full telomerase activity independent of the passage level. Surprisingly, however, Schwann cells and OECs escaped from the growth arrest and underwent immortalization only after late passage transfection and cultivation in the presence of fibroblast growth factor-2 (FGF-2). Transfection of early passage cells did not induce immortalization but slowed down proliferation independent of FGF-2 (Techangamsuwan et al., 2009). This was accompanied by subtle differences in the expression of marker molecules. Transfection significantly altered the expression of O4 and GFAP of early passage cells and of O4 and A2B5 of late passage cells. This demonstrates that the altered growth rate and the changed molecular phenotype induced by hTERT transfection are dependent on the replicative age.
This shows for the first time that hTERT is able to reduce proliferation. So far, TERT expression has always been shown to stimulate cellular proliferation (Gorbunova and Seluanov, 2003; Jin et al., 2006; Smith et al., 2003; Xiang et al., 2002; Young et al., 2003). This unexpected finding might be the result of the early introduction of ectopic hTERT expression at a time long before growth arrest of the cells occurs. The vast majority of studies introduced TERT into cells whose telomere had already reached a critical length. Our observation might, therefore, be the basis to reveal not yet characterized additional functions of hTERT regarding cell cycle control.

The observation that non-transfected adult canine glia did not undergo spontaneous immortalization, maintained responsiveness to FGF-2 and grew throughout multiple passages before entering cellular senescence is reminiscent on human and different from rodent cells. Several studies have shown that normal human cells rarely undergo spontaneous immortalization (Di Donna et al., 2003; Kuroki and Huh, 1993) while this might occur in rodent cells after only a few serial subcultivations (Hoeben et al., 1995; Masamune et al., 2003) or after continued growth factor stimulation (Kotev-Emeth et al., 2002; Pringproa et al., 2008). There is only one report describing the spontaneous immortalization of canine cells. However, the immortalized canine cells were derived from the embryonic and not the adult stage (You et al., 2004).

Analysis of non-transfected Schwann cells and OECs demonstrated that both cell types constitutively expressed dog telomerase RNA (dTR) but were negative for dTERT indicating a lack of the minimum requirements necessary to gain full telomerase activity and to maintain telomere length of dividing cells. This is in agreement with previous findings in humans (Blackburn, 1992; Cairney and Keith, 2008; Mceachern et al., 2000; Shay and Wright, 2007) but in striking contrast to rodent glia. Rodents frequently express both components of the telomerase complex and lack replicative senescence (Hahn and Weinberg, 2002; Mathon et al., 2001; Prowse and Greider, 1995; Tang et al., 2001). As a consequence, adult canine Schwann cells and OECs used in this study proliferated well at early passages and slowed down division from passage 26 on even in growth factor-supplemented medium. This is different from the rodent glia that divide rigorously throughout multiple passages in the presence of mitogens and immortalize spontaneously (Mathon et al., 2001; Tang et al., 2001). In addition, the presence and absence of p53 and p16 mRNA expression was identical in both cell types and unaltered by transfection of cells from different passages. This may suggest that the pathway to senescence in adult canine glia involves a p53-mediated and telomere-dependent mechanism similar to human cells (Argyle and Nasir, 2003; Nasir, 2008).
Taken together, adult canine Schwann cells and OECs at late passage were successfully immortalized by ectopic telomerase transfection in combination with mitogen stimulation without substantial alterations of their morphological and molecular phenotype. The fact that observed alterations of growth rate and antigenic expression following transfection were identical in Schwann cells and OECs is strong evidence for a close relationship of both cell types, including comparable controls of cellular proliferation.

5.2 Similar behaviour and primate-like properties of adult canine Schwann cells and olfactory ensheathing cells in long-term culture

It is well established that non-myelin-forming Schwann cells and OECs are closely related glial cell types that share the morphological and antigenic phenotype, growth factor responsiveness in vitro, and the ability to myelinate a CNS axon after transplantation into the lesioned nervous system (Franklin et al., 1996; Smith et al., 2001; Wewetzer et al., 2002). The majority of studies so far focused on the rodent model. Large animal models, however, are required to translate basic research into human clinical practice (Wewetzer and Radtke, 2009). The adult dog is a relevant species in this context because it suffers from diseases similar to humans, including G_{M1}-gangliosidosis, Alzheimer’s disease and chronic kidney diseases (Aresu et al., 2008; Cotman and Head, 2008; Krakowka and Felsburg, 2005; Kreutzer et al., 2008; Opii et al., 2008). Moreover, the brain and spinal cord diameter of dogs are more comparable to humans and the animals live in the identical environment as humans. Finally, dogs develop malignant brain tumors and neurological disorders similar to humans (Jeffery et al., 2005, 2006; Kapil et al., 2008).

In the present study, the physiological in vitro properties of adult canine Schwann cells and OECs were comparatively analyzed. Main attention was paid to long-term proliferation, growth factor responsiveness and antigenic expression of cultured cells. Recently, differences in proliferation and cellular senescence were reported between rodent and primate OECs (Rubio et al., 2008). The major findings of the present study are that both cell types displayed long-term growth in the absence of mitogens and they did not enter senescence until 3 months culturing in vitro (Techangamsuwan et al., 2008). This is reminiscent of primate OECs that were shown to possess an extended life span in vitro and maintain proliferation in the absence of growth factors for more than 3 months (Rubio et al., 2008), but differs from rodent glia that is mitotically quiescent upon dissociation and culturing (Davis and Stroobant, 1990; Wewetzer et al., 2001; Yan et al., 2001) and requires mitogenic
Forskolin, increasing intracellular cAMP concentration, is well known for its capacity to stimulate proliferation of rodent cells alone or in combination with growth factors, such as heregulin-1ß or FGF-2. However, forskolin did not have any effects on adult canine Schwann cells and OECs. This is in accordance with previous studies in dogs (Krudewig et al., 2006), primates (Rubio et al., 2008) and humans (Barnett et al., 2000), but it is in striking contrast to rodent glia (Alexander et al., 2002; Jessen et al., 1991; Sobue et al., 1986; Wewetzer et al., 2001; Yan et al., 2001) underscoring the relationship of canine and human OECs. However, this is only true for the OECs since contradictory observations have been reported for human and monkey Schwann cells (Avellanada-Adalid et al., 1998; Levi et al., 1995; Monje et al., 2006).

The apparent differences in growth control between human and rodent cells have also consequences for the frequency of spontaneous immortalization events. As discussed previously, rodent but not human cells are likely to undergo immortalization in the continued presence of growth factors and/or during long-term cultivation (Bolin et al., 1995; Eccleston et al., 1991; Funk et al., 2007; Sonigra et al., 1996). In the present study, neither Schwann cells nor OECs underwent immortalization following prolonged mitogenic stimulation (Techangamsuwan et al., 2008). Moreover, the typical bi- to tripolar, spindle-shaped morphology of both canine cell types remained unchanged up to passage 25. At higher passages (>25) there was a transition to a more flattened phenotype. Interestingly, canine glial cells in long-term culture constantly expressed p75NTR. This is similar to primate but different from rodent OECs (Rubio et al., 2008; Techangamsuwan et al., 2008).

Taken together, it is demonstrated that canine glia closely resembles primate glia regarding in vitro properties, such as long-term growth and antigenic expression. This further underscores the suitability of the adult dog as a translational model to develop cell-transplantation-based novel therapeutic approaches to human diseases and injuries. Further investigations have to demonstrate how far the data on canine and primate OECs can be extrapolated to humans. The fact that Schwann cells and OECs behaved identically in vitro during long-term culture is further evidence for the close relationship of both cell types (Wewetzer and Brandes, 2006; Wewetzer et al., 2002).
5.3 Distinct cell tropism of canine distemper virus strains to adult olfactory ensheathing cells and Schwann cells in vitro

It is well established that OECs play an important role in the guidance of olfactory neuron axons from the nasal mucosa to the olfactory bulb during prenatal and postnatal development (Doucette, 1990). OECs, like Schwann cells, have been shown to display active phagocytosis (Bray et al., 1972; Chuah et al., 1995; Saada et al., 1996; Wewetzer et al., 2005) and to secrete pro- as well as anti-inflammatory cytokines (Cheepudomwit et al., 2008; Getchell et al., 2002; Li et al., 2005; Meyer zu Hörste et al., 2008; Rutkowski et al., 1999; Wewetzer et al., 2005). These observations suggested that both cell types may participate in immunoregulatory responses thereby protecting the nervous system from invading pathogens. Canine distemper virus (CDV), a member of the genus Morbillivirus of the family Paramyxoviridae, causes systemic and/or neurological signs (Baumgärtner and Alldinger, 2005; Beineke et al., 2009). Recently, it was shown that CDV enters the brain via the olfactory system in ferrets where transneuronal transmission along the olfactory axons occurs (Rudd et al., 2006). So far, investigations have focussed on olfactory receptor neurons and their role as vehicles for virus infection, while very little is known about the infection of OECs. Whether peripheral glia, such as Schwann cells act as a natural target for CDV has not been reported so far.

In the present study, cultures of adult canine Schwann cells and OECs were exposed to several attenuated (CDV-2544, CDV-R252, CDV-OndeGFP, CDV-Ond) and one mustelid virulent CDV strain (CDV-5804PeGFP). Both cell types were infected by CDV strains albeit to different levels. The cytopathic effect of affected cultures consisted of a few single necrotic cells and prominent monolayer detachment. Formation of multinucleated syncytial cells was absent in infected glial cultures. This is different from cell lines, such as African green monkey kidney cells (Vero cells), marmoset lymphoid cells (B95a) and subcutaneous fibroblasts which frequently show multinucleated syncytial formation following morbillivirus infection (Beineke et al., 2009; Gröne et al., 2002; Nishi et al., 2004). The percentage of CDV-infected cells increased until 10 days post infection as visualized by the co-localization of the cell type-specific marker p75$^{NTR}$ and the CDV nucleoprotein. In addition, all infected cultures produced infectious virus particles.

Following infection in vitro, both Schwann cells and OECs were affected by the different attenuated and virulent CDV strains, albeit to a variable extent. The CDV-2544, CDV-OndeGFP, and CDV-5804PeGFP strains infected significantly more OECs compared to Schwann cells, whereas the CDV-Ond strain mainly targeted Schwann cells. No difference was found between both cell types following CDV-R252 infection and between the virulent
CDV-5804PeGFP and attenuated CDV-OndeGFP strains. The differential susceptibility of the both closely related cell types can be interpreted as evidence for the presence of subtle molecular differences between Schwann cells and OECs. This is in agreement with recent investigations reporting significant differences in the gene expression profile between both cell types at the molecular level in rats (Franssen et al., 2008). However, though CDV can infect the CNS via transneuronal transmission along olfactory neuron axons (Rudd et al., 2006), the localization of CDV in OECs is difficult to demonstrate in situ since the cells have only thin processes encircling bundles of olfactory axons (Field et al., 2003; Raisman, 1985). Furthermore, no reliable cell type-specific markers for the identification of adult OECs in situ have become available (Wewetzer et al., 2002). Whether OECs become infected during CDV entry into the brain is not clear and has to be addressed by future studies.

The differential susceptibility of Schwann cells and OECs to CDV infection in vitro, as observed in the present study may have two main implications. Firstly, the increased susceptibility of the cells for CDV could be the results of cell isolation and culturing. Secondly, the differential infection of both closely related cell types could be interpreted as evidence for subtle differences in the molecular setup between both cell types. It was shown that the p75 neurotrophin receptor (p75NTR) is a functional but not essential receptor for the neurotropic rabies virus (Tuffereau et al., 1998, 2007). This observation is in agreement with a previous report showing the preferential infection of p75NTR-positive Schwann cell-like glial cells in adult canine brain in vitro by attenuated CDV strains (Orlando et al., 2008). In addition, the up-regulation of p75NTR expression observed in Schwann cells and OECs (Bock et al., 2007; Wewetzer et al., 2005) following cultivation, but its lack of expression in situ, would help to explain the discrepancy between in situ and in vitro CDV infection. Nevertheless, it still remains to be clarified whether OECs and Schwann cells are targets for CDV infection in situ.

To summarize, the non-virulent and virulent strains of CDV differentially infected Schwann cells and OECs in vitro. Assuming a possible involvement of p75NTR in CDV infection, future studies have to analyze quantitatively p75NTR expression and to study possible correlations between p75NTR expression and susceptibility to CDV infection as well as to determine whether OECs represent a crucial factor during CDV infection in vivo and distemper pathogenesis.
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SUMMARY

Immortalization and proliferation of adult canine Schwann cells and olfactory ensheathing cells and their infection with canine distemper virus

Sompong Techangamsuwan

A variety of studies have shown that Schwann cells and olfactory ensheathing cells (OECs) are closely related glial cell types that promote axonal growth and remyelination in vivo following transplantation into the lesioned nervous system. However, the vast majority of these studies have been done using the rodent model and it is not clear in how far the data can be extrapolated to large animals and humans. Canine distemper virus (CDV) infection of adult dogs is considered an alternative animal model to study demyelinating diseases, including multiple sclerosis. Recently, it was shown that CDV can enter the brain via infection of olfactory neurons. Whether associated OECs or Schwann cells of the peripheral nerve are natural targets for CDV infection has not been reported so far. Based on these considerations, Schwann cells and OECs were transfected with ectopic human telomerase reverse transcriptase (hTERT) to induce immortalization thereby establishing a stable source of regenerative adult canine glial cells. The proliferation and differentiation of transfected (1) and non-transfected adult canine glia (2) was analyzed to reveal putative alterations induced by the immortalization procedure and to characterize species-specific properties of non-transfected glia. Finally, Schwann cells and OECs were exposed to different CDV strains in order to study the susceptibility of both cell types to CDV infection in vitro (3).

(1) Both Schwann cells and OECs were successfully immortalized by hTERT transfection. However, both transfected cell types only displayed infinite growth in the presence of fibroblast growth factor-2 (FGF-2). Interestingly, adverse effects on cellular growth following hTERT transfection were observed. Though transfection of late passage Schwann cells and OECs induced FGF-2-dependent immortalization, transfection of early passage cells reduced proliferation independent of FGF-2. Immortalization was associated with subtle differences in the expression profile. Whereas the expression of p75 neurotrophin receptor (p75NTR), GFAP, p53, and p16 was unaltered, there were significant changes in the expression of O4 and A2B5. It is concluded that hTERT transfection is an efficient mean for establishing a stable source of adult canine glial cells and that hTERT plays distinct functional roles depending on the replicative age of the cultured cells.
(2) Adult canine non-transfected Schwann cells and OECs were analyzed regarding long-term proliferation, growth factor responses and antigenic expression. Both glial cell types increased proliferation in response to the same growth factors, including FGF-2 and heregulin-1ß, and displayed a similar expression of cell surface markers, such as p75NTR, O4 and A2B5. This underscored the assumption that both cell types are closely related to each other. Surprisingly, canine Schwann cells and OECs maintained long-term proliferation both under serum-containing and serum-free medium in the absence of any exogenous mitogens and underwent cellular senescence not until three months in culture. Expression of p75NTR was constantly observed during the cultivation period and neither Schwann cells nor OECs increased proliferation after elevation of the intracellular cAMP level using forskolin. These characteristics are in striking contrast to the rodent model and imply pronounced species-specific properties of both glial cell types. The fact that there is a similar in vitro growth behaviour of primate OECs further illustrates the suitability of the adult dog as a translational model system towards clinical application of regeneration-promoting glia in humans.

(3) Adult canine Schwann cells and OECs could be infected with several attenuated and one virulent CDV strain. While there were no differences in the infection rate between the attenuated and the virulent strain, distinct percentages of glial cells were infected by the different viruses. Whereas OECs were predominantly infected by CDV-2544, CDV-OndeGFP and CDV-5804PeGFP, the CDV-Ond strain mainly targeted Schwann cells. This is the first demonstration that Schwann cells and OECs are susceptible to CDV infection in vitro. Since p75NTR has been shown to function as a non-essential receptor for the neurotropic rabies virus, it might be interesting to study its role during CDV infection. Future investigations, therefore, have to reveal possible relations between susceptibility to CDV infection and expression levels of p75NTR. However, it remains to be clarified whether OECs play a pivotal role during CDV infection in situ. The observation that Schwann cells and OECs displayed distinct susceptibility to CDV is evidence for substantial differences of both cell types at the molecular level.
Immortalisierung und Proliferation von adulten kaninen Schwann-Zellen und olfaktorischen Hüllzellen und ihre Infektion mit dem Staupevirus

Somporn Techangamsuwan


Auf der Grundlage dieser Daten wurden Schwann-Zellen und OECs in der vorliegenden Arbeit zum Zweck der Immortalisierung und Etablierung einer stabilen Quelle regenerationsfördernder Gliazellen mit ektoper humaner Telomerase Reverse Transkriptase (human telomerase reverse transcriptase; hTERT) transfiziert. Die Differenzierung sowie die Proliferation transfizierter (1) und nicht-transfizierter (2) adulter kaniner Glia wurde mit dem Ziel untersucht, mögliche transfektionsabhängige Änderungen des Phänotyps zu beschreiben und darüber hinaus für die spätere therapeutische Anwendung wichtige Spezies-spezifische Eigenschaften zu identifizieren. Schließlich wurden Schwann-Zellen und OECs mit verschiedenen Staupevirusstämmen in vitro inkubiert und die Empfänglichkeit sowie Differenzierung analysiert (3).

und p16 unverändert exprimiert wurden, konnten signifikante Unterschiede bei der Expression von O4 und A2B5 beobachtet werden. Es ist festzuhalten, dass die hTERT Transfektions-vermittelte Immortalisierung glialer Zellen ein vielversprechender Ansatz zur Etablierung einer stabilen Quelle regenerationsfördernder Gliazellen dastellt, und, dass hTERT abhängig vom replikativen „Alter“ der kultivierten Zellen eine unterschiedliche funktionelle Bedeutung besitzt.


den einzelnen Staupevirusstämmen zeigen, ist ein wichtiger indirekter Hinweis für molekulare Unterschiede zwischen beiden Zelltypen.
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