TARGETING GROWTH FACTORS TO SITES OF INFLAMMATION: GENE THERAPY FOR MULTIPLE SCLEROSIS.

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Submitted to the University of London for the degree of Doctor of Philosophy

April 2012

The Bone and Joint Research Unit

Barts and The London School of Medicine and Dentistry

Queen Mary University of London

DECLARATION

This is to certify that

(i) This thesis comprises only my original work towards the PhD unless otherwise stated

(ii) Due acknowledgment has been made in the text to all other materials used

ACKNOWLEDGEMENTS

This thesis arose in part out of years of research that had been done since I joined the Bone and Joint Research Unit. During that time, I had worked with a number of people whose contributions to the research and to the making of this thesis deserve special mention. I would like to thank them wholeheartedly in my humble acknowledgments.

To **Professsor Yuti Chernajovsky** I would like to express my gratitude for his advice, supervision, support and guidance. His unflinching devotion to science and his perception of the fine details that have more than once sparked creative ideas, has inspired me to look and go further than I would have dreamed possible.

I gratefully acknowledge **Dr Alexander Annenkov** whose patience, supervision, guidance, understanding, advice and confidence have helped mould me into the scientist I am today. Without his focus and strength of character, I surely would have failed at every hurdle.

I wish to express my many thanks to **Dr Anne Rigby** who played a large part in my drive towards finishing this thesis–*Merci bien, nous vous sommes extrêmement reconnaissants*.

I gratefully thank **Dr Sarah Wythe**, **Mr Richard Rountree** and **Mr Arif Mustafa** for their help in caring for our furry friends.

I would like to express a special thank you to **Professor Gavin Giovannoni** for making me feel welcome and for the extension I received that enabled me to continue after the births of my children.

To my extended family of work colleagues over at Whitechapel: **Dr Sam Jackson** for his help, support, supervision and advice on all things concerning the experimental autoimmune encephalomyelitis (EAE) mouse model for MS, **Dr Sarah Al-Izki** for her help and instruction in dealing

with the chronic relapsing EAE (CREAE) model, and to **Dr Gareth Pryce** and **Prof David Baker** for developing the CREAE model and for answering any queries I had on it.

To my collegues **Dr Christina Subang**, **Mrs Rewas Fatah**, **Dr Taher Taher, Dr David Gould**, **Dr Tolis Koutsokeras** and especially **Dr Sandrine Vessillier** for all their support, advice and help in the lab and inmy life–I am eternally grateful; to **Mr Jules Kieswich** for his beautiful UT7 cells, and to **Dr Lisa Mullen** for her help, guidance, and unwaivering patience with me, and with the CHO-S cells.

Where would I be without my family? I am indebted in many ways to my mother **Wendy Anne Horn** for devoting her life to nurturing and caring for myself and my siblings. A special mention to my father **Roger Keith Horn** for his encouragement, laughter and support during my life, it was all 'character building'. Words cannot express my love for you both and I am externally indebted and grateful. To my children, **Fae** and **Griffin,** for their understanding when I couldn't go to the park to play or to whom I sacrificed time to be able to build a stronger and better future. I owe my deepest gratitude to my husband **Ryan Sclanders**. Words fail to express my appreciation for his faith, support, ability to listen, and especially his strength in helping me labour through this thesis–Thank you my love, we are finally at the end of a beginning. It is an honour for me to thank my aunt, **Mrs Jacqui Holding** for her keen eye and experienced suggestions which helped make this thesis a realization. To my in-laws, **Gail** and **Chicco Sclanders**, thank you from the bottom of my heart for all you have done to help me on my journey and for travelling so far to be there for me.

Finally, I would like to thank all that have made an impact in the creation of this thesis, and my deepest apologies for failing to mention you personally one by one.

ABSTRACT

Disease progression in Multiple Sclerosis (MS), an autoimmune disease of the CNS, is widely accepted to be due to persistent myelin loss (demyelination) coinciding with lost nerve cells and nerve fibres (neuroaxonal loss). Current treatments are immunomodulatory and do not address the neuroaxonal or demyelinating pathology of the disease. It is hypothesised that a lack of growth factors within the CNS may result in the failure of remyelination. Therefore, biologics such as recombinant therapeutic proteins used for gene therapy offer a promising therapeutic intervention to the progressive stages of the disease. However, due to the short half-lives of these therapeutics and their pleiotropic effects, there is cause for concern over their safety and efficacy. Using LAP technology (the fusion of the therapeutic protein with the latent associated peptide [LAP] of TGFβ), the half-life of the therapeutic protein can be increased and can be targeted to sites of inflammation and disease.

This study aimed to investigate the potential neuroprotective, remyelinating and anti-inflammatory effects of latent versions of the growth factors erythropoietin (EPO), insulin-like growth factor 1 (IGF1) and transforming growth factor beta (TGFβ) respectively. Firstly, using molecular cloning techniques, these growth factors were individually fused and linked to the LAP of TGFβ via a matrix metalloproteinase (MMP) cleavage site resulting in three latent growth factors. Secondly, these latent growth factors were shown to be expressed, and to be biologically active *in vitro* when released by MMP cleavage. Finally, syngeneic fibroblasts were engineered to express the latent growth factors. It was found that, in CREAE, the fibroblasts engineered to produce latent TGFβ significantly reduced the disease clinical score as compared to controls whilst latent EPO produced by transduced fibroblasts failed to exert a statistically significant effect on disease progression. Nonetheless, this study demonstrates the feasibility of the latency platform technology to generate latent therapeutics with the ability to act as an intervention to disease progression in MS.

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ABBREVIATIONS AND SYMBOLS

A

A	**adenine**	**AMT**	**Adsorptive-mediated transport**
ABC	**ATP-binding cassette**	**APC**	**Antigen presenting cells**
AJs	**Adherens junctions**		

B

BBB	**Blood brain barrier**	**B104**	**Rat neuroblastoma cell line**
BCR	**B-cell receptor**	**bFGF**	**Basic fibroblast growth factor**
BDNF	**Brain-derived neurotrophic factor**	**BSA**	**Bovine serum albumin**

C

CA	**Capsid protein**		
CEC	**Cerebral endothelial cells**	**CIP**	**Calf intestinal alkaline phosphatase**
CEPO	**Carbamylated variant of EPO**	**CMV**	**Cytomegalovirus**
CFA	**Complete Freund's Adjuvant**	**CREAE**	**Chronic relapsing EAE**
CHO	**Chinese hamster ovary cell line**	**CSF**	**Cerebrospinal fluid**
CG4	**Rat oligodendrocyte progenitor cell line**	**cGMP**	**Cyclic guanosine monophosphate**

D

DCs	**Dendritic cells**	**DMEM**	**Dulbecco's Modified Eagle's Medium**
DHFR	**Dihydrofolate Reductase**	**DTT**	**Dithiothreitol**

E

EAE	**Experimental autoimmune encephalomyelitis**	**EMP**	**Endothelial microparticles**
ECAMs	**Endothelial cell adhesion molecules**	**eNOS**	**Endothelial NOS**
		EPO	**Erythropoietin**

ECM	**Extracellular matrix**	**EPOR**	**EPO receptor**
E.coli	***Escherichia coli***	**ESL1**	**E-selectin ligand 1**
eGFP	**Enhanced green fluorescent protein**		

F

FBS	**Foetal bovine serum**	**FDCs**	**Follicular dendritic cells**

G

GA	**Glatiramer acetate**	**GPR17**	**G protein-coupled receptor 17**
GCs	**Germinal centres**	**GSK-3β**	**Glycogen synthase kinase-3β**
GM-CSF	**Granulocyte-macrophage colony-stimulating factor**		

H

HEK-293T	**Human Embryonic Kidney cell line containing the SV40 large T antigen**	**HIF-1**	**Hypoxia inducible factor-1**
HEV	**High endothelial venules**	**HLA-DR**	**Human leukocyte antigen-DR**

I

ICAM1	**Inter-cellular adhesion molecule 1**	**IN**	**Integrase**
IFA	**Incomplete Freund's Adjuvant**	**iNOS**	**Inducible NOS**
IFNγ	**Interferon gamma**	**INSR**	**Insulin receptor**
IGF1	**Insulin-like growth factor 1**	**iT_{Regs}**	**Induced T_{Regs}**
IGF1R	**IGF type I receptor**	**i.p.**	**Intraperitoneal**
IGFBPs	**IGF binding proteins**	**i.v.**	**Intravenous**
IL-	**Interleukin-**		

J & K

JAMs	**Junctional adhesion molecules**	**KA**	**Kainite**

L

LAP	**Latency-associated peptide**	**LTBPs**	**Latent TGFβ binding proteins**
L-Glut	**L-Glutamine**	**LTR**	**Long terminal repeats**
LFA-1	**Lymphocyte function-associated antigen 1**	**LPS**	**Lipopolysaccharide**

LLTC	**Large latent TGFβ complex**

M

MA	**Matrix protein**	**Mø**	**Macrophages**
MAdCAM-1	**Mucosal vascular addressin cell adhesion molecule 1**	**MOG**	**Myelin oligodendrocyte protein**
MAG	**Myelin-associated glycoprotein**	**MRF**	**Myelin gene regulatory factor**
MBP	**Myelin-basic protein**	**MRI**	**Magnetic resonance imaging**
mGluR5	**type 5 metabotropic glutamate receptors**	**MRPs**	**Multidrug resistance-associated proteins**
M-IFA	**Mycobacterium-supplemented incomplete Freund's Adjuvant**	**MS**	**Multiple Sclerosis**
miRNAs	**MicroRNAs**	**MTX**	**Methotrexate**
MLEC	**Mink lung epithelial cell line**		
MMP	**Matrix metalloproteinase**		

N

NC	**Nucleocapsid**	**NOS**	**Nitric oxide synthase**
Nef	**Negative factor**	**NRG1**	**Neuregulin 1**
NILVs	**Non-integrating lentiviral vectors**	**NS0**	**Mouse myeloma cells**
nNOS	**Neuronal NOS**	**nT_{Regs}**	**Natural T_{Regs}**

O

OD	**Optical density**	**OS**	**Oxidative stress**
OL/s	**Oligodendrocyte/s**		

P

PAI-1	**Plasminogen activator inhibitor-1**	**PPMS**	**Primary-progressive MS**
PBS	**Phosphate Buffered Saline**	**PPT**	**Polypurine tract**
PBST	**PBS with 0.05% Tween-20**	**PR**	**Protease**
PDGF	**Platelet-derived growth factor**	**PRMS**	**Progressive-relapsing MS**
PECAM1	**Platelet endothelial cell adhesion molecule 1**	**PSA-NCAM**	**Polysialylated-neural cell adhesion molecule**
Pen/Strep	**Penicillin/Streptomycin**	**PSGL1**	**P-selectin glycoprotein ligand 1**
Pgp	**P-glycoprotein**	**Psi**	**ψ**
PIC	**Preintegration complex**	**PTPα**	**Protein-tyrosine phosphatase alpha**
PLP	**Proteolipid protein**	**PVDF**	**Polyvinylidene fluoride**
PNGase F	**Peptide: N-Glycosidase F**	**PVE**	**Post–vaccinal encephalomyelitis**

R

RA	**Rheumatoid arthritis**	**R-SMAD**	**Receptor-regulated SMAD**
RMT	**Receptor-mediated transcytosis**	**RT**	**Reverse transcriptase**
ROS	**Reactive oxygen species**	**rtPA**	**Recombinant tissue plasminogen activator**
RRE	**Rev Response Element**		

S

s.c.	**Subcutaneous**	**SLTC**	**Small latent TGFβ complex**
SCH	**Spinal cord homogenate 33mg**	**SLCs**	**Solute carriers**
SDS	**Sodium dodecyl sulphate**	**SMAD**	**Sma and Drosophila Mad**
SDS-PAGE	**SDS-polyacrylamide gel electrophoresis**	**SPMS**	**Secondary-progressive MS**
SEM	**Standard error of the mean**	**SU**	**Surface glycoproteins**
SFFV	**Spleen focus forming virus promoter**		
Shh	**Sonic hedgehog homolog**		
SILT	**Stable isotope labelling tandem mass spectrometry**		

T

T	**thymine**	**TH**	**Thyroid hormone**
Tar	**Tat-responsive**	**TIMPs**	**Tissue-inhibitors of metallo-proteinases**
TCF	**T cell factor**	**TJs**	**Tight junctions**
TCM	**Central memory T cells**	**TM**	**Transmembrane proteins**
TCR	**T cell receptor**	**T_{Reg}**	**regulatory T cells**
TEM	**Effector memory T cells**	**tsF**	**Temperature sensitive fibroblast**
TGFβ	**Transforming growth factor beta**	**TSP1**	**Thrombospondin-1**
TGFβRI	**TGFβ receptor type I**	**tu/ml**	**Transducing units/ml**
TGFβRII	**TGFβ receptor type II**		

U

U3	**U3 repeat sequence**	**U5**	**U5 repeat sequence**

V

VASP	**Vasodilator-stimulated phosphoprotein**	**VSV-G**	**Vesicular stomatitis virus G protein**
VCAM1	**Vascular cell adhesion molecule 1**	**VVOs**	**Vesiculo-vacuolar organelles**

VEGF	**Vascular endothelial growth factor**
Vif	**Virion infectivity factor**

W

WPRE	**Woodchuck hepatitis B posttranscriptional regulatory element**

CHAPTER 1

INTRODUCTION

THE CNS IS 'IMMUNOPRIVILEGED'

The term immunological privilege was originally coined in 1953 (Billingham and Boswell) but was observed as early on as 1921 by a group in Japan (Galea et al., 2007). These and other groups (Byrnes et al., 1996, Stevenson et al., 1997) observed the lack of a systemic immune response when antigens were transplanted and retained within the brain parenchyma. Initially, this 'immunological privilege' was thought to be absolute and pertain wholly to the CNS. However, today it is agreed to be confined to the CNS parenchyma (and not to the CNS in its entirety) as the immune response in the ventricles and meninges is similar to that of a peripheral response (i.e. it is not absolute), and is mostly a result of the specialisation of the afferent arm of the adaptive immune response (Carson et al., 2006, Galea et al., 2007). Adaptive privilege of the afferent arm involves the development of antigen presentation to naïve T cells in the form of antigen presenting cells (APCs) that result in T cell priming and activation, and the ensuing emigration of these APCs to the lymph nodes—an important process in the transport of antigen (Galea et al., 2007). The loss of immune privilege is the consequence of increased permeability of the endothelial blood brain barrier (BBB) junctions, a dilution in the immunosuppressive effects of the cellular microenvironment, and the local activation of APCs resulting in an up-regulation of immunostimulatory growth factors and chemokines.

Immune Homeostasis

The immune system protects its host from a diverse variety of foreign antigens and pathogens, as well as transformed cells that could prove to be harmful. The identification and removal of these antigens, is mediated by immune cells (such as T and B cells). It occurs through several processes such as immunological tolerance formation, lymphocyte recirculation and immune surveillance. Once a potential threat (such as an antigen or virus) is recognised, the immune system elicits a response that eliminates the threat.

There are two types of immune response that occur: innate and adaptive responses. Innate responses are fleeting singular events in which macrophages (Mø), natural killer cells (NK cells), complement proteins, mast cells, dendritic cells (DCs) and neutrophils respond immediately and directly to pathogen-specific compounds, 'molecular signatures' and types of biological molecules, and eradicate any foreign antigen or infection. Conversely, adaptive immunity is a slow but effective long term response to foreign antigens that involves the generation of clonal memory B- or T lymphocytes (B- or T cells respectively) that are antigen-specific and result in the formation of 'immune memory'. Lymphocytes are generated in the bone marrow of the primary or central lymphoid system before maturing in the thymus, generating T cells; or remain within the bone marrow, generating B-cells. Naïve lymphocytes recirculate through lymphoid organs such as lymph nodes where they encounter antigen, proliferate and become activated.

Like T cells (discussed later on), B cell activation requres two signals: an initial signal provided by an APC and a second signal provided by helper T cell. The initial stages of activation occur when antigen binds to the B cell receptor (BCR), a transmembrane receptor consisting of α and β Ig signalling subunits and an antigen-binding membrane immunoglobulin subunit. Once bound to the BCR, the antigen is internalised, processed into antigenic peptides, and loaded onto MHC Class II molecules to be presented on the B cell surface. If the B cell fails to receive a second signal, it is either eliminated or becomes functionally inactivated. The second signal is given by effector helper T cells. In a primary antibody response, naive T cells are activated in a peripheral lymphoid organ by binding to a specific antigen loaded onto MHC Class II peptides displayed by an APC. Only once this particular antigen:MHC Class II complex is encountered again on a particular B cell, will it be able to fully activate that B cell. This binding send the second signal to activate the B cell and initiate its differentiation and proliferation into either antibody secreting plasma cells, or memory B cells that do not secrete antibody. Once the foreign antigen has been eradicated, the majority of the plasma cells die leaving behind mainly memory B cells. If these memory B cells encounter that specific antigen again, they respond more rapidly than naïve B cells. As memory B cells they are able to act

as direct APCs and display that particular antigen:MHC Class II complex and activate helper T cells, of which they are the subsequent targets of. This mutual reinforcement of helper T cell and B cell action leads to an immune response that is highly specific and very intense. Once fully activated, plasma B cells proliferate to produce antibodies that opsonize (identify) foreign antigen for destruction by cells such as Mø (Alberts et al., 2008).

Additionally, antibodies can bind to viruses and neutralize them before they are able to infect a cell. Although B cells are very successful at defending against foreign antigen and viruses, their antibodies are only effective in the exterior environment of the cell or on its cell surface. Once a virus has gained entry into the cell, another type of defence is required i.e. T cells. T cells are similar to B cells in that they originate from the bone marrow, have a surface receptor (the remarkable T cell receptor (TCR)) and undergo clonal selection once activated. The TCR is made from small snippets of DNA that are randomly scrambled and rearranged yielding around 10^{10} variations of the TCR, enough variations to recognise practically anything including self-antigen, but the body has several crucial checkpoints to remove potentially devastating self-reactive T cells. T cells mature in the thymus and can only recognise antigen if it is properly presented by another cell, an antigen-presenting cell (APC) such as DCs, B cells, Mø or microglia. T cells are subdivided into $CD8^+$ cytotoxic cells (also known as the killer T cells), or into $CD4^+$ cells. $CD8^+$ T cells detect virus infected T cells and initiate the cells apoptotic pathways. $CD4^+$ T cells on the other hand, further differentiate into either T helper cells (Th cells) or into regulatory T cells (T_{Reg}).

Th cells (such as Th1, Th2 and Th17 classes) are involved in directing and activating other immune cells, such as activating and (in some cases) determining B cell Ig class switching and in the activation of $CD8^+$ T cells (Martin-Orozco et al., 2009, Mitsdoerffer et al., 2010). The Th phenotype is determined by the type of T-cell activating growth factors secreted by APCs, with IL-12 (along with T-bet and IFN-γ) inducing Th1 cells; IL-4 and GATA-3 inducing Th2 T cells; and the retinoic acid receptor-related orphan nuclear receptor (RORγt) along with IL-17 inducing Th17 cells. Th1 T cells

are preferentially stimulated by Mø and DCs and secrete pro-inflammatory growth factors such as IFNγ, IL-2, TNFα and TNFβ, and promote cell mediated immunity, such as delayed hypersensitivity reactions (Murphy and Reiner, 2002, Fabry et al., 2003, Takatori et al., 2008). Conversely, Th2 cells are preferentially stimulated by B cells and secrete anti-inflammatory growth factors such as IL-4, IL-5, IL-10, IL-13 and TGFβ, growth factors known to inhibit cell-mediated immunity, including responses to autoantigens which lead to immunopathology in MS. As discussed earlier, helper T cells need to bind to a particular antigen:MHC Class II complex displayed by B cells in order to activate them. Secreted signals from helper T cells can also help B cells to proliferate and differentiate, and as mentioned above, to switch Ig class. For example interleukin 4 (IL-4) enables the switch to IgE antibody production (Alberts et al., 2008).

T_{Regs} have a lower antigen activation threshold than conventional T cells, they supress the immune response by supressing the function of activated T cells, and mediate peripheral tolerance (Takatori et al., 2008, Stockis et al., 2009, Tran et al., 2009b), playing a crucial role in immune homeostasis and in the prevention of inflammatory disease (Gao et al., 2012). Natural T_{Regs} (nT_{Regs}) develop in the thymus during positive and negative selection, whereas inducible or adaptive T_{Regs} (iT_{Regs}) are non-regulatory $CD4^+$ $FOXP3^+$ T cells that acquire their CD25 expression upon maturation in the periphery, possibly through interaction with latent TGFβ , cell–cell contact with neurons, or through unknown soluble factors secreted by astrocytes (Trajkovic et al., 2004, Takatori et al., 2008, Tran et al., 2009a). FOXP3 (forkhead box P3) a transcription factor is a master regulator for the development and the function of Treg cells, ultimately modulating the destiny of Tregs by possibly interacting with transcription factors which define the polarization of other Th subsets such as FOXP3 binding with the T-box transcription factor T-bet (Tbx21), to drive Th1 differentiation; or through reprogramming of Treg cells such as the generation of non-suppressive cells such as Th17 cells through induction by IL-6 to $FOXP3^+$ T cells; or even the differentiation of Th2 cells through the loss of FOXP3 which may lead to the induction of IL-4 (Haiqi et al., 2011). Recently, studies have shown these $FOXP3^+$ Treg cells

can be reprogrammed into proinflammtory cells for example in patients with relapsing remitting multiple sclerosis who exhibit Th1-like FOXP3$^+$ cells(Dominguez-Villar et al., 2011).

As mentioned before, T cells cannot directly interact with or recognise antigen. As in the case of antigen presentation on B cells, antigen must be presented in the form of antigen complexed to MHC molecules on APCs such as DCs, before it can interact with the TCR. The involvement of the glycoprotein co-receptors, CD4 and CD8, with the antigen: MHC complex allows for the activation of naïve T cells, and their subsequent differentiation and proliferation. CD8$^+$ and CD4$^+$ T cells recognise MHC Class I and Class II molecules respectively. Signalling from this trimolecular complex and in the presence of costimulatory molecules such as CD40L, CD2, CD28, and CTLA-4; integrins such as VLA-4; and certain adhesion molecules such as CD44, B7, ICAM-1, lymphocyte function-associated antigen 1 (LFA-1) and LFA-3, and vascular cell adhesion molecule 1 (VCAM-1), activates the T cell. In the absence of costimulatory molecules the T cells fail to produce IL-2, do not proliferate, become anergic and fail to elicit an immune response to antigen. Only when fully activated, can they recognise any cells that present its specific antigen: MHC complex.

The interaction between blood borne naïve T cells expressing homing receptors (such as CD62L) and cell adhesion molecules (such as CD54 or CD102) found on high endothelial venules (HEV), leads to tethering and rolling of the T cell along the endothelial wall. Once engaged, the chemokine receptor CCR7 binds the ligands CCL21 and/or CCL19 inducing a conformational change that promotes firm adhesion and transmigration of the naïve T cells into the lymph node. Lymphocytes are then able to leave the lymph node by either transcellular (piercing the cellular membrane) or by paracellular (migrating around the HEV cells) routes.

The two types of memory T cells that arise from naïve T cells have distinct homing patterns and effector functions. Effector memory T cells (TEM) cells are a readily available pool of antigen-primed T cells lacking CCR7 and CD62L that act immediately after activation expressing growth factors such as IL-4, IL-5, IFN-γ and perforin (a cytolytic protein); whereas central memory T cells (TCM) express

CCR7 and CD62L, are able to travel to lymphoid organs, can stimulate DCs when challenged and help B cells in generating new effector cells but lack immediate effector functions (Elgert, 2009). Memory T cells differentiate from naïve T cells in a stepwise manner in which effector differentiation—the proliferation and differentiation of antigen-activated T cells into effector T cells—is not, as originally thought, a prerequisite (Manjunath et al., 2001).

Most mature T cells continuously circulate through the body via the lymphoid organs from blood to tissue into lymph and back to blood. This lymphocyte recirculation ensures the effective recognition of the vastly dynamic repertoire of foreign antigens at all the possible sites of infection. Tumour immunoediting (also known as immune surveillance (Dunn et al., 2002)) is the ability of the immune system to detect and eradicate cancerous and precancerous cells. It plays an important role in immune homeostasis and in the control of cancer. It is mediated by lymphocytes looking specifically for tumour antigens or molecules expressed on cancerous or pre-cancerous cells and involve three phases: elimination, equilibrium and escape. During the elimination phase, the immune system either totally eliminates all potential cancerous cells, or only partially eliminates them. If the latter is true, the immune system proceeds to exert selective pressure on the progression of the tumour cells by eliminating cells where possible, whilst the tumour cells themselves continue to accumulate further changes such as DNA mutations. Although this selective pressure is sufficient to keep the tumour from progressing rapidly, if the immune system continues to fail in eliminating the entire population of cancerous cells, tumour cells that are able to resist, avoid or suppress the immune systems antitumor response will proliferate and escape.

The immune system is highly adept at initiating an immune response against pathogens. But in order to maintain the boundaries between antigens that are actually foreign and those that are self-antigens, lymphocytes undergo positive and negative selection at critical checkpoints, allowing a cell to be ‘tolerant of self-antigens’. Tolerance to self-antigens is an essential feature of the immune system, as loss of tolerance results in the development of autoimmune diseases. Within T cell

development, tolerance to self-antigens is achieved through central (utilizing negative and positive selection) and peripheral tolerance (including immunological ignorance, deletion, inhibition and suppression).

A critical checkpoint during thymic development of T cells occurs when the thymocytes are dubbed 'double positive' (DP) for CD4CD8. Those thymocytes that exhibit weak interaction to self-MHC complexes, expressed by thymic cortical epithelial cells, are selected to differentiate further into single-positive (SP) thymocytes expressing either CD4 or CD8. These SP thymocytes then undergo negative selection (another critical checkpoint in maintaining immune homeostasis)—those thymocytes that exhibit strong self antigen:MHC complex interactions to antigen expressed by DC's and thymic medullary epithelial cells, are made funtionally inactive or are deleted. Thymocytes that exhibit only intermediate reactivity mature in the thymus before exiting to the periphery (positive selection). The elimination of the majority of autoantigen i.e. pathogenic cells bearing high affinity for self-reactive TCRs, constitutes a major checkpoint in immune homeostasis called central tolerance. However, not all autoantigens are expressed in the thymus thus mechanisms are required in the periphery to maintain self-tolerance (Curiel, 2007, Kim and Rudensky, 2006).

Autoreactive T cells circulating within the peripheral lymphoid tissues are observed within normal individuals without autoimmune attack and are maintained through a form of self-tolerance known as immunological ignorance. Several mechanisms can induce immunological ignorance: the antigen threshold may be too low to induce reactivity, the antigens may be physically separated from T cells (e.g. by the BBB) or antigens are presented in the absence of co-stimulatory molecules thus rendering them anergic. Peripheral tolerance through T cell deletion can occur through inadequate amounts of vital growth factors, or through T cell death mediated by Fas and its ligand—engagement of the Fas receptor induces apoptosis in Fas-positive cells. As T cells express both Fas and its ligand when activated, apoptosis can be induced if they engage. Full T cell activation can also be inhibited through the interaction of the transiently expressed adhesion receptor CTLA-4 (also known as

CD152) found on Th cells, and the adhesion molecules B7-1 (CD80) and B7-2 (CD86) found on APCs and B cells. Normally CD80 and CD86 interact with the T cell costimulatory molecule CD28, but CTLA-4 has a higher affinity for these adhesion molecules and its interaction with B cells inhibits the full activation of T cells and renders them anergic.

AUTOIMMUNITY

Autoimmunity is an immune response to self-antigens. It arises from the breakdown of tolerance within the body through the triggering of receptors directly by self-antigen, through the breakdown of the BBB, or by virtue of cross reaction between a foreign and a self-antigen. Autoimmune diseases can be autoantibody mediated (such as in celiac disease), or T cell mediated (such as in rheumatoid arthritis (RA), insulin-dependent diabetes mellitus and Multiple Sclerosis (MS)). Autoaggressive T cells that have evaded tolerance and are pathogenic, participate in the development of autoimmune diseases.

The Blood Brain Barrier

The BBB is a physical barrier created by the close association of the cerebral endothelial cells (CEC) that form the capillaries in the brain, and the BBB interactions with pericytes, astrocytes, microglia and neurons make up the CNS microenvironment. The BBB is developed during foetal life and by birth is well formed. Under normal physiological conditions, the BBB maintains CNS integrity through the strict control of leukocyte entry, the exclusion of macromolecules such as pro-thrombin, albumin and plasminogen which could cause damage to the neural tissue, the regulatation of glutamate levels (a neuroexcitatory amino acid within the brain), the prevention of 'cross-talk' between neurotransmitter pools within the PNS and CNS, and the regulation of the ions K^+, Ca^{2+} and Mg^{2+} through specific ion channels and transport systems, thus maintaining a microenvironment that is conducive to efficient synaptic signalling. The phenotype of the BBB is created by the endothelial

cells lining the microvasculature to maintain and regulate homeostasis of the brain and to prevent neural disruption by circulating toxins (Abbott, 2002). This phenotype is strictly regulated by the CNS microenvironment as *ex vivo* culture of these cells results in its loss until the addition of astrocytes or their incubation in astrocyte-conditioned medium (cited in Abbott et al., 2006).

The BBB is both structurally and functionally supported by astrocytes, pericytes, microglia and nerve terminals through the release of soluble factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), angiopoietin-1 and transforming growth factor 1β (TGFβ1). The CNS microenvironment also regulates the trafficking of leukocytes into the CNS across the BBB through the suppression of selectins and the secretion of bFGF and VEGF. bFGF tightens the adherens junctions (AJs) and tight junctions (TJs) and induces the expression of the multidrug resistance proteins involved in ABC transporters. VEGF increases BBB permeability by lowering the expression of occludin and VE-cadherin. Both are produced by and released from pericytes (Rucker et al., 2000).

The BBB is formed during foetal life and maintained in adult life by the interactions of several cell types and the expression of junction proteins and transporter proteins. The junctional complexes between the endothelial cells comprise AJs and TJs. AJs span the intercellular space with cadherins that are attached to the scaffolding proteins α, β and γ catenin. AJs are essential for the formation of TJs and act as structural support for the tissue. TJs are sites where the cellular membranes of endothelial cells come into close association with one another. TJs are responsible for the severe restriction of the paracellular diffusional pathway that would otherwise allow for the entry of ions and other molecules, as well as for blocking the penetration of macromolecules into the brain. The TJ complex spans the intercellular cleft and consists of occludin and the claudins 3 and 5 that are linked to the scaffolding proteins ZO-1, ZO-2 and ZO-3, as well as cingulin and junctional adhesion molecules (JAMs). The scaffolding proteins regulate the effectiveness of TJs by linking the occludin and the claudins of the separate tight junctional complexes via cingulin and the actin cytoskeleton.

TJs also generate a high *in vivo* electrical resistance to the BBB (~ 1800 Ω/cm^2) (Butt et al., 1990) resulting in a low conductance of the barrier, thus reducing the movement of ions such as K^+, Ca^{2+} and Mg^{2+}. Additionally, the TJs function to segregate the transport proteins in the membranes from the lipid rafts thus preventing them from moving freely and therefore maintain barrier polarity. The BBB is regulated by changes in the TJs or by changes in the expression and activity of transporter proteins, thus matching the activities of the BBB with the requirements of the brain.

The BBB does not exclude all macromolecules from entering the brain as many essential amino acids and nutrients are needed for neural metabolism, therefore several transporter systems expressed by the endothelial cells are available to these molecules. They include solute carriers (SLCs), ATP-binding cassette (ABC) transcytosis transporters (such as the efflux transporters P-glycoprotein (Pgp) and multidrug resistance-associated proteins (MRPs)), and transcytosis. As the TJs confer the properties of a continuous cell membrane on the BBB, the entry and exit of polar molecules is dependent on its lipophilicity (the molecules ability to move through the lipid rafts), and the orientation of the transport proteins in the luminal or abluminal membranes of the endothelial cell allows for preferential transportation of these substrates from blood to brain, or from brain to blood. Similarly, SLCs such as GLUT1 for glucose or MCT8 for thyroid hormone allow the endothelial cells to mediate the flux of nutrients into or away from the brain. ABC transporters function mainly as efflux transporters of potentially neurotoxic endogenous or xenobiotic molecules and are thus neuroprotective and detoxifying. Molecules that are greater than 450 Daltons are generally restricted from entering the brain by the BBB. Large macromolecules such as glycosylated proteins and cationic molecules are transported across the BBB by endocytotic mechanisms such as receptor-mediated transcytosis (RMT) and adsorptive-mediated transport (AMT) respectively. During RMT, the binding of ligands to specific receptors initialises their endocytosis where they are internalised, and then exocytosed on the opposite pole of the cell. This transcytosis of intact peptides and macromolecules is achieved by avoiding the degradative lysosomal compartment, and as this type of routing does not occur in peripheral endothelial cells, it is considered to be a specialised feature of

the BBB. During AMT, a molecule displaying excess positive charges (cationic) interacts with a cell surface binding site, in turn inducing endocytosis and subsequent transcytosis.

Wnt signalling is a very important signalling pathway not only in the development and maturation of the BBB, but also in its maintenance. Wnt proteins are glycosylated proteins that bind to the frizzled family of transmembrane proteins as well as other heterologous receptors such as PDGFRα (Fancy et al., 2009). The Wnt signal is transduced to the cytoplasmic protein 'dishevelled' following receptor binding. Dishevelled inhibits the activity of glycogen synthase kinase-3β (GSK-3β), a serine-threonine kinase involved in neural cell development. In the absence of Wnt signalling, GSK-3β phosphorylates β-catenin (a protein involved in the makeup of AJs as well as an anchor for the actin cytoskeleton) resulting in its degradation. β-catenin regulates Wnt gene transcription by translocating to the nucleus and associating with the T cell factor (TCF) and lymphoid enhancer factor (LEF) family of transcription factors, which in turn bind to enhancers of T cell specific genes. In a normal noninflammed barrier, Wnt signalling down-regulates the expression of plasmalemma vesicle associated protein-1 (Plvap), a marker of leaky brain vessels, whilst under pathological conditions it is up-regulated by BBB microvessels. An increase in Wnt signalling results in an increase in expression of the junctional protein claudin-3 in a β-catenin dependent manner. A loss of β-catenin results in an increase in paracellular permeability and a deficit in cell-cell contact allowing the entry of activated lymphocytes into the CNS, an intangible beginning to MS.

The breakdown of the BBB is a major hallmark of MS. Indeed, one of the earliest events in the formation of a MS lesion, is the deposition of the serum protein fibrinogen through the rupture of the BBB. Fibronogen is a serum glycoprotein involved in blood clot formation, and has been shown to be a key regulator of inflammation (Davalos and Akassoglou, 2012). Interestingly it is also able to activate microglia, even without activated T cells. Additionally, the ECM proteins fibronectin and vitronectin are found within MS lesions, proteins that have similar functions to fibrinogen i.e. they bind to integrins and are able to induce the activation and expression of microglia and MMP-9

(Watzlawik et al., 2010). The disruption of the BBB also allows for the activation of OPCs and therefore the facilitation of remyelination as several factors have been shown an increase in expression following BBB breakdown i.e. NG2, the transcription factors Olig2 and Nkx2.2, and chondroitin sulfate proteoglycans, factors involved in oligodengrogenesis (Watzlawik et al., 2010).

MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is an autoimmune, demyelinating disease characterised by the breakdown of the BBB, the infiltration and accumulation of inflammatory cells in the CNS and the destruction of the myelin sheath, a process known as demyelination. The areas of destruction are termed lesions and occur most commonly in the periventricular white matter (such as the cerebellum, the brain stem, the spinal cord and the optic nerves) and it is the location of these lesions that dictates the symptoms that a patient experiences. The aetiology of the disease is, as yet, unclear.

History

Arguably, the first documented case of MS was of Saint Lidwina of Schiedam (1380–1433) who presented symptoms consistent with MS following an ice-skating fall in 1396. The first illustrations of MS plaques were documented by Robert Carswell (1793–1857) in 1838 in his collection Pathological Anatomy: Illustrations of the Elementary Forms of Disease. The term “sclérose en plaque disséminée” was coined by the physician-scientist Edmé Vulpian (1826–1887) in 1866. He later worked with the neurologist Jean-Martin Charcot (1825–1893) who, in 1868, was the first to systematically describe MS from his observations on the deceased. Jean-Martin Charcot also provided the first evidence of CNS damage and identified features for diagnosis and pathology. He also outlined the disease progression and noted the destruction of the myelin sheaths and the preservation of the axon (Herndon, 2003, Iezzoni, 2010).

Epidemiology

MS is the most common neurological condition that affects young adults today. In the general UK population, MS affects more females than males (2:1), has an incidence of seven per 100 000 every year, a prevalence of 83–120 per 100 000 (Compston and Coles, 2002, Pugliatti et al., 2006), and it presents between the ages 15 and 60 years.

There are four clinical stages of MS defined by their clinical course and not on known differences in genetics or pathogenesis (Figure 1.1, Rudick, 2003). The most commonly observed stage, relapsing-remitting MS (RRMS), affects around 85% of patients confirmed with MS and is characterised by distinct attacks or relapses that are followed by periods of remission or improvement. Primary-progressive MS (PPMS), affecting around 10% of patients confirmed with MS, shows deterioration from the start with no obvious clinical relapses or periods of improvement. Secondary-progressive MS (SPMS), to which the majority of RRMS patients progress, is characterised by a slow and gradually progressive deterioration that accounts for most of the disease impact. Finally, the relatively rare progressive-relapsing MS (PRMS) falls within PPMS in that some relapses may occur, but do little for the long term outcome (Keegan and Noseworthy, 2002).

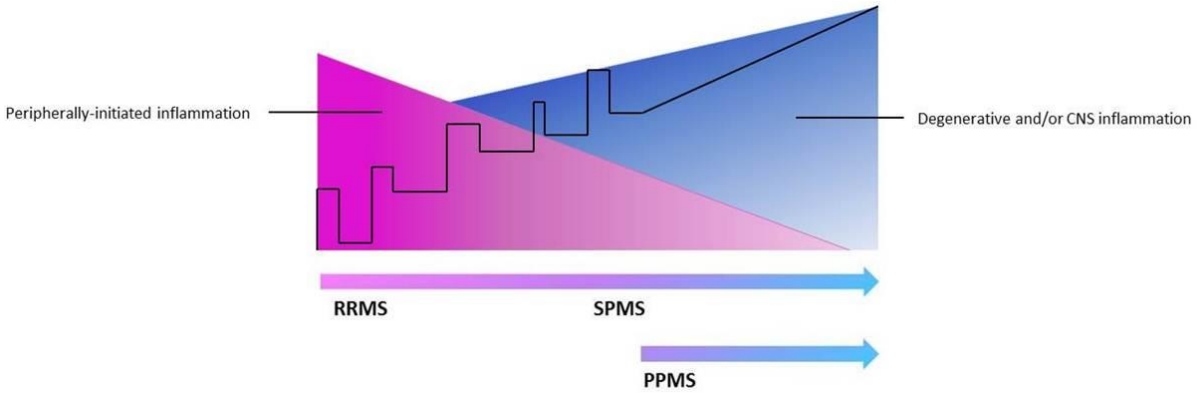


Figure 1.1. The stages of MS.
The clinical course of MS is indicated with a black line with stepwise increases in disability in early disease caused by relapses (RRMS) and, later, by gradual progression of disability in SPMS and from disease onset in PPMS. Early in the disease, peripherally-initiated activation and infiltration of lymphocytes drives the inflammatory processes that manifest clinical relapses and remissions (pink shading). As the disease progresses, the accumulation of disability becomes apparent in the form of continual disability with no remissions and degeneration and/or CNS inflammation predominates (blue shading). RRMS=relapsing-remitting multiple sclerosis, SPMN=secondary-progressive multiple sclerosis, PPMS=primary-progressive multiple sclerosis.

Diagnosis of MS

The current acceptable diagnostic criteria for MS are the McDonald criteria (McDonald et al., 2001). It essentially dictates that an individual must have a minimum of two or more neurological attacks, which correspond to two or more different sites of lesions within the CNS, thus providing evidence of dissemination in both time and space. Supporting clinical evidence in the absence of this dissemination in time and space can be provided by radiological and laboratory investigations such as magnetic resonance imaging and the analysis of the cerebrospinal fluid (CSF). The CSF is tested for increased IgG antibody production and oligoclonal banding (as compared to serum controls) (Anderson et al., 1992) as studies have shown that in over 90% of clinically definite MS patients, elevated levels of the CSF IgG antibody and the appearance of discreet oligoclonal bands have been found.

Possible Causes of MS?

MS is the result of complex interactions between genetics and environmental factors but its aetiology is, as yet, unknown (Compston and Coles, 2002, Ascherio and Munger, 2007a, Ascherio and Munger, 2007b). The support for a genetic component is significant. Twin studies have shown an increased risk of 30% for identical twins but only 4% for fraternal twins. First degree siblings show an increased risk of 3% which is significant when compared to the risk of 0.1% in the general population (Sadovnick and Ebers, 1993). The likely genes involved in contributing to the development of MS have been narrowed down to the DR15 and DQ6 markers of the MHC gene complex, with a protective effect conferred by the HLA-C5 allele (reviewed in Compston and Coles, 2008).

However, the distribution of MS cannot be explained by population genetics alone as there is a distinctive geographic distribution of MS. MS occurs mainly in individuals of north European ancestry where persons of Caucasian decent are twice as likely to develop the disease. Interestingly, migration of large numbers of people also alters the distribution of MS. Migration from a high risk region to a low risk region during childhood is associated with a reduced risk, and vice versa. Conversely, if migration occurs after childhood, the risk of the previous region is retained (reviewed by Ebers and Sadovnick, 1993). However, conflicting data has been recently published stating that although a geographical gradient of incidence does exist for Australia and New Zealand, it doesn't exist for Europe and North America (Koch-Henriksen and Sorensen, 2010).

Environmental components to MS are "strongly presumed but not yet proven" and remain enigmatic (Iezzoni, 2010). During the 1980's, it was discovered that lymphocytes in MS patients produced subnormal amounts of the warning proteins IFN-α and IFN-β, which are usually secreted by cells that have encountered viruses, leading researchers to investigate the involvement of viruses as acute viral infection has been shown to result in an impairment in the lymphocyte response to viral antigens (Haahr et al., 1983). Since then many viruses have been implicated in the development of MS and several including measles, mumps, rubella, rabies, herpes simplex virus, parainfluenza 1

and human retrovirus (Iezzoni, 2010) have been investigated to no avail. A possible exception is that of Epstein Barr Virus which might be involved in early MS pathogenesis (Salvetti et al., 2009).

Other factors and theories have been thought to influence the development of MS including sunlight levels (as UV radiation has demonstrated a suppressive effect), the effect vitamin D has on regulatory T cells, and the lack of vitamin D correlating to the gradient of incidence observed in MS cases (reviewed in Smolders et al., 2008).

Whatever the initial cause may be, it is the migration of activated T cells across an impaired BBB that initiates the pathway of autoimmune neuroinflammation.

PATHWAY OF AUTOIMMUNE NEUROINFLAMMATION

The pathway of autoimmune T cell neuroinflammation is thought to occur in two phases (Flugel et al., 2001, Platten and Steinman, 2005): the first phase occurs with the priming and activation of naïve $CD4^+$ T cells by DCs in the periphery; the second involves the transendothelial migration and entry of these activated $CD4^+$ T cells into the CNS through a process called the leukocyte adhesion cascade, and their subsequent reactivation by activated microglia (Figure 1.2). The leukocyte adhesion cascade comprises several distinct stages: tethering and rolling, arrest, intravascular crawling, and paracellular and transcellular transmigration that allows the activated lymphocytes to gain entry across the BBB.

The tethering and rolling of leukocytes along the blood vessel wall can be mediated through either selectins (a family of cell adhesion molecules) or through integrins. Leukocytes interact with E-, L-, and P-selectins (expressed by endothelial, leukocyte, and platelet cells respectively) via the cell surface ligands P-selectin glycoprotein ligand 1 (PSGL1) or other glycosylated ligands such as CD44 and E-selectin ligand (ESL1). However, CEC do not express either E- or P-selectin, neither do they store P- nor E-selectin in their Weibel-Palade bodies (cell-specific storage granules). Presumably,

leukocyte tethering and rolling in CECs occurs through the interactions of the leukocyte integrins $\alpha_4\beta_1$ (also known as very late antigen-4 or VLA-4) and $\alpha_4\beta_7$, and the endothelial ligands VCAM-1 and mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) respectively (Baron et al., 1993, Engelhardt et al., 1997).

During inflammation, endothelial cells are induced to express chemokine receptors (such as CCR3 and CXCR4) and chemoattractants (such as CCL5, CXCL4 and CXCL5) on their luminal surface by activated platelets and Mø. Arrest is rapidly triggered by these chemokines and chemoattractants and is mediated through leukocyte rolling. Following arrest, the final process of transendothelial migration involves intravascular crawling and either paracellular or transcellular migration into inflamed tissue.

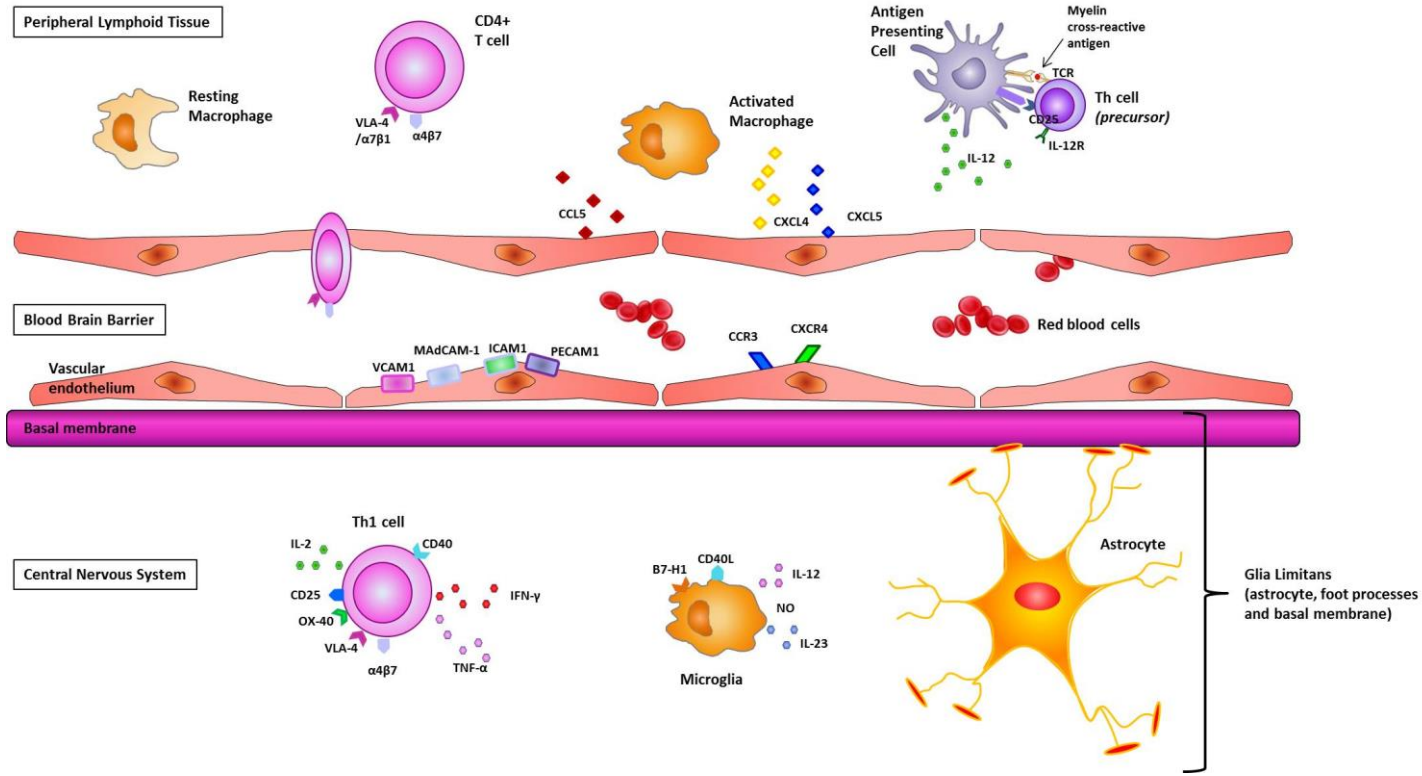


Figure 1.2: Diagrammatic representation of the pathway of autoimmune inflammation in MS.
The pathway of autoimmune inflammation is thought to occur in two stages: a priming stage in which antigen presenting cells (APC) activate naïve $CD4^+$ T cells via the T cell receptor (TCR) and through the expression of T cell activating growth factors such as IL-12. The activated T cells, in turn, induce the endothelial cells of the blood brain barrier (BBB) to express cell adhesion molecules, such as ICAM1, VCAM1, PECAM1, and MAdCAM-1. The migration of the activated T cell through the BBB involves a cascade of tethering, crawling, rolling and transmigration across or through the cell using cell-cell interactions such as $\alpha_4\beta_7$ and Very-late antigen 4 (VLA-4; also known as $\alpha_7\beta_1$) with their respective ligands MAdCAM1 and VCAM1 on the endothelial cells. Additionally, activated macrophages induce endothelial cells to express chemokine receptors (such as CCR3 and CXCR4) and chemoattractants (such as CCL5, CXCL4 and CXCL5) which trigger leukocyte rolling. Once these interactions occur, the leukocytes form projections that allow it entry through the BBB and into the CNS. Once they have entered the CNS, they are reactivated resulting in a phenotypic change and an upregulation of receptors and markers such as OX-40, and CD25, as well as the expression of pro-inflammatory molecules such as IFNγ, TNFα and IL-2. The exposure of endothelial cells to IFNγ and TNFα results in their release of nitric oxide (NO) and other oxygen radicals. This leads to an overall decrease in BBB integrity. During the initial wave of infiltrating leukocytes, macrophages (Mø) down regulate T cell growth factor production through its expression of the B7-H1 protein whose interaction with T cell programmed death proteins results in either immunological ignorance or anergy. However, following a second wave of infiltrating leukocytes, these Mø become activated and act as APCs expressing the T cell activating growth factors IL-12, IL-23 and NO thus propagating the autoimmune reaction.

Role of adaptive immunity

Prior to crossing the BBB, the activated T cells undergo alterations with a down-regulation in the activation markers IL-2 receptor α-chain (CD25) and OX-40. The cells then acquire surface bound MHC class II antigens and up-regulate the chemokine receptors CCR-1, CCR-2b, CCR-3, CCR5 and CXCR4 expressed on various cell types (such as endothelial, Mø, microglia, astrocytes, B cells, T cells and DCs) with a resulting phenotype called 'migratory membrane phenotype'. This increase in chemokines results in the alteration of selectin-mediated leukocyte interactions with endothelial cells to one that is integrin-mediated with high affinity that leads to transcellular migration of monocytes, lymphocytes and Mø (Ley et al., 2007).

Once in the CNS, the majority of these $CD4^+$ T cells are reactivated as suggested by the upregulation of OX-40, CD25 and the pro-inflammatory growth factors IFNγ, TNFα, and IL-2, and a down-regulation in the TCR/CD3 complex. Exposure of endothelial cells and astrocytes to IFNγ and TNFα respectively results in a decrease in occludin expression and its dissociation from the Z0-1 complex. This dissociation through increased tyrosine phosphorylation, the phosphorylation of the junction-associated protein vasodilator-stimulated phosphoprotein (VASP) and of VE-cadherin through induction of nitric oxide (NO) by TNFα, all result in a decrease in BBB integrity by increasing endothelial permeability.

Microglia are the resident Mø of the brain parenchyma and during the initial wave of T cell infiltration through the BBB, they negatively regulate the T cell growth factor production. They do this by expressing B7-H1 (a homologue of the co-stimulatory protein B7 found on activated APCs), which interacts with the T cell programmed death proteins, resulting in either immunological ignorance or anergy. During the autocrine feedback loop created, the microglial cells themselves become activated and act as APCs during the second wave of infiltrating T cells thus propagating the autoimmune response (Duncan and Miller, 2011). Antigen-presentation on microglia is a multistep

activation process that involves stimulation through growth factors GM-CSF and IFN-γ and cognate signalling of B7/CD28 and CD40L/CD40 ligand interactions (Matyszak et al., 1999). These activated microglia release T cell activating growth factors such as IL-12, IL-23 and toxic mediators such as NO and oxygen radicles (Platten and Steinman, 2005), thus playing an important role in maintaining a pro-inflammatory environment, both through IFNγ secretion and T cell proliferation (via IL-23) and Th1 induction. Therefore any tissue damage inflicted by the immune response within the CNS may be as a result of the second wave of infiltrating lymphocytes directed against self-antigens.

B cells on the other hand do not require entry across the BBB to exert a pathological response. They secrete destructive antibodies that can diffuse across the BBB and induce damage through terminal membrane attack complex formation, a product that (through complement activation) forms transmembrane channels that cause disruption of the cell membrane and cell lysis. As mentioned previously, the apperance of oligoclonal bands in the CSF of a patient is a diagnostic hallmark of MS and occurs in over 95% of patients. During late progression of MS, lymphoid-like structures (also referred to as ectopic lymphoid follicles) have been identified that contain B cells and plasma cells capable of expansion and maturation and possibly the source of the increased IgG and oligoclonal bands (Serafini et al., 2004).

PATHOLOGY OF MS

The pathology of MS involves both inflammation and demyelination with associated axonal loss, which results in plaque scarring or lesions. Archetypical lesions (active, chronic active and inactive/chronic inactive lesions) are sharply outlined grey and translucent areas scattered throughout the white and grey matter and are the most characteristic hallmarks of MS. They are defined by inflammatory cell infiltration, a level of demyelination and oligodendrocyte (OL) loss, microglial/macrophage activation and astrocyte scarring (Yuan et al., 2008). As discussed earlier, perivascular monocytes secrete growth factors and chemokines that alter adhesion molecule

function leading to the attraction and infiltration of non-specific and myelin-specific immune cells such as activated T cells. This process of inflammation initiates the demyelinating process in the newly developed inflammatory lesion (Arredondo, 2003).

Within an initial active lesion, a solid mass of foamy Mø secreting toxic mediators such as reactive oxygen species (ROS), NO, glutamate, TNFα and MMPs (Anthony et al., 1998, Perry and Anthony, 1999, Stuckey et al., 2005), is observed with infiltrating leukocytes, activated astrocytes and small numbers of OLs. This lesion progresses to a chronic active lesion that develops outwards as the myelin in the centre becomes progressively degraded and phagocytosed as demonstrated by the rim of Mø containing the lipid debris of the myelin sheath, such as myelin basic protein (MBP). Finally, the lesion dies out leaving a hypocellular scar-like lesion known as an inactive or chronic lesion in which no demyelination occurs but may contain some remnant Mø (Bruck et al., 1995).

The spontaneous remyelination of axons within a lesion does occur in the RRMS but appears to fail within progressive stages of the disease. Remyelinating lesions are identified by their thin myelin sheaths and pronounced infiltration of Mø and lymphocytes. In late remyelinating lesions, only a small degree of inflammation is observed with marked astrocyte proliferation and gliosis (Bruck et al., 1995). The normal appearing white matter (NAWM) appears normal through light microscopy with no visible demyelination or phagocytosis. However, although there appears to be no demyelinating event occurring, axonal degradation and loss have been observed in early lesions (Trapp et al., 1998, Bitsch et al., 2000).

Additionally, a preactive lesion has been defined (De Groot et al., 2001). Preactive lesions are round or oval regions that are demarcated by the clustering of activated microglia/macrophages (Maeda and Sobel, 1996). These activated microglia/Mø express increased levels of the human leukocyte antigen-DR (HLA-DR), CD68 and the benzodiazepine receptor. These lesions lack any detectable demyelination, leukocyte infiltration, gliosis or hypertrophic astrocytes, but occasionally display a small number of $CD45^+$ cells within the perivascular space. Although they do not display any inciting

agents such as viral or bacterial, their vicinity to activated OLs suggests an unknown soluble factor that mediates the communication between these OLs and activated microglia. Activated microglia/Mø show increased expression of the small heat shock protein αβ-crystallin, a protein that is secreted into the CSF and its accumulation is linked to the activation of OLs in these preactive lesions (van Noort et al., 2010). Most notably the expression of αβ-crystallin is induced by antibodies, specifically myelin surface protein antibodies (Duvanel et al., 2004), thus linking the abundance of intrathecal antibodies observed in MS and these preactive lesions. Additionally, the number of preactive lesions observed is significantly more than the number of full blown active lesions that occur later in those same regions. This suggests that some intrinsic regulation allows the lesion to resolve without any subsequent damage (van der Valk and Amor, 2009).

The inflammatory component of MS: immune-mediated demyelination.

The immunopathology of MS is not wholly understood but is generally regarded to be the initial penetration of the CNS by activated immune cells (such as $CD4^+$ and $CD8^+$ T cells, B cells and plasma cells, monocytes and Mø expressing MHC Class II (Bitsch et al., 2000)) that results in inflammatory destruction followed by demyelination. The exact order of events is as yet unclear, however recent studies have demonstrated axonal degradation early on in the disease in the absence of inflammation and demyelination, causing researchers to look more closely at the interactions between cellular and molecular processes (Trapp et al., 1998, Bitsch et al., 2000, Herz et al., 2010, Tallantyre et al., 2010).

In EAE (experimental autoimmune encephalomyelitis, the animal model for MS) the spatial distribution of a lesion and its immune cell profile can differ depending on the antigen used to induce the disease. For example, the immunisation of rats with the myelin-specific antigen MOG (myelin oligodendrocyte glycoprotein) incurs lesion damage in the cerebellar white matter, whereas astroglial antigens (such as s100β and glial fibrillary acidic protein) elicit severe inflammation in the grey matter (Huizinga et al., 2008). Immunisation of Biozzi ABH mice with the neurofilament light

(NF-L) protein induces symptoms of MS such as spastic paresis and paralysis, presumably due to axonal autoimmunity (Huizinga et al., 2008). Spinal cord lesions taken from these mice were compared with the spinal cord lesions taken from MOG-immunised Biozzi ABH mice. The lesions from NF-L-immunised mice had increased numbers of macrophages in the thoracic level compared to MOG-immunised mice. MOG-immunised mice had increased levels of T and B cells in the meninges compared to NF-L-immunised mice with similar levels in the parenchyma. Activated endothelial cells, glial cells and occasionally astrocytes also play important roles in mediating this inflammatory event. The subsequent demyelination of neurons is driven by Mø that leads to the eventual destruction and loss of OLs.

The oligodendrocyte-neuron unit

The cytoarchitecture of the CNS is maintained by astrocytes and OLs that support the neural axon both structurally and functionally. OLs are important for several reasons: (1) they support neurons within the CNS through the induction of sodium channel clusters required for efficient saltatory conduction, a highly efficient method of signal transmission (Kaplan et al., 1997); (2) they increase axonal stability, and induce the accumulation and phosphorylation of neurofilaments within the axon (Sanchez et al., 1996); and (3) they myelinate axons by wrapping their cytoplasmic processes around the axon generating myelin sheaths.

Oligodendrogenesis

Oligodendrocyte precursor cells (OPCs) arise from multipotent neuronal stem and progenitor cells (MNSC) in the subventricular zones of the embryonic neural tube and from progenitor cells in the dorsal spinal cord and hindbrain (Miller, 1996, Vallstedt et al., 2005). OPCs migrate to various regions of the brain prior to proliferating and differentiating into neurons, astrocytes or OLs (McKay, 1997, Temple, 2001). OPCs are morphologically bipolar with short processes and have the ability to

proliferate and migrate, whereas OLs are multipolar with long extended processes, they do not proliferate and have very little or no migratory abilities (Stangel and Hartung, 2002).

OPCs proliferate mainly in response to mitogens including platelet-derived growth factor (PDGF) and bFGF (Bogler et al., 1990) expressed by astrocytes. Removal of PDGF results in early differentiation of OLs whereas overexpression results in a transient increase in OPCs. These precocious and excessive cells are later rectified by normal developmental processes such as programmed cell death and/or cell cycle control by p27 (Richardson et al., 1988, Calver et al., 1998) resulting in a 'steady state' of OPCs differentiating into OLs (Barres et al., 1992, Calver et al., 1998, Chen et al., 2009). However, the signals involved in OL differentiation and myelin formation are not wholly understood.

OPC differentiation

Only once all neuronal components of the developing CNS are in place, and when a critical threshold of OPCs have been recruited or reached, can the stages of OL differentiation begin. The different stages of development within the OPC lineage are governed by local trophic factors and feedback cues expressed by both OPCs and mature, myelinating OLs (Figure 1.3) (Zhang and Miller, 1996, Calver et al., 1998, Chen et al., 2009, Yang et al., 2011).

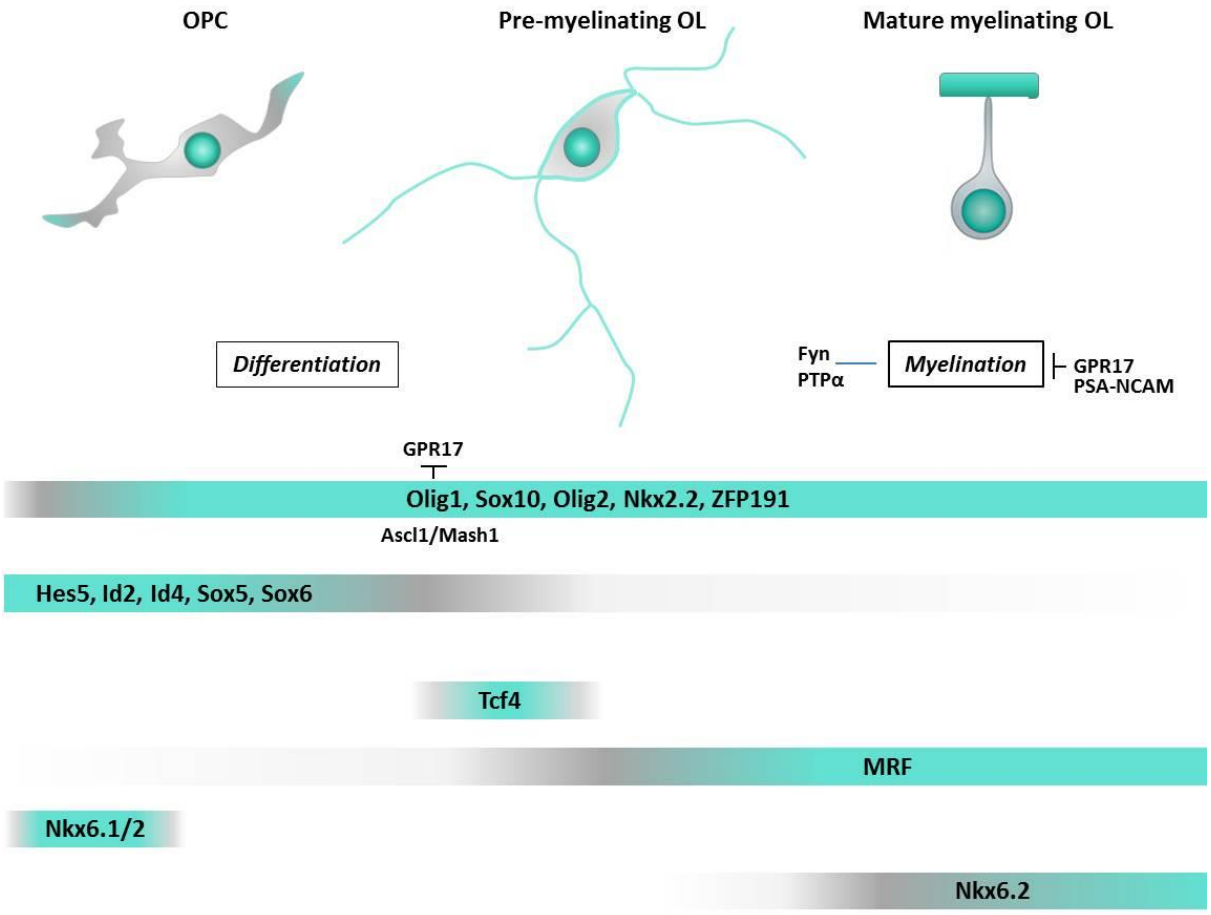


Figure 1.3. Transcriptional regulation of OPC development and differentiation into mature myelinating oligodendrocytes.
Several transcription factors including Nkx6.1/6.2 and Olig2 in response to sonic hedgehog homolog (Shh) (reviewed in Richardson et al., 2006, Merchan et al., 2007) are involved in the commitment of MNSC to an OL lineage with Olig2 being essential. Once the lineage has been committed to develop into OLs, the expression of Sox5, Sox6 and the basic helix-loop-helix inhibitory proteins Hes5, ID2 and ID4 (Wang et al., 2001), and the Olig1 regulated G protein-coupled receptor 17 (GPR17) (Chen et al., 2009) maintain the level of OPCs and inhibit their differentiation into OLs (Emery, 2010). During early lineage stages the transcription factors Hes5, Sox5 and Sox6, as well as the DNA binding inhibitory proteins Id2 and Id4 are expressed, but when signaled to undergo differentiation, the transcription activator Tcf4 associtaes with the Wnt/β-catenin pathway to target and inhibit Id2 and Id4. However, when Tcf4 associates with members of the Groucho/Tle family, it acts as a transcriptional repressor.Nkx2.2, Olig 1 and Sox10 function only as mediators in OPC differentiation and maturation but not on the expression of the myelin genes required for myelination. The maturation of OPCs and the expression of the myelin genes is driven by the transcriptional factor, MRF. During the initial differentiation stage, Nkx6.2 is upregulated, followed by down-regulation during the intermediatary stages, with further upregulation (for the development of the paranodes between the myelin sheaths) during the final 'fine-tuning' stage of OL maturation. Additionally, although myelination is inhibited by GPR17 and polysialylated-neural cell adhesion molecule (PSA-NCAM), it is also stimulated by the SRC family tyrosine kinase Fyn that is activated and regulated by the receptor type protein-tyrosine phosphatase alpha (PTPα). Furthermore, GPR17 is inhibited by the expression of Olig1 whose expression is required by the basic helix loop helix protein Ascl1/Mash1 for the generation of mature OL and for myelination. Adapted from Emery, 2010

The timing of development of myelinating OLs depends on the intrinsic and extrinsic environment, the cellular timers and on cell–cell interactions. Similar to myoblasts and erythroid precursor cells, OL lineage cells appear to utilise an intrinsic timing mechanism that requires both mitogens (such as PDGF) and lipid-soluble mediators (such as retinoic acid, dexamethasone or thyroid hormone (TH)) to operate and differentiate normally (Barres et al., 1994). This timing mechanism consists of two components: a counting component that is involved in OPC proliferation and operates independently of lipid-soluble mediators; and an effector component involved in OL differentiation that is dependent on the hydrophobic signals. It appears to count by measuring time and not cell cycle divisions (Gao et al., 1997), possibly through positive regulators of the cell cycle (such as the transcriptional activator AP-1) and by an accumulation of the cell-cycle inhibitor p27. The gradual inhibition of AP-1 by an increasing accumulation of thyroid hormone and p27 act concomitantly to slow the cell cycle enabling the cell to withdraw from it and to differentiate into myelinating OLs. The heterchronic genes (genes that regulate the timing of developmental events) responsible for the timing of OLs are as yet, unknown.

The development of myelinating OLs also depends on cell-cell interactions. Notch1 receptors, part of a signalling family that regulates the interactions between adjacent cells, are expressed on OPCs and interact with the ligand Jagged1 expressed, in large amounts, by OLs. When a threshold of OLs is reached, Jagged1/Notch1 interactions signal to create feedback loops that restrict OPCs from differentiating (Wang et al., 1998). OPCs also express small amounts of this ligand, which may be autocrine, generating a small population of surplus OPCs that can be recruited at a later time if needed such as restoring damaged cells following injury in MS, or following demyelination and OL depletion. Interestingly, Notch1 activation has the ability to induce continual proliferation of OPCs when mitogens are withdrawn suggesting that OPC proliferation operates separately to the counting component of the intrinsic timing mechanism.

The specification and maintenance of OPCs is under transcriptional control. Several transcription factors including Nkx6.1/6.2 and Olig2 in response to sonic hedgehog homolog (Shh) (reviewed in Richardson et al., 2006, Merchan et al., 2007) are involved in the commitment of MNSC to an OL lineage with Olig2 being essential. Once the lineage has been committed to develop into OLs, the expression of Sox5, Sox6 and the basic helix-loop-helix inhibitory proteins Hes5, Id2 and Id4 (Wang et al., 2001), and the Olig1 regulated G protein-coupled receptor 17 (GPR17) (Chen et al., 2009) maintain the level of OPCs and inhibit their differentiation into OLs (Figure 1.3; Emery, 2010).

The transcription factor Tcf4 (also known as Tcf7L2) acts as a transcriptional activator in OPC maintenance when associated with Wnt/β-catenin signalling that paradoxically targets the DNA-binding inhibitors Id2 and Id4; and as a transcriptional repressor when associated with members of the Groucho/Tle family (Figure 1.3). It is an effector molecule involved in the intrinsic timing mechanism of the cell and is required for cell cycle exit and therefore, the onset of OL differentiation. It is found in MS lesions but not in normal white matter suggesting a role in remyelination (Fancy et al., 2009) . The transcription factors Nkx2.2, Sox10 and Olig1 are expressed early on in the development of the lineage but are only required for OL differentiation and maturation, and the expression of myelin genes. The myelin gene regulatory factor (MRF) presumably acts directly on Sox10 and Olig1 to drive OL maturation and up-regulate the majority of the myelin genes including myelin-basic protein (MBP) and proteolipid protein (PLP). During the late stages of OL differentiation, the basic helix-loop-helix proteins Ascl1/Mash1 are required by Olig1 for the generation of myelinating OLs and ZFP191 is essential for myelination although, the mechanism of ZFP191 in this process is not yet clear. Olig1 inhibits the actions of the differentiation-inhibitory molecule GPR17 by binding directly to its promoter thus promoting OL maturation and myelination (Chen et al., 2009). Myelination by OLs is inhibited by GPR17 and the polysialylated-neural cell adhesion molecule (PSA-NCAM) (Charles et al., 2000, Charles et al., 2002) but stimulated by Fyn, an SRC family tyrosine kinase, that is activated and regulated by the receptor type protein-tyrosine phosphatase alpha (PTPα) and is essential in OL differentiation, morphology and myelination

(Sperber and McMorris, 2001, Umemori et al., 1994, Wang et al., 2009). The correct assembly of myelin requires the myelin proteins MBP and PLP for the compaction of the sheaths, the protein MAG for axon-glia recognition (Stangel and Hartung, 2002) PTPα may also regulate survival cues, proliferation cues or even the cells withdrawal from the cell cycle (Wang et al., 2009). During the intermediate stages of OL differentiation the transcription factor Nkx6.2 is down-regulated but in the final stages it becomes up-regulated (Figure 1.3) and is required for the proper development of the myelin paranodes, but not for the expression of the myelin genes or for myelination suggesting that the fine-tuning of OL maturation is under separate transcriptional control. OL development is also under tight post-transcriptional control by microRNAs (miRNAs). Their main function is to act as negative feedback loops that define the transition between OPC maintenance and OL differentiation (Emery, 2010).

In vitro studies have demonstrated the expression of myelin proteins and of myelin-like processes extending from OLs that have not yet encountered amyelinated axons (Lubetzki et al., 1993). Axons that express the appropriate recognition signals attract these myelin-like processes stimulating them to begin wrapping and compacting, forming mature myelin sheaths around the axon. The initial processes that ensheath an axon dictate what diameter of axon subsequent processes from the same OL can myelinate (Fanarraga et al., 1998). Although the degree of myelination does not vary in time and results in a constant G-ratio (the ratio of axon diameter to myelin fibre (Lubetzki et al., 1993)), it does depend on size as axons less than 0.5 μm remain amyelinated (Charles et al., 2000). Furthermore, the act of ensheathing an axon with myelin results in rapid axonal expansion presumably through these trans-interactions and their recognition signals (Sanchez et al., 1996).

Myelination

Myelination is heavily invested in the neuronal/axonal network for structural support and is dependent on the neuronal axon for its expression of both recognition and survival signals. Several OLs are required to myelinate a single axon whilst a single OL can myelinate up to 50 axons (Kiernan

and Barr, 2004). This myelination enables saltatory conduction to occur as the electrical impulses utilise the Nodes of Ranvier, the gaps formed between the myelin sheaths along the nerve axon, to 'jump' across the axonal junctions rather than travel the entire length of the axon. This significantly increases the conduction speed of an impulse. For example, myelinated skeletal muscle fibres can propagate a signal at a speed of 70–120 m/sec, whereas an unmyelinated nociceptive nerve fibre can propagate a signal at a speed around 0.5–2.5 m/sec (Kiernan and Barr, 2004).

The mature neuron/axon cytoskeleton is built up from two neurofilament pools: a moving (or transport) pool that contains the newly synthesized unphosphorylated neurofilament molecules that, when induced by the myelin sheath to be phosphorylated, move into a stationary pool containing the phosphorylated units of neurofilament. This turnover of unphosphorylated neurofilament units into phosphorylated units increases the calibre of the axon and axonal conduction velocity (Trapp et al., 1998, Lovas et al., 2000). This increase in axon calibre may prove neuroprotective once a certain size is reached, as smaller calibre axons are preferentially degenerated in MS plaques and in NAWM (Lovas et al., 2000).

The myelin sheath profits from this axonal wrapping by inducing axonal recognition signals such as type III neuregulin, an axonal recognition signal that does not initiate or induce myelination. It acts solely by regulating the degree of myelination and the expression of all CNS myelin components, and possibly the action of process extension and wrapping around an axon through signalling interactions with erbB receptors (Taveggia et al., 2008). The expression of axonal recognition signals contribute to the co-ordination of myelination by not only dictating the degree/thickness of compact myelin, but also by acting to enhance OL differentiation (Taveggia et al., 2008, Sanchez et al., 1996).

Survival signals become apparent postnatally and encourage axon ensheathment as well as mediating MNSC homing to damaged regions of the CNS initiating the process of remyelination (Calver et al., 1998).

Demyelination and remyelination

Demyelination is a major underlying factor to the symptoms experienced by MS patients. The loss of myelin can occur via a variety of pathways/routes, most notably through axonal degeneration and/or transection leading to axonal loss; or through the loss of the myelin-producing cell, the OL. Therefore, an obvious therapeutic strategy is one that enables the remyelination of demyelinated axons, and/or the protection of the axon from injury and subsequent loss.

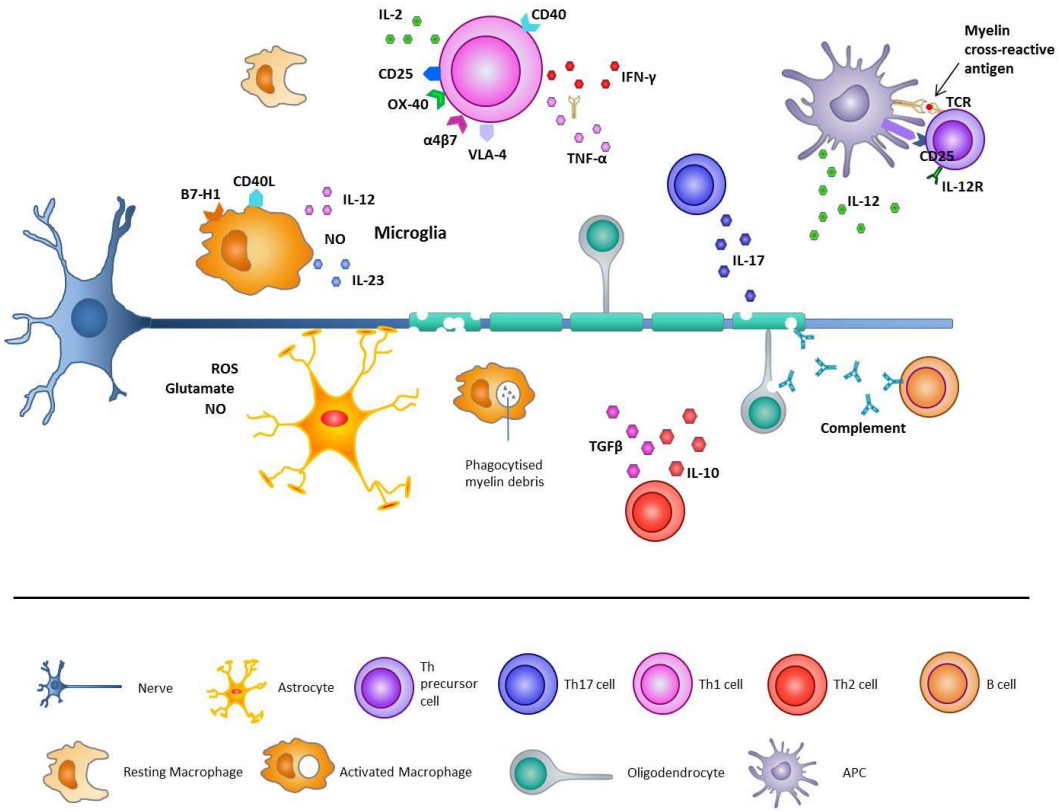
The death of oligodendrocytes and the subsequent loss of myelin

Reactive oxygen species (ROS) are free radicals and related molecules that are produced by normal aerobic metabolism and neutralised by cellular antioxidants. Under pathological conditions, an increase in ROS may exceed the cells antioxidant mechanisms leading to oxidative stress (OS), the biological imbalance between the production and appearance of ROS, and the cells ability to detoxify or its ability to repair the damage produced. As well as containing the highest concentration of antioxidants, astrocytes also supply neurons with substrates for antioxidants such as glutathione, an antioxidant that can act directly on ROS. Within MS patients and lesions, elevated levels of ROS and decreased levels of glutathione have been observed (LeVine, 1992, Langemann et al., 1992).

Glutamate, the precursor of glutathione, is a major excitatory neurotransmitter normally taken up by astrocytes to prevent excitotoxicity (Chen et al., 2001). During an excitatory crisis, as seen following CNS trauma, reactive astrocytes and microglia exposed to high levels of potassium release glutamate via reverse glutamate transport leading to the accumulation of pathological levels of glutamate within the extra-cellular space (Gilgun-Sherki et al., 2004, Pekny and Nilsson, 2005). This glutamate excitotoxicity may lead to OL loss through activation of the α-amino-3-hydroxy-5-methyl-4-isoxasole propionic acid (AMPA)/kainate receptors (Tekkok and Goldberg, 2001, Pitt et al., 2000). Antagonists of these receptors result in an increase in OL survival and a decrease in dephosphorylation of neurofilament H, an indicator of axonal damage, leading to disease amelioration in a model of EAE (Pitt et al., 2000, Smith et al., 2000).

NO, a highly reactive molecule with a variety of pleiotropic physiological actions, may also contribute to axonal damage in chronic lesions. NO is synthesized from the amino acid L-arginine through the action of the enzyme nitric oxide synthase (NOS). NOS is normally expressed in very low nanomolar quantities in the CNS in the forms of endothelial and neuronal NOS (eNOS and nNOS respectively). A third form inducible NOS (iNOS) not normally found in the CNS has been found to be expressed within inflammatory lesions of EAE (De Groot et al., 1997, Kim et al., 2000) and by endothelial cells and cells of a microglial/Mø lineage. Reactive astrocytes and glial cells also produce NO, possibly via growth factor-mediated induction of NOS (Dawson and Dawson, 1998, Xiao et al., 1996).

Within chronic lesions, activated microglia produce iNOS and (indirectly) NO which has been found to reversibly block axonal conduction *in vitro* (Redford et al., 1997, Jones et al., 2010, Shrager et al., 1998). The exact mechanism of this blockage is still unclear but is thought to involve impairment of the Na^+, K^+ or Ca^+ channels that are exposed following demyelination, or by depolarisation of the axon by cyclic guanosine monophosphate (cGMP) or the Na^+ pump. Axonal demyelination may also involve a positive feedback loop that is initiated by NO-mediated lipid peroxidation (the oxidation of lipid by NO) resulting in the expression of the macrophage scavenger receptor. This receptor mediates the endocytosis of modified low density lipoprotein (LDL) and foreign negatively charged macromolecules. The expression of this receptor therefore stimulates Mø to phagocytose myelin, which in turn leads to Mø-activated NO production (Smith and Lassmann, 2002) (Figure 1.4).



Mø = macrophages; APCs = antigen-presenting cell; NO = nitric oxide; TCR = T-cell receptor; ROS = reactive oxygen species.

Figure 1.4. Schematic representation of the pathological interactions that occur during inflammation in MS.

Following the initial wave of leukocyte infiltration and activation, Mø express the co-stimulatory ligand B7-H1 that down regulates the production of T cell growth factors and directs them towards immunological ignorance or anergy. However, during a second wave of infiltration, the Mø themselves become activated and act as APCs that express IL-12, IL-23 and NO. Once these activated Mø encounter the infiltrating activated T cells, they cause a phenotypic change in the T cell. The formation of the trimolecular complex (i.e. the interaction between the TCR, the MHC Class II molecules and the inciting antigen presented by the APC) in association with co-stimulatory molecules (such as B7-H1) triggers specific responses of the activated T cell. The growth factor IL-12 is a key growth factor that drives the differentiation of CD4+ T cells to a Th1 phenotype, releasing proinflammatory growth factors such as TNFα and IFNγ, growth factors that when exposed to endothelial cells, cause them to release NO and other oxygen radical species. The growth factor milieu also includes IL-4, which drives the development of Th2 cells that secrete anti-inflammatory growth factors (such as TGFβ and IL-10). These growth factors have the potential to down regulate the expression of the Th1 growth factors, of MHC class II antigens and co-stimulatory molecules found on activated Mø. These activated T cells also release the growth factor IL-17, which induces the differentiation of Th17 cells, thought to be involved in the pathogenesis of MS. NO released by activated Mø, astrocytes and endothelial cells induces the production of IL-2 in activated T cells, which in turn, induces expression of the T cell activation markers CD25 and OX-40. OX-40 when engaged to its ligand OX-40L, leads to cell proliferation and cell survival. The presence of TNFα and IFNγ is toxic to both myelin and to their OLs and leads to the activation of phagocytic Mø, identified through the presence of lipid debris from the myelin sheath, mainly the remnants of MBP. During normal physiological conditions, astrocytes take up toxic levels of ROS, glutamate and NO. However, within a lesion, reactive astrocytes reverse this uptake and release the molecules into the cellular environment, causing death and destruction of the myelin sheath and of its OLs and surrounding microglia. B cells also play a role in the destruction of the myelin sheath. When activated, they release destructive antibodies that diffuse the BBB and induce damage through the terminal attack complex and complement activation. Abbreviations:

The degenerative component of MS: immune-mediated neurodegeneration

Once an axon has undergone demyelination, it is subject to insult and injury that may result in transection of the axon itself. When a demyelinated axon is transected, the functionality (i.e. the axons ability to conduct impulses efficiently) is lost. Wallerian degeneration follows the transection of the axon and is a major component of axonal pathology in MS lesions (Dziedzic et al., 2010). Following Wallerian degeneration, the nerve cell body may survive forming a terminal axonal ovoid and may attempt to repair the axon, typically once the insult has dissipated. Within an acute MS lesion, an abundant number of these axonal ovoids have been observed (11 000/mm^3 of lesion area; (Trapp et al., 1998)). This suggests that axons, in particular small calibre axons (Lovas et al., 2000), are vulnerable to inflammatory environments such as an active MS lesion, and that this axonal injury is mediated by microglia, Mø and $CD8^+$ T cells (Bitsch et al., 2000) possibly through the expression of inflammatory factors such as proteolytic enzymes, growth factors, oxidative agents and free radicals (Anthony et al., 1998, Trapp et al., 1998, Perry and Anthony, 1999, Stuckey et al., 2005, Dutta and Trapp, 2011).

Growing evidence suggests that axonal degeneration or loss, is the pathological substrate for the progressive disability of the disease (De Stefano et al., 1998, Ceccarelli et al., 2008, Tallantyre et al., 2010), occurs independent to demyelination (Bitsch et al., 2000, Lovas et al., 2000, Huizinga et al., 2008, Siffrin et al., 2010, Tallantyre et al., 2010) and is considered to be the most devastating aspect of the disease. MS patients experience a variety of neurological symptoms at various times during their disease course. They include spasticity, fatigue, bladder weakness, cognitive dysfunction, depression and pain. These symptoms may appear and disappear without an apparent pattern, making MS diagnosis difficult. Recent studies comparing MS patients with different clinical symptoms have shown a significant loss of grey matter volume and cortical thinning when compared to healthy controls (Ceccarelli et al., 2008). This cortical thinning may be attributed to axonal

degeneration following axonal injury (Sailer et al., 2003). Importantly, this study suggests that the areas of grey matter affected could explain the type of clinical symptoms experienced (Ceccarelli et al., 2008). For example, it was noted that in SPMS patients whose clinical phenotype includes a common occurrence of sensory deficits, grey matter loss was more 'pronounced' in the post central gyrus in the cortex. Damage to the post central gyrus can cause sensory deficits such as agnosia, the inability to interpret sensations and ultimately to recognise things (Soanes and Stevenson, 2006). These finding have been corroborated by other studies that have found a significant relationship between axon loss and disability in MS patients (Lovas et al., 2000, Siffrin et al., 2010, Tallantyre et al., 2010).

TREATMENTS FOR MS

Six therapeutic agents have been approved for the treatment of RRMS, with some overlap in the possible treatment of PPMS. Unfortunately, not all therapeutics that demonstrated promise in animal models have successfully been translated into human clinical trials (Wiendl et al., 2009, Ulzheimer et al., 2010). Nevertheless, the data generated from these trials is by no means wasted. Indeed, it has provided valuable insight into the complexity this disease. For example, several studies have demonstrated an inhibition of EAE by the anti-inflammatory growth factors TGFβ1 and TGFβ2 (Johns et al., 1991, Kuruvilla et al., 1991, Fabry et al., 1995). However, when administering the active recombinant growth factor, no effects on the clinical score (i.e the expanded disability status scale (EDSS)) or on the MRI lesion load, were observed during a phase 1 trial of TGFβ2 (Calabresi et al., 1998). However, it did identify safety concerns for its use in humans such as renal toxicity as the long-term treatment with constitutively active TGFβ1 has proven harmful by inducing liver fibrosis and glomerulosclerosis (Clouthier et al., 1997).

Disease-modifying therapies

Significant developments in the treatment of RRMS were made following clinical trials during the 1980s and 1990s using interferons, particularly IFNβ. The first approved treatment for RRMS was Betaseron® (IFN-β1b; Bayer Healthcare; subcutaneous injection [s.c]) in 1993, followed by Avonex® (IFN-β1a; Biogen Idec; intramuscular injection [i.m]) in 1996 and Rebif® (IFN-β1a; Serono (UK) Pfizer (US); s.c) in 1998 (Rudick, 2005). IFN-β inhibits autoimmune inflammation in the CNS by suppressing pathogenic Th1 growth factor production, reducing the production of matrix metallo-proteinases (MMPs) and decreasing the expression of adhesion molecules by T cells and endothelial cells thus affecting their entrance into the CNS (Nagai et al., 2003, Yushchenko et al., 2003, reviewed by Billiau et al., 2004). Glatiramer acetate (GA, also known as Copaxone®, Teva Pharmaceutical Company, Ltd.) was approved by the FDA in 1996 for the reduction of relapses in MS patients with RRMS, and was the first non-interferon based treatment for MS (Johnson et al., 1998). GA activates antigen-specific T suppressor cells and shifts them towards a non-pathogenic Th2 response. These activated T cells then cross over the BBB where they are reactivated by myelin antigens prompting them to release anti-inflammatory growth factors (Table 1.1) (Dhib-Jalbut et al., 2006).

Table 1.1: Current treatments for MS in use or in clinical trials.

Agent	Clinical Stage	Regime	Mechanism of Action	Immunologic effects	Neuroprotective effects	Data from completed phase II/III trials	Side effects	Future trials	Clinical Trial Identifier
Oral Therapies									
FTY720 (Fingolimod; Gilenya®)	RRMS	Once daily; 1.25mg / 0.5mg/day	Targets the receptors for the potent signalling lipid sphingosine-1-phospate (S1P) used for the migration of lymphocytes from secondary lymphoid organs to periphery.	Sequesters naïve and central memory T cells in the lymph nodes; Increases endothelial barrier integrity; restricts the ability of lymphocytes to infiltrate the periphery.	Improves myelination and reverses demyelination.	Phase II - 12 month (1.25mg/0.5mg/day): ARR reduction 53-55%; Gd+ lesion reduction 43-62% Phase III **TRANSFORMS** - 24 month (1.25mg/0.5mg/day): ARR reduction 39-52% Phase III **FREEDOMS** – 24 month (0.5/1.25mg/day vs. placebo): ARR reduction 54-60%	Bradycardia, relapse, basal-cell carcinoma.	Phase III: **FREEDOMS** II for RRMS in over 1080 patients Phase IIIb: **INFORMS** for PPMS in over 650 patients	http://clinicaltrials.gov/ct2/show/NCT00355134 http://clinicaltrials.gov/ct2/show/NCT000731692
Laquinimod	RRMS	Once daily; 0.6mg/day	Through the suppression of the NFκB pathway	Growth factor shift to Th2 profile; through suppression of inflammatory pathways and enhanced apoptosis; suppression of antigen-presenting mechanisms followed by decrease in chemotaxis and adhesion.	Reduction in myelin destruction and axonal damage (indirect)	Phase IIb (0.3mg/0.6mg/day): Gd+ reduction 7(n/s)-40.4% Phase III **ALLEGRO** – 24 month (0.6mg/day): ARR reduction 23%; reduction in disease progression 36%; reduced brain atrophy 33.8%	Back pain and abdominal pain, elevations in liver enzymes but no serious liver damage.	Phase III **BRAVO** – 24 month (0.6mg/day vs. IFNβ-1a [Avonex®, Biogen Idec] for RRMS in over 1200 patients.	http://clinicaltrials.gov/ct2/show/NCT00605215
Cladribine	RRMS	Once daily per 5 day course for a total of 10-20	Causes accumulation of deoxynucleotides in lymphocytes.	Immune cell depletion; reduces levels of proinflammatory growth factors and	None	Phase III **CLARITY** (5.25mg/3.5mg/kg): ARR reduction 55-58%; reduction in disease progression 31-33%;	Headaches, upper respiratory tract infections, nasopharyngitis, nausea, lymphopenia;	Phase II **ONWARD** – 104 weeks (0.875mg/kg/cycle [1 cycle is the daily treatment of cladribine for 4 to 5 days consecutively] for up to 4 cycles) vs. IFNβ-1a	http://clinicaltrials.gov/ct2/show/NCT00436826

Agent	Clinical Stage	Regime	Mechanism of Action	Immunologic effects	Neuroprotective effects	Data from completed phase II/III trials	Side effects	Future trials	Clinical Trial Identifier
Cladribine cont.		days per year treatment		chemokines; lymphocyte supression		Gd+ reduction 73-88%	malignancies, choriocarcinoma.	[Rebif®, Serono Pfizer] for RRMS and SPMS with relapses in over 200 patients.	
BG00012	RRMS	240mg/tid (three times a day)	Not fully understood; Activates nuclear factor-E2-related factor 2 (Nrf2) transcriptional pathway whose downstream factors are involved in the expression of detoxification agents and may upregulate antioxidant players	Upregulates Th2 response	Reduction in myelin destruction and axonal damage (indirect)	Phase II (240mg/tid vs. placebo): ARR reduction 32% (n/s) Gd+ reduction 69%	None	Phase III **DEFINE** – 24 month (480mg/day vs. 720mg/day vs. placebo) for RRMS in over 1230 patients. Phase III **CONFIRM** – 24 month (480mg/day vs. 720mg/day vs. Glatiramer acetate [Copaxone®, Teva Pharmaceutical Industries, Ltd.] 20mg/day s.c vs. placebo) for RRMS in over 1400 patients. Phase III **EXPLORE** – 8 month (240mg/tid + IFNβ vs. 240mg/tid + Copaxone 20mg/day s.c) for RRMS in over 100 patients.	http://clinicaltrials.gov/ct2/show/NCT00420212 http://clinicaltrials.gov/ct2/show/NCT00451451 http://clinicaltrials.gov/ct2/show/NCT01156311
Teriflunomide (Aubagio®, Genzyme)	RRMS	Once daily, oral 7mg or 14mg.	Reversibly inhibits dihydroorotate dehydrogenase thus impairing DNA synthesis	Supresses inflammatory factors, inhibits tyrosine kinase activity, impede T cell activation and APC interaction	None	Phase II TEMSO (7mg/day vs. 14mg/day vs. placebo): ARR reduction 28-32% Gd+ reduction 60-40%	Increase in ALT, alopecia, diarrhea, influenza, nausea and paresthesia	Phase III **TOPIC** – 40 month (7mg/day vs. 14mg/day vs. placebo) for RRMS in over 1110 patients. Phase III **TOWER** – 24 month (7mg/day vs. 14mg/day vs. placebo) for CIS in over 780 patients. Phase III – 24 weeks (7mg/day + Copaxone® 20mg/day s.c vs. 14mg/day + Copaxone® 20mg/day s.c vs. Copaxone® 20mg/day s.c)	http://clinicaltrials.gov/ct2/show/NCT00622700 http://clinicaltrials.gov/ct2/show/NCT00751881 http://clinicaltrials.gov/ct2/show/NCT00475865

Agent	Clinical Stage	Regime	Mechanism of Action	Immunologic effects	Neuroprotective effects	Data from completed phase II/III trials	Side effects	Future trials	Clinical Trial Identifier
Teriflunomide cont.								for RRMS, SPMS, PRMS in over 120 patients. Phase III – 48-172 weeks (7mg/day + IFNβ i.m. vs. 14mg/day + IFNβ i.m. vs. IFNβ i.m.) for RRMS, in over 1455 patients.	http://clinicaltrials.gov/ct2/show/NCT01252355
Injectable Therapies									
IFNβ-1a (i.m., Avonex®, Biogen Idec)	RRMS	30mcg once a week	Unknown at present	Slows down immune response, possibly by interfering with T cell activation and infiltration into CNS, apoptosis of autoreactive T cells, growth factor shift from Th1 to Th2, restoration of Treg activity	Indirect effects possibly through the suppression of MMP9, T cell activation and infiltration and a shift in the growth factor profile to generate a pro-myelinating environment and to prevent myelin and axonal damage.	Decrease in exacerbation rate (0.67 per year in AVONEX-treated group vs. 0.82 per year in the placebo-treated group (p=0.04). Significant decrease in frequency of exacerbations (p=0.03). Significant Gd+ reduction (p≤0.05)	Flu-like symptoms including chills, fever, myalgia, and asthenia.	Phase III **BIIB017 (PEGylated interferon beta-1a) –** 24 month BIIB017 125 mcg s.c. vs. PBO s.c. (every 2-4 wks) for RRMS in over 1260 patients. **Combinational Trials** Phase III **Daclizumab High Yield Process (DAC HYP) vs. interferon beta-1a (Avonex®, Biogen Idec)** – 96 to 144 weeks (Avonex 30 mcg/week i.m. + placebo s.c. vs. DAC HYP 150 mg/every 4 weeks s.c. + placebo i.m. for RRMS in over 1500 patients. Phase III **CombiRx study** – 36 month (Avonex 30 mcg/week i.m. + Copaxone 20 mg/d s.c. vs. Avonex + placebo s.c. vs. Copaxone + placebo i.m.for RRMS in over 1000 patients. Phase IV **TOP MS** – 24	http://clinicaltrials.gov/ct2/show/NCT00906399 http://clinicaltrials.gov/ct2/show/NCT01064401 http://clinicaltrials.gov/ct2/show/NCT00211887 http://clinicaltrials.gov/ct2

Agent	Clinical Stage	Regime	Mechanism of Action	Immunologic effects	Neuroprotective effects	Data from completed phase II/III trials	Side effects	Future trials	Clinical Trial Identifier
IFNβ-1a (i.m., Avonex®, Biogen Idec) cont.								month (Avonex 30 mcg/week i.m. vs. Rebif 44 mcg tiw s.c. vs. Betaseron 250 mcg q.o.d s.c. vs. Copaxone 20 mg/d s.c. for active RRMS in over 3000 patients. Phase III **BRAVO** – 24 month (0.6mg/day vs. IFNβ-1a [Avonex®, Biogen Idec] for RRMS in over 1200 patients.	/show/NCT00819000 http://clinicaltrials.gov/ct2/show/NCT00605215
IFNβ-1a (s.c, Rebif®, Serono Pfizer)	RRMS	22mcg or 44mcg tiw (three times a week)	Unknown at present	Slows down immune response, possibly by interfering with T cell activation and infiltration into CNS, apoptosis of autoreactive T cells, growth factor shift from Th1 to Th2, restoration of Treg activity	Indirect effects possibly through the suppression of MMP9, T cell activation and infiltration and a shift in the growth factor profile to generate a pro-myelinating environment and to prevent myelin and axonal damage.	Decrease in exacerbations in Rebif-treated groups vs. placebo-treated group (29% and 32% in study 1 [22mcg tiw and 44 mcg tiw]; p≤0.001). Proton-density/T2-weighted MRI: Significant reduction in lesion area after 2 years (p<0.0001). Significant reduction in relapse rate after 24 weeks (p<0.001) and 48 weeks (p=0.009).	Flu-like symptoms including chills, fever, myalgia, and asthenia. Serious adverse reactions were psychiatric disorders including depression, suicidal ideation and attempt.	**Combinational Trials** Phase III **CARE-MS I, CAMMS 323** – 24 month (Alemtuzumab [Genzyme Corp.] 12 mg/day i.v. for 5 days vs. Rebif 44 mcg tiw s.c. for RRMS in over 580 patients. Phase III **CARE-MS II, CAMMS 324** – 24 month (Alemtuzumab [Genzyme Corp.] 12 mg/day i.v. for 5 days at month 0 and 3 days at month 12 vs. alemtuzumab 24 mg/day i.v. for 5 days at month 0 and 3 days at month 12 vs. Rebif 44 mcg tiw s.c. for RRMS in over 700 patients. Phase II **ONWARD** – 104 weeks (0.875mg/kg/cycle [1 cycle is the daily treatment of cladribine for 4 to 5 days consecutively] for up to 4 cycles) vs. IFNβ-1a	http://clinicaltrials.gov/ct2/show/NCT00530348 http://clinicaltrials.gov/ct2/show/NCT00548405 http://clinicaltrials.gov/ct2/show/NCT00436826

Agent	Clinical Stage	Regime	Mechanism of Action	Immunologic effects	Neuroprotective effects	Data from completed phase II/III trials	Side effects	Future trials	Clinical Trial Identifier
IFNβ-1a (s.c, Rebif®, Serono Pfizer) cont.								[Rebif®, Serono Pfizer] for RRMS and SPMS with relapses in over 200 patients. Phase II **Interferon beta-1a (Rebif®, EMD Serono and Pfizer Inc.) + estroprogestins** – 24 month (Rebif 44 mcg tiw s.c. vs. Rebif + desogestrel 150 mcg p.o. + etinilestradiol 20 mcg p.o. vs. Rebif + desogestrel 25 mcg + etinilestradiol 40 mcg for RRMS women in over 180 patients. Phase IV **TOP MS** – 24 month (Avonex 30 mcg/week i.m. vs. Rebif 44 mcg tiw s.c. vs. Betaseron 250 mcg q.o.d s.c. vs. Copaxone 20 mg/d s.c. for active RRMS in over 3000 patients.	http://clinicaltrials.gov/ct2/show/NCT00151801 http://clinicaltrials.gov/ct2/show/NCT00819000
IFNβ-1b (s.c, Betaseron®, Bayer Healthcare)	RRMS	250mcg (μg) q.o.d (every other day)	Unknown at present.	Slows down immune response, possibly by interfering with T cell activation and infiltration into CNS, apoptosis of autoreactive T cells, growth factor shift from Th1 to Th2, restoration of Treg activity	Indirect effects possibly through the suppression of MMP9, T cell activation and infiltration and a shift in the growth factor profile to generate a pro-myelinating environment	Significant change in MRI area (-1.1% for the 0.25mg Betaseron-treated group vs. +16.5% for the placebo-treated group)	Depression, risk to pregnancy, allergic reactions, injection site problems (i.e. skin reactions). Flu-like symptoms including chills, fever, myalgia, and asthenia. Serious adverse reactions were psychiatric disorders	**Long term study** **BEST study** – 5 years (250 mcg q.o.d s.c. for RRMS in over 3560 patients) **Combinational Trials** Phase IV **TOP MS** – 24 month (Avonex 30 mcg/week i.m. vs. Rebif 44 mcg tiw s.c. vs. Betaseron 250 mcg q.o.d s.c. vs. Copaxone 20 mg/d s.c. for active RRMS in over 3000 patients.	http://clinicaltrials.gov/ct2/show/NCT00819000

Agent	Clinical Stage	Regime	Mechanism of Action	Immunologic effects	Neuroprotective effects	Data from completed phase II/III trials	Side effects	Future trials	Clinical Trial Identifier
IFNβ-1b (s.c, Betaseron®, Bayer Healthcare) cont.					and to prevent myelin and axonal damage.		including depression, suicidal ideation and attempt.		
Glatiramer acetate (Copaxone®, Teva Pharmaceutical Company, Ltd.)	RRMS	20mg once a day	Bystander suppression through activation of T cells as an altered peptide ligand that would shift towards antiinflammatory profile; increase expression of Foxp3 in CD4+ T cells.	Generating suppressor cells, inducing tolerance, expanding Treg populations, and altering APC presentation mechanisms.	None			**Combinational Trials** Phase III **CombiRx study** – 36 month (Avonex 30 mcg/week i.m. + Copaxone 20 mg/d s.c. vs. Avonex + placebo s.c. vs. Copaxone + placebo i.m.for RRMS in over 1000 patients. Phase IV **TOP MS** – 24 month (Avonex 30 mcg/week i.m. vs. Rebif 44 mcg tiw s.c. vs. Betaseron 250 mcg q.o.d s.c. vs. Copaxone 20 mg/d s.c. for active RRMS in over 3000 patients. Phase III – 24 weeks (7mg/day + Copaxone® 20mg/day s.c vs. 14mg/day + Copaxone® 20mg/day s.c vs. Copaxone® 20mg/day s.c) for RRMS, SPMS, PRMS in over 120 patients.	http://clinicaltrials.gov/ct2/show/NCT00211887 http://clinicaltrials.gov/ct2/show/NCT00819000 http://clinicaltrials.gov/ct2/show/NCT00475865

ARR= Annual Relapse Rate n/s= not-significant s.c.= subcutaneous i.v.=intravenous i.m.=intramuscular p.o= orally q.o.d=every other day t.i.w=three times a week

Monoclonal antibody Therapies						
Monoclonal Ab	Targeted molecule/Cell expression	Type of antibody (Blocking/Signalling)	Mechanism of Action	Data from completed phase II/III trials	Future Trials	Clinical Trial Identifier
Alemtuzumab (Campath-1H, Genzyme Corporation)	Targets CD52 found on all T and B cells, NK cells, Mø, DCs, and tissue of the male reproductive system.	Signalling Ab	Complete depletion of CD52-bearing cells. Indirectly the stabilization of the BBB	Phase II: 12mg/day vs. 24mg/day i.v. in annual cycles in conjunction with 44mcg 3 t.i.w. IFNβ s.c. – reduction in accumulated disability – 9% vs 29.2% with improved clinical scores 0.39pts in alemtuzumab group vs. a worsening by 0.38pts in the IFNβ group. Due to serious adverse effects such as intracerebral haemorrhage, Grave's disease, Goodpasture syndrome and melonoma's, the safety of the drug in under investigation.	Phase III **CARE-MS I**, **CAMMS 323** – 24 month (Alemtuzumab [Genzyme Corp.] 12 mg/day i.v. for 5 days vs. Rebif 44 mcg tiw s.c. for RRMS in over 580 patients. Phase III **CARE-MS II**, **CAMMS 324** – 24 month (Alemtuzumab [Genzyme Corp.] 12 mg/day i.v. for 5 days at month 0 and 3 days at month 12 vs. alemtuzumab 24 mg/day i.v. for 5 days at month 0 and 3 days at month 12 vs. Rebif 44 mcg tiw s.c. for RRMS in over 700 patients.	http://clinicaltrials.gov/ct2/show/NCT00530348 http://clinicaltrials.gov/ct2/show/NCT00548405
Daclizumab (Zenapax)	Targets CD25 (IL-2Rα) found on activated T cells, FoxP3+ Treg cells, some NK cells, activated Mø, activated DCs and OLs.	Blocking Ab	Blocks CD25, the IL-2 binding epitope for the IL-2R, exact MoA unknown suspected to limit T cell expansion. Widely used to prevent allogeneic tissue transplant rejection.	Phase II: **CHOICE** – (2 mg/kg s.c. every 2 weeks vs. 1 mg/kg s.c. every 4 weeks (alternates with placebo every 2 weeks) + IFNβ vs. placebo s.c. + IFNβ). For active relapsing MS in 230 patients over 72 weeks. Gd+ reduction – 72% in 2mg group and 25% in 1mg group with no change in T-, B-, NK cells but $CD56^{bright}$ NK cells were found to be 7-8x higher in daclizumab group.	Phase II **ZAP MS study** – 20.5 month (1 mg/kg/month i.v. vs. placebo) for RRMS in 15 patients. Phase III **Daclizumab High Yield Process (DAC HYP) vs. interferon beta-1a (Avonex®, Biogen Idec)** – 96 to 144 weeks (Avonex 30 mcg/week i.m. + placebo s.c. vs. DAC HYP 150 mg/every 4 weeks s.c. + placebo i.m. for RRMS in over 1500 patients.	http://clinicaltrials.gov/ct2/show/NCT00071838 http://clinicaltrials.gov/ct2/show/NCT01064401
Natalizumab (Tysabri, Biogen Idec and Elan)	Targets CD49d (VLA-4) for on all T and B cells, NK cells, most Mø and monocytes, and most granulocytes (except neutrophils)	Blocking Ab	Is a selective adhesion molecule inhibitor bwhose target is CD49d, the α4 subunit of VLA-4 receptor, thus preventing cell adhesion and infiltration into CNS.	Best for early inflammation, not later degenerative stages. **SENTINEL** Trial: Combination therapy with Avonex – 24% reduction in disease progression vs. IFNβ alone. **AFFIRM** Trial: Monotherapy – 42% reduction in disease progression and ARR reduction by 68%.	**Phase IV:** To test effect of treatment on vaccination response in 46 RRMS patients over 8 month. *Dosage*: Tysabri 300 mg i.v. every 4 weeks for at least 9 months, along with 3 immunisations of keyhole limpet hemocyanin s.c. at Day 168, 182, 196 and immunisation of tetanus diphtheria vaccine i.m. at Day 168 vs. 3 immunisations of keyhole limpet hemocyanin s.c. at Day 0, 14, 28 and immunisation with tetanus diphtheria vaccine i.m. at Day 0. **Phase IV: ENER-G study** – To reduce fatigue in over 200 patients with relapsing MS.	http://clinicaltrials.gov/ct2/show/NCT00536120 http://clinicaltrials.gov/ct2/show/NCT00464074

Natalizumab (Tysabri, Biogen Idec and Elan) cont.					*Dosage:* Tysabri 300 mg every 4 weeks i.v. **Phase IV: TYGRIS** – Tysabri Global ObseRvational Program In Safety in 5111 patients with relapsing forms of MS for 5 years (Long term safety trial). *Dosage:* 300 mg every 4 weeks i.v.	http://clinicaltrials.gov/ct2/show/NCT00477113
Rituximab (Rituxan,	Targets CD20 found on B cells (not plasma cells) and induces B-cell lysis.	Signalling Ab	Complete but transient depletion of B cells early on in the disease.	Phase II: 26 patients with RRMS that is unresponsive to standard immunomodulatory drugs. Done over 12 months at a dosage of 375 mg/m^2 i.v. (4 times). No change in EDSS observed but found depletion of T cells as well as B cells. Phase II/III: 435 patients with PPMS over 30 months were treated with 1g/day i.v. vs. placebo. No difference in the time to disease progression observed. Interestingly, in sub-group analysis patients younger than 51 years of age time to disease progression was significantly increased.	**None**	

ARR= Annual Relapse Rate n/s= not-significant s.c.= subcutaneous i.v.=intravenous i.m.=intramuscular p.o.= orally q.o.d.=every other day t.i.w.=three times a week

Further treatments for MS have been developed including oral therapies (such as FTY720, Laquinimod, Cladribine, BG00012 and teriflunomide) and monoclonal antibodies (such as Alemtuzumab, Daclizumab, Natalizumab, and Rituximab) (Table 1.1), all of which have entered phase III/IV trials. Additionally, combination therapy has accumulated a great deal of interest. However, current treatments are aimed at reducing relapse rates, limit or reduce lesion loads, prevent the development of new lesions or target specific cell types based on the immune-mediated arm of the disease. They focus on reducing the occurrence of inflammatory demyelinating lesions by inhibiting the immune system, but they do not target tissue repair or remyelination of the CNS. As mentioned before, recent studies have demonstrated the importance of neurodegeneration in the pathology of MS i.e. it is the primary cause of irreversible neural damage and disease progression in MS (Chandran et al., 2008, Tallantyre et al., 2010) highlighting the importance of studies into neuroprotective factors and factors and molecules that initiate or increase remyelination (Ben-Hur, 2010).

Remyelination as a therapeutic target

The current disease-modifying therapies predominantly target the inflammatory arm of the disease and are insufficient in targeting the degenerative aspects of the disease (Bitsch et al., 2000). Therefore remyelination, the reinvesting of new myelin around demyelinated axons, is important because it not only repairs saltatory conduction but is also neuroprotective (i.e it protects the neuronal axon by preventing degeneration (Chandran et al., 2008)), and for promoting axonal survival.

Remyelination follows many of the developmental stages of myelination and begins with the recruitment of OPCs that differentiate into mature myelin producing OLs that remyelinate the naked axon. Following a demyelination event, resident astrocytes and microglia are activated and release

factors that recruit monocytes from the surrounding blood vessels. The microglia and the monocytes differentiate into Mø that remove the myelin remnants left over from the dissolution and degradation of the myelinated axon. These Mø also release growth factors that cause the OPCs recruited from the subventricular zones and surrounding areas around the lesion, to differentiate into mature myelin producing OLs that engage the demyelinated axons and establish new myelin that appears thinner, a characteristic feature of remyelination (Dangond, 2004).

Why does remyelination fail in MS?

During the progressive stages of MS, this spontaneous remyelination appears to fail. Although demyelinating plaques of MS contain a significant number of immature OLs as well as viable axons (Wilson et al., 2006) why remyelination fails is, at present, unclear. It has been suggested to be the result of impaired processes such as OPC proliferation, migration and differentiation, or due to poor axonal responsiveness (Olek, 2004). It may also be due to the loss of axonal recognition and survival signals through axonal degeneration (Lovas et al., 2000), or through the existence of inhibitory factors and/or mechanisms (Charles et al., 2002, Olek, 2004, Chandran et al., 2008) such as the polysialated form of the neural cell adhesion molecule (PSA-NCAM) (Charles et al., 2000) that negatively regulates myelination.

Myelin repair

The therapeutic avenues to remyelination can be simplified into two areas. Exogenous repair involves transplantation-mediated remyelination strategies, whereas endogenous repair involves the promotion of a multifaceted pro-myelinating environment (Stangel and Hartung, 2002, Dangond, 2004, Billinghurst et al., 1998).

Exogenous repair

Exogenous remyelination strategies have been investigated for many years in various animals models such as the myelin-deficient shiverer mouse (Yandava et al., 1999, Mitome et al., 2001) and

in a toxic demyelination model (Allamargot et al., 2001). The cell types available for this type of transplantation-mediated strategy include neural stem cells (NSC), OPCs, Schwann cells and olfactory ensheathing cells.

NSCs are highly proliferative, pluripotent cells that are easily manipulated *in vitro* allowing researchers to engineer specific cell types for use in cell-transplantation therapies. NSCs have demonstrated the ability to ameliorate disease in both mouse and marmoset EAE models for MS (Pluchino et al., 2003, Pluchino et al., 2009). However, a concern for NSC transplantation is the inability to direct their migration *in vivo* and therefore, into areas that require the replenishment of differentiated neural cells (such as OPCs) not knowing if they would target regions of inflammation and demyelination. Pluchino et al., (2003) were able to detect transduced NSCs (containing the *E.coli*-derived *lacZ* gene directed to the nuclear compartment) in areas of CNS damage such as demyelination and axonal loss for up to 30 days after transplantation intravenously or intracerebroventricularly. However, the *in vivo* transplantation of NSCs labelled with luciferase and green fluorescent protein in EAE mice failed to migrate to areas of CNS damage, and were destroyed soon after transplantation (Reekmans et al., 2011). These studies highlight the complexity of the use of cell transplantation as a therapeutic tool.

An alternative to NSCs are Schwann cells. Schwann cells are myelinating cells of the peripheral nervous system that can be isolated from the sural nerve (found in the ankle and biopsied under local anaesthetic) and expanded *in vitro*. This cell type can remyelinate the CNS and has the benefit of being autologous to the patient, perhaps enabling them to escape the immune attack against CNS myelin (Dangond, 2004). Another autologous cell type capable of remyelination and that can be isolated from the patient are olfactory ensheathing cells. Olfactory ensheathing cells are cells that wrap around the axon of the first cranial nerve and have demonstrated myelination of axons in a rat model of spinal cord injury (Sasaki et al., 2004).

Within any transplant-mediated remyelination strategy, one must consider the normal cellular environment and whether the transplanted cells interact in a positive or negative manner with the endogenous glial cells: astrocytes and OLs. Indeed, the transplantation of Schwann cells lead to the induction of reactive astrocytes as indicated through an increase in glial fibrillary acidic protein expression within normal white matter of the spinal cord of adult Fisher rats (Lakatos et al., 2003). The reciprocal transplantation of olfactory ensheathing cells did not induce such increased reactivity in astrocytes (Lakatos et al., 2003). Interestingly, it was the transplantation of these Schwann cells that demonstrated a more beneficial effect regarding locomotor recovery than their olfactory ensheathing cell counterparts (Lakatos et al., 2003). Therefore, a therapeutic outcome must be carefully considered before the introduction of exogenous cells.

Endogenous repair

In EAE , the endogenous promotion of remyelination has been achieved through various approaches such as the administration of specific compounds associated with myelination such as thyroid hormone (Fernandez et al., 2004), platelet-derived growth factor (PDGF) (Allamargot et al., 2001), and IGF1 (Yao et al., 1995) ; the promotion of OPC proliferation and differentiation (Mi et al., 2009); the inhibition of inflammation (Piraino et al., 2005) and through intravenous immunoglobulins such as rHIgM22 (Pirko et al., 2004) and PEGylated anti-LINGO-1 Li33 antibody (Pepinsky et al., 2011).

Neuroprotective Therapies

Growth factors are soluble proteins that act as local mediators of communication between cells of the immune system (Nissim et al., 2004, Chitnis and Khoury, 2003). They include factors produced by mononuclear Mø (monokines), lymphocytes (lymphokines), colony-stimulating factors and interleukins (factors that are produced by monocytes and T cells and act on other lymphocytes). All growth factor receptors are ubiquitously expressed in most tissues and cells. Growth factors have been used therapeutically in many diseases. For example IL-2, IFN-α and IFN-γ in cancer; IFN-α in viral infection; IL-11 in post-chemotherapy induced thrombocytopenia; and IFN-β in MS. The growth

factors EPO, TGFβ and IGF1 have also been assessed for their therapeutic potential in the EAE mouse model of MS.

Erythropoietin

Until recently, EPO was known primarily as a renal protein involved in the generation and maintenance of erythroid cells, a process known as erythropoiesis. However, recent studies have demonstrated that EPO is a neuroprotective, anti-apoptotic, anti-inflammatory, anti-oxidative growth factor that is therapeutically promising for the treatment of several diseases including MS (Siren et al., 2001a, Agnello et al., 2002, Celik et al., 2002, Chong et al., 2003, Erbayraktar et al., 2003, Villa et al., 2003, Brines et al., 2004, Avasarala and Konduru, 2005, Zhang et al., 2005, Genc et al., 2006, Savino et al., 2006, Mengozzi et al., 2008, Yoo et al., 2009).

EPO Expression

The EPO molecule is a 34 kDa glycoprotein produced in the foetal liver and in the adult kidney that promotes the formation of red blood cells. It also acts in response to ischemia or hypoxia, a condition where there is a decrease in the oxygen supply to tissue. It contains one O-linked and three N-linked glycosylated chains important for its production, secretion and biological activity (Gentry et al., 1988, Miyazono and Heldin, 1989, Wasley et al., 1991, Su et al., 2010). EPO mRNA and the protein for EPO and the EPOR are found in various regions of the brain including the cerebellum, hypothalamus, hippocampus, internal capsule, cortex and midbrain (Masuda et al., 1993, Yamaji et al., 1996, Bernaudin et al., 2000, Brines et al., 2000, Dame et al., 2000, Siren et al., 2001b, Sugawa et al., 2002, Mengozzi et al., 2008, Rabie and Marti, 2008, Kang et al., 2009). Under hypoxic conditions *in vitro*, EPO has been found to be expressed by neurons, OLs, microglia, CEC, inflammatory cells and reactive astrocytes. This expression stimulates erythropoiesis, the production of new red blood cells in the bone marrow, in order to maintain an adequate supply of oxygen to the tissue. Its expression is driven by the activation of the hypoxia inducible factor-1α (HIF-1α) or HIF-2 genes by stimuli such

as insulin, hypoxia, hypoglycaemia, ROS and IGF (Minet et al., 2000, Rabie and Marti, 2008, Lemus-Varela et al., 2010).

EPO Receptors

Recent studies have shown that the molecular versatility of EPO is mediated through the interaction of portions of the helices A, C and D and the loop connecting helices A and B of EPO with the $(EPOR)_2$ (Brines et al., 2008). The classical erythropoietic homodimer receptor $(EPOR)_2$, is involved in erythropoiesis, and the heteromer receptor $(EPOR)_2$/βcR, is involved in mediating its tissue-protective effects.

The erythropoietic effects of EPO are mediated through $(EPOR)_2$. $(EPOR)_2$ is a member of the single-chain growth factor type I receptor family and comprises two transmembrane EPOR molecules present on the cell surface of erythroid colony-forming units, that spontaneously associates to form a homodimer via a leucine zipper bridge within the cell membrane (Rabie and Marti, 2008). EPO, readily produced by the kidneys for use in erythropoiesis, is found circulating within the body in concentrations within the picomolar range (1–7 pmol/L) and interacts with $(EPOR)_2$ with high affinity (100–200 pmol/L) (Brines and Cerami, 2008).

On the contrary, the tissue-protective effects of EPO have been shown to be mediated through the EPOR and the beta common receptor (βcR), the signalling part of the receptor complex also used by IL-3, GM-CSF and IL-5 (Brines et al., 2004). This $(EPO)_2$/βcR unit, normally expressed at low levels, is transiently upregulated in response to injury such as ischemia, or to hypoxia. Additionally, locally produced EPO is then expressed by the surrounding environment undergoing this stress or injury. The $(EPO)_2$/βcR unit has a low affinity for EPO (1–20 nmol/L) and therefore requires higher concentrations of EPO (a minimum dose of 500IU/kg bw is required in a rat stroke model (Wang et al., 2007)) to initiate tissue-protection, yet it only requires a brief exposure to initiate sustained response (Brines and Cerami, 2008, Brines et al., 2008). Indeed, when a skin flap model for permanent ischemia is treated with a high (3µg/kg body weight [bw] equivalent to 300IU/kg bw) but

not a low (0.3µg/kg bw equivalent to 30 IU/kg bw EPO, a purely haematopoietic dose of EPO) dose of EPO, an increased rate of healing was observed (Erbayraktar et al., 2009).

However, in the event that the βcR concentration is very low or absent, the redundant system for tissue-protection (such as anti-apoptosis) would not occur through the dimerization of EPOR (Um et al., 2007) and the newly engineered nonerythropoietic EPO analogues such as CEPO, asialoEPO and ARA290 (discussed below) would not be able to initiate the necessary signalling pathways.

EPO Signalling Pathways

The binding of EPO ligand leads a conformational change that activates the expression and phosphorylation of Janus tyrosine kinase 2 (JAK2) that is physically attached to the cytoplasmic portion of the receptors. This in turn leads to downstream activation of several transduction signalling pathways pertaining to the erythropoietic, neurotrophic and anti-apoptotic actions of EPO. For example, phosphorylation of JAK2 leads to the activation of the signal transducer and activator of transcription 5 (STAT5), which dimerizes and translocates to the nucleus inducing the transcription and translation of the anti-apoptotic protein Bcl-x_L. This results in the survival of erythrocyte precursor cells (known as colony-forming units) that in turn give rise to mature erythrocytes (Brines and Cerami, 2008, Rabie and Marti, 2008).

Additionally, the phosphorylation of JAK2 leads to the activation of phosphatidyl inositol 3 kinase (PI3K) which in turn activates Akt protein kinase B Akt/PKB. The activation of Akt/PKB leads to the inhibition of glycogen synthase kinase 3β (GSk-3β), Bcl2-associated agonist of cell death (BAD), and caspase activation resulting in a reduction in apoptosis. It also induces the activation of NFκB which inhibits apoptosis (Jelkmann and Hellwig-Burgel, 2001, Jelkmann, 2004, Brines and Cerami, 2008, Rabie and Marti, 2008).

EPO and EAE

Following on from the discovery of EPOs tissue-protective abilities, researchers looked at the possibility of EPO being neuroprotective. Initially they believed that the large glycoprotein was unable to cross the BBB and therefore injected it intracranially, a procedure that would not be ethically feasible in human trials. It was therefore fortuitous that Ehrenreich et al., (2004) discovered the migration of EPO across the BBB when looking at EPO as an add-on therapeutic treatment for schizophrenia. This opened the way for EPO to be considered a therapeutic treatment for other neurological diseases, such as MS. Within the EAE model, EPO treatment has demonstrated a reduction in clinical score, neurological deficit duration, inflammatory cell infiltrate, demyelination and reactive gliosis in the EAE rodent models and in a model of focal cerebral ischemia (Brines et al 2000; Agnello et al 2002; Savino et al 2006; Yuan et al 2008; Sirén 2001).

A variety of studies have demonstrated that EPO is anti-inflammatory (Brines and Cerami 2008). Within the EAE model, EPO has demonstrated these anti-inflammatory effects by reducing the proliferation of autoreactive T cells and suppressing the production of the pro-inflammatory growth factors TNF-α and IFN-γ in negative feedback loops: inflammation induces TNFα and IFNγ which inhibit endogenous EPO induction, whilst the HIF-1α-induced expression of EPO by inflammatory stimuli inhibits the production of TNFα and IFNγ (Agnello et al., 2002, Mengozzi et al., 2008, Savino et al., 2006, Villa et al., 2003). EPO has also been shown to down-regulate TNF-α induced IL-6, IL1β, CXCR4 and IL-1α gene expression in CEC (Agnello et al., 2002, Avasarala and Konduru, 2005) thus limiting the ability of activated lymphocytes from crossing the BBB. These effects have been reported to persist for at least 20 days after EPO treatment cessation (Agnello et al., 2002, Savino et al., 2006). Additionally, EPO pre-treatment results in inhibition of VEGF, a multipotent growth factor up-regulated in acute and chronic MS lesions thought to promote the localised inflammatory response by inducing local BBB breakdown (Fan et al., 2009, Martinez-Estrada et al., 2003). Remarkably, these anti-inflammatory effects of EPO are not replicated in an adjuvant-induced model

of arthritis or in systemic lupus erythematosus (Agnello et al., 2002) and is therefore said to only occur in the setting of neuronal degeneration (Villa et al., 2003) and is CNS specific.

The neuroprotective action of EPO has been demonstrated *in vitro* against kainite (KA)-induced excitotoxicity and cell death (Yoo et al., 2009), as well as *in vivo* in an ischemia spinal cord injury model (Brines et al., 2000, Celik et al., 2002) and in EAE (Brines et al., 2000, Zhang et al., 2005).

Within the CNS, OLs and neurons are especially vulnerable to excitotoxicity, cytotoxicity and oxidative stress. EPO pre-treatment showed significant reductions in the expression of nitrite levels (a stable by-product generated by rapid NO oxidation) *in vitro* induced by IFN-γ and bacterial liposaccharide (LPS) in primary rat OL cultures. Pursuant to EPO pre-treatment, significant decreases in iNOS- and NO-induced oxidative stress by a downregulation in iNOS mRNA affords OLs and neurons its neuroprotection (Genc et al., 2006).

EPO post-treatment also exhibits a neuroprotective effect by suppressing caspase-3 proteolytic activity and Bax expression, both key players in the process of programmed cell death, apoptosis (Siren et al., 2001a, Yoo et al., 2009). In parenchymal cells of the CNS, EPO treatment increased the expression of brain-derived neurotropic factor (BDNF). BDNF (a neurotrophin required for cell survival and regeneration) has been shown to suppress apoptosis of myelinating OLs as well as stimulating the proliferation of OPCs that may lead to an influx of new, mature OLs and remyelination of stripped axons (Zhang et al., 2005).

Reactive gliosis observed in EAE is markedly reduced following EPO treatment (Agnello et al., 2002), resulting in a decrease in the production of ECM molecules including chondroitin and keratin sulphate proteoglycans released by reactive astrocytes (Silver and Miller, 2004, Canning et al., 1996, Zeinstra et al., 2003). These molecules are known to inhibit axonal regeneration (Canning et al., 1996) and their reduction could improve the outcome of OL axon survival following demyelination and possibly transection.

Interestingly, astrogliosis (the proliferation of astrocytes in response to injury, also referred to as reactive gliosis) that was induced by KA injury is only partially reduced following EPO treatment and is not affected by EPOR blocking antibody. This is suggestive of an alternative pathway, not of the classical EPOR, for the reduction in reactive gliosis by EPO. Astrocyte proliferation in response to excitotoxicity may be mediated through activation of type 5 metabotropic glutamate receptors (mGluR5) which are found to be enhanced in astrocytes participating in reactive gliosis and which increase glutamate uptake (Yoo et al., 2009). Furthermore, the anti-inflammatory effects exerted by EPO are also thought not to be due to its direct intervention on the EPOR, but rather due to the prevention of the molecular signals released by injured neurons i.e. it is anti-inflammatory due to its neuroprotection (Villa et al., 2003).

EPO presents a compelling case for use as a therapeutic agent in the treatment of MS. However, the issue of over-expression resulting in serious side-effects such as vascular perfusion defects, hypertension and thrombosis have caused researchers and doctors to be wary of its use. It was therefore crucial that the neuroprotection, anti-apoptotic, anti-oxidant, and anti-inflammatory actions of this molecule be separated from its haematopoietic properties or that it be modified such that the potential side-effects are negligible.

EPO Derivatives that are nonerythropoietic but are tissue-protective

In recent years, several derivatives of EPO have been produced that have the ability to be neuroprotective but not erythropoietic. The erythropoietic nature of EPO can be abolished through chemical modification i.e. carbamylation of the lysines (CEPO) or through amino acid substitution (asialoEPO). Erbayraktar et al., (2003) produced a variant of the EPO growth factor, asialoerythropoietin (asialoEPO) which was completely devoid of sialic acids. They found that even though it fails to increase erythrocyte mass, it is fully protective in animal models of stroke, spinal cord injury and peripheral neuropathy. It demonstrated a very short life in comparison to natural EPO (1.4 min vs. 5.6 hr), had a similar affinity to EPOR, was able to cross the BBB and has a

neuroprotective range between 5–50 µg/kg. The short half-life of the protein prevented any significant interactions occurring with the EPOR that may have initiated haematopoiesis as it was degraded so quickly, however once it crossed the BBB, it degraded much slower thus allowing EPOR/βcR binding and interaction that led to the initiation of neuroprotection (Rabie and Marti, 2008).

A carbamylated variant of EPO (CEPO) was produced by Leist et al. (2004) by changing all lysines to homocitruline. This carbamylation of the lysines of the EPO peptide profoundly altered the protein conformation resulting in a failure of CEPO to bind the EPOR dimer but retained its ability to cross the BBB. CEPO also had no effect on haematocrit even at high concentrations in the plasma and demonstrated reduced inflammatory response. The protective effect, also observed between the ranges of 5–50µg/kg, was retained as demonstrated by rescuing P19 mouse teratocarcinoma cells and hippocampal cells from apoptosis.

Interestingly these alterations did not affect the tissue-protective aspects of EPO. Through analysis of the tertiary structure of EPO in aqueous media, it was determined that the helix B of EPO was the region of neuroprotective capabilities. A 25-mer peptide synthesized from helix B (HBP) was shown to be a potent tissue-protective molecule that was not erythropoietic both *in vitro* (in the UT-7 cell line) and *in vivo* (in a rat motoneuron model) (Brines et al., 2008). Notably, additional doses of HBP did not increase the level or degree of tissue-protection, corroborating the findings that a single signalling event is required to initiate sustained tissue-protection. Additionally, the aqueous face of the EPO molecule was obtained from crystallographic studies and an 11-mer peptide was synthesized from those amino acids of the aqueous face of EPO along with the three amino acids found within the proximal portion of the BC loop. This peptide (HBSP also known as ARA290: QEQLERALNSS) was completely unrelated to the EPO sequence but mimicked a particular section of its tertiary structure, was shown to have a very short half-life (~ 2min), was nonerythropoietic (as determined *in vitro* using the UT7-EPO cell line and *in vivo* in the rat) and was highly protective (as

demonstrated by a reduced degree of injury in a sciatic nerve crush model and an increase in the rate of healing in a model of non-ischemic wounding) (Brines et al., 2008, Erbayraktar et al., 2009).

Transforming Growth Factor β

Mature TGFβ is a 25 kDa multifunctional growth factor that is ubiquitously expressed in normal tissue and cell lines including mitogen-activated T cells, B cells, monocytes and fibroblasts (Rollnik et al., 1997). It is a member of a family of growth, differentiation, and morphogenesis autocrine and paracrine factors (Schultz-Cherry and Murphy-Ullrich, 1993).

TGFβ Expression

TGFβ is initially synthesized as a precursor peptide consisting of a signal peptide, a latency-associated peptide (LAP) and the mature TGFβ molecule that are bound in conformation by covalent and noncovalent interactions. The signalling peptide of TGFβ is cleaved from the precursor peptide at amino acid residues Gly^{29} and Leu^{30} during transit through the rough endoplasmic reticulum. Once cleaved it is glycosylated at Asn^{82}, Asn^{136} and Asn^{176} within the Golgi complex with sialated oligosaccharides (Gentry et al., 1988). Two 12.5 kDa polypeptide chains of mature TGFβ form a homodimeric TGFβ complex bound by a single disulphide bond (Shi et al., 2011). The C-terminal of the homodimeric TGFβ complex associates by non-covalent interactions to the N-terminal pro-domain of its LAP (this structure is referred to as pro-TGFβ). LAP plays a variety of roles in the production and secretion of TGFβ by binding to various proteins during its transportation and processing. Importantly, LAP dimizes to create a 'shell' around TGFβ (forming the prodomain) thus conferring latency onto TGFβ. This latency regulates the bioavailability of TGFβ by controlling is activity and rendering it biologically inactive, and making it unable to engage the TGFβ receptor at its binding site. Interestingly, although the various isoforms of TGFβ (i.e. TGFβ1, TGFβ2 and TGFβ3) are structurally conserved, their LAPs differ in their amino acid sequences, perhaps contributing to their isoform-specific actions. Two polypeptide chains of pro-TGFβ, forming a dimer through a single disulphide bond, are attached to LAP by hydrophobic contacts to form a dimer called the Small

Latent TGFβ complex (SLTC) (also referred to as latent TGFβ) (Miyazono et al., 1988, Shi et al., 2011). It is in this form that TGFβ is secreted from the cell following processing with the Golgi network.

Latent TGFβ also associates with latent binding proteins (LTBP's), members of the LTBP/fibrillin-family of ECM proteins prior to secretion via the forth cysteine residue, to form the Large Latent Complex (LLTC) before trafficking to the ECM (Shi et al., 2011). LTBP's are important in the folding, activation and localisation of these protein complexes to the ECM. The release of the LLTC from the ECM is thought to occur at the hinge region i.e. a protease-sensitive region within the N-terminal domain of the LTBP found between the domains responsible for the binding of the molecule to the ECM and the SLTC (Koli et al., 2001). Intracellularly, mature TGFβ can be cleaved from the SLTC during transit within the Golgi network through proteolytic cleavage by Furin, a Ca^{2+} dependent serine endopeptidase that cleaves the protein at the RX(K/R)R motif found immediately upstream of the NH_2-terminal of mature TGFβ (Dubois et al., 1995). This cleavage however, does not result in the release of the mature protein. TGFβ is maintained within the LAP prodomain dimers via electrostatic and ionic interactions. *In vitro* the electrostatic interactions can be broken through a number of actions such as extremes in pH, high temperatures, sodium dodecyl sulphate (SDS), urea, or by some endopeptidases. Liberation of the mature TGFβ peptide can also be achieved by altering the protein conformation, by direct interaction with integrins or thrombospondin-1 (TSP1) (Schultz-Cherry and Murphy-Ullrich, 1993), through exposure of the TGFβ receptor type II binding site of the cell surface, or through receptor binding of LAP (Miyazono and Heldin, 1989). LAP contains 3 N-linked glycosylation sites, two of which have mannose-6-phosphate residues that are thought to act in the activation of the latent protein by binding to mannose-6-phosphate receptors.

TGFβ Receptor and Signalling Pathways

Many cells have TGFβ receptors and the protein is expressed by most cells. Within the CNS, TGFβ is produced by T cells, LPS-activated mononuclear phagocytes, astrocytes, microglia and neurons. The receptor consists of two proteins, TGFβ receptor type I (TGFβRI) and TGFβ receptor type II (TGFβRII).

Once active TGFβ binds to its receptor, TGFβRII signals through a serine/threonine kinase domain. This signals the phosphorylation of the *Caenorhabditis elegans* Sma and Drosophila Mad (SMAD) proteins, whilst forming a receptor-ligand heterodimeric complex. This results in the auto-phosphorylation of the receptor followed by the phosphorylation of the receptor-regulated SMAD (R-SMAD). This leads to the activation of homo-oligomeric and hetero-oligomeric complexes that translocate to the nucleus to associate with various molecules such as DNA-binding molecules and transcriptional co-activators/co-repressors to regulate the activity of the target genes (Mirshafiey and Mohsenzadegan, 2009).

TGFβ has demonstrated a pivotal role in the functioning of the immune system. It has been found to inhibit pro-inflammatory growth factor expression such as TNF-α, IFN-γ, IL-1, IL-2 and lymphotoxin, block growth factor induction of adhesion molecules, and to prevent entrance of sensitised T cells into the CNS by inhibiting the adhesive ability of endothelial cells (Gamble and Vadas, 1991, Santambrogio et al., 1993, Rollnik et al., 1997, Mirshafiey and Mohsenzadegan, 2009). It also plays a significant role in immune tolerance through the differentiation of $CD4^+$ T cell into T_{Reg} cells ($CD4^+$, $CD25^+$, $FoxP3^+$) (Fantini et al., 2004), inhibition of T cell proliferation by blocking the expression of pro-inflammatory growth factors such as IL-2 (Li and Flavell, 2006), by blocking growth factor induction on adhesion molecules and by maintaining APCs in an immature state (Mirshafiey and Mohsenzadegan, 2009). TGFβ has also recently been demonstrated having a role in differentiation of the autoreactive and disease promoting $CD4^+$ T cell, Th17, which secretes the pro-inflammatory growth factor IL-17 (Veldhoen and Stockinger, 2006). These properties highlighted TGFβ as a potential regulatory molecule for the treatment of the EAE.

TGFβ and EAE/MS

Initial studies utilizing TGFβ as a therapeutic molecule in EAE demonstrated inhibition of the disease in T cell adoptive transfer induced EAE mouse models. TGFβ also reduced the degree of perivascular inflammatory cell infiltrates and prevented demyelination in the CNS (Racke et al., 1991). Further

studies demonstrated that treatment with TGFβ activated T cells inhibit the secretion of the pro-inflammatory growth factors TNF-α and IFN-γ, and suppress its capacity to induce EAE. TGFβ1 has also demonstrated disease amelioration through the proliferation of the protective Th3 cell type.

TGFβ has also demonstrated significant neuroprotective effects both *in* vitro and *in vivo* (Flanders et al., 1998). It has demonstrated anti-apoptotic effects via the suppression of Bad expression and an increase in Bad phosphorylation (Zhu et al., 2002); and in murine cortical cells and rat hippocampal neurons, TGFβ prevents excitotoxity-induced neuronal death (Prehn et al., 1994, Ruocco et al., 1999). Additionally TGFβ, when injected into the CNS of Biozzi ABH mice as a DNA-cationic liposome complex, inhibited the development of clinical EAE (Croxford et al., 1998).

In a phase I clinical trial involving 11 patients with SPMS, five patients treated with active TGFβ2 had a reversible decline in the glomerular filtration rate (the flow rate of filtered fluid through the kidney giving an indication of the kidney's state). In addition, 8 patients displayed a decrease in haemoglobin; however, to what extent this was attributable to the drug is unclear. Furthermore, no notable change was observed in EDSS scores but, as the trial was designed to access the safety of the drug and not its efficacy, no definite conclusions could be made (Calabresi et al., 1998).

On the other hand, studies on EAE mice models using TGFβ have demonstrated disease amelioration, as well as a reduction in demyelination and proinflammatory growth factors (Johns et al., 1991). Furthermore as increased levels of total TGFβ are seen in the cerebrospinal fluid but are reduced as biologically active TGFβ in the serum, it can be said that TGFβ may act as an indicator of disease limitation (Rollnik et al., 1997). With these points in mind, TGFβ still remains a promising therapeutic agent for MS.

Insulin-like Growth Factor 1 (IGF1)

IGF1 Expression and Regulation

IGF1 is a single chain polypeptide of approximately 17 kDa. It is produced by many tissues including the CNS and the liver (Cohick and Clemmons, 1993, van Buul-Offers and Kooijman, 1998). There are four splice variants of the IGF1 gene: Types 1 and 2 and classes A and B are all derived from alternative splicing and from the two transcriptional start sites found on either exon 1 or 2 (Figure 1.5). At the time of writing, no studies that compare the two isoforms IGF1A and IGF1B and their functions within the CNS have been reported.

IGFs bind to the IGF type I receptor (IGF1R) and can also activate the insulin receptor (INSR). These receptors are $\alpha_2\beta_2$ (tetrameric) transmembrane glycoproteins that auto-phosphorylate following ligand interaction (Cohick and Clemmons, 1993). The IGF1R is expressed on lymphocytes, myelinating OLs, and brain capillary endothelium (van Buul-Offers and Kooijman, 1998, Liu et al., 1994, Komoly et al., 1992, Wilczak et al., 2008).

The IGF system includes the IGF binding proteins (IGFBPs) of which 6 have been identified (IGFBP1-6). The IGFBPs are carrier proteins and are responsible for the transport of IGFs to their target cells, for prolonging their half-life and for diminishing their hypoglycaemic effect (Wilczak et al., 2008, van Buul-Offers and Kooijman, 1998). They also have the ability to sequester IGFs away from their receptors. These 25–45 kDa binding proteins have phosphorylation and glycosylation sites that facilitate their binding to IGF's with varying affinities (van Buul-Offers and Kooijman, 1998). IGF1 and IGF2 circulate predominantly in growth hormone-dependent ternary complexes of high molecular weight that consist of IGFBP, IGF and an acid-labile glycoprotein subunit that does not bind IGF. IGFBP3 is the most abundant IGF-binding subunit of the growth hormone-dependent binding complex found in the serum. It has a very high-affinity for both IGF1 and IGF2, and over 95% of free IGF1 is found bound to IGFBP3 (Cohick and Clemmons, 1993).

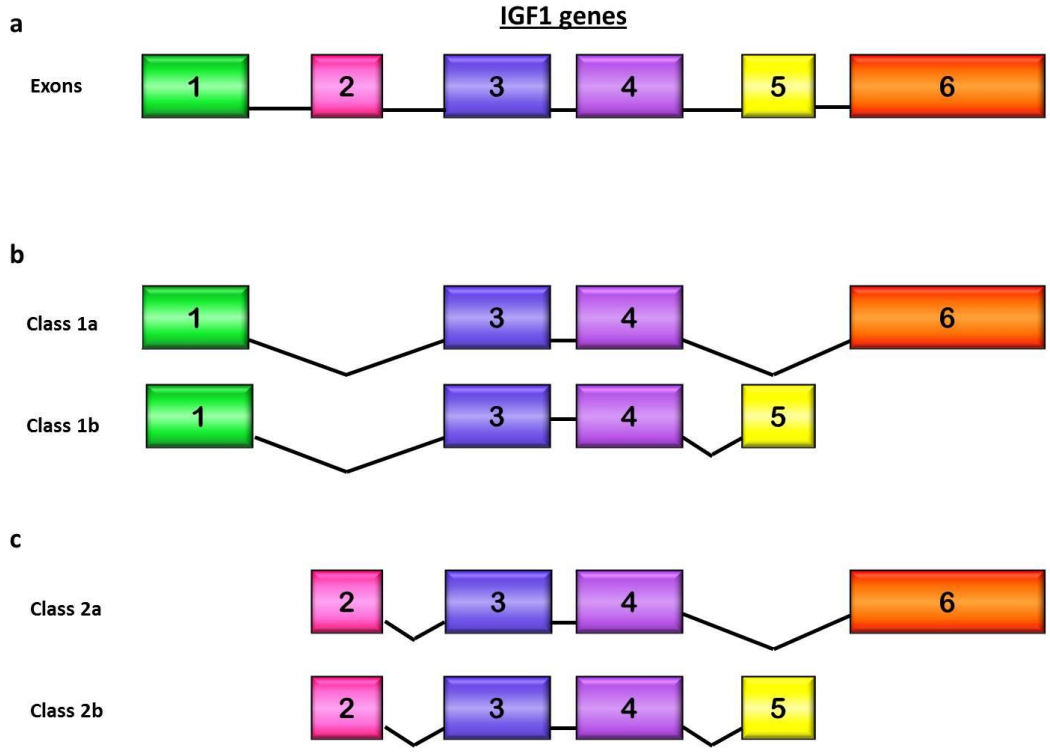


Figure 1.5: Schematic diagram indicating IGF1 alternative splicing.
(A) Alternative splicing of IGF1 mRNA. The IGF1 gene contains six exons that, through alternative splicing, generate four isoforms of IGF1: two classes and two types. **(B and C)** Alternative splicing of exons 1, 2, 5 and 6 leads to four isoforms of IGF1. Exon 1 and 2 encode distinct leader peptide sequences defining the class (i.e. 1 or 2), as well as part of the signal peptide and are known as the leader sequences. Exons 3 and 4 are identical in all isoforms. Exon 3 encodes the remainder of the signal peptide along with part of the mature peptide. Exon 4 encodes the remainder of the mature peptide and part of the amino-terminal end of the E-peptide. Exons 5 and 6 encodes distinct carboxy-terminal ends and the 3' UTR that define the type (i.e. a or b) of the IGF1 isoform. The initiation of transcription at exon 1 and excition of exon 2 results in the class 1 isoforms; whereas class 2 isoforms are transcribed from exon 2 leading to the exclusion of exon 1 during transcription. Stop codons and polyadenylation sites within exon 5 result in type 'b' isoforms, whereas the excision of exon 5 coupled with stop codons and polyadenylation sites within exon 6 result in type 'a' isoforms.

IGF1 and EAE/MS

In vitro IGF1 has been shown to be neuroprotective by promoting oligodendrocyte development, maturation and growth; and has demonstrated myelination and regeneration (Mozell and McMorris, 1991). *In vivo,* IGF1 is expressed in remyelinating lesions by reactive astrocytes, OLs and Mø, presumably induced by the proinflammatory growth factor IL-1β, and by OLs (Komoly et al., 1991, Lee et al., 1992, Liu et al., 1994, Yao et al., 1995, Mason et al., 2000, Mason et al., 2001, Wilkins et al., 2001, O'Leary et al., 2002, Wilczak et al., 2008). Expression of IGFBP1 and IGFBP6 by OLs at the edge of demyelinated lesions and of IGFBP2 by astrocytes in the CNS of EAE mice is reported to

coincide with IGF1 expression (Liu et al., 1994, Yao et al., 1995, Wilczak et al., 2008). Additionally, its effects are enhanced when coupled to a growth factor complex (Finkelman et al., 1993). Indeed, when IGF1 was coupled to IGFBP3 *in vitro* and injected i.p. into EAE mice, the onset of disease was delayed. This initial delay in onset of disease may be attributable to IGF1 preventing disease-causing cells from entering the CNS. However, those mice injected with the IGF1/IGFBP3 complex eventually went on to develop more severe disease symptoms. It was discovered that not only did the IGF1/IGFBP3 complex fail to inhibit the transfer of disease by adoptive transfer, it also enhanced the proliferation of those encephalitogenic T cells (Lovett-Racke et al., 1998). This is corroborated by the study by Ye et al., (1995) demonstrating that IGF1-induced cell survival and myelination is inhibited by IGFBP1 which may account, at least in part, for the failure of IGF1 to enhance CNS myelin repair during autoimmune demyelination or the lack of remyelination in chronic demyelinated lesions (Cannella et al., 2000).

IGF1 is a potent inducer of OL development and of myelination *in vitro*, and a promoter of neural cell proliferation and differentiation during development (McMorris et al., 1986, McMorris and Dubois-Dalcq, 1988, Mozell and McMorris, 1991, Shinar and McMorris, 1995, Annenkov, 2009). IGF1 has demonstrated enhanced synthesis of myelin proteins MBP and PLP in a monophasic model of EAE, as well as a reduction in lesion size and number and an improvement in clinical signs (Yao et al., 1995). This improvement in clinical signs in EAE has been confirmed by other studies in the acute phase (Cannella et al., 2000, Li et al., 1998, Lovett-Racke et al., 1998) including the lack of demyelination observed during the chronic phase following IGF1 treatment (Cannella et al., 2000, Frank et al., 2002, O'Leary et al., 2002). The initial appearance of myelin proteins during early disease may be due to a distinct mechanism involving multiple factors not directly influential on OLs that IGF1 alone cannot replicate within the chronic demyelinated lesion (Komoly et al., 1992, O'Leary et al., 2002).

However, when patients with secondary progressive MS were treated with recombinant IGF1 for a period of 24 weeks in an open-label early phase II crossover trial design, no evidence of remyelination or even demyelination were detectable by MRI studies (Frank et al., 2002).

Although the delivery of IGF1 within the EAE model and within the pilot study has been s.c, intraperitoneal (i.p), or systemic, the efficacy (or lack of it) may be in part due to the limited delivery of the protein to the CNS or the OLs. Additionally, due to the pleotropic nature of the protein, systemic release may directly influence the immune response (Liu et al., 1997) resulting in the exacerbation of the disease and unwanted side effects. The delivery of IGF1 through retrograde transport by an adeno-associated viral vector in a mouse amyotrophic lateral sclerosis (ALS) model, demonstrated local sustained delivery of the protein to the CNS without eliciting an immune response (Kaspar et al., 2003, Genoud et al., 2005). However, no clinical recovery was observed and moreover, symptoms were exacerbated when IGF1 was delivered after disease onset (Lovett-Racke et al., 1998, Genoud et al., 2005). These studies used dose levels that were acceptable in treating endocrine disorders which, when delivered locally, may be overwhelming and negative.

Gene Therapy

The introduction of genetic material into the cells of a patient with the intention of alleviating or treating a disease is known as somatic gene therapy. It is the lack of effective conventional treatments for disease that is the driving force for the development of novel gene therapies. Conventional treatments such as protein therapies are expensive to produce, involve proteins with short half-lives, and require systemic administration in high concentrations in order to elicit a local response in biologically relevant concentrations. This systemic administration may also result in unwanted side-effects. Gene therapy on the other hand, has the potential for long-term expression that is not only safe (proteins can be engineered to have short half-lives or indeed to be latent until reaching sites of disease (Adams et al., 2003, Chernajovsky et al., 2004)) but also have the ability to

be locally regulated by transcriptionally regulated plasmids that are modulated in response to pharmalogical agents (e.g. tetracycline), or to pathological conditions (e.g. in inflammation).

The use of viral vectors is the adaptation of a virus' natural ability to infect a host cell. They have been modified *in vitro* to be replication-deficient and are therefore unable to proliferate. This increases the safety of using viral vectors as therapeutic tools. Further strategies used for improving the safety of viral vectors includes the use of gutless adenoviral vectors (adenoviral vectors in which most of the viral genes have been removed in order to avoid immunogenicity), and the deletion of 3' long-terminal repeats (LTR). Infection of cells with therapeutic genes boosts the ability of utilizing ones own cells to produce the therapeutic molecules i.e. to become *in vivo* factories of recombinant proteins.

The approach taken for gene therapy of a particular disease is dependent on the molecular target, for example with the reconstitution of insulin expression in type-1 diabetes. Another approach may be the compensation of reactive immune cells expressing pro-inflammatory growth factors with the expression of anti-inflammatory growth factors such as TGFβ. The choice of vector to use in gene therapy is dependant on the size of the therapeutic gene needing to be introduced, the immunogenicity of the vector (less important if the expression is only required for a short duration), the longevity of expression, as well as the type and location of the cells targeted for infection. Transcriptionally regulated plasmids (such as the Tet-On system) are beneficial for diseases that require the cessation of a therapeutic treatment during periods of remission, such as in MS. Tet-On is a method of inducible expression in which transcription of the therapeutic gene is reversibly turned on or off in the presence of the tetracycline analogue doxycycline. The vector contains the gene encoding the transactivator protein rtTA (reverse-tetracycline-regulated transactivator) which, in the presence of doxycycline, envokes a conformational change allowing it to bind to the promoter leading to transcription of the therapeutic gene. In the absence of doxycycline, TetR (a eukaryotic repressor, Tetracycline repressor) binds strongly to the gene preventing transcription, but when

exposed to doxycycline, is removed. Immunogenecity of the vector can also be modified, for example through the addition of poly-glycine-alanine repeats of the Epstein-Barr virus nuclear antigen 1 (EBNA1) which prevents the degradation of the protein by the proteasome (Chernajovsky et al., 2004). The type of viral vector used will also affect the type of cell that can be infected such as the use of a lentiviral vector in order to infect cells that are non-dividing. Additionally, the location is an important consideration for determining the route of gene therapy administration. For example, gene therapy for MS involves targeting the CNS, an environment that is preferential in its entry to therapeutic molecules. The modification of a therapeutic gene to secrete latent growth factors has the potential to gain entry into this privileged area (Adams et al., 2003).

A successful therapeutic approach for MS should be able to deliver the therapeutic molecules directly into the CNS, not be immunogenic, be able to rescue surviving OLs and induce OL migration and differentiation into demyelinating areas (Martino, 2003) resulting in tissue regeneration and repair. Such approaches have been taken in the EAE model of MS, for example through the systemic delivery of genetically modified encephalitogenic T cells expressing either the α-subunit of PDGF (platelet-derived growth factor) or of NGF (nerve growth factor) into the CNS ; and by direct introduction into the CNS via intraparenchymal and intrathecal implantation of TGFβ and dTNFR respectively (Chen et al., 1998, Croxford et al., 2000, Martino, 2003, Chernajovsky et al., 2004).

The Role of MMPs in the pathology of MS

MMPs are zinc-containing, calcium-dependent endopeptidases (Meraz-Cruz et al., 2006) involved in the degradation and remodelling of the extracellular matrix (ECM). They perform a pivotal role in physiological conditions such as wound healing, angiogenesis, bone remodelling, and in pathological conditions such as tumour invasion, inflammation, BBB disruption, and MS. The family of MMPs consists of at least 23 members comprising collagenases, gelatinases A and B (MMP2 and MMP9 respectively), stromelysins (such as MMP3), matrilysin (MMP7), metalloelastase and membrane-type metallo-proteinases. T cells, Mø, neurons, astrocytes and OLs all express MMPs (Anthony et al.,

1998, Cossins et al., 1997, Maeda and Sobel, 1996). MMP activity is tightly regulated at three distinct levels: gene-transcription, pro-enzyme activation and by the activity of tissue-inhibitors of metallo-proteinases (TIMPs). Gene transcription of MMPs can be supressed or induced by growth factors such as TNFα, IL-1 and TGFβ respectively. MMPs are initially secreted as inactive enzymes containing a zinc-molecule bound to a cysteine residue in its catalytic domain. Activation occurs via proteolytic cleavage by other MMPs or other proteinases such as plasmin. This cleavage disrupts the cysteine-zinc interaction, modifies the enzyme conformation and exposes the active catalytic site. After activation, MMPs are regulated through inhibition by TIMPs. TIMPs are ubiquitously expressed in the extracellular environment and form a complex of 1:1 ratio with the MMP. This regulation may be overcome by a decrease in inhibitors such as α1-antitrypsin, a serine proteinase inhibitor that has the ability to inhibit TNFα and MMP in alveolar Mø in response to thrombin and cigarette smoke extract (Kieseier et al., 1999, Churg et al., 2003), or an excess in MMP production and activation leading to tissue damage and cell invasion.

MMPs play a role in the pathogenesis of inflammatory demyelinating diseases such as MS. Increased MMP expression has been demonstrated in animal models of EAE (Anthony et al., 1998, Proost et al., 1993) and in the CSF of MS patients, as well as in preactive and demyelinating lesions (van Horssen et al., 2006, Gold et al., 2000, Maeda and Sobel, 1996, Cossins et al., 1997) with MMP7 and MMP9, expressed by Mø within the lesion (Anthony et al., 1998), coinciding with peak disease severity (Clements et al., 1997). MMPs are expressed within CECs and open the BBB through hydrolysis of ECM components such as type IV collagen, fibronectin and laminin in the basal membrane, facilitating the trafficking of leukocytes into the CNS (Rosenberg et al., 1992, Vince et al., 1999, Costello et al., 1994). This degradation of the BBB and the subsequent transmigration of T cells across the endothelium are normally blocked by TIMPs activity, as is the activation of pro–inflammatory growth factors such as TNFα by MMPs. However, MMPs can be stimulated by pro–inflammatory growth factors (Han et al., 2001, Gearing et al., 1995).

MMPs can also degrade components of the myelin sheath such as MBP with the resulting peptide fragments viewed as a foreign antigen (Chandler et al., 1995). This may elicit an immune response that could contribute to the preservation of the inflammatory response within a lesion and to its demyelination. The ability of MMPs to activate latent enzymes through proteolytic cleavage, and their increased expression during inflammation is an attractive combination for the development of a therapeutic molecule that if initially latent can be rendered active at sites of increased MMP expression such as in MS lesions.

LAP TECHNOLOGY

Even after 150 years since the first formal classification of the disease, MS does not have any answers that shed light on its epidemiology, risks or aetiology. Many therapeutics have been investigated and proposed for the treatment of MS. Unfortunately none have yet been identified that arrest the destruction and degeneration of the CNS or restore functionality to those areas of the CNS that have been affected.

The growth factors EPO, TGFβ and IGF1 have demonstrated therapeutic potential in the EAE model for the treatment of MS. However, they exhibit several undesirable characteristics such as a short half-life, unwanted and potentially harmful side-effects in the high concentrations needed for treatment, and have ubiquitous and widespread biological activity. In order to develop a therapeutic molecule whose biological activity is inhibited but that can be unblocked at the site of disease, Adams et al. (2003) produced a latent growth factor. They encapsulated IFN-β within a shell provided by LAP of TGFβ. LAP was fused to the growth factor via a MMP cleavage site. The LAP shell is disulphide bonded closing the shell and rendering the growth factor latent (Figure 1.6). Activation of the growth factor was achieved by cleavage of the LAP, from the growth factor, by the action of the endogenous MMPs that are found at sites of inflammation and tissue remodelling. By adopting the LAP from a native protein, they were able to bypass the side effects normally seen from systemic

administration of a novel protein that is not latent. This LAP technology increases the half-life of peptides and growth factors and enables their specific delivery to sites of disease through the targeted release of local MMPs.

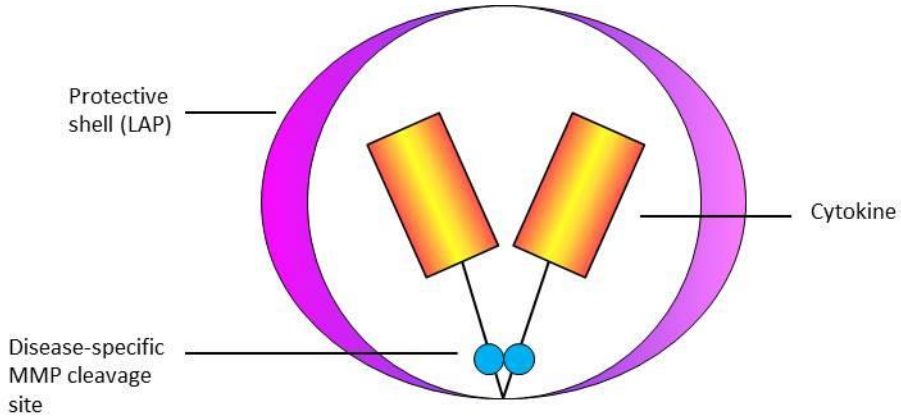


Figure 1.6 Latent growth factor fusion-protein structure.
The protective shell surrounding the growth factor is derived from the latency-associated peptide (LAP) of TGF (transforming growth factor)-β and serves to prevent growth factor interaction with its receptors, increasing the half-life of the growth factor *in vivo*. The cleavable matrix metalloproteinase (MMP) site can be tailored to be sensitive to the MMPs that are abundant in certain pathological conditions. Cleavage by MMP releases the growth factor or other therapeutic agent so that it acts locally, at the site of disease.

AIM

The aim of this study was to utilise the latency associated peptide technology to render each of the growth factors EPO, TGFβ and IGF1 latent in order to localise effective concentrations to sites of inflammation and damage found within the CNS of the EAE mouse model of MS. I hypothesise that the latent rendering of these growth factors will inhibit any interaction with the respective receptors until they have been released within regions of up-regulated MMP activity such as within demyelinating lesions. Once released, the biologically active growth factor can exert its neuroprotective and/or anti-inflammatory influence on its targets in the immediate microenvironment. As the concentrations of the growth factor are therapeutically effective but localised, they should not lead to systemic side effects.

This thesis details the structural design, production and *in vivo* testing of this novel therapeutic tool. In chapter 3, the construction and *in vitro* characterisation of these latent growth factors is

addressed. Chapter 4 details the delivery of these growth factors by cells genetically modified *in vitro* using lentiviral vector. The final chapter, chapter 5, assesses the therapeutic potential of the latent growth factors EPO and TGFβ in the EAE mouse model of MS.

CHAPTER 2

MATERIALS AND METHODS

MOLECULAR CLONING

All chemical reagents came from Sigma-Aldrich®, Dorset, UK, unless otherwise stated. All DNA restriction enzymes and DNA modifying enzymes came from New England Biolabs (NEB), Hitchin, UK, unless otherwise stated. All cell culture reagents came from Cambrex, Workingham, UK unless otherwise stated.

PCR amplification

In order to generate the DNA sequences required for subcloning into an expression vector, the growth factor specific DNA was amplified using growth factor specific primers (Table 2.1) that incorporated specific restriction enzyme recognition sequences to both the 5' and the 3' end of the required DNA sequence. The DNA was amplified by 30 cycles of amplification using the Bio-Rad Gene Cycler Thermal Cycler® PCR machine. Optimal conditions were determined by performing PCR at different concentrations of $MgCl_2$ (1–2.5mM) and different annealing temperatures (45°C–65°C).

huTGFβ PCR amplification

The human TGFβ gene (huTGFβ) was amplified by Platinum® *Pfx* DNA polymerase (Invitrogen, Paisley, UK)–a DNA polymerase possessing proofreading capabilities–using plasmid huTGFβ-Babe neo (-5'UTR) (Chernajovsky et al., 1997) as a template. Based on the gene sequence of huTGFβ, oligonucleotide primers were synthesized to amplify the intact DNA sequence region of mature TGFβ. The forward primer (A) 5'-CGCGGCCGCAGCCCTGGACACCAACTATTGC-3' introduced a *NotI* restriction site (underlined) just prior to initiation of the mature protein antisense. The reverse primer (B) 5'-CGCTCCTGCAAGTGCAGCTGACTCTAGACC-3' is complementary to the nucleotides positioned prior to the stop codon and introduced an *XbaI* (underline) restriction site just downstream to the stop codon. The PCR mixture consisted of 10µl *Pfx* amplification buffer (1x final), 2µl dNTP mix (200µM of each final), 1.5µL each of forward and reverse primers (0.3µM each primer

final), 0.5µl of Platinum *Pfx* DNA polymerase (1 unit), and 200ng of template in a total volume of 50µl. The PCR was performed with the following cycling profile: initial denaturation at 95°C for 2 min, followed by 30 cycles of 1 min denaturation at 95°C, annealing at 50°C for 1 min, and extension at 72°C for 2 min. The time for the final extension step was 5 min at 72°C. After PCR the product was pooled and purified using either the Qiagen Qiaquick PCR purification kit (Qiagen, Cawley, UK) following the manufacturer's protocol; or by phenol: chloroform extraction and then by DNA precipitation either using 0.3M sodium acetate pH 5.2, or 2M ammonium acetate pH 4.8, and 2.5-3 volumes ethanol, prior to separation using agarose gel electrophoresis, to a final volume of 50µl.

mIGF1 PCR amplification

The mature IGF1A or IGF1B genes were initially amplified by *Taq* polymerase using the cDNA from mouse brain as a template. The difference in the isotypes IGF1A and IGF1B is the inclusion or exclusion of exon 5. Based on this, the oligonucleotide primers were synthesized to amplify the intact IGF1B and a truncated IGF1A product. The forward primer (W) 5'-CGCGGCCGCAGGACCAGAGACCCTTTGC-3', introduced a *NotI* restriction site (underlined) just prior to initiation of the mature protein. The reverse primer 5'-CTACTTGTGTTCTTC-3' is complimentary to the nucleotides positioned before a stop codon within exon 6, exon 6 has the same nucleotide sequence for both isoforms mIGF1A and mIGF1B. These primers correspond to the boundaries of the coding sequence for both isoforms mIGF1A and mIGF1B from translation initiation to a termination codon found near the beginning of exon 6. The PCR mixture consisted of 5µl reaction *Taq* buffer (1x final), 1.5µl dNTP mix (0.3µM of each final), 1.5µL each of forward and reverse primers (0.3µM each primer), 0.5µl of *Taq* DNA polymerase (5U/µl), and 100ng of template in a total volume of 50µl. The PCR was performed with the following cycling profile: initial denaturation at 94°C for 5 min, followed by 30 cycles of 1 min denaturation at 94°C, annealing at 55°C for 2 min, and extension at 72°C for 3 min. The time for the final extension step was 5 min at 72°C. After PCR the product was pooled and purified using either the Qiagen Qiaquick PCR purification kit (Qiagen, Cawley, UK) following the

manufacturer's protocol; or by phenol: chloroform extraction and then by DNA precipitation either using sodium acetate or ammonium acetate and 2.5-3 volumes ethanol, prior to separation using agarose gel electrophoresis. Once separated the PCR fragments were excised using a sharp scalpel and purified (see below).

However, once the products were sequenced, it was found that the mIGF1A PCR product was truncated at the 3' end. The difference between the two isoforms mIGF1A and mIGF1B is due to alternative splicing of exon 5. Inclusion of this exon in mIGF1B generates a frame shift in the reading frame resulting in the stop codon used in the design of the original reverse primer. However mIGF1A does not contain exon 5 and therefore this stop codon does not exist within its reading frame, its (mIGF1A) stop codon occurs further downstream towards the end of exon 6. By using the reverse oligonucleotide primer (AJ) 5'-TATGCATCTAGACTACATTCTGTAGGTCTTGTTTCCTGCACTTCCT**CTACTT GTGTTCTTCAAATGTAC**-3' that is complimentary (bold nucleotides) to the nucleotides positioned towards the 3' end of the truncated IGF1A product, and which additionally contains the remaining nucleotides required to complete the mature IGF1A DNA sequence and introduces an *XbaI* restriction site (underlined) just downstream to the stop codon, the sequence was elongated and completed. The elongation of the truncated IGF1A product was performed using the Platinum® *Pfx* DNA (Invitrogen) polymerase to ensure correct amplification. The PCR product was purified using either the Qiagen Qiaquick PCR purification kit (Qiagen, Cawley, UK) following the manufacturer's protocol; or by phenol: chloroform extraction and then by DNA precipitation either 0.3M sodium acetate pH 5.2, or 2M ammonium acetate pH 4.8, and 2.5-3 volumes ethanol, prior to separation using agarose gel electrophoresis. Once separated the PCR fragments were excised using a sharp scalpel and purified.

Table 2.1 Sequences of PCR primers and annealing oligonucleotides.
Restriction sites used for cloning are underlined. The base pairs added for better efficiency of restriction enzyme cutting are in small, plain letters. Those nucleotides in bold, of the antisense IGF1 (AJ) primer, represent the nucleotides that anneal to the beginning of exon 6 on the IGF1 DNA sequences, with the remaining nucleotides until the XbaI restriction site filling in the missing remainder of the exon. The nucleotides in italics indicate the in-frame stop codon introduced within the stuffer sequence.

Primer	Sequence	Restriction site
mIGF1	Sense (W) 5'-CGCGGCCGCAGGACCAGAGACCCTTTGC Antisense (M) 5'- AGATCTCTACTTGTGTTCTTCA	*NotI* *XbaI*
mIGF1	Antisense (AJ) 5'-TATGCATCTAGACTACATTCTGTAGGTCTTGTTTCCTGCACTTCCT**CTACTTG TGTTCTTCAAATGTAC**	*XbaI*
huTGFβ	Sense (1) 5'-CGCGGCCGCAGCCCTGGACACCAACTATTGC Sense (2) 5'-TAGACTAGATCTATGCCGCCCTCCGGGCTGC Antisense (1) 5'- GGTCTAGAGTCAGCTGCACTTGCAGGAGCG Antisense (2) 5'-TAGACTTACGTATTCGAACTAGTCAGCTGCACTTGCAGGAGCGCACG	*NotI* *XbaI* *BglII* *SpeI/ BstBI/ SnaBI*
GlySer linker	Sense 5'-AATTCGGGTGGGGGCGGTTCGGGTGGCGGGGGCTCGGGCGGGGGTGGCTC AGGC Antisense 5'-TCGAGCCTGAGCCACCCCCGCCCGAGCCCCCGCCACCCGAACCGCCCCCACC CG	*EcoRI* *XhoI*
Stuffer nucleotides	Sense 5'-GGCCGCA*TGA*ATTCA Antisense 5'-CTAGTGAATTCATGC	*NotI overhang/ EcoRI* *SpeI overhang*

Separation of DNA fragments using agarose gel electrophoresis

The quantity and quality of the PCR product was assessed by agarose gel electrophoresis. DNA was diluted in either 1x Orange G (Sigma-Aldrich, Dorset, UK) or 1x bromophenol blue loading dye (6x stock: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol made up in distilled water) and loaded into a 1% (w/v) agarose gel (containing 0.5µg/ml ethidium bromide final) placed in 0.5x TAE (50x: 2M Tris-acetate, 100mM Na_2EDTA in dionized water, pH 8.3, National Diagnostics, Yorkshire, UK). Electrophoresis was done for ~45 min at 120V alongside 10µl of either 1 kb or 1 kb

Plus DNA ladder (Invitrogen), in 0.5x TAE running buffer. The DNA fragments were then visualised under UV light and the image captured using a gel documentation system (UviTec, Cambridge, UK).

Purification of DNA from agarose gel

Following separation of the DNA fragments by electrophoresis, gel slices containing bands of interest were purified using the Roche Agarose Gel DNA Extraction kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's' protocol. Briefly, the gel slice was solubilised at 65°C in solubilisation buffer containing a silica suspension. Following centrifugation, the pellet was resuspended in DNA binding buffer before washing and resuspending in deionised H_2O (dH_2O, Elix™ Purification system; Millipore, Watford, UK).

Purification of DNA from solutions

All centrifugation steps were done at room temperature in a bench top microfuge centrifuge unless otherwise stated.

For DNA extraction, an equal volume of water-saturated phenol: chloroform (Phenol:Chloroform:Isoamyl alcohol (25:24:1), Biogene, Cambs, UK) was added to the sample, vortexed for 1 min and then centrifuged (12 000 rpm, 2 min). The top aqueous phase containing the DNA was carefully removed and transferred into a sterile microfuge tube. Either 1µl of 3M Sodium Acetate pH5 was added to every 10 µl of sample, followed by the addition of 2.5 volumes absolute ethanol; or ammonium acetate was added to a final concentration of 2M, followed by the addition of 3 volumes absolute ethanol. The tube was gently inverted to mix and then placed at -20°C for 20 minutes to allow the DNA to precipitate. The sample was then centrifuged (12 000rpm, 10 minutes), the supernatant aspirated using a gentle vacuum, and the DNA resuspended in an appropriate amount of buffer (10mM Tris-Cl, 1mM EDTA, pH 8.5) and stored at 4°C until needed. DNA concentration was determined using either a GenQuant spectrophotometer (Pharmacia, Surrey, UK) or the NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, U.S.A.) at the

260 nm wavelengths. Alternatively, the Qiagen Qiaquick PCR purification kit (Qiagen, Cawley, UK) following the manufacturer's protocol was used.

TA® Cloning

The purified PCR products (huTGFβ, mIGF1A and mIGF1B) were then cloned into the pCR2.1 plasmid vector. PCR products generated using *Taq* polymerase have a non-template derived deoxyadenosine (A) addition to its 3' end, which the TA Cloning® kit exploits by enabling ligation of the PCR product to the pCR 2.1 plasmid vector–a linearised plasmid vector that contains a single 3' deoxythymidine (T) residue. Products amplified by *Pfx* DNA polymerase do not contain this additional deoxyadenosine as the enzyme removes the 3' A-overhangs in its proofreading activity. Addition of these 3' A-overhangs was achieved through incubation with *Taq* at the end of the reaction. The PCR reaction sample was incubated on ice and 1U of *Taq* polymerase was added and the sample mixed well. The sample was then incubated at 72°C for 10 minutes before immediate extraction via phenol: chloroform precipitation and was resuspended in an appropriate amount of deionized H_2O. For cloning of the PCR products into the pCR2.1 plasmid vector, the amount of PCR product required is calculated using the following equation:

$$\text{X ng PCR product} = \frac{\text{(y bp PCR product) (50ng pCR®2.1 vector)}}{\text{Size in bp of pCR®2.1 vector ~ 3 900bp}}$$

, where x ng is the amount of PCR product of y base pairs to be ligated for a 1:1 (vector: insert) molar ratio.

Digestion and Ligation

The latent growth factor constructs were produced by sequential cloning of the different growth factor fragments into, firstly, the pCR2.1 cloning vector followed by subcloning into the pcDNA3 expression vector.

All PCR products, as well as the plasmid vectors and viral vectors, were incubated overnight at 37°C with specific restriction enzymes (Table 2.1). The digested fragments were separated on 2% and 1% agarose gels according to fragment size with 0.5% TAE as described earlier. DNA fragments of interest were excised using a sharp scalpel, before purifying as described earlier.

The ligation stage was achieved using T4 DNA ligase (NEB) and was performed using the manufacturer's recommended protocol. The amount of DNA needed for a 3:1 (insert: vector) molar ratio is determined by the equation:

$$X \text{ ng product} = \frac{(y \text{ bp product})\ (z \text{ ng vector})}{\text{Size in bp of vector}}$$

followed by the addition of 1x T4 DNA ligation buffer (500mM Tris-HCl, 100mM $MgCl_2$, 10mM ATP, 100mM dithithreitol (DTT; pH 7.5) and 1U T4 DNA ligase enzyme to a final volume of 10µl, and incubated overnight at 4°C. This was followed by competent *Escherichia coli* (*E.coli*) DH5α bacterial transformation.

Klenow Blunt Ending

Blunt ending of DNA sequences containing a 3' overhang is carried out using the Klenow enzyme (Roche Applied Science, Mannheim, Germany)–the large fragment (Mr 75 000) of DNA Polymerase 1, that retains the 3'–5' exonuclease activity but which lacks the 5'–3' exonuclease activity.

A typical 20µl reaction comprised of 1µg DNA, 1mM of each oligonucleotide (final concentration), filling buffer (50mM Tris pH 7.5, 10mM $MgCl_2$, 1mM DTT, and 50µg/ml bovine serum albumin (BSA)) and 1U Klenow enzyme (2U/µl), followed by incubation for 15 min at 37°C. The reaction was stopped by the addition of 2µl 0.2M EDTA (pH 8.0) followed by incubation for 10 min at 65°C.

Oligonucleotide Annealing

Complementary strands of oligonucleotides (10µg of each oligo), used for the production of the control plasmid vector pcDNA3 (LAP-$(Gly_4Ser)_3$-mEPO) and the control lentiviral vector pHRSIN-CSGW (LAP-MMP), were annealed by boiling for 5 min with 10µl T4 DNA ligation buffer (10x stock) in a final volume of 100µl before allowing the bath to cool to room temperature overnight to facilitate annealing, followed by storage at 4°C until required.

Dephosphorylation of vectors

Calf intestinal alkaline phosphatase (CIP; NEB) catalyses the removal of 5' phosphate groups from DNA resulting in the failure of vector backbones to re-ligate during cloning DNA inserts.

1U CIP (10 000U/ml) was added per 2µg DNA diluted in 40µl 1x buffer (50mM Tris-HCl, 100mM NaCl, 10mM $MgCl_2$, 1mM DTT pH 7.9) and incubated for 60 min at 37°C. The DNA was purified using phenol extraction, its presence and size confirmed by agarose gel electrophoresis and quantified using spectrophotometry.

Preparation of Competent DH5α *E.coli* bacteria

DH5α *E.coli* cells are rendered competent for the uptake of DNA by a calcium chloride protocol (Sambrook et al., 1989). 5ml of LB broth (Merck, Nottingham, UK) was inoculated with *E.coli* DH5α bacterial cells and incubated overnight at 37°C. The culture was added to 500ml fresh LB broth and incubated at 37°C until the optical density (OD) measured at 600nm was within 0.4 and 0.6, corresponding to exponential growth. The culture was centrifuged (5000 rpm, 10 min, 4°C) and the supernatant discarded. The pellet was resuspended in 50ml of ice cold 0.1M $MgCl_2$ and centrifuged (2000 rpm, 10 min, 4°C). The supernatant was discarded, the pellet resuspended in ice cold 0.1M $CaCl_2$, incubated on ice for 20 minutes and then centrifuged at 2000rpm for 10 min at 4°C. The pellet was resuspended in 12.5ml ice cold 0.1M $CaCl_2$ containing 14% glycerol, aliquoted, snap frozen on dry ice and stored at -80°C.

Transformation of Bacteria

Following the successful ligation of vector and insert, the recombinant plasmid vectors were transformed into competent DH5α *E.coli* bacteria. A 50µl aliquot of competent DH5α bacteria *E.coli* suspension was thawed on ice before the addition of 2µl ligation mix. The mixture was incubated on ice for 30 min, followed by heat shocking at 42°C for 2 min before being returned to ice. 250µl LB broth was added to the cell mixture and incubated for 1 hour at 37°C with shaking at 220 rpm. 100µl of bacterial culture was spread on pre-warmed agar plates (Biogene) containing ampicillin (100ug/ml) with or without 1.6mg X-gal and incubated overnight at 37°C. When cloning DNA fragments into pCR2.1, which has the galactosidase gene, individual white colonies were picked and placed in a 7ml bijou containing 5ml LB broth and ampicillin, and incubated at 37°C for 8–18 hours with shaking at 220 rpm.

Extraction of Plasmid DNA

Following transformation, a small volume (1.5ml in a clean microfuge tube) of the bacterial culture was centrifuged (11 000g; 20 sec) and the plasmid DNA recovered using the QIAspin Miniprep kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The presence of the insert and its correct orientation within the plasmid vector was confirmed by restriction enzyme digestion, followed by agarose gel electrophoresis as described previously. Once a clone containing the correct insert size was identified, a sample was sent for complete DNA sequencing, and a large-scale culture prepared–1ml of the 5ml starter culture was incubated overnight at 37°C in 500ml LB with ampicillin (100µg/ml). The recovery of plasmid DNA was obtained using either the QIAprep MegaPrep Kit (Qiagen) or the PureLink™ HiPure Plasmid DNA Megaprep Kit (Invitrogen, Paisley, UK) following the manufacturer's protocols. Briefly, the bacterial culture is centrifuged and the pellet lysed under alkaline conditions. The DNA is absorbed onto a silica membrane column under high salt conditions before being washed and eluted under low salt conditions and stored at 4°C until required.

DNA Sequencing

All DNA sequencing was performed by the Genome Centre (WHRI, Queen Mary University, UK) using 400–800ng of either plasmid DNA or 200–600ng PCR product and 10pmoles/µl DNA primers.

Construction of the latent growth factor gene plasmid vectors

pcDNA3 (LAP-MMP)

This construct has been described and used before by our group (Adams et al., 2003). The LAP sequence of huTGFβ spanning Met1–Ser273 is followed by a linker sequence (GGGGS), a MMP cleavage site (PLGLWA) and a final flexible linker sequence (GGGGSAAA) and is cloned into the pcDNA3 plasmid.

Cloning of the MMP cleavage site:

A double-stranded deoxyoligonucleotide coding for the MMP cleavage site (GGGGS*PLGLWA*GGGGSAAA) was designed using the following deoxyoligonucleotides: sense 5'-AATTCGGGGGAGGCGGATCCCCGCTCGGGCTTTGGGCGGGAGGGGGCTCAGC-3'; antisense: 5'-GGCCGCTGAGCCCCCTCCCGCCCAAAGCCCGAGCGGGGATCCGCCTCCCCCG-3'. The annealed deoxyoligonucleotides were cloned into the pcDNA3 plasmid cut with *EcoRI* and *NotI*. This construct was named GS-MMP-GS.

Cloning of the pcDNA3(LAP-MMP) construct.

The signal peptide of huTGFβ LAP and with the restriction sites HindIII and EcoRI were cloned from the plasmid huTGFβ-Babe neo (-5'UTR) (Chernajovsky et al., 1997), using the following primers : sense 5'-CCAAGCTTATGCCGCCCTCCGGGCTGCGG-3'; antisense 5'-CCGAATTCGCTTTGCAGATGCTGGGCCCT-3'. This sequence (820bp) was cloned into the GS-MMP-GS construct cut with *HindIII* and *EcoRI*. This construct was named pcDNA3(LAP-MMP).

pcDNA3 (LAP-MMP-huTGFβ)

Human TGFβ with *NotI* and *XbaI* ends was cloned by PCR from the plasmid huTGFβ-Babe neo (-5'UTR) (Chernajovsky et al., 1997, Adams et al., 2003). The 358-base pair (bp) product was cloned into the pcDNA3 (LAP-MMP) plasmid vector cut with *NotI* and *XbaI*. The clone was named pcDNA3 (LAP-MMP-huTGFβ).

pcDNA3 (LAP-MMP-mIGF1)

Both isoforms of mIGF1 were cloned by PCR from mouse brain cDNA using the forward and reverse primers W and M. The original 294bp PCR product of mIGF1A was truncated at the 3' end. This was rectified by elongation of the product by PCR using a second reverse primer incorporating the missing nucleotides and an *XbaI* restriction site resulting in a 340bp fragment. The correctly cloned and sequenced product of mIGF1A was cloned into the pcDNA3 (LAP-MMP) plasmid cut with *NotI* and *XbaI*. The clone named pcDNA3 (LAP-MMP-mIGF1A).

However the 346bp product of mIGF1B did not contain a restriction site. This was overcome by exploiting the *XbaI* site found downstream of the PCR product insertion site of pCR2.1. Additionally, the pCR2.1 plasmid vector contained a *NotI* site downstream from mIGF1B (already containing a *NotI* site) and upstream from the *XbaI* site as illustrated in Figure 2.1 which needed to be removed. The pCR2.1 plasmid was cut with *EcoRV* and *XbaI* resulting in the linearised vector and a fragment containing the second *NotI* site. The *XbaI* overhang within the linearised vector was then blunt-ended with Klenow enzyme before religating back. The DNA product mIGF1B, cut with *NotI* and *XbaI* from the religated pCR2.1 plasmid vector, was cloned into the pcDNA3 (LAP-MMP) plasmid cut with *NotI* and *XbaI*. The clone was named pcDNA3 (LAP-MMP-mIGF1B).

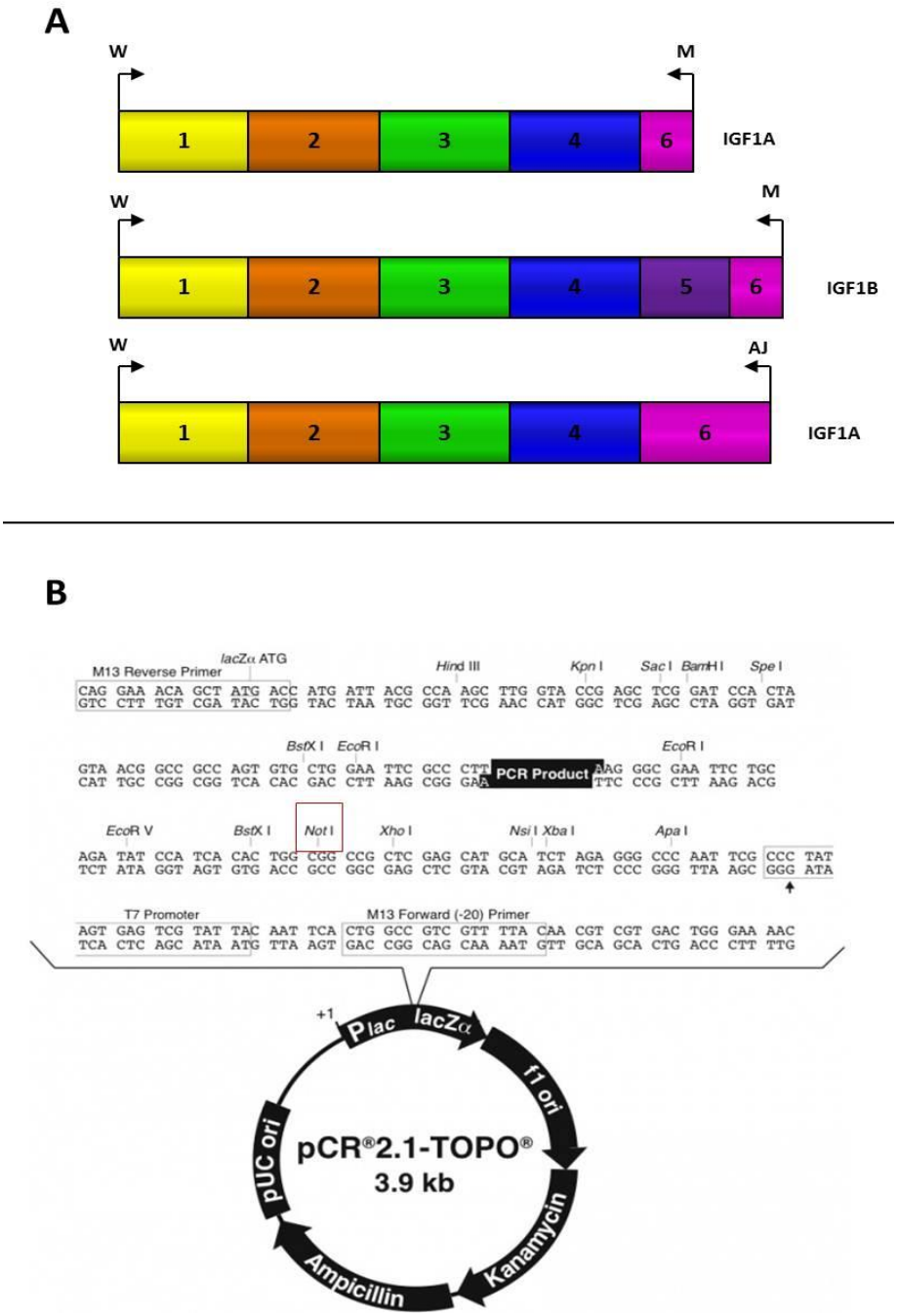


Figure 2.1: Construction of plasmids containing the IGF1A and IGF1B isoforms.
(A) Primers W and M bind to both isoforms of mouse IGF1 prior to initiation of the mature protein and at a termination codon within exon 6 for IGF1B and include a *NotI* restriction site at the 5' end. Elongation of the truncated IGF1A gene sequence performed using reverse primer AJ binding to complementary sequence at 3' end and extended to include a termination codon and an *XbaI* restriction site. **(B)** The plasmid pCR2.1 contains a *NotI* site (red square) downstream of the inserted PCR products but upstream from the *XbaI* site. This is only significant in the generation of the IGF1B plasmid, as the 3' primer (M) did not attach an *XbaI* site at the 3' end.

pcDNA3 (LAP-(Gly$_4$Ser)$_3$-mEPO)

The control vector, with the MMP site removed and replaced with a glycine-serine linker bridge, was produced by subcloning the annealed oligonucleotides sense: 5'-AATTCGGGGGAGGCGGATCCGGAGGTGGCGGTTCGGGTGGAGGGGGCTCAGC-3'; antisense: 5'-GGCCGCTGAGCCCCCTCCACCCGAACCGCCACCTCCGGATCCGCCTCCCCCG-3' containing *EcoRI* and *NotI* overhangs, into the pcDNA3 (LAP-MMP-mEPO) plasmid cut with *EcoRI* and *NotI.* The clone was named pcDNA3 (LAP-(Gly$_4$Ser)$_3$-mEPO).

Construction of lentiviral vectors with latent growth factor genes.

All lentiviral techniques were performed in Class II biological hoods using aseptic techniques. All biological waste was neutralised in Microsol 3 (Anachem, Bedfordshire, UK) and all surfaces decontaminated with Microsol 3 and 70% ethanol prior to and during use. All cell lines were incubated at 37°C with 10% CO_2 humidified atmosphere unless otherwise stated.

The self-inactivating lentiviral vector pHRSIN-CSGW was kindly provided by Dr Adrian Thrasher (Institute of Child Health, UCL, London, UK) and has been described previously (Demaison et al., 2002). The engineered constructs LAP-MMP-mEPO, LAP-(Gly$_4$Ser)$_3$-mEPO, LAP-MMP-huTGFβ, LAP-MMP-mIGF1A, LAP-MMP-mIGF1B, LAP-MMP and mEPO alone were subcloned into the pHRSIN-CSGW lentiviral vector between the *BamHI* and *NotI* restriction sites.

LAP-MMP-huTGFβ generic lentiviral vector

A generic lentiviral vector was produced for the insertion of other growth factors or trophic factors downstream of the LAP fragment. Following sequence confirmation of pcDNA3(LAP-MMP-huTGFβ), PCR amplification of the LAP-MMP-huTGFβ fragment was done using pcDNA3(LAP-MMP-huTGFβ) as a template. The oligonucleotide primers were synthesized to incorporate a 5' *BglII* restriction site and 3' *SnaBI*/*SpeI* restriction sites (Table 2.1). This sequence, when cut with *BglII* and *SnaBI*, has a complementary 5' GATC overhang to the *BamHI* sequence and a 3' blunt end. The LAP-MMP-huTGFβ

fragment with *BglII* and *SnaBI* ends was cloned into the pHRSIN-CSGW lentiviral vector cut with *NotI*, and blunt ended with Klenow. The lentiviral vectors were sequenced and the correct clone was named pHRSIN-CSGW-(LAP-MMP-huTGFβ).

LAP-MMP-mEPO, LAP-MMP-mIGF1A and LAP-MMP-mIGF1B lentiviral vectors

The pcDN3 plasmid vectors for LAP-MMP-mEPO, LAP-MMP-mIGF1A and LAP-MMP-mIGF1B were digested with *NotI* and *XbaI* and the fragments isolated through agarose gel electrophoresis and purification. The fragments mEPO, mIGF1A and mIGF1B with NotI and XbaI ends were cloned into the generic lentiviral vector pHRSIN-CSGW-(LAP-MMP-huTGFβ) pre-digested with *NotI* and *SpeI*– releasing the huTGFβ fragment and leaving 3' overhang compatible with *XbaI* digested ends. The resulting clones were sequenced and the correct clones named pHRSIN-CSGW-(LAP-MMP-mEPO) pHRSIN-CSGW-(LAP-MMP-mIGF1A), pHRSIN-CSGW-(LAP-MMP-mIGF1B)

Control lentiviral vectors

The mEPO and LAP-MMP control lentiviral vectors were produced in our lab by Dr Anne Rigby during my maternity leave.

LAP-MMP lentiviral vector

The pHRSIN-CSGW-(LAP-MMP) control lentiviral vector was produced by removal of the huTGFβ fragment from the generic pHRSIN-CSGW-(LAP-MMP-huTGFβ) lentiviral vector and replacing it with a stop codon. Synthetic oligonucleotides were designed to contain a stop codon between *NotI* and *SpeI* overhangs (Table 2.1). An additional *EcoRI* restriction site was included to perform diagnostic restriction digestion following ligation. The annealed oligonucleotides with *NotI* and *SpeI* ends were cloned into the pHRSIN-CSGW-(LAP-MMP-huTGFβ) lentiviral vector cut with *NotI* and *XbaI*. Correct clones were screened through *EcoRI* restriction enzyme digestion. The correct clone was named pHRSIN-CSGW-(LAP-MMP).

mEPO lentiviral vector

The control lentiviral vector mEPO has been described previously (Rigby, 2009). Briefly, the mouse EPO protein (complete with its signal peptide) was digested with *BamHI* and *SbfI* to release a fragment consisting of the signal peptide and the upstream portion of mEPO. The downstream portion of EPO was obtained through digestion with *SbfI* and *NotI* of the plasmid pHRSIN-CSGW containing scFV-EPO with a V5-His B tag for immunoblotting with the anti-V4 probe. The two fragments containing the upstream (with *BamHI* and *SbfI* ends) and downstream portions (with *SbfI* and *NotI* ends) of mEPO were then subcloned into the pHRSIN-CSGW lentiviral vector cut with *BamHI* and *NotI*. The lentiviral vector was named pHRSIN-CSGW-mEPO.

RECOMBINANT PROTEIN EXPRESSION

Once the sequences of the plasmid DNA were confirmed, various cell lines were transfected to produce the recombinant proteins, beginning with the dominant mammalian host for recombinant protein manufacturing, the DG44 CHO cell line.

Production of protein therapeutic agents in mammalian cells

Chinese hamster ovary (CHO) cell line expression system

The DG44 CHO cell line was deficient in DHFR (-/-) and required thymidine to enable growth. DHFR is responsible for the formation of tetrahydrofolic acid, a cofactor necessary for de novo DNA synthesis (Urlaub and Chasin, 1980). The enzyme is very sensitive to the tetrahydrofolate analog methotrexate and is useful for gene amplification through metabolic manipulation (Gasser et al., 1982). Transfection of CHO DHFR deficient cells, with a DHFR-expressing plasmid pSV_2DHF (Chernajovsky et al., 1984), resulted in clones that were resistant to methotrexate in a dose-dependent manner because these clones express different copy numbers of the plasmid. Clones with the highest copy number were selected for by applying high doses of methotrexate. Once co-transfected with the

gene of interest and the DHFR gene, these cells have the highest copy number of the gene of interest.

DG44 cells were routinely cultured and maintained in DG44 growth medium (Ham's F12 medium supplemented with Penicillin/Streptomycin (final concentration:100U/ml and 100µg/ml respectively; Pen/Strep; L-Glutamine (final concentration: 2mM; Glut); thymidine (final concentration: 30µg/ml; Sigma-Aldrich) and 10% (v/v) heat-inactivated foetal bovine serum (FBS; Invitrogen)) at 37°C with a humidified atmosphere of 10% CO_2 in air. Cells have a doubling time of 22–26 hours and were split when they reached confluency (80-100%) using trypsin-EDTA (2.5% final) incubated for 10 min and washed with FCS containing medium (800g, 5 min) before plating.

DG44 cells were permanently co-transfected with plasmids (20µg) expressing LAP-MMP-mEPO, LAP-MMP-huTGFβ, LAP-MMP-mIGF1A, LAP-MMP-mIGF1B or LAP-MMP, and the pSV_2DHFR gene vector (Chernajovsky et al., 1984) using a calcium phosphate DNA co-precipitation protocol (Sambrook et al., 1989). The cells were seeded at a density of $0.5x10^6$ per 10cm plate (BD Falcon®; BD Bioscience, Oxford, UK) in DG44 growth medium and incubated overnight. The medium was replaced with fresh medium for 3 hours the following day to induce cell proliferation. 20µg of plasmid DNA and 1µg pSV_2DHFR gene vector was made up to 450µl with dH_2O to which 500µl of 2x HBS (280mM NaCl, 50mM HEPES, 1.5mM $NaHPO_4$; pH 7.05) was added followed by the drop wise addition of 50µl of 2.5M $CaCl_2$. The solution was allowed to develop a precipitate for 30 min at room temperature.

The medium was removed by gentle vacuum aspiration, and the precipitate added to the side of the plates and tilted until the cells on the plates were completely covered, followed by repeated tilting every 5 min for a total of 30 min. The cells were incubated for 4 hours following the addition of 9 ml fresh DG44 growth medium, the medium removed by gentle vacuum aspiration before adding 1ml of glycerol shock solution (10% glycerol (v/v) in serum-free medium) and tilting continuously for 4 min. The cells were then washed twice with 10ml of Ham's F12 supplemented with 10% FBS, Pen/Strep and Glut, before incubating overnight in 10ml DG44 growth medium to recover.

The cells were left to recover for no more than 48 hours and the media was replaced with 10ml Geneticin® selection medium (Alpha-MEM medium supplemented with Penicillin/Streptomycin (100x = 100U/ml and 100µg/ml respectively, Pen/Strep; L-Glutamine (100x = 200mM; Glut); G418 (Geneticin® sulphate (100mg/ml; Invitrogen) and 10% (v/v) dialysed foetal bovine serum (dFBS; Invitrogen)). Cells that had been successfully transfected were resistant to G418 as the plasmids expressing the recombinant proteins contained the neomycin gene. Cells that were successfully transfected with the pSV_2DHFR gene vector were able to synthesize thymidine. In order for a cell to survive and proliferate in selection medium, it needs to be successfully transfected with both gene vectors. The selection medium was replaced with fresh selection medium twice a week. After approximately 10-14 days in selection medium, individual colonies of approximately 2mm in diameter were seen and expanded using ring cloning—a technique used to isolate and expanded populations of cells from an original clone. The cells were washed with 1x phosphate buffered saline (PBS) prior to coating the underside of cloning rings (d=6mm; Bellco Glass, New Jersey, U.S.A) with sterile silicon grease creating a seal between the plate and the ring, and then placed to encircle the marked colony. 100µl of 2.5% trypsin (v/v) diluted 1:4 in 1x PBS was added to the cells, and incubated for 1 min on the bench at room temperature before inactivation with 100µl growth medium. Cells were centrifuged (1200 rpm, 3 min) and resuspended in growth medium and seeded into wells of 24-well plate (BD Falcon®). The cells were left to recover before expanding and stocks generated.

Gene amplification of the transfected gene of interest was achieved through Methotrexate (MTX; Calbiochem, Nottingham, UK) amplification as described elsewhere (Gasser et al., 1982). The cell medium was removed and the cells washed once with 1x PBS before adding fresh selective medium supplemented with increasing doses of MTX, starting at 12nM followed by 25nM, 50nM and finally 250nM. The cells were maintained and grown in selective medium supplemented with 250nM MTX to maintain selective pressure.

DG44 cells were washed once with 1x PBS when their confluence reached 80%, CD CHO serum-free medium (Gibco, Invitrogen) added, and the cells incubated for 48 hours. The supernatant was removed, filtered through a 0.45µm filter (Nalgene filters, Fisher Scientific, Leicestershire, UK) to remove dead cells and, following the removal of a 1ml aliquot, EDTA was added to a final concentration of 20mM to inhibit any endogenous MMP enzyme activity before storage at -80°C.

HEK-293T cell line expression system

The Human Embryonic Kidney cell line containing the SV40 large T antigen, HEK-293T, was cultured and maintained in HEK-293T growth medium (Dulbecco's Modified Eagle's Medium (DMEM), supplemented with Pen/Strep, Glut and 10% FBS). HEK-293T cells have a doubling time of 20 hours, and were routinely spilt every 3-4 days. Cells were incubated with 5ml trypsin/PBS solution (1:4) at 37°C for 3–5 min before adding 5ml 10% FBS containing medium to inactivate the trypsin. The cell suspension was transferred to a sterile conical tube, centrifuged (800 g, 5 min) and the supernatant discarded. The cell pellet was gently resuspended in fresh growth medium before plating 1:10.

For transient transfection of the HEK-293T cell line with a latent growth factor gene plasmid (20µg), 1 ml of medium was removed by vacuum from the plates and the calcium phosphate precipitate (described in CHO cell line expression system) was added drop wise, followed by occasional tilting for 30 min. A further 9ml of growth medium was added and the cells incubated overnight. The following day, 1ml glycerol shock solution was added for 4 minutes before washing twice with FBS-containing medium. The medium was removed by gently aspirated and replaced with fresh HEK-293T growth medium. The culture supernatant was collected 48 hours later, centrifuged (800g, 5 min), filtered to remove cell debris, and stored at -80°C until required.

Freestyle™ CHO-S cell expression system

Freestyle™ CHO-S cells (Invitrogen) are suspension CHO cells adapted to be used in conjunction with the FreeStyle™ MAX CHO Expression System—an optimised system for transient transfection and protein production. CHO-S cells are grown in FreeStyle™ CHO Expression Medium and incubated on

an orbital shaker rotating at 120-135rpm at 37°C with 8% CO_2 humidified atmosphere. Cells are passaged the day before transfection at a density of 5–6x10^5 cells/ml at a volume of 30ml/125ml flask. On the day of transfection, the cells are diluted to a density of 1x10^6 cells/ml and 30ml of this cell suspension added to a new flask. pcDNA3(LAP-MMP-mEPO) plasmid DNA (37.5µg) was diluted in OptiPro™ SFM before adding to diluted Freestyle MAX Transfection reagent. The solution was allowed to develop a precipitate for 10 min at room temperature. The complex solution was added gently to the cell suspension whilst gently swirling, and the flask retuned to the orbital shaker (135rpm) and incubated for 48 hours. The cell culture was transferred to a sterile conical tube (BD Falcon™, Oxford, UK), centrifuged and the supernatant filtered (0.45µm) to remove cell debris before storing at -80°C until required.

Biozzi ABH mouse fibroblast cell expression system

Our lab previously generated temperature sensitive SV40 large T-antigen immortalised cell lines from fibroblast cells taken from the Biozzi ABH mouse (Croxford et al., 2000, Croxford et al., 2001). These cells are permanently transduced with a temperature sensitive simian virus 40 (SV40) large T-antigen that is rapidly degraded at body temperature (*in vivo*) or when grown at 37°C (*in vitro*). These cells were therefore designated tsF (temperature sensitive fibroblast) cells. They were transfected to express the latent growth factors LAP-MMP-mEPO, LAP-MMP-huTGFβ, LAP-MMP-mIGF1A, LAP-MMP-mIGF1B and the control LAP-MMP (partially engineered by Dr Anne Rigby), and were maintained in Geneticin® selection medium (DMEM supplemented with Pen/Strep, Glut, Geneticin® sulphate (0.5mg/ml, G418; Invitrogen) and 10% FBS) to maintain selection of the neomycin resistant gene present in the retroviral vector expressing the temperature sensitive SV40 large T antigen for approximately 3 weeks. Following this selection period, the cells were maintained rountinely in HEK-293T growth medium (Dulbecco's Modified Eagle's Medium (DMEM), supplemented with Pen/Strep, Glut and 10% FBS). The cells reached confluency at a similar rate as HEK-293T cells (personal observations) and were routinely split 1:10 every 3–4 days as carried out for HEK-293T cells. Stable transfection of the tsF cell line was carried out when cells reached a

confluency of 50%. After transduction and glycerol shock, the cells were left to recover for 48 hours before splitting. Culture supernatants were collected following recovery and stored at -80°C until required.

Production of lentiviral vector packaged particles for Gene Therapy

HEK-293T cells were seeded at a density of $7x10^6$ cells per 15cm tissue culture plate (BD Falcon) and incubated overnight in 15ml HEK-293T growth medium. One hour prior to transfection, 25µl of a 25mM chloroquine solution was added to the culture medium. 18µg of lentiviral vector DNA, 18µg of pCMVR8.2 packaging plasmid and 4µg of pMD.G envelope plasmid (containing the VSVG protein) were co-transfected into HEK-293T cells (Naldini et al., 1996). DNA was made up to 900µl with distilled water to which 1ml 2x HBS pH 7.1 was added, followed by the drop wise addition of 100µl $CaCl_2$ (as for CHO cell transfection) and the solution mixture left for 30 min at room temperature to form a precipitate. The mixture was added to the culture medium for 6—8 hours before the medium was replaced and the cells left to recover overnight. The medium was replaced the next day (15 ml) and the cells left for a further 24 hours. The viral supernatant was removed every 24 hours for the next 3 days, filtered to remove cell debris (0.45µm) and ultracentrifuged (30 000 x g, 2 hours, 4°C, Beckman XL90 rotor 70Ti) to concentrate. The stock viral pellet was resuspended in 100µl sterile 1x PBS and aliquoted (20µl; Nalgene) and stored at -80°C.

Lentiviral transduction

tsF cells were seeded at a density of $1.5x10^4$ cells per 15mm well of a 24-well tissue culture plate (BD Falcon) and incubated overnight. The cells were transduced the following day with 20µl concentrated virus in the presence of 6µg/ml polybrene (Sigma-Aldrich) in a total volume of 200µl for 16—18 hours. The medium was replaced and the cells left to recover before expanding and generating stocks. Protein production and secretion of recombinant protein was assessed by western blot analysis of collected supernatant taken from the stably transduced tsF cells. Cells that

failed to express sufficient quantities of recombinant protein were retransduced until sufficient quantities were achieved.

Generation of stably transducted tsF cell stocks

Once the stably transfected tsF cells reached confluency of 60%, the cells were trypsinised, washed with FBS-containing medium, centrifuged (800g, 5 min) and resuspended ($0.5x10^6$ cells/ml) in 1ml FBS containing 10% DMSO before slow cooling and storing at -80°C until required.

PROTEIN ANALYSIS

Transfected or transduced cells were washed once with 1x PBS when their confluence reached 60% and 80% respectively, followed by adding 15ml DMEM supplemented with Pen/Strep and Glut, or alternatively for DG44 cells, with CD CHO serum-free medium (Gibco, Invitrogen), and incubated for 48 hours. The supernatant was removed, filtered through a 0.45µm filter to remove dead cells and, following the removal of a 1ml aliquot, EDTA was added to a final concentration of 10mM to inhibit any endogenous MMP enzyme activity before storage at -80°C.

MMP1 Cleavage Assay

Serial dilutions of EDTA-free supernatant were incubated with 1x MMP1 buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 10mM $CaCl_2$, 50µM $ZnCl_2$, 0.05% Brij-35) and 30nM MMP1 ΔC (EC 3.4.24.7 kindly provided by Prof. H. Nagase, Imperial College, London) overnight. The reaction was stopped by adding EDTA to a final concentration of 20mM. Cleavage was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot analysis.

Western Blotting

Protein concentration was measured using the bicinchoninic acid assay (BCA; Pierce, Rockford, U.S.A), a biochemical assay similar to the Lowry or Bradford assays. All western blots were run under reducing conditions. Protein samples for SDS-PAGE were diluted in 1x denaturing buffer (4x = 10%

SDS, 40% glycerol, 125mM Tris-HCl pH 6.8, 0.002% bromophenol blue, containing 0.4M DTT or 10% final concentration of β-mercaptoethanol) and denatured by boiling in a water bath for 3 min. Samples were loaded alongside 8µl–10µl of pre-stained protein marker (Bio-Rad) on either 10% SDS-PAGE run at 100V (constant) or on a 4%-12% gradient NuPAGE® minigel (Invitrogen) run at 200V (constant) following the manufacturer's protocol, in 1x NuPAGE® MOPS SDS running buffer (Invitrogen). Proteins were transferred onto Polyvinylidene Difluoride (PVDF, GE Healthcare, Buckinghamshire, UK) membrane for 2 hours using either CAPS transfer buffer (10mM 3-(Cyclohexylamino) propanesulfonic acid pH 11) at 200mA (constant) or NuPAGE® (Invitrogen) transfer buffer and running at 30V (constant) for SDS-PAGE gels or NuPAGE™ Novex Bis-Tris (Invitrogen) minigels respectively. The membrane was rinsed three times with 1x PBS before incubating for 2 hours at room temperature in blocking buffer (5% (w/v) non-fat reconstituted milk in PBS) with agitation, followed by an overnight incubation at 4°C with primary antibody (goat anti-LAP (R&D systems, Abingdon, UK), goat anti-EPO (R&D systems), goat anti-IGF1 (R&D systems), rabbit anti-TGFβ (abcam, Cambridge, UK)) in 0.5x blocking buffer with agitation. The membrane was then rinsed with 1x PBS with 0.05% Tween-20 (PBST), followed by five incubations of 5 min each in 1x PBST with agitation. The membrane was then incubated for 2 hours at room temperature with agitation with secondary antibody (mouse anti-goat-IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology, Heidelberg, Germany), goat anti-rabbit-IgG-horseradish peroxidase conjugated (DAKO, Cambridge, UK) diluted in 0.25x blocking buffer, before rinsing 3 times in 1x PBST, followed by 5 x 5 min incubations in 1x PBST. The membrane was then incubated for 1 min in 10ml ChemiLucent™ Plus ECL Detection System solution (Chemicon, Hampshire, UK) before autoradiography using Hyperfilm ECL x-rays (Amersham, Buckinghamshire, UK) and developing using the AGFA Curix 60™ (AGFA Healthcare).

ELISA

Direct LAP ELISA

ELISA plates (Immuno 96 Microwell®; Nunc, Thermo Fisher Scientific, UK) were coated with serial dilutions of serum or supernatant, or recombinant LAP protein (0—30ng/ml; Sigma-Aldrich) in bicarbonate/carbonate coating buffer (100mM, pH 9.6) and incubated overnight at 4°C. The following day, the plate was washed 5 times with 200µl/well PBST and blocked with 200µl/well 2% casein (w/v) in PBS for 2 hours at room temperature. The plate was washed five times with 200µl/well PBST and incubated with 50µl/well goat anti-LAP primary antibody (0.4µg/ml, R&D Systems, Abingdon, UK) in 1% casein for 2 hours. Following five washes in PBST, the plate was incubated with 100µl/well mouse anti-goat HRP conjugated secondary antibody (0.8µg/ml; Santa Cruz Biotechnology, Middlesex, UK) in 1% casein for 1 hour. Following five washes, a 1:1 mixture of peroxidase B (H_2O_2) and tetramethylbenzidine (TMB; KLP, Maryland, U.S.A) at 100µl/well was added and incubated until the development of adequate colour. The reaction was stopped by adding 100µl/well 4.5M H_2SO_4 and the OD read at 450nm on an ELISA plate reader (Bio-kinetics reader; Bio-Tek instruments, EL3122).

Sandwich EPO ELISA

The EPO sandwich ELISA was kindly performed by Dr Anne Rigby using an ELISA kit (Quantikine mouse/rat immunoassay, R&D Systems, Oxon, UK). ELISA was carried out on serial dilutions of the supernatant from tsF transduced with rEPO (EPO.tsF) or with LAP-MMP-mEPO (LAP-MMP-mEPO.tsF). Linear regression analysis was performed on the commercial recombinant mouse EPO protein standards to derive a linear standard curve. Linear regression analysis was also performed on the supernatant sample dilutions to determine the linear portion of the supernatant samples dilution curve. Supernatant EPO concentrations were calculated using OD values from the linear portion of the supernatant dilutions and applying the protein standards linear regression analysis. An R^2 value was calculated for each regression performed.

Protein deglycosylation

N-Glycosidase F (PNGase F; NEB) an amidase that cleaves between the innermost GlcNAc and asparagine residues of N-linked glycoproteins, was used to deglycosylate the synthesised variants of the latent EPO glycoprotein. Denaturation of the protein was carried out in a 10µl reaction using 1—20µg protein and denaturing buffer (0.5% SDS, 40mM DTT), followed by incubation for 10 min at 100°C. Deglycosylation was performed in a 20µl reaction volume by adding G7 Reaction Buffer (50mM sodium phosphate, pH 7.5), 1% NP-40, PNGase F (500U/µl), followed by incubation for 1 hour at 37°C.

Inhibition of protein glycosylation

The antibacterial and antifungal agent Tunicamycin (MW = 840.0) is an N-linked glycosylation inhibitor that blocks dolichol monophosphate-linked oligosaccharide biosynthesis. Asparagine (N) linked glycoproteins fail to be synthesised in the ER during treatment with tunicamycin. Lyophilised tunicamycin (Sigma-Aldrich) dissolved in DMSO to a concentration of 1mg/ml was filter sterilised (0.45µm) and stored at 4°C.

HEK-293T cells were seeded at a density of $0.5x10^6$ cells per 10cm plate (BD Flacon®) and incubated for 16—18 hours. They were transiently transfected with the pcDNA3 (LAP-MMP-mEPO) plasmid DNA for 5 hours before the medium was replaced with medium containing tunicamycin (0—5µg/ml). The medium was removed after 3 days, centrifuged (1200 rpm, 5 min) and filtered (0.25µm) before being aliquoted and stored at -80°C. The cells were lysed (1% NP-40 containing proteinase inhibitors) on ice for 15 min and centrifuged (3900 rpm, 10 min, 4°C). The supernatant was filtered (0.25µm), aliquoted and stored at -80°C.

CHO-S cells were seeded at a density of $1x10^6$ cells/ml in a total volume of 20ml per Erlenmeyer flask (Sigma-Aldrich), containing 2.5–5µg/ml tunicamycin and left for 16–18 hours. 50µg DNA was diluted in polyethyleneimine (1mg/ml, PEI; Sigma-Aldrich) and NaCl (150mM) to a final volume of 1ml and left for 10 min at room temperature. An additional 5µg/ml tunicamycin was added before adding the

transfection mixture to the cells. The cells were incubated for 3 days at 37°C in an 8% CO_2 humidified atmosphere on an orbital shaker platform rotating at 135 rpm before the cell suspension was centrifuged (1200 rpm, 5 min) and the supernatant stored at -80°C.

BIOLOGICAL ACTIVITY ANALYSES

Detection of EPO biological activity using UT-7 cells

The human megakaryoblastic cell line, UT-7, is dependent on the factors EPO or GM-CSF for survival and proliferation as hown by an increase in cell number in response to EPO or GM-CSF, in a dose-dependent manner. The CellTiter-Glo® assay (Promega; Southampton, UK) is based on the measurement of cellular ATP content, which is used as the equivalent of viable cell number because ATP content of a given cell type remains constant. All cell cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere in air unless otherwise stated.

The UT-7 cell line (kindly donated Dr Julian Kieswich, Queen Mary, UK) was cultured in UT-7 growth medium (RPMI medium supplemented with 10% FBS, Pen/Strep, L-glut and human recombinant GM-CSF (10ng/ml)) and replaced on alternate days. The cells were collected and centrifuged (800 rpm, 5 min), resuspended in GM-CSF-free UT-7 growth medium and incubated for 16–24 hours prior to assay. The cells were then centrifuged (800 rpm, 5 min), counted and resuspended to $4x10^5$ cell/ml in the same medium. Each 6mm well of a 96-well plate was seeded with 50µl cell suspension corresponding to $2x10^4$ cells. Duplicates of either recombinant mouse EPO (0–10ng/ml; R&D systems), or cell culture supernatant (following incubation for 16–18 hours in the presence or absence of MMP1 enzyme) were diluted in the same medium to a 2x final concentration. Each of the dilutions (50µl) was then added to the cells and incubated for 48–72 hours prior to the CellTiter-Glo® assay. The cells were allowed to equilibrate to room temperature for 30 min prior to the addition of 100µl CellTiter-Glo® reagent. The cells were incubated on a platform rotor for 10 min before the samples were transferred to assembly strips of white microtiter plates (Thermo Electron

Corporation, Vantaa, Finland) and the luminescence read using the Revelation MLX 4.06 software of the MLX Microtiter® Plate Luminometer (Dynex Technologies).

Detection of TGFβ growth inhibition biological activity using MLEC cells

huTGFβ has been shown to stimulate the expression of plasminogen activator inhibitor-1 (PAI-1) in the mink lung epithelial cell line (MLEC). Abe, Harpel et al. (1994) stably transfected MLEC cells with a construct consisting of a truncated PAI-1 promoter fused, at the 5' flanking region, to the firefly luciferase reporter gene (MLEC (PAI/L)). Stimulation of these transfected cells with huTGFβ results in a dose-dependent increase in luciferase activity. Using the Luciferase Assay System (Promega, Southampton, UK), the luciferase generated by the MLEC (PAI/L) cells in response to huTGFβ exposure, catalyses the oxidation of luciferin to oxyluciferin, resulting in a flash of light that can be read. All cell cultures were incubated at 37°C in a 10% CO_2 humidified atmosphere in air.

The MLEC (PAI/L) cell line (kindly donated by Dr M. Abe, New York University Medical Centre) was cultured in Geneticin® selection medium (DMEM medium supplemented with 10% FBS, Pen/Strep, L-glut and G418 (1mg/ml)). Following Trypsin/EDTA cell detachment, the cells were centrifuged (1200 rpm, 5 min), resuspended in MLEC PAI/L selection medium and counted. 1.6 x 10^4 cells were seeded per 6mm well of a 96-well plate and allowed to attach for 3 hours. Duplicates of either recombinant human TGFβ (0–350pg/ml; Peprotech, London, UK), or cell culture supernatant (acid activated followed by incubation for 16–18 hours in the presence or absence of MMP1 enzyme) were diluted in serum-free MLEC PAI/L selection medium containing 0.1% BSA (Sigma-Aldrich). The cells were washed twice with 1xPBS and each of the dilutions (100µl) added to the cells and incubated for 14–16 hours prior to the Luciferase Assay. Cells were washed once with 1x PBS before the addition of 50µl 1x Passive Lysis Buffer (Promega) and subsequent incubation for 15 min at room temperature on a platform rocker. The cells were then placed at -80°C for 30 min prior to thawing at room temperature for 10 min. The freeze/thaw procedure was repeated twice more, followed by titration before transferring 25µl of the cell lysate to assembly strips of white microtiter plates (Thermo

Electron Corporation). The luminescence was read using the Revelation MLX 4.06 software of the MLX Microtiter® Plate Luminometer following the addition of 50µl of the Luciferase Assay Reagent.

Detection of IGF1 biological activity using CG4 cells

The CG4 cell line contains rat CNS glia precursors derived from bipotential oligodendrocyte-type 2 astrocyte progenitor cells. Using conditioned medium taken from the neuroblastoma cell line B104, CG4 cells can be maintained in culture indefinitely as oligodendrocyte-type 2 astrocyte cells. Withdrawal of the mitogens expressed by B104 cells in the conditioned medium, or from the additive bFGF and PDGF mitogens in normal growth medium, induces the differentiation of the CG4 cells into oligodendrocytes. However replacement of the mitogens with 20% FBS results in the differentiation of CG4 cells into type-2 astrocytes. Following differentiation, OL or type-2 astrocyte cells can be maintained in 5% FBS for several weeks (Louis et al., 1992).

Cultures of B104 neuroblastoma cells were maintained in B104 growth medium (DMEM supplemented with 10% FBS, pen/strep and glut). CG4 cells were maintained in CG4 growth medium (Ham's F12/DMEM [1:1] supplemented with B104 conditioned medium, N1 supplement (100x = Progesterone (0.73µg/ml), Putricine (1.6mg/ml), Sodium Selenite (0.5µg/ml), Insulin (0.5mg/ml) and Transferrin (0.5mg/ml), 10ng/ml biotin, 5ng/ml bFGF, 1ng/ml PDGF). All cells were maintained at 37°C in a 5% CO_2 humidified atmosphere in air.

Preparation of B104-conditioned medium for growth of CG4 cells

Preparation of B104 cells

B104 cell were grown in B104 growth medium until 70% confluency, washed twice with PBS-without Mg^{2+}/Ca^{2+} (6ml/75 cm flask). The cells were allowed to detach by incubation at room temperature in PBS (6ml) with gentle tapping. B104 growth medium (20ml) was added and the cells centrifuged (200g, 3 min). The supernatant was gently aspirated, the pellet resuspended in B104 growth medium (25ml/75 cm flask) before plating 1:5 and adding B104 growth medium to a final volume of 20ml.

Growth of B104 cell depends on the concentration of FCS and cell grown in 10% FCS have a doubling time of 27 hours, whereas cells grown in serum-free medium have a doubling time of 51.5 hours (Luo and Miller, 1997). Medium was replaced every 3 days with fresh medium and cell routinely passaged (1:5) once confluence reached 70%.

Preparation of N1 conditioned medium

Once the B104 cells reach confluency, the cells are washed twice in serum-free DMEM and incubated for 4 days in N1 medium (15ml/75 cm flask; 1x N1 supplement [5ml 100x, Sigma-Aldrich], 1x Pen/Strep [5ml 100x, Sigma-Aldrich], to final volume 500ml with DMEM). After 4 days, the medium was removed, centrifuged (250 x g, 5 min), sterile filtered (0.25µM) before storing at -80°C in aliquots of 15ml labelled N1-conditioned medium.

Preparation of CG4 cells

Cultures of the OPC line CG4 (kindly donated by Huseyin Mehmet, Imperial College London, UK) were seeded onto poly-L-ornithine coated dishes (10ml of 15µg/ml sterile poly-L-ornithine solution added to 100mm tissue culture dishes and incubated for 1—18 hours at 37°C, washed 3x with PBS; BD Falcon) and grown in CG4 growth medium until confluent. The medium was replaced initially after 3 days and thereafter at alternate days. Cells were harvested by trypsin for 1 min before inactivation with soy bean trypsin inhibitor (Sigma-Aldrich). The cell suspension was centrifuged (800 rpm, 5 min) and resuspended in pre-warmed medium before plating onto pre-coated polyornithine dishes.

CG4 biological response assay

CG4 proliferation was assessed using cultures established on pre-coated polyornithine 96-well plates. Once the cells were at confluence, they were detached by trypsin, and seeded at a density of $1x10^4$ cells in CG4 growth medium (50µl per well of a 96-well plate). Replicates of cell culture supernatant (following incubation for 16–18 hours in the presence or absence of MMP1 enzyme) were diluted in DMEM to a 2x final concentration, ranging between 3 two–fold dilutions (2, 4 and 8)

and 3 ten–fold dilutions (20, 200, and 2000). Controls included insulin (100nM); DMEM/F12 medium alone (1:1) and HEK-293T conditioned DMEM medium. Each of the dilutions (50µl) were added to the cells and incubated for 3 days prior to the Cell Titer-Glo® Assay and the luminescence read using the Revelation MLX 4.06 software of the MLX Microtiter® Plate Luminometer.

IN VIVO STUDIES

Induction of EAE

Biozzi AB/H mice (both male and female) were obtained from Harlan UK Ltd. (Oxon, UK) and maintained according to UK Home Office and institutional guidelines.

Preparation of antigen used for immunisation

Biozzi AB/H spinal cords (age/sex indifferent) were expelled from the lumbar region of the severed spinal column by hydraulic pressure using a 20ml syringe fitted with a 20G needle. The spinal cords were then homogenised using a glass hand homogeniser and freeze dried at -70°C overnight, before being diced into a fine powder and stored at -20°C. A stock solution of *Mycobacterium*-supplemented incomplete Freund's Adjuvant (M-IFA) was prepared by adding 16mg *Mycobacterium tuberculosis* H37Ra and 2mg *M.butyicum* (8:1) to 4ml incomplete Freund's Adjuvant (IFA) and stored at 4°C for no longer than 1 month. The complete Freund's adjuvant (CFA) was prepared by adding 1ml M-IFA to 11.5ml IFA and mixed well. Lyophilised spinal cord homogenate (33mg; SCH) was reconstituted in 5ml sterile PBS before being added to 5ml CFA and sonicated in a water bath sonicator (Bransonic Ultrasonic cleaner; Sigma-Aldrich) for 10 min at room temperature. The solution was emulsified using a 1ml syringe following retraction and replacement of the plunger to generate a suspension that was thick enough as not to disperse in water. Sterile 1ml syringes fitted with 16mm 25G needles were then loaded with the sonicated emulsion in preparation for inoculation. Female Biozzi AB/H mice 6–8 weeks of age were inoculated subcutaneously at two sites in the flank with a total volume of 0.3ml sonicated emulsion on days 0 and 7. Each animal received

1mg of SCH and 60µg *Mycobacterium* on each inoculation occasion. All animals were allowed to proceed into a spontaneous relapse once the acute phase had resolved.

The mice were fed on RM1(E) pellets and water *ad libitum*. When clinical signs were evident, these pellets were softened in warm water and placed inside the cage, and longer spouted water bottles used.

Clinical Scoring of EAE

The animals were weighed and scored daily from day 11 onwards. Clinical signs are evident after an initial overnight weight loss of >1.5g. A six point score system was followed: 0 = normal; 1 = limp tail; 2 = loss of inverted righting reflex; 3 = partial hind limb paralysis; 4 = complete hind limb paralysis; 5 = moribund or death, at which point the animal was sacrificed. Animals were also sacrificed if the weight loss exceeded 35% from the original day 11 weight, or if the animal remained at the graded score 4 for more than 4 days during the acute phase, or 10 days during the relapsing phase. All clinical signs that were of a lesser degree than the defined graded scores were scored 0.5 lower.

Blood sampling

All blood obtained was done so either by tail snipping or by terminal cardiac puncture. Animals were warmed for a minimum of 15 min under a heating lamp prior to tail sniping. 1mm off the tip of the tail was taken using a clean, sterile scalpel whilst the animals were held in mouse restraints (VetTech, UK). The tail was then gently 'milked' and the blood collected using a heparinised micro haematocrit capillary tubule (d = 1.15mm, l = 75.00mm, GMBH & Co. KG, Wertheim, Germany). One end was sealed with a naked flame to prevent sample loss. Terminal cardiac puncture was performed at the end of the experiment. Animals were euthanized by CO_2 overdose. The sternum was exposed using straight surgical scissors. Following puncture of the heart using scissors, the blood was collected via sterile disposable pipettes into microfuge tubes and heparinised micro haematocrit capillary tubules (GMBH & Co. KG, Wertheim, Germany)

Haematocrit measurements

Samples were centrifuged at 14 000 x g at room temperature for 10 min using a micro haematocrit centrifuge (HaematoSpin 1400, 24 place rotor 01971-00; Hawkley, Sussex, UK). Using a metric ruler, the ratio between total blood volume and haematocrit volume was determined.

STATISTICAL ANALYSES

Statistical analyses were performed using the GraphPad Prism software (version 5.0; GraphPad Software, California, U.S.A.).

Values are presented as mean ± standard error of the mean (SEM). The value of 'n' refers to animals/group. The differences between groups were determined by ANOVA, followed by Tukey post-hoc test for multiple comparisons. Scores and weights are presented as a daily mean ± SEM per treatment group and all differences between groups were assessed using the non-parametric test, Mann-Whitney, followed by Tukey post-hoc test for multiple comparisons. P values ≤ 0.05% were considered statistically significant.

CHAPTER 3

CONSTRUCTION AND *IN VITRO* CHARACTERISATION OF LATENT GROWTH FACTORS

INTRODUCTION

TGFβ is a multifunctional growth factor expressed ubiquitously in normal tissue and cell lines. Gene regulation of TGFβ is, as with other growth factors, controlled at the level of gene transcription and posttranslational processes that tightly control its expression. One of these processes includes the rendering of the growth factor latent by its association with its LAP preventing interaction with cellular receptors. Growth factors have demonstrated great potential in treating various diseases but have pleiotropic effects, short half-lives, and are administered systemically and often in high doses in order to obtain a therapeutic result. The prospect of rendering a therapeutic growth factor latent whilst targeting it to specific target areas is extremely appealing as the delivery of a therapeutic molecule to a target tissue without substantial toxicity is the cornerstone to successful therapy (Kay et al., 2001).

Adams, Vessillier et al. (2003) first described a latent growth factor that functions at a specific site. They demonstrated that the growth factor IFN-β could be rendered latent by the addition of LAP to its N terminal end. They also determined that by rendering IFN-β latent, they were able to extend its half-life 37 times than that of IFN-β alone (55.02 hrs vs. 1.46 hrs respectively). This was even longer than when IFNβ had undergone pegylation (10.82 hrs). The addition of a MMP cleavage site (Vessillier et al., 2004) meant that the latent protein could not interact with its cellular receptor/s unless in areas where inflammation or tissue remodelling processes involving MMPs was taking place. MMPs are found within diseases such as rheumatoid arthritis, atherosclerosis and MS and their expression is tightly regulated.

CHAPTER HYPOTHESIS AND AIMS

If the growth factors mEPO, mIGF1A, mIGF1B and huTGFβ were to be linked to the LAP peptide linked with a MMP cleavage site, then we hypothesised that they too, should become latent and only once incubated with MMP enzymes, should the peptide become biologically active and functional.

The aims of this chapter were to determine first, these peptides could be constructed *in vitro*; second, they were able to be secreted and expressed by a mammalian cell ine; and third, that they demonstrated latent biological activity until cleavage occurred using the MMP enzyme, at which point the peptide became biologically active.

RESULTS

Construction of latent growth factors

The latent growth factor gene constructs (Figure 3.1) were engineered by fusing the LAP of human TGFβ at the N-terminus followed by an MMP site linked to a growth factor, either mouse EPO (mEPO), human TGFβ (huTGFβ), mouse IGF1A or mouse IGF1B (mIGF1A or mIGF1B) as described previously (Adams et al., 2003). The control plasmid vector LAP-MMP was used previously by our group (Adams et al., 2003) and the LAP-MMP-mEPO plasmid vector was previously produced by our group (unpublished work).

Growth factors are interchangeable in a generic expression vector

Constructs were produced by sequential cloning of different growth factor cDNA into the pcDNA3 (LAP-MMP) plasmid as illustrated in Figure 3.1. The pcDNA3 (LAP-MMP-mEPO) had previously been cloned by Prof. Yuti Chernajovsky and Mrs Gillian Adams (unpublished work). The cDNA from mouse

thymus, brain and spleen was kindly provided by Dr Alexander Annenkov. Primers were designed to enable in-frame ligation of PCR amplified DNA fragments, and to incorporate specific restriction sites at either the 5' or 3' ends of the product, into the pcDNA3 plasmid or pHRSIN-CSGW viral vector (Chapter 2: Table 2.1). Additionally, the primers were designed to amplify only the mature peptide sequence so that the biologically active mature peptide was secreted through the signal domain of the LAP to which they are fused. The sequence of the peptide linker $(Gly_4Ser)_3$ has been previously described (Annenkov and Chernajovsky, 2000) and was produced by oligonucleotide annealing (Chapter 2: Table 2.1).

Following bacterial transformation and plasmid vector purification, all plasmid DNA was diagnostically digested with restriction enzymes to confirm sequence insertion and orientation before proceeding with further subcloning or recombinant protein expression (Figure 3.2). A schematic representation of the recombinant proteins and their respective sizes–as calculated from their amino acid sequences–is shown in Figure 3.3. DNA sequence analysis confirmed DNA sequence and the annotated sequences can be found in the Appendix.

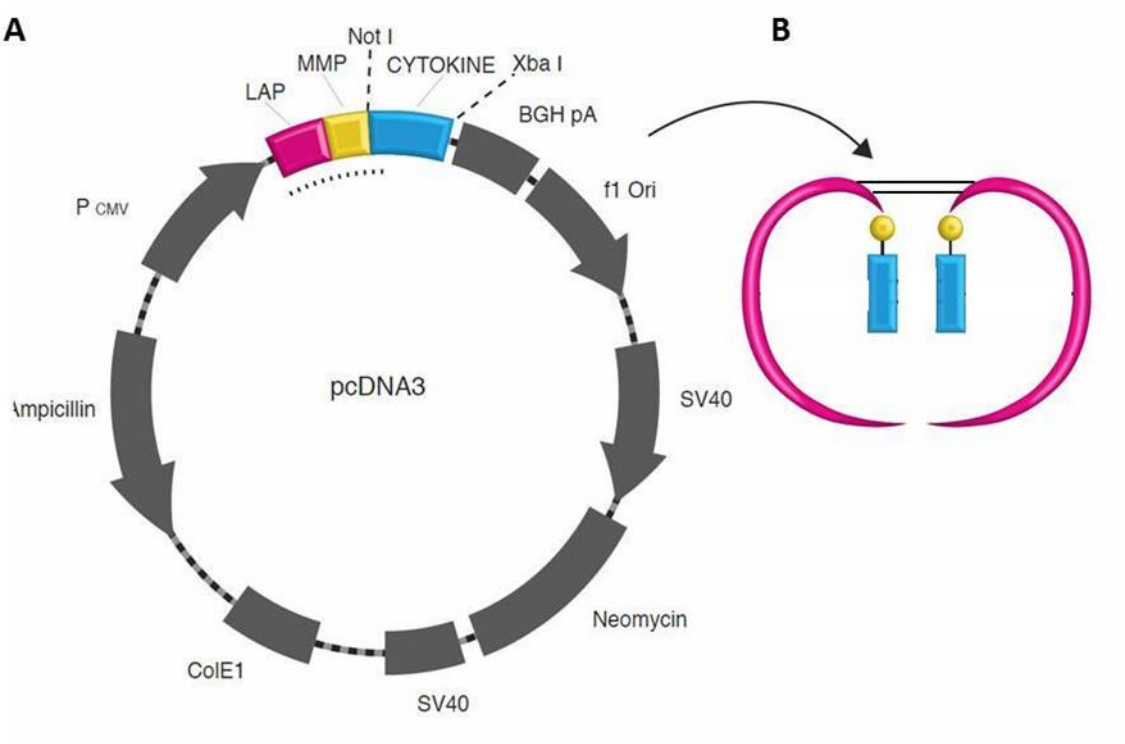


Figure 3.1: A generic gene expression vector map for the construction of a latent growth factor.
(A) A generic template containing the LAP fused to a growth factor linked to a MMP site. The growth factor is replaced with another protein of interest/growth factor between the *NotI* and *XbaI* restriction sites. The pcDNA3 plasmid contains ampicillin for selection in *E.coli* and neomycin resistance under the SV40 promoter for selection in mammalian cells. The expression vector also includes a cytomegalovirus (CMV) promoter for high efficiency expression of the recombinant protein in mammalian cells. **(B)** Expression of the gene expression vector should result in the secretion of a latent recombinant fusion protein that is biologically active when the mature growth factor is released at the MMP site by an MMP enzyme.

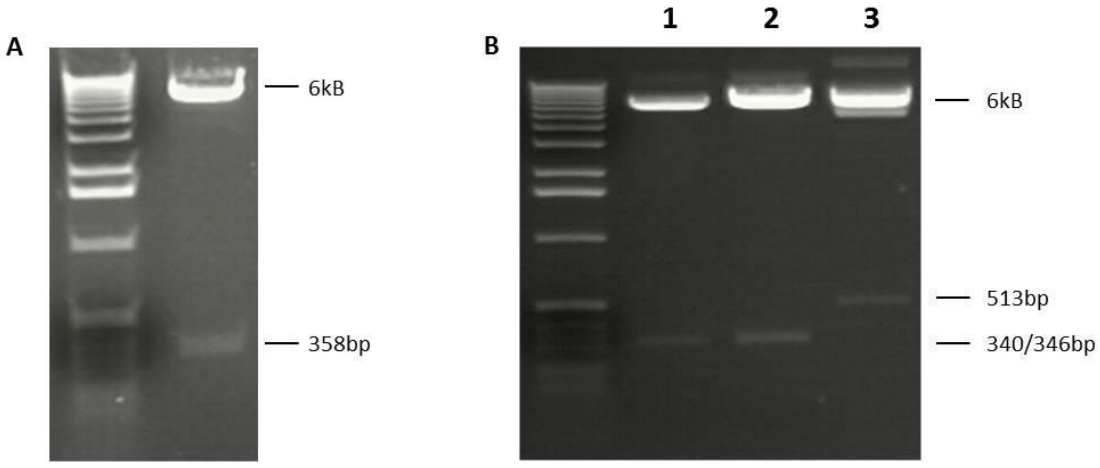


Figure 3.2: Restriction analysis of the latent growth factor constructs.
The PCR amplified growth factors huTGFβ, mIGF1A, and mIGF1B with *NotI* and *XbaI* ends were cloned into the pcDNA3 plasmid vector cut with *NotI* and *XbaI*. Clones with insert were screened by diagnostic digestion using *NotI* and *XbaI* enzymes. **(A)** A 358bp huTGFβ fragment is released from pcDNA3(LAP-MMP-huTGFβ); **(B)** 340bp mIGF1A (lane 1), 346bp mIGF1B (lane 2) and 513bp mEPO (lane 3) fragments are released from pcDNA3(LAP-MMP-mIGF1A), pcDNA3(LAP-MMP-mIGF1B) and pcDNA3(LAP-MMP-mEPO) plasmids respectively.

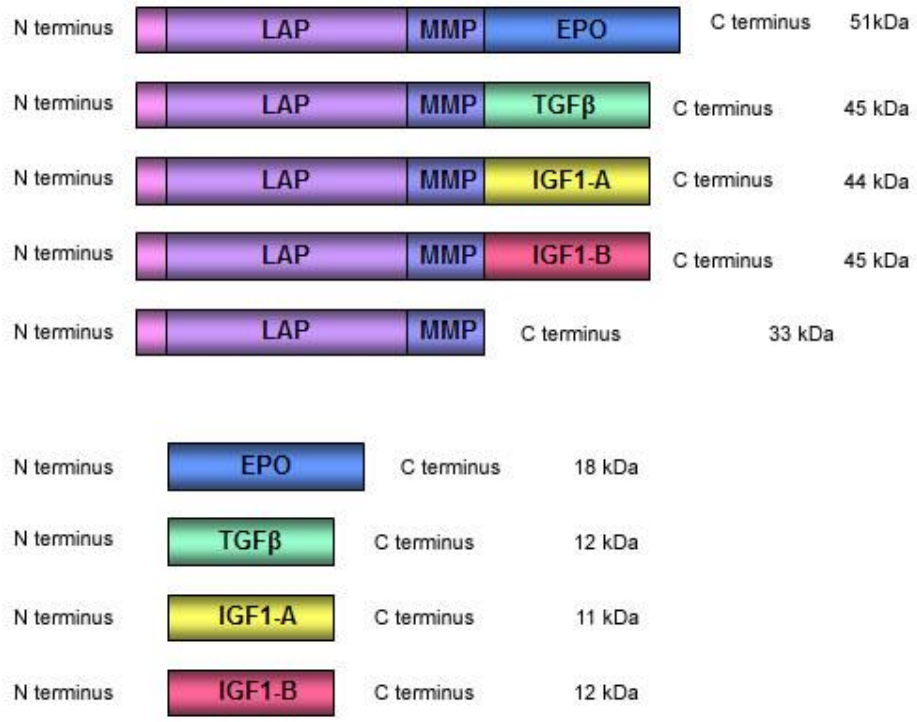


Figure 3.3: A schematic representation of recombinant latent growth factors and mature growth factors.
(A) Linkage of the growth factors mEPO, huTGFβ, mIGF1A and mIGF1B by a MMP cleavage site with the LAP of huTGFβ renders then latent. **(B)** Cleavage at this site releases the mature peptide from the latent 'shell' allowing it to interact with its corresponding receptor. The molecular weights as estimated from its amino acid sequence are shown on the right of each growth factor.

Expression of latent growth factors

LAP genetically fused to the sequences of the mature mEPO, huTGFβ, mIGF1A or mIGF1B cDNA via an MMP cleavage site, produced four distinct latent growth factor proteins. The control protein LAP-MMP has been used by our group previously (Adams et al., 2003). The latent growth factor constructs were produced by sequential cloning of LAP-MMP and the pertinent growth factor fragment, excised by restriction enzyme digestion, into the mammalian expression plasmid vector pcDNA3 and the correct orientation verified by DNA sequencing.

Latent recombinant proteins are secreted from CHO and HEK-293T cells

Following calcium phosphate transfection of the constructs into the CHO DG44 and HEK-293T cell lines, western blot analysis was used to determine if the constructs LAP-MMP-mEPO, LAP-MMP-huTGFβ, LAP-MMP-mIGF1A, LAP-MMP-mIGF1B or LAP-MMP are expressed and secreted as latent

recombinant proteins. A combination of polyclonal LAP antibody and the appropriate primary growth factor antibody confirmed the expression and the secretion of the latent growth factors. The predicted molecular weight of the LAP-MMP control recombinant protein, according to the amino acid sequence, is 32 kDa but due to glycosylation at the amino acid residues $Asn^{82, 136, \text{and } 176}$ (Brunner et al., 1992), the detected band is ~ 37-40 kDa when probed with LAP antibody (Figure 3.4). LAP-MMP-huTGFβ was detected around 45 kDa as calculated by its amino acid sequence when probed with anti-TGFβ antibody (Figure 3.5C). The cleaved growth factor mEPO is documented to be highly glycosylated with 3 N-glycosylation sites at $Asn^{24, 38, \text{and } 83}$ and an O-glycosylation site at Ser^{126} (Dube et al., 1988) and although LAP-MMP-mEPO is calculated to be 51 kDa based on its amino acid sequence, a band is detected at ~70-75 kDa when probed with anti-EPO antibody (Figure 3.5D). The molecular weights of the LAP-MMP-mIGF1A and LAP-MMP-mIGF1B recombinant proteins were calculated to be 44 kDa and 45 kDa respectively, but were detected at ~50 kDa when probed with the anti-IGF1 antibody (Figures 3.5 A and B).

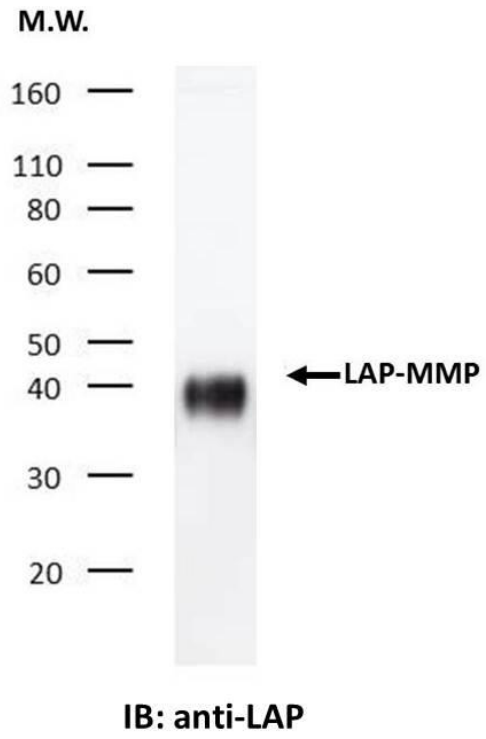


Figure 3.4: The CHO cell line DG44 secretes the control recombinant protein LAP-MMP.
DG44 CHO cells were stably transfected with the pcDNA3(LAP-MMP) plasmid vector and the culture supernatants collected after 48 hours, and analyzed by western blotting with antibodies against anti-LAP. The molecular weight is higher than expected (an observed ~37-40 kDa vs. the expected 32 kDa as determined by the amino acid sequence) possibly due to post-translation modification, e.g glycosylation. Abbreviation: IB=immunoblot, M.W.=molecular weight.

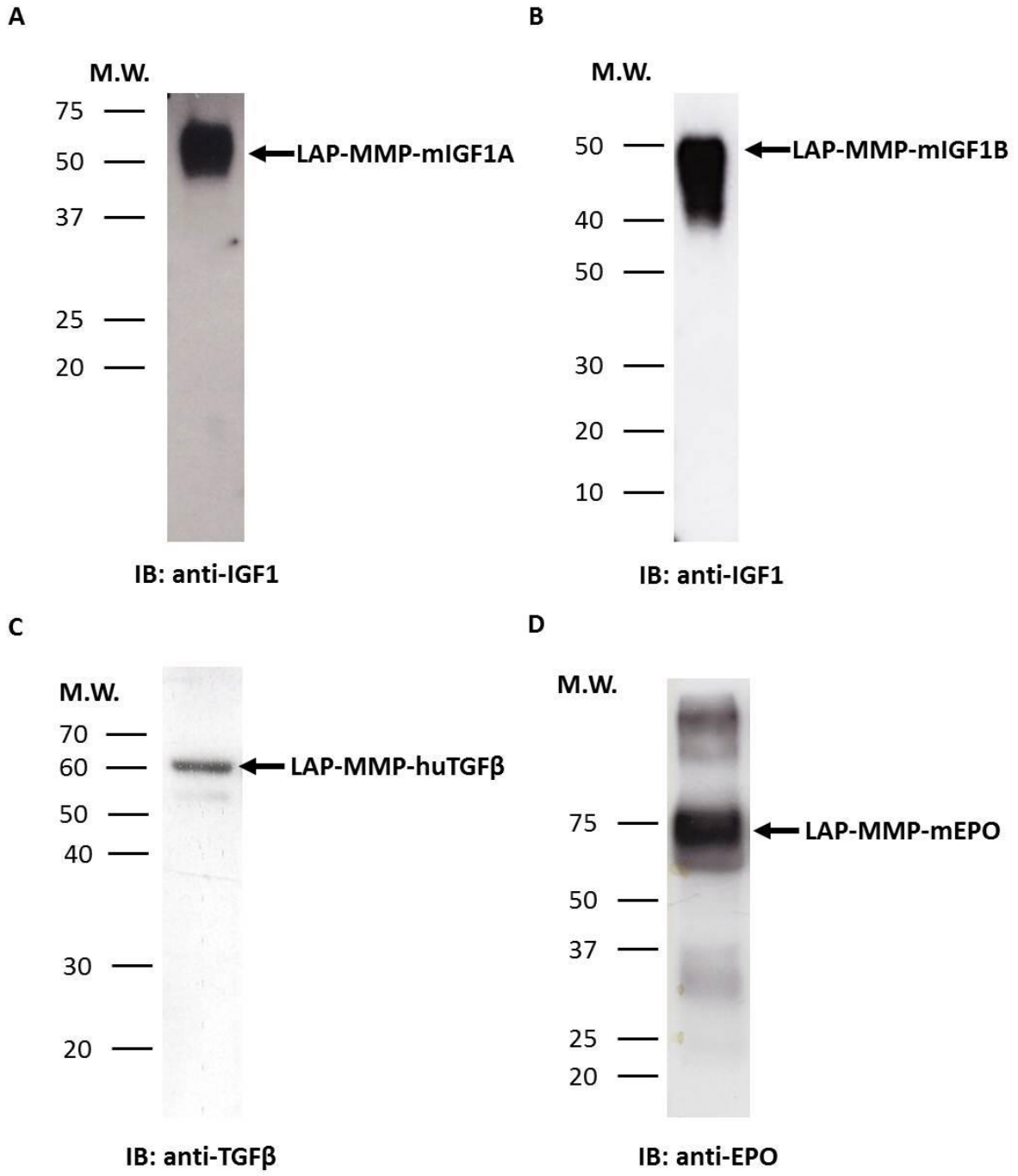


Figure 3.5: Latent recombinant proteins are secretable when expressed by CHO DG44 cells.
CHO DG44 cells were stably transfected with **(A)** pcDNA3(LAP-MMP-mIGF1A), **(B)** pcDNA3(LAP-MMP-mIGF1B), **(C)** pcDNA3(LAP-MMP-huTGFβ) and **(D)** pcDNA3(LAP-MMP-mEPO) plasmid vectors and the culture supernatants collected after 48 hours, and analyzed by western blotting with anti-IGF1 **(A and B)**, anti-TGFβ **(C)** and anti-EPO **(D)** antibodies. All bands ran higher than expected, possibly due to post-translation modifications such as glycosylation. Abbreviation: M.W.=molecular weight, IB = immunoblot.

MMP cleavage of latent growth factors

To assess whether the latent recombinant protein releases the cleaved growth factor when incubated with recombinant MMP1, culture supernatants were incubated with MMP1 overnight and analysed by western blot using the appropriate growth factor antibody and/or the LAP antibody.

Analysis of cleavage by MMP1 of recobimant proteins secreted by DG44 CHO cells.

The cleaved mIGF1A and mIGF1B molecules, from LAP-MMP-mIGF1A and LAP-MMP-mIGF1B respectively, were detected at ~12 kDa as predicted from their amino acid sequence (Figure 3.6A and B, Figure 3.6 C and D respectively). Unexpectedly, two bands smaller than 10kD were also observed. The LAP-MMP molecule, when probed with LAP antibody, was detected at approximately 37 kDa following cleavage by MMP1 (Figure 3.7B). Cleavage of LAP-MMP-huTGFβ by MMP1 releases huTGFβ with the resulting LAP peptide seen at approximately 37 kDa (Figure 3.7A). The recombinant protein LAP-MMP-mEPO, when secreted by DG44 CHO cells, was also incubated overnight in 30nM MMP1 and the supernatant run on SDS-PAGE and analysed by western blot. However, no release of mEPO was observed when probed with the anti-LAP antibody (Figure 3.8). This was unexpected as the four other ecombinant proteins (LAP-MMP, LAP-MMP-huTGFβ, LAP-MMP-mIGF1A and LAP-MMP-mIGF1B) all demonstrated cleavage and growth factor release when incubated with MMP1.

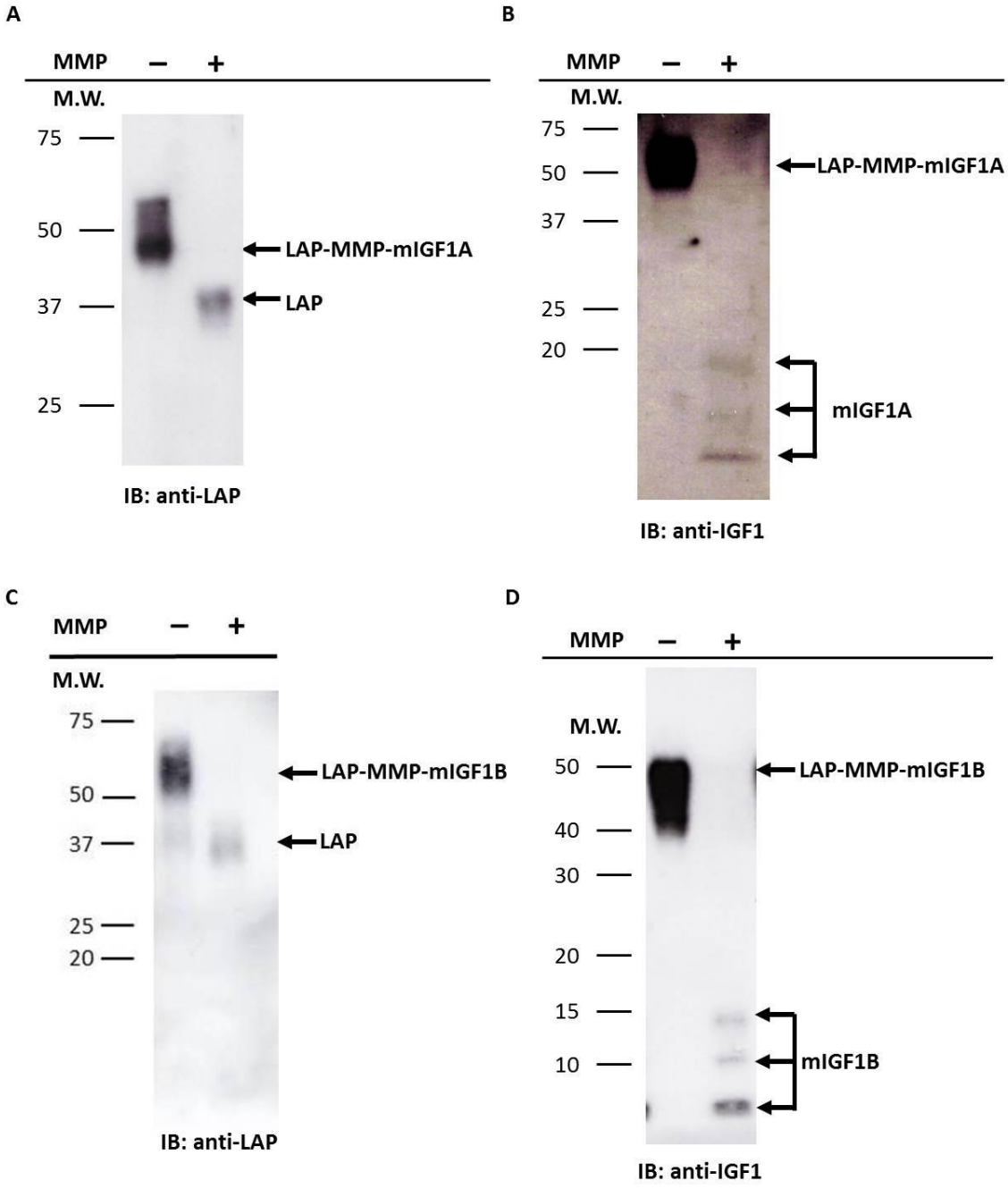


Figure 3.6: Latent recombinant proteins LAP-MMP-mIGF1A and LAP-MMP-mIGF1B are cleavable by MMP1 when expressed by DG44 CHO cells.

DG44 CHO cells were stably transfected with pcDNA3(LAP-MMP-mIGF1A) **(panels A and B)** and pcDNA3(LAP-MMP-mIGF1B) **(panels C and D)** plasmid vectors and the culture supernatants collected after 48 hours, incubated with and without MMP1 for 16–18 hours, and analyzed by western blotting with antibodies against both anti-LAP and anti-IGF1 antibodies. Abbreviation: M.W.=molecular weight, IB = immunoblot. **–** indicates absence of MMP1 during incubation overnight; **+** indicates presence of MMP1 enzyme during incubation overnight.

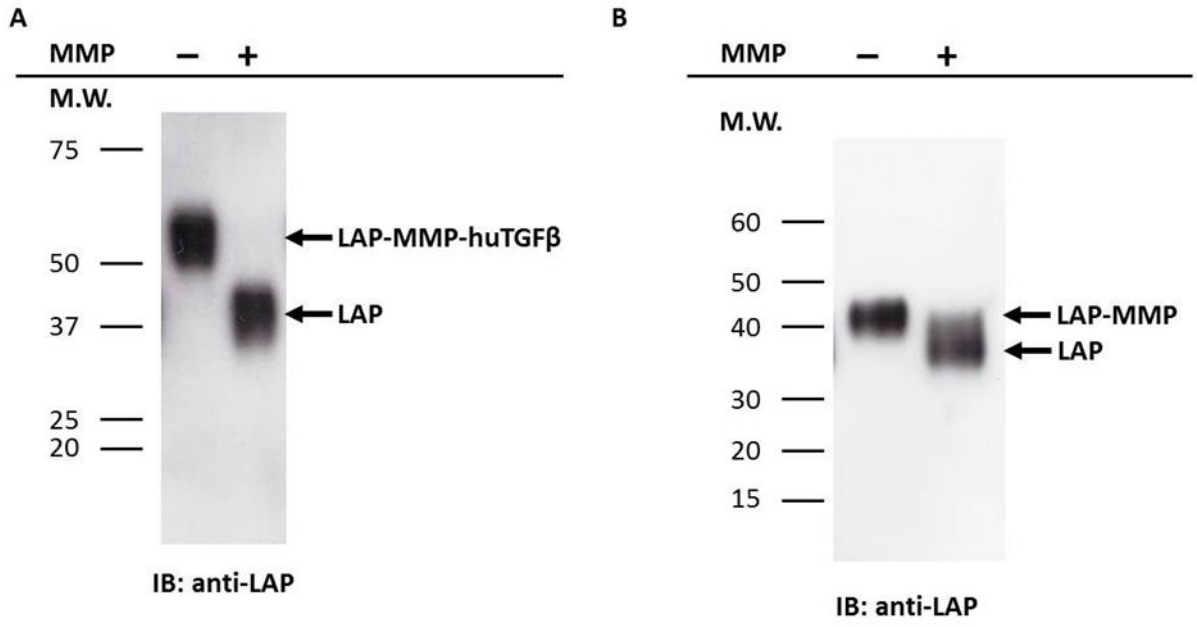


Figure 3.7: The latent recombinant proteins LAP-MMP and LAP-MMP-huTGFβ cleaved by MMP1 when expressed by DG44 CHO cells.
DG44 CHO cells were stably transfected with pcDNA3(LAP-MMP-huTGFβ) (A) and pcDNA3(LAP-MMP) (B) plasmid vectors and the culture supernatants collected after 48 hours, incubated with and without MMP1 for 16–18 hours, and analyzed by western blotting with antibodies against anti-LAP. Abbreviation: M.W.=molecular weight, IB = immunoblot. **–** indicates absence of MMP1 during incubation overnight; **+** indicates presence of MMP1 enzyme during incubation overnight.

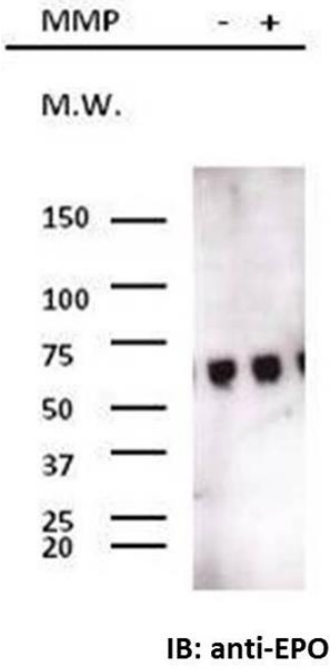


Figure 3.8: MMP1 fails to cleave the recombinant protein LAP-MMP-mEPO secreted by DG44 CHO cells.
DG44 CHO cells were stably transfected with the pcDNA3(LAP-MMP-mEPO) plasmid vector and the culture supernatant was collected after 48 hours, incubated with and without 30nM MMP1 for 16–18 hours, and analysed by western blotting with antibodies against anti-EPO. Abbreviation: M.W.=molecular weight, IB = immunoblot. **–** indicates absence of MMP1 during incubation overnight; **+** indicates presence of MMP1 enzyme during incubation overnight.

Analysis of cleavage by MMP1 of recobimant proteins secreted by HEK-293T cells.

Cleavage of the latent recombinant proteins secreted the HEK-293T cell line was observed as with DG44 CHO cells. Interestingly, the LAP-MMP-mEPO recombinant protein was able to be cleaved by MMP1, and when analysed by western blot and probed with anti-EPO antibody, the growth factor mEPO (initially predicted to be 18 kDa as calculated from the amino acid sequence) was detected at a higher molecular weight of approximately 70–75 kDa (Figure 3,9). As previously discussed, mEPO has several glycosylation sites therefore it is not surprising that, when LAP-MMP-mEPO was incubated with MMP1, the size of the cleaved mEPO protein was bigger (~34 kDa) (Figure 3.9).

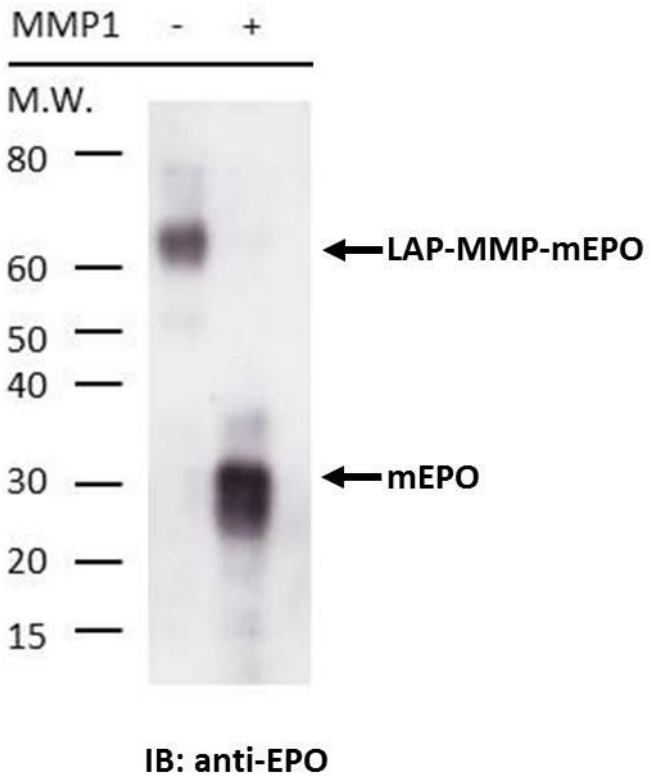


Figure 3.9. When secreted by HEK-293T cells, MMP1 is able to cleave the recombinant protein LAP-MMP-mEPO.
HEK-293T cells were stably transfected with the pcDNA3(LAP-MMP-mEPO) plasmid vector and the culture supernatant was collected after 48 hours, incubated with and without MMP1 for 16–18 hours, and analyzed by western blotting with anti-EPO antibody. Abbreviation: M.W.=molecular weight, IB = immunoblot. **–** indicates absence of MMP1 during incubation overnight; **+** indicates presence of MMP1 enzyme during incubation overnight.

In order to determine if the failure of MMP1 to release the cleaved mEPO protein, when secreted from the DG44 CHO cell line, was due to protein production from a single CHO clone population, the CHO suspension cell line CHO-S, a sub-clone of the parental CHO cell line that has been adapted to grow in suspension under serum-free conditions, was transiently transfected with LAP-MMP-mEPO, the supernatant collected and incubated overnight in the presence of MMP1. Although the cell line expressed and secreted LAP-MMP-mEPO as determined by western blot analysis, it too failed to release the cleaved mEPO protein (Figure 3.10, lanes 3 and 4) as compared to LAP-MMP-mEPO secreted from HEK-293T cells and incubated with MMP1 (Figure 3.10, lanes 1 and 2).

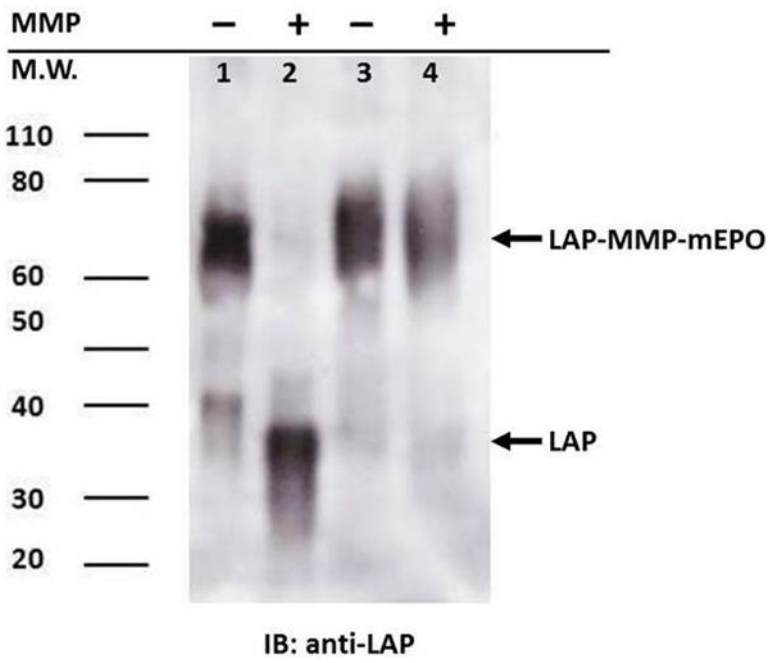


Figure 3.10: The recombinant protein LAP-MMP-mEPO is cleaved by MMP1 when secreted by HEK-293T cells but not from CHO-S cells.
HEK-293T and CHO-S cells were transiently transfected with LAP-MMP-mEPO and the culture supernatant was collected after 48 hours, incubated in the presence of MMP1 enzyme overnight at 37°C, assessed by western blot analysis and probed with anti-LAP antibody. The supernatant from HEK-293T cells (lanes 1 and 2) demonstrates cleavage whereas supernatant taken from CHO-S cells (lanes 3 and 4) does not. Some degradation of the protein is observed. Arrow indicates the released LAP peptide. Abbreviation: M.W.=molecular weight, IB = immunoblot. **–** indicates absence of MMP1 during incubation overnight; **+** indicates presence of MMP1 enzyme during incubation overnight.

It is possible that this lack of cleavage was due to different glycosylation patterns between the human HEK-293T cell line and the hamster CHO cell line. Incubation of CHO-S cells expressing LAP-MMP-mEPO with the antibiotic Tunicamycin should result in the inhibition of N-glycosylation. Tunicamycin interrupts the synthesis of glycoproteins by blocking uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) and Dol-P, enzymes used by glycosyltransferases to transfer N-acetylglucosamine to specific sites on a protein molecule. Tunicamycin was added to culture medium of CHO-S cells producing LAP-MMP-mEPO at concentrations 5µg/ml and the cells were incubated for 48 hours. However, no cleavage of the protein was observed when incubated with MMP1 enzyme (data not shown). Importantly, LAP-MMP-mEPO is cleaved by MMP1 when expressed in mouse Biozzi/ABH fibroblasts that were used for therapeutic purposes (see below Figure 4.3)

Cleavage of latent mEPO is dependent on the MMP site

To investigate the latency of the latent mEPO, a glycine-serine flexible linker bridge genetically replaced the MMP cleavage site. The glycine-serine linker is a cryptic site that is not sensitive to MMP1 cleavage and should not result in cleavage when in the presence of the enzyme, thus conferring permanent latency to the recombinant protein. Conditioned medium from HEK-293T cells transfected with either the construct LAP-$(Gly_4Ser)_3$-mEPO or LAP-MMP-mEPO, was incubated for 6 hours with increasing dilutions of MMP1, and assessed by western blot analysis for cleavage of the LAP as probed using the LAP antibody. Diluting the MMP1 enzyme will rule out the possibility that an excess of enzyme, resulting in non-specific opportunistic cleavage, releases any cleaved mEPO growth factor. MMP incubation resulted in the cleaved LAP molecule from LAP-MMP-mEPO with an expected size of 37 kDa. A faint band corresponding to 37 kDa was seen for LAP-$(Gly_4Ser)_3$-mEPO (Figure 3.11).

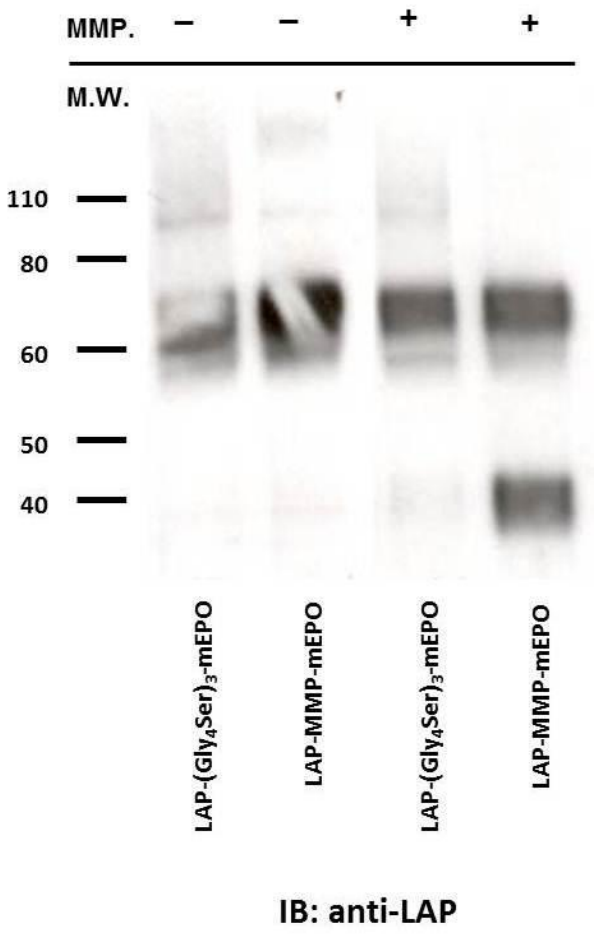


Figure 3.11 Cleavage of mEPO by MMP1 from the recombinant protein is dependent on the MMP site.
HEK-293T cells were transiently transfected either with pcDNA3(LAP-MMP-mEPO) or pcDNA3(LAP-$(Gly_4Ser)_3$-mEPO0. 24 hours after transfection, the conditioned supernatant was collected and incubated for 6 hours with 75pM MMP1, before analyses by western blot and probing with anti-LAP antibody. Abbreviation: M.W.=molecular weight, IB = immunoblot. **–** indicates absence of MMP1 during incubation overnight; **+** indicates presence of MMP1 enzyme during incubation overnight.

Biological activity of the latent growth factors

LAP-MMP-huTGFβ

Using MLEC cells the biological activity of latent huTGFβ, before and after MMP1 cleavage, was tested. huTGFβ can stimulate the expression of plasminogen activator inhibitor-1 (PAI-1) in MLEC cells (Abe et al., 1994). Stimulation of MLEC cells stably transfected with a construct consisting of a truncated PAI-1 promoter fused to the Firefly luciferase reporter gene (MLEC (PAI/L)), with huTGFβ resulted in a dose-dependent increase in luciferase activity.

TGFβ is secreted as a latent protein comprising the disulphide linked homodimer of mature TGFβ that is associated via non-covalent bonds to LAP and LTBP, associations that are required for localisation and binding to the extracellular matrix (Miyazono et al., 1988, Wakefield et al., 1988, Lyons and Moses, 1990). Several activation mechanisms release the biologically active TGFβ from the SLTC and the LLTC including the dissociation of the LTBP from LAP via acid activation, and the proteolytic cleavage of LAP via proteases such as plasmin (Lyons et al., 1988, Lyons et al., 1990).

Conditioned medium collected from HEK-293T cells transiently transfected with pcDNA3(LAP-MMP-huTGFβ) was acid treated following incubation with MMP1 enzyme, releasing the mature homodimer huTGFβ required for biological assessment. The experimental conditions tested were cleaved and uncleaved LAP-MMP-huTGFβ, with or without acid activation (Figure 3.12). Without acid activation, the cleaved huTGFβ protein elicited a luciferase output that was significantly higher than that of uncleaved LAP-MMP-huTGFβ ($p < 0.0001$). Interestingly, uncleaved LAP-MMP-huTGFβ still exhibited a small degree of luciferase activity. When activated by acid, the luciferase output of cleaved huTGFβ was significantly enhanced ($p < 0.0001$) in comparison to the uncleaved LAP-MMP-huTGFβ. Additionally, the luciferase output of acid-activated uncleaved LAP-MMP-huTGFβ was of a similar intensity as that of the cleaved huTGFβ protein that did not undergo acid activation (Figure 3.12), possibly due to endogenous MMPs found in the medium of the transfected or the MLEC cells.

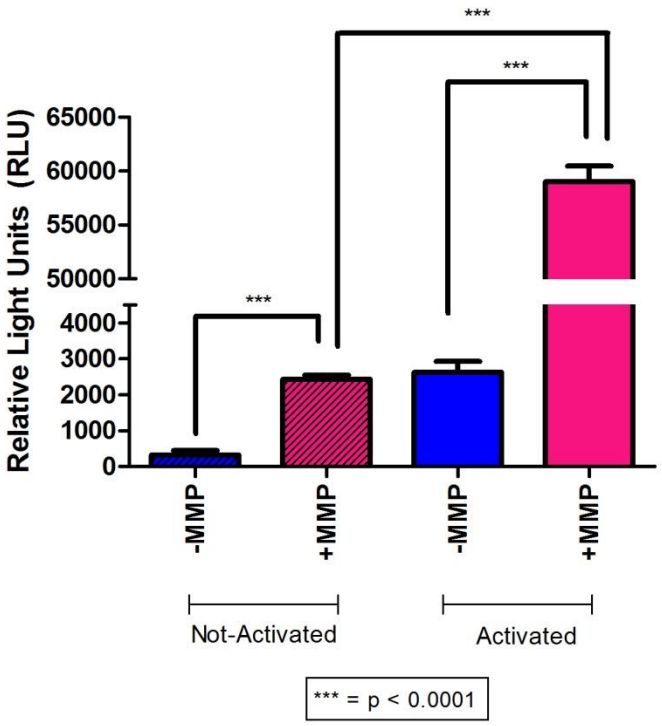


Figure 3.12: Latent huTGFβ remains biologically inactive until cleaved with MMP1 enzyme.
MLEC (PAI/L) cells were transiently transfected with pcDNA3(LAP-MMP-huTGFβ) and the supernatant collected after 48 hours. Following incubation of the supernatant with or without MMP1, the sample were either activated or not by acid. The luciferase output was read, and translated into relative light units (RLU). A significant increase in RLU was detected following MMP1 cleavage between uncleaved LAP-MMP-huTGFβ and cleaved huTGFβ, as well as between the samples with and without acid activation. Statistical analysis was performed using a one-way ANOVA followed by Tukeys post-hoc test for multiple comparrisons.

LAP-MMP-mIGF1A

To test the biological activity of the LAP-MMP-mIGF1A and cleaved mIGF1A proteins, I used the OPC line CG4. CG4 cells proliferate in response to growth factors and mitogens secreted from the neuronal cell line B104. Withdrawal of this conditioned medium and replacement with a serum-free medium supplemented with IGF1 results in cell proliferation and survival, rather than differentiation. Supernatant taken from HEK-293T cells transfected with pcDNA3(LAP-MMP-mIGF1A) was incubated with MMP1 and the biological activity of cleaved mIGF1A assessed. When incubated with cleaved mIGF1A protein, a significant number of cells survived as compared to those incubated with the LAP-MMP-mIGF1A ($p < 0.001$) (Figure 3.13).

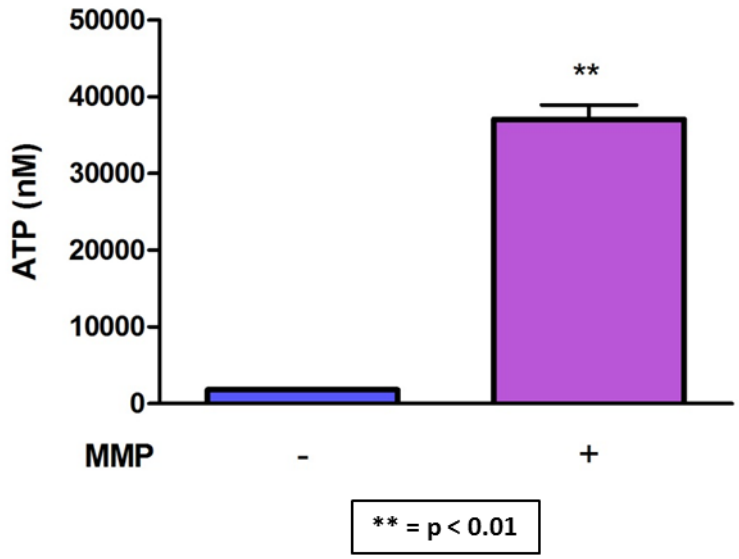


Figure 3.13. Incubation of CG4 cells with LAP-MMP-mIGF1A after MMP1 cleavage significantly increases cell proliferation.
HEK-293T cells were transiently transfected with pcDNA3(LAP-MMP-mIGF1A). After 48 hours, the supernatant was collected and incubated with 30 nM MMP1 overnight prior to assaying with Cell-Titer Glo (Promega). Statistical significance was determined by paired t-test.

LAP-MMP-mEPO

To test the biological activity of the latent mEPO protein before and after MMP1 cleavage, the human megakaryoblastic leukaemia cell line UT-7 was used. UT-7 cells are able to proliferate in the presence of IL-3, GM-CSF or EPO (Komatsu et al., 1991). HEK-293T cells were transiently transfected with pcDNA3(LAP-MMP-mEPO), and after 48 hours the supernatant was collected and incubated overnight with 30nM MMP1. The supernatant was added to the UT-7 cells and then assayed using the Cell Titer Glo assay (Promega). The LAP-MMP-mEPO (without MMP1) demonstrated a small amount of UT-7 proliferation but when cleaved with MMP1, proliferation doubled ($p < 0.0001$) (Figure 3.14A).

To determine if the increase in cell number observed in Figure 3.14A for LAP-MMP-mEPO was due to endogenous MMP activity found either within the HEK-293T conditioned medium or from the UT-7 cells themselves, the UT-7 cells were incubated with conditioned medium from HEK-293T cells transfected with pcDNA3(LAP-$(Gly_4Ser)_3$-mEPO) with or without MMP1 incubation, and with supernatant collected from HEK-293T cells transfected with pcDNA3(LAP-MMP-mEPO). Similar levels

of stimulation of UT7 cells was observed when the UT7 cells were exposed to supernatant collected from HEK-293T cells transfected with pcDNA3(LAP-$(Gly_4Ser)_3$-mEPO) (with and without MMP1 incubation) and supernatant collected from HEK-293T cells transfected with pcDNA3(LAP-MMP-mEPO) but without MMP1 incubation. Background levels from UT-7 cells incubated with MMP enzyme only (no recombinant protein) were negligible and subtracted from the values of the assay. These results indicate that the inferred 'background' level of cell proliferation seen when exposed to the latent recombinant protein, LAP-MMP-mEPO, is unlikely to be due to release of cleaved EPO at the MMP site (Figure 3.14B).

To test whether this background level of activity could be abolished, a broad-spectrum MMP inhibitor TAPI-2 was used. Incubation of the latent mEPO protein with TAPI-2 resulted in a significant decrease in background activity ($p < 0.05$) but failed to completely eliminate it, possibly indicating an additional pathway for protein cleavage that results in the exposure of the mEPO protein to its receptor on the UT-7 cell. Unsurprisingly, a significant reduction in cell proliferation ($p < 0.0001$) occurred when the cleaved mEPO protein was incubated with TAPI-2 (Figure 3.14B).

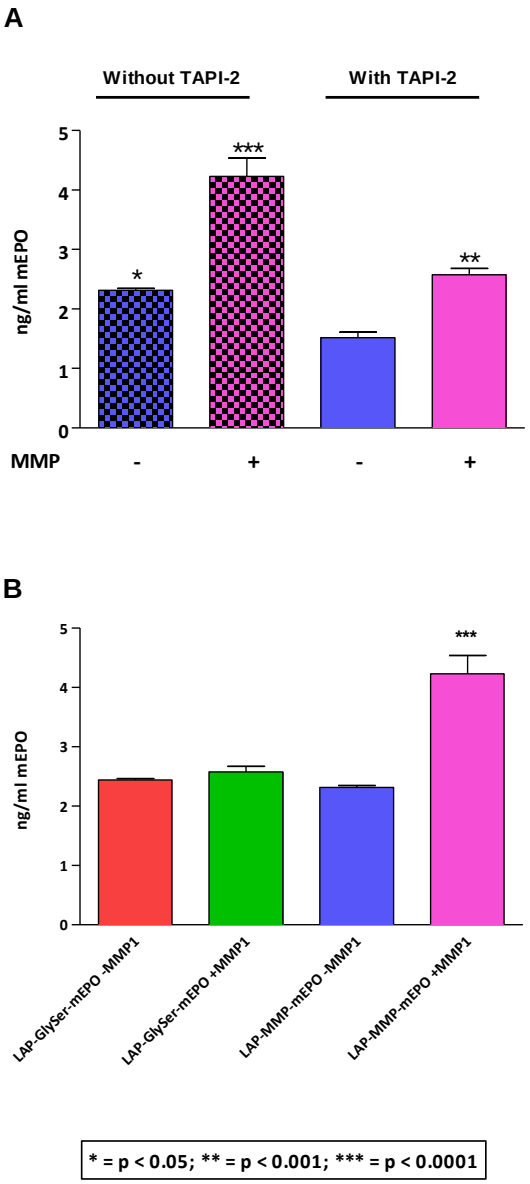


Figure 3.14: Incubation of UT-7 cells with broad-spectrum MMP inhibitor significantly reduces endogenous MMP activity on latent EPO.

(A) Supernatant taken from HEK-293T cells transiently transfected with pcDNA3(LAP-MMP-mEPO) were incubated with and without MMP1, and with or without the broad spectrum MMP inhibitor TAPI-2 prior to proliferation assay. Initial results without TAPI-2 and without MMP1 incubation show a degree of proliferation. In the presence of TAPI-2, the background is reduced as are the samples incubated with MMP1. Significance was determined by one-way ANOVA with Tukey post-hoc tests. **(B)** Latency was investigated using a control containing a glycine serine linker in place of the MMP cleavage site and the latent recombinant EPO protein. Protein quantiy was determined by direct LAP elisa and equal amounts used for the assay. Both sample supernatants were incubated in the presence of 30nM MMP1 overnight before assaying. Significance was determined by one-way ANOVA with Tukey post-hoc tests.

DISCUSSION

The results presented here describe the design, genetic construction and *in vitro* characterisation of the latent growth factors mEPO, huTGFβ, mIGF1A and mIGF1B. Adams et al. (2003) originally described this latency platform technology. They ascertained that the growth factor IFN-β could be made latent through fusion with the LAP of TGFβ. Activation of IFN-β occurred through cleavage of the cleaved protein from the LAP protective 'shell' at an engineered MMP site incorporated between the two molecules. The latent growth factor demonstrated a lack of biological activity until the cleaved protein was released. Interestingly, they also noted that the cleaved protein was released when incubated with the CSF taken from patients suffering from neurological diseases such as multiple sclerosis. By exploiting this technology, these modular building blocks, the LAP shell fused to the MMP cleavage site, and growth factors can be cloned into expression plasmids that are able to express recombinant proteins (with a longer half-life and the ability to become active in areas of increased MMP activity) in a variety of cell lines, for use as therapeutic agents in MS. Growth factors such as mEPO, mIGF1 and huTGFβ target more the neurodegenerative component of the disease rather than the immune-mediated component that the approved therapies do.

Expression and secretion of all four latent growth factors and the control (LAP-MMP) was investigated in both the CHO and the HEK-293T mammalian cell lines and analysed using western blotting. Methotrexate amplification of the CHO cell clones led to several clonal populations to secrete high concentrations of latent growth factor protein that were isolated and expanded in order to generate a stable, continuous and consistent production of the latent growth factor protein. The expression, secretion and cleavage of the latent growth factors from the CHO cell line were successful for all but one latent protein (LAP-MMP-mEPO). The producer of the latent LAP-MMP-mEPO protein in the CHO cell line was a population of an original clone obtained through ring cloning techniques. Therefore, it was hypothesised that the failure of the MMP enzyme to cleave

LAP-MMP-mEPO was due to a cellular defect with that particular clone. However, MMP once again failed to cleave LAP-MMP-mEPO produced by a suspension-based CHO cell population. As cleavage was obtained in the HEK-293T cell line, the inability for MMP to cleave latent EPO was theorised to be down to the cell-specific post-translational modifications (such as glycosylation, Raju et al., 2000) resulting in altered protein structure or conformation through the interaction of the glycosylation sites of EPO and LAP.

The EPO and LAP proteins are highly glycosylated with 4 N-linked glycosylation sites (Asn^{24}, Asn^{38} and Asn^{83}) and 1 O-linked glycosylation site (Ser^{126}) on the EPO molecule, and 3 N-linked glycosylation sites (Asn^{82}, Asn^{136} and Asn^{176}) on the LAP molecule (Wasley et al., 1991, Purchio et al., 1988). Tunicamycin is an antibiotic that interrupts the synthesis of glycoproteins by blocking UDP-GlcNAc and Dol-P, enzymes used by glycosyltransferases to transfer N-acetylglycosamine to specific sites on a protein molecule. To determine whether the glycosylation pattern of the proteins generated by the CHO cells may be the inhibitory factor in the ability of the MMP1 enzyme to cleave LAP-MMP-mEPO, pcDNA3(LAP-MMP-mEPO) transfected CHO-S were cultured in the presence of tunicamycin before the conditioned supernatant was incubated with MMP1. Although the CHO-S cells were able to express and secrete unglycosylated LAP-MMP-mEPO, they failed to release mEPO when incubated with MMP1. Surprisingly, our lab has demonstrated that when human EPO (huEPO) is fused to LAP and expressed by CHO DG44 cells, MMP1 is able to cleave the protein and release huEPO (unpublished data, Dr. Sandrine Vessilier). As to the reason MMP1 fails to cleave mEPO when fused to LAP is, at present, unclear. Further studies on the recombinant protein structure and composition (such as mass spectrometry) may be able to shed some light onto a possible reason for MMP1 failure.

CHO cells are the dominant cell line for the production of glycoprotein therapeutics, although other cell lines such as HEK-293T cells are being engineered to produce high-expression cell lines (Hossler et al., 2009). This addition of oligosaccharides to either the side chain of asparagine (*N*-linked

glycans) or to the side chain of serine/threonine (*O*-linked glycans) plays a crucial role in protein function and half-life and therefore in the pharmacokinetics of the protein. For example, brain derived EPO is smaller in size than serum EPO but is more active *in vitro* at low concentrations, factors all pertaining to the degree of sialylation (i.e. glycosylation) (Genc et al., 2004). This was best demonstrated in the recombinant protein asialylated EPO (Erbayraktar et al., 2003). The plasma half-life of rEPO, usually 5–6 hours, is reduced to 2 min when asialylated. Moreover, the addition of two *N*-linked glycosylation sites into the recombinant protein darbopoetin-α increased the protein half-life (and ultimately its *in vivo* activity) significantly, even when given at a ten-fold higher dose of rEPO to darbopoeitic-α (Elliott et al., 2003). Methotrexate amplification is a widely used method of gene amplification in CHO cells for the large-scale production of recombinant protein, and an increase in gene copy number increases the specific productivity of recombinant proteins from the CHO cell line (Kim et al., 1998). However, even after methotrexate amplification, the CHO cell line stably transfected with the recombinant protein vectors produced significantly smaller levels of latent protein than that found from the conditioned medium collected from HEK-293T cells transfected with those same recombinant vectors. The purification of latent proteins is problematic due to the adherence of the LAP protein to the purification filters (unpublished data, Dr Sandrine Vessillier) coupled with small initial concentrations, this method of protein production was abandoned in favour of transient transfection of the HEK-293T cell line for these studies.

The HEK-293T cell line correctly expressed and secreted the latent growth factors and the MMP1 enzyme successfully released the cleaved protein. Interestingly, the latent mIGF1 proteins, when incubated with MMP1, released three protein molecules rather than the expected single cleaved protein. This may be due to a MMP enzyme cleaving the mature IGF1 peptide at amino acids Pro^{63} and Leu^{64}, which correspond to amino acids found within the engineered MMP cleavage recognition site (PLGLWA) resulting in a peptide fragment around 8 kDa. Another possibility may be cleavage of the amino acids Gln^{15}, Phe^{16} and Val^{17} within the IGF1 protein, which are found within the self-activation site of MMP1 (GVPDVA**QFV**LTE) resulting in a peptide fragment around 5 kDa (Vessillier et

al., 2004). The mutation of the amino acids Pro^{63} and Leu^{64}, or of Gln^{15}, Phe^{16} and Val^{17} within LAP-MMP-mIGF1 to ones that do not constitute a recognition site may enable us to confirm this theory. Within our biological assay, a significant increase in cell proliferation was observed when supernatant containing secreted LAP-MMP-mIGF1 was incubated with MMP and applied to CG4 cells. It may be that although the MMP enzyme cleaves the mature protein into three fragments, each of these fragments is biologically active. Alternatively, only a small amount of IGF1 may be required to stimulate proliferation of the CG4 cells and that, although the MMP enzyme has cleaved the mature protein into three peptides, the amount of biologically active protein is sufficient to induce proliferation. The protein fragments would initially require purification in order to separate them into their individual sizes. This can be achieved by running the cleaved proteins through SDS-PAGE, the gel silver stained to identify the relevant bands, followed by excision and electroelution of the protein bands. Once the bands have been isolated, they can be used individually in the CG4 biological assays to determine whether they are all biologically active; or if not all are biologically active, those that are can be titered to determine the minimum concentration required to initiate proliferation of CG4 cells.

By engineering the latent control peptide (LAP-$(Gly_4Ser)_3$-mEPO), through the replacement of the MMP cleavage site with a flexible glycine-serine linker, the ability of LAP-MMP-mEPO to be cleaved by the MMP1 enzyme could be investigated. When incubated with MMP1, a faint band corresponding to the LAP fragment of this control is present; however, it is negligible in comparison to that of the LAP protein cleaved from the LAP-MMP-mEPO protein. A recent study has demonstrated the ability of the proteinase MMP2 to cleave the LAP peptide *in vitro* in mouse embryo fibroblasts (Ge and Greenspan, 2006). It is therefore possible that the latent LAP-MMP-mEPO peptide contains a cleavage site for both MMP2 and MMP1 in the absence of the preferential engineered MMP site resulting in the faint band observed when the MMP site is absent. Alternatively, it may be that the addition of MMP1 to the conditioned medium collected from transfected cells activates other MMPs found within the supernatant and secreted by the cell, such

as MMPs secreted by CHO cells (Adams et al., 2003). This substantiates the finding that cleavage is dependent on an MMP site.

The human megakaryoblastic leukaemia cell line UT-7 proliferates in the presence of EPO and was used to assess the biological activity of LAP-MMP-mEPO before and after cleavage by MMP1. A significant increase in cell proliferation following MMP1 cleavage confirmed its latency; although in the absence of MMP1 incubation the latent protein did exert a notable increase in cell number. The MMP cleavage site incorporated into the latent recombinant proteins (LAP-MMP-mEPO, LAP-MMP-huTGFβ, LAP-MMP-mIGF1A and LAP-MMP-mIGF1B) is a consensus sequence and is not exclusive for cleavage by MMP1, but has been found to be cleaved by other MMPs such as MMP2, -3, -7 and -9 (Adams et al., 2003). Inclusion of LAP-$(Gly_4Ser)_3$-mEPO demonstrated similar levels of proliferation that was substantially reduced when the broad-spectrum MMP inhibitor TAPI-2 to added to the sample. One theory is that other proteases may play a part in the release of cleaved mEPO; another is that (as seen from the lack of MMP1 cleavage in CHO generated LAP-MMP-mEPO protein) the tertiary structure is somewhat altered allowing the EPO peptide access to its receptor on the cell surface even without release in the cleaved form by a proteinase such as MMP1.

An assay assessing the biological activity of LAP-MMP-mIGF1A before and after incubation with MMP1 demonstrated a significant increase in the proliferation of CG4 cells in response to cleaved mIGF1A indicating that the LAP-MMP-mIGF1A recombinant protein is cleaved by MMP1 and the cleaved mIGF1A peptide is biologically active and able to induce proliferation of CG4 cells and not their differentiation in type-2 astrocytes. Once again, a small degree of background level of cell proliferation proliferation was observed without MMP cleavage. This is more than likely due to the release of cleaved mIGF1A from LAP-MMP-mIGF1A by endogenous MMPs secreted by the producing cell line, HEK-293T.

Utilisation of the MLEC cell line integrated with a luciferase reporter gene demonstrated a significant increase in luciferase output when incubated with cleaved huTGFβ as compared to LAP-MMP-

huTGFβ. Human TGFβ is secreted as a latent protein where the TGFβ homodimer—bound by disulphide bonds—is attached by non-covalent bonds to LAP (Miyazono et al., 1988, Wakefield et al., 1988, Lyons et al., 1990, Shi et al., 2011). The *in vitro* application of acid activation commissions the dissociation of these non-covalent bonds allowing for the release of huTGFβ bound by non-covalent bonding to the LAP, visualized by a significant increase in luciferase output. Unsurprisingly, the amount of luciferase output for acid activated LAP-MMP-huTGFβ without exposure to MMP1 enzyme appeared to be similar in intensity to that of inactivated but cleaved huTGFβ. This is because although acid activation allows the TGFβ peptide to be exposed, it is still unable to access its receptor. Similarly although cleaved TGFβ is released from the LAP, LAP still provides latency to it through its association by non-covalent bonds.

In vitro experiments confirmed the expression, secretion, latency and cleavability of the engineered latent growth factors mEPO, huTGFβ, mIGF1A and mIGF1B and their biological activities confirmed when cleaved with MMP1. These characteristics demonstrate the appeal of this technology as a therapeutic avenue for growth factors as it aims to circumvent the issues of the growth factors pleiotropism and toxicity typically observed following systemic administration. The next chapter discusses the method of protein delivery.

CHAPTER 4

A CELL-BASED GENE-VECTOR APPROACH TO THERAPY OF EAE

INTRODUCTION

Treatment of MS, gained from studies performed in the mouse model EAE, with the growth factors TGFβ, EPO and IGF1 shows promise. However, the limited access of growth factors to the CNS, and their pleiotropic nature when administered systemically hampers their development as therapeutic molecules. In animal studies, delivery of these growth factors has been through either direct intracerebral injection into the CNS, or via direct gene transfer for example using the Herpes Simplex Virus vector. The administration of a therapeutic agent directly into the human brain is obviously not possible and as such, alternative delivery methods must be devised. Delivery via a lentiviral vector system is extremely attractive due to its natural proficiency to evade the host immune system and integrate within a host cell genome. Viruses are highly evolved biological machines that gain access to a host cell and exploit the cellular machinery to facilitate their propagation and replication. The introduction into, and the replication of the proviral genome within the host cell, is the key to utilizing lentiviral vectors as therapeutic delivery vehicles.

The Lentivirus Life Cycle

The lentivirus life cycle consists of two phases: the early phase involving the entry and integration of the virus into the host cell; and the late phase involving the expression of the proviral genome, and all its processes up to and including viral budding and maturation (Coffin et al., 1997).

The Early Phase

During the early phase, the virus gains access to the host by binding to a specific receptor on the cell surface. The binding of the viral surface glycoproteins (SU, also known as gp120) with the transmembrane proteins (TM, also known as gp41), followed by the binding of that complex with CD4 and then CXCR4 or CCR5, thus triggering a conformational change that results in the fusion of the two membranes (the viral and the host cell's membrane) and the generation of a "fusion-pore"

(Coffin et al., 1997, Turner and Summers, 1999). It is through this pore that the virus injects its genetic material into the host cell's cytoplasm.

The Late Phase

The late phase begins with the injection of the viral genome into the cytoplasm of the host cell. An infectious virus has a pseudodiploid RNA genome (two identical copies of which one is a backup in case the template is damaged) which enters the cytoplasm as a nucleoprotein complex that is uncoated and reverse transcribed before translocating to the nucleus via the preintegration complex (PIC) for integration into the host genome. The ability of a virus to reverse transcribe its RNA into DNA for integration into the hosts genome is a fundamental and crucial step in the viral life cycle. Transcription results in a single linear DNA duplex (co-linear with the RNA template) containing coding genes, *cis*-acting genes and LTR required for the transcription of the viral DNA to RNA for packaging (Coffin et al., 1997, Lever et al., 2004).

The *cis*-acting elements are the unique 3' LTR (U3), the unique 5' LTR (U5), the R, the primer binding site (PBS), the polypurine tract (PPT) and the LTR's including the ψ (Psi) packaging sequence or encapsidation sequence (E). The R is the 'Repeat' at both ends of the viral RNA found within U3 and U5 and containing the polyadenylation signal sequence (AAUAAA). The PBS is a tRNA primer for reverse transcriptase binding site situated adjacent to U5. The PPT is a purine rich sequence situated immediately upstream of the 5' U3 that is cleaved during reverse transcription to generate the RNA primer for the synthesis of the positive strand of viral DNA. The PPT, PBS and R sequences enable efficient reverse transcription to occur. This transcription begins at the 5' LTR promoter/enhancer U3 region and ends at the 3' LTR transcription termination and polyA tail U5 region (Coffin et al., 1997, Lever et al., 2004).

There are three principal groups of viral proteins, translated from the coding genes, which are required for efficient and effective infection and replication: Gag, Gag-Pro-Pol, and Env. The Env proteins (encoded by the *env* gene) are responsible for the infection and interaction of the virus with

the cell surface receptors. Processing of the Env polypeptide generates the SU and TM proteins responsible for the fusion of the viral and host cell membranes and in defining the tropism of the virus through the host-cell receptor interaction. The Gag proteins (encoded by the *gag* gene) are nonglycosylated structural proteins of the matrix (MA), capsid (CA) and nucleocapsid (NC). The Pro-Pol proteins (encoded by the *pro* and *pol* genes) are polymerase proteins responsible for reverse transcription and integration. They include reverse transcriptase (RT) and RNase H (responsible for RNA/DNA hybrid degradation), integrase (IN) and protease (PR) and, in addition to the NC, are essential for viral replication. The Gag proteins are responsible for directing viral budding at the plasma membrane. The incorporation of the Pro-Pol proteins into the viral bud occurs through linkage with the Gag proteins. The viral bud then undergoes a metamorphosis into a mature, infectious virus with a core shaped like a truncated cone (Lever et al., 2004).

Lentiviruses are members of the retrovirus family of which HIV-1 is an example. In addition to the *gag*, *pro, pol* and *env* genes, lentiviruses also contain additional regulatory genes (*tat* and *rev*) that are essential for the expression of the genome. Tat, a low molecular weight protein, activates transcription by binding to a highly structured stem-loop region of R that is Tat-responsive (Tar). Rev is a protein that binds to the Rev Response Element (RRE), a highly structured sequence found within the lentiviral genome, to aid in the transportation of partially spliced and unspliced RNA from the cell nucleus to the cytoplasm (Coffin et al., 1997).

Primate lentiviruses also produce other accessory proteins (Nef, Vpr, Vpu, Vpx and Vif) that are not required for viral growth but are crucial for replication and pathogenesis. Negative factor (Nef) is an intracellular protein that acts on the signal transduction pathways leading directly to the loss of CD4 receptors on the cell surface. It increases the infectivity of viral particles and may activate RT either directly or indirectly. It also stimulates infected cells to divide. The Vpr protein (Viral protein r) is required for the targeting of viral DNA to the nucleus via its connection to the cellular import machinery. The karyophilic nature of the PIC (pre-integration complex) allows it to utilise the nuclear

import machinery of the host cell to gain access to its cytoplasm. This allows the virus to infect non-dividing cells as well as those undergoing mitosis making them a very attractive option for gene therapy. Vpr is crucial for efficient replication as it causes infected cells to arrest in the G2 phase of the cell cycle. It may also promote transport of the PIC into the nucleus after reverse transcription. The PIC consists of the viral DNA, the IN and RT enzymes, the MA and Vpr proteins and the cellular protein HMG-I(Y) which markedly enhances the integration activity of the PIC. Vpu, a small integral membrane protein, indirectly decreases the levels of the CD4 receptor by binding to them and signalling their degradation. It also promotes the release of the viral bud at the plasma membrane (Turner and Summers, 1999, Kay et al., 2001, Coffin et al., 1997). Vif (Virion infectivity factor) is a largely intracellular protein that affects the infectivity of the mature viral particles and is required for the efficient viral replication (Figure 4.1).

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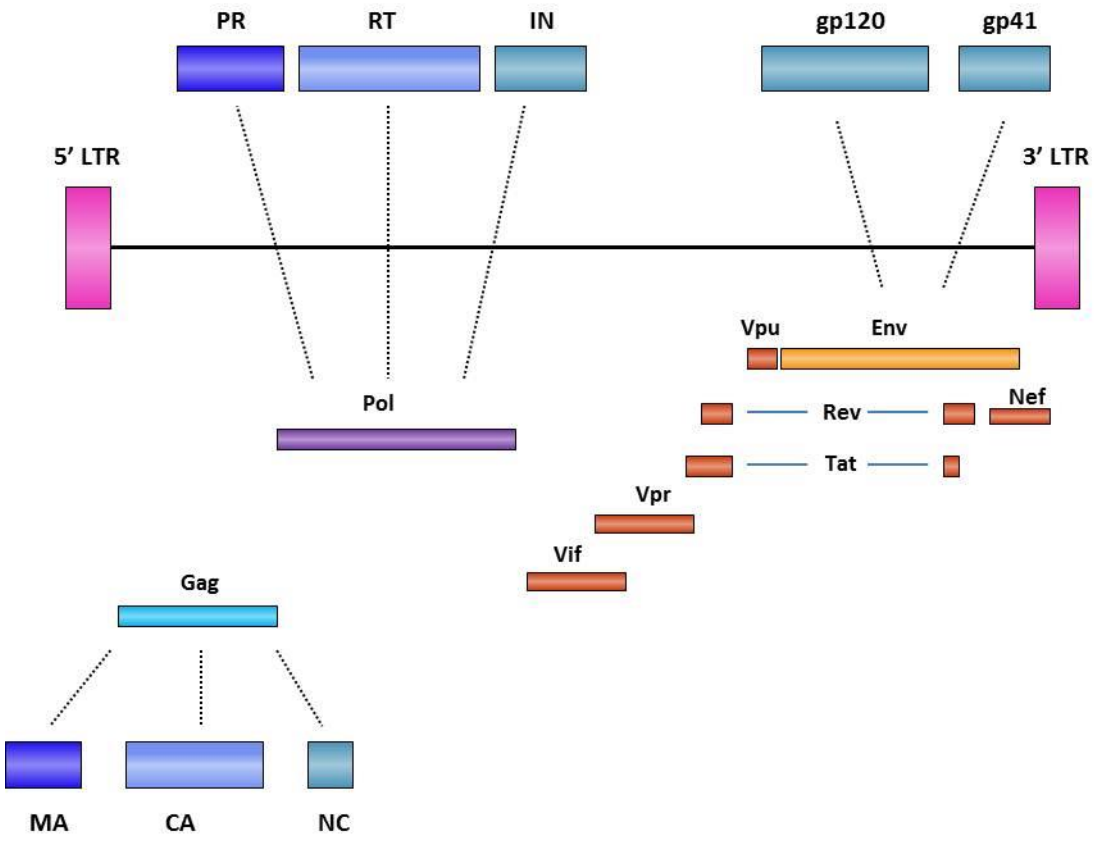


Figure 4.1: Organisation of the HIV-1 genome.
The long terminal repeat (LTR) regions, the genes and proteins encoded by the viral genome are indicated. The three principal viral protein groups Gag, Env and Pol are originally translated as polypeptides and are essential for the infection, integration and replication of the virus. The Gag peptide is cleaved into its relative protein subunits by the viral protease (PR) into the matrix (MA), capsid (CA) and nucleocapsid (MC) proteins. The Gag-Pol precursor peptide undergoes processing to generate the Gag protein subunits MS, CA and NC as well as the viral protease PR, and the enzymes reverse transcriptase (RT) and integrase (IN). The Env glycoprotein is processed by a cellular enzyme to generate the surface glycoprotein (SU, gp120) and the transmembrane glycoprotein (TM, gp41). The lentiviral accessory proteins (Nef, Vpr, Vpu and Vif) are also indicated and are crucial for the replication and pathogenesis of the virus. A lentivirus also contains additional regulatory genes (Tat and Rev) that are essential for the expression of the viral genome. The protein and gene sizes are not to scale.(Adapted from Freed, 2004 with permission).

HIV-1

HIV-1 binds to the CD4 receptors amino-terminal immunoglobulin domain expressed on Mø, T_H cells, monocytes and other phagocytic cells. It undergoes reverse transcription before utilizing nuclear localisation signals to move through the nucleopores of the intact nuclear membrane and replicating. HIV-derived lentiviral vectors were the first recombinant lentiviral vector system to be developed and have been extensively modified from their wild-type counterparts in an attempt to improve their performance and their biosafety (Escors and Breckpot, 2010). They have since

expanded to include non-human lentiviruses (such as simian, equine, feline, and bovine) in an attempt to circumvent the issue regarding the human immune response as they (the non-human lentiviruses) are not infectious to humans (Shibata et al., 2009, Grinshpun et al., 2010, Kachi et al., 2009, Mitomo et al., 2010). These vectors may be advantageous in human treatments. For example, the feline immunodeficiency-virus is able to effeciently and stably transduce several human cell lines (such as hepatocytes), but has inefficient replication capabilities due to the negligible transcriptional activity of the FIV LTR (Grinshpun et al., 2010).

Lentiviral Vectors for Gene Therapy

The advantages of a lentiviral vector system in gene therapy include a wide tropism as the viral envelope is pseudotyped with the VSV-G envelope protein, the lack of viral protein production after infection, integration of the therapeutic gene into the host genome and the long-term expression of the transgene at high levels. The use of a lentiviral vector has the additional advantage of genomic integration without mitosis. However, the genetic sequences inserted into the viral vector must be compatible to the viral life cycle i.e. must not contain introns (Coffin et al., 1997, Cockrell and Kafri, 2007).

The design of the lentiviral vector exploits the spatial segregation of the coding genes and the *cis*-acting genes by placing them into different plasmids. This enables researchers to manipulate and control the performance and biosafety of the virus depending on the application required. For example the replacement of the wild-type *env* gene with that of another virus—a process known as pseudotyping—can either broaden or narrow the range of host cells that a recombinant virus can infect; by modifying the *integrase* gene through directional mutagenesis within the packaging vector, one can generate non-integrating lentiviral vectors (NILVs) (Escors and Breckpot, 2010). The generation of self-inactivating (SIN) lentiviral vectors, the removal of non-essential viral proteins and the separation of the coding and *cis*-acting genes onto isolated plasmids have facilitated the increase in biosafety by increasing the number of recombinational events required to generate a replicative-

competent virus. The expression of the *rev* gene on a separate plasmid further enhances the safety of the lentiviral vector (Coffin et al., 1997).

The transgene vector contains all the *cis*-acting elements, such as the ψ packaging sequence, a promoter and polyadenylation signal, the PBS, R and LTR's, required for packaging, reverse transcription, and integration. Modifications such as the inclusion of the PPT and the addition of the woodchuck hepatitis B posttranscriptional regulatory element (WPRE) enhance the expression and stability of the transgene. The addition of cell-specific or tissue-specific promoters are also advantageous in that they can target that particular cell-type of tissue type and are less sensitive to promoter inactivation. By removing the four accessory proteins, deleting the *tat* gene and using a strong tat-independent constitutive promoter such as the cytomegalovirus (CMV) promoter (Dull et al., 1998) no negative effects on vector expression were noted (Gruber et al., 2000, Zufferey et al., 1997) adding to the safety profile of the developing lentiviral vector, however some target cells can silence the CMV promoter (Loser et al., 1998).

Implementing the assembly and packaging of a virus within a packaging cell line is followed by transduction. A lentiviral vector is a replication deficient virus which infects a target cell but which cannot replicate and produce viral particles. The envelope-encoding plasmid contains the Env proteins that determine the tropism of the virus. Pseudotyping of the HIV-1 lentiviral vector with the G protein of the vesicular stomatitis virus (VSV-G) allows for a wider host cell range. Additionally it results in a more stable vector that can generate viral titers exceeding $1x10^{10}$ t.u/ml (transducing units/ml) and which can endure concentration by ultracentrifugation (Kay et al., 2001, Coffin et al., 1997, Naldini et al., 1996). The number of transducing units (or infectious viral particles) as well as the total viral particle number (particle titer) is important as impurities and variations in infectious activity can lead to toxicity and can influence infectivity and immunogenicity.

HIV vectors stably integrate into the host genome in a random manner. This can result in the insertional activation of oncogenes by the random integration of the proviral genome, the

interruption and inactivation of an important gene due to this insertion and the possibility of generating a replication-competent virus through recombinational events (Miyoshi et al., 1998, Hanawa et al., 2005, Hacein-Bey-Abina et al., 2003). A SIN vector overcomes these problems as it is generated through the deletion of the transcriptional enhancers or the enhancers and promoters found within the U3 region or the 3' LTR, or by placing direct repeats on either side of the ψ packaging sequences such that the packaging sequence is deleted during reverse transcription (Hanawa et al., 2005, Coffin et al., 1997, Kay et al., 2001). The use of a SIN vector significantly increases the biosafety of the virus as activation of neighbouring genes cannot occur due to the removal of the enhancer elements.

Cells as transgene carriers

Gene therapy requires the delivery of an appropriate amount of a therapeutic gene into the target tissue without substantial toxicity to be successful. The use of syngeneic cells has the benefit of not eliciting an immune response. Autologous skin fibroblasts are ideal as *ex vivo* gene therapy as they are easy to obtain through biopsy, are easy to propagate and engineer *in vitro*, are able to be transplanted *in vivo* using simple procedures such as intraperitoneal injection, and once transduced are able to express the therapeutic protein at appropriate amounts. Many studies utilizing this approach have demonstrated the efficiency and effectiveness of transplanting genetically engineered cells in treating diseases concerning the CNS such as Parkinson's, Alzheimer's and MS; and for diseases involving inflammation such as arthritis (Fisher et al., 1993, Triantaphyllopoulos et al., 1999, Croxford et al., 2001, Levivier et al., 1995).

CHAPTER HYPOTHESIS AND AIMS

If these latent proteins could be manufactured from the transfection of mammalian cells with a DNA plasmid containing the latent protein DNA, then we hypothesised that the latent protein DNA could

also be incorporated into a lentiviral vector. Moreover, we hypothesised that this lentivirus could infect a mammalian cell line and cause it to manufacture, express and secrete latent proteins that are only biologically active once incubated with MMP enzyme.

The aim of this study was to determine if lentiviral vectors containing our transgenes of interest are able to transduce syngeneic cells that were conditionally immortalised and permanently transduced (tsF cells) *in vitro*. Once transduced and the ability of the expressed latent recombinant proteins to be cleaved by MMP1 *in vitro*, the *in vivo* biological activity was assessed following *ex vivo* gene delivery.

RESULTS

Lentiviral vector production and transduction of syngeneic fibroblasts

Dr Anne Rigby carried out the creation of the LAP-MMP lentiviral control vector, the production of the lentiviral particles LAP-MMP-mEPO, LAP-MMP-mIGF1A, LAP-MMP-mIGF1B and LAP-MMP and the transduction of the tsF cells during my maternity leave.

Lentiviral vector production and transduction of AB/H Biozzi tsF cells

The HIV-1-derived lentiviral vector pHRSIN-CSGW (Figure 4.2A) has been previously described (Demaison et al., 2002). Deletion of the TATA box and the 3' LTR U3 region (LTR/ΔU3) rendered the lentiviral vector SIN. The addition of a strong spleen focus forming virus (SFFV) promoter upstream and a WPRE downstream of the enhanced green fluorescent protein (eGFP) drives efficient transgene expression originally described by Zufferey et al. (1999).

To transduce the conditionally (SV40 large T) immortalised Biozzi/ABH fibroblast (tsF) cell line with the different transgenes including the original eGFP vector, HEK-293T cells were co-transfected with the transgene of interest within the self-inactivating transfer lentiviral vector, along with the pCMVR8.2 packaging plasmid and the VSVG envelope plasmid (Figure 4.2). The supernatant was collected, cleared by filtration and centrifugation before concentrating by ultracentrifugation.

Transduction of the tsF cell line was done in the presence of polybrene for 4 days—polybrene has been shown to improve the transduction efficiency of lentiviruses in a wide range of cells although its mechanism of enhancement is, as yet, unclear (Davis et al., 2002). Transduction with each of the lentiviral constructs resulted in stable permanently transduced cells designated LAP-MMP-mEPO.tsF, LAP-MMP-huTGFβ.tsF, LAP-MMP-mIGF1A.tsF, LAP-MMP-mIGF1B.tsF, LAP-MMP.tsF (acting as a negative control) and mEPO.tsF (acting as a positive growth factor control). The transduction

efficiency has previously been shown to be approximately 100% as all cells seen were eGFP-positive (Rigby, 2009).

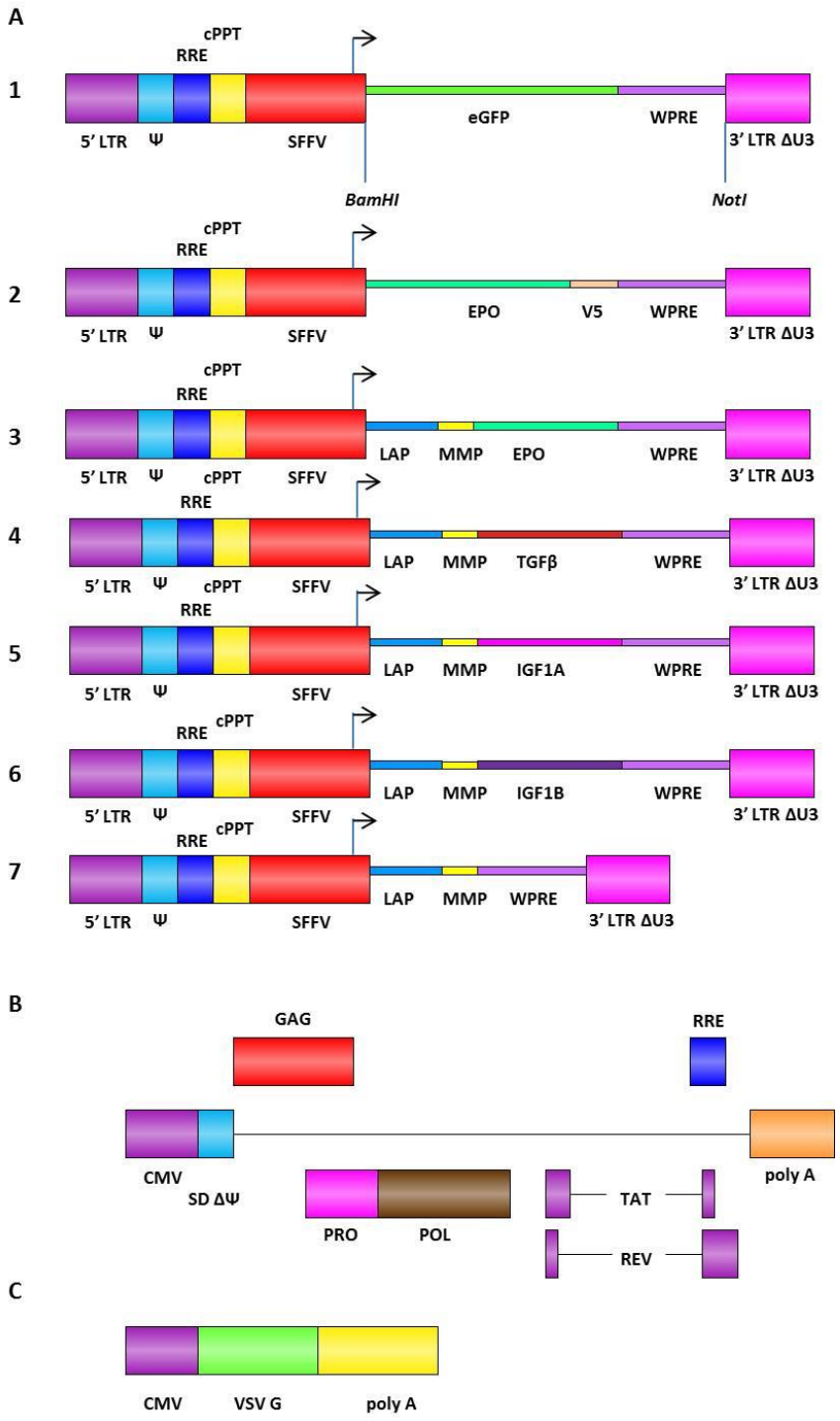


Figure 4.2: DNA lentiviral vector constructs.
(A) The HIV- 1 derived lentiviral vector pHRSIN-CSGW was originally described by Demaison (2002), is rendered self-inactivating (SIN) through the deletion of the TATA box and the 3' LTR U3 region (LTR/ΔU3). The addition of a strong spleen focus-forming virus (SFFV) promoter upstream and a WPRE downstream of the enhanced green fluorescent protein (eGFP) drives efficient transgene expression. **1-7**| pHRSIN-CSGW-eGFP (1), EPO with its native signal peptide (2), LAP-MMP-mEPO (3), LAP-MMP-huTGFβ (4), LAP-MMP-mIGF1A (5), LAP-MMP-mIGF1B (6), LAP-MMP (7). **(B)** Packaging construct (pCMVΔ8.2). **(C)** Envelope construct (pMD.G).

High levels of in vitro expression following stable lentiviral transduction

In order to determine whether transduction of the tsF cell line with the various lentiviruses resulted in stable expression and secretion of each latent fusion protein, ELISAs were done using the supernatant taken 24 hours after transduction of tsF cells with the various constructs.

A sandwich ELISA for EPO quantified the protein concentration for both the growth factor control protein mEPO secreted by the mEPO.tsF transduced cells, and the LAP-MMP-mEPO proteins secreted by the LAP-MMP-mEPO.tsF transduced cells (Figure 4.3). The transduced mEPO.tsF cells acting as the control, produced approximately 2400ng mEPO/mL/10^6 cells/24hours whereas the LAP-MMP-mEPO.tsF transduced cells produced approximately 330ng mEPO/mL/10^6 cells/24hours (Figure 4.3B), a 7-fold difference in expression levels. However, using a direct LAP ELISA the concentration of LAP from the LAP-MMP-mEPO.tsF transduced cells produced approximately 2329ng LAP/ml/10^6 cells/24hours (Figure 4.4). When testing the relative therapeutic efficacy of these proteins, this variation in expression was taken into account.

The protein concentration for the latent fusion proteins LAP-MMP-huTGFβ, LAP-MMP-mIGF1A, LAP-MMP-mIGF1B, or the LAP-MMP control was quantified using a direct LAP ELISA (Figure 4.4). The transduced cells LAP-MMP-huTGFβ.tsF, LAP-MMP-mIGF1A.tsF, LAP-MMP-mIGF1B.tsF and the control LAP-MMP.tsF produced approximately 1679ng, 79ng, 48ng and 68ng LAP/mL/10^6 cells/24hour respectively. A second infection of the LAP-MMP-mIGF1A.tsF, LAP-MMP-mIGF1B.tsF and LAP-MMP.tsF transduced cells led to a significant increase in protein production for LAP-MMP-mIGF1A and LAP-MMP control, with no increase in LAP-MMP-mIGF1B expression: 132, 170 and 43 ng LAP/mL/10^6 cells/24hour respectively (Figure 4.5). This may be due to a lack of viable lentiviral

vector particles able to infect the cells to express a greater concentration of recombinant protein as no titration of viral particles was done, only a set volume of viral particle supernatant used to infect the relevant cells(20µl). It is important to note that the levels shown here are the levels of LAP protein as determined by the direct LAP ELISA. It is possible, indeed plausible, that a small amount of the latent fusion protein is cleaved immediately after secretion from the cell due to endogenous MMP enzymes. Therefore some LAP proteins quantified by the ELISA may no longer be a latent fusion protein, but only the LAP portion of the fusion protein.

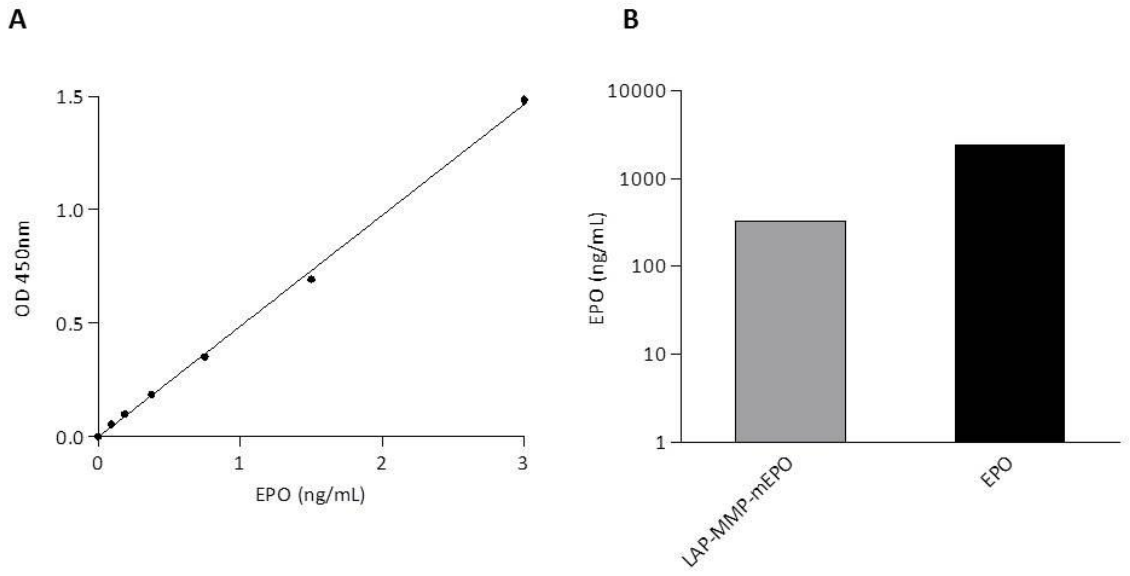


Figure 4.3: Quantification of EPO and LAP-MMP-mEPO production by tsF transduced cells.
The tsF cell line was infected with the pHRSIN-CSGW-(LAP-MMP-mEPO) or pHRSIN-CSGW-EPO lentiviral vectors producing the LAP-MMP-mEPO.tsF and EPO.tsF transduced cells respectively. **(A)** After 24 hours, the culture supernatant was collected to measure expression of the LAP-MMP-mEPO or the native EPO molecule with an EPO-specific sandwich ELISA assay standardised with recombinant EPO protein. The results are expressed as a function of protein concentration (ng/ml). **(B)** Mean of two replicate OD interpolated from the standard curve for concentrations of LAP-MMP-mEPO and mEPO protein production by LAP-MMP-mEPO.tsF and mEPO.tsF transduced cells respectively and expressed in ng/ml EPO.

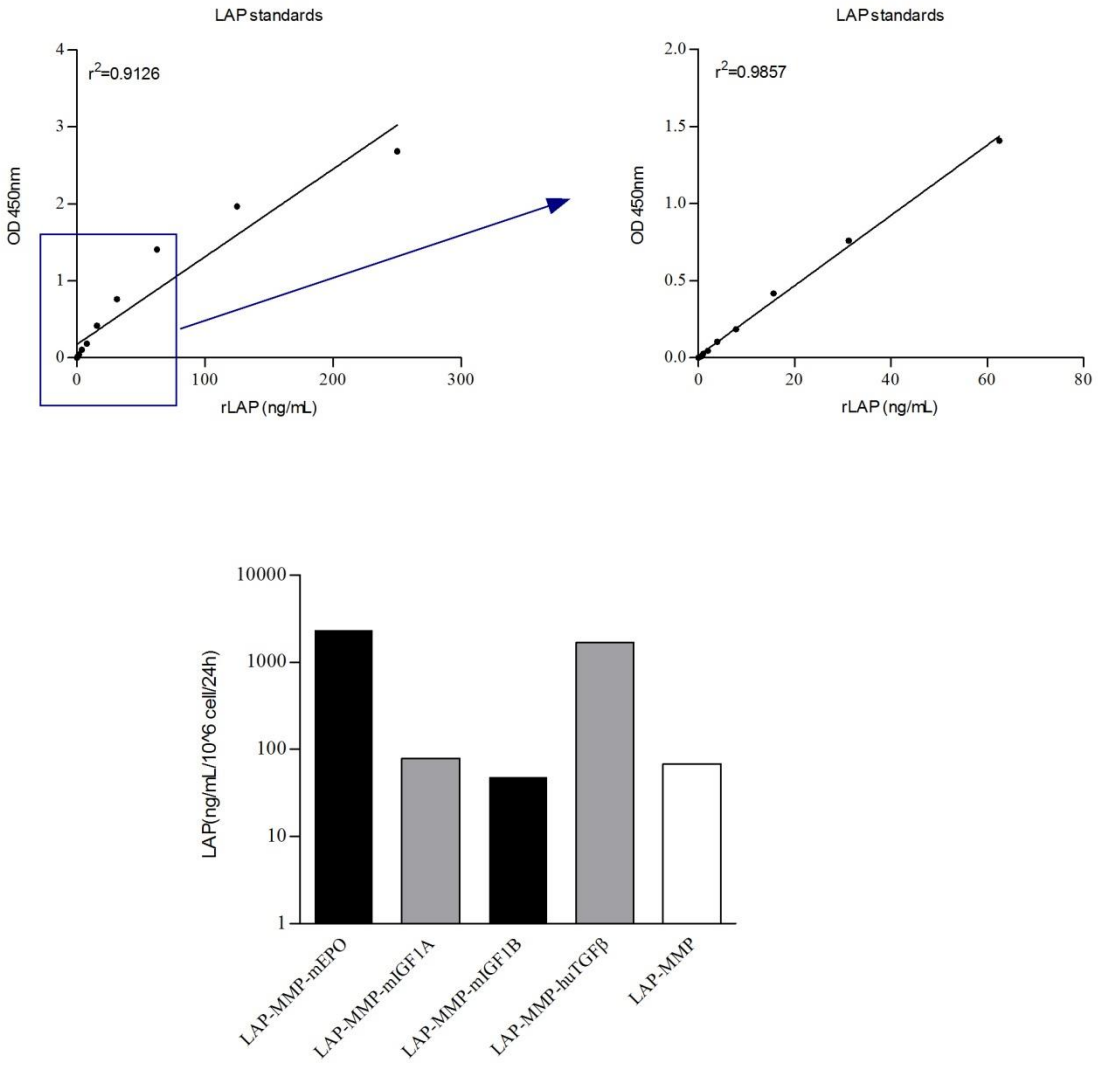


Figure 4.4. Quantification of latent protein production by tsF transduced cells.
The tsF cell line was infected with the pHRSIN-CSGW-(LAP-MMP-mEPO), pHRSIN-CSGW-(LAP-MMP-huTGFβ), pHRSIN-CSGW-(LAP-MMP-mIGF1A), pHRSIN-CSGW-(LAP-MMP-mIGF1B) or pHRSIN-CSGW-(LAP-MMP) lentiviral vectors. After 24 hours, the culture supernatant was collected to measure expression of the recombinant proteins LAP-MMP-mEPO, LAP-MMP-mIGF1A, LAP-MMP-mIGF1B, LAP-MMP-huTGFβ, or LAP-MMP with a direct LAP ELISA assay standardised with recombinant LAP protein. **(A)** Standard curve generated through duplicate samples of recombinant LAP ranging in concentration from 250ng/ml–0ng/ml. Linear regression analysis was performed and the r^2 displayed. Values falling within the linear range have been refit in a second standard curve (blue insert) and used to interpolate protein values. Results are expressed as a function of recombinant LAP protein concentration (ng/ml). **(B)** Mean of two replicate OD measurements interpolated from the standard curve for concentrations of LAP-MMP-mEPO, LAP-MMP-mIGF1A, LAP-MMP-mIGF1B, LAP-MMP-huTGFβ, and LAP-MMP protein production by LAP-MMP-mEPO.tsF, LAP-MMP-mIGF1A.tsF, LAP-MMP-mIGF1B.tsF, LAP-MMP-huTGFβ.tsF and LAP-MMP.tsF cells respectively and expressed in ng/ml/10^6 cells/24 hrs recombinant LAP.

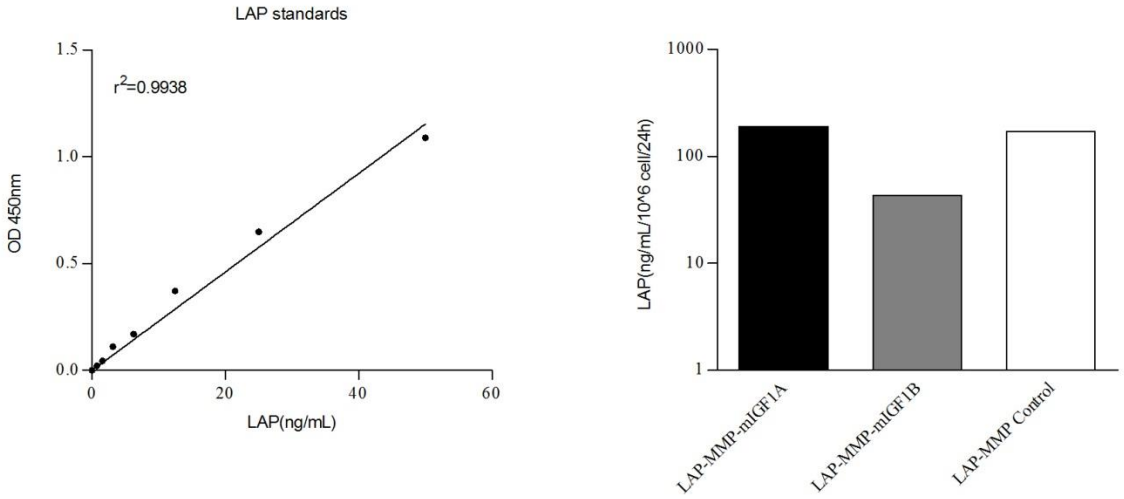


Figure 4.5. Quantification of latent protein production by tsF transduced cells following a second round of transduction. The LAP-MMP-mIGF1A.tsF, LAP-MMP-mIGF1B.tsF and LAP-MMP.tsF transduced cells were infected for a second time following low expression levels after initial infection. After 24 hours, the culture supernatant was collected to measure expression of the recombinant proteins LAP-MMP-mEPO, LAP-MMP-mIGF1A, LAP-MMP-mIGF1B, LAP-MMP-huTGFβ, or LAP-MMP with a direct LAP ELISA assay standardised with recombinant LAP protein. **(A)** Standard curve generated through duplicate samples of recombinant LAP. Linear regression analysis was performed and r^2 displayed. **(B)** Mean of two replicate OD measurements interpolated from the standard curve for concentrations of LAP-MMP-mIGF1A, LAP-MMP-mIGF1B, and LAP-MMP protein production by LAP-MMP-mIGF1A.tsF, LAP-MMP-mIGF1B.tsF and LAP-MMP.tsF cells respectively and expressed in ng/ml/10^6 cells/24 hrs recombinant LAP.

Growth factor release is achieved following MMP cleavage

Cleavage of the latent fusion proteins at the MMP site by a MMP enzyme is imperative for the release of the growth factor from the LAP. This was demonstrated by incubating the supernatant taken from the transduced tsF cell lines with 30nM MMP1, and the presence of the cleaved growth factor assessed by western blotting analysis using the anti-LAP antibody (Figure 4.6). The fusion proteins LAP-MMP-mEPO, LAP-MMP-huTGFβ and LAP-MMP were detected around 50 kDa and 40 kDa respectively, consistent with the data seen from 239T and CHO protein production (Figure 4.6 A, D and E). The latent fusion proteins LAP-MMP-mIGF1A and B exhibited several forms of the glycosylated protein with the dominant protein size at 50 kDa (Figure 4.6 B and C). Cleavage of the latent proteins with the MMP enzyme releases the LAP peptide, which was detected at 37 kDa as expected (Figure 4.6).

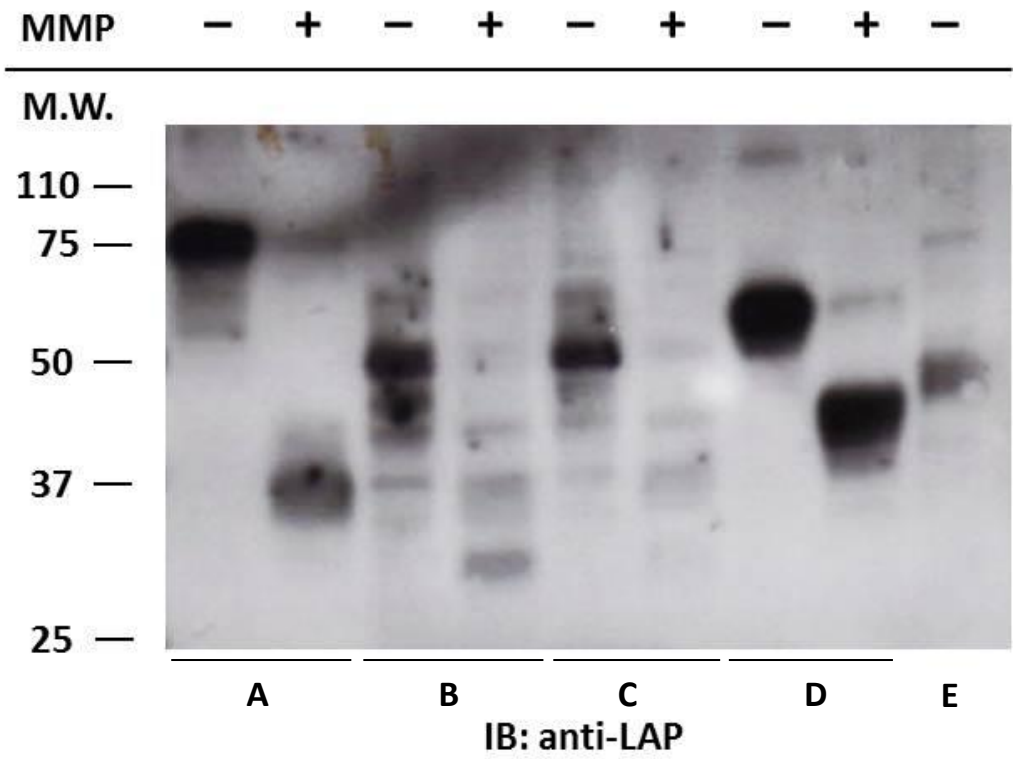


Figure 4.6: MMP Cleavage of supernatant taken from transduced tsF cell lines.
The tsF cell line was infected once with lentiviral vector particles for LAP-MMP-mEPO, and LAP-MMP-huTGFβ, and three times for the lentiviral vector particles for LAP-MMP-mIGF1A, LAP-MMP-mIGF1B, and LAP-MMP. The supernatant was collected after 24 hours and incubated overnight with 30nM MMP1 enzyme. 35µl supernatant samples for **(A)** LAP-MMP-mEPO (diluted 1:2), **(B)** LAP-MMP-mIGF1A, **(C)** LAP-MMP-mIGF1B, **(D)** LAP-MMP-huTGFβ (diluted 1:3), **(E)** LAP-MMP control were electrophoresed down a reducing SDS-PAGE gel and probed with anti-LAP antibody. The protein molecular weights are represented in kDa on the left hand side of the Figure. The LAP element of the LAP-MMP-huTGFβ appers to be running higher than expected and as previously visualised. This is suspected to be a combination of two factors. First, the edge of the gel was not buffered properly as loading dye was not added to the end and unused wells, causing a distortion in the running of the protein through the gel; and second overloading of the lane with excess protein has compound this distortion.

In vivo biological activity is achieved following *ex vivo* gene delivery

In order to validate that the delivery of a biologically active growth factor occurs *in vivo* following the transplantation of permanently transduced cells expressing our latent fusion protein, tsF cells (transduced or untransduced) were injected i.p into AB/H CREAE mice (10^7 cells/mouse) 27 days after EAE innoculation (after the acute phase but one day before a third inoculation was given to induce a relapse) and the haematocrit measured after 7, 14 and 21 days post-injection (Figure 4.7). Haematocrit levels were significantly elevated following exposure to transduced tsF cells expressing LAP-MMP-mEPO (56.7% ± 1.1) or mEPO (57.27% ± 3.2) as compared to untransduced cells (47.1%

±1.65, $P \leq 0.05$) on day 7. During the course of the next 7 days, the level of haematocrit of the untransduced tsF group decreased slightly representing the slight anaemia the mice undergo during a relapse. The haematocrit levels of the treated groups mEPO.tsF and LAP-MMP-mEPO.tsF however remained unchanged demonstrating the ability of the mEPO and LAP-MMP-mEPO transduced tsF cells to secrete biologically functional EPO that is involved in haematopoiesis. As expected, these observations demonstrate that the delivery of permanently transduced, fusion protein expressing tsF cells can induce and sustain *in vivo* expression and biological activity of the fusion proteins corroborating the *in vitro* data.

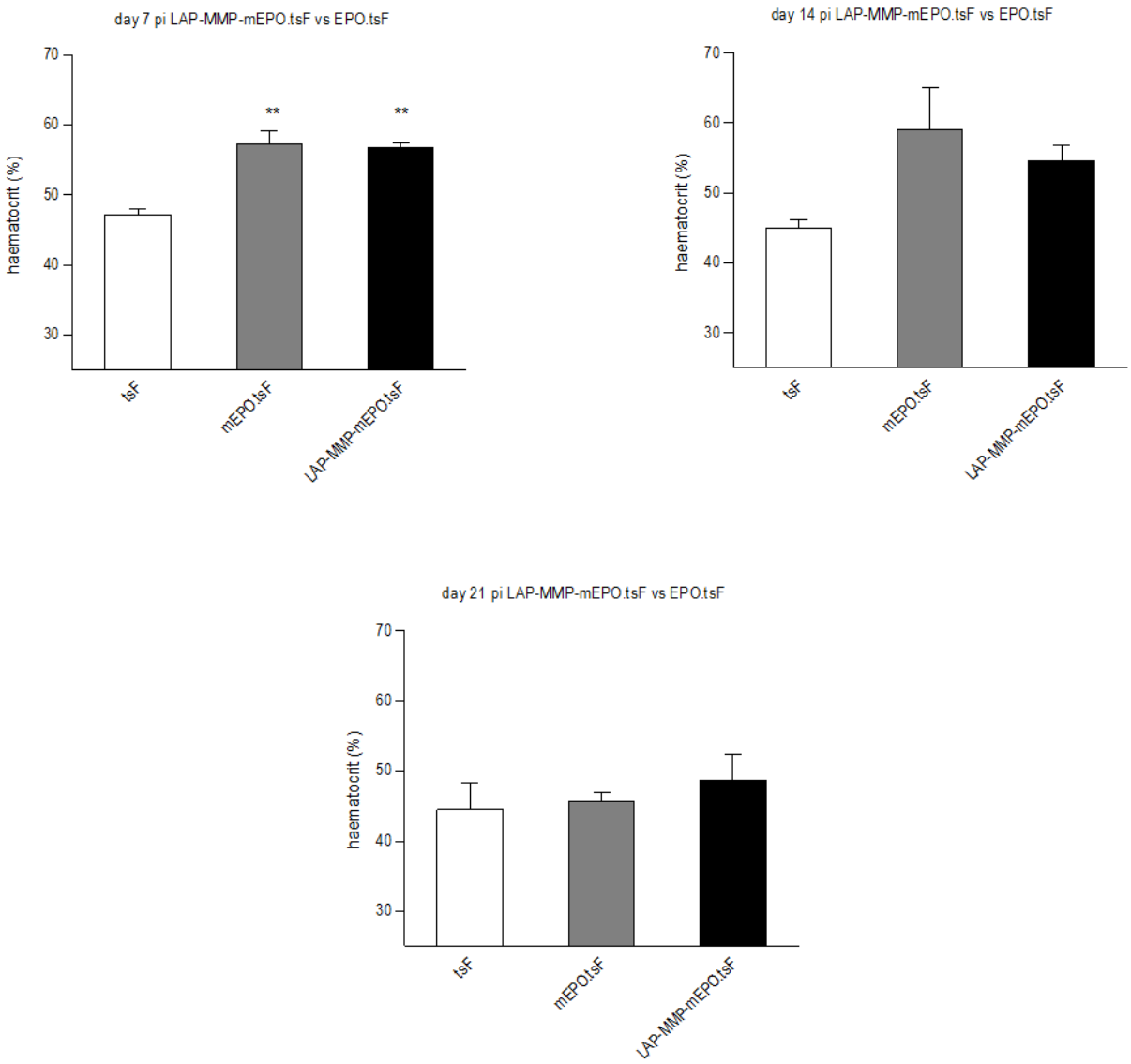


Figure 4.7 Haematocrit levels following ex vivo gene delivery.
AB/H Biozzi CREAE mice were injected i.p. 27 days after initial innoculation with a syngeneic fibroblast cell line (10^7 cells/mouse), which was either untransduced (tsF), transduced with mEPO (mEPO.tsF) or transduced with LAP-MMP-mEPO (LAP-MMP-mEPO.tsF). The number of transduced cells injected was adjusted to EPO expression level and compensated with untransduced cells to obtain both equivalent EPO expression and equal total number of cells injected. Haematocrit levels were measured 7, 14 and 21 days after injection of the cells. The results represent mean haematocrit levels ± SEM of all animals within the group (n= 2-4, **p ≤ 0.05 compared with control mice, 1-way Anova followed by Tukeys post-hoc test).

DISCUSSION

The aim of this study was to determine if an *ex vivo* approach to the delivery of LAP-MMP-mEPO, LAP-MMP-huTGFβ, LAP-MMP-mIGF1A and LAP-MMP-IGF1B *in vivo* was feasible. Syngeneic fibroblasts taken from the AB/H mouse strain were immortalised and sustained in culture (Croxford et al., 2000) before transduction using the lentiviral vector pHRSIN-CSGW. The use of a lentiviral vector is advantageous over the use of a retroviral vector due to its ability to integrate into the host genome of even non–dividing cells, whereas a retroviral system requires the cells to be actively dividing in order to access its nucleus. Previous *in vitro* experiments using LAP-MMP-mEPO, LAP-MMP-mIGF1A, LAP-MMP-mIGF1B, LAP-MMP-huTGFβ and LAP-MMP secreted by the HEK-293T cell line demonstrated the ability of the enzyme MMP1 to release the mEPO, mIGF1A, mIGF1B, huTGFβ proteins from LAP-MMP-mEPO, LAP-MMP-mIGF1A, LAP-MMP-mIGF1B and LAP-MMP-huTGFβ respectively following incubation overnight, and that the released growth factor proteins retained their biological activities when assessed using the appropriate biological assays. Additionally, incubation of LAP-MMP-mEPO, LAP-MMP-mIGF1A, LAP-MMP-mIGF1B, LAP-MMP-huTGFβ and LAP-MMP (secreted from transduced tsF cells) with the MMP1 enzyme, released the growth factor protein *in vitro* as measured using ELISA. Levels of expression of the latent fusion proteins ranged from 48—2400ng/ml/10^6 cells/24 hours depending on the fusion protein. Although the levels of expression differed between proteins, they were still significantly higher than the expression levels achieved (1.7 and 3.3ng/ml/10^6 cells/24 hours for dTNFR.tsF and IL-10.tsF respectively) using the retroviral vector system described elsewhere (Croxford et al., 2001, Croxford et al., 2000). The same ELISA protocol quantified these levels therefore allowing direct comparisons. The rationale for utilizing an *ex vivo* approach as the delivery method for our therapeutic proteins is to address the question as to whether our therapeutic gene can be delivered *in vivo*. Measurements of the haematocrit of AB/H mice injected with mEPO.tsF and LAP-MMP-mEPO.tsF cells showed a significant increase in the haematocrit levels corresponding to biologically active EPO as compared to tsF cells

alone. The difference in the haematocrit levels was no longer observed 14 days after cell injection and the animals exhibited no observable side effects. This was, at the time of the experiment, unexpected. However, another study (Rigby, 2009) later found that when serum taken from naïve mice or mice injected with transduced tsF cells, was probed for binding antibodies using an ELISA and flow cytometry, a notable increase of binding was observed from the serum of the mice injected with the transduced tsF cells. Further experiments determined that the inciting antigen was that of the BSA used in the growth medium of the tsF cells. It is therefore thought that the lack of increase in haematocrit levels after 14 days is due to the gradual destruction of the cells. Although the author tried to grow the transduced tsF cells in a serum-free medium for 48 hours prior to intra-peritoneal transplantation, loss of expression and immunogenicity agianst the BSA antigen was still observed. It should be possible to adapt these cells to serum-free conditions by sequential adaptation or weaning where cells are switched to from serum-containing medium to serum-free medium in several steps, however this was not attempted during this study.

As previously discussed, the erythropoietic effects of EPO require a mere 15 units of EPO to increase erythropoiesis [where one unit elicits the same erythropoiesis-response in fasted rats as five µmoles of cobaltous chloride (Jelkman 2009)] and these effects are mediated through the homodimer $(EPOR)_2$ to which EPO has a high affinity. Although the fusion of EPO to the LAP peptide confers latency, endogenous MMPs (such as those found within the kidney where endogenous EPO is produced) may cleave the protein thus releasing a small amount of biologically active mEPO from LAP-MMP-mEPO. Therefore an increase in the haematocrit of healthy AB/H Biozzi mice transplanted with LAP-MMP-mEPO.tsF cells would be a reflection of the release of mEPO from LAP-MMP-mEPO. This data validates *in vivo* what was demonstrated *in vitro* as discussed in the previous chapter i.e. that our recombinant protein is secreted by the tsF cells and the peptide released is biologically active. The fact that the release of the mEPO protein from LAP-MMP-mEPO was observed after the injection of LAP-MMP-mEPO.tsF cells into healthy mice is not unexpected as endogenous EPO (used for the generation of new red blood cells) is produced mainly in the kidney, an organ known to

express a variety of MMPs such as MMP1, -2, -3, -9, -13, -14, -24, 25, -27, and -28 (Catania et al., 2007). Thus this cell-based gene-vector approach is ideal for testing our fusion proteins into the EAE mouse model.

CHAPTER 5

THE THERAPEUTIC EFFECTS OF LAP-MMP-mEPO IN CHRONIC RELAPSING EAE

EAE

EAE Origins and Development

In 1885 Louis Pasteur developed the first rabies vaccine comprising an emulsion of dried spinal cords of rabbits inoculated intracranially with the rabies virus. Treatment was generally accepted and consisted of 10-day courses of the vaccine. However, in around 30% of the vaccinated patients sporadic paralysis occurred followed by mortality. Histological analysis confirmed that they had developed post–vaccinal encephalomyelitis (PVE) which was easily distinguishable from rabies by the lymphoid infiltrates and demyelination around the blood vessels of the CNS, and the relative sparing of the nerve fibres and nerve cell bodies.

In the 1930's, Thomas Rivers investigated those cases of paralysis by the Pasteur vaccine as the neurological symptoms resembled those shown by patients infected with smallpox, vaccinia and measles. In his experiments on monkeys and rabbits that had received repeated injections of rabbit brain (Schwentker and Rivers, 1934, Rivers et al., 1933, Rivers and Schwentker, 1935), he discovered varying titers of brain-specific antibodies and the number of affected animals varied according to the antigenicity of the emulsion given. The disease was therefore caused by factors in the spinal cord and not by the inactivated virus (Rivers et al., 1933, Wisniewski and Bloom, 1975, Kabat et al., 1946). By including the bacterium *Mycobacterium tuberculosis* in the emulsion—utilizing an adjuvant strategy—the production of antibodies was boosted and the number of injections required to induce the disease in the animals was reduced. The results were an autoimmune encephalomyelitis, now known as EAE.

The dominant characteristic of EAE was that of a relapsing remitting course of the illness. An animal would become blind, followed by recovery of its vision only to return to blindness several days later. Paralysis of one limb would subside followed by paralysis of another appendage. Histological studies

found acute lesions adjacent to blood vessels, and infiltration of the vessel walls with leukocytes, and chronic lesions demonstrating an increasing number of lymphocytes and proliferation of microglia (Baxter, 2007).

EAE As We Know It

EAE is one of the most studied models for autoimmune-mediated diseases. It was initially termed experimental allergic encephalomyelitis as its autoimmune origins were still under debate. This debate was resolved with the development of EAE by the adoptive transfer of sensitised T cells into naïve syngeneic rats and the term changed to 'experimental autoimmune encephalomyelitis' (Schluesener and Wekerle, 1985, Ben-Nun and Cohen, 1981). EAE has since been replicated in several species including guinea pigs, rabbits, pigs, goats, mice, hamsters, dogs, sheep, marmosets and even chickens (Gold et al., 2000). A number of variations of EAE have been developed by altering the species and strain and what type of antigen was used for its induction, for example models for optic neuritis, and relapsing–remitting MS and progressive MS.

The EAE Mouse Model

No single animal model exists that mimics all the features of the human demyelinating diseases especially for MS but EAE is not one single model rather a collection of models that vary in their disease course, animal species and strain, antigen used for induction and in the type of adjuvant employed. Mice are the preferred animal for the development of an animal model of human disease due to the vast diversity of molecular biology techniques and reagents available. Mice are easy to genetically manipulate and generate transgenics and gene knock-outs, and there is a wealth of genetic information that is available especially regarding their immune system.

The immunisation of mice to actively induce EAE is either by the injection of CNS tissue such as brain or spinal cord homogenates, of purified components of the CNS myelin – such as MBP (Bernard and Carnegie, 1975), PLP, MOG, and myelin-associated glycoprotein (MAG), with the exception of S-100

which is a component of glial cells (Gold et al., 2000), or through the adoptive transfer of sensitised $CD4^+$ T-cells (Kennedy et al., 1990, Schluesener et al., 1988, Bernard et al., 1976).

Initially only a monophasic acute form of EAE could be induced in which most animals experienced perivascular inflammation and minimal demyelination with spontaneous recovery. Monophasic EAE is best suited for studying the mechanisms involved in down-regulation of the immune response evident in the vast perivascular accumulation of monocytes, lymphocytes and Mø (Lublin et al., 1981). By adapting the right induction protocol or strain a relapsing-remitting disease or a chronic relapsing EAE (CREAE) model could be induced (Kozlowski et al., 1987, Lublin et al., 1981, Brown and McFarlin (1981) cited in Amor et al., 2005). Chronic EAE models have persistent paralytic disease that consists of an acute phase followed by spontaneous remittance, then further relapses and are characterised by inflammation, demyelination and axonal damage (Lublin et al., 1981, Kozlowski et al., 1987). Animals often do not fully recover and remain with some degree of permanent neurologic deficit (Lublin et al., 1981, Ben-Hur, 2010). The CREAE model is best suited for studying the mechanisms involved in inflammation mediated neuronal degeneration such as MS as both the EAE mouse model and MS are “inflammatory demyelinating diseases of the CNS whose development and course varies according to several genetic and environmental determinants” (Arredondo, 2003)

EAE vs. MS

Multiple Sclerosis is a disease characterised by neuroinflammation, demyelination, astrogliosis, remyelination, and neuronal injury, whose clinical history follows a pattern of relapses with periods of complete recovery. However, over time these periods of relapses are marked by recovery periods that are incomplete and residual disabilities persist and accumulate, until the disease progresses without remission. Unlike the multiphasic nature of MS, EAE on the other hand is mostly monophasic, with few models demonstrating the secondary progressive characteristics of MS such as progressive disability following relapses and remissions, demyelination, gliosis, remyelination and neuronal injury. Dependant on the type of species, strain, and immunising agent, the EAE model has

the ability to resemble MS in many respects, leading to it being the sole model for the study of MS. It displays several features that are found in MS such as demyelination of nerve fibres, axonal sparing, lesions of the CNS that differ in both space and time, the maturation of these lesions from inflammation to demyelination through to remyelination and gliosis, and the presence of immunoglobulin in both the CNS and cerebrospinal fluid (CSF) (Baxter, 2007), making EAE an extremely informative and invaluable animal model for the disease. Indeed, the drugs used in the treatment of MS (e.g. Copaxone, mitoxantrone and Natalizumab) were originally founded through their ability to improve the clinical symptoms of EAE (Lisak et al., 1983, Ridge et al., 1985, Mauch et al., 1992, Yednock et al., 1992, Aharoni et al., 1999). And although some clinical trials emerging from studies done on EAE models have failed (such as Infliximab, Sulfasalazine and Linomide (Ziemssen, 2008)), it still remains an excellent model for studying MS.

It is precisely the ability of the model to be flexible and adaptive that enables it to be the 'prototypical autoimmune model' (Sriram and Steiner, 2005). For example $CD4^+$ T lymphocytes were originally thought to play a pivotal role in the pathogenesis of MS but recent evidence suggests that $CD8^+$ T lymphocytes play an equally crucial role (Mars et al., 2011). If the model is induced with MBP or PLP, then the role of $CD4^+$ lymphocytes and their putative role in the pathology of EAE can be elucidated, similarly if induced with MOG, then the role of $CD8^+$ lymphocytes can be investigated.

EAE has the ability to mimic the phases associated with MS depending on the strain of mouse or species and the type of antigen used to induce EAE. The acute phase can be induced in SJL, PL/J, C57BL/6 and the Biozzi AB/H mouse strains, to name a few, using the CNS antigens previously mentioned. The relapsing phase can be induced in the Lewis rat model using monoclonal antibodies specific for MOG (Linington et al., 1992). The CREAE model can be induced in the Biozzi AB/H mouse using spinal cord homogenate without the need for pertussis vaccine usually given to induce reliable relapses. The monophasic model is best suited for studying the mechanisms involved in down-regulation of the immune response; the relapsing model is best suited for studying the mechanisms

involved in relapses; and the chronic progressive model is best suited for studying the mechanisms involved in inflammation mediated neuronal degeneration (Voskuhl, 2010).

Biozzi Mice

The AB mouse was originally engineered through selection of outbred random populations of the Swiss Albino mouse by Guido Biozzi giving two stains of high (AB/H) and low (AB/L) antibody lines (Biozzi et al., 1972). AB/H mice have high titre antibody responses to certain antigens such as sheep erythrocytes and are able to be induced with a number of experimental diseases such as autoimmune collagen-induced arthritis and EAE (Biozzi et al., 1972, Amor et al., 2005).

Baker and Colleagues (1990) first described the induction of EAE in the Biozzi mouse through an increase of mycobacterium levels in the spinal cord homogenate leading to the development of a reproducible and predictable form of CREAE with high incidence and virtually no mortality. Onset of the disease occurs between 15 and 20 days following inoculation with relapses occurring between 33 and 39 days following inoculation. Mice are induced either with an additional inoculation generating synchronised relapses, or left to undergo spontaneous relapses. Young and old, male and female mice are equally susceptible. This high degree of susceptibility may be due to the large numbers of antigen specific T cells capable of inducing delayed–type hypersensitivity (Biozzi 1985 cited in Baker et al., 1990) and why once the disease has been initiated, it is able to trigger further autoimmune relapses (Figure 5.1).

As mentioned before, the majority of EAE models tend to follow a monophasic display of acute inflammation without subsequent relapse-remission leading to irreversible disability, a characteristic of chronic MS. They inadequately model the sequence of events that occurs pathologically in time and clinical outcome (Baker et al., 1990, Hampton et al., 2008). The AB/H CREAE model however, demonstrates little demyelination within the CNS during the acute phase of the disease, with inflammation and demyelination being major pathological features within the relapsing phase, a pattern that closely mimics secondary progressive MS (Hampton et al., 2008). Additionally, it

behaves in a predictable relapse-remission pattern, with a period of relapse that progressively results in disability. The accumulation of these disabilities is known to include tremors and spasticity, characteristics that are found in secondary progressive MS (Baker et al., 2000). Inflammation is characterised by the infiltration of mononuclear cells such as Mø containing myelin debris and lipid deposits and $CD4^+$ T cells expressing αβ receptors and low levels of IL-2 in areas of pro-inflammatory growth factors as seen in MS (Hofman et al., 1986, Allen et al., 1993, Baker et al., 1990, Baker et al., 1991). The expression of adhesion molecules such as ICAM-1 on CNS endothelial cells provides evidence for cellular infiltration (Baker et al., 1991). Another pathological characteristic of MS is that of demyelinating and inflammatory lesions developing within the optic nerves. EAE induced in the Biozzi AB/H mouse exhibits similar demyelinating lesions (O'Neill et al., 1998). During the chronic phase, the AB/H mouse induced with EAE follows a predictable course of relapse-remission, followed by progressive disability (such as tremors and spasticity) without remission that is accumulative (Baker et al., 1990, Hampton et al., 2008). The Biozzi AB/H CREAE model also demonstrates significant demyelination and remyelination, hypercellularity, a marked increase in the astrocytic marker glial fibrillary acidic protein (GFAP) relating to astrocytic gliosis, axonal and neuronal loss—distinctive characteristics found within MS, therefore making the Biozzi AB/H CREAE the preferred model for this study.

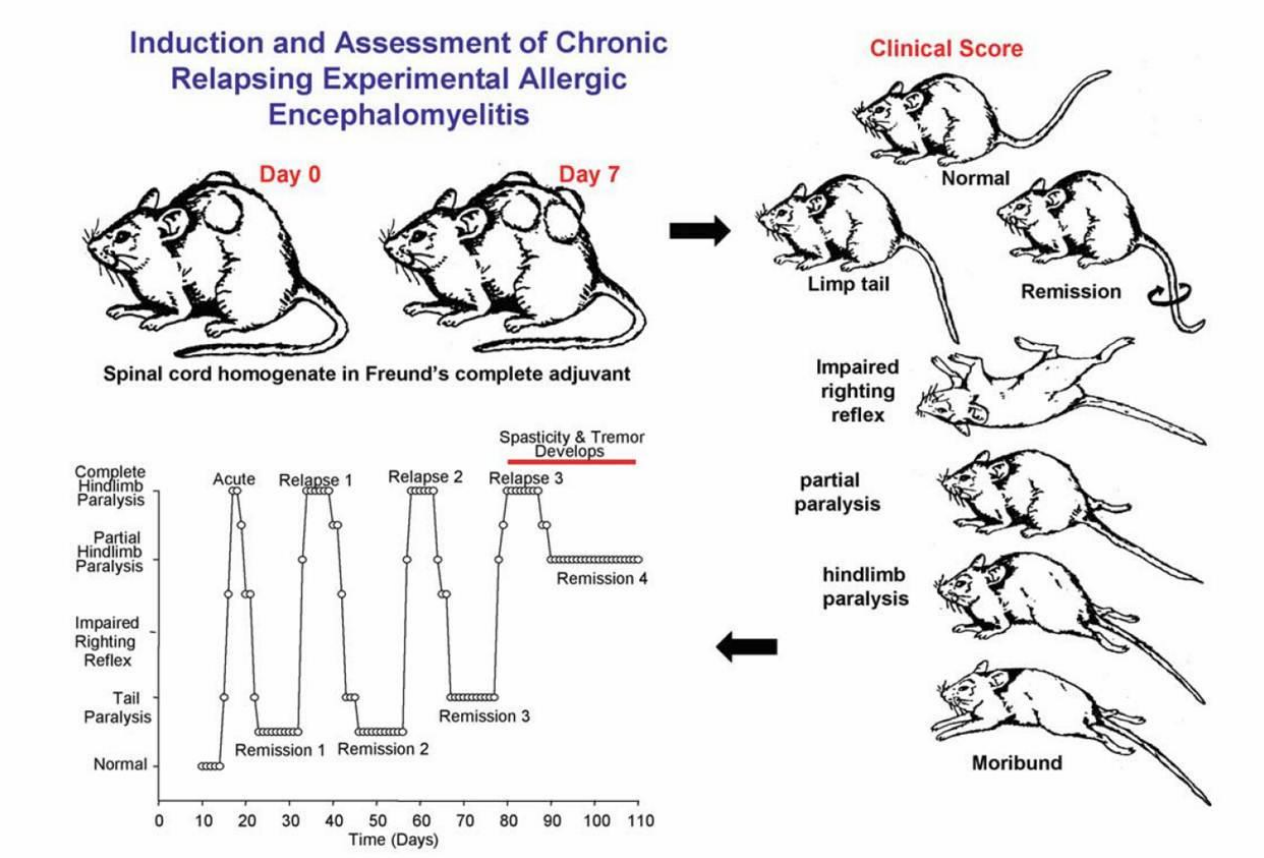


Figure 5.1. Induction and clinical course in an EAE model. Taken from (Baker and Jackson, 2007).

CHAPTER HYPOTHESIS AND AIMS

The previous chapter showed that a lentiviral vector could be constructed to contain the DNA of the latent protein mEPO and that this protein remained latent until the MMP cleavage site was cleaved by MMP enzymes. The following experiments tested the hypothesis that when permanently transduced LAP-MMP-mEPO.tsF cells are injected into mice affected by CREAE, the latent peptide LAP-MMP-mEPO is cleaved and becomes biologically active. Moreover, that this biologically active mEPO does not cause any of the unwanted side effects noted with high-dose systemic peptide exposure. Additionally, it is able to reduce the progression of the disease and its clinical score.

The aim of this study was to assess the therapeutic potential for the *in vivo* delivery of *ex vivo*-transduced fibroblasts in the CREAE mouse model. Following the injection of permanently transduced tsF cells expressing LAP-MMP-mEPO and mEPO proteins into AB/H with CREAE, the progression and/or remission of disease and its effect on weight loss/gain were assessed. The biological activity of EPO released from the latent fusion protein secreted from the transplanted LAP-MMP-mEPO.tsF transduced cells, was assessed indirectly through measurements of the haematocrit.

RESULTS

After establishing that the *in vivo* delivery of LAP-MMP-mEPO.tsF transduced cells resulted in the biological activity of EPO as seen in the increase in haematocrit levels (see Chapter 4), and that expression of luciferase in the biological assay for TGFβ using MLEC cells was detected using LAP-MMP-huTGFβ, the therapeutic potential of LAP-MMP-huTGFβ and LAP-MMP-mEPO in the CREAE model was investigated.

Preliminary LAP-MMP-huTGFβ and LAP-MMP-mEPO Response in AB/H CREAE Model

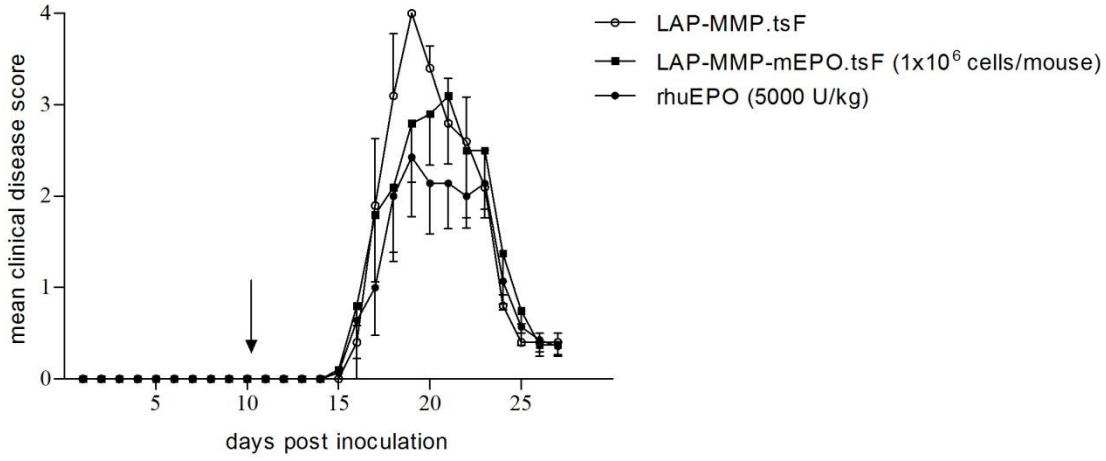
Dr Anne Rigby performed these preliminary experiments using LAP-MMP-huTGFβ.tsF and LAP-MMP-mEPO.tsF cells during my maternity leave and includes the figures 5.2 and 5.3.

Induction of the AB/H CREAE mouse model occurred on days 0 and 7 with spinal cord homogenate and Freund's Complete Adjuvant by s.c injection. Five groups, each consisting of eight mice, were injected with a total of 10^7 tsF cells/mouse on day 11 post-inoculation. Three treatment groups were injected i.p. with $1x10^6$ cells/mouse of LAP-MMP-mEPO.tsF transduced cells (Figure 5.2), or either 1- or $3x10^6$ cells/mouse LAP-MMP-huTGFβ.tsF transduced cells (Figure 5.3) expressing ~2330ng/ml mEPO/10^6 cells/24 hours or ~1700ng/ml huTGFβ/10^6 cells/24 hours respectively, or 5000IU/kg bw rhEPO, made up to a total of 10^7 cells with untransduced tsF cells. The control group consisted of CREAE induced mice that received an i.p. injection of 10^7 LAP-MMP.tsF cells. In this particular model of EAE, the mice can be made to undergo synchronised relapses through additional inoculations of 1mg syngeneic spinal cord homogenate and 60µg of mycobacteria, however these mice were allowed to undergo spontaneous relapse.

The scoring of the groups followed the system used by Baker et al (1990): The treatment groups ($1x10^6$ LAP-MMP-mEPO.tsf, $1x10^6$ LAP-MMP-huTGFβ.tsF and $3x10^6$ LAP-MMP-huTGFβ.tsF transplanted cells) entered the acute phase from day 15 with clinical symptoms peaking to 3.9±0.7,

3.3±0.9 and 3.7±0.6 respectively, and dropping to 0.5±0.0 during remission. The rhEPO control group entered the acute phase on day 15 with a disease severity peaking at a clinical score of 3.2±0.8, falling to 0.5±0.0 during remission.

Although no significant difference between the treatment groups was observed, the LAP-MMP-mEPO.tsF group displayed lower clinical scores as compared to the LAP-MMP.tsF control group, albeit not at levels coinciding with rhEPO. Conversely, in the LAP-MMP-huTGFβ.tsF treatment group injected with $1x10^6$ cells/mouse, a significant difference ($P \leq 0.001$) in clinical score was observed as compared to the LAP-MMP.tsF control group. However, when the number of cells injected was



increased to $3x10^6$ cells/mouse this significance was lost.

Figure 5.2. Therapeutic effects of LAP-MMP-mEPO.tsF cells in acute EAE.
AB/H Biozzi mice were immunised by subcutaneous injection of 1mg spinal cord homogenate in complete Freund's adjuvant at day 0 and 7. Animals were injected i.p. on day 10 (arrow) with $1x10^6$ cells/mouse of either LAP-MMP.tsF transduced cells (negative control cells) or LAP-MMP-mEPO.tsF transduced cells (treatment cells). A positive control group of mice was injected i.p, 3 times a week, with 5000IU/kg bw of recombinant human EPO (rhuEPO) protein (administration of 5000IU/kg bw of rhuEPO has been shown to increase the haematocrit (Agnello et al., 2002). The results represent the mean clinical disease score ± SEM of all animals within the group (n=8). (**P ≤ 0.005 compared to LAP-MMP.tsF injected mice, 2 way ANOVA followed by Tukeys post-hoc test).

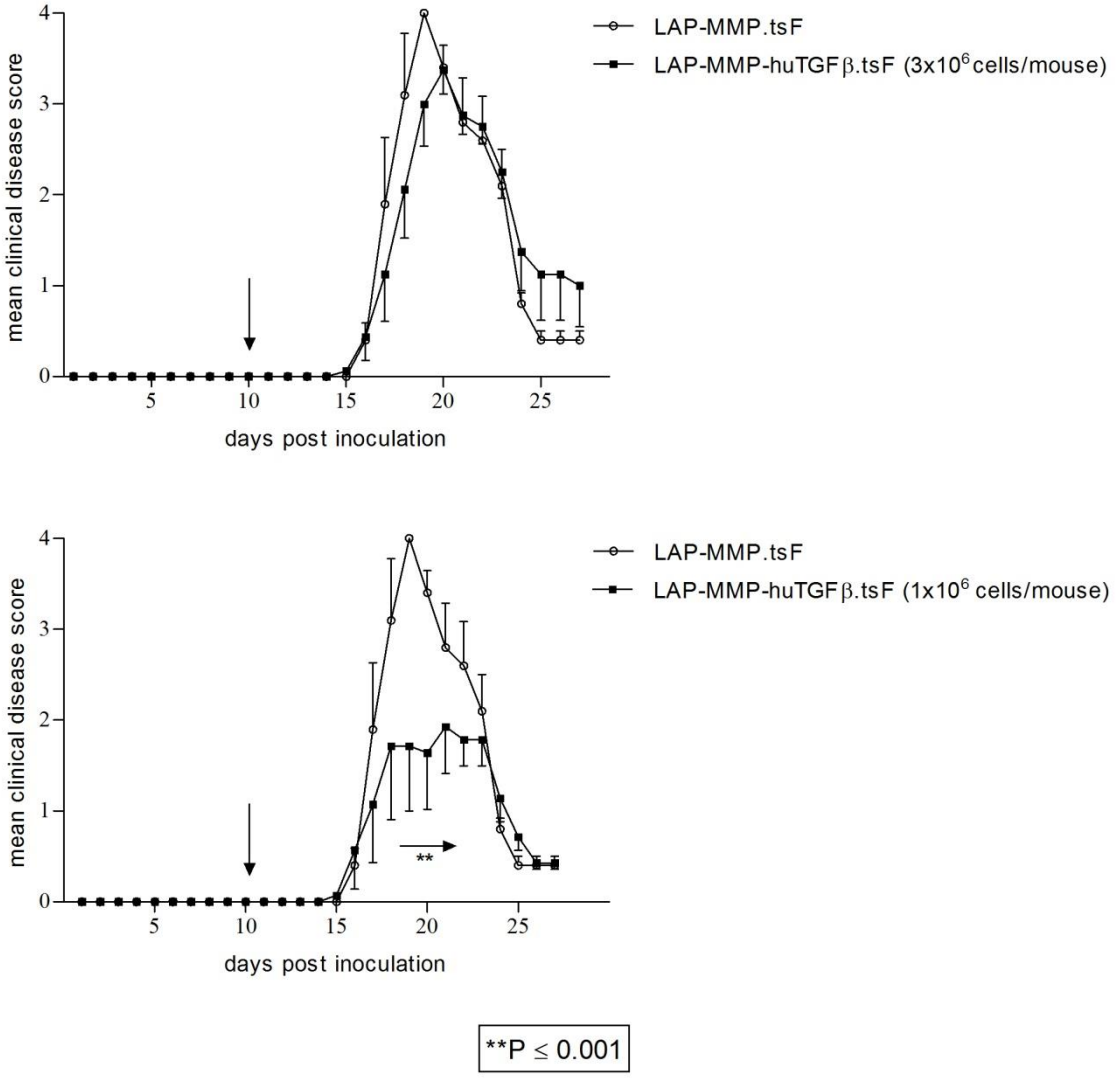


Figure 5.3 Therapeutic effects of LAP-MMP-huTGFβ.tsF cells in acute EAE.
AB/H Biozzi mice were immunised by subcutaneous injection of 1mg spinal cord homogenate in complete Freund's adjuvant at day 0 and 7. Animals were injected i.p. on day 10 (arrow) with a syngeneic fibroblast cell line (either 1- or $3x10^6$ cells/mouse) which was transduced with either construct: control LAP-MMP (LAP-MMP.tsF) or LAP-MMP-huTGFβ (LAP-MMP-huTGFβ.tsF). The results represent the mean clinical disease score ± SEM of all animals within the group (n=8). (**P ≤ 0.005 compared to LAP-MMP.tsF injected mice, 2 way ANOVA followed by Tukeys post-hoc test).

Latent EPO Dose Response in AB/H CREAE Model

Induction of the AB/H CREAE mouse model occurred on days 0 and 7 with spinal cord homogenate and Freund's Complete Adjuvant by s.c injection. Four groups, each consisting of ten mice, were injected with a total of 10^7 tsF cells/mouse on day 11 post-inoculation. Three treatment groups were injected i.p. with tsF cells permanently transduced with LAP-MMP-mEPO.tsF at concentrations of either $1x10^6$, $2x10^6$ or $3x10^6$ cells/mouse, expressing 2µg/ml mEPO/10^6 cells/24 hours made up to a total of 10^7 cells with untransduced tsF cells. The control group consisted of CREAE induced mice

that did not receive an injection of tsF cells. In this particular model of EAE, the mice can be made to undergo synchronised relapses through additional inoculations of 1mg syngeneic spinal cord homogenate and 60µg of mycobacteria, however these mice were allowed to undergo spontaneous relapse.

Induction of CREAE

A characteristic of the Biozzi CREAE model is its high incidence of induction. This was consistent in this study with 35 out of 40 mice (87.5%) experiencing an acute phase. Of those mice that developed acute EAE, 28 (86% of $1x10^6$ group, 70% of $2x10^6$ group, 80% of the $3x10^6$ group and 75% of the EAE control group) experienced spontaneous relapses (Table 5.1).

Clinical symptoms for the treatment groups, $1x10^6$, $2x10^6$, $3x10^6$ and the EAE Control were preceded by an initial weight loss of 4.44%±3.04, 6.20%±2.83, 3.74%±5.33 and 3.31%±2.13 respectively and continued to decrease as the severity of clinical symptoms increased. This was followed by an increase in weight gain and a reduction in the severity of clinical symptoms indicating entering into a phase of remission in agreement with the literature (Baker et al., 1990). As expected, the mice remitted to a low clinical score demonstrating a small neurological deficit evident in the animals inability to raise its tail (grade (1)). The onset of a relapse was indicated by an increase in the severity of clinical symptoms and associated weight loss.

Measurement of Biologically Active EPO in vivo

The indirect measurement of released, biologically active EPO from the latent EPO expressing tsF cells was done through haematocrit measurements on days 10 and 35 post cell transplantation (days 21 and 46 post-inoculation respectively). The haematocrit values demonstrated a dose response following the transplantation of $3x10^6$ LAP-MMP-mEPO.tsF cells, $2x10^6$ LAP-MMP-mEPO.tsF cells, and $1x10^6$ LAP-MMP-mEPO.tsF cells when compared to the EAE control (61.2%±3.2, 56.4%±3.2, 53.5%±5.2 and 42.3%±3.0 respectively) on day 10, following cell transplantation. The values were significantly elevated when compared against the EAE control ($P \leq 0.0001$ using one-way ANOVA

with Tukey post-hoc test) as well as the highest and lowest doses demonstrating a significant difference when compared against one another (P ≤ 0.001) (Figure 5.4).

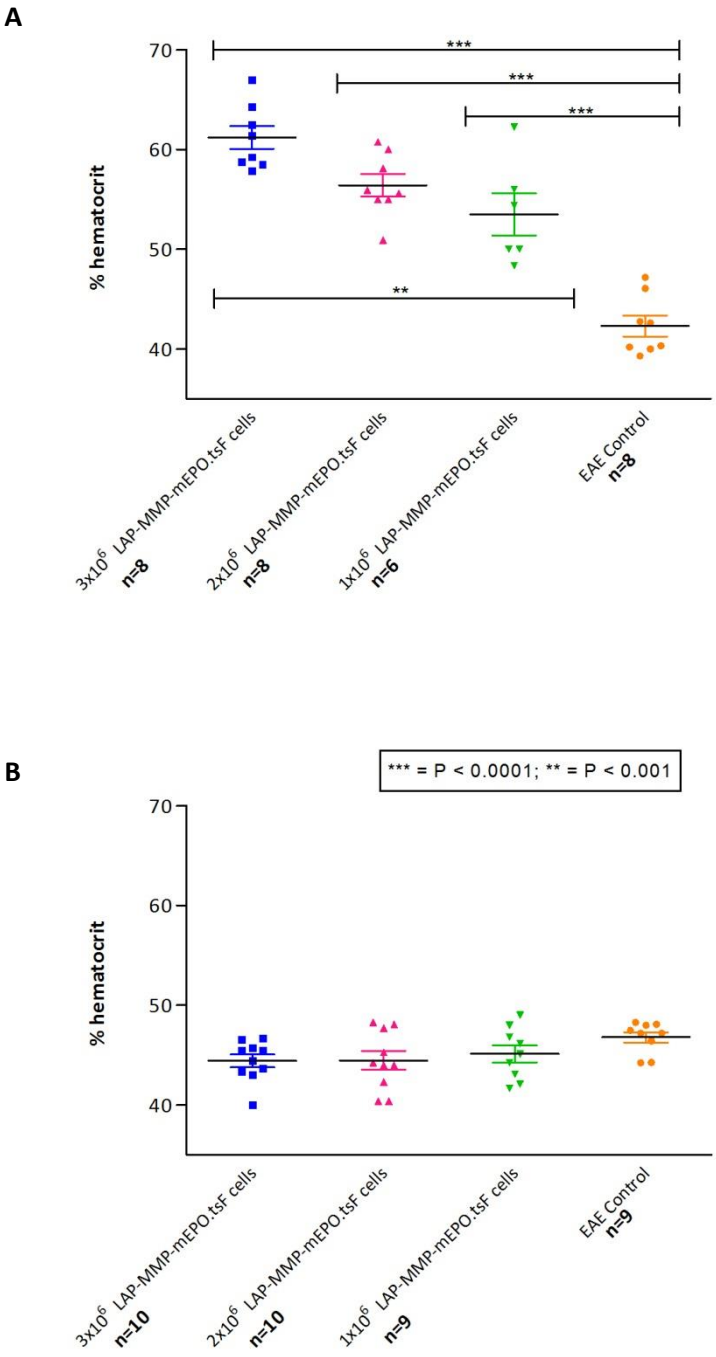


Figure 5.4: Biological activity of latent EPO is observed 10 days after tsF cell transplantation but is absent after 35 days. Following the i.p. injection of LAP-MMP-mEPO.tsf cells expressing up to 6µg/ml latent mEPO/ml/24 hours/mouse, the biological activity is assessed by haematocrit measurements taken on days 10 **(A)** and day 35 **(B)** following cell transplantation. A significant difference is seen as compared to the EAE control (***P≤ 0.0001) and between the highest dose of $3x10^6$ cells (6µg latent mEPO/ml/24 hours/mouse) and the lowest dose of $1x10^6$ cells (2µg latent mEPO/ml/24 hours/mouse) (**P ≤ 0.001). Significance was determined using a one-way ANOVA followed by Tukey post-hoc test.

Latent EPO Dose Effect on EAE Development

The scoring of the groups followed the system used by Baker et al (1990): The treatment groups ($1x10^6$ LAP-MMP-mEPO.tsf, $2x10^6$ LAP-MMP-mEPO.tsF and $3x10^6$ LAP-MMP-mEPO.tsF transplanted cells) entered the acute phase from day 11 with clinical symptoms peaking to 3.8±0.3, 3.5±0.4 and 3.7±0.3 respectively, and dropping to 0.5±0.0 during remission (Table 5.1). The EAE control group entered the acute phase on day 13 with a disease severity peaking at a clinical score of 3.7±0.3, falling to 0.5±0.0 during remission (Table 5.1).

The onset of first relapse was sharper in the control group than in the treatment groups (Table 5.1; Figure 5.5). Clinical symptoms peaked at 3.6±0.7, 3.3±0.7 and 3.1±0.6 respectively for the first relapse with the EAE control peaking at 3.3±1.1. Symptoms fell to 1.0±0.4, 0.8±0.3 and 0.8±0.3 respectively compared to the control at 0.8±0.3 during first remission. During the second relapse, a score of 3.5±0.7 (n=2), 2.7±0.3 (n=3) and 3.1±0.7 (n=5) for the treated groups and a score of 3.3±1.1 (n=2) for the EAE control was reached (Figure 5.5). Although no statistically significant difference between the treatment groups was observed, a delayed but milder relapse was observed for the treatment groups. I hypothesize that this delay occurs only because of the distruction of the injected tsF cells and that, if the experiment were to be repeated with transduced tsF cells that had been adapted to be grown in serum-free conditions, without the immunogenicity (previously determined by (Rigby, 2009) the clinical score would remain mild and the relapse would be supressed. The treatment groups $1x10^6$ LAP-MMP-mEPO.tsF and $3x10^6$ LAP.MMP.mEPO.tsF each had one mouse that had to be euthanized to comply with animal welfare regulations (Table 5.1).

Table 5.1: Clinical signs in Biozzi AB/H CREAE mouse model transplanted with EPO-secreting fibroblast cells.
The mice were injected subcutaneously with spinal cord adjuvant in CFA on days 0 and 7. They were weighed and checked daily for a maximum of 60 days post-inoculation for clinical signs. The disease course was divided into different disease phases: the acute phase = onset of clinical symptoms (graded (1)–5 and *) occurring after an initial weight loss of >1.5g (disease onset day 11); remission phase = decline in clinical symptoms following disease phase and an increase in weight; and relapse phase = the reappearance of clinical symptoms (graded (1)–5 and *) following period of remission. Mean disease scores were calculated for each disease phase by including all susceptible animals in each group.

		Number of animals in each disease phase					
		Acute	**Remission**	**Relapse**	**Remission**	**Relapse**	**Remission**
1x10^6 LAP-MMP-mEPO.tsF cells							
Complete Paralysis	(4)–4	6	-	4	-	1	-
Partial Paralysis	(3)–3	-	-	1	-	3	-
Inverted Righting Reflex (IRR)	(2)–2	-	-	1	1	-	-
Limp tail	1	-	-	-	5	-	-
Limp tail with Tone	(1)	-	6	-	1	-	-
Normal	0	-	-	-	-	-	-
*Mean score (n=6)**		*3.8±0.3*	*0.5±0.0*	*3.6±0.7*	*1.0±0.4*	*3.5±0.7*	*0.0*[a]
2x10^6 LAP-MMP-mEPO.tsF cells							
Complete Paralysis	(4)–4	5	-	3	-	-	-
Partial Paralysis	(3)–3	2	-	3	-	1	-
Inverted Righting Reflex (IRR)	(2)–2	-	-	-	-	-	1
Limp tail	1	-	-	-	1	-	1
Limp tail with Tone	(1)	-	7	-	2	-	-
Normal	0	-	-	-	-	-	-
Mean score (n=7)		*3.5±0.4*	*0.5±0.0*	*3.3±0.7*	*0.8±0.3*	*2.7±0.3*	*1.3±0.4*
3x10^6 LAP-MMP-mEPO.tsF cells							
Complete Paralysis	(4)–4	9	-	4	-	2	-
Partial Paralysis	(3)–3	-	-	4	-	3	-
Inverted Righting Reflex (IRR)	(2)–2	-	-	1	1	-	-
Limp tail	1	-	-	-	4	-	2
Limp tail with Tone	(1)	-	9	-	4	-	-
Normal	0	-	-	-	-	-	-
*Mean score (n=9)**		*3.7±0.3*	*0.5±0.0*	*3.1±0.6*	*0.8±0.3*	*3.1±0.7*	*1.0±0.0*
EAE Control							
Complete Paralysis	(4)–4	6	-	3	-	1	-
Partial Paralysis	(3)–3	-	-	3	-	1	-
Inverted Righting Reflex (IRR)	(2)–2	-	-	-	-	-	-
Limp tail	1	-	-	-	4	-	1
Limp tail with Tone	(1)	-	6	-	2	-	-
Normal	0	-	-	-	-	-	-
Mean score (n=6)		*3.7±0.3*	*0.5±0.0*	*3.2±0.6*	*0.8±0.3*	*3.3±1.1*	*1.0±0.0*

a No mouse had entered remission by end of experiment for this group.
* Loss of mouse

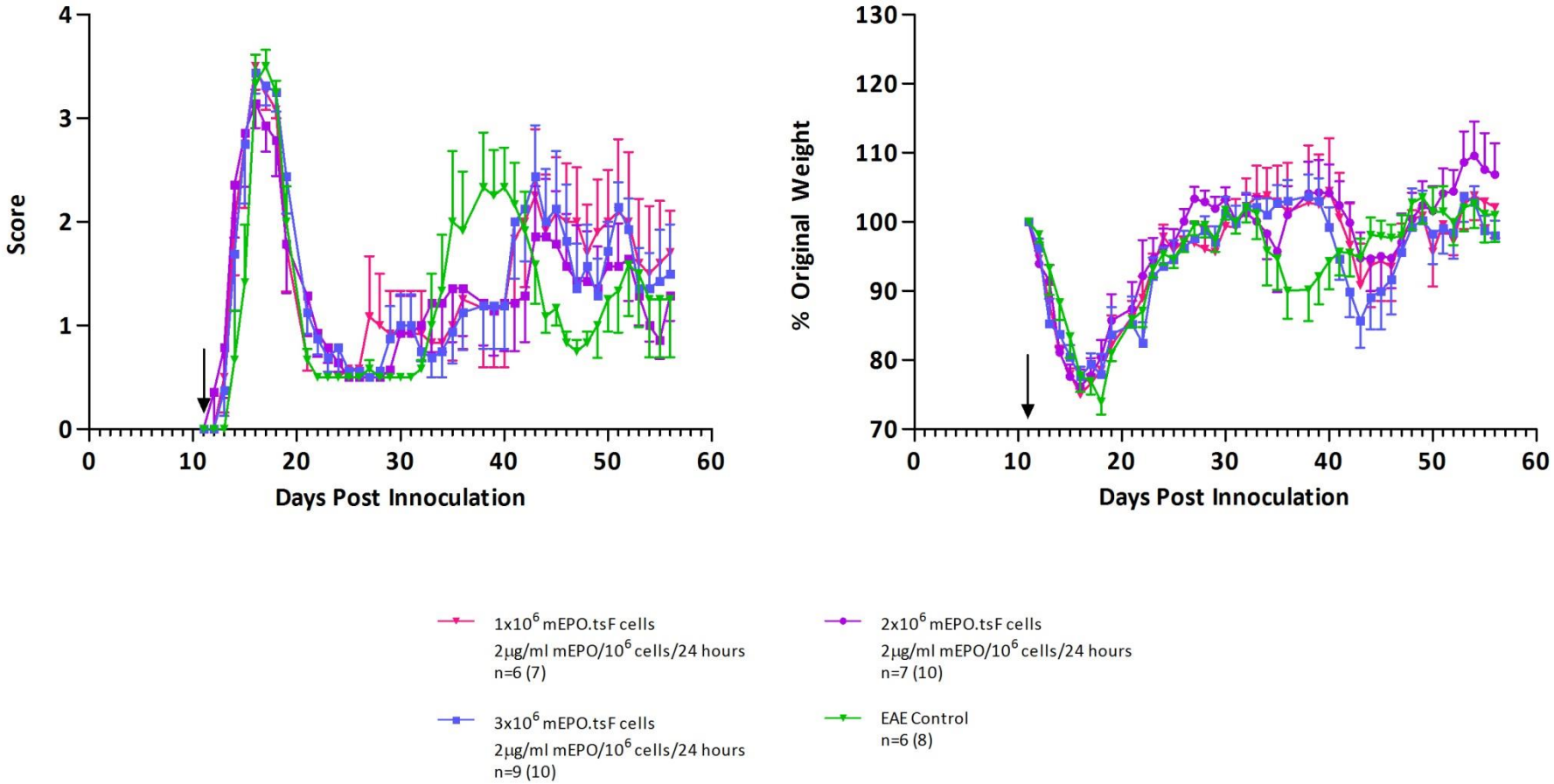


Figure 5.5: A milder, delayed relapse in the AB/H EAE mouse model is observed following dose response of latent EPO expressing tsF cells.
I.p. injection of 10^7 tsF cells expressing up to 6µg/ml of latent EPO per day displayed no significant change in disease score and/or weight loss/gain, but rather a milder delayed relapse. The number in brackets corresponds to the number of animals that underwent an acute phase with 'n' numbers corresponding to the number of those animals that underwent at least one spontaneous relapse. The arrow indicates the day of cell transplantation.

Table 5.2: Day of onset for first relapse in the Biozzi AB/H CREAE mouse model.
Values are for mice with an initial onset of relapse that occurred prior to day 31, during days 31 and 43, or after day 43 post-inoculation. The percentages are in relation to the number of mice within the individual treatment group ('n' in brackets).

Day of Relapse	>31	31-43	>43
$1x10^6$ LAP-MMP-mEPO.tsF cells (n=6)			
Number of mice	1	4	1
% of group entering relapse	*17%*	*67%*	*17%*
$2x10^6$ LAP-MMP-mEPO.tsF cells (n=7)			
Number of mice	1	4	2
% of group entering relapse	*14%*	*57%*	*29%*
$3x10^6$ LAP-MMP-mEPO.tsF cells (n=9)			
Number of mice	2	5	2
% of group entering relapse	*22%*	*56%*	*22%*
EAE Control (n=6)			
Number of mice	0	6	0
% of group entering relapse	*0%*	*100%*	*0%*

Dose Response of Latent EPO Fails to (Significantly) Ameliorate Disease.

When comparing groups, no significant difference in day of onset (Table 5.2), disease severity (Table 5.1) or degree of weight loss was observed within the acute phase in any of the groups as compared to the control group (Figure 5.5).

However, the treatment group that received $2x10^6$ LAP-MMP-mEPO.tsF cells (expressing a total of 4µg/ml latent mEPO/24 hours/mouse) exhibited better clinical scores during the acute phase and second relapse than either of the other groups, with scores comparable to the control during the initial relapse and both remissions (Tables 5.1). This experiment was therefore repeated using the dosage of 4µg/ml with additional control groups (LAP-MMP.tsF and untransduced tsF cells) (Figure 5.6).

The untransduced tsF control group developed acute EAE on day 13, followed by the treatment group LAP-MMP-mEPO.tsF and the control groups LAP-MMP.tsF and EAE on day 14. The degree of

severity of the clinical score during the acute phase was 3.6±0.5 for the LAP-MMP-mEPO.tsF treated group and the EAE control; and 3.6±0.6 and 3.4±0.6 for the LAP-MMP and untransduced tsF cells respectively (Table 5.3). All groups demonstrated a low clinical score during remission (0.6±0.2 for the treated group, untransduced tsF and LAP-MMP control groups; and 0.8±0.3 for EAE control) and fell into first relapse with clinical scores of 3.7±0.7, 3.7±1.0, and 3.1±0.5 for the treated, LAP-MMP and EAE control groups respectively. No significant difference between groups was observed. However the tsF control showed a lower overall clinical score of 2.9±0.5 reflected in the score and weight loss graphs (Table 5.3; Figure 5.6). At the time of experiment end, the group appeared to be relapsing and it can be said that this group experienced a delay in first relapse. One mouse control group that received 10^7 LAP-MMP.tsF cells died during relapse as indicated by the asterisk (Figure 5.6). However, this was the only mouse in the experiment that died as a result and was gaining weight in the preceding days.

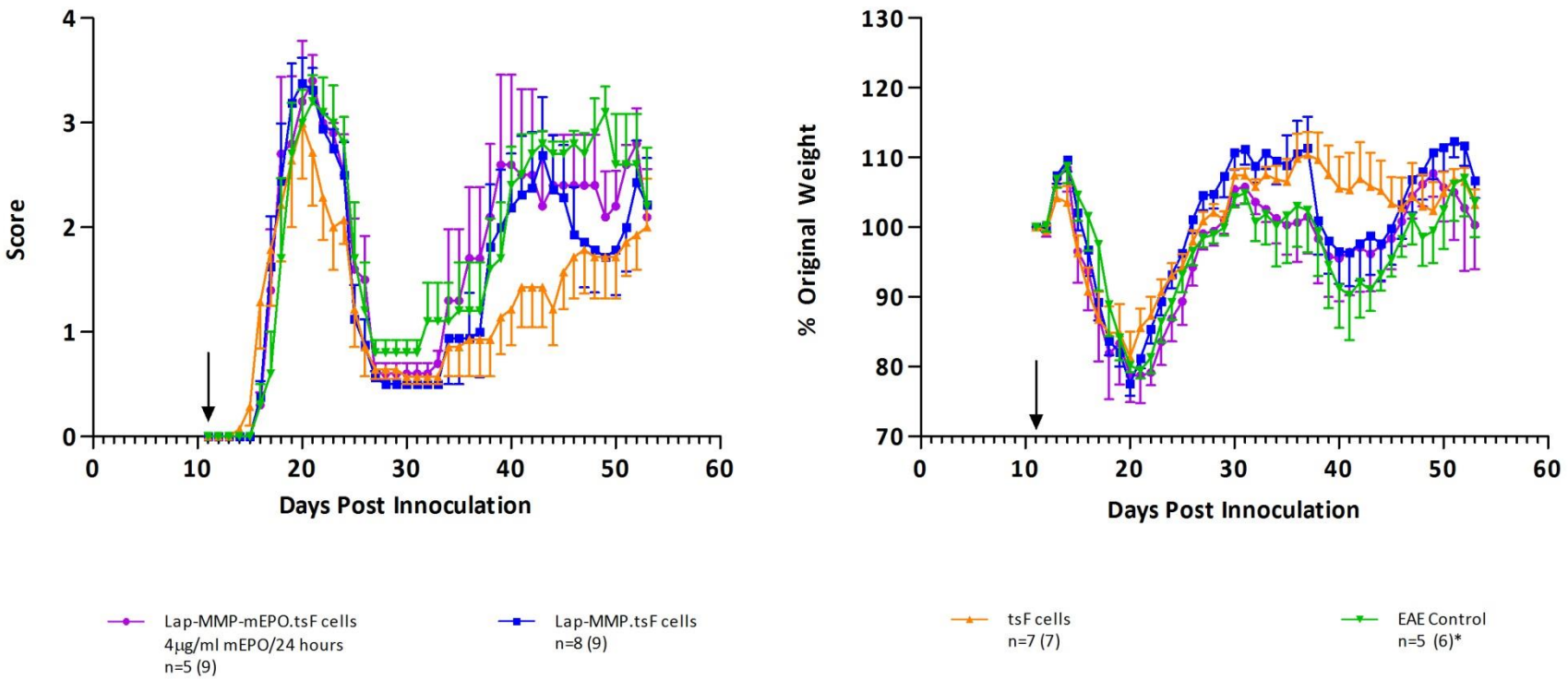


Figure 5.6 Treatment of chronic EAE transplantated with tsF cells.
I.p. injection of 10^7 tsF cells expressing 4µg/ml of latent EPO per day displayed no significant change in disease score, weight loss/gain or to day of disease onset. The untransduced tsF control group displayed a tendency for reduced clinical score and less weight loss compared to the other groups. The number in brackets corresponds to the number of animals that underwent an acute phase with 'n' numbers corresponding to the number of those animals that underwent at least one spontaneous relapse. The day of cell transplantation is indicated by the arrow. Asterisk indicates death of a mouse.

Table 5.3: Clinical signs in Biozzi AB/H CREAE mouse model transplanted with fibroblast cells secreting 4µg/ml LAP-MMP-mEPO.

The mice were injected subcutaneously with spinal cord adjuvant in CFA on days 0 and 7. LAP-MMP-mEPO.tsF cells (10^7 cells/mouse) were injected on day 11 post-innoculation. They were weighed and checked daily for a maximum of 60 days post-inoculation for clinical signs. The disease course was divided into different disease phases: the acute phase = onset of clinical symptoms (graded (1)–5 and *) occurring after an initial weight loss of >1.5g (disease onset day 11); remission phase = decline in clinical symptoms following disease phase and an increase in weight; and relapse phase = the reappearance of clinical symptoms (graded (1)–5 and *) following period of remission. Mean disease scores were calculated for each disease phase by including all susceptible animals in each group. Asterisk indicates death of mouse within group.

		Number of animals in each disease phase			
		Acute	**Remission**	**Relapse**	**Remission**
2µg/ml LAP-MMP-mEPO/24 hours/mouse					
Complete Paralysis	(4)–4	3	-	4	-
Partial Paralysis	(3)–3	2	-	1	2
Inverted Righting Reflex (IRR)	(2)–2	-	-	-	-
Limp tail	1	-	1	-	1
Limp tail with Tone	(1)	-	4	-	-
Normal	0	-	-	-	-
Mean score (n=5)		*3.6±0.5*	*0.6±0.2*	*3.7±0.7*	*1.9±1.0*
LAP-MMP Control					
Complete Paralysis	(4)–4	5	-	5	-
Partial Paralysis	(3)–3	3	-	1	3
Inverted Righting Reflex (IRR)	(2)–2	-	-	1	1
Limp tail	1	-	1	-	1
Limp tail with Tone	(1)	-	7	-	-
Normal	0	-	-	-	-
Mean score (n=8)		*3.6±0.6*	*0.6±0.2*	*3.7±1.0*	*2.1±0.7*
Untransduced tsF cells					
Complete Paralysis	(4)–4	3	-	1	-
Partial Paralysis	(3)–3	4	-	6	1
Inverted Righting Reflex (IRR)	(2)–2	-	-	-	2
Limp tail	1	-	1	-	-
Limp tail with Tone	(1)	-	6	-	2
Normal	0	-	-	-	-
Mean score (n=7)		*3.4±0.6*	*0.6±0.2*	*2.9±0.5*	*1.5±0.9*
EAE Control					
Complete Paralysis	(4)–4	3	-	1*	-
Partial Paralysis	(3)–3	2	-	4	1
Inverted Righting Reflex (IRR)	(2)–2	-	-	-	-
Limp tail	1	-	3	-	2
Limp tail with Tone	(1)	-	2	-	-
Normal	0	-	-	-	-
*Mean score (n=5)**		*3.6±0.5*	*0.8±0.3*	*3.1±0.5*	*1.8±0.5*

LAP-MMP-mEPO initiates early disease onset

Although no significant difference was observed for the degree of clinical severity during the relapse between groups, a significant difference was observed in the average day of relapse onset between the mice transplanted with the LAP-MMP-mEPO.tsF cells (28.4±9.3) and either the untransduced tsF cells (43±6.5; $P \leq 0.001$) or the LAP-MMP control (40.9±6.7; $P \leq 0.05$) (Figure 5.7). No difference in day of onset was observed when comparing the treatment group LAP-MMP-mEPO.tsF, LAP-MMP.tsF or the untransduced tsF cells with the EAE control group (37.4±3.8).

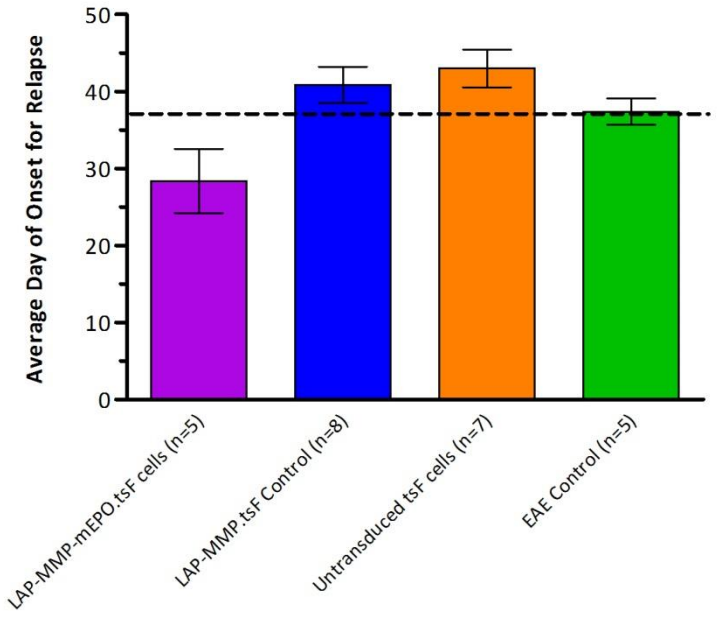


Figure 5.7: Disease onset in CREAE mice transplanted with LAP-MMP-mEPO.tsF cells.
The i.p. injection of 10^7 tsF cells expressing 4µg/ml of latent EPO per day displayed a significant change in day of relapse onset when compared to LAP-MMP.tsF cells ($P \leq 0.05$) or untransduced tsF cells ($P \leq 0.001$). Significance was determined using One-Way ANOVA with the Tukey post-hoc test. Dashed line indicates the average day of onset for the EAE control which displayed no significant difference with either treatment group.

Amongst the mice that underwent an acute phase, 72% fell into relapse between day 31 and 43. Of the mice that received the transduced LAP-MMP-mEPO.tsF cells, 40% fell into relapse before day 31 with none relapsing after day 43. However, of those mice that received either the LAP-MMP.tsF transduced cells or untransduced cells, none experienced an early onset of relapse compared to the EAE control. Furthermore, 75% and 57% respectively fell into relapse between days 31 and 43, with 25% and 43% respectively falling into relapse after day 43.

As observed in the previous experiment, no statistically significant difference in disease day of onset, day of relapse onset, weight loss or clinical score was observed when the CREAE model was injected with LAP-MMP-mEPO.tsF cells expressing 4µg/ml latent mEPO/24 hours/mouse as compared to the EAE, LAP-MMP.tsF and untransduced tsF controls (Figure 5.6).

Syngeneic untransduced tsF cells may suppress disease.

When comparing the untransduced tsF groups against the EAE control group, a significant difference in the clinical score during relapse ($P \leq 0.05$) was seen. This significance was however not reflected in its corresponding weight loss (Figure 5.6). Altogether, the mice in the tsF treated group appeared to maintain their weight above that in which they began the experiment.

Latent EPO fails to ameliorate disease or delay onset of relapse.

When the day of disease onset between all treatment groups and all control groups over both experiments were synchronised, no discernable difference was observed between severities of clinical scores or delay of relapse onset. It can however be said, that the treatment of mice with LAP-MMP-mEPO.tsF cells expressing up to 4µg/ml latent mEPO/ 24 hours/mouse displayed a trend towards relapse suppression prior to day 30 (Figure 5.5). However, this effect was either reduced or removed after day 30 resulting in a delay in relapse peak and remission. This effect will need to be investigated further by checking the haematocrit levels on day 30 and every 3 days thereafter. Interestingly, the tsF cells alone (untransduced cells) appeared to prolong the onset of the disease. Potentially, the amount of endogenously expressed TGFβ from the tsF cells observed by our lab (unpublished data), may be in sufficient concentrations to act on the induction of the disease.

DISCUSSION

The aim of this study was to determine if the *ex vivo* transduction of tsF cells with lentivirus engineered to express the latent proteins LAP-MMP-mEPO and LAP-MMP-huTGFβ, would result in a biologically active protein that exerts a therapeutic and regulatory effect *in vivo* in the Biozzi CREAE mouse model. The Biozzi ABH mouse has proven itself to be a valuable strain in inducing a relatively predictable and reproducible model for relapsing-remitting disease (Baker et al., 1990). Guido Biozzi originally selected outbred Swiss albino mice for studies into the susceptibility of infection, due to their ability to produce mice with either high antibody (ABH) or low antibody (ABL) titers (Biozzi et al., 1972). Inbred strains of these mice allowed researchers to study susceptibility of autoimmunity (Baker et al., 1990). Induction of CREAE occurs through the repeated injection of syngeneic spinal cord homogenate in CFA in ABH mice without the need for pertussis toxin, but not in the ABL strain. Further research found that the myelin oligodendrocyte glycoprotein (MOG) peptide could induce similar disease in these animals, but with accentuated demyelination and antibody deposition (Morris-Downes et al., 2002). Other studies found that severe relapsing-remitting disease could be induced by the myelin protein PLP but not with another myelin protein, MBP (Amor et al., 1993, Amor et al., 1996) and so did myelin-associated glycoprotein (MAG). Other non-myelin peptides that have been found to induce EAE in this strain include αβ crystallin (Thoua et al., 2000) and glial fibrillary acid protein (GFAP) (Amor et al., 2005).

The growth factor EPO has previously been shown to result in disease amelioration in other models of EAE (Agnello et al., 2002, Zhang et al., 2005, Savino et al., 2006), and the use of TGFβ has shown both disease amelioration and disease exacerbation in the EAE model (Mirshafiey and Mohsenzadegan, 2009). Interestingly, the injection of TGFβ plasmid DNA in the periphery failed in ameliorate disease progression, however when TGFβ plasmid DNA was coupled to a cationic-liposome complex, a reduction in EAE development was observed (Croxford et al., 1998). The

increase in efficiency of a therapeutic when injected directly into the CNS has also been observed with other molecules such as TNFα (Baker et al., 1994).

Preliminary experiments injected LAP-MMP-huTGFβ.tsF, LAP-MMP-mEPO.tsF, and LAP-MMP.tsF cells i.p. into the CREAE mouse model, along with a group injected with rhEPO (5000IU/kg body weight [bw]). Those mice treated with LAP-MMP-huTGFβ.tsF cells demonstrated a lower disease score during initial attack when compared to the LAP-MMP.tsF control group. The groups injected with LAP-MMP-mEPO or rhEPO demonstrated a trend towards a lower clinical score although this was not statistically significant when compared against the LAP-MMP.tsf control group. It has recently been shown that the erythropoietic and tissue-protective effects of EPO function through two different receptor units and at different concentrations (tissue protection only occurs at high concentrations whereas erythropoiesis at low concentrations). It was hypothesized that although a trend to lower the clinical score of EAE was observed when CREAE mice were injected with LAP-MMP-mEPO.tsF, the levels of EPO (once released by MMP cleavage) were not high enough to initiate tissue protection. Therefore a subsequent experiment was designed to inject 3 groups of mice with either 1-, 2- or $3x10^6$ LAP-MMP-mEPO.tsF cells expressing 2μg/ml mEPO/10^6 cells/24hrs as these levels of mEPO are within the range of tissue-protection. However in the AB/H CREAE model, the LAP-MMP-mEPO protein failed to demonstrate disease amelioration or a delay in disease onset or relapse. The initial attack began 2 days earlier in those groups treated with the latent EPO expressing tsF cells when compared against the EAE control. Interestingly, once the acute phase was resolved, the treated groups underwent a milder and presumably delayed relapse compared to the control group. It is hypothesised that this relapse is actually the suppression of the original relapse by the exogenous EPO generated by tsF cells that secreted the latent EPO protein, but that are destroyed by day 32 post–transplantation, as demonstrated by an increase of clinical symptoms; or that the relapse was delayed due to the tsF cells themselves. These hypotheses need further investigation.

From the haematocrit profiles, biologically active EPO release from the latent protein was in circulation when the acute phase resolved but was absent when measured on day 35 post–cell transplantation. This infers either the loss of the transplanted tsF cells or the loss of latent EPO production by the transplanted tsF cells. It is unlikely that the latter is true as the cells were manitained in culture for one month and their expression tested prior to transplantation. A possible reason for the former may be in the production of antibodies against either the tsF cells or against the fusion protein. A study by Rigby et al. (2009) tested serum taken from EAE mice transplanted with untransduced tsF cells, tsF cells expressing fusion proteins and from naïve mice for binding antibodies. High levels of antibodies were found in the serum from mice that received the transplantation of tsF cells (both transduced and untransduced), but very few were found in the serum from naïve mice. This was unexpected as the cells are syngeneic to the mice and should not exert an immunological response. As the serum from untransduced tsF cells cultured *in vitro* exhibited antibodies against it, it is unlikely that the neo gene product is responsible for the clearing of these cells *in vivo.* As mentioned earlier, these cells are permanently transduced with a temperature sensitive SV40 large T-antigen that is rapidly degraded at body temperature (*in vivo*) or when grown at 37°C (*in vitro*) and therefore unlikely to be a source of immunogenicity. The growth of the tsF cell line *in vitro* is dependent on the presence of serum in the medium. BSA was therefore a likely candidate for the antigenicity demonstrated. Indeed, upon further investigation, it was determined that BSA did in fact induce the production of antibodies. The immunological response against the BSA present in the transplanted tsF cells may be the cause of the eventual loss of latent protein expression through systematic destruction of the cells, possibly by peritoneal Mø found at the site of injection. The ability of tsF cells to be grown *in vitro* in serum-free conditions was investigated but the cells failed to survive for longer than 48 hours. The use of an adaptation protocol (Sinacore 2000) may allow for tsF cells to be grown in serum–free conditions whilst still expressing the latent fusion proteins. A repeat of the study with tsF cells adapted to serum-free growth conditions would provide strong evidence that the BSA contained in serum–containing

growth medium may have induced the production of the antibodies thus leading to the possible destruction of the tsF cells *in vivo* evident at day 35 post-transplantation.

Although the disease score did not differ significantly when comparing the controls with the mice that received $2x10^6$ LAP-MMP-mEPO.tsF cells (4µg/ml latent mEPO/24 hours), using a One way ANOVA with the Tukey post-hoc test to compare the groups, the overall status of the animals was improved in the LAP-MMP-mEPO.tsF group, as it is evident from the weight loss curves reported in Figure 5.7, where at the end of the experiment on day 54 the body weight loss was observably less in mice treated with LAP-MMP-mEPO.

The low number of mice that underwent an acute phase and experienced a spontaneous relapse in the treated groups resulted in high SEM. This was resolved by repeating the experiment using the dose of 4µg/ml latent mEPO/24 hours/mouse (equivalent to $2x10^6$ LAP-MMP-mEPO.tsF cells) and including additional controls LAP-MMP.tsF cells and untransduced tsF cells, thereby increasing the number of mice for statistical analysis. The data generated was synchronised to day of disease onset to allow for biological variability. However, no significant difference in disease onset or disease amelioration was observed. Once again prior to day 29 following disease onset, the mice treated with the latent mEPO protein entered relapse but the clinical severity appeared to be suppressed. On day 30 this effect was gone leading to a continued relapse. It is therefore highly plausible that, in combination with the haematocrit data stating that the EPO effect was negligible by day 35 post transplantation, the cells were destroyed due to the immunogenic effect discovered by Dr Rigby in the days leading up to the onset of relapse. The replication of this experiment with transduced tsF cells expressing the latent EPO protein that had been grown in serum–free conditions, may demonstrate a different outcome. Therefore it cannot be conclusively stated that the latent EPO protein does not ameliorate EAE and further studies need to be performed with frequent haematocrit readings taken.

Surprisingly, the control group injected with untransduced tsF cells showed significant disease amelioration when compared with the EAE control, and the LAP-MMP-mEPO.tsF and LAP-MMP.tsF treatment groups. Untransduced tsF cells have been found to express low levels of TGFβ (2.1ng/ml/10^6 cells/24hrs (Croxford et al., 2000)) which potentially act to inhibit the onset of EAE. Indeed even 3ng/ml active TGFβ has been shown to inhibit the proliferation of over 92% of T cells and can inhibit the development of EAE if given prior to disease onset (Racke et al., 1991) Conversely, several studies have shown that TGFβ can exacerbate EAE for example, through the increase in Th17 production and subsequently the expression of the proinflammatory growth factor IL17 (Mirshafiey and Mohsenzadegan, 2009). It would therefore be beneficial if investigations were made into whether TGFβ has a 'therapeutic window' in which levels over and above a certain threshold switch it from having a positive effect in MS to a negative one. It may be that the low levels of TGFβ expressed by the untransduced tsF cells is within the threshold of beneficial effects, whereas the higher levels of TGFβ expressed by LAP-MMP-huTGFβ cells may be on the cusp of beneficial vs. deleterious effects of the protein, where an increase in expression levels actually lead to exacerbation of the disease rather than amelioration. This appears to indeed be the case as CREAE mice injected with 3 times as many LAP-MMP-huTGFβ.tsF cells show little difference when compared to the LAP-MMP.tsF control group.

CHAPTER 6

GENERAL DISCUSSION

Growth factors have a multitude of functions and interact with receptors in a diverse number of organs and tissues, and as such are able to elicit serious side effects when found in high concentrations. For example, the growth factor EPO is produced in the foetal liver and adult kidney and interacts with receptors found throughout the body, including the brain. But as it promotes the formation of new red blood cells, high concentrations lead to serious complications such as thrombosis and heart failure. Interestingly, studies have shown that treatment of EAE with EPO leads to a decrease in clinical severity, however the concentrations of EPO required to deliver these therapeutic effects are high and therefore unsuitable for human clinical trials. This study utilized the LAP technology originally described by Adams et al. (2003) to render EPO latent through its fusion to an MMP cleavage site attached to the LAP peptide of human TGFβ. The resulting LAP-MMP-mEPO fusion protein was shown to be inactive until incubated with recombinant MMP1, whereby the released mEPO protein was found to be biologically active *in vitro.* This demonstrates the ability of the technology to overcome the limitations of most growth factors, the implications of which are highly significant. The design and construction of the DNA vectors used to manufacture the latent growth factors is modular i.e. the LAP peptide, the MMP cleavage site, and the protein of interest site are all easily removable, and the protein of interest can be removed or inserted simply through two common restriction sites (*NotI* and *XbaI*), making it very attractive to 'chop-and-change' therapeutic protein targets as the need or research arises. The mere fact that a growth factor can be released into the body of an animal and not interact with the multitude of receptors available to it is, in itself, a remarkable achievement. It is this versatility to confer latency to a number of other growth factors that lends itself to a broad range of clinical applications, such as cancer, atherosclerosis and other autoimmune diseases.

MS is an autoimmune disease that is characterised by lesions in the brain, areas demarcated by demyelination and leukocyte infiltrates. Although growth factor therapy has shown therapeutic potential in EAE models for MS, the high concentrations needed and the location of where the growth factor is required presents serious problems in their future consideration as a therapeutic

molecule. The LAP technology has advantages as a targeted drug delivery system. The latent fusion protein remains inactive until it is released at sites of inflammation, areas demonstrating high levels of MMP activity such as lesions in MS. This technology therefore presents itself as a potential solution to overcome the difficulty in accessing the CNS without surgical procedure, or the administration of high concentrations of therapeutic drug to elicit the therapeutic effect. This study has shown that this modular design can be used to generate lentivirus capable of infecting syngenic cells, cells that are able to produce the latent fusion protein *in vivo*, an extension of the LAP technology that has the potential to act as a self-sustaining treatment for a number of clinical applications.

MS is one of the most common neurological diseases affecting the young population today. It is defined as an autoimmune demyelinating disease whose aetiology is unknown but both environmental and genetic factors influence its susceptibility and/or severity. Pathologically it is characterised by an increase in inflammatory cell infiltration, OL loss and demyelination, microglial activation and astrocyte scarring within the CNS. The order of destruction is, as yet, unclear as to whether it is initially inflammation-driven followed by demyelination, or demyelinating followed by inflammation, with the former being the most generally accepted hypothesis. Nevertheless, the presence of monocytes, T lymphocytes (both $CD4^+$ and $CD8^+$) and B lymphocytes, and Mø provides a diverse and dynamic puzzle board on which to work with, both therapeutically and aetiologically.

Treatments have been aimed at resolving the disease from the perspective of autoimmunity with little success when targeting the demyelinating arm of the disease. The majority of these treatments and their failed counterparts were initially investigated for efficacy in the EAE model for MS. This model has been called the 'prototypical autoimmune model' as it can be manipulated to demonstrate specified points of the disease. MS is a disease that progresses through stages of relapse and remission to which point EAE can be induced to simulate, dependent on species, strain and antigen used for induction. Furthermore, these stages can be somewhat 'compartmentalised' in

their inflammatory and demyelinating aspects. For example, the induction of monophasic acute EAE in the Lewis rat with CNS proteins or purified myelin components is mainly inflammatory with very little demyelination, whereas the CREAE model in Biozzi AB/H mouse, induced with syngeneic spinal cord homogenate, demonstrates inflammation during the acute and initial relapsing phases with demyelination and remyelination, in addition to inflammation, occurring in the chronic phase (Hampton et al., 2008). It is through our research in these models, that we have become adept at inhibiting relapsing EAE. However, once the inflammatory component has been eliminated, the neurodegenearative component remains and is unresponsive to immunosuppression. The typical models of C57BL/6 mice and SJL mice used in the inflammatory studies are inadequate in studying the progressive phase of the disease, characterised by the neurodegenerative component. The C57BL/6 model is monophasic and primarily inflammatory, whilst the SJL EAE-induced model has a clinical course that is aggressive and has a variable frequency of relapses. The AB/H mouse model is very robust with a reproducible disease course with easily distinguishable relapses and is amenable to disease manipulation. It is easier to handle and the disease slowly evolves into progressive EAE with associated neurological damage. It shows persistent demyelination, gliosis and glial cell activation, remyelination as well as neuronal and axonal loss. In particular, it is possible to stop further relapse autoimmunity at any stage of the disease through immune tolerance induction allowing researchers to monitor long-term repair strategies (Pryce et al., 2005, Jackson et al., 2009).

But EAE is not MS; it does not evolve spontaneously, it requires a strong immunological kick-start (i.e. through the use of Freund's adjuvant) that is unlikely to exist during physiological conditions and is studied in thoroughly inbred animals, quite unlike the spontaneously evolving disease that appears in the genetically heterogenous human population. Despite these limitations, most of the principle processes of brain inflammation have been elucidated through studies on EAE and without them, the pathogenesis of MS and future therapies for its treatment could not be developed.

The use of growth factors for the treatment of disease is inherently problematic due to the pleiotropic nature of the proteins, their short half-life, the systemic administration of high concentrations to attain therapeutic levels in the targeted tissue, and their inevitable side effects as a result of such high concentrations. The construction of a latent growth factor utilizing the LAP of human TGFβ fused via a MMP site to the growth factor (Adams et al., 2003) circumvents many of these drawbacks. By encasing the biologically active growth factor IFNβ within LAP, its pleiotropic nature was silenced until its release through cleavage by MMP enzymes. The inclusion of the MMP site ensures that the release of the growth factor occurs predominantly at sites of elevated MMP activity such as areas of inflammation. The inclusion of the growth factor within the LAP allows not only for the targeting of growth factor release to these areas, but also attributes a longer half-life, for example LAP-MMP-VIP has a half-life of 31 hours as compared to 1.5 min for free VIP (Vessillier et al., 2012). Interestingly cleavage of the latent IFNβ occurred when incubated with CSF from rhesus monkeys with EAE but not from monkeys with collagen-induced arthritis. As increased MMP activity has been found within active lesions of multiple sclerosis and in animal models of EAE, this LAP technology presents a novel method for the treatment of MS with growth factors that have demonstrated ameliorating effects in the EAE model.

Three proteins in particular, EPO, TGFβ and IGF1, have demonstrated potential in the treatment of MS as elucidated from EAE data. The growth factor EPO has demonstrated neuroprotective and anti-inflammatory properties in several models of EAE through the suppression of pro-inflammatory growth factors and the inhibition of proliferating autoreactive T cells. Treatment of EAE with TGFβ has demonstrated anti-inflammatory effects by blocking the expression of growth factor induced endothelial adhesion molecules, by inhibiting pro-inflammatory growth factors and through its role in preventing the entry of autoreactive T cell into the CNS (Johns et al., 1991, Racke et al., 1991, Prehn et al., 1994, Croxford et al., 1998, Ruocco et al., 1999, Zhu et al., 2002). IGF1 is intricately involved in the remyelination aspect of the disease with its expression coinciding with the expression of IGFBP by OLs and astrocytes. It has also demonstrated an involvement in neural cell proliferation

and differentiation during development (McMorris et al., 1986, McMorris and Dubois-Dalcq, 1988, Mozell and McMorris, 1991, Shinar and McMorris, 1995, Annenkov, 2009).

By utilizing engineered latent growth factors mEPO, huTGFβ and isoforms IGF1A and B, I was able to determine that they could be made latent whilst still retaining their respective biological activities, activities which are only functional following MMP cleavage. The production of therapeutic proteins on a large scale by transforming DHFR- CHO cells to $DHFR^{+}$ transgene expressing CHO clones is routinely used and was initially attempted in this study to express the latent proteins. CHO cells are currently the "premier workhorse" for the production of therapeutic proteins with over 70% of all recombinant therapeutic proteins being produced by CHO cells (Jayapal et al., 2007). The therapeutic proteins Aranesp (Amgen) and Epogen/Procrit (Amgen/Ortho Biosci) used in the treatment of anaemia are recombinant EPO proteins produced by CHO cells. Mammalian cell are mainly used for the production of therapeutic proteins as they are able to produce biologically active forms that are correctly folded with appropriate post-translational modifications, predominantly glycosylation, unlike microbial hosts like *E.coli.* Interestingly, when coupled to the LAP shell containing the MMP cleavage site, latent EPO produced by CHO cells was not cleaved by MMP1. LAP and EPO are both highly glycosylated with N-linked glycosylation sites (3 and 4 sites respectively) that play pivotal roles in their production, secretion and biological activities. It may be that the pattern of carbohydrate moieties of EPO, coupled with the LAP shell, interferes with the folding of the latent protein in such a way that either the MMP enzyme cannot access its binding site or that the binding site is structurally hidden. The failure of MMP to cleave latent EPO was unexpected and the role of glycosylation was investigated. Unglycosylated latent EPO, expressed by CHO-S cells exposed to tunicamycin, succeeded in releasing the mature EPO molecule following MMP cleavage. Nonetheless, for latent EPO to be biologically active and therefore therapeutic, it would have to be glycosylated that, if produced by CHO cells, would render it uncleavable and therefore useless. Interestingly, the use of human EPO fused to the cleavable LAP-MMP shell has demonstrated cleavage when expressed by CHO DG44 cells (unpublished data, Dr. Sandrine Vessillier). Why MMP is

able to cleave EPO when it is the human homologue but not the mouse homologue is unclear. Further experiments mutating the EPO molecule should shed some light in this regard. Additionally, proteins rendered latent through the addition of the LAP shell are unable to reach a degree of purity that would allow *in vivo* testing. Therefore an alternative method of protein production was needed. Other mammalian cell lines such as those derived from baby hamster kidney, mouse myeloma and HEK-293T cells are regulatory approved cells for the production of therapeutic proteins (Wurm, 2004). The transient transfection of HEK-293T cells with latent growth factor constructs consistently produced high concentrations of secreted, cleavable and biologically active latent growth factors (including LAP-MMP-mEPO) as assessed by western blot analysis and the appropriate biological assays.

The use of protein therapy has been used extensively for the treatment of various diseases such as the treatment of MS with IFNβ. However, the prolonged systemic delivery of therapeutic proteins *in vivo* may be of more benefit than serial injections. Great success has been achieved in ameliorating or inhibiting EAE in the mouse model by transplanting *ex vivo* transduced syngeneic fibroblasts expressing TNF inhibitors or IL-10 (Croxford et al., 2000, Croxford et al., 2001). The transplantation of tsF cells from the AB/H mouse is an attractive method of cellular gene therapy as fibroblasts are easily cultured from skin biopsies thereby minimising any potential allogeneic immune response, whilst the cells additionally contain a temperature-sensitive T antigen mutant that is easily degraded at body temperature. The transduction of tsF cells with lentiviral vectors expressing our latent growth factors resulted in constitutively produced latent proteins that were able to be cleaved by MMP1 *in vitro*.

During a preliminary study of the tsF response *in vivo*, LAP-MMP-huTGFβ.tsF cells demonstrated a significant reduction in disease score of mice induced with EAE. It is well known and documented that TGFβ is a potent anti-inflammatory growth factor. It has demonstrated disease amelioration in previous studies of EAE. The reason as to why LAP-MMP-huTGFβ appeared to work less effectively at

higher does is unclear at this moment; no paper to date has been published stating the TGFβ produceses a dose-dependent bell curve. However these cells are not inert, they secrete various other factors that may interact with TGFβ such as BMP7 that could regulate TGFβ signalling.

When transplanted into AB/H CREAE mice, the LAP-MMP-mEPO protein demonstrated biological activity as seen by an increase in haematocrit values comparable to the literature (Agnello et al., 2002). However these values had subsided by day 35 after transplantation, presumably through the systematic destruction of the LAP-MMP-mEPO.tsF cells. Previous studies have shown that both the AB/H mouse and the DBA/1 mouse do not mount an immune response to these transduced tsF cells (Dr Anne Rigby, Dr Marinela Mendez-Pertuz, unpublished data). However it was determined that they were initiating an immune response to the BSA that had been absorbed on the cell membrane leading to there distruction (Rigby, 2009). The cells were transplanted on day 11 to coincide with the onset of clinical symptoms. The group treated with LAP-MMP-mEPO.tsF cells displayed no significant amelioration of disease or delay of onset compared to that of the control. Current literature has shown that amelioration of disease occurs when EPO is injected preventatively but not therapeutically (Savino et al., 2006). Additionally, onset of relapse appears to be expedited when treated with LAP-MMP-mEPO whilst demonstrating a reduction, albeit not a significant one, in clinical score that increased after a period of apparent suppression. It is plausible that the destruction of the tsF cells initiated the end of this period of suppression resulting in a consistent decrease in LAP-MMP-mEPO production, and that this effect is be due to the molecular and cellular environment (i.e. the types of cells surrounding them and their interactions with them, as well as the proteins and other molecules secreted through these interactions) surrounding the transplanted cells as the tsF control group demonstrated a similar delay in relapse.

However, the levels of serum EPO in the mice were not tested and therefore it cannot be said that the levels of recombinant EPO were sufficient to engage the tissue-protective receptor $(EPOR)_2/\beta cR$ necessary for sustained tissue-protection. Additionally, the expression of $(EPOR)_2/\beta cR$ during the

course of EAE has not yet been assessed. It may be expressed at very low levels, or may even be absent within lesions thereby interacting with the classical $(EPOR)_2$ receptor. Nonetheless, the worsening of clinical symptoms was not reflected in the weight of the mice suggesting an improvement in the overall status of the mice treated with latent EPO.

Interestingly, the group treated with untransduced tsF cells demonstrated significant disease amelioration during relapse as compared to the EAE control that was not seen when treated with latent EPO. It is known that the transduced fibroblasts secrete small endogenous levels of TGFβ which may account for the reduction in disease score. Furthermore, clinical symptoms appear to escalate following the surmised period of tsF cell destruction further substantiating the theory that it is the loss of the tsF cells that results in the return of clinical symptoms. Further experiments will need to be done in order to verify this.

Although fibroblasts were transduced with LAP-MMP-mIGF1A and LAP-MMP-mIGF1B, time and funding were critical and therefore only a limited amount of *in vivo* experiments could be performed. Futher funding would allow another researcher to continue this work, to determine whether IGF1A or IGF1B ameliorate EAE and provide neuroprotection during the relapse phase.

FURTHER EXPERIMENTS

The Biozzi AB/H CREAE model is the best representative of progressive MS available to date. It displays spontaneous relapses, a high degree of incidence and an acute phase that is predominantly inflammatory followed by periods of remission and relapse with inflammation and demyelination. Additionally, it displays demyelinating lesions within the optic nerves, a pathological characteristic of MS (O'Neill et al., 1998).

This study was undertaken using the AB/H CREAE model based on these characteristics, utilizing the general scoring system currently used for the majority of EAE murine models, with statistically acceptable numbers and additional control groups to account for untreated EAE, delivery of untransduced tsF cells, vehicle delivery (LAP-MMP) and dosage.

With these elements in place, the study was scientifically sound and corroborated the data generated from different rodent strains and induction protocols. Systemic administration of EPO has demonstrated a delay in disease onset with a reduction in clinical score, a reduction in disease severity and duration and in reduced body weight loss (Agnello et al., 2002, Savino et al., 2006, Li et al., 2004). This study demonstrated a tendency to increase the relapse rate albeit with reduced clinical score and severity that was reflected in a reduction in body weight loss when treated with latent EPO expressed by *ex vivo* transduced tsF cells. It can be suggested that should the transduced tsF cells be grown in serum-free conditions and a repeat of the study done whilst giving consistent results, that the data generated could translate into human MS subjects.

The tissue-protective properties of EPO are only initiated when EPO expression is at high levels as the tissue-protective receptor unit $(EPOR)_2/\beta cR$ has a low affinity for the ligand. Therefore any LAP-MMP-mEPO induced neuroprotection would need to occur at high levels locally. Therefore the inferred latency given by the fusion of LAP to EPO is ideal for the delivery of a high level of EPO

without the activation of the erythropoietic receptor $(EPOR)_2$ systemically, prior to receptor initiation within the CNS. However, at these levels the appearance of unwanted side effects such as thrombosis are possible as the EPO protein is not contained within the CNS, and the CNS does express $(EPOR)_2$ able to initiate erythropoiesis. Therefore the next step in utilizing this latency technology, would be to add LAP fused to the MMP cleavage site to the nonerythropoietic, EPO-mimicking peptide ARA290, thus creating a latent peptide that has no possibility of these aforementioned side-effects, but which is neuroprotective and only engages the $(EPOR)_2/\beta cR$ unit resulting in a high efficiency of neuroprotective signalling.

Modifications in the experimental design

This study could not conclusively demonstrate any therapeutic benefit of latent EPO in the treatment of MS. However, if the study were to be repeated utilizing the following modifications in its design, a conclusive outcome may be obtained. The modified experimental design should include weekly tail vein blood sampling under anaesthesia for determining a change in the haematocrit ratios, and three different 'therapeutic windows'—preventative, therapeutic and late therapeutic schedule—where the transduced EPO.tsF cells are transplanted on day of induction (day 0), day 10 and day 25 respectively to determine if the latent protein is most beneficial on a preventative basis as demonstrated by Savino (2006). Additionally, as tsF cells are grown under serum-containing conditions, it should be investigated as to whether these cells can be adapted for serum-free culturing conditions to avoid the targeted destruction of these cells due to their BSA antigens (Rigby, 2009). Should this prove to be unachievable, the direct infection of the mice with the lentivirus encoding latent EPO expression should be considered. It was identified that the transplantation of untransduced tsF cells ameliorated EAE. This was very unexpected and should be investigated and a growth factor/protein profile generated using protein array (chip) technology (capture array). This will enable us to quantify the growth factors expressed by the conditionally immortalised fibroblasts

and detect any that differ in their expression levels from control fibroblasts that have not been immortalised i.e. fibroblasts that have not been immortalised.

Alternatively, another attractive technology for the introduction of the tsf cells (transduced to secrete our therapeutic latent growth factor) into the CREAE mouse model, is the encapsulation of the cells. The encapsulation of the cells in a hydrogel, a cross-linking polymer, creates a physical barrier for transplanted cells against the host immune response through the prevention of immune cell and antibody infiltration. The cells are immobilized in a polymer matrix surrounded by a semipermeable membrane. The mesh structure is small enough to prevent immune cells and antibodies from entering, but large enough to allow the diffusion of oxygen, nutrients and other molecules. The choice of polymer used to encapsulate the cells needs to be biocompatible as it influences the rate of biodegradation (http://cdn.intechopen.com/pdfs/19720/InTech-The_therapeutic_potential_of_cell_encapsulation_technology_for_drug_delivery_in_neurological_disorders.pdf; http://homepages.cae.wisc.edu/~bme200/microencapsulation_fall05/reports/BME_400_Microencapsulation_Final_Report.pdf). Additionally the mesh structure created by the polymer determines the molecular weight cut-off. By generating a mesh structure with a molecular weight cut-off of 100kDa, antibodies (at around 150kDa) and the larger leukocytes are excluded from entering, offering the encapsulated cells immunoprotection, whilst allowing the cell secretions (such as the latent growth factors) to diffuse out of the capsule, and target the sites of inflammation. A problem encountered with cell encapsulation is cell necrosis brought about by large diffusion distances. Small diffusion distances of 100µm can avoid hypoxia of the cells. Additionally, the site of injection of the encapsulated cells can play an important part in the avoidance of cell necrosis for example subcutaneous injection, as it presents a better oxygen supply than peritoneal injection. Through the use of a hydrogel polymer, the encapsulation of the transduced tsF cells would allow for their immunoprotection whilst still allowing the secretion of the latent growth factors for diffusion to sites of inflammation within the brain. The cost of protein therapies becomes increasingly more with

inceased treatments. The continuous and sustained production of the latent growth factors *in vivo* afforded by the low rate of biodegradation may dramatically reduce the therapeutic costs involved (http://cdn.intechopen.com/pdfs/19720/InTech-The_therapeutic_potential_of_cell_encapsulation_technology_for_drug_delivery_in_neurological_disorders.pdf; http://homepages.cae.wisc.edu/~bme200/microencapsulation_fall05/reports/BME_400_Microencapsulation_Final_Report.pdf).

Quantification of *in vivo* protein production and clearance rate

The expression and secretion of the latent therapeutic EPO protein has been confirmed directly *in vitro* and indirectly *in vivo* in the haematocrit ratios. However, the precise concentration of protein produced is highly variable dependent on the number of surviving and viable tsF cells. It is imperative that the dose of latent protein produced *in vivo* in the EAE mouse model be determined. Assuming the levels of protein released within the CREAE model are within the detection range, a standard direct or sandwich ELISA can be performed. With a sandwich ELISA, two different antibodies of different species recognising two different epitopes of the growth factor protein can be used to identify the released growth factor with a high degree of specificity. However, if the levels of MMP expression within the CREAE model are not sufficient as to release a concentration of the growth factor detectable by ELISA or that the protein is degraded swiftly *in vivo* such that the serum levels are below the detection threshold of an ELISA (i.e. protein levels in the fentomol range) than an alternative method of protein detection is required, such as stable isotope labelling tandem mass spectrometry (SILT) (Bateman et al., 2007). This technique utilises the incorporation of the naturally occurring isotope carbon-13 into the amino acid leucine. Following immunoprecipitation of the protein from the CSF, blood and/or urine, the protein is digested with trypsin and the tryptic peptides quantified using tandem mass spectrometry. It is reproducible, robust, can be used *in vitro* or *in vivo*, and has the advantage of being able to correctly identify the protein analysed through

tandem mass spectrometry ions. Most importantly, it is able to quantify low abundance protein levels and clearance rates, as would be the case with our latent protein, that are beyond the detection limits of other established *in vivo* analytical approaches such as Gas Chromatography/Mass Spectrometry or Gas Isotope Ratio Mass Spectrometry.

Clinical Trials

The recent results of a clinical trial using EPO in the early treatment of acute ischemic stroke have highlighted an alarming increase in the risk of death and of further complications such as intracerebral haemorrhage, brain oedema and other thromboembolic events when combined with recombinant tissue plasminogen activator (rtPA) (Ehrenreich et al., 2009). It is possible that these effects are due to a dysfunction in the endothelium of vessels (Dame, 2010). Endothelial microparticles (EMP) are "submicron particles that are shed from plasma membranes in response to cell activation, injury and/or apoptosis" found in increasing circulatory levels during a worsening of endothelium-dependent vasodilation making them useful markers of endothelial dysfunction (Chironi et al., 2009). They are also the main carriers of circulating tissue factor (see below) (Horstman et al., 2009).

Currently, clinical trials of recombinant EPO (48000 IU i.v) in conjunction with methylprednisolone (1g i.v) in two stages (weekly administration for 12 weeks and bi-weekly for 12 weeks) are underway at the Rigshospitalet in Denmark (The Danish MS Research Centre; Rigshospitalet. The Effects of Erythropoietin on Clinical Disability and Brain Pathology in Patients With Progressive Multiple Sclerosis (EPO-ProgMS). In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2010 Oct 16]. Available from: http://clinicaltrials.gov/ct2/show/NCT01144117 NLM Identifier: NCT01144117). The side effects noted in the Acute Stroke Trial may occur during the EPO trial in Denmark as increased circulatory levels of EMP, associated with endothelial dysfunction, have been found in MS during exacerbations but not during remission (Minagar et al., 2001). EPO has been found to increase the expression of tissue factor on the extracellular matrix of endothelial

cells, leading to an increase in platelet adherence and therefore its thrombogenecity (Fuste et al., 2002). The neuroprotective properties of EPO occur through the interaction of the $(EPOR)_2/\beta cR$, similarly demonstrated by the neuroprotective action of EPO in tissue repair after stroke (Sautina et al., 2010). It may be that the side effects highlighted in the stroke trial are modulated through the homodimer EPOR, rather than $(EPOR)_2/\beta cR$ (Brines et al., 2004, Brines and Cerami, 2008, Tufekci and Genc, 2010). Furthermore, non-erythropoietic EPO derivatives such as asialoEPO or CEPO may circumvent those side effects as they have different procoagulant and vasoactive activities, however it has been found that derivatives of recombinant proteins may elicit an immunogenic response (Sauerborn et al., 2010, Coleman et al., 2006). Latent EPO is expected to be therapeutically useful at concentrations considerably lower than that required if delivered systemically as a fully active therapeutic molecule. Therefore it is unlikely that the increased side-effect profile or immunogenic response would pertain to latent EPO, however a small safety study should be done to verify this.

The potential benefit of EPO in the treatment of progressive MS cannot be conclusively drawn. Although there is a tendency to suppress the clinical severity of symptoms in the mice that underwent EPO treatment by ex vivo transduced tsF cells, it cannot be said that it is the role of EPO that affords this benefit as the relapse eventually does occur.

The multimodal protein TGFβ has been shown to have paradoxical effects in MS (Mirshafiey and Mohsenzadegan, 2009). Whether these effects are triggered through different ligand threshold concentrations is, as yet, unknown. When LAP-MMP-huTGFβ.tsF cells are injected into the CREAE mouse model of MS, the low ($1x10^6$ cells/mouse) but not the high ($3x10^6$ cells/mouse) dose resulted in disease amelioration. However it remains to be determined what levels of TGFβ are required to transfer the balance of positive effects in EAE (for example the generation of TH3 cells) to one of negative effects.

The remyelinating properties of IGF1 have highlighted this protein as a promising therapeutic molecule for MS. Although treatment with IGF1 has demonstrated both positive (the lack of

demyelination and an improvement in clinical score in EAE (Genoud et al., 2005, Cannella et al., 2000)) and negative results (such as unwanted side effects (Lovett-Racke et al., 1998)), the latency inferred by LAP would allow the protein to be released in small amounts that are specifically released at sites of increased MMP activity such as within the CNS. Additionally, as with EPO, IGF1 may act within a particular therapeutic window where outside those threshold levels its effects are negative. Unfortunately, due to time and monetary constraints, further experiments utilising LAP-MMP-mIGF1A and LAP-MMP-mIGF1B in the CREAE could not be done. Additionally, there have been no studies done (at the time of writing) investigating the functionality of the different isoforms IGF1A and IGF1B within EAE, or even within the CNS. This information would help us understand the further molecular versatility of this protein within the CNS and within MS.

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APPENDICES

APPENDIX 1

Annotated sequence of LAP-MMP-huTGFβ in pcDNA3.

```
>CMV Promoter (3' end)                                  >T7                                HindIII >LAP
|                                                       |                                  |       |
G TCT ATA TAA GCA GAG CTC TCT GGC TAA CTA GAG AAC CCA CTG CTT ACT GGC TTA TCG AAA TTA ATA CGA CTC ACT ATA GGG AGA CCC Aag ctT ATG CCG  < 900
                                                                                                                    M   P
>AarI
|
CCC TCC GGG CTG CGG CTG CTG CCG CTG CTG CTA CCG CTG CTG TGG CTA CTG GTG CTG ACG CCT GGC CGG CCG GCC GCG GGA CTA TCC ACC TGC AAG ACT A  < 1000
P   S   G   L   R   L   L   P   L   L   L   P   L   L   W   L   L   V   L   T   P   G   R   P   A   A   G   L   S   T   C   K   T   I

tc gac atg gag ctg gtg aag cgg aag cgc atc gag gcc atc cgc ggc cag atc ctg tcc aag ctg cgg ctc gcc agc ccc ccg agc cag ggg gag gtg cc  < 1100
D   M   E   L   V   K   R   K   R   I   E   A   I   R   G   Q   I   L   S   K   L   R   L   A   S   P   P   S   Q   G   E   V   P
Bsu36I
|
G CCC GGC CCG CTG CCC GAG GCC GTG CTC GCC CTG TAC AAC AGC ACC CGC GAC CGG GTG GCC GGG GAG AGT GCA GAA CCG GAG CCC GAG CCT GAG GCC GAC  < 1200
P   G   P   L   P   E   A   V   L   A   L   Y   N   S   T   R   D   R   V   A   G   E   S   A   E   P   E   P   E   P   E   A   D
AleI                          >RleAI
|                             |
TAC TAC GCC AAG GAG GTC ACC CGC GTG CTA ATG GTG GAA ACC CAC AAC GAA ATC TAT GAC AAG TTC AAG CAG AGT ACA CAC AGC ATA TAT ATG TTC TTC A  < 1300
Y   Y   A   K   E   V   T   R   V   L   M   V   E   T   H   N   E   I   Y   D   K   F   K   Q   S   T   H   S   I   Y   M   F   F   N
KpnI
Acc65I                                                                                  PmlI
|                                                                                       |
AC ACA TCA GAG CTC CGA GAA GCG GTA CCT GAA CCC GTG TTG CTC TCC CGG GCA GAG CTG CGT CTG CTG AGG CTC AAG TTA AAA GTG GAG CAG CAC GTG GA  < 1400
T   S   E   L   R   E   A   V   P   E   P   V   L   L   S   R   A   E   L   R   L   L   R   L   K   L   K   V   E   Q   H   V   E

G CTG TAC CAG AAA TAC AGC AAC AAT TCC TGG CGA TAC CTC AGC AAC CGG CTG CTG GCA CCC AGC GAC TCG CCA GAG TGG TTA TCT TTT GAT GTC ACC GGA  < 1500
L   Y   Q   K   Y   S   N   N   S   W   R   Y   L   S   N   R   L   L   A   P   S   D   S   P   E   W   L   S   F   D   V   T   G

GTT GTG CGG CAG TGG TTG AGC CGT GGA GGG GAA ATT GAG GGC TTT CGC CTT AGC GCC CAC TGC TCC TGT GAC AGC AGG GAT AAC ACA CTG CAA GTG GAC A  < 1600
V   V   R   Q   W   L   S   R   G   G   E   I   E   G   F   R   L   S   A   H   C   S   C   D   S   R   D   N   T   L   Q   V   D   I
BstXI
|
TC AAC GGG TTC ACT ACC GGC CGC CGA GGT GAC CTG GCC ACC ATT CAT GGC ATG AAC CGG CCT TTC CTG CTT CTC ATG GCC ACC CCG CTG GAG AGG GCC CA  < 1700
N   G   F   T   T   G   R   R   G   D   L   A   T   I   H   G   M   N   R   P   F   L   L   L   M   A   T   P   L   E   R   A   Q


EcoRI   >MMP site                                          BlpI   NotI       >huTGFB         <AjuI
|       |                                                  |      |          |               |
G CAT CTG CAA AGC GAA TTC GGG GGA GGC GGA TCC CCG CTC GGG CTT TGG GCG GGA GGG GGC TCA GCG GCC GCA GCC CTG GAC ACC AAC TAT TGC TTC AGC  < 1800
H   L   Q   S   E   F   G   G   G   G   S   P   L   G   L   W   A   G   G   G   S   A   A   A   A   L   D   T   N   Y   C   F   S
<BarI
|
TCC ACG GAG AAG AAC TGC TGC GTG CGG CAG CTG TAC ATT GAC TTC CGC AAG GAC CTC GGC TGG AAG TGG ATC CAC GAG CCC AAG GGC TAC CAT GCC AAC T  < 1900
S   T   E   K   N   C   C   V   R   Q   L   Y   I   D   F   R   K   D   L   G   W   K   W   I   H   E   P   K   G   Y   H   A   N   F

TC TGC CTC GGG CCC TGC CCC TAC ATT TGG AGC CTG GAC ACG CAG TAC AGC AAG GTC CTG GCC CTG TAC AAC CAG CAT AAC CCG GGC GCC TCG GCG GCG CC  < 2000
C   L   G   P   C   P   Y   I   W   S   L   D   T   Q   Y   S   K   V   L   A   L   Y   N   Q   H   N   P   G   A   S   A   A   P

G TGC TGC GTG CCG CAG GCG CTG GAG CCG CTG CCC ATC GTG TAC TAC GTG GGC CGC AAG CCC AAG GTG GAG CAG CTG TCC AAC ATG ATC GTG CGC TCC TGC  < 2100
C   C   V   P   Q   A   L   E   P   L   P   I   V   Y   Y   V   G   R   K   P   K   V   E   Q   L   S   N   M   I   V   R   S   C

XbaI                <SP6                                          >BGH polyadenylation signal
|                   |                                             |
AAG TGC AGC TGA CTC TAG AGG GCC CTA TTC TAT AGT GTC ACC TAA ATG CTA GAG CTC GCT GAT CAG CCT CGA CTG TGC CTT CTA GTT GCC AGC CAT CTG T  < 2200
K   C   S   *
```

Annotated sequence of LAP-MMP-mEPO in pcDNA3.

```
>CMV Promoter (3' end)                                  >T7                                HindIII
|                                                       |                                  |
G TCT ATA TAA GCA GAG CTC TCT GGC TAA CTA GAG AAC CCA CTG CTT ACT GGC TTA TCG AAA TTA ATA CGA CTC ACT ATA GGG AGA CCC AAG CTT ATG CCG  < 900

>LAP
|
CCC TCC GGG CTG CGG CTG CTG CCG CTG CTG CTA CCG CTG CTG TGG CTA CTG GTG CTG ACG CCT GGC CGG CCG GCC GCG GGA CTA TCC ACC TGC AAG ACT A  < 1000
                                                                                  L   S   T   C   K   T   I
TC GAC ATG GAG CTG GTG AAG CGG AAG CGC ATC GAG GCC ATC CGC GGC CAG ATC CTG TCC AAG CTG CGG CTC GCC AGC CCC CCG AGC CAG GGG GAG GTG CC  < 1100
D   M   E   L   V   K   R   K   R   I   E   A   I   R   G   Q   I   L   S   K   L   R   L   A   S   P   P   S   Q   G   E   V   P
Bsu36I
|
G CCC GGC CCG CTG CCC GAG GCC GTG CTC GCC CTG TAC AAC AGC ACC CGC GAC CGG GTG GCC GGG GAG AGT GCA GAA CCG GAG CCC GAG CCT GAG GCC GAC  < 1200
P   G   P   L   P   E   A   V   L   A   L   Y   N   S   T   R   D   R   V   A   G   E   S   A   E   P   E   P   E   P   E   A   D
AleI
|
TAC TAC GCC AAG GAG GTC ACC CGC GTG CTA ATG GTG GAA ACC CAC AAC GAA ATC TAT GAC AAG TTC AAG CAG AGT ACA CAC AGC ATA TAT ATG TTC TTC A  < 1300
Y   Y   A   K   E   V   T   R   V   L   M   V   E   T   H   N   E   I   Y   D   K   F   K   Q   S   T   H   S   I   Y   M   F   F   N
KpnI
Acc65I                                                                                  PmlI
|                                                                                       |
AC ACA TCA GAG CTC CGA GAA GCG GTA CCT GAA CCC GTG TTG CTC TCC CGG GCA GAG CTG CGT CTG CTG AGG CTC AAG TTA AAA GTG GAG CAG CAC GTG GA  < 1400
T   S   E   L   R   E   A   V   P   E   P   V   L   L   S   R   A   E   L   R   L   L   R   L   K   L   K   V   E   Q   H   V   E
G CTG TAC CAG AAA TAC AGC AAC AAT TCC TGG CGA TAC CTC AGC AAC CGG CTG CTG GCA CCC AGC GAC TCG CCA GAG TGG TTA TCT TTT GAT GTC ACC GGA  < 1500
L   Y   Q   K   Y   S   N   N   S   W   R   Y   L   S   N   R   L   L   A   P   S   D   S   P   E   W   L   S   F   D   V   T   G
GTT GTG CGG CAG TGG TTG AGC CGT GGA GGG GAA ATT GAG GGC TTT CGC CTT AGC GCC CAC TGC TCC TGT GAC AGC AGG GAT AAC ACA CTG CAA GTG GAC A  < 1600
V   V   R   Q   W   L   S   R   G   G   E   I   E   G   F   R   L   S   A   H   C   S   C   D   S   R   D   N   T   L   Q   V   D   I
BstXI
|
TC AAC GGG TTC ACT ACC GGC CGC CGA GGT GAC CTG GCC ACC ATT CAT GGC ATG AAC CGG CCT TTC CTG CTT CTC ATG GCC ACC CCG CTG GAG AGG GCC CA  < 1700
N   G   F   T   T   G   R   R   G   D   L   A   T   I   H   G   M   N   R   P   F   L   L   L   M   A   T   P   L   E   R   A   Q

EcoRI   >MMMP Site                                          BlpI   NotI      >mEPO
|       |                                                   |      |         |
G CAT CTG CAA AGC GAA TTC GGG GGA GGC GGA TCC CCG CTC GGG CTT TGG GCG GGA GGG GGC TCA GCG GCC GCA CTC TGT GCT CCC CCA CGC CTC ATC TGC  < 1800
H   L   Q   S   E   F   G   G   G   G   S   P   L   G   L   W   A   G   G   G   S   A   A   A   L   C   A   P   P   R   L   I   C
PpumI
|
GAC AGT CGA GTT CTG GAG AGG TAC ATC TTA GAG GCC AAG GAG GCA GAA AAT GTC ACG ATG GGT TGT GCA GAA GGT CCC AGA CTG AGT GAA AAT ATT ACA G  < 1900
D   S   R   V   L   E   R   Y   I   L   E   A   K   E   A   E   N   V   T   M   G   C   A   E   G   P   R   L   S   E   N   I   T   V
<AjuI
<BaeI
|   |
TC CCA GAT ACC AAA GTC AAC TTC TAT GCT TGG AAA AGA ATG GAG GTG GAA GAA CAG GCC ATA GAA GTT TGG CAA GGC CTG TCC CTG CTC TCA GAA GCC AT  < 2000
P   D   T   K   V   N   F   Y   A   W   K   R   M   E   V   E   E   Q   A   I   E   V   W   Q   G   L   S   L   L   S   E   A   I
BmtI
SbfI                    NheI                                                                                    <BarI
|                       |                                                                                       |
C CTG CAG GCC CAG GCC CTG CTA GCC AAT TCC TCC CAG CCA CCA GAG ACC CTT CAG CTT CAT ATA GAC AAA GCC ATC AGT GGT CTA CGT AGC CTC ACT TCA  < 2100
L   Q   A   Q   A   L   L   A   N   S   S   Q   P   P   E   T   L   Q   L   H   I   D   K   A   I   S   G   L   R   S   L   T   S

CTG CTT CGG GTA CTG GGA GCT CAG AAG GAA TTG ATG TCG CCT CCA GAT ACC ACC CCA CCT GCT CCA CTC CGA ACA CTC ACA GTG GAT ACT TTC TGC AAG C  < 2200
L   L   R   V   L   G   A   Q   K   E   L   M   S   P   P   D   T   T   P   P   A   P   L   R   T   L   T   V   D   T   F   C   K   L
XbaI                    <SP6
|                       |
TC TTC CGG GTC TAC GCC AAC TTC CTC CGG GGG AAA CTG AAG CTG TAC ACG GGA GAG GTC TGC AGG AGA GGG GAC AGG TGA GGT CTA GAG GCC CCT ATT CT  < 2300
F   R   V   Y   A   N   F   L   R   G   K   L   K   L   Y   T   G   E   V   C   R   R   G   D   R   *
```

Annotated sequence of LAP-MMP-mIGF1A in pcDNA3.

```
>CMV Promoter (3' end)                                  >T7                                HindIII >LAP
|                                                       |                                  |       |
G TCT ATA TAA GCA GAG CTC TCT GGC TAA CTA GAG AAC CCA CTG CTT ACT GGC TTA TCG AAA TTA ATA CGA CTC ACT ATA GGG AGA CCC AAG CTT ATG CCG  < 900
M   P
>AarI
|
CCC TCC GGG CTG CGG CTG CTG CCG CTG CTG CTA CCG CTG CTG TGG CTA CTG GTG CTG ACG CCT GGC CCG CCG GCC GCG GGA CTA TCC ACC TGC AAG ACT A  < 1000
P   S   G   L   R   L   L   P   L   L   L   P   L   L   W   L   L   V   L   T   P   G   P   P   A   A   G   L   S   T   C   K   T   I
TC GAC ATG GAG CTG GTG AAG CGG AAG CGC ATC GAG GCC ATC CGC GGC CAG ATC CTG TCC AAG CTG CGG CTC GCC AGC CCC CCG AGC CAG GGG GAG GTG CC  < 1100
D   M   E   L   V   K   R   K   R   I   E   A   I   R   G   Q   I   L   S   K   L   R   L   A   S   P   P   S   Q   G   E   V   P
BsrGI                                                                 Bsu36I
|                                                                     |
G CCC GGC CCG CTG CCC GAG GCC GTG CTC GCC CTG TAC AAC AGC ACC CGC GAC CGG GTG GCC GGG GAG AGT GCA GAA CCG GAG CCC GAG CCT GAG GCC GAC  < 1200
P   G   P   L   P   E   A   V   L   A   L   Y   N   S   T   R   D   R   V   A   G   E   S   A   E   P   E   P   E   P   E   A   D
AleI
|
TAC TAC GCC AAG GAG GTC ACC CGC GTG CTA ATG GTG GAA ACC CAC AAC GAA ATC TAT GAC AAG TTC AAG CAG AGT ACA CAC AGC ATA TAT ATG TTC TTC A  < 1300
Y   Y   A   K   E   V   T   R   V   L   M   V   E   T   H   N   E   I   Y   D   K   F   K   Q   S   T   H   S   I   Y   M   F   F   N
Acc65I
>LAP                KpnI                                                                                                  PmlI
|                   |                                                                                                     |
AC ACA TCA GAG CTC CGA GAA GCG GTA CCT GAA CCC GTG TTG CTC TCC CGG GCA GAG CTG CGT CTG CTG AGG AGG CTC AAG TTA AAA GTG GAG CAG CAC GT  < 1400
T   S   E   L   R   E   A   V   P   E   P   V   L   L   S   R   A   E   L   R   L   L   R   R   L   K   L   K   V   E   Q   H   V
G GAG CTG TAC CAG AAA TAC AGC AAC AAT TCC TGG CGA TAC CTC AGC AAC CGG CTG CTG GCA CCC AGC GAC TCG CCA GAG TGG TTA TCT TTT GAT GTC ACC  < 1500
E   L   Y   Q   K   Y   S   N   N   S   W   R   Y   L   S   N   R   L   L   A   P   S   D   S   P   E   W   L   S   F   D   V   T
GGA GTT GTG CGG CAG TGG TTG AGC CGT GGA GGG GAA ATT GAG GGC TTT CGC CTT AGC GCC CAC TGC TCC TGT GAC AGC AGG GAT AAC ACA CTG CAA GTG G  < 1600
G   V   V   R   Q   W   L   S   R   G   G   E   I   E   G   F   R   L   S   A   H   C   S   C   D   S   R   D   N   T   L   Q   V   D
BstXI
|
AC ATC AAC GGG TTC ACT ACC GGC CGC CGA GGT GAC CTG GCC ACC ATT CAT GGC ATG AAC CGG CCT TTC CTG CTT CTC ATG GCC ACC CCG CTG GAG AGG GC  < 1700
I   N   G   F   T   T   G   R   R   G   D   L   A   T   I   H   G   M   N   R   P   F   L   L   L   M   A   T   P   L   E   R   A


EcoRI   >MMP site   BamHI                                          NotI       >mIGF1A
|       |           |                                             |          |
C CAG CAT CTG CAA AGC GAA TTC GGG GGA GGC GGA TCC CCG CTC GGG CTT TGG GCG GGA GGG GGC TCA GCg gcc gca gga cca gag acc ctt tgc ggg gct  < 1800
Q   H   L   Q   S   E   F   G   G   G   G   S   P   L   G   L   W   A   G   G   G   S   A   A   A   G   P   E   T   L   C   G   A
EcoNI
|
gag ctg gtg gat gct ctt cag ttc gtg tgt gga ccg agg ggc ttt tac ttc aac aag ccc aca ggc tat ggc tcc agc att cgg agg gca cct cag aca g  < 1900
E   L   V   D   A   L   Q   F   V   C   G   P   R   G   F   Y   F   N   K   P   T   G   Y   G   S   S   I   R   R   A   P   Q   T   G
BspEI
|
gc att gtg gat gag tgt tgc ttc cgg agc tgt gat ctg agg aga ctg gag atg tac tgt gcc cca ctg aag cct aca aaa gca gcc cgc tct atc cgt gc  < 2000
                          I   V   D   E   C   C   F   R   S   C   D   L   R   R   L   E   M   Y   C   A   P   L   K   P   T   K   A   A   R   S   I   R   A
c cag cgc cac act gac atg ccc aag act cag aag gaa gta cat ttg aag aac aca agt aga gga agt gca gga aac aag acc tac aga atg tag AGG AAG  < 2100
Q   R   H   T   D   M   P   K   T   Q   K   E   V   H   L   K   N   T   S   R   G   S   A   G   N   K   T   Y   R   M   *

XbaI                <SP6                                          >bGH PA
|                   |                                             |
TGC AGG AAA CAA GAC CTA CAG AAT GTA GTc tag AGG GCC CTA TTC TAT AGT GTC ACC TAA ATG CTA GAG CTC GCT GAT CAG CCT CGA CTG TGC CTT CTA G  < 2200
```

Annotated sequence of LAP-MMP-mIGF1B in pcDNA3.

```
>CMV Promoter (3' end)                                  >T7                                HindIII >LAP
|                                                       |                                  |       |
G TCT ATA TAA GCA GAG CTC TCT GGC TAA CTA GAG AAC CCA CTG CTT ACT GGC TTA TCG AAA TTA ATA CGA CTC ACT ATA GGG AGA CCC AAG CTT ATG CCG  < 900
                                                                                                                          M   P
>AarI  <BcgI
|      |
CCC TCC GGG CTG CGG CTG CTG CCG CTG CTG CTA CCG CTG CTG TGG CTA CTG GTG CTG ACG CCT GGC CCG CCG GCC GCG GGA CTA TCC ACC TGC AAG ACT A  < 1000
P   S   G   L   R   L   L   P   L   L   L   P   L   L   W   L   L   V   L   T   P   G   P   P   A   A   G   L   S   T   C   K   T   I
TC GAC ATG GAG CTG GTG AAG CGG AAG CGC ATC GAG GCC ATC CGC GGC CAG ATC CTG TCC AAG CTG CGG CTC GCC AGC CCC CCG AGC CAG GGG GAG GTG CC  < 1100
D   M   E   L   V   K   R   K   R   I   E   A   I   R   G   Q   I   L   S   K   L   R   L   A   S   P   P   S   Q   G   E   V   P
BsrGI                                                                   Bsu36I
|                                                                       |
G CCC GGC CCG CTG CCC GAG GCC GTG CTC GCC CTG TAC AAC AGC ACC CGC GAC CGG GTG GCC GGG GAG AGT GCA GAA CCG GAG CCC GAG CCT GAG GCC GAC  < 1200
P G   P   L   P   E   A   V   L   A   L   Y   N   S   T   R   D   R   V   A   G   E   S   A   E   P   E   P   E   P   E   A   D
AleI
|
TAC TAC GCC AAG GAG GTC ACC CGC GTG CTA ATG GTG GAA ACC CAC AAC GAA ATC TAT GAC AAG TTC AAG CAG AGT ACA CAC AGC ATA TAT ATG TTC TTC A  < 1300
Y   Y   A   K   E   V   T   R   V   L   M   V   E   T   H   N   E   I   Y   D   K   F   K   Q   S   T   H   S   I   Y   M   F   F   N
KpnI
Acc65I                                                                                                  PmlI
|                                                                                                       |
AC ACA TCA GAG CTC CGA GAA GCG GTA CCT GAA CCC GTG TTG CTC TCC CGG GCA GAG CTG CGT CTG CTG AGG AGG CTC AAG TTA AAA GTG GAG CAG CAC GT  < 1400
T   S   E   L   R   E   A   V   P   E   P   V   L   L   S   R   A   E   L   R   L   L   R   R   L   K   L   K   V   E   Q   H   V
G GAG CTG TAC CAG AAA TAC AGC AAC AAT TCC TGG CGA TAC CTC AGC AAC CGG CTG CTG GCA CCC AGC GAC TCG CCA GAG TGG TTA TCT TTT GAT GTC ACC  < 1500
E   L   Y   Q   K   Y   S   N   N   S   W   R   Y   L   S   N   R   L   L   A   P   S   D   S   P   E   W   L   S   F   D   V   T
GGA GTT GTG CGG CAG TGG TTG AGC CGT GGA GGG GAA ATT GAG GGC TTT CGC CTT AGC GCC CAC TGC TCC TGT GAC AGC AGG GAT AAC ACA CTG CAA GTG G  < 1600
G   V   V   R   Q   W   L   S   R   G   G   E   I   E   G   F   R   L   S   A   H   C   S   C   D   S   R   D   N   T   L   Q   V   D
BstXI
|
AC ATC AAC GGG TTC ACT ACC GGC CGC CGA GGT GAC CTG GCC ACC ATT CAT GGC ATG AAC CGG CCT TTC CTG CTT CTC ATG GCC ACC CCG CTG GAG AGG GC  < 1700
I   N   G   F   T   T   G   R   R   G   D   L   A   T   I   H   G   M   N   R   P   F   L   L   L   M   A   T   P   L   E   R   A


EcoRI   >MMP site   BamHI                                          NotI        >mIGF1B
|       |           |                                             |           |
C CAG CAT CTG CAA AGC GAA TTC GGG GGA GGC GGA TCC CCG CTC GGG CTT TGG GCG GGA GGG GGC TCA GCg gcc gca GGA CCA GAG ACC CTT TGC ggg gct  < 1800
Q   H   L   Q   S   E   F   G   G   G   G   S   P   L   G   L   W   A   G   G   G   S   A   A   A   G   P   E   T   L   C   G   A
EcoNI
|
gag ctg gtg gat gct ctt cag ttc gtg tgt gga ccg agg ggc ttt tac ttc aac aag ccc aca ggc tat ggc tcc agc att cgg agg gca cct cag aca g  < 1900
E   L   V   D   A   L   Q   F   V   C   G   P   R   G   F   Y   F   N   K   P   T   G   Y   G   S   S   I   R   R   A   P   Q   T   G
BspEI
|
gc att gtg gat gag tgt tgc ttc cgg agc tgt gat ctg agg aga ctg gag atg tac tgt gcc cca ctg aag cct aca aaa gca gcc cgc tct atc cgt gc  < 2000
I   V   D   E   C   C   F   R   S   C   D   L   R   R   L   E   M   Y   C   A   P   L   K   P   T   K   A   A   R   S   I   R   A
<RpaB5I
|
c cag cgc cac act gac atg ccc aag act cag aag tcc ccg tcc cta tcg aca aac aag aaa acg aag ctg caa agg aga agg aaa gga aGT ACA TTT GAA  < 2100
Q   R   H   T   D   M   P   K   T   Q   K   S   P   S   L   S   T   N   K   K   T   K   L   Q   R   R   R   K   G   S   T   F   E
XbaI                <SP6
|                   |
GAA CAC AAG TAG AGG AAG TGC AGG AAA CAA GAC CTA CAG AAT GTA GTc tag AGG GCC CTA TTC TAT AGT GTC ACC TAA ATG CTA GAG CTC GCT GAT CAG C  < 2200
E   H   K   *   R   K   C   R   K   Q   D   L   Q   N   V   V   *   R   A   L   F   Y   S   V   T   *   M   L   E   L   A   D   Q   P
```

Annotated sequence of LAP-MMP-huTGFβ

```
>LAP
|
C TCC GAC AGA CTG AGT CGC CCG GGG GGG ATC TCC ATG CCG CCC TCC GGG CTG CGG CTG CTG CCG CTG CTG CTA CCG CTG CTG TGG CTA CTG GTG CTG ACG  < 600
M   P   P   S   G   L   R   L   L   P   L   L   L   P   L   L   W   L   L   V   L   T
>AarI             >ApyPI
|                 |
CCT GGC CGG CCG GCC GCG GGA CTA TCC ACC TGC AAG ACT ATC GAC ATG GAG CTG GTG AAG CGG AAG CGC ATC GAG GCC ATC CGC GGC CAG ATC CTG TCC A  < 700
P   G   R   P   A   A   G   L   S   T   C   K   T   I   D   M   E   L   V   K   R   K   R   I   E   A   I   R   G   Q   I   L   S   K
AG CTG CGG CTC GCC AGC CCC CCG AGC CAG GGG GAG GTG CCG CCC GGC CCG CTG CCC GAG GCC GTG CTC GCC CTG TAC AAC AGC ACC CGC GAC CGG GTG GC  < 800
L   R   L   A   S   P   P   S   Q   G   E   V   P   P   G   P   L   P   E   A   V   L   A   L   Y   N   S   T   R   D   R   V   A
Bsu36I                              AleI                          >RleAI
|                                   |                             |
C GGG GAG AGT GCA GAA CCG GAG CCC GAG CCT GAG GCC GAC TAC TAC GCC AAG GAG GTC ACC CGC GTG CTA ATG GTG GAA ACC CAC AAC GAA ATC TAT GAC  < 900
G   E   S   A   E   P   E   P   E   P   E   A   D   Y   Y   A   K   E   V   T   R   V   L   M   V   E   T   H   N   E   I   Y   D
Acc65I
KpnI
|
AAG TTC AAG CAG AGT ACA CAC AGC ATA TAT ATG TTC TTC AAC ACA TCA GAG CTC CGA GAA GCG GTA CCT GAA CCC GTG TTG CTC TCC CGG GCA GAG CTG C  < 1000
K   F   K   Q   S   T   H   S   I   Y   M   F   F   N   T   S   E   L   R   E   A   V   P   E   P   V   L   L   S   R   A   E   L   R
<BpuEI                      PmlI                                                  >AlfI
|                           |                                                     |
GT CTG CTG AGG CTC AAG TTA AAA GTG GAG CAG CAC GTG GAG CTG TAC CAG AAA TAC AGC AAC AAT TCC TGG CGA TAC CTC AGC AAC CGG CTG CTG GCA CC  < 1100
L   L   R   L   K   L   K   V   E   Q   H   V   E   L   Y   Q   K   Y   S   N   N   S   W   R   Y   L   S   N   R   L   L   A   P
C AGC GAC TCG CCA GAG TGG TTA TCT TTT GAT GTC ACC GGA GTT GTG CGG CAG TGG TTG AGC CGT GGA GGG GAA ATT GAG GGC TTT CGC CTT AGC GCC CAC  < 1200
S   D   S   P   E   W   L   S   F   D   V   T   G   V   V   R   Q   W   L   S   R   G   G   E   I   E   G   F   R   L   S   A   H
TGC TCC TGT GAC AGC AGG GAT AAC ACA CTG CAA GTG GAC ATC AAC GGG TTC ACT ACC GGC CGC CGA GGT GAC CTG GCC ACC ATT CAT GGC ATG AAC CGG C  < 1300
C   S   C   D   S   R   D   N   T   L   Q   V   D   I   N   G   F   T   T   G   R   R   G   D   L   A   T   I   H   G   M   N   R   P

>MMP_site
>EciI      >BsrBI       <PspOMII          BlpI
|     |    |       |    |         |
CT TTC CTG CTT CTC ATG GCC ACC CCG CTG GAG AGG GCC CAG CAT CTG CAA AGC GAA TTC GGG GGA GGC GGA TCC CCG CTC GGG CTT TGG GCG GGA GGG GG  < 1400
F   L   L   L   M   A   T   P   L   E   R   A   Q   H   L   Q   S   E   F   G   G   G   G   S   P   L   G   L   W   A   G   G   G
>MaqI
<AjuI        <AcuI         >TspGWI                            <BarI
|            |             |                                  |   |
C TCA GCG GCC GCA GCC CTG GAC ACC AAC TAT TGC TTC AGC TCC ACG GAG AAG AAC TGC TGC GTG CGG CAG CTG TAC ATT GAC TTC CGC AAG GAC CTC GGC  < 1500
S   A   A   A   A   L   D   T   N   Y   C   F   S   S   T   E   K   N   C   C   V   R   Q   L   Y   I   D   F   R   K   D   L   G

>BssSI                                                                    >huTGFB
|                                                                         |
TGG AAG TGG ATC CAC GAG CCC AAG GGC TAC CAT GCC AAC TTC TGC CTC GGG CCC TGC CCC TAC ATT TGG AGC CTG GAC ACG CAG TAC AGC AAG GTC CTG G  < 1600
W   K   W   I   H   E   P   K   G   Y   H   A   N   F   C   L   G   P   C   P   Y   I   W   S   L   D   T   Q   Y   S   K   V   L   A
CC CTG TAC AAC CAG CAT AAC CCG GGC GCC TCG GCG GCG CCG TGC TGC GTG CCG CAG GCG CTG GAG CCG CTG CCC ATC GTG TAC TAC GTG GGC CGC AAG CC  < 1700
L   Y   N   Q   H   N   P   G   A   S   A   A   P   C   C   V   P   Q   A   L   E   P   L   P   I   V   Y   Y   V   G   R   K   P
MreI
<BsaXI                                                    SgrAI                          BsaBI   >NlaCI
|                                                         |                              |       |
C AAG GTG GAG CAG CTG TCC AAC ATG ATC GTG CGC TCC TGC AAG TGC AGC TGA TGA CGC CGG CGT CCA AGC CGA ATT CTG CAG ATA TCC ATC ACA CTG GCG  < 1800
K   V   E   Q   L   S   N   M   I   V   R   S   C   K   C   S   *   *
```

Annotated sequence of LAP-MMP-mEPO

```
>LAP
|
C TCC GAC AGA CTG AGT CGC CCG GGG GGG ATC TCC ATG CCG CCC TCC GGG CTG CGG CTG CTG CCG CTG CTG CTA CCG CTG CTG TGG CTA CTG GTG CTG ACG  < 600
S   D   R   L   S   R   P   G   G   I   S   M   P   P   S   G   L   R   L   L   P   L   L   L   P   L   L   W   L   L   V   L   T
<BcgI      >ApyPI
|           |
CCT GGC CGG CCG GCC GCG GGA CTA TCC ACC TGC AAG ACT ATC GAC ATG GAG CTG GTG AAG CGG AAG CGC ATC GAG GCC ATC CGC GGC CAG ATC CTG TCC A  < 700
P   G   R   P   A   A   G   L   S   T   C   K   T   I   D   M   E   L   V   K   R   K   R   I   E   A   I   R   G   Q   I   L   S   K
AG CTG CGG CTC GCC AGC CCC CCG AGC CAG GGG GAG GTG CCG CCC GGC CCG CTG CCC GAG GCC GTG CTC GCC CTG TAC AAC AGC ACC CGC GAC CGG GTG GC  < 800
L   R   L   A   S   P   P   S   Q   G   E   V   P   P   G   P   L   P   E   A   V   L   A   L   Y   N   S   T   R   D   R   V   A
Bsu36I                              AleI                          >RleAI
|                                   |                             |
C GGG GAG AGT GCA GAA CCG GAG CCC GAG CCT GAG GCC GAC TAC TAC GCC AAG GAG GTC ACC CGC GTG CTA ATG GTG GAA ACC CAC AAC GAA ATC TAT GAC  < 900
G   E   S   A   E   P   E   P   E   P   E   A   D   Y   Y   A   K   E   V   T   R   V   L   M   V   E   T   H   N   E   I   Y   D
Acc65I
KpnI
|
AAG TTC AAG CAG AGT ACA CAC AGC ATA TAT ATG TTC TTC AAC ACA TCA GAG CTC CGA GAA GCG GTA CCT GAA CCC GTG TTG CTC TCC CGG GCA GAG CTG C  < 1000
K   F   K   Q   S   T   H   S   I   Y   M   F   F   N   T   S   E   L   R   E   A   V   P   E   P   V   L   L   S   R   A   E   L   R
<BpuEI                      PmlI                                                    >AlfI
|                           |                                                       |
GT CTG CTG AGG CTC AAG TTA AAA GTG GAG CAG CAC GTG GAG CTG TAC CAG AAA TAC AGC AAC AAT TCC TGG CGA TAC CTC AGC AAC CGG CTG CTG GCA CC  < 1100
L   L   R   L   K   L   K   V   E   Q   H   V   E   L   Y   Q   K   Y   S   N   N   S   W   R   Y   L   S   N   R   L   L   A   P
LpnI
HaeII
|
C AGC GAC TCG CCA GAG TGG TTA TCT TTT GAT GTC ACC GGA GTT GTG CGG CAG TGG TTG AGC CGT GGA GGG GAA ATT GAG GGC TTT CGC CTT AGC GCC CAC  < 1200
S   D   S   P   E   W   L   S   F   D   V   T   G   V   V   R   Q   W   L   S   R   G   G   E   I   E   G   F   R   L   S   A   H
TGC TCC TGT GAC AGC AGG GAT AAC ACA CTG CAA GTG GAC ATC AAC GGG TTC ACT ACC GGC CGC CGA GGT GAC CTG GCC ACC ATT CAT GGC ATG AAC CGG C  < 1300
C   S   C   D   S   R   D   N   T   L   Q   V   D   I   N   G   F   T   T   G   R   R   G   D   L   A   T   I   H   G   M   N   R   P


PspOMI
ApaI                              >MMP site   BamHI
BaeGI                                    >EciI       >BsrBI        <PspOMII           BlpI
|                                 |       |   |       |             |                  |
CT TTC CTG CTT CTC ATG GCC ACC CCG CTG GAG AGG GCC CAG CAT CTG CAA AGC GAA TTC GGG GGA GGC GGA TCC CCG CTC GGG CTT TGG GCG GGA GGG GG  < 1400
F   L   L   L   M   A   T   P   L   E   R   A   Q   H   L   Q   S   E   F   G   G   G   G   S   P   L   G   L   W   A   G   G   G


DraIII     >mEPO
|          |
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SspI               <BaeI
|                  |   |
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BmtI
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|                                                     |                               |
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SnaBI                               >BmrI
|   |                               |
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I   D   K   A   I   S   G   L   R   S   L   T   S   L   L   R   V   L   G   A   Q   K   E   L   M   S   P   P   D   T   T   P   P
>EarI
>SapI                       <AquIV
| |                         |
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A   P   L   R   T   L   T   V   D   T   F   C   K   L   F   R   V   Y   A   N   F   L   R   G   K   L   K   L   Y   T   G   E   V   C
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R   R   G   D   R   *
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Annotated sequence of LAP-MMP-mIGF1A

```
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>AarI           >ApyPI                              >CdiI                 <Bsp24I
|               |                                   |                     |
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BsrGI
|
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Bsu36I                              AleI
|                                   |
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Acc65I
KpnI
|
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K   F   K   Q   S   T   H   S   I   Y   M   F   F   N   T   S   E   L   R   E   A   V   P   E   P   V   L   L   S   R   A   E   L   R
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TaiI
PmlI
>BdaI      <BpuEI                      BsaAI                                                                  >AlfI
|          |                           ||                                                                     |
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PspOMI                      >MMP site   >EciI
|                           |         |   |                 |
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>mIGF1A                                  >EarI
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<BsmI    >BplI     EcoNI                                  BspEI                                  HpyCH4III
|        |         |                                      |                                      |
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|                                          |                                      |
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G   S   A   G   N   K   T   Y   R   M   *
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Annotated sequence of LAP-MMP-mIGF1B

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|      |                                              |                     |
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BsrGI
|
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|                        |                                   |
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Acc65I
KpnI
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BsaAI
>BdaI      <BpuEI                    PmlI                                                                  >AlfI
|          |                         ||                                                                    |
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PspOMI                        >MMP site       BamHI               <PspOMII
ApaI                                    >EciI
|                             |           |   |                   |       |
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>MIGF1B                                        >EarI
<BsaI                                  >SapI                  >TaqII
|      |                               ||                     |
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|                                              |
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MreI
<HpyAV                                       SgrAI                            BsaBI   >NlaCI
|                                            |                                |       |
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T   K   L   Q   R   R   R   K   G   S   T   F   E   E   H   K   *
```

APPENDIX 2

NCBI Accession Numbers

Table A.1: NCBI accession numbers.

Protein Definition	Reference Seq (DNA)	Size	Reference Seq (Protein)	Size
huTGFβ transforming growth factor beta-1 precursor [Homo sapiens]	NM_000660	2217 bp	NP_000651	390 aa
mEPO erythropoietin precursor [Mus musculus]	NM_007942	715 bp	NP_031968	192 aa
mIGF1A insulin-like growth factor I isoform 4 preproprotein [Mus musculus]	NM_001111275	7069 bp	NP_001104745	153 aa
mIGF1B insulin-like growth factor I isoform 1 preproprotein [Mus musculus]	NM_010512.4	7121 bp	NP_034642	159 aa

APPENDIX 3

Aggregating cultures of rat brain cells

Hypothesis

The *in vitro* model of aggregating neural cell cultures consists of primary three-dimentional neural cells that have spontaneously aggregated following physical dissociation. They are able to differentiate and develop specialized structures such as synapes and myelinated neurons when cultured under the right conditions. Experiments using this model were anticipated to test the hypothesis that the latent recombinant proteins LAP-MMP-mEPO, LAP-MMP-huTGFβ and LAP-MMP-mIGF1 help prevent myelin loss and/or aid in the process of remyelination following incubation with MMP enzyme.

Analysis of Aggregating Neural Cell Cultures

Due to the high reproducibility of the cultures, and the possibility of producing numerous replicates of the cultures, a wide variety of analysis such as immunocytochemistry, histochemistry, electron microscopy, autoradiography, and biochemical analysis is possible. Biochemical analyses in this model allows for the testing of enzymatic activities, total protein and DNA content; mitotic activity; protein expression; ELISA and radioimmunoassay; and subcellular fractions (e.g. for the quantitative extraction of myelin).

Materials

Pucks D1 solution

NaCL	8g/L
KCl	400mg/L
$Na_2HPO_4 \cdot 7H_2O$	45mg/L
KH_2PO_4	30mg/L
D-Glucose	1g/L
Sucrose, pH 7.4, 340 mOsm	20g/L

Store at 4°C until required then add 25mg/L gentamicin-sulfate, pH 7.4

Serum-free culture medium

The protocol for the preparation of 10L serum-free culture medium is as follows:

1) Purchase powdered DMEM containing high glucose (4.5g/L) and L-glutamine, without bicarbonate and pyruvate for 10L.
2) Supplement with choline chloride (1.35g), L-Carnitine (20mg), Lipoic acid (2mg) and Vitamin B12 (13.6mg).
3) Add 1ml of each of the following 10^4-fold concentrated stock solutions (stored frozen at -20°C)

$CdSO_4 \cdot 8H_2O$	50µM
$CuSO_4 \cdot 5H_2O$	100µM
$MnCl_3 \cdot 4H_2O$	50µM
$Na_2 \cdot SeO_3$	150 µM
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	5µM
$NiSO_4 \cdot 6H_2O$	2.5µM
$SnCl_2 \cdot 2H_2O$	2.5µM
$ZnSO_4 \cdot 7H_2O$	50µM

4) Adjust the pH close to the final pH with NaOH (1N).
5) Add sodium bicarbonate (3.7g/L) and gas immediately with 10% CO_2 in air until pH is adjusted to 7.4.
6) Adjust the osmolarity of the medium to 340 ± 2mOsm by adding water.
7) Sterilize and store at 4°C in the dark in 500ml bottles.
8) Prior to use, add the following supplements to complete the medium:

Basal Medium (Eagle) [BME] vitamins (100x stock)	
Transferrin	1mg/L final concentration
Triiodothyronine	30nM final concentration
Insulin	5mg/L final concentration
Hydrocortisone-21-phosphate	20nM final concentration
Linoleic acid	3mg/L final concentration
Gentamicin sulfate	25mg/L final concentration

Barbitone buffer

The protocol for the preparation of barbitol buffer is as follows:

1) Dissolve barbital sodium (12g) in 800ml distilled water.
2) Dissolve barbital (4.4g) in 150ml distilled water.
3) Mix both solutions together and adjust pH to 8.2 with NaOH (1N).
4) Add merthiolate (0.15g) and adjust final volume to 1L.

Methods

Embryo's were removed from pregnant Sprague-Dawley rats on embryonic day 16 following decapitation and placed into ice cold Puck's D1 solution. The brain was removed and the telencephalon dissected out and placed into ice-cold sterile D1 solution. The telencephalons were progressively dissociated by sequential sieving through 200μm nylon mesh fitted with a glass funnel, followed by 100μm nylon mesh (Nybolt, Zurich Switzerland).

The filtrates were washed twice with ice-cold D1 solution and centrifuged (300 x***g***, 15 min, 4°C). The pellet was resuspended by trituration in cold serum-free culture medium and cell viability determined by 0.1% Trypan Blue exclusion before diluting to a density of $2.5x10^7$ cells per 25 ml flask. The cultures were incubated at 37°C at a 10% CO_2 humidified atmosphere under constant rotation (68rpm to 70rpm; Kuhner Shaker, Philip Harris Scientific). The rotation speed was increased during the first week to a maximum rotation of 80rpm to ensure optimum cell aggregation. The initial day of seeding is termed day *in vitro* 0 (DIV=0). The culture was transferred to 50ml culture flasks on DIV=2 and the total culture medium volume doubled to 8ml.

Regular replenishment of the culture medium was done every third day from DIV=5 to DIV=14, and every other day thereafter. The cell cultures were subdivided on DIV=20 due to the increase in metabolic activity of the maturing aggregates. An equivalent number of culture flasks were placed into the incubator 1 hour prior to subculturing. The aggregates were resuspended by gentle aspiration using a pipette before being placed into new prewarmed flasks and the total volume doubled to 8ml.

Either Z12 derived anti-MOG antibody in PBS (31.3μg/ml) plus complement (25μg/ml guinea pig serum [GPS]) were added to cultures on DIV=26 for 4 days with a medium feed and fresh addition of anti-MOG antibody after 48 hours, or lentivirus (20μl/8ml; LAP-MMP-mEPO or LAP-MMP) was added once and the culture left for 4 days before replacing with fresh medium. The demyelinating agents were removed by replacing the culture medium twice with fresh prewarmed culture medium. Control cultures received subclass specific IgG2a (Sigma-Aldrich) plus GPS. Samples of aggregates were taken at DIV=26, 28 and 48 and washed in sterile barbitone buffer before being stored at -20°C. Prior to analyses by ELISA, the aggregates were resuspended in 500μl Tissue Protein Extraction Reagent (T-PER; Pierbio Science UK, Northumberland, UK) containing HALT® Protein Inhibitor Cocktail (Pierbio Science UK, Northumberland, UK) and homogenised for 30sec. The total protein concentration was assayed using the BCA protein concentration assay as described previously.

Results

Unfortunately the samples were lost before performing the ELISA during the move of laboratories. The cost involved in performing this experiment and the time required prevented me from repeating this experiment.