1 List of abbreviations

AT-MSCs	adipose tissue-derived MSCs
BDNF	brain derived neurotrophic factor
BHA	butylated hydroxyanisole
BME	b-mercaptoethanol
BM-MSCs	bone marrow-derived MSCs
BMP	bone morphogenetic protein
CD	cluster of differentiation
CK	cytokeratin
CNS	central nervous system
ConA	concanavalin A
COX-2	cyclooxygenase-2
DC	dendritic cells
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ESCs	embryonic stem cells
bFGF	basic fibroblast growth factor
GAD	glutamic acid decarboxylase
HGF	hepatocyte growth factor
IDO	indoleamine 2,3-dioxygenase
IFN-gamma	interferon gamma
IGF-1	inzulin-like growth factor 1
IL	interleukin
iPSCs	induced pluripotent stem cells
LSCs	limbal stem cells
LSCD	limbal stem cell deficiency
MHC	major histocompatibility complex
MSCs	mesenchymal stem cells

Nf-L	neurofilament light polypeptide
NGF	nerve growth factor
NK	natural killer cells
Nf-M	neurofilament medium polypeptide
NSCs	neural stem cells
PGE2	prostaglandin E2
qPCR	quantitative polymerase chain reaction
RA	retinoic acid
SDF-1	stromal cell-derived factor-1
SH	sonic hedgehog
SYP	synaptophysin
TACs	transient amplifying cells
T-cell	T lymphocyte
TGF-beta	transforming growth factor beta
Th	helper T lymphocyte
TNF-alpha	tumor necrosis factor alpha
T-reg	regulatory T lymphocyte
TUBB3	beta-III tubulin
UC-MSCs	umbilical cord-derived MSCs
VEGF	vascular endothelial growth factor

2 Introduction

2.1 Stem cells

Stem cells are undifferentiated cells, described as self-renewing and capable of differentiation into other types of cells. These properties are termed "stemness" (Ebrahimi *et al*., 2009, Zomer *et al.*, 2015). Stem cells normally function in the quiescent or slow cycling state, dividing only to maintain the stem cell pool. When tissue regeneration is needed, they undergo the process of asymmetrical cell division. This asymmetrical cell division results in two cell types: self-renewing stem cells, which maintain the reservoir of stem cells, and cells beginning the differentiation, transient amplifying cells (TACs) (Ebrahimi *et al*., 2009). TACs, unlike stem cells, divide frequently and continue to differentiate up to terminally differentiated, mature cells (Ghosh *et al.*, 2011.) The more matured the cell is, the lower is its differentiation potential. The first embryonic cells which evolve after fertilization - totipotent stem cells - are capable of creating any kind of cells of the embryo and extra-embryonic tissue. One of the early embryonal stages, blastocyst, is a source of pluripotent embryonic stem cells (ESCs), i.e. they are capable of forming any kind of cells in three embryonic germ layers, but not extra-embryonic tissue. During a lifetime, many types of adult multipotent stem cells persist in the body; these cells are able to self-renew or to differentiate into several types of mature cells, forming tissues or organs of specific developmental lineage, for example neural stem cells (NSCs), mesenchymal stem cells (MSCs), limbal stem cells (LSCs). As the differentiation process continues, this multipotency is reduced into unipotency and former stem cells become the direct progenitor of one cellular type. An alternative to ESCs is represented by induced pluripotent stem cells (iPSCs). iPSCs are artificially redirected from mature, fully differentiated cells, back to pluripotent stem cells, and then differentiated into the required cell type. Redirection can be performed by genetic reprogramming, inserting pluripotency-associated transcription factors into the nucleus, where

they restart signaling pathways which lead to broadened differentiation potential. Their disadvantage, similarly to ESCs, is the risk of mutagenicity and teratoma formation (Liu *et al.*, 2013, Gong *et al.*, 2014). Recently, protocols using chemical induction by small molecules in epigenetic reprogramming of somatic cells have been published (Higuchi *et al.*, 2015, Cao *et al.*, 2016, Zhang *et al.,* 2016).

2.1.1 Tissue-specific stem cells

Adult, somatic, tissue-specific stem cells are characterized by their multipotency, i.e. they can differentiate into several types of cells, regenerating specific organs and tissues. For example, MSCs regenerate the mesodermal connective tissues, NSCs differentiate into neurons and glia and LSCs regenerate the corneal epithelium. Adult stem cells reside in their specific environment, reffered to as stem cell niche. Stem cell niche has a protective and regulative character; it participates in maintaining the stem cell pool through the self-renewal process as well as in stem cell differentiation into more matured cells (Ghosh *et al.*, 2011).

Adult stem cells can be found all over the body, though they vary substantially in their accessibility. Hematopoietic stem cells and MSCs are easy to obtain and cultivate *in vitro*; on the contrary LSCs or NSCs are quite rare, difficult to obtain and cultivate.

Transdifferentiation can be explained as the process of switching the developmental lineage of the adult stem cells from its natural course to another, resulting in a cell type of unrelated tissue (Krabbe *et al.*, 2005). Basically, this result may be achieved through two ways: by direct nuclear reprogramming, or by indirect, external way, where the environmental stimuli provoke the stem cells to react by transcription changes which lead to transdifferentiation. For example, Dr. Meyer-Blazejewska transdifferentiated hair follicle-derived stem cells into cells of corneal epithelial phenotype, to substitute the absent LSCs in ocular injury (Meyer-Blazejewska *et al.*, 2011).

2.2 Mesenchymal stem cells

MSCs are plastic-adherent cells, with fibroblast-like morphology and the ability of chondrogenic, osteogenic and adipogenic differentiation (Dominici *et al.,* 2006). This type of stem cells was described by A. J. Friedenstein, as a subpopulation of bone marrow cells with osteogenic potential (Friedenstein, 1990).

MSCs are adult, multipotent stem cells which are obtained mostly from bone marrow (BM-MSCs), adipose tissue (AT-MSCs) and umbilical cord (UC-MSCs), however they can be found in all tissues and organs of the adult organism, mostly in the perivascular niche (da Silva *et al.*, 2006). It is estimated that the total number of MSCs in human body is cca one million of cells (Bae *et al.*, 2011).

MSCs naturally differentiate into connective tissues of mesodermal lineage: cartilage, bone, adipose and muscular tissue. By *in vitro* transdifferentiation, they can be switched into cells of ectodermal (Tropel *et al.,* 2006) or endodermal lineage (Uccelli *et al*., 2008), e.g. hepatic cells, photoreceptor cells, epidermal cells, endothelial cells, corneal epithelial-like and neuron-like cells (Krabbe *et al.*, 2005, Catacchio *et al.*, 2013, Kyurkchiev *et al.*, 2014). Although the possibility of *in vivo* transdifferentiation of MSCs is considered to be controversial (Uccelli *et al.*, 2008), an increasing number of authors suggest that it is possible (Parr *et al.*, 2007, Khoo *et al.*, 2011).

Since MSCs lack unique specific markers, they are characterized by their ability of plastic adherence, a set of positive (CD73, CD90, CD105) and negative (hematopoietic lineage markers CD34, CD45, CD11b, CD14 and co-stimulatory molecules CD80, CD86 and CD40) immunophenotypic markers, and by their ability of adipogenic, chondrogenic and osteogenic differentiation (Dominici *et al.,* 2006, Kyurkchiev *et al.,* 2014).

Apart from their natural role in connective tissue regeneration, MSCs also play an important role in hematopoiesis, forming a protective microenvironmental niche for blood cell progenitors and producing growth and differentiation factors (Méndez-Ferrer *et al.*, 2010). Significant MSCs characteristics are their immunomodulatory properties: they are able to prevent graft rejection in allogenic recipients (Griffin *et al.*, 2010), they naturally express very low levels of major histocompatibility complex (MHC) molecules and do not express co-stimulatory molecules in a non-stimulated state. Generally, MSCs are not considered to be immunogenic; they can evade the immune control of the recipient and actively support immunosuppression (Griffin *et al.*, 2010). Another important MSC property is their anti-inflammatory and anti-apoptotic effect, which has been proven *in vitro* and *in vivo*, their ability to lower oxidative stress and to migrate directly to the injured tissue (Yagi *et al.*, 2010, Strioga *et al.*, 2012, Li *et al.*, 2013). This unique combination of homing, tissue protective and immunosuppressive properties makes MSCs highly valuable in tissue engineering and regeneration (Mougiakakos *et al.*, 2011). MSCs are also known to be "licensed" by their environment; for example in a pro-inflammatory milieu they obtain anti-inflammatory properties. In this way, they actively participate in maintaining the immunological balance (Krampera *et al.*, 2006, 2011). The anti-inflammatory licensing process in MSCs is mainly performed by interferon gamma (IFN-gamma), supported by interleukin 1 (IL-1) and tumour necrosis factor alpha (TNF-alpha), which is followed by up-regulation in gene expression for prostaglandin E2 (PGE2), cyclooxygenase-2 (COX-2) etc. (Mougiakakos *et al.*, 2011). Finally, licensed MSCs are strongly immunosuppressive.

The immunomodulatory abilities of MSCs are manifested through direct cell-cell interaction and through the production of cytokines and growth factors (Kyurkchiev *et al.,* 2014). Direct, cell-cell contact of MSCs is targeted on natural killer (NK) cells, dendritic cells (DC), T lymphocytes (T-cells) and other immunocytes.

In vitro co-cultivation with NK cells shows that MSCs are rather resistant to NK-mediated cellular lysis. MSCs are also able to suppress NK cell secretion of IFN-gamma and to inhibit NK cell differentiation through PGE2 and indoleamine 2,3-dioxygenase (IDO) secretion (Griffin *et al.*, 2010, Yagi *et al.*, 2010).

MSCs suppress the DC maturation process and support immature DCs, through the inhibition of maturation markers' expression and inhibition of antigen presentation. DCs under MSC influence show changes in cytokine expression, such as up-regulation of IL-10 production and down-regulation of IL-12 production. DCs also become tolerogenic: their contact with naive T lymphocytes results in T lymphocyte anergy (Griffin *et al.*, 2010, Yagi *et al.*, 2010). Moreover, under MSC influence, DCs become supportive to regulatory T lymphocytes (T-regs) (Griffin *et al.*, 2010). Also, MSCs themselves (without DCs) can induce the T-regs through secreting PGE2 and transforming growth factor beta (TGF-beta) and cell-cell contact (Griffin *et al.*, 2010, Yagi *et al.*, 2010, Mougiakakos *et al.*, 2011).

The indirect, contact-independent immunomodulatory effects of MSCs are due to the secretion of a substantial number of cytokines and growth factors, mainly PGE2, IDO, IL-6, IL-10 and TGF-beta (Mougiakakos *et al.*, 2011). PGE2 is produced constitutively by MSCs, and its production is further increased in an inflammatory environment. PGE2 is considered to inhibit the inflammation by shifting T lymfocyte response from type 1 reaction (which is performed by type 1 helper T lymphocytes (Th1)) to type 2 reaction (performed by type 2 helper T lymphocyte (Th2)). PGE2 also inhibits the maturation of DCs and supports the production of IL-10 by macrophages (Ma *et al.*, 2013). PGE2 synthesis in MSCs is induced by COX-2 enzyme. COX-2 is strongly up-regulated after MSC licensing in a pro-inflammatory environment by IFN-gamma, supported by IL-1 and TNF-alpha (Mougiakakos *et al.*, 2011).

Another key player in the immunosuppressive activity of MSCs is IDO. Human MSCs produce IDO after stimulation with IFN-gamma (Yagi *et al.*, 2010). IDO metabolises the essential amino acid tryptophan into kynurenine. Both molecules have an impact on T-cell proliferation. Tryptophan is essential for the early development of T-cells, while kynurenine inhibits their proliferation. Thus, by employing IDO, MSCs have a twofold suppressive effect on T-cells (Yagi *et al.*, 2010). In mice, a similar immunosuppressive role in T-cell inhibition is performed by iNOS (Ma *et al.*, 2013).

Pleiotropic IL-6 is mostly considered to be pro-inflammatory cytokine (Scheller *et al.*, 2011). However, in MSCs, which constitutively produce IL-6, it seems to have a similar immunosuppressive effect as the above-mentioned PGE2 and IDO, since it suppresses the antigen presentation in DCs and thus helps to establish a tolerogenic DC population (Yagi *et al.*, 2010, Li *et al.*, 2013, Kyurkchiev *et al.,* 2014).

The production of anti-inflammatory IL-10 by MSCs is questionable, as both the positive (Ma *et al.*, 2013) and negative (Kyurkchiev *et al.,* 2014) evidence exists. However, there is no doubt that MSCs support IL-10 production in several types of immune cells, for example in macrophages and DCs (Kyurkchiev *et al.,* 2014).

TGF-beta is another pleiotropic cytokine constitutively produced by MSCs. MSCs use TGF-beta in several ways to suppress T and B lymphocyte proliferation, differentiation and effector functions; through the TGF-beta-mediated inhibition of IL-2 production, inhibition of Th1 and Th2 transcription factors T-bet and GATA-3, inhibition of IFN-gamma production, Fas ligand and perforin expression and through the suppression of IL-12 signalization (Kyurkchiev *et al.,* 2014). TGF-beta also supports the induction of FOXP3, transcription factor of T-regs (Griffin *et al.*, 2010, Kyurkchiev *et al.,* 2014) and it has supportive effect on wound healing.

Generally, the character of MSCs (proliferation and differentiation potential, transcriptome, proteome, immunomodulatory properties) slightly vary according to their origin and location (Sakaguchi *et al.*, 2005, Koci *et al.*, 2014, Holan *et al.,* 2015, Li *et al.*, 2015, Heo *et al.*, 2016).

2.3 Neural stem cells

The nervous system comprises generally two types of neural cells: neurons and glia. Neurons represent the active, signal transmitting and electrically conductive component, while glial cells (astrocytes and oligodendrocytes) are supportive, protective, non-conductive regulators of the extracelullar environment. Cooperation between neurons and glia is vital for the physiological functions of the nervous system (Fields *et al.*, 2002). In contrast to former opinions, both of these types of cells can be regenerated in the adult body by multipotent NSCs (Gage *et al.*, 2013). NSCs were first described in embryonic rat forebrain by Temple (Temple, 1989) and isolated by Reynolds and Weiss, using a special isolation protocol termed neurosphere assay (Reynolds and Weiss, 1992). The way of differentiation of NSCs is similar to other tissue-specific stem cells; they divide and differentiate into TACs – neural progenitors, which gradually lose the capability of self-renewal. Neural progenitors migrate and differentiate into more mature cells. Their regional migratory patterns are specific for every resulting neural cell type and depend also on the actual developmental state of the cell.

As with other types of tissue-specific stem cells, NSCs are able to transdifferentiate, for example into blood cells or myocytes (Vescovi *et al.,* 2001). There are two niches in the adult brain where NSCs can be found: the subventricular zone in the lateral ventricles and the subgranular zone in the hippocampus (Kennea *et al.*, 2002, Gage *et al.*, 2013). Since NSCs are not easily available, the transplantations of NSCs are used mostly in experimental neural injuries in animal models. Here, NSC transplantations resulted in functional improvement (Shoichet *et al.*, 2008). In some cases human fetal NSC were transplanted in animal models (Einstein *et al.*, 2008, Kerkis *et al.*,

2015). However, in the clinical therapy of neural injuries it is not ethically acceptable to apply fetal NSCs, as well as ESC-derived neural precursors (Lee-Kubli *et al.*, 2015). For this purpose, other cellular sources for the develpoment and replacement of NSCs / neural progenitors are examined, including iPCSs and MSCs (Shoichet *et al.*, 2008).

There are several common characteristics among various neural injuries. The first phase of injury is characterised by mechanical and vascular damage of the tissue, which is manifested by ischemia, edema and the release of cytotoxic compounds. This is soon followed by secondary damage, including the activation of inflammatory, necrotic and apoptotic processes, which damage the environment suitable for surviving cells (Dasari *et al.*, 2014). This leads to neuronal death (caused by ischemia, which results from damaged blood supply), demyelination (caused by oligodendrocyte loss, which results from necrotic and apoptotic processes in damaged neural tissue) and glial scar formation (Beck *et al.*, 2008, Almad *et al.,* 2011, Dasari *et al.*, 2014). Neural injuries can be divided into CNS and spinal cord-affecting injuries, and peripheral neural injuries, with significant differences in regeneration potential. The peripheral nerve system has a relatively good regenerative potential; its growth-promoting environment (damaged tissue is promptly eliminated by macrophages, cellular growth is supported by secretion of neurotrophins) supports axonal regrowth (which is enabled by the up-regulation of regeneration-associated genes). CNS regenerative potential is significantly lower, due to obstacles in the axonal regrowth activation pathway, together with a growth-inhibiting environment (CNS myelin-associated inhibitors and scar-associated molecules and non-efficient elimination of debris) (Purves *et al.*, 2001, Huebner *et al.*, 2009). The low regeneration potential in CNS can be boosted by changes to the environment, towards a more supportive niche for neuroprotection and neuroregeneration, with some differences in strategies for brain and spine cord (Shoichet *et al.*, 2008). Thus, the main goal in the therapy of neural injuries is the establishment of an environment suitable for

cellular regeneration and replacement, by the prevention of apoptotic and inflammatory processes (Dasari *et al.*, 2014).

2.3.1 Neural differentiation of pluripotent stem cells

Generally, there are ways to generate neurons or neuron-like cells artificially: through the directed differentiation of ESCs, through the generation of iPSCs and through the transdifferentiation of tissue-specific stem cells (using chemicals, growth factors or protocols based on neurosphere assay). Recently, a novel approach using epigenetic reprogramming by small molecules was applied on adult somatic cells, which were reprogrammed into NSC-like cells (Zhang *et al.*, 2016).

Pluripotent ESCs were first isolated and *in vitro* differentiated into all three developmental lineages from mice (Evans *et al.*, 1981) and later from humans (Thomson *et al.*, 1998). For the application of ESCs in neural differentiation it is necessary to suppress the differentiation into entoderm and mesoderm and support the establishment of neuroectoderm. During directed neural differentiation, ESCs *in vitro* undergo similar process as in *in vivo* development (Hu *et al.,* 2010). The process of *in vitro* directed neural differentiation can be described in two steps. First, ESCs are neurally induced to form neurospheres with multipotent NSCs, by above-mentioned suppression of the signaling pathways for other two developmental lineages. This can be performed for example through the inhibition of bone morphogenetic protein (BMP) signalization by noggin transcription factor (Dottori, 2008). Secondly, NSCs are patterned into particular types of neurons and glia through activation appropriate morphogens, transcription factors and signaling pathways, using for example Sonic Hedgehog (SH), fibroblast growth factor beta (bFGF), retinoic acid (RA) (Zhang *et al.*, 2001, Dhara *et al.*, 2008). Based on this general description, numbers of protocols for directed neural differentiation of ECSc have been established (Tropepe *et al.,* 1999, Reubinoff *et al.,* 2001, Tropepe *et al.,* 2001, Dhara *et al.*, 2008,

Woo *et al.,* 2009, Bentz *et al.,* 2010, Hayat, 2013, Li *et al*., 2014, Romanyuk *et al.,* 2015). However, the use of ECSs derived from blastocysts remains ethically controversial.

iPSCs represent attractive alternative in pluripotent cells, since they can be directly obtained from reprogrammed somatic cells of individual patients (Li *et al.*, 2014). The genetic reprogramming is based on forced expression of pluripotency-associated factors OCT4, SOX2, KCF4, c-MYC, Nanog and Lin28 (Takahashi and Yamanaka, 2006, Kim *et al.*, 2014, Yap *et al.,* 2015). Resulting iPSCs have the same characteristics as ESCs, with the same morphology, expression patterns, transcriptional network and morphogen effects and they are recently considered to be highly similar to ESCs (Yap *et al.,* 2015). Thus, the differentiation protocols for neural differentiation of ESCs are often successfully applied on iPSCs differentiation (Muratore *et al.*, 2014). However, when the results of the same differentiation protocol in ESCs and iPSCs were compared (Hu *et al.,* 2010), they found that the efficiency of neural differentiation in iPSCs is lower and variability of resulting cells is higher than in ESCs. Moreover, iPSCs as well as ESCs can form *in vivo* teratomas with uncontrolled growth (Gong *et al.*, 2014, Yap *et al.,* 2015).

2.3.2 Neuronal transdifferentiation of multipotent stem cells

At the level of tissue-specific stem cells, MSCs are the most frequently used cellular source for neuronal transdifferentiation experiments. However, other types of tissue-specific stem cells are also used; for example dental pulp-derived stem cells (Ariffin *et al.*, 2013), hair follicle bulge-derived stem cells (Gho *et al.*, 2015), hematopoietic stem cells (Roybon *et al.,* 2006), hepatic oval cells (Deng *et al.*, 2003), olfactory mucosa-derived stem cells (Murrell *et al.*, 2008). MSCs can be applied in several ways in the field of neural injuries: not only as a cell source for transdifferentiation and cell replacement, but often for regenerative, supportive and immunomodulatory capabilities, both in animal model and in human (Hu *et al.*, 2004, Lu *et al*., 2006, Parr *et al.*, 2007). The important property of MSCs is their broad spectrum of

neurotrophic and growth factors, which they secrete (constitutively or in response to environmental stimuli); for example bFGF, epidermal growth factor (EGF), nerve growth factor (NGF), brain-derived growth factor (BDNF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) (Chen *et al.*, 2002). In an experimental model of a stroke undifferentiated human BM-MSCs were applied to rodent brains. When these MSCs were transplanted, they migrated into the infarction site and started to express early developmental neuronal and glial markers, while they also maintained some characteristics of stem cells. Transplanted animals showed an improvement in neurological functions (Zhao *et al.,* 2002). However, the level of such *in vivo* transdifferentiation of MSCs is very low, and cannot explain the functional improvements, which probably resulted from proteins secreted by MSCs (Chen *et al.*, 2002). Thus, prior *in vitro* or *ex vivo* priming or transdifferentiation is required to increase the regenerative impact of MSCs in treatment of neural injuries.

The first evidence of the neuronal transdifferentiation ability of MSCs *in vitro* was proved in 2000, using rodent and human MCSs (Woodbury *et al*., 2000). However, the process of neuron-like morphological changes occurred several hours after the application of differentiation agents and following electrophysiological measurements did not confirm successful transdifferentiation into functional neurons. Since this type of transdifferentiation was based on application of chemical agents (beta-mercaptoethanol (BME), dimethyl sulfoxide (DMSO) or butylated hydroxyanisole (BHA)), the resulting neuron-like morphology and promising changes in relative gene expression of neuron-specific markers in differentiated cells are considered to be done by chemical damage to the cells, rather than by transdifferentiation process (Woodbury *et al.*, 2000, Bertani *et al.*, 2005, Parr *et al.*, 2007).

Shortly after Woodbury´s work, protocols based on other ways of neuronal transdifferentiation were published: applications of cytokines and growth factors and protocols using neurosphere assay, or combinations of above-mentioned approaches. Neurosphere assay is an *in vitro* tool for

isolation and characterization of NSCs which was established by Reynolds and Weiss in 1992. Basically, neural tissue samples containing NSCs are cultured in a serum-free medium with bFGF and EGF, which only allows the survival and division of NSCs; all differentiated cells are removed (Reynolds and Weiss, 1992, Casarosa *et al.*, 2014). In this way, the neurosphere assay-based transdifferentiation approach converts MSCs into clonogenic NSCs-like populations with floating, neurosphere-like structure. As in NSCs, MSCs formed heterogeneic cellular aggregates, which were able to differentiate into neurons and glial cells (Wislet-Gendebien *et al.*, 2003, Hermann *et al.*, 2004, Fu *et al.*, 2008). In a similar way as in ESCs-based differentiation, these protocols can be described as two step process. In the first step MSCs are converted into NSCs-like cells; this correlates with higher expression of early neuroectodermal markers, for example nestin or musashi-1. In the second step NSCs-like cells differentiate into specific neural cell types (Wislet-Gendebien *et al.*, 2003, Hermann *et al.*, 2004, Fu *et al.*, 2008, Khoo *et al.*, 2011, Heo *et al.*, 2013).

The first application of growth factors in the neural transdifferentiation of MSCs was performed in 2000 (Sanchez-Ramos *et al.,* 2000). The authors applied EGF, RA and BDNF as neural differentiation agents to mouse BM-MSC cultures, which were cocultured with fetal midbrain cells. Other works showed the differentiation effect of pretreatment with EGF and bFGF on both mouse and human AT-MSCs (Tropepe *et al.*, 1999, Safford *et al.,* 2002). Moreover, bFGF was used as a single neuronal differentiation agent in mouse BM-MSCs differentiation protocol, only combined with low seeding density of cells on poly-lysine coated surface (Tropel *et al.*, 2006). The role of low seeding density in neuronal transdifferentiation of MSCs is confirmed also by other authors (Keilhoff *et al.,* 2006).

The development of neuronal differentiation protocols for human MSCs followed the trend established in mouse MSC transdifferentiation. Firstly, chemically-based protocols were published (Jeong *et al.*, 2004) and, with time, they were replaced by growth factor-based

applications and neurosphere assays or their combinations (Jang *et al.,* 2010, Khoo *et al.*, 2011), since the above-mentioned chemical damage could be risky in humans (Bae *et al.*, 2011). Soon, other factors were incorporated into neuronal differentiation protocols: for example the application of 3D scaffolds, which offer 3D extracellular support for the successful spacial growth of axons and dendrites. Promising results were obtained when nanoimprinted gratings were used as a surface for BM-MSCs, seeded at a low density, with RA as differentiation agent (Yim *et al.*, 2007). This illustrated the significance of surface topography on neuronal transdifferentiation in MSCs. In another study, BM-MSCs were grown on caprolactone- and collagen-based nanofibrous scaffolds and transdifferentiation was induced by combination of BME, bFGF, EGF, NGF and BDNF (Prabhakaran *et al.*, 2009). Another team grew placenta-derived MSCs on electrically conductive gold-coated collagen nanofibres, thus implementing into the differentiation protocol another factor: electrical stimulation (Orza *et al.*, 2011). The electrophysiological properties of MSCs have been proven to be suitable for differentiation into electrically excitable cells (Heubach *et al.*, 2004, Bai *et al.*, 2007). Electrical stimulation in physiological conditions affects the intracellular Ca^{2+} oscillations and thus the activation of transcription factors and signaling pathways and it increases the permeability of cellular membrane for growth and differentiation factors.

The promising results from the application of undifferentiated MSCs in transplantation models confirmed that the injured neural environment transmits damage and inflammatory signals to MSCs. MSCs react by paracrine neurotrophic secretion, activation of regenerative and supportive processes and by regulation of inflammation (Chen *et al.*, 2002, Chhabra *et al.,* 2015). Thus, the extracts from injured neural tissue or supernatants obtained from injured cells were used in neuronal differentiation protocols to stimulate the above-mentioned reactions in MSCs (Chen *et al.*, 2002, Moviglia *et al.*, 2006, Bentz *et al.*, 2010, Nicaise *et al.,* 2011, Zhou *et al.*, 2012, Jahromi *et al.*, 2015).

Recently the most often-used MSCs in neuronal transdifferentiation have been BM-MSCs. Some authors have used AT-MSCs (Zuk *et al.*, 2002, Jang *et al.*, 2010), UC-MSCs (Divya *et al.,* 2012) or amniotic fluid MSCs (Kim *et al.*, 2014); an example of an uncommon type of MSCs in neuronal differentiation is the application of tonsil-derived MSCs (Patel *et al.*, 2015).

The properties which MSCs offer for applications in neuronal transdifferentiation slightly vary, depending mainly on the MSC source tissue; variation can be found in ratios of cytokine production and in immunomodulatory or differentiation potential. However, the results of direct comparisons of MSCs properties according to the source of tissue are ambiguous. One study suggested that AT-MSCs may have better immunomodulatory properties than BM-MSCs (greater IDO activity), according to the results of mixed lymphocyte assay (Li *et al.,* 2015). On the other hand, another recent paper found BM-MSCs to have stronger immunomodulatory properties (higher production of anti-inflammatory cytokines IL-10 and TGF-beta) compared to AT-MSCs in the same co-cultivation experiment with activated T-lymfocytes (Heo *et al.*, 2016). Similarly, in *in vivo* models of neural injuries both AT- and BM-MSCs supported functional recovery. This support was occurred mainly due to the release of trophic factors and reduction of the inflammatory and apoptotic processes, particularly through inhibition of the negative impact of astrocytes and microglia (Urdzikova *et al.*, 2006, Dasari *et al.*, 2014).

AT-MSCs, unlike BM-MSCs, when differentiated under the same neuronal differentiation protocol (Arboleda *et al.*, 2011), expressed P2Y purinergic receptor (Forostyak *et al.*, 2016). The P2Y receptor mobilises intracellular Ca^{2+} influx under physiological concentration of ATP (James and Butt, 2001). This can make Ca^{2+} signalization and following neuronal differentiation in AT-MSCs less energy-demanding when compared to BM-MSCs. Another work found that AT-MSCs seem to be more effective in production of bFGF (Li *et al.*, 2015). Among *in vivo* models, it was found that application of AT-MSCs in the therapy of spinal cord injury can be more appropriate than application of BM-MSCs (Zhou *et al.*, 2013).

UC-MSCs are considered to have greater proliferative potential than AT- and BM-MSCs, as there is no donor-age dependent inhibition (Joyce *et al.*, 2012). They are able to produce large amounts of neurotrophic and growth factors (Dasari *et al.*, 2014). However, their ion channel equipment seems to be very similar to BM-MSC equipment (Pillozzi and Becchetti, 2012) and their neuronal transdifferentiation ability could be decreased, compared to AT-MSCs.

2.4 Limbal stem cells

Unlike MSCs, which can be found in every tissue of the body, the location of LSCs is strictly bound to the peripheral part of the cornea – the limbus. The limbus is situated on the border-line between cornea and sclera, where LSCs can be found in the basal layer of limbal epithelium, known as the palisades of Vogt. The limbal *niche* for LSCs is formed by stromal keratocytes, sometimes termed limbal fibroblasts. Stromal keratocytes create the extracellular matrix of the corneal stroma, which is the crucial component of corneal transparency (Katikireddy *et al.*, 2014). The *niche* environment preserves the stemness of LSCs, as well as keeping the conjunctival epithelium out of the cornea (Ebrahimi *et al.*, 2009, He *et al.*, 2014). LSCs can be defined as multipotent, self-renewing, slowly cycling cells with proliferative capability. They form several percent (no more than 5%) of the total number of limbal cells (Krulova *et al.*, 2008). When activated by signals from the cornea, LSCs differentiate into TACs. TACs migrate centripetally from the limbus to the periferal cornea, replacing the more matured TACs, which concurrently differentiate into terminally differentiated suprabasal cells of corneal epithelium and are finally removed by natural shedding (Ebrahimi *et al.*, 2009, Ahmad, 2012, He *et al.*, 2014). To date, there is no known reliable specific marker for LSCs, except for general stem cell markers, for example p63 or ABCG2. However, corneal and limbal epithelial cells can be distinguished by the expression of cornea-specific cytokeratins (CK3 and CK12), which are not present in cells of limbal epithelium. The strategy for the identification of LSCs is often based on

the presence of stem cell markers and absence of differentiation-related markers (Krulova *et al.*, 2008, Ebrahimi *et al.*, 2009, Trosan *et al.*, 2012, He *et al.*, 2014).

The limbus can be damaged by chemical injuries or burns or absent due to genetic disorders, for example aniridia (Ahmad, 2012). Then the unique reservoir of LSCs is lost, which often leads to the development of limbal stem cell deficiency (LSCD) syndrome: the cornea becomes keratopathic and vascularised due to the invasion of the conjunctive epithelium; vision is impaired due to reduced corneal transparency and eventually it is lost totally (Yoon *et al.*, 2014). This deficiency can only be treated by limbal / LSCs transplantation. Unilateral chemical injuries or burns can be cured by autologous transplantation. In the case of bilateral damage or genetic disorder allogeneic transplantation is necessary. However, this treatment is not easily available due to the limited number of limbal transplants. Also, the results are compromised by the poor survival rate of allografts (Chan *et al.*, 2013, Sareen *et al.*, 2014).

2.4.1 Corneal epithelial differentiation of pluripotent stem cells

There is a strong need for an alternative cell source for treatment of LSCD. ESCs can be *in vitro* differentiated into corneal epithelial cells (Ahmad *et al.*, 2007) or into stromal keratocytes (Chan *et al.*, 2013), but when seeded on corneas *in vivo*, ESCs may fail to stratify (Sareen *et al.*, 2014). The risk of tumorigenesis and the ethical dilemma remain objections to ECS application.

iPSCs as a source of cells for differentiation into corneal epithelial cells are easily expandable, bankable and can be reprogrammed from cells of individual patients (Hayashi *et al.*, 2012, Sareen *et al.*, 2014). iPSCs can be differentiated into corneal epithelial cells by co-cultivation with keratocytes, which compensate the effect of the LSC niche (Guo and Chen, 2015).

2.4.2 Corneal epithelial transdifferentiation of multipotent stem cells

All common types of MSCs have been used for corneal epithelial transdifferentiation and corneal regeneration; for example BM-MSCs were transdifferentiated into corneal epithelial cells (Rohaina *et al.*, 2014), into corneal keratocytes (Liu *et al.*, 2012). AT-MSCs were transdifferentiated into corneal epithelial cells (Nieto-Miguel *et al.,* 2013), into corneal keratocytes (Du *et al.,* 2010) and UC-MSCs into corneal endothelium (Joyce *et al.,* 2012) or into corneal epithelium (Reza *et al.*, 2011). Also other, less common types of adult stem cells have recently been used as sources for transdifferentiation into corneal epithelium: for example dental pulp stem cells (Gomes *et al.*, 2010), hair follicle bulge cells (Meyer-Blazejewska *et al.*, 2011) or oral mucosa epithelial cells (Nakamura *et al.*, 2011).

Apart from transdifferentiation potential, MSCs in corneal regeneration can contribute also by their secretory and anti-inflammatory properties (Holan *et al.,* 2015). Moreover, there is also a similarity in the immunoregulatory behaviour of LSCs and MSCs (Zajicova *et al.,* 2010). Another similarity which supports the application of MSCs in corneal regeneration can be found between MSCs and corneal keratocytes, which originated from neural crest-derived periocular MSCs (Joyce *et al.,* 2012, Li *et al.,* 2012, Chan *et al.,* 2013, Katikireddy *et al.*, 2014). Indeed, it is also possible to isolate MSCs from limbal tissue, to expand them in vitro and to use them in corneal regeneration (Basu *et al.*, 2014). Based on this resemblance, MSCs are able to substitute the effect of the limbal *niche* (Du *et al.,* 2010) or LSCs (Holan *et al.*, 2015).

2.5 Other applications and perspectives of stem cell-based therapy

MSCs have recently been used in cell-based therapy – at preclinical or clinical levels - for several different diseases, due to their differentiation and immunomodulatory potential, together with their good engraftment and healing properties.

Apart from neural injuries MSCs are used in diabetes therapy as a source for transdifferentiation into insulin-producing cells, as supportive cells and immunomodulators in islet-cell transplantations and also in treatment of non-healing diabetic wounds and ulcers. These wounds, similarly to severe burns, are characterized by damage at several tissue levels, where the sef-regenerative potential of tissue is severely decreased. Here MSCs actively participate in every phase of wound healing, utilizing their anti-inflammatory, antimicrobial, immunomodulatory and regenerative properties (Maxson *et al.,* 2012). Although various types of MSCs are used in diabetes therapy, the most often applied are BM- and AT-MSCs. AT-MSCs seem to have a greater differentiation and proliferative potential than BM-MSCs in these applications (Hashemian *et al.*, 2015). However, there are differences and ambiguities in the impact of the hyperglycaemic environment on MSCs. Some authors claim that this environment supports the differentiation of MSCs into insulin-producing cells, while others consider the hyperglycaemic environment to be harmful for MSCs (Koci *et al.*, 2014, Hashemian *et al.*, 2015).

In the therapy of osteoarthritis, MCSs can contribute to cartilage repair through their chondrogenic differentiation potential. Usage of MSCs (non-differentiated or pre-differentiated) is possible both in autologous and allogeneic applications (Gupta *et al.*, 2012). In this case, BM-MSCs seem to be more appropriate, compared to AT-MSCs, as their differentiation potential and abundant protein secretion (stromal cell-derived factor-1 (SDF-1) and HGF) are higher (Li *et al.*, 2015). In the area of liver diseases, MSCs can be applied not only due to their capacity to differentiate into functional hepatocyte-like cells, but also as potent producers of HGF, a growth

factor with anti-fibrotic effect (Beradis *et al.*, 2015). BM-MSCs are the most-commonly used cells, but also AT- or UC-MSCs have been applied (Meier *et al.*, 2013). MSCs are also used in cardiac infarction therapy, as they contribute to cardiomyogenesis and angiogenesis, and AT-MSCs seem to be more appropriate for this particular application (Mazo *et al.*, 2012). MSCs are also used in the treatment of graft-versus-host disease, where they modulate the functions of immune effector cells. In clinical studies the application of BM-MSCs strongly prevails (Amorin *et al.*, 2014). Other applications of MSCs, recently tested, are in the therapy of haematological diseases, Crohn's disease, multiple sclerosis etc. (Patel *et al*., 2013, Wei *et al*., 2013, Gupta *et al.*, 2014).

As mentioned above, MSCs represent robust, easily handled population of cells, but their properties vary slightly according to their origin and location (Sakaguchi *et al.,* 2005, Kern *et al*., 2006, Koci *et al.*, 2014). These differences may not be of great importance *in vitro*, but may become important in clinics, where the proper type of MSCs has to be carefully selected, to gain maximum effects according to the demands of the particular situation (Kern *et al.,* 2006, Strioga *et al.*, 2012).

3 Aims of thesis

Main aims of thesis

3.1 The development of an *in vitro* transdifferentiation protocol for generating neuron-like cells from mouse MSCs.

MSCs generally have the potential to transdifferentiate into neuron-like cells. Since the environment has an important effect on stem cell behaviour, the protocol was designed to simulate the impact of injured neural tissue on stem cell transdifferentiation. This protocol included the effect of growth factors, brain tissue extract, supernatant from concanavalin A (ConA) stimulated splenocytes, electrical stimulation in physiological conditions and extracellular support by nanofiber scaffolds. The protocol was optimized in terms of:

- the passage number of seeded cells, seeding density and duration of differentiation protocol
- selection and dosage of growth factors
- preparation and dosage of brain tissue extract and supernatant from ConA-stimulated splenocytes
- intensity, frequency, duration and timing of electrical stimulation in physiological conditions
- selection of 3D extracellular nanofiber support

3.2 The characterization of the immunomodulatory properties of neuron-like cells.

MSCs are able to modulate immune reactions. As a consequence of their previous licension by the inflammatory environment, they are able to inhibit inflammation. Thus, the inflammatory environment, established in transdifferentiation protocol by supernatants from ConA-stimulated splenocytes and brain tissue extracts, should trigger anti-inflammatory properties in MSCs differentiating into neuron-like cells.

3.3 The optimization of the transdifferentiation protocol for human AT-MSCs.

Since the original differentiation protocol was designed for mouse AT-MSCs, it can be presumed that human AT-MSCs could need some optimizations in passage numbers, seeding density and differentiation components doses, to obtain optimal differentiation results.

Other aims of the thesis

3.4 The characterization of the role of growth factors in the differentiation of LSCs into corneal epithelium.

The impact of growth factors on particular types of stem cells´ behaviour can vary depending on the surrounding environment. The effect of commonly used cytokines on LSC differentiation and proliferation is to be defined.

3.5 The comparison of the potential of AT- and BM-MSCs to support or substitute LSCs in cornea regeneration.

MSCs seem to be an alternative stem cell source in corneal regeneration. To estimate the most appropriate MSCs for this application, the comparison of the characteristics of AT- and BM-MSCs was performed.

4 List of publications

4.1 Thesis publications

Chudickova M, Bruza P, Zajicova A, Trosan P, Svobodova L, Javorkova E, Kubinova S, Holan V. Targeted neural differentiation of murine mesenchymal stem cells by a protocol simulating the inflammatory site of neural injury. J Tissue Eng Regen Med. 2015; doi: 10.1002/term.2059. [Epub ahead of print]. IF=5,199

Trosan P, Svobodova E, **Chudickova M**, Krulova M, Zajicova A, Holan V. The key role of insulin-like growth factor I in limbal stem cell differentiation and the corneal wound-healing process. Stem Cells Dev. 2012;21(18):3341-3350. IF=3,727

Holan V, Trosan P, Cejka C, Javorkova E, Zajicova A, Hermankova B, **Chudickova M**, Cejkova J. A comparative study of the therapeutic potential of mesenchymal stem cells and limbal epithelial stem cells for ocular surface reconstruction. Stem Cells Transl Med. 2015;4(9):1052-1063. IF=5,709

4.2 Other publications

Koci Z, Turnovcova K, Dubsky M, Baranovicova L, Holan V, **Chudickova M**, Sykova E, Kubinova S. Characterization of human adipose tissue-derived stromal cells isolated from diabetic patient's distal limbs with critical ischemia. Cell Biochem Funct. 2014;32(7):597-604. IF=2,005

Hermankova B, Zajicova A, Javorkova E, **Chudickova M**, Trosan P, Hajkova M, Krulova M, Holan V. Suppression of IL-10 production by activated B cells via a cell contact-dependent cyclooxygenase-2 pathway upregulated in IFN-γ-treated mesenchymal stem cells. Immunobiology 2016;221(2):129-136. IF=3,044

Holan V, **Chudickova M**, Trosan P, Svobodova E, Krulova M, Kubinova S, Sykova E, Sirc J, Michalek J, Juklickova M, Munzarova M, Zajicova A. Cyclosporine A-loaded and stem cell-seeded electrospun nanofibers for cell-based therapy and local immunosuppression. J Control Release. 2011;156(3):406-412. IF=7,705

Holan V, Zajicova A, Javorkova E, Trosan P, **Chudickova M**, Pavlikova M, Krulova M. Distinct cytokines balance the development of regulatory T cells and interleukin-10-producing regulatory B cells. Immunology. 2014;141(4):577-586. IF=3,795

Zajicova A, Javorkova E, Trosan P, **Chudickova M**, Krulova M, Holan V. A low-molecular-weight dialysable leukocyte extract selectively enhances development of $CD4^{+}ROR\gamma t^{+}$ T cells and IL-17 production. Folia Biol (Praha). 2014;60(6):253-260. IF=1,000

Bilkova B, Albrecht T, **Chudickova M**, Holan V, Pialek J, Vinkler M. Application of Concanavalin A during immune responsiveness skin-swelling tests facilitates measurement interpretation in mammalian ecology. Ecol Evol. 2016; doi: 10.1002/ece3.2211. IF=2,320 in press

5 Results

5.1 Targeted neural differentiation of murine mesenchymal stem cells by a protocol simulating the inflammatory site of a neural injury.

Chudickova M., Bruza P., Zajicova A., Trosan P., Svobodova L., Javorkova E., Kubinova S., Holan V.

J Tissue Eng Regen Med. 2015; doi: 10.1002/term.2059. [Epub ahead of print]

The complex environment of neural injury includes a lot of factors, which have various effects on stem cell behaviour and regenerative potential. Due to these environmental effects, the *in vivo* regeneration of central nervous system is complicated and frequently unsuccessful. However, generally it is possible; for example in the more supportive environment of an injured peripheral nervous system. This work was aimed to design an *in vitro* transdifferentiation protocol which would mimic the stimulatory impacts of neural injury on stem cells, while regulating its negative effects. In this way the stem cells could be pre-differentiated into neuron-like cells which could persist and operate in the environment of a neural injury. The protocol was based on the effect of growth factors, brain tissue extract, supernatant from ConA-stimulated splenocytes, electrical stimulation in physiological conditions and extracellular support by nanofiber scaffolds. Neuron-like cells resulting from this protocol acquired neuron-like morphology, expressed the genes for neuron-specific markers, for example neurofilament light polypeptide (Nf-L), neurofilament medium polypeptide (Nf-M), synaptophysin (SYP), glutamic acid decarboxylase (GAD), beta-III tubulin (TUBB3), and were positive for Nf-L polypeptide.

JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE RESEARCH ARTICLE
J Tissue Eng Regen Med 2015.
Published online in Wiley Online Library (wileyonlinelibrary.com) DOI: 10.1002/term.2059

Targeted neural differentiation of murine mesenchymal stem cells by a protocol simulating the inflammatory site of neural injury

Milada Chudickova[1,2], Petr Bruza[3], Alena Zajicova[1], Peter Trosan[1,2], Lucie Svobodova[1], Eliska Javorkova[1,2], Sarka Kubinova[1] and Vladimir Holan[1,2]*
[1]Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Prague, Czech Republic
[2]Faculty of Science, Charles University, Prague, Czech Republic
[3]Faculty of Biomedical Engineering, Czech Technical University in Prague, Kladno, Czech Republic

Abstract

Damaged neural tissue is regenerated by neural stem cells (NSCs), which represent a rare and difficult-to-culture cell population. Therefore, alternative sources of stem cells are being tested to replace a shortage of NSCs. Here we show that mouse adipose tissue-derived mesenchymal stem cells (MSCs) can be effectively differentiated into cells expressing neuronal cell markers. The differentiation proto-col, simulating the inflammatory site of neural injury, involved brain tissue extract, fibroblast growth factor, epidermal growth factor, supernatant from activated splenocytes and electrical stimulation un-der physiological conditions. MSCs differentiated using this protocol displayed neuronal cell morphol-ogy and expressed genes for neuronal cell markers, such as neurofilament light (Nf-L), medium (Nf-M) and heavy (Nf-H) polypeptides, synaptophysin (SYP), neural cell adhesion molecule (NCAM), glutamic acid decarboxylase (GAD), neuron-specific nuclear protein (NeuN), βIII-tubulin (Tubb3) and microtubule-associated protein 2 (Mtap2), which are absent (Nf-L, Nf-H, SYP, GAD, NeuN and Mtap2) or only slightly expressed (NCAM, Tubb3 and Nf-M) in undifferentiated cells. The differentiation was further enhanced when the cells were cultured on nanofibre scaffolds. The neural differenti-ation of MSCs, which was detected at the level of gene expression, was confirmed by positive im-munostaining for Nf-L protein. The results thus show that the simulation of conditions in an injured neural tissue and inflammatory environment, supplemented with electrical stimulation under physiological conditions and cultivation of cells on a three-dimensional (3D) nanofibre scaffold, form an effective protocol for the differentiation of MSCs into cells with neuronal markers.

Received 15 July 2014; Revised 19 January 2015; Accepted 29 April 2015

Keywords mouse; adipose-derived mesenchymal stem cells; neural differentiation; neural injury; nanofibre scaffold; electrical stimulation

1. Introduction

Damage to the nervous system is dangerous and can cause injuries that compromise the quality of life; thus, enormous efforts are made to enable its regeneration and recovery. This is naturally secured by neural stem cells (NSCs), a rare cell population, which is difficult to obtain and grow in vitro. Therefore, there is a need for alternative cell sources that are easy to make available and handle while robust enough for the demands of tissue engineering. These requirements are met by mesenchymal stem cells (MSCs); they can be obtained from various sources, such as bone marrow or adipose tissue, can be isolated according to their adherence to plastic surfaces and propagated in vitro to obtain sufficient quantities of cells (Dominici et al., 2006). These properties make them a promising tool in tissue engineering and reconstruction. Besides their general beneficial impact on wound healing

*Correspondence to: V. Holan, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic. E-mail: holan@biomed.cas.cz

by the production of growth and differentiation factors, and by their immunomodulation properties, they are able to differentiate and transdifferentiate into many types of cells, including neuron-like cells (Sanchez-Ramos et al., 2000; Tropel et al., 2006; Jackson et al., 2007; Jang et al., 2010). Since the factors and signals from the damaged tissue activate stem cells to differentiate into particular cell types and to contribute to regeneration (Trosan et al., 2012), we have made attempts to simulate the inflammatory environment in damaged neural tissue and to differentiate MSCs into cells with neuron-like properties and characteristics. It has been shown that extracts from damaged neural tissue support stem cells to differentiate into neuron-like cells (Chen et al., 2002; Bentz et al., 2010; Nicaise et al., 2011) and some of the factors supporting neural differentiation have been identified (Tropepe et al., 1999; Safford et al., 2002; Arnhold et al., 2006; Tate et al., 2010; Khoo et al., 2011). Furthermore, the factors produced by activated splenocytes infiltrating the inflamed site of a neural wound also contribute to the neural differentiation of stem cells (Armstrong et al., 2003; Moviglia et al., 2006). Another important factor supporting neural differentiation is a type of extracellular matrix. It has been shown that three-dimensional (3D) scaffolds support the differentiation of stem cells into neuron-like cells (Yim et al., 2007; Prabhakaran et al., 2009; Orza et al., 2011). Finally, electrical stimulation, under physiological conditions that are characteristic for the nervous system, has also been shown to be another factor increasing the neural differentiation of stem cells (Woo et al., 2009; Orza et al., 2011).

In this study we used mouse adipose tissue-derived MSCs and brain tissue extract, defined recombinant growth factors, supernatants from activated splenocytes, electrical stimulation and 3D nanofibre scaffolds as differentiation factors. The study was aimed at simulating the effects of an inflammatory environment at the site of neural injury on stem cell differentiation. Individual components of the differentiation protocol, when used separately, induced only a limited or no significant differentiation of MSCs into neuron-like cells. However, the combination of all differentiation factors created a superior environment and resulted in a highly effective transdifferentiation of MSCs. Since adipose tissue-derived MSCs represent an adult type of stem cells, which can be obtained from a particular patient and applied therapeutically as autologous cells, the ethical and biological problems associated with the application of embryonic stem cells or induced pluripotent stem cells are avoided. Thus, predifferentiated MSCs growing on nanofibre scaffold may represent a perspective cell source for neural tissue repair and regeneration.

2. Materials and methods

2.1. Mice

Mice of the inbred strain BALB/c, aged 7–10 weeks, were obtained from the breeding unit of the Institute of Molecular Genetics, Prague. Use of the animals was approved by the local Animal Ethics Committee of the Institute of Experimental Medicine.

2.2. Isolation and culture of MSC

Subcutaneous adipose tissue from the dorsal part of the body was collected in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal calf serum (FCS; Sigma), antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) and 10 mM HEPES buffer. The tissue was cut into small pieces with scissors and incubated in 1 ml Hanks's balanced salt solution containing 10 mg/ml collagenase type I (Sigma) for 60 min at 37 °C with gentle agitation. Then the collagenase was diluted with complete DMEM, the cells were filtered and centrifuged at 250 × g for 8 min. The upper adipose layer was removed, the cells were centrifuged again, resuspended in 6 ml complete DMEM and seeded in 25 cm^3 tissue culture flasks (Corning, Schiphol-Rijk, The Netherlands). After incubation for 48 h, the cells were washed with medium to remove non-adherent cells and cell debris. The adherent cells were cultured for 3 weeks in complete DMEM, with a regular medium exchange and passaging cells to keep an optimal cell concentration. After 3 weeks of cultivation (3–5 passages), the cells were collected by trypsinization.

2.3. Characterization of MSCs

MSCs were rinsed with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and labelled on ice with: fluorescein isothiocyanate (FITC)-labelled monoclonal antibody (mAb) anti-CD44 (clone IM7, BD Pharmingen, San Jose, CA, USA); FITC-labelled anti-CD90.2 (clone 30-H12, BioLegend, San Diego, CA, USA); phycoerythrin (PE)-labelled anti-CD105 (clone TY/11.8, eBioscience, San Diego, CA, USA); allophycocyanin (APC)-labelled anti-CD11b (clone M1/70, BioLegend); PE-labelled anti-CD31 (clone MEC 13.3, BD Pharmingen); or FITC-labelled anti-CD45 (clone 30-F11, BioLegend) for 30 min. Cells stained with PE-labelled rat IgG2a (clone RTK2758, BioLegend), APC-labelled rat IgG2b (clone RTK4530, BioLegend) or FITC-labelled rat IgG2b (clone RTK4530, BioLegend) were used as negative controls. Dead cells were stained with Hoechst 33258 (1 μg/ml, Sigma), added to the samples for the last 15 min before flow-cytometry analysis. Data were collected using an LSRII cytometer (BD Bioscience) and analysed using FlowJo software (Tree Star, Ashland, OR, USA).

2.4. Preparation of brain tissue extracts

Brain tissue from adult mice was collected in serum-free DMEM on ice, cut with scissors, homogenized with a glass homogenizer in a volume of approximately one brain/ml DMEM and frozen at −80 °C. The homogenate was thawed

 DOI: 10.1002/term

and frozen three times and centrifuged at 3000 x g for 8 min. The supernatant was filtered through a 0.45 μm filter (Millipore, Billerica, MA, USA) and stored in aliquots at −80 °C.

2.5. Preparation of supernatants from Concanavalin A (ConA)-stimulated splenocytes

A single-cell suspension of mouse spleen cells (1×10^6 cells/ml) was stimulated in RPMI 1640 medium (Sigma) containing 10% FCS, antibiotics and 10 mM HEPES buffer for 48 h at 37 °C with 1.5 μg/ml ConA (Sigma). The supernatants were harvested, centrifuged at 3000 × g for 8 min, filtered through 0.45 μm filter (Millipore) and stored in aliquots at −80 °C. We have shown previously that supernatants from Con A-stimulated spleen cell cultures contained a number of pro-inflammatory cytokines, such as interleukin (IL)-2, IL-6 or interferon-γ (IFNγ) (Holan et al., 2010).

2.6. Nanofibre scaffolds

Polycaprolactone (PCL) nanofibre scaffold was prepared using the electrospinning technique, as we have described (Zajicova et al., 2010). Briefly, PCL polymer was dissolved in chloroform at 7 weight percent (wt%) and the solution was completed by 1,2-dichlorethane (29 wt%) and ethyl acetate (10 wt%) (both from Penta, Prague, Czech Republic). To prepare the nanofibres, modified needleless Nanospider technology was used (Zajicova et al., 2010). The mass/unit area of nanofibre material used in this study was 5 g/m^2; the nanofibres had a diameter of 283–520 nm. The morphology of PCL nanofibres was characterized by scanning electron microscopy (data not shown).

To use nanofibres as a cell scaffold, the nanofibres were cut into pieces (1.5 × 1.5 cm^2) and fixed into CellCrown™ 24 inserts (Scaffdex, Tampere, Finland).

2.7. Electrical stimulation

To perform the electrical stimulation experiments, a system comprising a pulse generator, an amplifier front-end and a data acquisition subsystem was designed, as depicted in Figure 1. The signal from the high-impedance pulse generator HM8150 (Hameg Instruments, Mainhausen, Germany) output was buffered using the voltage follower OP27 (Analog Devices, Norwood, MA, USA) and guided to the positive electrode. The negative electrode was con-nected to the low-impedance current-to-voltage amplifier AD549 (Analog Devices) in order to monitor the current flowing in the cell culture. The pulse generator and current-to-voltage amplifier signals were digitalized by a USB6009 data acquisition device (DAQ, National Instru-ments, Austin, TX, USA) and stored in a computer. The experimental cell cultures were subjected to a 4 Hz positive monophasic pulse wave with a 2.5 ms pulse duration and an amplitude of 1.1 V. The silver (Ag) electrodes were fixed to a cell culture dish with Teflon clamps.

2.8. Differentiation protocol

MSCs were seeded at a low concentration (2×10^3 cells/cm^2) in 35 mm Petri dishes (Corning) or on PCL nanofibres fixed to inserts, in accordance with similarly low concentrations of cells used in previous studies (Safford et al., 2002, Tate et al. 2010, Khoo et al., 2011). The inserts with cell-seeded nanofibres were placed into Petri dishes and the cells were cultured in 3 ml complete DMEM. Half of the culture medium was removed on days 2, 5 and 8 and replaced by the same volume of a mixture that consisted of five volume parts of complete DMEM, four parts of supernatant from ConA-stimulated spleen cells and one part of brain tissue extract. Recombinant murine fibroblast growth factor-basic (FGF-b; Peprotech, Rocky Hill, NJ, USA) was added to the cultures on days 2, 4, 5 and 8 to make a final concentration of 10 ng/ml. Recombinant murine epidermal growth factor (EGF; Peprotech) at a final concentration of 10 ng/ml was added to the cultures on day 8. The electrical stimulation was applied on days 3, 4, 5 and 8, with the following parameters: strength of electric field 0.33 V/cm, which corresponds to an applied voltage of 1.1 V, frequency of pulses 4 Hz, for 5 min. On day 9, the nanofibres with cells were collected in 500 μl TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and used for the detection of gene expression. Our pilot experiments have revealed that the best results were obtained after at least 9 days of differentiation.

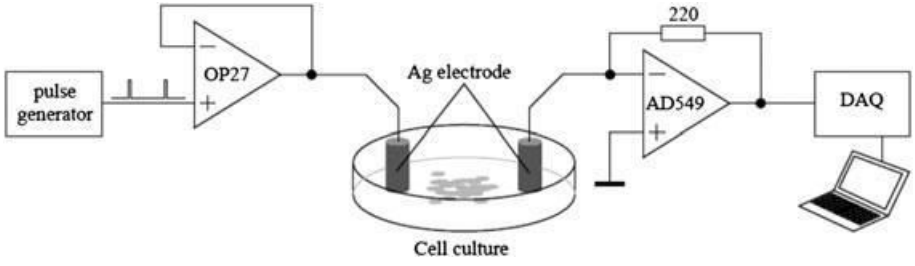

Figure 1. Electrical stimulation set-up. The electrical current generated by a single output of direct current power supply (not depicted in the figure), modulated by a pulse generator and buffered by an OP27 voltage follower, is guided to the positive electrode. The negative electrode is connected to an AD549 current-to-voltage amplifier. The signals from the pulse generator and the current-to-voltage amplifier are digitalized by a DAQ device and stored in a computer

DOI: 10.1002/term

2.9. Detection of gene expression by real-time PCR

Total RNA was isolated from cells using TRI Reagent with Polyacryl Carrier (Molecular Research Center) augmentation, according to the manufacturer's instructions. Total RNA (1 μg) was pretreated using deoxyribonulease I (Promega, Madison, WI, USA) and subsequently used for reverse transcription. The first strand of cDNA was synthetized using M-MLV Reverse Transcriptase and random primers (Promega) in a total reaction volume of 25 μl. Quantitative real-time PCR was performed on a StepOne Plus Cycler (Applied Biosystems, Warrington, UK), using Power SYBR Green (Applied Biosystems) according to the manufacturer's instructions. The primers used for amplification of genes for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), neurofilament light polypeptide (Nf-L), neurofilament medium polypeptide (Nf-M), neurofilament heavy polypeptide (Nf-H), synaptophysin (SYP), neural cell adhesion molecule (NCAM), glutamic acid decarboxylase (GAD), neuron-specific nuclear protein (NeuN), βIII tubulin (Tubb3) and microtubule-associated protein 2 (Mtap2) (Table 1) were obtained from Generi-Biotech (Hradec Kralove, Czech Republic). The PCR parameters involved denaturation at 95 °C for 3 min, then 40 cycles at 95 °C for 20 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. Fluorescence data were collected at each cycle after an elongation step at 80 °C for 5 s and were analysed on a StepOnePlus detection system (Applied Biosystems). CT numbers were used for CT calculation, with GAPDH as the housekeeping gene. Murine brain tissue cDNA was used as a positive control for neuron-specific markers. Each experiment was done in duplicate.

2.10. Immunocytochemistry

MSCs from control cultures or cultures undergoing a differentiation protocol were grown on microscope slides, which were sterilized with 70% ethanol, coated with 10 μg/ml laminin solution (Sigma) for 20 min, rinsed with sterile water and sterilized with UV light. On day 9, cells growing on slides were fixed with 4% paraformaldehyde for 10 min, then soaked three times for 10 min into PBS and permeabilized with 0.3% solution of Tween 20 for 20 min. Cells were treated for 2 h at room temperature with Chemiblocker (1 : 10) and 0.1% solution of Tween 20 in PBS to block non-specific staining.

Neurofilament-L specific mAb (1:100; clone MA5-14981, Thermo Fisher Scientific, Rockford, IL, USA) diluted in 0.1 M PBS containing Chemiblocker (1:6) and Tween 20 (0.1%) was applied to the cells for 16 h at 4 °C. This step was followed by incubation with a secondary goat anti-rabbit IgG antibody conjugated with AlexaFluor 488 (1:250; Invitrogen, Eugene, OR, USA) diluted in 0.1 M PBS with Chemiblocker (1:10) and Tween 20 (0.1%) for 2 h at room temperature. The slides were fixed to glass with Mowiol 4-88 (Calbiochem, San Diego, CA, USA) containing the nuclear dye 4′,6′-diamidino-2-phenylindol (DAPI; 1:1000; Life Technologies, Eugene, OR, USA). The samples were stored in the dark at 4 °C and assessed using a fluorescent microscope (Leica, Wetzlar, Germany).

2.11. Statistical analysis

The statistical significance of differences between individual samples was calculated using Student's t-test and multiple comparisons were analysed by ANOVA.

3. Results

3.1. Characterization of MSCs

The expression of cell surface markers on adipose tissue-derived MSCs cultured for 3 weeks was determined by flow cytometry. The cells were negative for CD11b, CD45 and CD31 and positive for CD44, CD90 and CD105 (Figure 2). In addition, the cells had typical fibroblast-like cell morphology and were capable of differentiating into adipogenic and osteogenic lineages (results not shown).

MSCs grown under standard culture conditions were tested by quantitative PCR (qPCR) for the expression of genes for the neuron-specific markers Nf-L, Nf-M, Nf-H, SYP, NCAM, GAD, Tubb3, NeuN and Mtap2. Undifferentiated MSCs did not express detectable levels of the listed markers, except for a low expression of Nf-M, NCAM and Tubb3 (Figure 3).

Table 1. Primers used for real-time PCR

Gene	Sense primer	Antisense primer
GAPDH	CCCAACGTGTCTGTCGTG	CCGACCCAGACGTACAGC
Nf-L	GGACATCGAGATTGCAGCTT	CTGGTGAAACTGAGCCTGGT
Nf-M	GAATACCAGGATCTCCTTAACGTC	TTCCCCCTCTAGGAGTTTCC
Nf-H	CATTGAGATTGCCGCTTACA	ACTCGGACCAAAGCCAATC
SYP	CTGGCAGACATGGACGTG	CTTGACCACCCGGAACTG
NCAM	CACTTTGTGTTCAGGACCTCAG	AAAAGCAATGAGACCAAGGTG
GAD	ATACAACCTTTGGCTGCATGT	TTCCGGGACATGAGCAGT
NeuN	CACAGCAACCTACAGCATCG	CACAGCAACCTACAGCATCG
Tubb3	GCGCATCAGCGTATACTACAA	TTCCAAGTCCACCAGAATGG
Mtap2	GCTCCAAGTTTCACAGAAGGAG	AGGTTGGTTCAGATCAATATAAATAGG

3.2. Differentiation effect of neural tissue extract, supernatant from activated splenocytes, recombinant growth factors, electrical stimulation and nanofibre scaffold

The differentiation protocol for targeted neural differentiation of MSCs was designed with regard to simulating the physiological conditions in the environment of wounded neural tissue. The protocol involved brain tissue extract, supernatant from stimulated splenocytes, FGF-b and EGF, and was supported by electrical stimulation and nanofibre scaffolds. For a more detailed analysis, the neuron-specific markers with the highest expression were chosen (i.e. Nf-L, Nf-M, SYP and GAD).

Cells treated with growth factors only (Figure 4, group G) did not express detectable levels of neuron-specific markers SYP and GAD and only low levels of Nf-M, compared to untreated cells (Figure 4, group ctrl). Similar results were obtained for cells treated with growth factors and supernatant from activated splenocytes (Figure 4, group G + S). The levels of all neuron-specific markers in cells treated with growth factors and brain tissue extract (Figure 4, group G + E) were slightly increased in comparison to untreated cells. A significant increase in the expression levels of all tested neuron-specific markers was observed in cells treated with a combination of

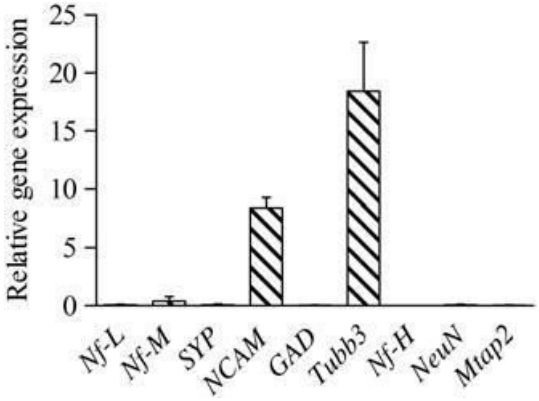

Figure 3. Relative gene expression of neuron-specific markers in undifferentiated MSCs. The cells did not express detectable levels of Nf-L, Nf-H, SYP, GAD, NeuN or Mtap2 and only low expressions of Tubb3, NCAM and Nf-M were detected. Each bar represents mean ± SE from three independent determinations

growth factors, brain tissue extract and supernatant from activated splenocytes (Figure 4, group G + E + S).

Electrical stimulation alone had no differentiation effect (results not shown). However, when electrical stimulation was involved in the basic differentiation protocol, composed of brain tissue extract, supernatant and growth factors, further increase in the expression of neuron-specific markers was observed (Figure 5). The electrical

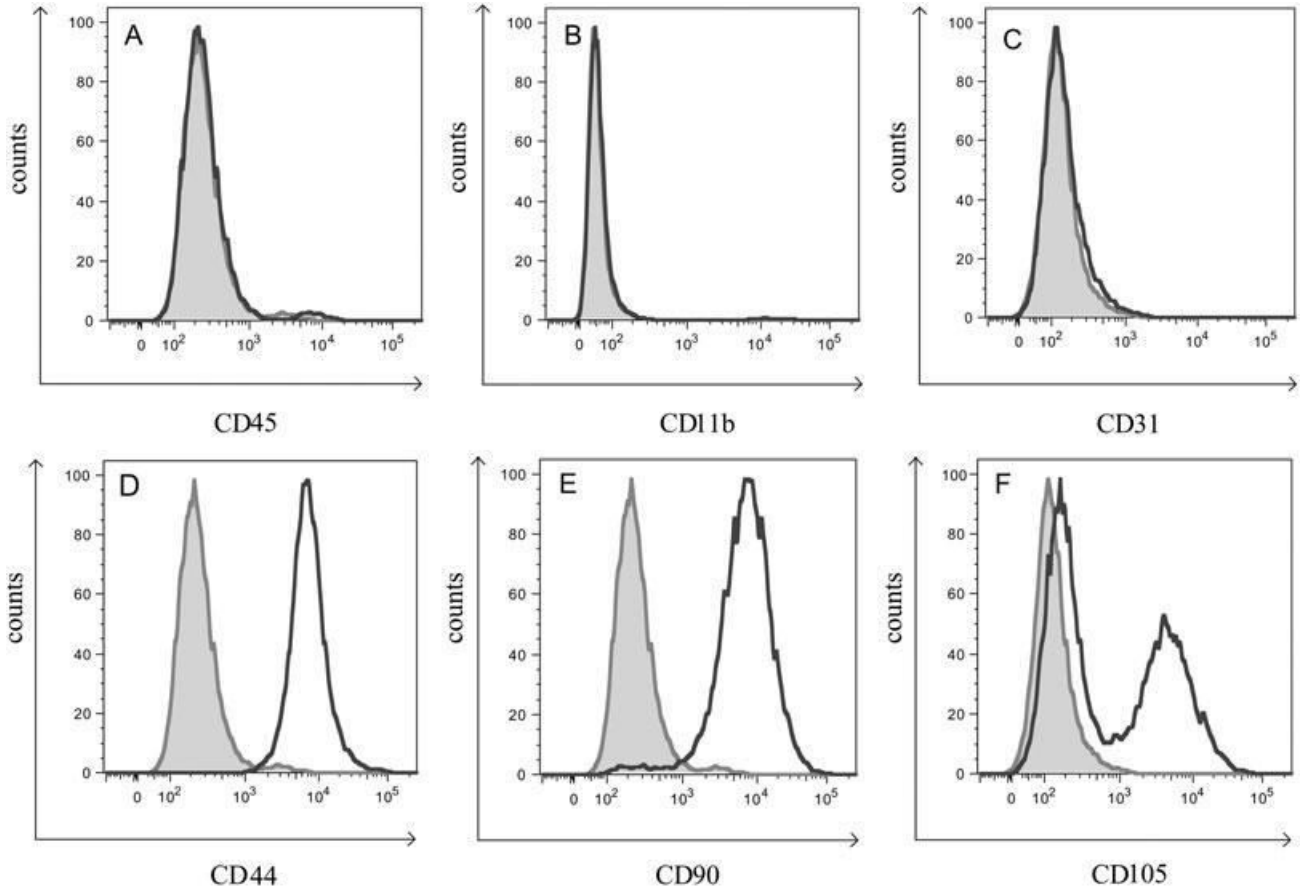

Figure 2. Flow-cytometry analysis of undifferentiated MSCs; the cells were stained with Hoechst 33258 dye and by antibodies against CD45 (A), CD11b (B), CD31 (C), CD44 (D), CD90 (E) and CD105 (F). MSCs stained with appropriate isotype control were used as a negative control (grey colour). A typical result from three determinations is shown

DOI: 10.1002/term

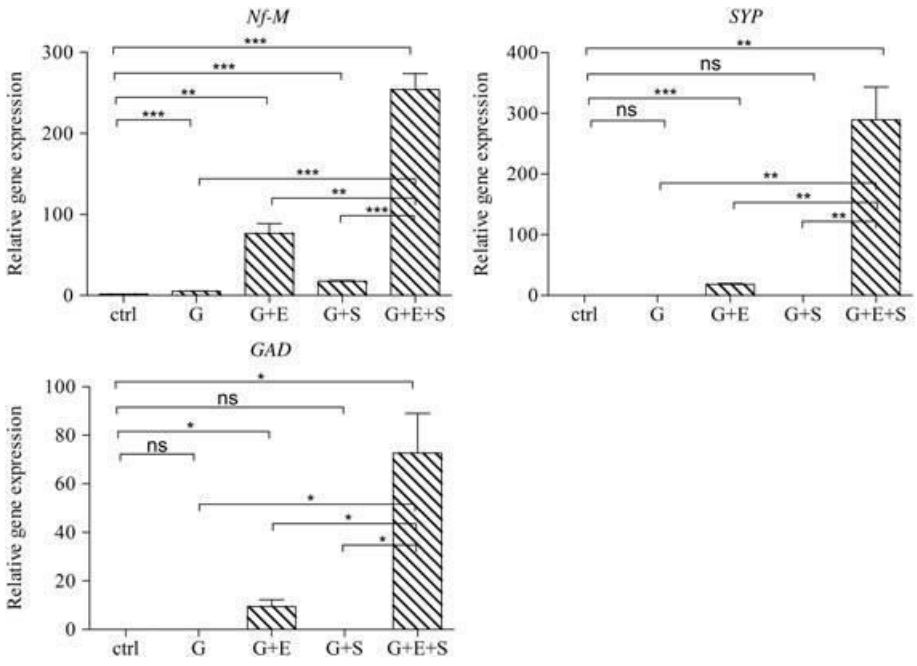

Figure 4. The effect of growth factors, brain tissue extract and supernatant from activated splenocytes on MSCs differentiation. The differentiation effect of growth factors FGF-b and EGF (group G), growth factors and brain tissue extract (G + E), growth factors and supernatant from activated splenocytes (G + S) or a combination of the growth factors, extract and supernatant together (G + E + S) on neural differentiation of MSCs was characterized according to the expression of genes for the neuron-specific markers Nf-M, SYP and GAD; Ctrl is a negative control – untreated MSCs. Expression of the genes Nf-M, SYP and GAD was detected by real-time PCR. Each bar shows mean ± SE from three independent determinations; *statistically significant differences; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

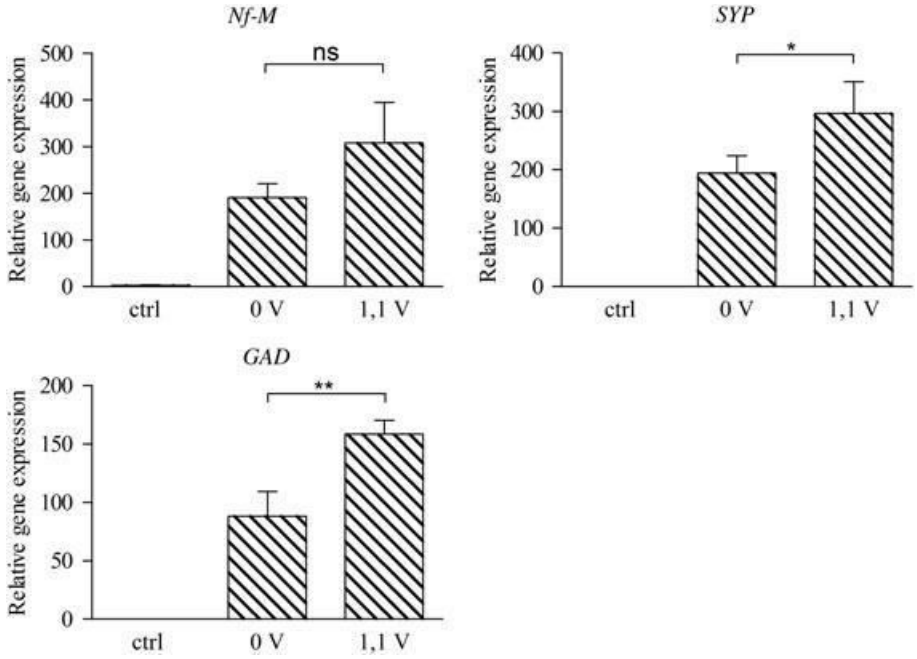

Figure 5. Effect of electrical stimulation on differentiation of MSCs, which were cultured untreated (group Ctrl) or were differenti-ated with brain tissue extract, growth factors and supernatant from activated splenocytes. The cell cultures were grown without elec-trical stimulation (group 0 V) or with electrical stimulation of 1.1 V involved in the differentiation protocol (group 1.1 V). Expression of the neuron-specific markers Nf-M, SYP and GAD was determined by real-time PCR. Each bar represents mean ± SE from three in-dependent determinations; *statistically significant differences; *$p < 0.05$, **$p < 0.01$

stimulation in these experiments used a voltage of 1.1 V, which was recognized in our preliminary experiments as an optimal dose (data not shown).

Nanofibre scaffolds were used to provide extracellular support, mimic the natural 3D environment and enable a possible transfer of in vitro differentiated cells for their prospective therapeutic application. We found that PCL nanofibre scaffold itself did not have any significant effect on neural differentiation (Figure 6, group Ctrl + N). However, in MSCs differentiated with growth factors and

DOI: 10.1002/term

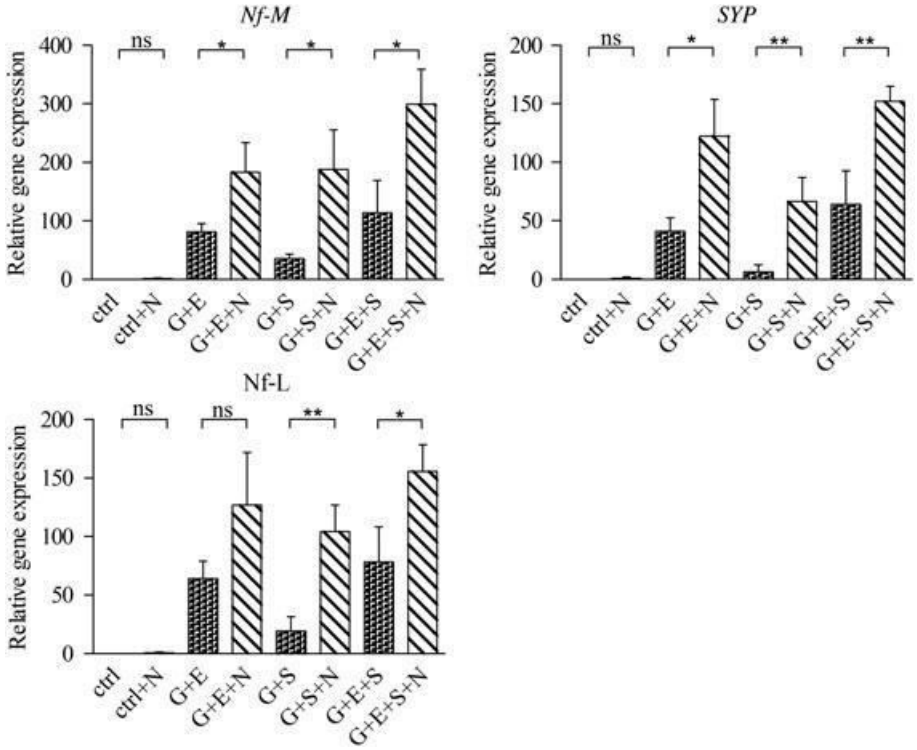

Figure 6. The effect of nanofibre scaffold on differentiation of MSCs, which were cultured untreated on plastic (group Ctrl) or on PCL nanofibres (Ctrl + N), or were differentiated with growth factors FGF-b and EGF and brain tissue extract on plastic (G + E), or on nanofibres (G + E + N), with growth factors and supernatant from activated splenocytes on plastic (G + S) or on nanofibres (G + S + N), or with growth factors, extract and supernatant on plastic (G + E + S), or on nanofibres (G + E + S + N). All groups except Ctrl and Ctrl + N were treated by electrical stimulation with a voltage of 1.1 V. Expression of the neuron-specific markers Nf-M, SYP and Nf-L was determined by real-time PCR. Each bar represents mean ± SE from three independent determinations; *statistically significant differences in gene expression; *$p < 0.05$, **$p < 0.01$

extract (group G + E), growth factors and supernatant (group G + S) or with growth factors, extract and supernatant (group G + E + S) (with the involvement of electrical stimulation in all groups), the expression of neuron-specific markers was significantly enhanced in samples cultured on nanofibre scaffold when compared to the samples differentiated in the same way on plastic surfaces (Figure 6).

Finally, using the WST-1 assay, we compared the viability of non-differentiated MSCs and MSCs undergoing the complex differentiation protocol for 9 days. We found that differentiated cells had a slightly decreased (roughly by half) viability in comparison with undifferentiated cells (data not shown). However, none of the components of the protocol, when used separately, had any toxic effect on the growth of MSCs.

3.3. Detection of Nf-L protein by immunocytochemistry

Immunocytochemistry was used to demonstrate the presence of Nf-L protein (as one of selected markers with the highest expression) in differentiated MSCs. While control samples of non-differentiated MSCs stained with mAb against Nf-L did not show the presence of this protein (Figure 7A), samples of MSCs differentiated in the presence of brain tissue extract, growth factors, supernatant from stimulated spleen cells and subjected to electrical stimulation were clearly positive for Nf-L protein (Figure 7B–D).

The morphology of differentiated and undifferentiated MSCs was compared in both fluorescence (Figure 8A, C) and phase (Figure 8B, D) channels at the same time. The differentiated MSCs were positive for Nf-L protein (Figure 8A) and showed neurite-like morphology (Fig-ure 8B), while undifferentiated MSCs did not show the presence of Nf-L protein (Figure 8C) and maintained their fibroblast-like shape (Figure 8D).

4. Discussion

Various distinct protocols have been used to differentiate stem cells into cells expressing neuronal cell markers and characteristics (Sanchez-Ramos et al., 2000; Tropel et al., 2006; Prabhakaran et al., 2009). These protocols include defined recombinant factors (Jang et al., 2010; Khoo et al., 2011), chemicals (Sanchez-Ramos et al., 2000; Safford et al., 2002), neural tissue extracts (Chen et al., 2002; Bentz et al., 2010; Nicaise et al., 2011), supernatants from acti-vated lymphocytes (Armstrong et al., 2003; Moviglia et al., 2006), electrical stimulation (Woo et al., 2009; Orza et al., 2011) or different matrices or scaffolds to support cell growth (Yim et al., 2007; Prabhakaran et al., 2009; Orza et al., 2011). However, the physiological

DOI: 10.1002/term

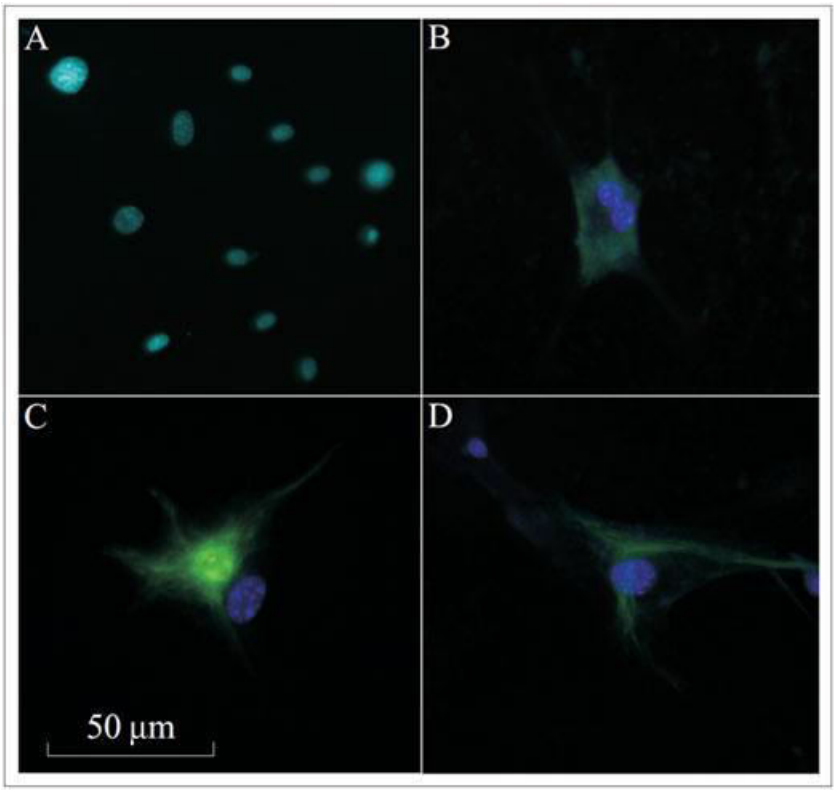

Figure 7. Detection of Nf-L protein (green) in differentiated MSCs by immunocytochemistry. MSCs were cultured untreated (A) or were differentiated using a protocol involving brain tissue extracts, growth factors, supernatants from stimulated splenocytes and electrical stimulation (B, C, D). The nuclei are stained by DAPI (blue); magnification = ×400; representative figures from three independent experiments are shown

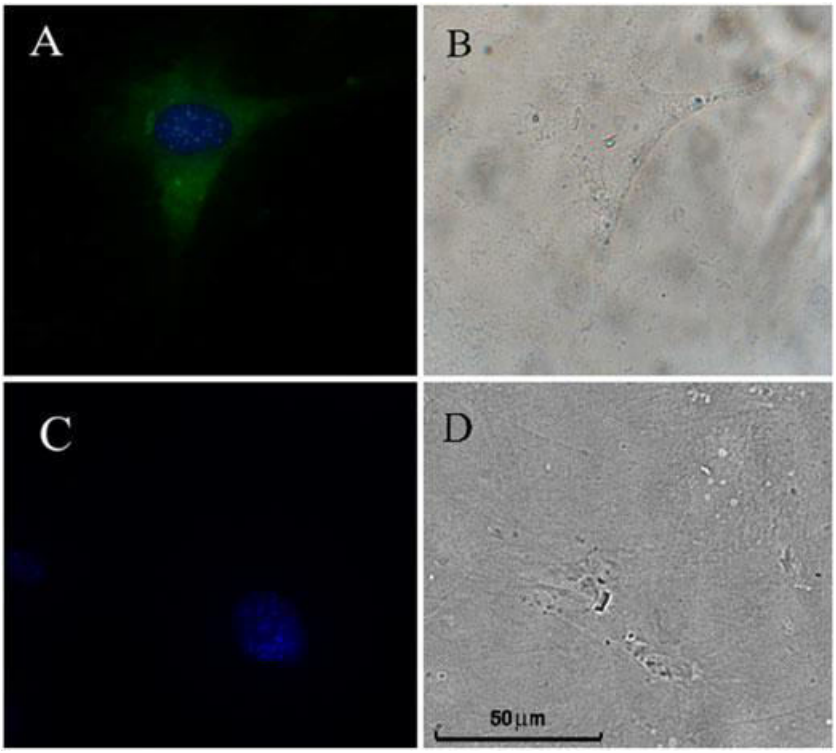

Figure 8. Comparison of morphology of differentiated and undifferentiated MSCs in fluorescence and phase channel. MSCs were differentiated using a protocol involving brain tissue extracts, growth factors, supernatants from stimulated splenocytes and electrical stimulation (A, B) or were cultured untreated (C, D). Nf-L protein (green) was detected by immunocytochemistry; the nuclei were stained by DAPI (blue) (A, C); morphology of the cells is shown in phase channel (B, D); magnification = ×400

DOI: 10.1002/term

complexity of damaged tissue and an inflammatory environment at the site of neural injury involve all the above factors, which can influence stem cells differentiation in the vicinity of the injury. To simulate these complex conditions, we combined neural tissue extract, defined growth factors, supernatants of activated splenocytes, electrical stimulation and a 3D nanofibre scaffold.

Our results showed that the complex approach for MSC differentiation was more effective than separate application of individual components of the protocol. Individual components contributed unequally, but each of them had a benefit to the final cell differentiation. For example, electrical stimulation itself did not induce the expression of neuronal markers, but it enhanced the expression of neuron-specific genes in MSCs differentiated by a combination of tissue extract, supernatant from activated splenocytes and growth factors. Similarly, involvement of nanofibre scaffolds in the differentiation protocol further enhanced the expression of neuronal genes in differentiated MSCs.

As a source of stem cells in this study, we used adipose tissue-derived MSCs, which can be obtained and handled easily and which are known to be a suitable source of cells for neural differentiation (Zuk et al., 2002; Jang et al., 2010). MSCs generally express undetectable or low levels of neuron-specific markers in a non-induced state (Sanchez-Ramos et al., 2000; Deng et al., 2006) and their electrophysiological properties enable them to differentiate into excitable cells (Heubach et al., 2004).

Brain tissue extracts and supernatants from ConA-stimulated spleen cells were used to simulate the physiological conditions at the site of the neural injury and inflammation and to stimulate MSCs by molecules produced in an inflamed environment. Brain tissue extracts, as the differentiation agents, have been tested before in many differentiation protocols, where the brain tissue was impaired by a model injury or disease (Moviglia et al., 2006; Bentz et al., 2010; Nicaise et al., 2011). In our study, the inflammatory immune reaction was simulated by the supernatant from mitogen-stimulated spleen cells.

Electrical stimulation of MSCs under physiological conditions seems to have a supportive rather than a determinative role in our differentiation protocol. We observed that electrical stimulation itself (without other components of the differentiation protocol) did not induce expression of neuronal markers in MSCs. However MSCs undergoing the differentiation protocol under electrical stimulation express higher levels of neuron-specific markers than cells differentiated without electrical stimulation. It has been shown previously that external electric stimulation leads to hyperpolarization of the cell membrane, which is closely connected with differentiational processes (Sundelacruz et al., 2009; Wu et al., 2011).

According to the intensity and frequency of electrical stimulation, this approach induces changes in the intracellular concentration of calcium ions (Sun et al., 2006; Sun et al., 2007). Such a change influences the expression pattern of numerous transcriptional factors in the nucleus, which affects the differentiation of stem cells (Sun et al., 2007; Sundelacruz et al., 2009; van Vliet et al., 2010). In addition, electrical stimulation increases the permeability of the cell membrane to soluble growth and differentiation factors (Titushkin et al., 2010).

It has been shown that different scaffolds used for cell growth represent another factor which significantly influences stem cell differentiation (Yim et al., 2007; Prabhakaran et al., 2009; Orza et al., 2011). Nanofibre scaffolds mimic a natural 3D environment and provide extracellular support for cell growth. Stem cells growing on nanofibres have a higher differentiation capacity than cells growing on plastic surfaces (Yim et al., 2007). In our study, a 3D nanofibre scaffold itself did not induce neural differentiation in untreated MSCs. However, the differentiation of MSCs using complex differentiation and growth on nanofibre scaffolds yielded superior results and induced the highest expression of neuronal cell markers, when compared to cells differentiated on plastic.

Although MSCs undergoing the differentiation protocol had a lower proliferation rate than undifferentiated cells, the difference seems to be a consequence of cell differentiation rather than a toxic effect of individual components of the differentiation protocol. In our preliminary experiments, none of the components of the differentiation protocol had any cytotoxic or cytostatic effect on MSC proliferation.

Altogether, our results have shown that the use of a complex differentiation protocol is an effective way to differentiate MSCs into cells expressing neuron-specific markers. MSCs can be easily obtained from the fatty tissue of a particular patient, predifferentiated by the described protocol and used therapeutically as autologous adult stem cells. We have recently shown the effectiveness of MSCs growing on a nanofibre scaffold for the treatment of ocular surface injuries in mouse and rabbit models (Zajicova et al., 2010; Cejkova et al., 2013). When being mindful of the ethical problems associated with the use of embryonic stem cells (Robertson, 2001; Lo and Parham, 2009), or when considering the immunogenicity of induced pluripotent cells (Dhodapkar et al., 2010; Wu and Hochedlinger, 2011; Zhao et al., 2011), we suggest that MSCs pre-activated by a complex differentiation protocol, and easily handled when grown on a nanofibre scaffold, could represent a suitable tool for cell-based therapy of various neural injuries and neurological disorders.

Acknowledgements

This study was supported by the Grant Agency of the Czech Repub-lic (Grant Nos P304/11/0653, 14-12580S and P301/11/1568), Biocev (Grant No. CZ.1.05/1.1.00/02.0109), the Ministry of Education, Youth and Sports of the Czech Republic (Grant No. NPU I: LO1309), and Charles University (Grants No. UNCE 204013 and No. SVV 260206).

Conflict of interest The authors declare no conflicts of interest.

References

Armstrong BD, Hu Z, Abad C et al. 2003; Lymphocyte regulation of neuropeptide gene expression after neuronal injury. J Neurosci Res 74: 240–247.

Arnhold S, Klein H, Klinz FJ et al. 2006; Human bone marrow stroma cells display certain neural characteristics and integrate in the subventricular compartment after injection into the liquor system. Eur J Cell Biol 85: 551–565.

Bentz K, Molcanyi M, Schneider A et al. 2010; Extract derived from rat brains in the acute phase following traumatic brain injury im-pairs survival of undifferentiated stem cells and induces rapid differentiation of surviving cells. Cell Physiol Biochem 26: 821–830.

Cejkova J, Trosan P, Cejka C et al. 2013; Suppression of alkali-induced oxidative injury in the cornea by mesenchymal stem cells growing on nanofiber scaffolds and transferred onto the damaged corneal surface. Exp Eye Res 116: 312–323.

Chen X, Katakowski M, Li Y et al. 2002; Human bone marrow stromal cell cultures conditioned by traumatic brain tissue extracts: growth factor production. J Neurosci Res 69: 687–691.

Deng J, Petersen BE, Steindler DA et al. 2006; Mesenchymal stem cells spontaneously express neural proteins in culture and are neurogenic after transplantation. Stem Cells 24: 1054–1064.

Dhodapkar KM, Feldman D, Matthews P et al. 2010; Natural immunity to pluripotency antigen OCT4 in humans.Proc Natl Acad Sci U S A 107: 8718–8723.

Dominici M, Le Blanc K, Mueller I et al. 2006; Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8: 315–317.

Heubach JF, Graf EM, Leutheuser J et al. 2004; Electrophysiological properties of human mesenchymal stem cells. J Physiol 554: 659–672.

Holan V, Pokorna K, Prochazkova J et al. 2010; Immunoregulatory properties of mouse limbal stem cells. J Immunol 184: 2124–2129.

Jackson L, Jones DR, Scotting P et al. 2007; Adult mesenchymal stem cells: differentiation potential and therapeutic applica-tions. J Postgrad Med 53: 121–127.

Jang S, Cho HH, Cho YB et al. 2010; Functional neural differentiation of human adipose tissue-derived stem cells using bFGF and forskolin. BMC Cell Biol 11: 25–38.

Khoo MLM, Tao H, Meedeniya ACB et al. 2011; Transplantation of neuronal-primed human bone marrow mesenchymal stem cells in hemiparkinsonian rodents. PLoS One 6: 5.

Lo B, Parham L 2009; Ethical issues in stem cell research. Endocr Rev 30: 204–213.

Moviglia GA, Varela G, Gaeta CA et al. 2006; Autoreactive T cells induce in vitro BM mes-enchymal stem cell transdifferentiation to neural stem cells. Cytotherapy 8: 196–201.

Nicaise C, Mitrecic D, Pochet R. 2011; Brain and spinal cord affected by amyotrophic lateral sclerosis induce differential growth factors expression in rat mesenchymal and neural stem cells. Neuropathol Appl Neurobiol 37: 179–188.

Orza A, Soritau O, Olenic L et al. 2011; Electrically conductive gold-coated collagen nanofibres for placental-derived mesenchymal stem cells enhanced differentiation and proliferation. ASC Nano 5: 4490–4503.

Prabhakaran MP, Venugopal JR, Ramakrishna S. 2009; Mesenchymal stem cell differentiation to neuronal cells on electrospun nanofibrous substrates for nerve tissue engineering. Biomaterials 30: 4996–5003.

Robertson JA. 2001; Human embryonic stem cell research: ethical and legal issues. Nat Rev Genet 2: 74–78.

Safford KM, Hicok KC, Safford SD et al. 2002; Neurogenic differentiation of murine and hu-man adipose-derived stromal cells. Biochem Biophys Res Commun 294: 371–379.

Sanchez-Ramos J, Song S, Cardozo-Pelaez F et al. 2000; Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 164: 247–256.

Sun S, Liu Y, Lipsky S et al. 2007; Physical manipulation of calcium oscillation facilitates osteodifferentiation of human mesenchymal stem cells. FASEB J Res Commun 21: 1472–1480.

Sun S, Titushkin I, Cho M. 2006; Regulation of mesenchymal stem cells adhesion and ori-entation in 3D collagen scaffold by electrical stimulus. Bioelectrochemistry 69: 133–141.

Sundelacruz S, Levin M, Kaplan DL. 2009; Role of membrane potential in the regulation of cell proliferation and differentiation. Stem Cell Rev Rep 5: 231–246.

Tate CC, Fonck C, McGrogan M et al. 2010; Human mesenchymal stromal cells and their derivative, SB623 cells, rescue neural cells via trophic support following in vitro ischemia. Cell Transpl 19: 973–984.

Titushkin I, Sun S, Shin J et al. 2010; Physicochemical control of adult stem cell differentiation: shedding light on potential molecular mechanisms. J Biomed Biotech 2010: 14, doi: 10.1155/2010/743476.

Tropel P, Platet N, Platel JC et al. 2006; Functional neuronal differentiation of bone marrow-derived mesenchymal stem cells. Stem Cells 24: 2868–2876.

Tropepe V, Sibilia M, Ciruna BG et al. 1999; Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. Dev Biol 208: 166–188.

Trosan P, Svobodova E, Chudickova M et al. 2012; The key role of insulin-like growth factor I in limbal stem cell differentiation and the corneal wound-healing process. Stem Cells Dev 21: 3341–3350.

van Vliet P, de Boer TP, van der Heyden MA et al. 2010; Hyperpolarization induces differentiation of human cardiomyocyte progenitor cells. Stem Cell Rev 6: 178–185.

Woo DG, Shim MS, Park JS et al. 2009; The effect of electrical stimulation on the differentiation of hESCs adhered onto fibronectin-coated gold nanoparticles. Biomaterials 30: 5631–5638.

Wu SM, Hochedlinger K. 2011; Harnessing the potential of induced pluripotent stem cells for regenerative medicine. Nat Cell Biol 13: 497–505.

Wu W, Zhao H, Xie B et al. 2011; Implanted spike wave electric stimulation promotes survival of the bone marrow mesenchymal stem cells and functional recovery in the spinal cord injured rats. Neurosci Lett 491: 73–78.

Yim EKF, Pang SW, Leong KW. 2007; Synthetic nanostructures inducing differentiation of human mesenchymal stem cells into neuronal lineage. Exp Cell Res 313: 1820–1829.

Zajicova A, Pokorna K, Lencova A et al. 2010; Treatment of ocular surface injuries by limbal and mesenchymal stem cells growing on nanofiber scaffolds. Cell Transpl 19: 1281–1290.

Zhao T, Zhang ZN, Rong Z et al. 2011; Immunogenicity of induced pluripotent stem cells. Nature 474: 212–215.

Zuk PA, Zhu M, Ashjian P et al. 2002; Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13: 4279–4295

J Tissue Eng Regen Med 2015.

DOI: 10.1002/term

5.2 The characterization of the immunomodulatory properties of neuron-like cells – the unpublished results

According to licensing theory, MSCs are able to develop anti-inflammatory activity as a reaction to an inflammatory environment. During the transdifferentiation protocol MSCs were cultured in an inflammatory environment which mimics the environment of a neural injury. This environment could support the transdifferentiation of MCSs into neuron-like cells and induce the anti-inflammatory properties of transdifferentiating cells. After the completion of the transdifferentiation protocol the resulting neuron-like cells and non-differentiated AT-MSCs controls were co-cultivated for 24 and 48 hours with ConA-stimulated mouse splenocytes and their cytokine secretion profiles were compared by qPCR and ELISA test. The experiments were performed both in mixed co-cultivation and in physically separated conditions; to identify the cells correctly the groups were physically isolated by nanofiber scaffolds, and to exclude possible bias due to lack of cell-cell contact the experiments were performed also without these separating conditions. The neuron-like cells maintained the relative gene expression of neuron-specific makers during the co-cultivation with the activated splenocytes. Both the non-differentiated AT-MSCs controls and neuron-like cells were comparably able to suppress the relative gene expression for IFN-gamma in splenocytes stimulated by ConA, which was also confirmed on the protein production level by an ELISA test. Both neuron-like cells and non-differetiated AT-MSCs similarly supported the relative expression of the gene for IL-10 in splenocytes stimulated by ConA. Neuron-like cells co-cultivated with activated splenocytes expressed lower levels of the gene for IL-6 compared to non-differentiated AT-MSCs co-cultivated with activated splenocytes, which was also confirmed on the protein production level by an ELISA test. The neuron-like cells were able to suppress the inflammatory behaviour of activated splenocytes comparably to non-differentiated AT-MSCs. Finally, the neuron-like cells could participate in the regeneration and restoration processes in neural injury.

5.3 The optimization of the differentiation protocol for human AT-MSCs – the unpublished results

The transdifferentiation protocol for AT-MSCs was originaly designed for mouse cells. For human AT-MSCs the protocol was optimized by several changes, due to the higher viability and proliferation rates in human cells compared to mouse cells. The human cells showed the optimal results of differentiation when they were used in an earlier passage (3^{rd} passage compared to 5^{th} passage, which was used in mouse cells) and in a lower seeding density (500 cells/cm^2, compared to 2000 cells/cm^2 in mouse cells). Consequently, human tissue-derived differentiation agents were used in lower doses. In the case of human AT-MSCs, the 3D extracellular support was performed by hydrogel (combination of collagen and hyaluronic acid). The resulting human neuron-like cells acquired neuron-like morphology, expressed the genes for neuron-specific markers and showed positivity for proteins Nf-L, Nf-M and TUBB3.

5.4 The key role of insulin-like growth factor I in limbal stem cell differentiation and the corneal wound-healing process

Trosan P., Svobodova E., **Chudickova M.**, Krulova M., Zajicova A., Holan V.

Stem Cells Dev. 2012; 21(18):3341-50. doi: 10.1089/scd.2012.0180.

The specific environment in injured cornea activates LSCs to proliferate, migrate and differentiate into corneal epithelial cells. To determine the participating cytokines an animal model of a wounded cornea was established. The epithelial layer of the central cornea was mechanically damaged by sharp needle. The samples of cornea and limbus were excised at different time intervals after the injury (3, 6, 9, 12, 24 and 48 hours) and their cell proliferation, growth factor production and relative gene expression were detemined. The injured cornea significantly up-regulated the gene expression for insulin-like growh factor 1 (IGF-1), EGF, bFGF and other cytokines. It was found that IGF-1, but not EGF nor bFGF, supports the differentiation of LSCs into corneal epithelial cells. By contrast, the proliferation of LSCs was supported by EGF and bFGF, but not by IGF-1.

STEM CELLS AND DEVELOPMENT Volume 21, Number 18, 2012
Mary Ann Liebert, Inc. DOI: 10.1089/scd.2012.0180

The Key Role of Insulin-Like Growth Factor I in Limbal Stem Cell Differentiation and the Corneal Wound-Healing Process

Peter Trosan,[1,2] Eliska Svobodova,[1,2] Milada Chudickova,[1,2] Magdalena Krulova,[1,2] Alena Zajicova,[1] and Vladimir Holan[1,2]

Limbal stem cells (LSC), which reside in the basal layer of the limbus, are thought to be responsible for corneal epithelial healing after injury. When the cornea is damaged, LSC start to proliferate, differentiate, and migrate to the site of injury. To characterize the signaling molecules ensuring communication between the cornea and LSC, we established a mouse model of mechanical corneal damage. The central cornea or limbal tissue was excised at different time intervals after injury, and the expression of genes in the explants was determined. It was observed that a number of genes for growth and differentiation factors were significantly upregulated in the cornea rapidly after injury. The ability of these factors to regulate the differentiation and proliferation of limbal cells was tested. It was found that the insulin-like growth factor-I (IGF-I), which is rapidly overexpressed after injury, enhances the expression of IGF receptor in limbal cells and induces the differentiation of LSC into cells ex-pressing the corneal cell marker, cytokeratin K12, without any effect on limbal cell proliferation. In contrast, the epidermal growth factor (EGF) and fibroblast growth factor-b (FGF-b), which are also produced by the damaged corneal epithelium, supported limbal cell proliferation without any effect on their differentiation. Other factors did not affect limbal cell differentiation or proliferation. Thus, IGF-I was identified as the main factor stimulating the expression of IGF receptors in limbal cells and inducing the differentiation of LSC into cells expressing corneal epithelial cell markers. The proliferation of these cells was supported by EGF and FGF.

Introduction

The impaired or otherwise damaged cornea is thought to be healed by the cells that originate from the limbus.

Although some degree of self-regenerative capacity of the corneal epithelium has been described in both the mouse [1] and in humans [2,3], the majority of cells migrating to the site of injury originate from limbal stem cells (LSC). It has been observed that in the case of LSC deficiency, the cornea cannot heal properly and its healing is associated with conjunctivilization, neovascularization, chronic inflammation, and persistent epithelial defects that may result in a loss of vision [4,5]. Conversely, such defects can be treated by the transplantation of limbal tissue or LSC [6,7]. It has been shown that after corneal damage, LSC start to proliferate and differentiate into transient amplifying cells, which migrate to the site of injury and give rise to cells expressing corneal epithelial cell markers [4,8]. The molecular basis of the interplay between the cornea and the limbus remains mostly unknown. Jia et al. [9] demonstrated that a large number of genes in corneal cells are upregulated after mechanical or chemical damage of the ocular surface. Among them, the genes coding for growth and differentiation factors represent a significant group. It was shown that the transforming growth factor-a (TGF-a), TGF-b, epidermal growth factor (EGF), hepatocyte growth factor (HGF), and fibroblast growth factor-b (FGF-b), which are produced by corneal epithelial cells, can modulate the proliferation and growth of cells on the ocular surface [10–12]. Another factor produced by the corneal epithelium, insulin-like growth factor-I (IGF-I), has been shown to support the proliferation of keratinocytes [13], enhance the production of the adherens-junction protein N-cadherin [14], stimulate the formation of the extracellular matrix [15], and increase the synthesis of collagen by keratinocytes [16]. The IGF signaling pathway has been shown to be involved in cell proliferation and differentiation, and it has an important role in growth regulation at both cellular and organism levels [17]. It suggests that IGF-I and other factors produced by the damaged corneal epithelium can be involved in the regulation of LSC differentiation, prolifer-ation, and migration.

[1]Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic.
[2]Faculty of Science, Charles University, Prague, Czech Republic.

The identification of cornea-associated phenotypic markers, such as cytokeratins K3 and K12 or connexin 43, which are expressed by corneal cells, but are absent in the limbus [18,19], enables monitoring the differentiation of LSC into cells with the characteristics of corneal epithelial cells and the characterization of those factors responsible for the differentiation and proliferation of limbal cells. Previous studies have shown that supernatants from corneal cell cultures induce the differentiation of various types of stem cells, including hair follicule stem cells, mesenchymal stem cells (MSC), and embryonic stem cells, into cells expressing the corneal epithelial cell marker, K12 [20–23].

However, the key differentiation factor present in the corneal cell supernatants has not yet been identified. In the present study, a model of mechanically damaged cornea in the mouse has been used to characterize factors that are produced by the corneal epithelial cells after corneal injury, and which are responsible for differentiation and proliferation of LSC. Since these factors are produced by corneal cells after a superficial corneal epithelial damage and they induce the expression of the corneal cell marker, K12, in limbal cells, we suggest that these factors are involved in the regulation of corneal epithelial healing after the injury.

Materials and Methods

Animals

Mice of the inbred strain BALB/c of both sexes were used in the experiments at the age of 2–4 months. The animals were obtained from the breeding unit of the Institute of Molecular Genetics, Prague. The use of animals was approved by the local Animal Ethics Committee of the Institute of Molecular Genetics.

A model of corneal injury

The recipient BALB/c mice were deeply anesthetized by an intramuscular injection of a mixture of xylazine and ketamine (Rometar, Spofa, Prague, Czech Republic). The surface of the central cornea (only the epithelial layer) of the left eye was damaged by the epithelial debridement with a sharp needle (G23). The damage involved only the region in the central cornea (with a diameter 1 mm) and was made with a care to avoid injury of the corneal stroma or the limbus. The extent of damage can be observed under a microscope after a dissection of the cornea and mechanical separation of individual corneal layers. At different time intervals after the injury (3, 6, 9, 12, 24, or 48 h), the central cornea (the diameter 1 mm) and limbal tissue from control and damaged eyes were excised and tested for cell proliferation, factor production, or gene expression.

Preparation of a single-cell suspension from limbal tissue

A single-cell suspension from limbal tissues was obtained by enzyme digestion, as we have described [24]. In brief, limbal tissues (a narrow tissue between the cornea and the conjunctiva) from 10–12 BALB/c mice were cut out with scissors and subjected to 10 short (10 min each) trypsinization cycles. The released cells were harvested after each cycle, finally centrifuged (8 min at 250 g), and resuspended in the Dulbecco's modified Eagle's medium (DMEM; Sigma Corp., St. Louis, MO) containing 10% fetal calf serum (FCS; Sigma), antibiotics (100 U/mL of penicillin, 100 mg/mL of streptomycin), and 10 mM HEPES buffer (a complete DMEM).

Preparation of supernatants from cultures of corneal explants

Explants of the central cornea (1 mm in diameter, without any limbal tissue) from control and damaged eyes (24 h after the damage) were cultured in the complete DMEM (one cornea explant per 125 mL of medium) in 48-well tissue culture plates (Nunc, Roskilde, Denmark). After a 24-h incubation period, the supernatants were harvested and centrifuged at 400 g for 10 min, filtered through 0.22 m filters, and stored at - 80LC until used.

Determination of limbal cell proliferation

Limbal explants from individual eyes were incubated in a volume of 200 mL of complete DMEM in 96-well tissue culture plates (Nunc), and cell proliferation was determined by adding ^{3}H-thymidine (1 mCi/well, Nuclear Research Institute, Rez, Czech Republic) for the last 6 h of the 48-h incubation period. To determine the effects of recombinant factors on limbal cell proliferation, limbal cells ($25 \cdot 10^{3}$ cells/ mL) from healthy eyes were cultured in a volume of 200 mL of complete DMEM in 96-well tissue culture plates in the absence or the presence of recombinant mouse (m)EGF, mFGF-b, human (h)TGF-b1, human keratinocyte growth factor (KGF), mHGF, or mIGF-1 (all factors were purchased from PeproTech, Rocky Hill, NJ, and were tested at concentrations ranging from 0.1 to 500 ng/mL). Cell proliferation was determined by measuring ^{3}H-thymidine incorporation after a 48-h incubation period using a Tri-Carb 2900TR scintillation counter (Packard, Meridien, CT).

Detection of gene expression by real-time polymerase chain reaction

The expression of genes for EGF, FGF-b, TGF-b1, TGF-b2, KGF, HGF, IGF-I, IGF-II, IGF-IR, and IGF-IIR in corneal and limbal tissue or in cultured limbal cells was detected using quantitative real-time polymerase chain reaction (PCR). The central cornea or the limbus was excised using Vannas scissors at the indicated time intervals after the injury and transferred into Eppendorf tubes containing 500 mL of TRI Reagent (Molecular Research Center, Cincinnatti, OH). Total RNA was extracted using the TRI Reagent according to the manufacturer's instructions. One mg of total RNA was trea-ted using deoxyribonuclease I (Sigma) and used for subse-quent reverse transcription. The first-strand cDNA was synthesized using random primers (Promega, Madison, WI) in a total reaction volume of 25 mL using M-MLV Reverse Transcriptase (Promega). Quantitative real-time PCR was performed in an iCycler (BioRad, Hercules, CA), as previously described [25,26]. The primers used for amplification are shown in Table 1. The PCR parameters included denaturation at 95LC for 3 min, then 40 cycles at 95LC for 20 s, annealing at 60LC for 30 s, and elongation at 72LC for 30 s. Fluorescence data were collected at each cycle after an elongation step at 80LC for 5 s and were analyzed on the iCycler Detection system, Version 3.1. Each individual

Table 1. Mouse Primer Sequences Used for Real-Time Polymerase Chain Reaction

Gene	Sense primer	Antisense primer
GAPDH	AGAACATCATCCCTGCATCC	ACATTGGGGGTAGGAACAC
EGF	CATGCCCCACAGGATTTG	GGGCAGGAAACAAGTTCGT
FGF-b	TCTCATCAATCTCCAGTTCACAA	CTTGCGTTGATTGCTACTCCT
KGF	CGGCTCTACTGCAAGAACG	TGCTTGGAGTTGTAGTTTGACG
HGF	CACCCCTTGGGAGTATTGTG	GGGACATCAGTCTCATTCACAG
TGF-b1	TGGAGCAACATGTGGAACTC	CAGCAGCCGGTTACCAAG
TGF-b2	TGGAGTTCAGACACTCAACACA	AAGCTTCGGGATTTATGGTGT
K12	GTGAGTCCGCTGGTGGTAAC	CATCAGCACAGCAGGAAGTG
IGF-I	TCGGCCTCATAGTACCCACT	ACGACATGATGTGTATCTTTATTGC
IGF-IR	GAGAATTTCCTTCACAATTCCATC	CACTTGCATGACGTCTCTCC
IGF-II	CGCTTCAGTTTGTCTGTTCG	GCAGCACTCTTCCACGATG
IGF-IIR	CCTTCTCTAGTGGATTGTCAAGTG	AGGGCGCTCAAGTCATACTC

EGF, epidermal growth factor; FGF, fibroblast growth factor; KGF, keratinocyte growth factor; HGF, hepatocyte growth factor; TGF, transforming growth factor; IGF-I, insulin-like growth factor-I.

experiment was done in triplicate. A relative quantification model was applied to calculate the expression of the target gene in comparison to GAPDH used as an endogenous control.

Immunostaining

Limbal cells or corneal epithelial cells ($25 \cdot 10^3$ cells/mL) were cultured for 48 h in a volume of 1 mL of complete DMEM in 24-well tissue culture plates (Nunc) in the absence or the presence of IGF-I (100 ng/mL). Dead cells were marked using Hoechst 33258 dye (Invitrogen, Carlsbad, CA) and sorted out using a BD Influx Cell Sorter (BD Biosciences, Franklin Lakes, NJ). The remaining cells were fixed for 20 min with 4% paraformaldehyde and permeabilized for 10 min with 0.1% Triton X-100. The samples were incubated with goat polyclonal anti-K12 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature, then with a secondary donkey anti-goat IgG conjugated with Alexa Flour 594 (Invitrogen). The cells were rinsed with phosphate-buffered saline containing 0.05% TWEEN and fixed on glass slides with Mowiol 4-88 (Calbiochem, San Diego, CA) in the presence of the nuclear dye 4¢,6¢-diamidino-2-phenylindole (DAPI). Visualization of the fluo-rescent label was performed using a fluorescent microscope (Leica, Wetzlar, Germany). Control samples were run without the primary antibody.

The effect of neutralization antibody anti-IGF-I on the ability of corneal cell supernatants to induce differentiation of LSC

Supernatants from cultures of explants from damaged corneas were preincubated for 2 h in the absence or presence of 10 mg/mL of neutralization goat anti-murine anti-IGF-I antibody (Peprotech, Rocky Hill, NJ) and were added into cultures of freshly isolated limbal cells ($25 \cdot 10^3$ cells/mL). After a 48-h cultivation period, the expression of K12 gene was determined by real-time PCR.

Statistical analysis

The statistical significance of differences between individual groups was calculated using the Student's t-test.

Results

Enhanced proliferative activity of limbal cells after corneal damage

The epithelium of the central cornea was mechanically damaged, and the limbal tissue was excised at the indicated time intervals after the injury. The limbal explants were cultured for 48 h, and the proliferation of cells was determined. As shown in Fig. 1A, limbal cells from the wounded eyes harvested 1 day after the injury proliferated significantly more strongly than did limbal cells from the control eyes. The proliferation of limbal cells on day 4 after the injury was, in contrast, rather decreased in comparison with limbal cells from control eyes and returned to a normal level 7 days after the injury.

To determine whether the enhanced proliferation of limbal cells observed after corneal damage was induced by a factor(s) produced by corneal cells, corneas from control or damaged eyes were cultured for 24 h. The supernatants from these cultures were added to cultures of limbal cells from control untreated mice. As shown in Fig. 1B, the supernatants from the cultures of corneas from damaged eyes induced a significantly stronger proliferation of limbal cells than supernatants from the cultures of corneal explants from control eyes.

Expression of genes in cells of the central cornea after injury

The epithelium of the central cornea was mechanically damaged and the corneas were excised at the indicated time intervals (0, 3, 6, 12, and 24 h) after the injury. The expression of genes for the tested factors was determined by real-time PCR. Figure 2 shows that the expression of genes for EGF, FGF-b, KGF, HGF, TGF-b1, IGF-I, and IGF-II was significantly upregulated already 3 or 6 h after the injury.

The effects of recombinant factors on limbal cell proliferation

A single-cell suspension of limbal cells from control mice was incubated in the absence or in the presence of recombinant factors that had been identified as being produced by

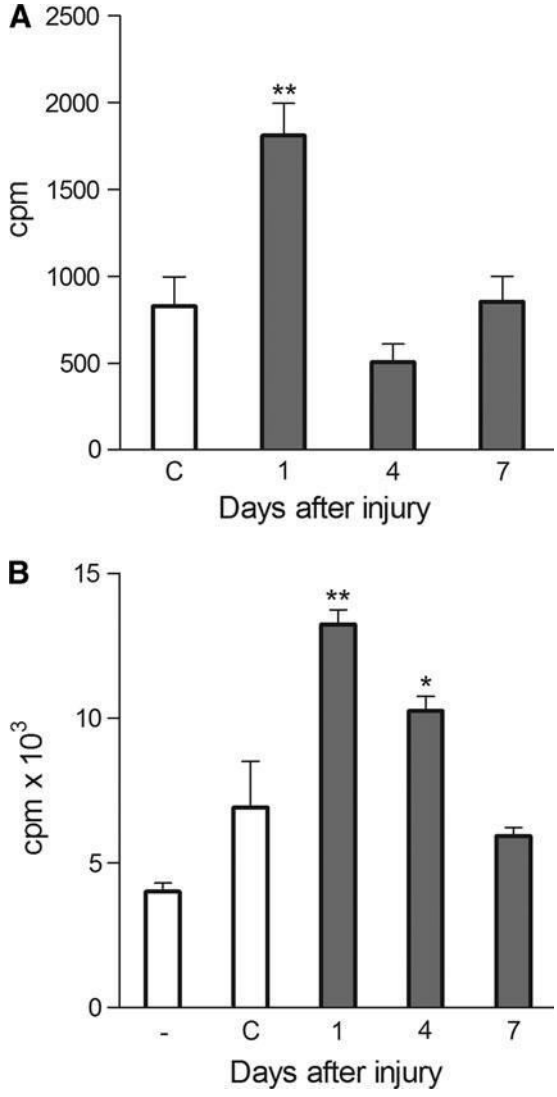

FIG. 1.Proliferation of limbal cells after corneal damage. (A) The central cornea was mechanically damaged, the limbal tissue was excised at the indicated time intervals after the injury, and the proliferation of cells of the limbal explants was determined. (B) The corneas from control or damaged eyes were excised at the indicated time intervals after the injury, and the corneal explants were cultured for 24 h to prepare supernatants. Proliferation of limbal cells in cultures without supernatants (-) or in the presence of supernatants from control corneas (C) or corneas from damaged eyes was determined. Each bar represents mean – SD from 3–5 determinations. Values with asterisks are significantly (*P < 0.01, **P < 0.001) different from the controls (i.e., the proliferation of limbal explants from control eyes or limbal cell proliferation in cultures without supernatants).

the damaged cornea. In preliminary experiments, all factors were tested over a wide range of concentrations (0.1–500 ng/mL); for the sake of brevity, the effects of only one concentration (10 ng/mL) are shown. As demonstrated in Fig. 3, EGF and FGF-b significantly stimulated the proliferation of limbal cells, KGF had less effect, and the other tested factors had only a marginal or no effect on limbal cell growth. Conversely, TGF-b, at higher concentrations, inhibited the proliferation of limbal cells (Fig. 3).

The effect of recombinant factors on limbal cell differentiation

Since cytokeratin K12 is a molecule that can distinguish corneal epithelial and limbal cells (K12 is strongly expressed in the corneal epithelium, but is absent in the limbus), we cultured limbal cells in the presence of factors that are produced by the damaged cornea. After a 48-h incubation, the expression of the K12 gene was detected by real-time PCR. As shown in Fig. 4, only IGF-I induced the significant expression of K12 mRNA, while all other tested factors did not enhance expression of the K12 gene above the level seen in control cells (cultured in the absence of factors).

The expression of K12 protein in limbal cells cultured in the presence of IGF-I

The differentiation potential of IGF-I was confirmed by immunostaining for the K12 protein. Limbal cells from con-trol mice were cultured in the absence or in the presence of recombinant IGF-I, and the expression of K12 protein was detected by immunostaining using an anti-K12 antibody. As demonstrated in Fig. 5, clear positivity for K12 protein was detected in limbal cells incubated in the presence of IGF-I. Cultured corneal epithelial cells served as a positive control.

Kinetics of IGF-IR and IGF-IIR gene expression in the cornea and limbus after corneal injury

Although a number of genes in the corneal epithelium are rapidly upregulated after corneal injury (Fig. 2), the expression of genes for IGF-IR and IGF-IIR in the central cornea was simultaneously decreased (Fig. 6A). In contrast to the cornea, the expression of genes for IGF receptors in the limbus was significantly enhanced after corneal injury (Fig. 6B).

Regulation of IGF receptor expression in limbal cells by IGF-I

Next, we tested whether the enhanced expression of genes for IGF-IR and IGF-IIR that was observed in limbal cells after corneal injury was induced by a factor produced by corneal cells. Limbal cells from healthy eyes were incubated in supernatants from cultures of corneal explants, and the expression of genes for IGF-IR and IGF-IIR was determined. As demonstrated in Fig. 7A, the supernatants from cultures of corneal explants from damaged eyes significantly increased the expression of genes for IGF-IR and IGF-IIR. When limbal cells were cultured in the presence of recombinant IGF-I, a significant upregulation of IGF-IR gene expression was observed, without stimulation of IGF-IIR gene expression (Fig. 7B).

The effect of neutralization antibody anti-IGF-I on LSC differentiation

To prove that IGF-I present in the corneal cell supernatants is responsible for differentiation of limbal cells, the supernatants from cultures of explants from damaged corneas were preincubated with the neutralization antibody anti-IGF-I, and then added to the culture of limbal cells. Figure 8 shows that the expression of gene for K12 in limbal

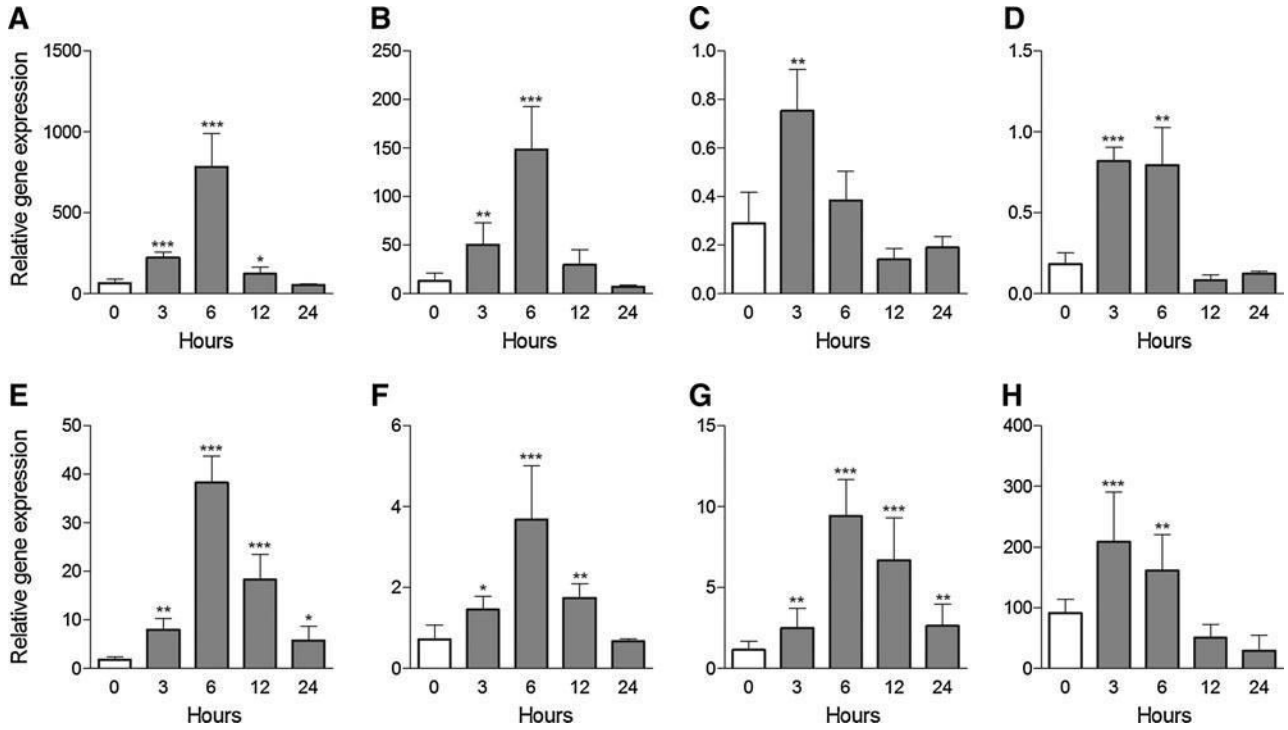

FIG. 2. The expression of genes for growth and differentiation factors in corneal epithelial cells after corneal injury. Corneas were mechanically damaged and the central cornea was excised 0 (untreated eye), 3, 6, 12, or 24 h after the injury. The expression of genes for TGF-b1 (A), TGF-b2 (B), EGF (C), HGF (D), KGF (E), FGF-b (F), IGF-I (G), and IGF-II (H) was determined by real-time PCR. Each bar represents mean – SD from 3 determinations. Values with asterisks represent a statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001) increase in gene expression. EGF, epidermal growth factor; FGF, fibroblast growth factor; KGF, keratinocyte growth factor; HGF, hepatocyte growth factor; TGF, transforming growth factor; IGF-I, insulin-like growth factor-I; PCR, polymerase chain reaction.

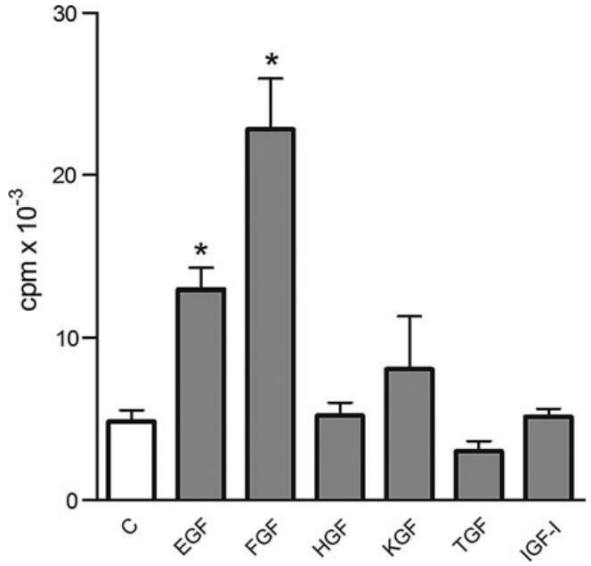

FIG. 3. The effects of recombinant factors on limbal cell proliferation. A single-cell suspension was prepared by enzymatic digestion from the limbal tissue of control eyes, and the cells were cultured for 48 h without factors (C) or in the presence of 10 ng/mL of EGF, FGF-b, HGF, KGF, TGF-b, or IGF-I. Cell proliferation was determined according to ^{3}H-thymidine incorporation. Each bar represents mean – SD from 4 determinations. Values with an asterisk are significantly (*P < 0.001) different from the control cultures (cells cultured without added factors).

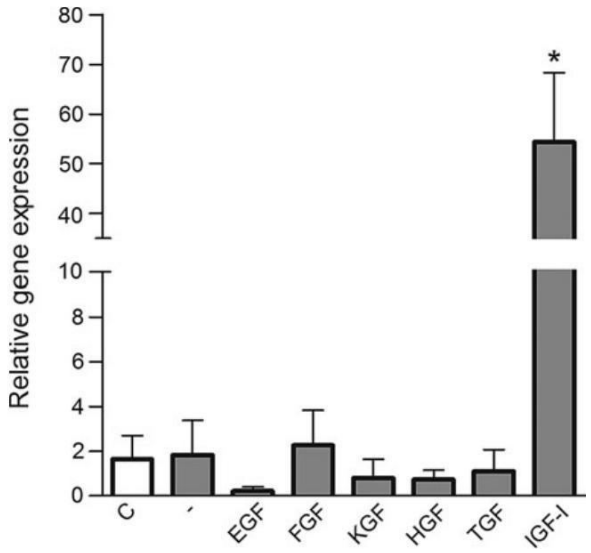

FIG. 4. The effect of recombinant factors on K12 mRNA expression in cultured limbal cells. A single-cell suspension of limbal cells prepared from control eyes was cultured for 48 h without factors (-) or in the presence of the indicated recombinant factors, and the expression of the K12 gene was determined by real-time PCR. The level of K12 mRNA expression in fresh (nonstimulated) cells (C). Each bar represents mean – SD from 3 determinations. The value with an asterisk is significantly (*P < 0.001) different from the control value (cells cultured without added factors).

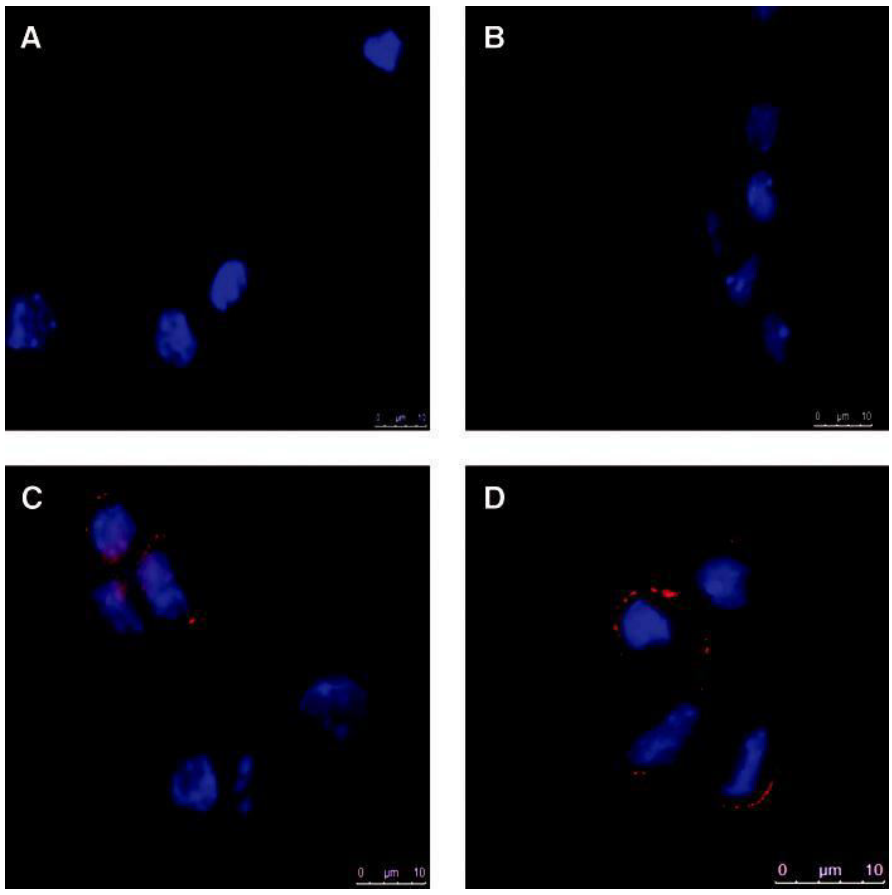

FIG. 5. Immunostaining for K12 protein in limbal cells treated with IGF-I. A single-cell suspension prepared from the limbal tissue of control mice was incubated for 72 h with or without IGF-I (100 ng/mL), and the cells were stained with a goat antibody against mouse K12. The nuclei were stained with DAPI (blue). The limbal cells were cultured without IGF-I (A), with IGF-I, but with the primary antibody omitted during the staining procedure (B) or with IGF-I and with the K12 protein stained using anti-K12 antibody (C). As a positive control, cultured corneal epithelial cells were stained with the anti-K12 antibody (D). One representative experiment of 4 similar ones is shown. DAPI, 4¢,6¢-diamidino-2-phenylindole.

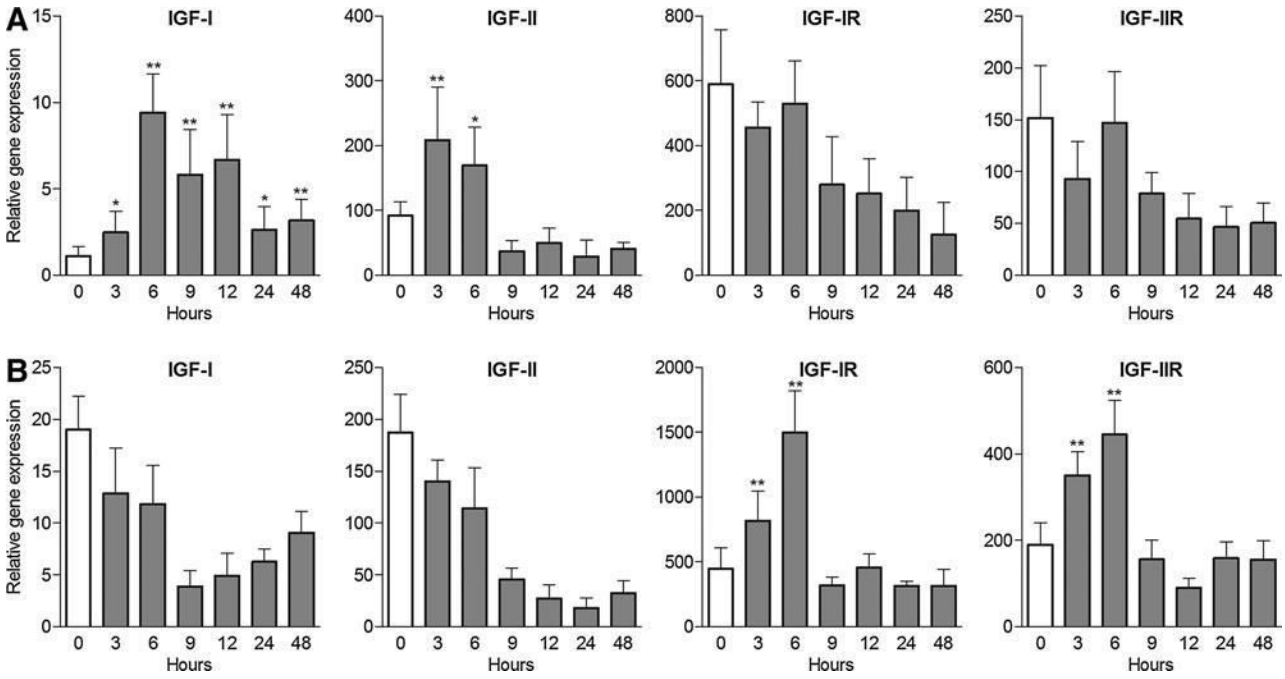

FIG. 6. Kinetics of IGF-IR and IGF-IIR mRNA expression in the cornea and in the limbus after corneal injury. The cornea was mechanically damaged and the expression of IGF-IR and IGF-IIR mRNA in the explants of the central cornea (A) or the limbus (B) was determined by real-time PCR. Each bar represents mean – SD from 3 determinations. Values with asterisks represent a statistically significant (*P < 0.01, **P < 0.001) increase in gene expression.

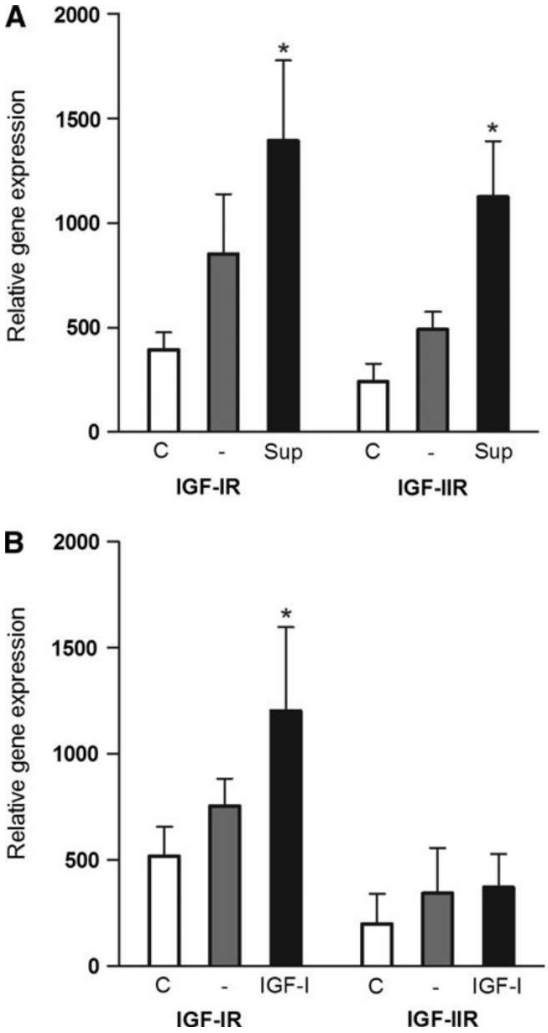

FIG. 7. Regulation of IGF receptor expression by IGF-I. Limbal cells isolated from healthy eyes were cultured in the presence of supernatant from cultures of corneal explants (A) or with recombinant IGF-I (B), and the expression of IGF-IR and IGF-IIR mRNA was determined. The expression of IGF-IR or IGF-IIR mRNA was analyzed in freshly isolated limbal cells (C), in cells incubated for 6 h in culture medium without factors (-), in medium containing 20% of supernatant from cultures of corneal explants from damaged eyes (Sup) or in medium containing 250 ng/mL of IGF-I (IGF-I). Each bar represents mean – SD from 3 determinations. Values with an asterisk represent a statistically significant (*P < 0.001) increase in gene expression (in comparison with cultures without supernatant or without IGF-I).

cells was significantly increased after incubation of cells with supernatants from damaged corneas and this increase was abrogated by preincubation of supernatants with the antibody, anti-IGF-I.

Discussion

The impaired healing of the corneal epithelium in the case of LSC deficiency suggests that the corneal epithelium is regenerated from stem cells that reside in the limbus [27].

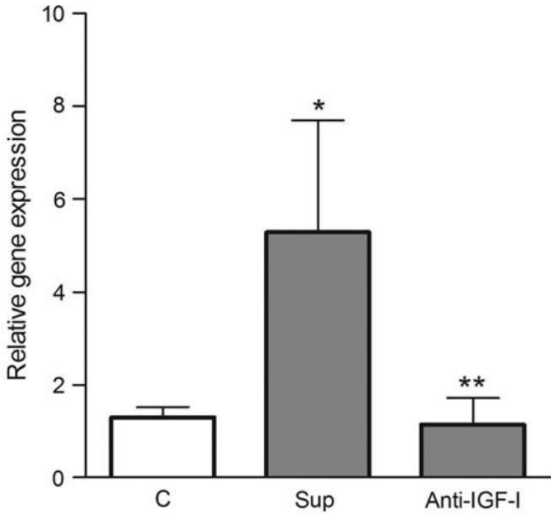

FIG. 8. The effect of neutralization antibody anti-IGF-I on corneal cell supernatant-induced limbal stem cells differentiation. Limbal cells were cultured for 2 days in medium (C), in medium with supernatant from cultures of cells from damaged corneas (Sup) or in medium with supernatants, which were preincubated for 2 h with neutralization anti-body anti-IGF-I (Anti-IGF-I). The expression of the gene for K12 was determined by real-time PCR. Each bar represents mean – SD from 3 determinations. The value with an asterisk is significantly (P < 0.01) different from C, the value with 2 asterisks is significantly (P < 0.05) different from a positive control (Sup).

This suggestion is supported by the observation that corneal regeneration can be improved by the transplantation of limbal tissue or by a transfer of LSC [6,7]. These cells represent, in the healthy eye, a quiescent and slowly dividing cell population located in a specific niche that preserves their stemness [28]. LSC can be distinguished from corneal epithelial cells by phenotypic markers as well as by morphological and behavioral properties. Even though these stem cells are effectively protected in their niche, there must be a factor(s) produced by corneal cells after an injury that activates LSC and induces their proliferation, differentiation, and migration into the site of injury [8]. However, the molecular characteristics and the nature of those factors that overcome stemness and induce LSC differentiation remain unknown.

Previous studies that have described the inductive properties of limbal or the corneal cell-conditioned medium on the differentiation of stem cells into corneal epithelial lineages focused on embryonic stem cells [22,29], MSC [21,23], or adult tissue-specific stem cells [20]. These studies pointed out the role of the extracellular matrix of the stem cell niche and demonstrated the importance of factors present in the conditioned media. It was shown that it is possible to induce the expression of K12, a corneal cell-specific marker, in stem cells cultured in supernatants from corneal cells. However, the particular factors responsible for stem cell activation and differentiation have not been identified.

Here, we show that a number of genes for growth and differentiation factors are rapidly upregulated in cells of the corneal epithelium after an injury. These factors are able to

reach the limbal region through the tear film, aqueous fluid, or the epithelial layer of the cornea. In the limbus, they induce the increased proliferative activity of LSC. We observed that limbal cells harvested from the eyes 1 day after a corneal injury have a significantly higher proliferative activity than limbal cells from the healthy eyes. To identify the molecules responsible for the activation of cell proliferation and differentiation, we cultured limbal cells in the presence of factors that had been found to be produced by cells of the damaged corneal epithelium. Among these factors, IGF-I was the only one that induced the expression of the gene for K12, a marker of terminally differentiated corneal epithelial cells. This marker is not expressed by limbal cells and has been used in previous studies to monitor the transdifferentiation of stem cells cultured in corneal cell supernatants into corneal epithelial cells [20–23]. Since IGF-I is produced by corneal epithelial cells rapidly after an injury, it may be the main candidate molecule responsible for the activation and differentiation of LSC into corneal epithelial cells. The production of IGF-I and IGF-II by corneal cells has been documented [14,30], and their multiple effects on ocular surface cells have been demonstrated [13–16,30]. Our study, thus extends the list of IGF functions, and we suggest that IGF-I is the main factor produced by the wounded cornea that induces the activation and differentiation of LSC into cells expressing corneal epithelial markers.

IGF-I is a member of the IGF signaling pathway, which regulates a number of cellular functions [17]. The system involves peptide hormones (IGF of type I and II), cell surface receptors (insulin receptor, IGF receptors of type I and II), and circulating binding proteins (IGF binding proteins of type 1–6). IGF-I is produced by the liver as a growth hormone and is synthesized also locally by many cell types under basal conditions and in response to inflammatory signals [31,32]. The effects of IGF-I and IGF-II are mediated through the IGF-I receptor, which initiates signaling cascades leading to regulation of a number of biological processes.

Our results also showed that the expression of IGF receptors in corneal cells is significantly downregulated after corneal injury. This mechanism can prevent the binding of locally synthesized IGF to corneal cells, and thus supports the penetration of IGF into the limbal region, where it stimulates the expression of IGF receptors and LSC differentiation. We demonstrated that supernatants from cultures of corneal cells stimulate IGF-IR and IGF-IIR expression in limbal cells, and IGF-I was identified as the factor that stimulates the expression of its own receptors in limbal cells. We also showed that IGF-I present in supernatants from corneal cell cultures is a molecule responsible for differentiation of limbal cells into cells expressing K12. The neutralization antibody anti-IGF-I completely abrogated the differential potential of corneal cell supernatants.

Our study also showed that IGF-I induces limbal cell differentiation, but it has no effect on limbal cell proliferation in the mouse. Previous studies have demonstrated that IGF-I and IGF-II can support the proliferation of keratinocytes [13] and, together with substance P, promote the growth of rabbit corneal epithelial cells [33,34]. A similar growth-promoting effect of KGF and HGF on limbal epithelial cells in the rabbit was described by Cheng et al. [10]. In our mouse model, IGF-I, which induced the differentiation of LSC, did not support the proliferation of limbal cells, even though it was over a wide range of concentrations. Similarly, KGF also had only a weak promoting effect on limbal cell proliferation. In contrast, 2 other factors produced by corneal epithelial cells after injury, EGF and FGF-b, stimulated the significant proliferation of limbal cells although they did not induce K12 expression. Thus, distinct factors regulate limbal cell proliferation and differentiation in the mouse.

It has been recently shown that IGF significantly improves the differentiation of MSC into hepatocyte-like cells [35], promotes the myogenic differentiation of mouse myoblasts [36,37], enhances the insulinogenic differentiation of human eyelid adipose stem cells [38], and supports the activation and differentiation of human cardiac stem cells [39,40]. Our results suggest that the differentiation-promoting activities of IGF are more general and also involve effects on stem cell differentiation in the eye.

The identification of differentiation and growth factors for LSC may have an impact on the targeted differentiation of stem cells for therapeutic purposes. Recently, the cultivation of LSC in vitro and their transfer onto the damaged ocular surface has become a prospective way to treat severe corneal injuries and LSC deficiencies [7,41]. The implementation of appropriate factors into cultures of LSC may increase the yield of the appropriate cell type for reparative and regenerative medicine.

Acknowledgments

This work was supported by grants P304/11/0653, P301/ 11/1568, and 310/08/H077 from the Grant Agency of the Czech Republic, grant KAN200520804 from the Grant Agency of the Academy of Sciences, projects MSM0021620858 and SVV 265211 from the Ministry of Education of the Czech Republic, and project RVO 68378050 from the Academy of Sciences of the Czech Republic.

Author Disclosure Statement

No competing financial interests exist.

References

1. Majo F, A Rochat, M Nicolas, GA Jaoude and Y Barrandon. (2008). Oligopotent stem cells are distributed throughout the mammalian ocular surface. Nature 456:250–254.
2. Chang C-Y, CR Green, CNJ McGhee and T Sherwin. (2008). Acute wound healing in the human central cor-neal epithelium appears to be independent of limbal stem cell influence. (2008). Invest Ophthalmol Vis Sci 49: 5279–5286.
3. Dua HS, A Miri, T Alomar, AM Yeung and DG Said. (2009). The role of limbal stem cells in corneal epithelial mainte-nance: testing the dogma. Ophthalmology 116:856–863.
4. Tseng SC. (1996). Regulation and clinical implication of corneal epithelial stem cells. Mol Biol Rep 23:47–58.
5. Gomes JA, MS dos Santos, MC Cunha, VL Mascaro, JN Barros and LB de Sousa. (2003). Amniotic membrane transplantation for partial and total limbal stem cell defi-ciency secondary to chemical burn. Ophthalmology 110: 466–473.
6. Du Y, J Chen, JL Funderburgh, X Zhu and L Li. (2003). Functional reconstruction of rabbit corneal epithelium by human limbal cells cultured on amniotic membrane. Mol Vis 9:635–643.
7. Rama P, S Matuska, G Paganoni, A Spinelli, M De Luca and G Pellegrini. (2010). Limbal stem-cell therapy and long-term corneal regeneration. N Engl J Med 363:147–155.
8. Lehrer MS, TT Sun and RM Lavker. (1998). Strategies of epithelial repair: modulation of stem cell and transit ampli-fying cell proliferation. J Cell Sci 111:2867–2875.

9. Jia C, W Zhu, S Ren, H Xi, S Li and Y Wang. (2011). Comparison of genome-wide gene expression in suture- and alkali burn-induced murine corneal neovascularization. Mol Vis 17:2386–2399.
10. Cheng CC, DY Wang, MH Kao and JK Chen. (2009). The growth-promoting effect of KGF on limbal epithelial cells is mediated by upregulation of DeltaNp63alpha through the p38 pathway. J Cell Sci 122:4473–4480.
11. Li DQ and SC Tseng. (1995). Three patterns of cytokine expression potentially involved in epithelial-fibroblast interactions of human ocular surface. J Cell Physiol 163: 64–79.
12. Wilson SE, YG He, J Weng, JD Zieske, JV Jester and GS Schultz. (1994). Effect of epidermal growth factor, hepato-cyte growth factor, and keratinocyte growth factor, on pro-liferation, motility and differentiation of human corneal epithelial cells. Exp Eye Res 59:665–678.
13. Jester JV and J Ho-Chang. (2003). Modulation of cultured corneal keratocyte phenotype by growth factors/cytokines control in vitro contractility and extracellular matrix con-traction. Exp Eye Res 77:581–592.
14. Ko JA, R Yanai and T Nishida. (2009). IGF-1 released by corneal epithelial cells induces up-regulation of N-cadherin in corneal fibroblasts. J Cell Physiol 221:254–261.
15. Hassell JR and DE Birk. (2010). The molecular basis of cor-neal transparency. Exp Eye Res 91:326–335.
16. Etheredge L, BP Kane and JR Hassell. (2009). The effect of growth factor signaling on keratocytes in vitro and its rela-tionship to the phases of stromal wound repair. Invest Ophthalmol Vis Sci 50:3128–3136.
17. Pollak M. (2009). Insulin and insulin-like growth factor sig-nalling in neoplasia. Nat Rev Cancer 8:915–928.
18. Liu CY, G Zhu, R Converse, CW Kao, H Nakanuta, SC Tseng, MM Mui, J Seyer, MJ Justice, ME Stech, et al. (1994). Characterization and chromosomal localization of the cor-nea-specific murine keratin gene Krt1.12. J Biol Chem 269: 24627–24636.
19. Matic M, IN Petrov, S Chen, C Wang, SD Dimitrijevich and JM Wolosin. (1997). Stem cells of the corneal epithelium lack connexins and metabolite transfer capacity. Differentiation 61:251–260.
20. Blazejewska EA, U Schlo¨tzer-Schrehardt, M Zenkel, B Bachmann, E Chankiewitz, C Jacobi and FE Kruse. (2009). Corneal limbal microenvironment can induce transdiffer-entiation of hair follicle stem cells into corneal epithelial-like cells. Stem Cells 27:642–652.
21. Gu S, C Xing, J Han, MO Tso and J Hong. (2009). Differ-entiation of rabbit bone marrow mesenchymal stem cells into corneal epithelial cells in vivo and ex vivo. Mol Vis 15: 99–107.
22. Homma R, H Yoshikawa, M Takeno, MS Kurokawa, C Masuda, E Takada, K Tsubota, S Ueno and N Suzuki. (2004). Induction of epithelial progenitors in vitro from mouse embryonic stem cells and application for reconstruction of damaged cornea in mice. Invest Ophthalmol Vis Sci 45: 4320–4326.
23. Jiang TS, L Cai, WY Ji, YN Hui, YS Wang, D Hu and J Zhu. (2010). Reconstruction of the corneal epithelium with induced marrow mesenchymal stem cells in rats. Mol Vis 16:1304–1316.
24. Krulova M, K Pokorna, A Lencova, A Zajicova, J Fric, M Filipec, JV Forrester and V Holan. (2008). A rapid separation of two distinct populations of corneal epithelial cells with limbal stem cell characteristics in the mouse. Invest Oph-thalmol Vis Sci 49:3903–3908.
25. Holan V, K Pokorna, J Prochazkova, M Krulova and A Zajicova. (2010). Immunoregulatory properties of mouse limbal stem cells. J Immunol 184:2124–2129.
26. Svobodova E, M Krulova, A Zajicova, J Prochazkova, P Trosan and V Holan. (2012). The role of mouse mesenchy-mal stem cells in differentiation of naive T cells into anti-inflammatory regulatory T cell and proinflammatory helper T-cell 17 population. Stem Cells Dev 21:901–910.
27. Schermer A, S Galvin and TT Sun. (1986). Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. J Cell Biol 103:49–62.
28. Li W, Y Hayashida, Y-T Chen and SCG Tseng. (2007) Niche regulation of corneal epithelial stem cells at the limbus. Cell Res 17:26–36.
29. Ahmad S, R Stewart, S Yung, S Kolli, L Armstrong, M Stojkovic, F Figueiredo and M Lako. (2007). Differentiation of human embryonic stem cells into corneal epithelial-like cells by in vitro replication of the corneal epithelial stem cell niche. Stem Cells 5:1145–1155.
30. Izumi K, D Kurosaka, T Iwata, Y Oguchi, Y Tanaka, Y Mashima and K Tsubota. (2006). Involvement of insulin-like growth factor-I and insulin-like growth factor binding protein-3 in corneal fibroblasts during corneal wound healing. Invest Ophthalmol Vis Sci 47:591–598.
31. Maiter D, LE Underwood, M Maes and JM Ketelslegers. (1988). Acute downregulation of the somatogenic receptors in rat liver by a single injection of growth hormone. En-docrinology 122:1291–1296.
32. Smith TJ. (2010). Insuline-like growth factor-I regulation of immune function: a potential therapeutic target in autoim-mune diseases? Pharmacol Rev 62:199–236.
33. Nakamura M, TI Chikama and T Nishida. (2000). Char-acterization of insulin-like growth factor-1 receptors in rab-bit corneal epithelial cells. Exp Eye Res 70:199–204.
34. Yamada N, R Yanai, M Nakamura, M Inui and T Nishida. (2004). Role of the C domain of IGFs in synergistic promo-tion, with a substance P-derived peptide, of rabbit corneal epithelial wound healing. Invest Ophthalmol Vis Sci 45: 1125–1131.
35. Ayatollahi M, M Soleimani, B Geramizadeh and MH Im-anieh. (2011). Insulin-like growth factor 1 (IGF-I) improves hepatic differentiation of human bone marrow-derived mesenchymal stem cells. Cell Biol Int 35:1169–1176.
36. Matheny RW, Jr. and BC Nindl. (2011). Loss of IGF-IEa or IGF-IEb impairs myogenic differentiation. Endocrinology 152:1923–1934.
37. Shima A, J Pham, E Blanco, ER Barton, HL Sweeney and R Matsuda. (2011). IGF-I and vitamin C promote myogenic differentiation of mouse and human skeletal muscle cells at low temperatures. Exp Cell Res 317:356–366.
38. Kang HM, S Park and H Kim. (2011). Insulin-like growth factor 2 enhances insulinogenic differentiation of human eyelid adipose stem cells via the insulin receptor. Cell Prolif 44:254–263.
39. D'Amario D, MC Cabral-Da-Silva, H Zheng, C Fiorini, P Goichberg, E Steadman, J Ferreira-Martins, F Sanada, M Piccoli, et al. (2011). Insulin-like growth factor-1 receptor identifies a pool of human cardiac stem cells with superior therapeutic potential for myocardial regeneration. Circ Res 108:1467–1481.
40. Ellison GM, D Torella, S Dellegrottaglie, C Perez-Martinez, A Perez de Prado, C Vicinanza, S Purushothaman, V Galuppo, C Iaconetti, et al. (2011). Endogenous cardiac stem cell acti-vation by insulin-like growth factor-1/hepatocyte growth factor intracoronary injection fosters survival and regenera-tion of the infarcted pig heart. J Am Coll Cardiol 58:977–986.
41. Zajicova A, K Pokorna´, A Lencova, M Krulova, E Svobodova, S Kubinova, E Sykova, M Pradny, J Michalek, et al. (2010). Treatment of ocular surface injuries by limbal and mesenchymal stem cells growing on nanofiber scaffolds. Cell Transplant 19:1281–1290.

Address correspondence to:
Dr. Vladimir Holan
Institute of Molecular Genetics
Academy of Sciences of the Czech Republic
Videnska 1083
142 20 Prague 4
Czech Republic

E-mail: holan@img.cas.cz

Received for publication April 4, 2012 Accepted after revision

August 5, 2012 Prepublished on Liebert Instant Online August

8, 2012

5.5 A comparative study of the therapeutic potential of mesenchymal stem cells and limbal epithelial stem cells for ocular surface reconstruction

Holan V., Trosan P., Cejka C., Javorkova E., Zajicova A., Hermankova B., **Chudickova M.,** Cejkova J.

Stem Cells Transl Med. 2015; 4(9):1052-63. doi: 10.5966/sctm.2015-0039.

Differences in amounts and rates of cytokine production among MSCs can be further amplified by the impact of environment and resulting effects can be substantially different. This is important especially on the clinical level. A rabbit model of alkali-injured eye was used to compare the potential of AT-MSCs and BM-MSCs to support or replace LSCs in therapy of limbal stem cell deficiency. AT-MSCs, BM-MSCs and LSCs were grown on nanofiber scaffolds and transferred onto the injured corneal surface. After three days, the nanofiber scaffolds were removed from ocular surface. The animals were sacrificed and healing properties of AT-MSCs, BM-MSCs and LSCs were compared, based on their growth, differentiation and secretion. It was found that therapeutic effect of BM-MSCs is comparable to effect of tissue-specific LSCs and that AT-MSCs are less effective in regeneration of the injury. Thus, BM-MSCs seem to be a good alternative cell source in corneal healing and regeneration, in cases of limbal stem cell deficiency.

Received March 3, 2015; accepted for publication June 15, 2015.
©AlphaMed Press 1066-5099/2015/$20.00/0 http://dx.doi.org/10.5966/sctm.2015-0039

A Comparative Study of the Therapeutic Potential of Mesenchymal Stem Cells and Limbal Epithelial Stem Cells for Ocular Surface Reconstruction

VLADIMIR HOLAN,a,b PETER TROSAN,a,b CESTMIR CEJKA,a,c ELISKA JAVORKOVA,a,b ALENA ZAJICOVA,aBARBORA HERMANKOVA,a,b MILADA CHUDICKOVA,a,b JITKA CEJKOVAa

Key Words. Limbal stem cells x Mesenchymal stem cells x Alkali-injured ocular surface x Corneal regeneration x Stem cell-based therapy

ABSTRACT

Growth, differentiation, and secretory properties of mesenchymal stem cells (MSCs) and limbal epithelial stem cells (LSCs) were compared and their therapeutic potential characterized in a rabbit model of eye injury. Results show that MSCs from bone marrow had a therapeutic effect on healing of the injured corneal surface comparable to that of tissue-specific LSCs.Stem cell-based therapy has become an attractive and promising approach for the treatment of severe injuries or thus-far incurable diseases. However, the use of stem cells is often limited by a shortage of available tissue-specific stem cells; therefore, other sources of stem cells are being investigated and tested. In this respect, mesenchymal stromal/stem cells (MSCs) have proven to be a promising stem cell type. In the present study, we prepared MSCs from bone marrow (BM-MSCs) or adipose tissue (Ad-MSCs) as well as limbal epithelial stem cells (LSCs), and their growth, differentiation, and secretory properties were compared. The cells were grown on nanofiber scaffolds and transferred onto the alkali-injured eye in a rabbit model, and their therapeutic potential was characterized. We found that BM-MSCs and tissue-specific LSCs had similar therapeutic effects. Clinical characterization of the healing process, as well as the evaluation of corneal thickness, re-epithelialization, neovascularization, and the suppression of a local inflammatory reaction, were comparable in the BM-MSC- and LSC-treated eyes, but results were significantly better than in injured, untreated eyes or in eyes treated with a nanofiber scaffold alone or with a nanofiber scaffold seeded with Ad-MSCs. Taken together, the results show that BM-MSCs' therapeutic effect on healing of injured corneal surface is comparable to that of tissue-specific LSCs. We suggest that BM-MSCs can be used for ocular surface regeneration in cases when autologous LSCs are absent or difficult to obtain.

STEM CELLS TRANSLATIONAL MEDICINE 2015;4:1–13

SIGNIFICANCE

Damage of ocular surface represents one of the most common causes of impaired vision or even blindness. Cell therapy, based on transplantation of stem cells, is an optimal treatment. However, if limbal stem cells (LSCs) are not available, other sources of stem cells are tested. Mesenchymal stem cells (MSCs) are a convenient type of cell for stem cell therapy. The therapeutic potential of LSCs and MSCs were compared in an experimental model of corneal injury and healing was observed following chemical injury. MSCs and tissue-specific LSCs had similar therapeutic effects. The results suggest that bone marrow-derived MSCs can be used for ocular surface regeneration in cases when autologous LSCs are absent or difficult to obtain.

INTRODUCTION

Severe injuries or defects of the cornea represent are among the most common causes of decreased quality of vision or even blindness. In many cases, penetrating keratoplasty is performed as the first treatment option. However, if the corneal damage is more extensive and the limbal region is involved, the defect can lead to limbal stem cell deficiency (LSCD). In such cases, corneal transplantation alone is not a sufficient treatment method. The only effective way to treat LSCD is by the transplantation of whole limbal tissue or the transfer of limbal epithelial stem cells (LSCs). Although beneficial effects of limbal transplantation have been reported [1–3], the shortage of limbal tissue and a strong immune response to a limbal allograft are the main obstacles to such treatment protocols. Therefore, a more promising treatment method is offered by LSC transplantation. The first encouraging results from LSC transplantation have been published [4–6]. Since LSCs represent a relatively small population of limbal cells that are difficult to isolate and prepare in sufficient quantities, other stem cell sources are being explored and tested to treat LSCD.

An alternative source of stem cells for ocular surface regeneration and the treatment of LSCD is mesenchymal stem cells (MSCs). These cells can be obtained relatively easily in a sufficient amount from various types of tissues (e.g., bone marrow, adipose tissue) and expanded in vitro for autologous application. It has been shown that MSCs retain their differentiation potential during in vitro expansion and that they can differentiate into various cell types [7], including cells expressing corneal epithelial cell markers [8, 9]. The first results of using MSCs for ocular surface healing in small animal models have been published [9, 10]. We have shown in mice [11] and rabbits [12] that MSCs grown on a nanofiber scaffold and transferred onto the damaged ocular surface significantly inhibit the local inflammatory reaction and support the healing process. Although LSCs and MSCs have different origins, they share comparable immunoregulatory properties in vitro [13]. Similarly, numerous common properties have been described for tissue-specific stem cells isolated from different organs [14]. Comparative studies on MSCs prepared from bone marrow and other sour-ces have shown many similarities but also some differences [15, 16]. For the treatment of ocular surface injuries and LSCD, both tissue-specific LSCs and MSCs isolated from the bone marrow (BM-MSCs) or adipose tissue (Ad-MSCs) have been proposed and tested. In these studies, MSCs proved to be a promising cell type to support the healing of the damaged ocular surface [9–12, 17–19]. However, so far, there is no direct evidence that MSCs can support the healing and regeneration of damaged corneal tissue as effectively as the tissue-specific LSCs. Therefore, in the present study, we used a well-established model of the alkali-damaged ocular surface in rabbits and directly compared the regenerative and reparative potential of tissue-specific LSCs and MSCs derived from bone marrow or adipose tissue. On the basis of several evaluated parameters, we show the therapeutic potential of BM-MSCs for the treatment of damaged ocular surface is comparable to that of tissue-specific LSCs, and, thus, BM-MSCs can be used therapeutically as a convenient source of stem cells to support healing of the wounded cornea.

MATERIALS AND METHODS

Animals and Alkali-Induced Corneal Damage

Adult, female New Zealand white rabbits (2.5–3.0 kg) obtained from Velaz Ltd. (Prague, Czech Republic, http://www.velaz.cz) were used in the experiments. Rabbits were anesthetized by an intramuscular injection of a 1:1 mixture of xylazinum hydrochloridum 2% (0.2 ml/kg body weight; Rometar; Spofa, Prague, Czech Republic) and ketaminum hydrochloridum 5% (1 ml/kg body weight; Narkamon; Spofa). The right corneas of anesthetized rabbits were injured by dropping 0.25N sodium hydroxide (NaOH) on the corneal surface (10 drops during 1 minute, alkali injuring the whole cornea, including the limbal region), then the eyes were immediately rinsed with an excess of tap water. After the alkali injury and awakening from the anesthesia, the rabbits were treated with analgesia (ketoprofen, 1.0 mg/kg i.m.) 2 times daily for 5 days.

All experiments were conducted according to the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research and were approved by the local ethics committee.

Isolation of LSCs and MSCs

LSCs were obtained by the enzymatic digestion of limbal tissue, as we have described in a mouse model [20]. In brief, limbal tissue was cut with scissors and subjected to 10 short (10 minutes each) trypsinization cycles. The released cells were harvested after each cycle, centrifuged (8 minutes at 250g), and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich Corp., St. Louis, MO, http://www.sigmaaldrich.com) containing 10% fetal calf serum (FCS; Sigma-Aldrich), antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin), and 10 mM HEPES buffer. The cells were seeded in 25-cm2 tissue culture flasks (Corning Inc., Schiphol-Rijk, The Netherlands, http://www.corning.com). For characterization of the cells and for their transfer onto a nanofiber scaffold, cells grown in vitro for 2–3 weeks (third passage) were used.

BM-MSCs were isolated from the femurs of rabbits. The bone marrow was flushed out, a single-cell suspension was prepared by homogenization, and the cells were seeded at a concentration of 4 3 106 cells per milliliter in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing 10% FCS, antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin), and 10 mM HEPES buffer in 25-cm2 tissue culture flasks (Corning). After a 48-hour incubation, the nonadherent cells were washed out and the adherent cells were cultured with a regular exchange of the medium and passaging of the cells to maintain their optimal concentration. The cells were characterized and used at the third passage.

Ad-MSCs were isolated from subcutaneous adipose tissue. The tissue was cut into small pieces with scissors and incubated in 1 ml of Hanks' balanced salt solution containing 10 mg/ml collagenase type I (Sigma-Aldrich) for 60 minutes at 37°C with gentle agitation. Then the collagenase was diluted with complete DMEM. The cells were filtered and centrifuged at 250g for 8 minutes. The upper adipose layer was removed, the cells were centrifuged, resuspended in 6 ml complete DMEM (4 3 106 cells per milliliter), and seeded in 25-cm2 tissue culture flasks (Corning). After incubation for 48 hours, the cells were washed with medium to remove nonadherent cells and cell debris, and cultured under standard conditions. Ad-MSCs were used in passages 3 and 4.

Stem Cell Growth, Differentiation, and Gene Expression

To show the morphology of MSCs and LSCs, the cells were grown on glass cover slips, fixed with paraformaldehyde, and incubated with Alexa Fluor 568 phalloidin (Invitrogen/Thermo Fisher Scientific Inc., Paisley, U.K., http://www.thermoscientific.com) to label F actin. The nuclei were visualized by using 49,6-diamidino-2-phenylindole (DAPI) fluorescent dye (Invitrogen). Images were taken by a laser scanning confocal microscope (Zeiss International, Jena, Germany, http://www.zeiss.com). For characterization of their growth properties, cells were seeded (1 3 104 cells per well) in 500 ml of complete DMEM in 48-well tissue culture plates (Nunc/Thermo Fisher Scientific Inc., Roskilde, The Netherlands, http://www.thermoscientific.com), and the growth of the cells was determined after 3-, 24-, and 48-hour cultivation using the WST assay, as we have described [21]. In brief, WST-1 reagent (Roche Diagnostics, Man-nheim, Germany, http://www.roche.de) was added to each well to form formazan. The plates were then incubated for

another 4 hours, and the absorbance was measured by spectrophotometry. The assay is based on the ability of living cells to use mitochondrial dehydrogenases to cleave tetrazolium salts into water-soluble formazan, which is then measured by spectrophotometry. To compare the growth of stem cells on plastic or on a nanofiber scaffold, MSCs and LSCs were seeded (4 3 104 cells per well) in 700 ml DMEM in 24-well tissue culture plates (Corning) directly into wells or onto a nanofiber scaffold fixed into CellCrown TM24 inserts (Scaffdex Ltd., Tampere, Finland, http://www.scaffdex.com). The growth of cells was determined after 48 hours by the WST assay. The ability of stem cells to differentiate into adipocytes was determined using specific adipogenic medium containing 0.1 mM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 0.1 mM indomethacine, and 0.5 mg/ml insulin, as we described previously [22]. The differentiation of the cells was confirmed by staining with Oil Red O and by quantifying the expression of the adipocyte-specific genes for adiponectin (ADPC) and peroxisome proliferator-activated receptor g (PPARg) using real-time polymerase chain reaction (PCR). The expression of genes for the immunoregulatory molecules indoleamine-2,3-dioxygenase (IDO-2), cyclooxygenase-2 (Cox-2) and inducible nitric oxide synthase (iNOS), and for hepatocyte growth factor (HGF), transforming growth factor-b (TGF-b), and vascular endothelial growth factor (VEGF) was determined in unstimulated and lipopolysaccharide (LPS)-stimulated MSCs and LSCs. In these experiments, the cells (4 3 104 cells per well) were cultured in 700 ml of DMEM for 48 hours in 24-well tissue culture plates (Corning) with or without 5 mg/ml LPS, and the expression of the genes was determined by real-time PCR, as described below.

Nanofiber Scaffolds

Nanofiber scaffolds were prepared from the biocompatible polymer poly(L-lactic) acid (PLA) by a needleless electrospinning procedure, as we have described [23]. In brief, PLA polymer was dissolved in chloroform and two other solvents, 1,2-dichlorethane and ethyl acetate, were added to this solution. The mixture was stirred until a homogenous polymer solution was obtained. The modified needleless Nanospider technology (Elmarco s.r.o., Liberec, Czech Republic, http://www.elmarco.com), in which polymeric jets are spontaneously formed from liquid surfaces on a rotating spinning electrode, was used for the preparation of the nanofibers. In this study, nanofiber material with a mass per unit area of 10 g/m2 and with a nanofiber diameter ranging from 290 to 539 nm was used. The morphology of the nanofibers and their nanofibrous architecture were analyzed using scanning electron microscopy and shown previously [11, 21].

Stem Cell Growth on Nanofiber Scaffold and Cell Transfer

Nanofiber scaffolds were cut into squares (approximately 1.5 3 1.5 cm) and fixed into CellCrown TM24 inserts (Scaffdex). The inserts with nanofibers were transferred into 24-well tissue culture plates (Corning).

Stem cells (3 3 105) in 700 ml of complete DMEM were transferred into each well. The plates were incubated for 24 hours to allow the cells to adhere to the scaffold. For stem cell transfer, nanofiber scaffolds seeded with stem cells were transferred within 1 hour after the injury with the cell side facing down on the damaged ocular surface. The scaffolds were sutured to the conjunctiva with four interrupted sutures using 11.0 Ethilon (Ethicon, Johnson & Johnson, Livingston, U.K., http://www.ethiconproducts.co.uk). The eyelids were closed by tarsorrhaphy using 1 suture of Resolon 7.0 (Resorba Medical GmbH, Nuremberg, Germany, http://www.resorba.com) for 72 hours. An ophthalmic ointment compound containing bacitracin and neomycin (Ophthalmo-Framykoin; Zentiva Group, Prague, Czech Republic, http://www.zentiva.com) was applied on the oc-ular surface for 3 days. The nanofiber scaffolds were removed from the ocular surface on day 3 after the operation. The animals were sacrificed following an i.v. injection of thiopental anesthesia (30 mg/kg thiopental; Spofa) after premedication with an intra-muscular injection of xylazinum hydrochloridum/ketaminum hydrochloridum. Each experimental group involved six rabbits (i.e., six experimental eyes). In all experiments with alkali injury, the corneas of healthy rabbit eyes served as controls.

Immunohistochemistry

After sacrificing the animals, the eyes were enucleated and the anterior eye segment dissected out and quenched in light petroleum chilled with an acetone-dry ice mixture. Sections were cut on a cryostat and transferred onto glass slides. Subsequently, the cryostat sections were fixed in acetone at 4°C for 5 minutes. For the immunohistochemical detection of cells staining for CD3, iNOS, VEGF, or the cytokeratins K3 and K12 (K3/12), the following primary monoclonal antibodies (mAbs) were used: anti-CD3 (Abcam, Cambridge, U.K., http://www.abcam.com), anti-iNOS (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com), anti-caspase-3 (Abcam), anti-K3/12 (Abcam), and anti-VEGF (Abcam). The binding of the primary antibodies was demon-strated using the horseradish peroxidase/3,3'-diaminobenzidine (HRP/DAB) Ultra Vision detection system (Thermo Fisher Scientific) following the instructions of the manufacturer. Individual steps involved the following: hydrogen peroxide block (15 minutes), ultra V block (5 minutes), incubation with the primary antibody (60 minutes), incubation (10 minutes) with biotinylated goat anti-mouse IgG secondary antibody (Thermo Fisher Scientific) and peroxidase-labeled streptavidin incubation (10 minutes). Visualization was performed using a freshly prepared DAB substrate-chromogen solution. Cryostat sections in which the primary antibodies were omitted from the incubation media served as negative controls. Some sections were counterstained with Mayer's hematoxylin.

The counting of cells positive for CD3 and iNOS in the corneal stroma and for caspase-3 in the corneal epithelium was performed by an examiner without prior knowledge of the experimental procedure. Three randomly chosen fields of corneal sections (of the same field size and the same microscope magnification) from six corneas of each experimental animal group were used. For each cornea, the mean value from the three fields was calculated.

Determination of Corneal Thickness

Changes of corneal transparency after the injury and during healing were examined according to the measurement of the central corneal thickness (taken as an index of corneal hydra-tion). The central corneal thickness was measured in anesthetized animals using an ultrasonic pachymeter SP-100 (Tomey

Corp., Nagoya, Japan, http://www.tomey.com) in the corneal center. The corneal thickness was measured in the same corneas before alkali injury (corneas of healthy eyes) and on days 5 and 12 after the injury (all experimental groups). Each cornea was measured four times and the mean value of the thickness (in mm) was computed.

Determination of Corneal Neovascularization and Re-epithelialization

For the evaluation of corneal neovascularization, the number of vessels was counted in each 60° sector of the corneal surface. The mean value and standard deviation were determined from six eyes in each group. To characterize corneal re-epithelialization, postfixed cryo-stat sections of the corneas were stained with a mAb directed against the corneal epithelial cell-associated cytokeratins K3 and K12, using hematoxylin and eosin stain for counterstaining. The images were evaluated microscopically. For the quantifica-tion of re-epithelialization, the expression of genes for K3 and K12 was determined by real-time PCR in healthy, injured and treated corneas.

Detection of Gene Expression by Real-Time PCR

The expression of genes in cultured cells or in control and treated corneas was determined by quantitative real-time PCR. The corneas or cultured stem cells were transferred into Eppendorf tubes containing 500 ml TRI Reagent (Molecular Research Center Inc., Cincinnati, OH, http://mrcgene.com). The details of RNA isolation, transcription, and the PCR parameters have been described previously [24]. In brief, total RNA was extracted using TRI Re-agent according to the manufacturer's instructions. Total RNA (1 mg) was treated using deoxyribonuclease I (Promega Corp., Madison, WI, http://www.promega.com) and subsequently used for reverse transcription. The first-strand cDNA was synthesized using random primers (Promega) in a total reaction volume of 25 ml using Moloney murine leukemia virus reverse transcriptase (Promega). Quantitative real-time PCR was performed using a StepOne-Plus real-time PCR system (Thermo Fisher Scientific). The relative quantification model with efficiency correction was applied to calculate the expression of the target gene in comparison with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as the housekeeping gene. The following primers were used for am-plification:

GAPDH: 59-CCCAACGTGTCTGTCGTG (sense), 59-CCGACCCAGACGTACAGC (antisense); K3: 59-GAACAAGGTCCTG-GAGACCA (sense), 59-TTGAAGTCCTCC ACCAGGTC (antisense); K12: 59-AGGAGGTGGTGAATGGTGAG (sense), 59-GTTGTTTC-CCAGGAGCAAAA (antisense); ADPC: 59-.ACCAGGACAAGAACGT-GGAC (sense), 59-TGGAGATGGAATCGTTGACA (antisense); PPARg: 59-AGTCGCCATCC GCATCTT (sense), 59-ATCTCATGGACGCCG-TACTTG (antisense); IDO-2: 59-GTTTC CTTGGCTCGTTGG (sense), 59-CCTTTTCTGAAAGGATAAACTCTCG (antisense); iNOS: 59-AGG-GAGTGTTGTTCCAGGTG (sense), 59-TCCTCAACCTGCTCCTCACT (antisense); Cox-2: 59-ACATCGTCAATAGCATTC (sense), 59-TAG-TAGGAGAGGTT GAGA (antisense); TGF-b1: 59-GCCTGCAAGTGCT-CAAGTTAC (sense), 59-TGCTG CATTTCTGGTACAGC (antisense); HGF: 59-AGGCAGCTATAAGGGAACAGTG (sense), 59-ATGGAACTC-CAGGGCTGAC (antisense); and VEGF: 59-CGAGACCTTGGTG GACATCT (sense), 59-ATCTGCATGGTGACGTTGAA (antisense).

The PCR parameters included denaturation at 95°C for 3 minutes, then 40 cycles at 95°C for 20 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds. Fluorescence data were collected at each cycle after an elongation step at 80°C for 5 seconds and were analyzed using StepOne Software, version 2.2.2 (Thermo Fisher Scientific). Each individual experiment was performed in triplicate.

Statistical Analysis

Analysis of the data showed a normal distribution and the results are expressed as mean 6 SD. Comparisons between two groups were made using Student t test, and multiple comparisons were analyzed by analysis of variance. A value of p , .05 was considered statistically significant.

RESULTS

Growth, Differentiation, and Gene Expression of Rabbit MSCs and LSCs

The morphology of BM-MSCs, Ad-MSCs, and LSCs growing on glass cover slips in vitro is shown in Figure 1A. All three cell types adhered to plastic and glass surfaces and had a typical fibrocyte-like shape. The cells had similar growth characteristics when cultured on plastic (Fig. 1B) and proliferated comparably on a nanofiber scaffold (Fig. 1C). When all three cell types were cultured in a specific adipogenic differentiation medium, the highest differentiation potential was recorded in BM-MSCs, as demonstrated microscopically (Fig. 1D) and also according to the expression of genes for the adipocyte markers ADPC and PPARg determined by real-time PCR (Fig. 1E). To test the ability of BM-MSCs, Ad-MSCs, and LSCs to produce basic immunoregulatory molecules and growth factors, the cells were cultured for 48 hours unstimulated or stimulated with LPS, and the expression of genes for IDO-2, Cox-2, iNOS, TGF-b, HGF, and VEGF was determined by real-time PCR. As shown in Figure 2, some of these genes were expressed spontaneously and comparably in all cell types, while other factors were produced preferentially in only some cell populations or only after stimulation with LPS.

Immunohistochemical Detection of CD3+, iNOS+, and Caspase-3+ Cells in Alkali-Injured and Stem Cell-Treated Corneas

The presence of CD3+ cells (Fig. 3A) or cells expressing iNOS (Fig. 3B) or caspase-3 (Fig. 3C) was very low or absent in healthy control corneas. After alkali injury, the corneas were strongly infiltrated with CD3+ cells (Fig. 3D) and the expression of iNOS (Fig. 3E) was high. Similarly, the number of apoptotic caspase-3+ cells was high in the remaining islands of the corneal epithelium (Fig. 3F). The infiltration of corneas with CD3+ cells (Fig. 3G) or the presence of iNOS+ (Fig. 3H) or caspase-3+ (Fig. 3I) cells was slightly decreased in injured corneas treated with cell-free nanofiber scaffolds. However, after the treatment of injured corneas with stem cell-seeded nanofibers (Fig. 3J–3R), the numbers of CD3+, iNOS+, or caspase-3+ cells were significantly decreased. The expression of caspase-3 in apoptotic cells, which was high in the remaining islands of the corneal epithelium in untreated injured corneas (Fig. 3F, arrow), was only weakly expressed in the epithelium of corneas treated with a nanofiber scaffold seeded with BM-MSCs (Fig. 3L, arrow) or LSCs (Fig. 3R, arrow). The number of CD3+ cells (Fig. 3V) as well as cells expressing iNOS (Fig. 3W) or caspase-3 (Fig. 3X) was counted in defined fields of corneal sections for

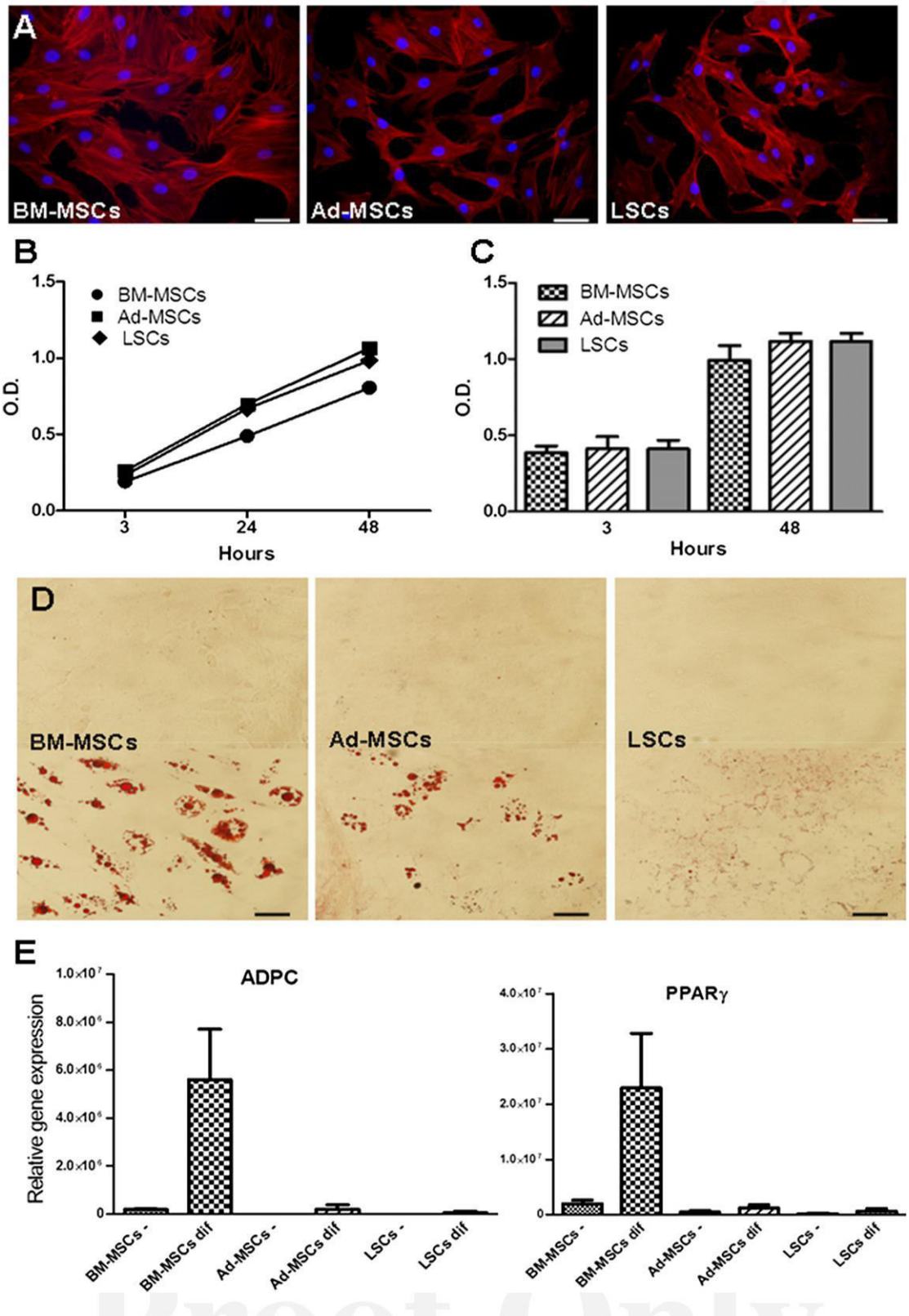

Figure 1. Characterization of BM-MSCs, Ad-MSCs, and LSCs. (A): The morphology of the cells is shown by staining for F actin with phalloidin (red filaments). The nuclei are blue (4′,6-diamidino-2-phenylindole [DAPI] staining). Scale bars = 200 mm. (B–E): The growth of cells on plastic (B) or on (Figure legend continues on next page.)

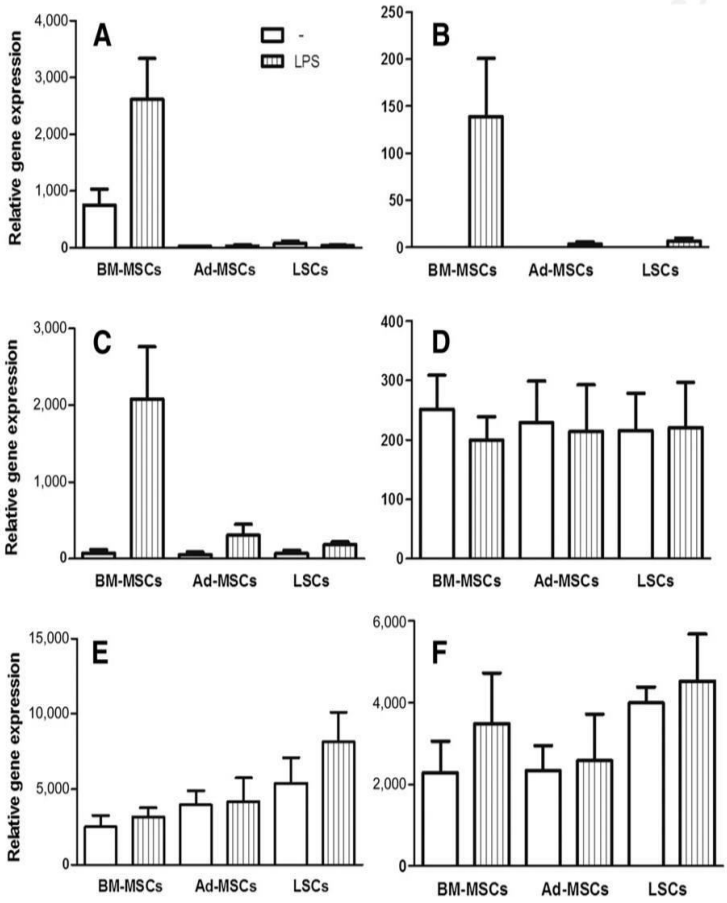

Figure 2. Expression of genes for immunoregulatory molecules and growth factors by BM-MSCs, Ad-MSCs, and LSCs. (A–F): The cells were cultured for 48 hours unstimulated or stimulated with LPS and the expression of genes for indoleamine-2,3-dioxygenase (A), cyclooxyge-nase-2 (B), inducible nitric oxide synthase (C), transforming growth factor-b (D), hepatocyte growth factor (E), and vascular endothelial growth factor (F) was determined by real-time polymerase chain reaction. Each bar represents the mean 6SD from four determinations. Abbreviations: Ad-MSC, adipose tissue-derived mesenchymal stem cell; BM-MSC, bone marrow-derived mesenchymal stem cell; LPS, lipopolysaccharide; LSC, limbal epithelial stem cell.

each experimental group. The graphs show that the numbers of $CD3^+$, $iNOS^+$, or caspase-3^+ cells, which were high in untreated injured corneas or corneas treated with cell-free nanofiber scaffolds, were significantly decreased in the groups treated with stem cell-seeded nanofibers.

Corneal Thickness After Alkali-Injury and Treatment With MSCs and LSCs

The central corneal thickness of healthy corneas was about 380 mm (Fig. 4). Shortly after alkali injury, the corneal thickness increased (as a result of hydration) more than twofold and remained high on day 5 in untreated corneas and in corneas treated with cell-free nanofiber scaffolds. In corneas treated with Ad-MSC-seeded nanofiber scaffolds, the corneal thickness slightly decreased, but it was significantly reduced already on day 5 in corneas treated with nanofiber scaffolds seeded with BM-MSCs or LSCs (Fig. 4). On day 12 after injury, the corneal thickness remained enhanced in untreated injured corneas but was significantly decreased in corneas treated with cell-free or Ad-MSC-seeded nanofiber scaffolds. In corneas treated with nanofiber scaffolds seeded with BM-MSCs or LSCs, the corneal thickness returned to the values observed before injury (Fig. 4).

Expression of VEGF and Neovascularization in Injured and Stem Cell-Treated Corneas

The expression of VEGF was very low in healthy control corneas (Fig. 5A). On day 12 after the injury, the expression of VEGF was

(Figure legend continued from previous page.)
a nanofiber scaffold (C) was determined by the WST assay. The ability of cells to differentiate into adipocytes was characterized microscopically (D, upper: undifferentiated cells; lower: cells in differentiation medium) or according to the expression of the ADPC and PPARg genes detected by real-time polymerase chain reaction (E). Each bar represents the mean 6 SD from three determinations. Abbreviations: Ad-MSC, adipose tissue-derived mesenchymal stem cell; ADPC, adiponectin gene; BM-MSC, bone marrow-derived mesenchymal stem cell; dif, differentiation medium; LSC, limbal epithelial stem cell; o.d., optical density (absorbance); PPARg, peroxisomeproliferator-activated receptor g.

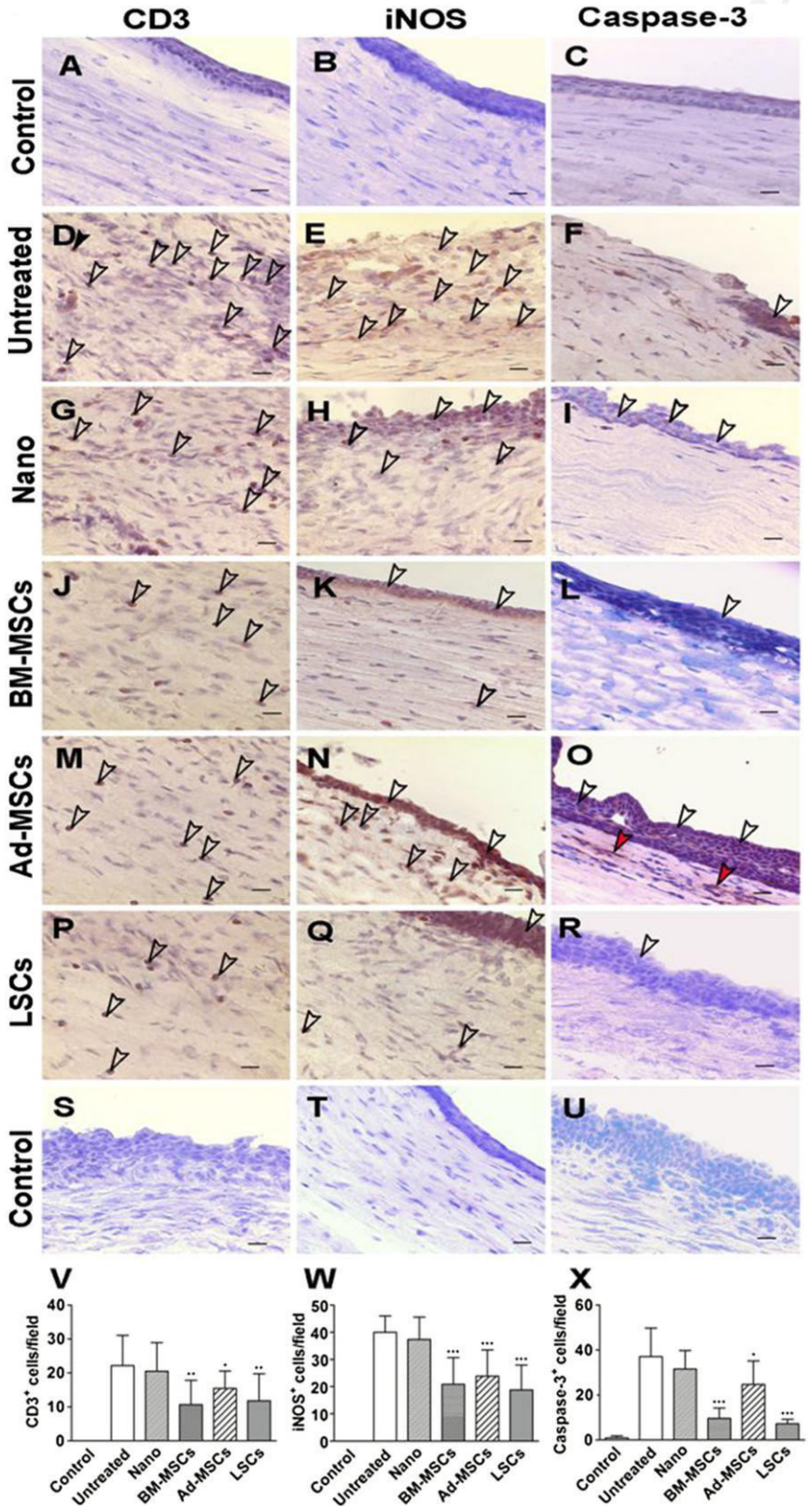

Figure 3. The immunohistochemical detection of $CD3^+$, $iNOS^+$, and caspase-3^+ cells in healthy, injured and stem cell-treated corneas. (A–C): Undetectable or very low numbers of $CD3^+$ (A), $iNOS^+$ (B), or caspase-3^+ (C) cells were found in healthy corneas. (D–I): On day 12 after alkali injury, the number of $CD3^+$ (D), $iNOS^+$ (E), and apoptotic caspase-3^+ (F) cells was significantly increased and remained high in corneas treated with a cell-free nanofiber scaffold (G–I). (J, M, P): The presence of $CD3^+$ cells was clearly decreased in corneas treated with nanofiber scaffolds seeded with (Figure legend continues on next page.)

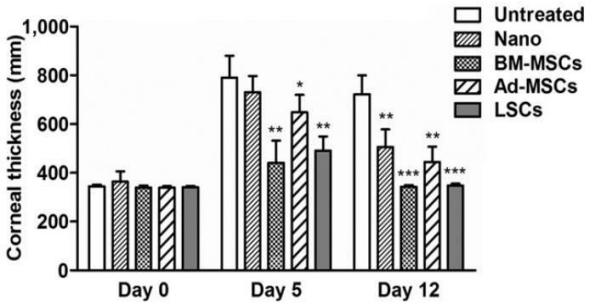

Figure 4. Central corneal thickness of healthy, alkali-injured and stem cell-treated corneas. The corneas were injured with alkali and then left untreated, treated with a nanofiber scaffold alone or treated with nanofiber scaffolds seeded with BM-MSCs, Ad-MSCs, or LSCs. The central corneal thickness was measured in the same rabbit before injury (day 0) and on days 5 and 12 after the injury. Each bar represents the mean 6 SD from six corneas. The values with asterisks for day 5 are significantly different from those of untreated injured corneas on day 5; similarly, the values with asterisks for day 12 are significantly different from those of untreated injured corneas on day 12 (p, p , .05; pp, p , .01; ppp, p , .001). Abbreviations: Ad-MSC, adipose tissue-derived mesenchymal stem cell; BM-MSC, bone marrow-derived mesenchymal stem cell; LSC, limbal epithelial stem cell; nano, nanofiber scaffold.

high in untreated corneas (Fig. 5B) and was only slightly decreased in corneas treated with cell-free nanofiber scaffolds (Fig. 5C). The treatment of injured corneas with Ad-MSC-seeded nanofiber scaffolds apparently reduced VEGF expression (Fig. 5E), but the greatest reduction in VEGF expression was seen in corneas treated with nanofiber scaffolds seeded with BM-MSCs (Fig. 5D) or LSCs (Fig. 5F). The quantification of corneal neovascularization is summarized in Figure 5H. The number of vessels was high in untreated injured corneas and was partially reduced in injured corneas treated with cell-free nanofibers. The treatment of injured corneas with nanofiber scaffolds seeded with all three types of stem cells significantly decreased neovascularization. The greatest decrease was found in injured corneas treated with nanofiber scaffolds seeded with BM-MSCs or LSCs.

Corneal Re-epithelialization After Treatment of Injured Eyes With MSCs or LSCs

The extent of corneal re-epithelialization was evaluated on day 12 after alkali injury by examining corneal sections stained with mAb anti-K3/K12. A typical image of a normal healthy cornea is shown in Figure 6A. In contrast, only rare and isolated islands of the epithelium were detected in untreated injured corneas (Fig. 6B). Covering the injured ocular surface with a cell-free nanofiber scaffold improved re-epithelialization and islands with epithelium covered about 20%–40% of the corneal surface (Fig. 6C, 6D). The transfer of stem cell-seeded nanofiber scaffolds onto the damaged ocular surface (Figs. 6E–6G) significantly improved corneal healing, with the best re-epithelialization observed in corneas treated with nanofiber scaffolds seeded with BM-MSCs (Fig. 6E) or LSCs (Fig. 6G). The expression of genes for the cytokeratins K3 and K12 in healthy, injured and treated corneas was quantified by real-time PCR (Fig. 6I, 6J). Cytokeratin gene expression was apparent in healthy corneas but was absent or very low in untreated injured corneas and in corneas treated with cell-free nanofiber scaffolds. In accordance with the immunohistochemical results, the treatment of injured corneas with BM-MSC- or LSC-seeded nanofiber scaffolds significantly enhanced the expression of both cytokeratin genes.

Corneal Opacity of Alkali-Injured and Stem Cell-Treated Eyes

Representative photographs of healthy, injured and treated eyes are shown in Figure 7. In comparison with healthy control eyes (Fig. 7A), the corneas of injured eyes became opalescent shortly after the injury and remained opalescent and highly vascularized on day 12 after injury (Fig. 7C). An eye on day 2 after injury and covered with a nanofiber scaffold is shown in Figure 7B. Only a weak improvement in the appearance of the corneas was observed on day 12 in the eyes treated with cell-free nanofiber scaffolds (Fig. 7D). In the eyes treated with stem cell-seeded nanofibers, corneal opacity was decreased and corneal neovascularization was less apparent (Fig. 7E–7G), with the best therapeutic effects seen with nanofiber scaffolds seeded with BM-MSCs (Fig. 7E) or LSCs (Fig. 7G).

DISCUSSION

Stem cell-based therapy holds great promise for the treatment of severe injuries as well as a number of thus-far incurable diseases. The best source of stem cells for tissue therapy is tissue-specific stem cells, but these cells are often rare in the body, difficult to isolate, and not easily handled in vitro. Therefore, research is focused on the search for alternative cell sources that could effectively replace tissue-specific stem cells.

One possibility has been offered by embryonic stem cells (ESCs), but the use of ESCs is limited by their uncontrolled growth, the risk of teratoma formation, and ethical problems associated with their isolation and use [25, 26]. Induced pluripotent stem cells, which could be used as autologous cells, initially appeared to offer great promise, but these cells turned out to be immunogenic even in syngeneic hosts [27] and frequently form teratomas after in vivo application [28]. MSCs represent a convenient type of stem cells with a wide spectrum of potential applications. These

(Figure legend continued from previous page.)
BM-MSCs (J), Ad-MSCs (M), or LSCs (P). (K, N, Q): Similarly, the number of iNOS$^+$ cells was decreased in corneas treated with BM-MSCs (K), Ad-MSCs (N), or LSCs (Q). (L, Q, R): The presence of caspase-3$^+$ cells, which were numerous in the remaining islands of the corneal epithelium in untreated injured corneas, was decreased in the corneas treated with BM-MSCs (L), Ad-MSCs (Q), or LSCs (R). (S–U): Cells expressing CD3 (S), iNOS (T), or caspase-3 (U) were absent in corneal sections stained only with counterstaining, where the primary antibody was omitted from the incubation medium (negative control). Scale bars = 50 mm. (V–X): The numbers of CD3$^+$ (V), iNOS$^+$ (W), and caspase-3$^+$ (X) cells counted in comparable fields of corneal sections were determined from six corneas in the individual experimental groups. The values with asterisks are significantly different (p, p , .05; pp, p , .01; ppp, p , .001) from those of untreated injured corneas. Abbreviations: Ad-MSC, adipose tissue-derived mesenchymal stem cell; BM-MSC, bone marrow-derived mesenchymal stem cell; iNOS, inducible nitric oxide synthase; LSC, lim-bal epithelial stem cell; nano, nanofiber scaffold.

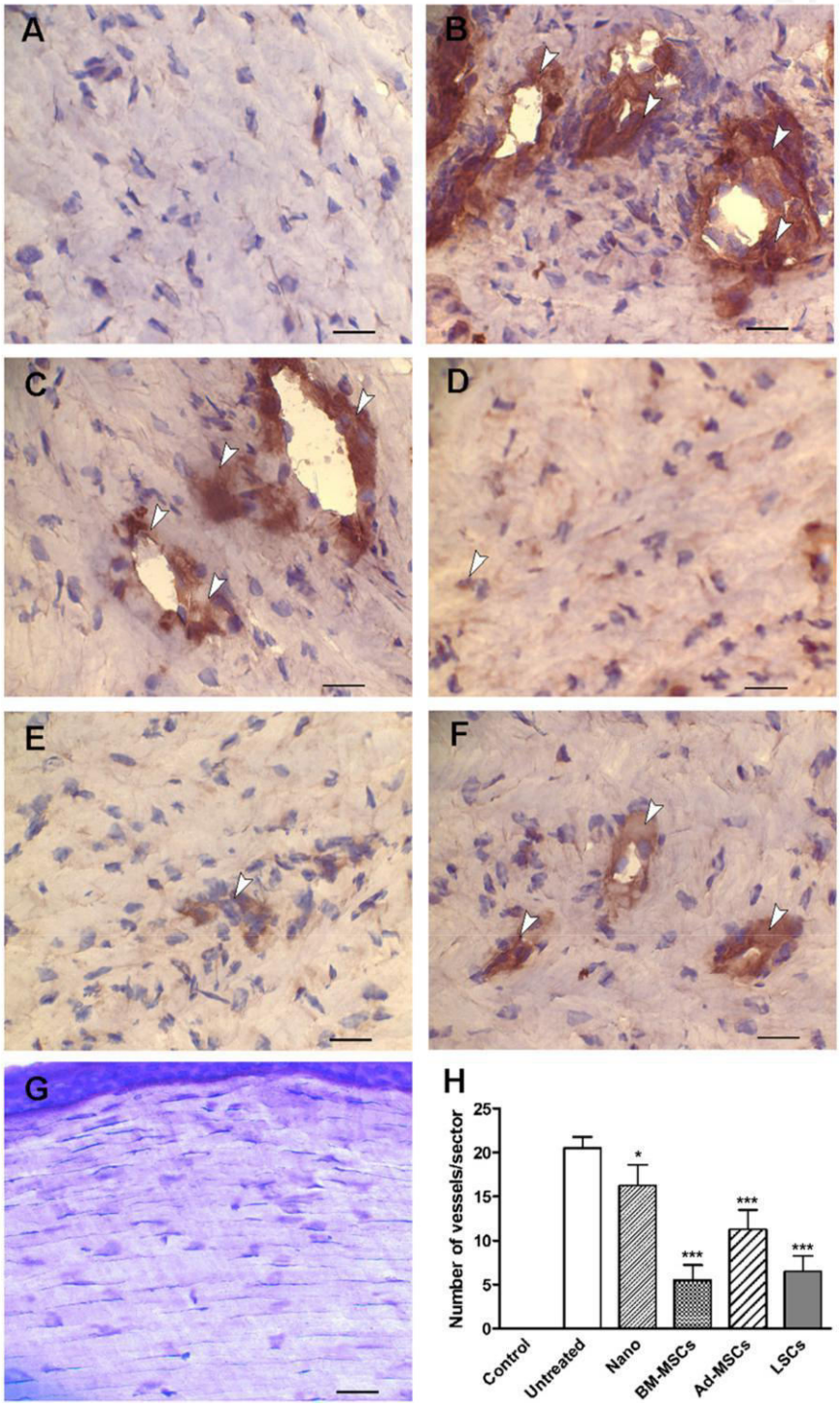

Figure 5. The expression of vascular endothelial growth factor (VEGF) and corneal neovascularization after alkali injury and treatment with stem cell-seeded nanofiber scaffolds. (A–F): The expression of VEGF was determined by immunohistochemistry in healthy corneas (A) and in alkali-injured corneas on day 12 that were untreated (B), treated with cell-free nanofibers (C); or treated with nanofiber scaffolds seeded with BM-MSCs (D), Ad-MSCs (E), or LSCs (F). (G): In the negative control, the sections were stained only with counterstaining (hematoxylin). Scale bars = 10 mm. (H): The quantification of corneal neovascularization was performed by counting the number of vessels in defined corneal sectors. Each bar represents the mean 6 SD from six corneas. The values with asterisks are significantly different (p, p , .05; ppp, p , .001) from those determined in untreated injured corneas. Abbreviations: Ad-MSC, adipose tissue-derived mesenchymal stem cell; BM-MSC, bone marrow-derived mesenchymal stem cell; LSC, limbal epithelial stem cell; nano, nanofiber scaffold.

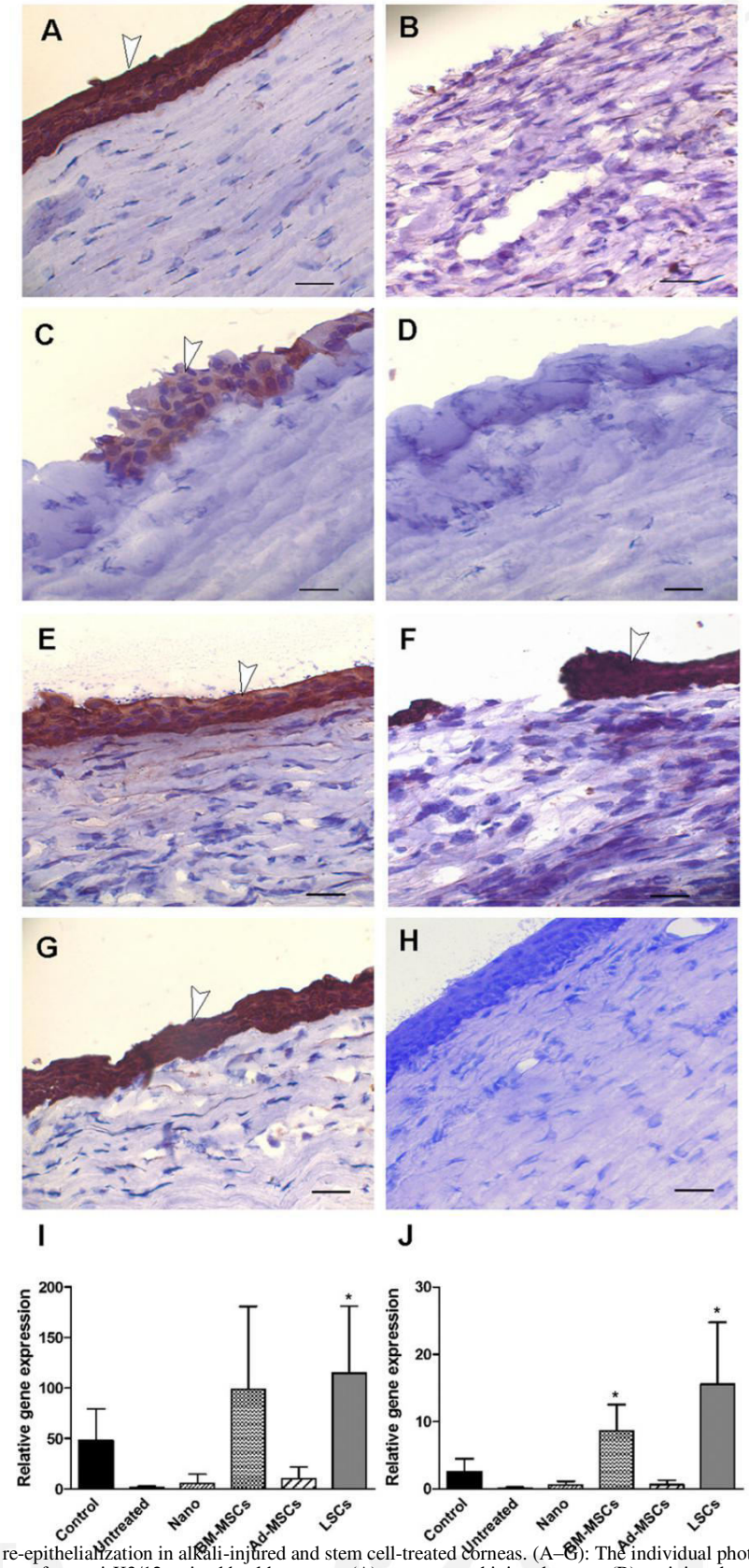

Figure 6. Corneal re-epithelialization in alkali-injured and stem cell-treated corneas. (A–G): The individual photographs show representative images of an anti-K3/12 stained healthy cornea (A), an untreated injured cornea (B), or injured corneas treated with cell-free nanofibers (C, D), (Figure legend continues on next page.)

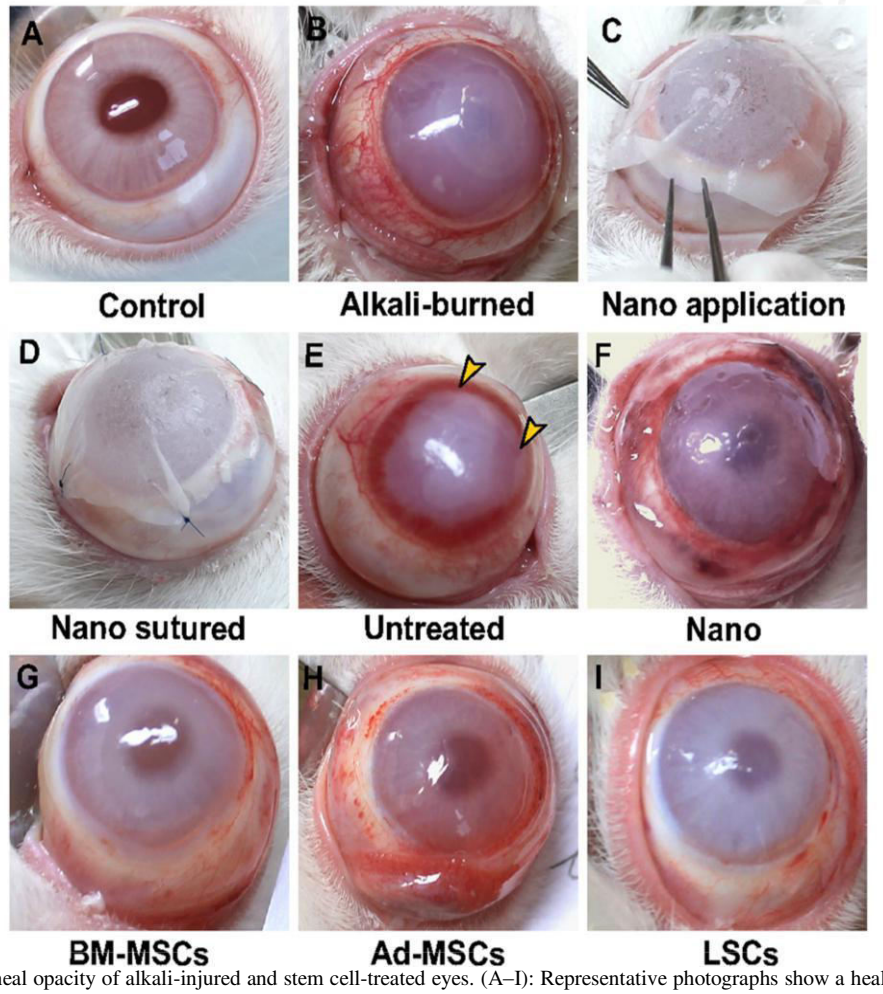

Figure 7. Corneal opacity of alkali-injured and stem cell-treated eyes. (A–I): Representative photographs show a healthy control eye (A), an alkali-injured eye (immediately after the injury) (B), an injured eye with nanofiber application (C), and a sutured nanofiber scaffold (immediately after the injury) (D); and injured eyes on day 12 that were either untreated (E), treated with a cell-free nanofiber scaffold (F), or treated with nanofiber scaffolds seeded with BM-MSCs (G), Ad-MSCs (H), or LSCs (I). (E, G, I): Corneal neovascularization was clearly visible expressed in untreated injured corneas (E, arrows) and strongly suppressed in corneas treated with nanofiber scaffolds seeded with BM-MSCs (G) or LSCs (I). Abbreviations: Ad-MSC, adipose tissue-derived mesenchymal stem cell; BM-MSC, bone marrow-derived mesenchymal stem cell; LSC, limbal epithelial stem cell; nano, nanofiber scaffold.

cells can be obtained in relatively sufficient numbers from an individual patient, can be easily propagated in vitro, and can then be used as autologous cells without requiring immunosuppression after their transplantation.

To treat ocular surface injuries or various types of LSCD, LSCs represent the optimal cell source, and LSC transplantation has resulted in the recovery of vision in blind patients [4, 6, 29]. However, the use of LSCs is limited by the absence of autologous LSCs in the case of bilateral LSCD and by a requirement for strong immunosuppression if allogeneic LSCs are used. To overcome these limitations, attempts have been made to use other cell sources for ocular surface regeneration, and the results of experimental studies using various cell types to treat LSCD have been published [30–32], but the majority of these studies have used MSCs [12, 17–19]. The rationale for the use of MSCs is based on their ability to differentiate into various cell types even apart from the mesodermal lineage from which they originate [7, 9], to produce numerous growth and trophic factors [33], and to inhibit harmful inflammatory reactions [12, 17]. Although an apparent improvement of corneal healing after the application of MSCs has been

(Figure legend continued from previous page.)
nanofibers seeded with BM-MSCs (E), Ad-MSCs (F), or LSCs (G). All injured corneas are shown on day 12 after injury. (H): The staining for the cytokeratins K3/12 was negative in corneal sections stained only with counterstaining, where the primary antibody was omitted from the incubation medium. Scale bars = 50 mm. (I, J): The expression of genes for K3 (I) and K12 (J) in individual experimental groups on day 12 after injury was determined by real-time polymerase chain reaction. Each bar represents the mean 6 SD from six individual corneas. The values with an asterisk represent a statistically significant (p , .05) difference from the values determined in untreated injured corneas. Abbreviations: Ad-MSC, adipose tissue-derived mesenchymal stem cell; BM-MSC, bone marrow-derived mesenchymal stem cell; LSC, limbal epithelial stem cell; nano, nanofiber scaffold.

observed in various models, a direct comparison of the therapeutic effects of MSCs and LSCs has not previously been made.

In this study, we prepared BM-MSCs, Ad-MSCs, and LSCs always from the same rabbit and we compared their growth, differentiation properties, and ability to produce immunoregulatory and growth factors and to support the healing of the damaged ocular surface. All of these cell types have similar fibrocytic morphology and comparable growth characteristics. After cultivation in adipogenic differentiation medium, the highest differentiation potential was observed in BM-MSCs. In accordance with the literature data [33–35], all three types of stem cells spontaneously, or after stimulation with LPS, expressed genes for a number of immunoregulatory and growth factors, but the secretion profiles were different among the individual cell types. Though we were not able to characterize the stem cell populations phenotypically because of the lack of species-specific antibodies for the rabbit model, our MSCs fulfilled other criteria for MSCs: their morphology, adherence to plastic, differentiation, and factor production [36]. Similarly, the population of LSCs was prepared by a standard method described for the preparation of mouse [20], rat [9], rabbit [37, 38], or human [5, 6] LSCs. We are aware that the LSC population contains a significant proportion of descendants of LSCs, such as transient corneal epithelial cells and corneal epithelial cells, in addition to LSCs. Indeed, we observed a gradual increase in CK3/CK12 expression during culture of rabbit LSCs (unpublished observation). Some reports suggest that MSCs can be expanded from the limbal tissue in vitro rather than corneal epithelial cells [39, 40]. For example, Basu et al. [40] described the expansion of mesenchymal stromal cells derived from human limbal biopsies and their use for the treatment of mouse corneal wounds. These authors also showed that the type of enzymatic digestion influences the preferential growth of cells with epithelial or mesenchymal morphologies. Thus, there may be species-specific and cell culture-dependent differences that support preferential growth of limbal MSCs or corneal epithelial cells.

We have shown previously in the mouse model that MSCs transferred onto the damaged ocular surface, using a nanofiber scaffold, migrate from the scaffold onto the ocular surface and inhibit the local inflammatory reaction [21]. In the present study, we used a model of the alkali-injured ocular surface in rabbits and compared the therapeutic potential of two types of MSCs and tissue-specific LSCs. The injury of the cornea with 0.25N NaOH induced damage of the corneal epithelium, an increase in corneal thickness, a strong infiltration with cells of adaptive (T lymphocytes) and innate (iNOS-expressing cells) immunity, an increase in the presence of apoptotic cells (caspase-3^+ cells), neovascularization, and corneal opacity associated with decreased corneal transparency. All these parameters characterizing the ocular injury were decreased in the treated eyes. The nanofiber scaffold itself slightly supported healing and decreased the harmful impacts of injury, similarly to what has been described after the treatment of a skin wound [41] or corneal injury [12]. The treatment of injured eyes with a nanofiber scaffold seeded with stem cells significantly decreased all of the harmful manifestations of the injury. The alkali injury strongly damaged the corneal epithelium (as demonstrated by immunohistochemistry and real-time PCR for K3 and K12), and treatment with a stem cell-seeded nanofiber scaffold improved re-epithelialization. The less-pronounced therapeutic effects of Ad-MSCs in comparison with BM-MSCs or LSCs could be due to the lower differentiation potential of Ad-MSCs and to the different spectrum of growth and immunoregulatory factors produced by these cells.

Conclusion

Taken together, our results show that BM-MSCs have comparable therapeutic effects to those of tissue-specific LSCs on the healing of corneal injury. Even though there are data on the direct differentiation of MSCs into corneal epithelial cells [8, 18], this trans-differentiation is probably not the main mechanism of the healing effect of MSCs [42]. We suggest that a more important role is represented by the production of numerous trophic and growth factors that can support the growth of residual corneal epithelial cells and LSCs [33–35], and by the ability of MSCs to suppress the local inflammatory reaction that could impede the healing process [12, 43]. All of these properties make BM-MSCs a promising candidate cell population for improving ocular surface healing in situations when autologous LSCs are difficult to obtain or are absent.

Acknowledgments

This work was supported by Grant 14-12580S from the Grant Agency of the Czech Republic, Grant NT/14102 from the Grant Agency of the Ministry of Health of the Czech Republic, Grants 889113 and 80815 from the Grant Agency of the Charles University, and Projects Biocev CZ.1.05/1.1.00/02.0109, NPUI: LO1309, UNCE 204013, and SVV 260206.

Author contributions:

V.H.: conception and design, financial support, manuscript writing; P.T., E.J., B.H., and M.C.: collection or assembly of data; C.C. and A.Z.: collection of data, data analysis and interpretation; J.C.: conception and design, collection or assembly of data, manuscript writing.

Disclosures

The authors indicated no potential conflict of interest.

References

1 Tan DT, Ficker LA, Buckley RJ. Limbal transplantation. Ophthalmology 1996;103:29–36.

2 Dua HS, Azuara-Blanco A. Allo-limbal transplantation in patients with limbal stem cell deficiency. Br J Ophthalmol 1999;83:414–419.

3 Cauchi PA, Ang GS, Azuara-Blanco A et al. A systematic literature review of surgical interventions for limbal stem cell deficiency in humans. Am J Ophthalmol 2008;146:251–259.

4 Rama P, Matuska S, Paganoni G et al. Limbal stem-cell therapy and long-term corneal regeneration. N Engl J Med 2010;363:147–155.

5 Marchini G, Pedrotti E, Pedrotti M et al. Long-term effectiveness of autologous cultured limbal stem cell grafts in patients with limbal stem cell deficiency due to chemical burns. Clin Experiment Ophthalmol 2012;40:255–267.

6 Basu S, Ali H, Sangwan VS. Clinical outcomes of repeat autologous cultivated limbal epithelial transplantation for ocular surface burns. Am J Ophthalmol 2012;153:643–650, 650.e1–650.e2.

7 Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–147.

8 Gu S, Xing C, Han J et al. Differentiation of rabbit bone marrow mesenchymal stem cells into corneal epithelial cells in vivo and ex vivo. Mol Vis 2009;15:99–107.

9 Jiang TS, Cai L, Ji WY et al. Reconstruction of the corneal epithelium with induced marrow mesenchymal stem cells in rats. Mol Vis 2010;16:1304–1316.

10 Reinshagen H, Auw-Haedrich C, Sorg RV et al. Corneal surface reconstruction using adult mesenchymal stem cells in experimental limbal stem cell deficiency in rabbits. Acta Ophthalmol 2011;89:741–748.

11 Holan V, Javorkova E. Mesenchymal stem cells, nanofiber scaffolds and ocular surface reconstruction. Stem Cell Rev 2013;9:609–619.

12 Cejkova J, Trosan P, Cejka C et al. Suppression of alkali-induced oxidative injury in the cornea by mesenchymal stem cells growing on nanofiber scaffolds and transferred onto the damaged corneal surface. Exp Eye Res 2013;116:312–323.

13 Holan V, Pokorna K, Prochazkova J et al. Immunoregulatory properties of mouse limbal stem cells. J Immunol 2010;184:2124–2129.

14 Di Trapani M, Bassi G, Ricciardi M et al. Comparative study of immune regulatory properties of stem cells derived from different tissues. Stem Cells Dev 2013;22:2990–3002.

15 Strioga M, Viswanathan S, Darinskas A et al. Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. Stem Cells Dev 2012;21:2724–2752.

16 Jin HJ, Bae YK, Kim M et al. Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. Int J Mol Sci 2013;14:17986–18001.

17 Oh JY, Kim MK, Shin MS et al. The anti-inflammatory and anti-angiogenic role of mesenchymal stem cells in corneal wound healing following chemical injury. Stem Cells 2008;26:1047–1055.

18 Ma Y, Xu Y, Xiao Z et al. Reconstruction of chemically burned rat corneal surface by bone marrow-derived human mesenchymal stem cells. Stem Cells 2006;24:315–321.

19 Yao L, Li ZR, Su WR et al. Role of mesenchymal stem cells on cornea wound healing induced by acute alkali burn. PLoS One 2012;7:e30842.

20 Krulova M, Pokorna K, Lencova A et al. A rapid separation of two distinct populations of mouse corneal epithelial cells with limbal stem cell characteristics by centrifugation on percoll gradient. Invest Ophthalmol Vis Sci 2008;49:3903–3908.

21 Zajicova A, Pokorna K, Lencova A et al. Treatment of ocular surface injuries by limbal and mesenchymal stem cells growing on nanofiber scaffolds. Cell Transplant 2010;19:1281–1290.

22 Svobodova E, Krulova M, Zajicova A et al. The role of mouse mesenchymal stem cells in differentiation of naive T-cells into anti-inflammatory regulatory T-cell or proinflammatory helper T-cell 17 population. Stem Cells Dev 2012;21:901–910.

23 Holan V, Chudickova M, Trosan P et al. Cyclosporine A-loaded and stem cell-seeded electrospun nanofibers for cell-based therapy and local immunosuppression. J Control Release 2011;156:406–412.

24 Trosan P, Svobodova E, Chudickova M et al. The key role of insulin-like growth factor I in limbal stem cell differentiation and the corneal wound-healing process. Stem Cells Dev 2012;21:3341–3350.

25 Gong SP, Kim B, Kwon HS et al. The co-injection of somatic cells with embryonic stem cells affects teratoma formation and the properties of teratoma-derived stem cell-like cells. PLoS One 2014;9:e105975.

26 Bobbert M. Ethical questions concerning research on human embryos, embryonic stem cells and chimeras. Biotechnol J 2006;1:1352–1369.

27 Zhao T, Zhang ZN, Rong Z et al. Immunogenicity of induced pluripotent stem cells. Nature 2011;474:212–215.

28 Nishimori M, Yakushiji H, Mori M et al. Tumorigenesis in cells derived from induced pluripotent stem cells. Hum Cell 2014;27:29–35.

29 Pellegrini G, Rama P, Di Rocco A et al. Concise review: hurdles in a successful example of limbal stem cell-based regenerative medicine. Stem Cells 2014;32:26–34.

30 Inatomi T, Nakamura T, Kojyo M et al. Ocular surface reconstruction with combination of cultivated autologous oral mucosal epithelial transplantation and penetrating keratoplasty. Am J Ophthalmol 2006;142:757–764.

31 Gomes JA, Geraldes Monteiro B, Melo GB et al. Corneal reconstruction with tissue-engineered cell sheets composed of human immature dental pulp stem cells. Invest Ophthalmol Vis Sci 2010;51:1408–1414.

32 Liu H, Zhang J, Liu CY et al. Cell therapy of congenital corneal diseases with umbilical mesenchymal stem cells: lumican null mice. PLoS One 2010;5:e10707.

33 Oh JY, Kim MK, Shin MS et al. Cytokine secretion by human mesenchymal stem cells cocultured with damaged corneal epithelial cells. Cytokine 2009;46:100–103.

34 Di Nicola M, Carlo-Stella C, Magni M et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 2002;99:3838–3843.

35 Najar M, Raicevic G, Fayyad-Kazan H et al. Immune-related antigens, surface molecules and regulatory factors in human-derived mesenchymal stromal cells: the expression and impact of inflammatory priming. Stem Cell Rev 2012;8:1188–1198.

36 Dominici M, Le Blanc K, Mueller I et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315–317.

37 Wan P, Wang X, Ma P et al. Cell delivery with fixed amniotic membrane reconstructs corneal epithelium in rabbits with limbal stem cell deficiency. Invest Ophthalmol Vis Sci 2011;52:724–730.

38 Samoilă O, Soriţău O, Totu L et al. Cultivation and characterization of limbal epithelial stem cells in rabbits. Rom J Morphol Embryol 2014;55:63–69.

39 Polisetty N, Fatima A, Madhira SL et al. Mesenchymal cells from limbal stroma of human eye. Mol Vis 2008;14:431–442.

40 Basu S, Hertsenberg AJ, Funderburgh ML et al. Human limbal biopsy-derived stromal stem cells prevent corneal scarring. Sci Transl Med 2014;6:266ra172.

41 Dubský M, Kubinová S, Sirc J et al. Nanofibers prepared by needleless electrospinning technology as scaffolds for wound healing. J Mater Sci Mater Med 2012;23:931–941.

42 Harkin DG, Foyn L, Bray LJ et al. Concise reviews: Can mesenchymal stromal cells differentiate into corneal cells? A systematic review of published data. Stem Cells 2015;33:785–791.

43 Abumaree M, Al Jumah M, Pace RA et al. Immunosuppressive properties of mesenchymal stem cells. Stem Cell Rev 2012;8:375–392.

6 Discussion

It is evident that the environment has an essential impact on the behaviour of stem cells and that the properties of stem cells vary substantially depending on the surrounding tissue. Therefore, to obtain the optimal effectiveness in stem cell-based therapy it is also necessary to estimate the proper type of stem cells for the particular application. For example, higher production of bFGF (Li *et al.*, 2015) and also the ability to express P2Y purinergic receptor (Forostyak *et al.*, 2016) can make AT-MSCs more successful in neuronal differentiation compared to BM-MSCs. However, bFGF supports the proliferation, but not the differentiation of LSCs (Trosan *et al.*, 2012) and the application of AT-MSCs in corneal regeneration can be less successful compared to BM-MSCs (Holan *et al.*, 2015).

The importance of selecting the proper stem cell type and the impact of the specific environment has been also shown in the protocol for transdifferentiation of mouse MCS into neuron-like cells. The nine-day protocol was designed to simulate the complex character of injured and inflamed neural tissue. This specific environment was modelled by the application of the following differentiation agents: the brain tissue extract and supernatant from ConA-stimulated splenocytes as signalization of injury and inflammation, and growth factors bFGF and EGF as supporters of neuronal transdifferentiation. Further, electrical stimulation under physiological conditions was applied, as it affects intracellular Ca^{2+} oscillations and the activation of transcription factors and signaling pathways, and also increases membrane permeability for differentiation agents.

Nanofiber scaffolds, fabricated from poly (L-lactid) acid or polycaprolactone polymers with mass/unit area of 5 g/cm^2, were used as 3D extracellular support. The resulting cell population after transdifferentiation appeared to be heterogenous. The differentiated neuron-like cells showed neuron-like morphology, relative gene expression of neuron-specific markers (for example Nf-L, Nf-M, SYP, GAD, TUBB3) and were positive for Nf-L protein. The differentiated cells with neuron-like morphology comprised approximately one third of total cell population and the cells positive for neurofilament light polypeptide comprised approximately

10% of total cell population, which was assessed microscopically. Comparison of transdifferentiation results in mouse AT-MSCs and BM-MSCs confirmed that AT-MSCs are a more convenient cell source for this transdifferentiation protocol.

To compare the immunomodulatory properties of non-differentiated AT-MSCs and neuron-like cells obtained by transdifferentiation, both groups of cells were co-cultivated for 24 and 48 hours with ConA-stimulated splenocytes and the profiles of cytokines were estimated by qPCR and ELISA test. The experiments were performed both in mixed co-cultivation and separately. The neuron-like cells maintained the expression of neuron-specific makers during co-cultivation with the ConA-stimulated splenocytes.

The relative gene expression for IFN-gamma in ConA-stimulated splenocytes decreased significantly when they were co-cultivated with non-differentiated AT-MSC controls and similarly with neuron-like cells. This result was also confirmed by ELISA. Further, both non-differentiated AT-MSC controls and neuron-like cells supported similarly the relative gene expression for IL-10 in ConA-stimulated splenocytes. Thus, the neuron-like cells were, similarly to non-differentiated AT-MSCs, able to suppress the inflammatory behaviour of ConA-stimulated splenocytes. This can be explained by the previous licensing process (Krampera *et al*., 2006, 2011) during neuron-like cells transdifferentiation, which was induced by the application of brain tissue extracts and supernatants from ConA-stimulated splenocytes. Another explanation is related to the heterogeneity of the resulting population of neuron-like cells. Cells without pronounced neuron-like character may provide this immunoregulatory effect.

Neuron-like cells co-cultivated with ConA-stimulated splenocytes showed a lower level of relative gene expression for IL-6, compared to levels expressed by non-differentiated AT-MSCs co-cultivated with ConA-stimulated splenocytes. This difference was also confirmed by an ELISA test on the protein production level. These results indicate that neuron-like cells

could be used *in vivo* in the regeneration of neural injury, both for their anti-inflammatory effect as well as for the replacement of lost neuronal cells.

For human AT-MSCs the protocol was optimized by several changes due to higher viability and proliferation rates in human cells compared to mouse cells. The optimal differentiation results in human cells were obtained when they were seeded in an earlier passage and in a lower seeding density. In the case of human AT-MSCs hydrogel was used as 3D extracellular support. The neuron-like character of differentiated cells was confimed by neuron-like morphological changes, by relative gene expression for neuron-specific markers and by the presence of neuron specific proteins (Nf-L, Nf-M, TUBB3). In human MSCs the transdifferentiation results of AT-, BM- and UC-MSCs were compared and AT-MSCs were found to be the most suitable cell source.

Comparison of the potential of AT- and BM-MSCs to support or even replace absent LSCs in the regeneration of injured rabbit cornea showed that BM-MSCs have slightly superior regenerative properties. This can be explained by the above-mentioned differences in the spectrum of growth factors and cytokines, which are secreted by AT- and BM-MSCs (Holan *et al.*, 2015). The relatively higher production of bFGF by AT-MSCs can be useful in neuronal differentiation (Li *et al.*, 2015). In the corneal environment bFGF seems to support the proliferation, but not the differentiation of LSCs (Trosan *et al.*, 2012). The differentiation of LSCs into corneal epithelial cells is supported by IGF-1 (Trosan *et al.*, 2012). However, IGF-1 is generally produced both by AT- and BM-MSCs, and its production by AT-MCSs seems to be higher than by BM-MSCs (Li *et al.*, 2015). It is probably the ratio in the production of all relevant cytokines which favours BM-MSCs over AT-MSCs in the specific corneal environment.

7 Conclusions

7.1 The development of an *in vitro* transdifferentiation protocol for generating neuron-like cells from mouse MSCs.

The *in vitro* transdifferentiation protocol was based on the simulation of the environment in neural injury (brain tissue extracts, supernatants from stimulated splenocytes, growth factors, electrical stimulation and 3D nanofibre scaffolds) and its impact on AT-MSC differentiation. The acquired neuron-like cells showed typical neuron-like morphology, expressed the genes for neuron-specific marker and were positive for neuron-specific protein.

7.2 The characterization of the immunomodulatory properties of neuron-like cells.

The behaviour of neuron-like cells co-cultivated with ConA-stimulated splenocytes was compared to non-differentiated AT-MSC controls. It was found that neuron-like cells have similar anti-inflammatory properties as non-differentiated AT-MSCs.

7.3 The optimization of the transdifferentiation protocol for human AT-MSCs.

The transdifferentiation protocol was optimized for human AT-MSCs by several modifications. Human cells showed their best differentiation results when earlier passages and lower seeding density were applied, compared to mouse cells. Hydrogel was found to be an optimal 3D extracellular support for human AT-MSCs.

7.4 The characterization of the role of growth factors in differentiation of LSCs into corneal epithelium.

Cytokines bFGF, EGF and IGF-1 were found to have important roles in LSC proliferation and differentiation. IGF-1 supports the differentiation of LSCs into corneal epithelial cells, while bFGF and EGF support the proliferation of LSCs.

7.5 The comparison of the potential of AT- and BM-MSCs to support or substitute LSCs in cornea regeneration.

Compared to AT-MSCs, BM-MSCs are more convenient to substitute LSCs in corneal injury healing.

8 References

Ahmad S, Stewart R, Yung S, Kolli S, Armstrong L, Stojkovic M, Figueiredo F, Lako M. Differentiation of human embryonic stem cells into corneal epithelial-like cells by in vitro replication of the corneal epithelial stem cell niche. Stem Cells. 2007;25(5):1145-1155.

Ahmad S. Concise Review: Limbal stem cell deficiency, dysfunction, and distress. Stem Cells Transl Med. 2012;1(2):110-115.

Almad A, Sahinkaya FR, McTigue DM. Oligodendrocyte fate after spinal cord injury. Neurotherapeutics. 2011;8(2):262-273.

Amorin B, Alegretti AP, Valim V, Pezzi A, Laureano AM, da Silva MA, Wieck A, Silla L. Mesenchymal stem cell therapy and acute graft-versus-host disease: a review. Human Cell. 2014;27(4):137-150.

Arboleda D, Forostyak S, Jendelova P, Marekova D, Amemori T, Pivonkova H, Masinova K, Sykova E. Transplantation of predifferentiated adipose-derived stromal cells for the treatment of spinal cord injury. Cell Mol Neurobiol. 2011;31(7):1113-1122.

Ariffin SHZ, Kermani S, Abidin IZZ, Megat Abdul Wahab R, Yamamoto Z, Senafi S, Zainal Ariffin Z, Abdul Razak M. Differentiation of dental pulp stem cells into neuron-like cells in serum-free medium. Stem Cells Int. 2013;2013:250740.

Bae KS, Park JB, Kim HS, Kim DS, Park DJ, Kang SJ. Neuron-like differentiation of bone marrow-derived mesenchymal stem cells. Yonsei Med J. 2011;52(3):401-412.

Bai X, Ma J, Pan Z, Song YH, Freyberg S, Yan Y, Vykoukal D, Alt E. Electrophysiological properties of human adipose tissue-derived stem cells. Am J Physiol - Cell Physiol. 2007;293(5):1539-1550.

Basu S, Hertsenberg AJ, Funderburgh ML, Burrow MK, Mann MM, Du Y, Lathrop KL, Syed-Picard FN, Adams SM, Birk DE, Funderburgh JL. Human limbal biopsy-derived stromal stem cells prevent corneal scarring. Sci Transl Med. 2014;6(266):266ra172.

Bentz K, Molcanyi M, Schneider A, Riess P, Maegele M, Bosche B, Hampl JA, Hescheler J, Patz S, Schäfer U. Extract derived from rat brains in the acute phase following traumatic brain injury impairs survival of undifferentiated stem cells and induces rapid differentiation of surviving cells. Cell Physiol Biochem. 2010;26(6):821–830.

Berardis S, Dwisthi Sattwika P, Najimi M, Sokal EM. Use of mesenchymal stem cells to treat liver fibrosis: Current situation and future prospects. World J Gastroenterol. 2015;21(3):742-758.

Bertani N, Malatesta P, Volpi G Sonego P, Perris R. Neurogenic potential of human mesenchymal stem cells revisited: analysis by immunostaining, time-lapse video and microarray. J Cell Sci. 2005;118(17):3925-3936.

Cao N, Huang Y, Zheng J, Spencer CI, Zhang Y, Fu JD, Nie B, Xie M, Zhang M, Wang H, Ma T, Xu T, Shi G, Srivastava D, Ding S. Conversion of human fibroblasts into functional cardiomyocytes by small molecules. Science. 2016;352(6290):1216-1220.

Casarosa S, Bozzi Y, Conti L. Neural stem cells: ready for therapeutic applications? Mol Cell Ther. 2014;2(1):1.

Catacchio I, Berardi S, Reale A, De Luisi A, Racanelli V, Vacca A, Ria R. Evidence for bone marrow adult stem cell plasticity: properties, molecular mechanisms, negative aspects, and clinical applications of hematopoietic and mesenchymal stem cells transdifferentiation. Stem Cells Int. 2013;2013:589139.

Chan AA, Hertsenberg AJ, Funderburgh ML, Mann MM, Du Y, Davoli KA, Mich-Basso JD, Yang L,Funderburgh JL. Differentiation of human embryonic stem cells into cells with corneal keratocyte phenotype. PLoS One. 2013;8(2):e56831.

Chen X, Li Y, Wang L, Katakowski M, Zhang L, Chen J, Xu Y, Gautam SC, Chopp M. Ischemic rat brain extracts induce human marrow stromal cell growth factor production. Neuropathology. 2002;22(4):275-279.

Chhabra HS, Sarda K. Stem cell therapy in spinal trauma: Does it have scientific validity? Indian J Orthopaedics. 2015;49(1):56-71.

Chudickova M, Bruza P, Zajicova A, Trosan P, Svobodova L, Javorkova E, Kubinova S, Holan V. Targeted neural differentiation of murine mesenchymal stem cells by a protocol simulating the inflammatory site of neural injury. J Tissue Eng Regen Med. 2015; doi: 10.1002/term.2059. [Epub ahead of print]

Dasari VR, Veeravalli KK, Dinh DH. Mesenchymal stem cells in the treatment of spinal cord injuries: A review. World J Stem Cells. 2014;6(2):120-133.

da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci. 2006;119(11):2204-2213.

Deng J, Steindler DA, Laywell ED, Petersen BE. Neural trans-differentiation potential of hepatic oval cells in the neonatal mouse brain. Exp Neurol. 2003;182(2):373-382.

Dhara SK, Stice SL. Neural differentiation of human embryonic stem cells. J Cell Biochem. 2008;105(3):633-640.

Divya MS, Roshin GE, Divya TS, Rasheed VA, Santhoshkumar TR, Elizabeth KE, James J, Pillai RM. Umbilical cord blood-derived mesenchymal stem cells consist of a unique population of progenitors co-expressing mesenchymal stem cell and neuronal markers capable of instantaneous neuronal differentiation. Stem Cell Res Ther. 2012;3(6):57.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A,Prockop Dj, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315–317.

Dottori M, Pera MF. Neural differentiation of human embryonic stem cells. Methods Mol Biol. 2008;438:19-30.

Du Y, Roh DS, Funderburgh ML, Mann MM, Marra KG, Rubin JP, Li X, Funderburgh JL. Adipose-derived stem cells differentiate to keratocytes in vitro. Mol Vis. 2010;16:2680-2689.

Ebrahimi M, Taghi-Abadi E, Baharvand H. Limbal stem cells in review. J Ophthalmic Vis Res 2009;4(1):40-58.

Einstein O, Ben-Hur T. The changing face of neural stem cell therapy in neurologic diseases. Arch Neurol. 2008;65(4):452-456.

Evans MJ, Kaufman MH: Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981;292(5819):154-156.

Fields RD, Stevens-Graham B. New insights into neuron-glia communication. Science. 2002;298(5593):556-562.

Forostyak O, Butenko O, Anderova M, Forostyak S, Sykova E, Verkhratsky A, Dayanithi G. Specific profiles of ion channels and ionotropic receptors define adipose- and bone marrow derived stromal cells. Stem Cell Res. 2016;16(3):622-634.

Friedenstein AJ. Osteogenic stem cells in bone marrow. In: Heersche JNM, Kanis JA, editors. Bone and Mineral Research. Amsterdam: Elsevier; 1990. pp. 243–272.

Fu L, Zhu L, Huang Y, Lee TD, Forman SJ, Shih CC. Derivation of neural stem cells from mesenchymal stemcells: evidence for a bipotential stem cell population. Stem Cells Dev. 2008;17(6):1109-1121.

Gage FH, Temple S. Neural stem cells: Generating and regenerating the brain. Neuron. 2013;80(3): 588-601.

Gho CG, Schomann T, de Groot SC, Frijns JH, Rivolta MN, Neumann MH, Huisman MA. Isolation, expansion and neural differentiation of stem cells from human plucked hair: a further step towards autologous nerve recovery. Cytotechnology. 2015. doi: 10.1007/s10616-015-9938-x. [Epub ahead of print].

Ghosh M, Helm KM, Smith RW, Giordanengo MS, Li B, Shen H, Reynolds SD. A single cell functions as a tissue-specific stem cell and the in vitro niche-forming cell. Am J Respir Cell Mol Biol. 2011;45(3):459-469.

Gomes JA, Geraldes Monteiro B, Melo GB, Smith RL, Cavenaghi Pereira da Silva M, Lizier NF, Kerkis A, Cerruti H, Kerkis I. Corneal reconstruction with tissue-engineered cell sheets composed of human immature dental pulp stem cells. Invest Ophthalmol Vis Sci. 2010;51(3):1408-1414.

Gong SP, Kim B, Kwon HS, Yang WS, Jeong JW, Ahn J, Lim JM. The co-injection of somatic cells with embryonic stem cells affects teratoma formation and the properties of teratoma-derived stem cell-like cells. PLoS One. 2014;9(9):e105975.

Griffin M, Ritter T, Mahon B. Immunological aspects of allogeneic mesenchymal stem cell therapies. Hum Gene Ther. 2010;21(12):1641-1655.

Guo X-L, Chen J-S. Research on induced pluripotent stem cells and the application in ocular tissues. Intl J Ophthalmol. 2015;8(4):818-825.

Gupta PK, Das AK, Chullikana A, Majumdar AS. Mesenchymal stem cells for cartilage repair in osteoarthritis. Stem Cell Res Ther. 2012;3(4):25.

Hashemian SJ, Kouhnavard M, Nasli-Esfahani E. Mesenchymal stem cells: rising concerns over their application in treatment of type one diabetes mellitus. J Diabetes Res. 2015;2015:675103.

Hayashi R, Ishikawa Y, Ito M, Kageyama T, Takashiba K, Fujioka T, Tsujikawa M, Miyoshi H, Yamato M,Nakamura Y, Nishida K. Generation of corneal epithelial cells from induced pluripotent stem cells derived from human dermal fibroblast and corneal limbal epithelium.

PLoS One. 2012;7(9):e45435.

Hayat, M.A. (Ed.) Stem cells and cancer stem cells, Volume 10. Therapeutic applications in disease and injury. Springer 2013

He H, Yiu SC. Stem cell-based therapy for treating limbal stem cells deficiency: A review of different strategies. Saudi J Ophthalmol. 2014;28(3):188-194.

Heo JS, Choi SM, Kim HO, Kim EH, You J, Park T, Kim E, Kim HS. Neural transdifferentiation of human bone marrow mesenchymal stem cells on hydrophobic polymer-modified surface and therapeutic effects in an animal model of ischemic stroke. Neuroscience. 2013;238:305-318.

Heo JS, Choi Y, Kim HS, Kim HO. Comparison of molecular profiles of human mesenchymal stem cells derived from bone marrow, umbilical cord blood, placenta and adipose tissue. Int J Mol Med. 2016;37(1):115-125.

Hermann A, Gastl R, Liebau S, Popa MO, Fiedler J, Boehm BO, Maisel M, Lerche H, Schwarz J,Brenner R, Storch A. Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. J Cell Sci. 2004;117(19):4411-4422.

Heubach JF, Graf EM, Leutheuser J, Bock M, Balana B, Zahanich I, Christ T, Boxberger S, Wettwer E, Ravens U. Electrophysiological properties of human mesenchymal stem cells. J Physiol. 2004;554(3):659–672.

Higuchi A, Ling QD, Kumar SS, Munusamy MA, Alarfaj AA, Chang Y, Kao SH, Lin KC, Wang HC, Umezawa A. Generation of pluripotent stem cells without the use of genetic material. Lab Invest. 2015;95(1):26-42.

Holan V, Pokorna K, Prochazkova J, Krulova M, Zajicova A. Immunoregulatory properties of mouse limbal stem cells. J Immunol. 2010;184(4):2124-2129.

Holan V, Trosan P, Cejka C, Javorkova E, Zajicova A, Hermankova B, Chudickova M, Cejkova J. A comparative study of the therapeutic potential of mesenchymal stem cells and limbal epithelial stem cells for ocular surface reconstruction. Stem Cells Transl Med. 2015;4(9):1052-1063.

Hu DZ, Zhou LF, Zhu JH. Marrow stromal cells administrated intracisternally to rats after traumatic brain injury migrate into the brain and improve neurological function. Chin Med J (Engl) 2004;117(10):1576–1578.

Hu BY, Weick JP, Yu J, Ma LX, Zhang XQ, Thomson JA, Zhang SC. Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. Proc Natl Acad Sci U S A. 2010;107(9):4335-4340.

Huebner EA, Strittmatter SM. Axon Regeneration in the Peripheral and Central Nervous Systems. Results Probl Cell Differ. 2009;48:339-351.

Jahromi IR, Mehrabani D, Mohammadi A, Dianatpour M, Tamadon A, Zare S, Seno MG, Khodabandeh Z. The effect of fetal rat brain extract on morphology of bone marrow-derived mesenchymal stem cells. Compar Clin Pathol. 2015;25(2): 343-349.

James G, Butt A M. Changes in P2Y and P2X purinoceptors in reactive glia following axonal degeneration in the rat optic nerve. Neuroscience Letters. 2001;312(1):33-36.

Jang S, Cho HH, Cho YB, Park JS, Jeong HS. Functional neural differentiation of human adipose tissue-derived stem cells using bFGF and forskolin. BMC Cell Biol. 2010;11:25-38.

Jeong JA, Gang EJ, Hong SH, Hwang SH, Kim SW, Yang IH, Ahn C, Han H, Kim H. Rapid neural differentiation of human cord blood-derived mesenchymal stem cells. Neuroreport. 2004;15(11):1731-1734.

Jin HJ, Bae YK, Kim M, Kwon SJ, Jeon HB, Choi SJ, Kim SW, Yang YS, Oh W, Chang JW. Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. Int J Mol Sci. 2013;14(9):17986–18001.

Joyce NC, Harris DL, Markov V, Zhang Z, Saitta B. Potential of human umbilical cord blood mesenchymal stem cells to heal damaged corneal endothelium. Mol Vis. 2012;18:547-564.

Katikireddy K, Dana R, Jurkunas U. Differentiation potential of limbal fibroblasts and bone marrow mesenchymal stem cells to corneal epithelial cells. Stem Cells. 2014;32(3):717-729.

Keilhoff G, Goihla A, Langnasea K, Fansa H, Wolf G. Transdifferentiation of mesenchymal stem cells into Schwann cell-like myelinating cells. Eur J Cell Biol. 2006;85(1):11–24.

Kennea NL, Mehmet H. Neural stem cells. J. Pathol. 2002;197(4):536–550.

Kerkis I, Haddad MS, Valverde CW, Glosman S. Neural and mesenchymal stem cells in animal models of Huntington's disease: past experiences and future challenges. Stem Cell Res Ther. 2015;6:232.

Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells. 2006;24(5):1294–1301.

Khoo MLM, Tao H, Ma DDF. Mesenchymal stem cell-based therapies for Parkinson's disease: progress, controversies and lessons for the future. J Stem Cell Res Ther. 2011;S2:005.

Kim E, Lee K, Kim M. The potential of mesenchymal stem cells derived from amniotic membrane and amniotic fluid for neuronal regenerative therapy. BMB Rep. 2014;47(3):135-140.

Kim DS, Ross PJ, Zaslavsky K, Ellis J. Optimizing neuronal differentiation from induced pluripotent stem cells to model ASD. Front Cell Neurosci. 2014;8:109.

Kočí Z, Turnovcová K, Dubský M, Baranovičová L, Holáň V, Chudíčková M, Syková E, Kubinová S. Characterization of human adipose tissue-derived stromal cells isolated from diabetic patient's distal limbs with critical ischemia. Cell Biochem Funct. 2014;32(7):597-604.

Krabbe C, Zimmer J, Meyer M. Neural transdifferentiation of mesenchymal stem cells--a critical review. APMIS. 2005;113(11-12):831-844.

Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, Santarlasci V, Mazzinghi B, Pizzolo G,Vinante F, Romagnani P, Maggi E, Romagnani S, Annunziato F. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem cells 2006;24(2):386-398.

Krampera M. Mesenchymal stromal cell 'licensing': a multistep process. Leukemia 2011;25(9): 1408-1414.

Krulova M, Pokorna K, Lencova A, Fric J, Zajicova A, Filipec M, Forrester JV, Holan V. A rapid separation of two distinct populations of mouse corneal epithelial cells with limbal stem cell characteristics by centrifugation on percoll gradient. Invest Ophthalmol Vis Sci. 2008;49(9):3903-3908.

Kyurkchiev D, Bochev I, Ivanova-Todorova E, Mourdjeva M, Oreshkova T, Belemezova K, Kyurkchiev S. Secretion of immunoregulatory cytokines by mesenchymal stem cells. World J Stem Cells. 2014;6(5):552-570.

Lee-Kubli CA, Lu P. Induced pluripotent stem cell-derived neural stem cell therapies for spinal cord injury. Neural Regen Res. 2015;10(1):10-16.

Li CY, Wu XY, Tong JB, Yang XX, Zhao JL, Zheng QF, Zhao GB, Ma ZJ. Comparative analysis of human mesenchymal stem cells from bone marrow and adipose tissue under xeno-free conditions for cell therapy. Stem Cell Res Ther. 2015;6:55.

Li G, Zhu Y, Xie H, Chen SY, Tseng SC. Mesenchymal stem cells derived from human limbal niche cells. Invest Ophthalmol Vis Sci. 2012;53(9):5686-5697.

Li P, Li SH, Wu J, Zang WF, Dhingra S, Sun L, Weisel RD, Li RK. Interleukin-6 downregulation with mesenchymal stem cell differentiation results in loss of immunoprivilege. J Cell Mol Med. 2013;17(9):1136–1145.

Li Y, Liu M, Yan Y, Yang ST. Neural differentiation from pluripotent stem cells: The role of natural and synthetic extracellular matrix. World J Stem Cells. 2014;6(1):11–23.

Liu H, Zhang J, Liu CY, Hayashi Y, Kao WW. Bone marrow mesenchymal stem cells can differentiate and assume corneal keratocyte phenotype. J Cell Mol Med. 2012;16(5):1114-1124.

Liu Z, Tang Y, Lü S, Zhou J, Du Z, Duan C, Li Z, Wang C. The tumourigenicity of iPS cells and their differentiated derivates. J Cell Mol Med. 2013;17(6):782–791.

Lu J, Moochhala S, Moore XL, Ng KC, Tan MH, Lee LK, He B, Wong MC, Ling EA. Adult bone marrow cells differentiate into neural phenotypes and improve functional recovery in rats following traumatic brain injury. Neurosci Lett. 2006;398(1-2):12–17.

Ma S, Xie , Li W, Yuan B, Shi Y, Wang Y. Immunobiology of mesenchymal stem cells. Cell Death Differ. 2013;21:216–225.

Maxson S, Lopez EA, Yoo D, Danilkovitch-Miagkova A, Leroux MA. Concise review: role of mesenchymal stem cells in wound repair. Stem Cells Transl Med. 2012;1(2):142-149.

Mazo M, Araña M, Pelacho B, Prosper F. Mesenchymal stem cells and cardiovascular disease: a bench to bedside roadmap. Stem Cells Int. 2012; Article ID 175979.

Meier RP, Müller YD, Morel P, Gonelle-Gispert C, Bühler LH. Transplantation of mesenchymal stem cells for the treatment of liver diseases, is there enough evidence? Stem Cell Res. 2013;11(3):1348-1364.

Méndez-Ferrer S, Michurina T, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, Scadden DT, Ma'ayan A, Enikolopov GN, Frenette PS. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature. 2010;466(7308):829–834.

Meyer-Blazejewska EA, Call MK, Yamanaka O, Liu H, Schlötzer-Schrehardt U, Kruse FE, Kao WW. From hair to cornea: toward the therapeutic use of hair follicle-derived stem cells in the treatment of limbal stem cell deficiency. Stem Cells. 2011;29(1):57-66.

Mougiakakos D, Jitschin R, Johansson C, Okita R, Kiessling R, Le Blanc K. The impact of inflammatory licensing on heme oxygenase-1–mediated induction of regulatory T cells by human mesenchymal stem cells. Blood. 2011;117(18):4826-4835.

Moviglia GA, Varela G, Gaeta CA, Brizuela JA, Bastos F, Saslavsky J. Autoreactive Tcells induce in vitro BM mesenchymal stem cell transdifferentiation to neural stem cells. Cytotherapy. 2006;8(3):196–201.

Muratore CR, Srikanth P, Callahan DG, Young-Pearse TL. Comparison and optimization of hiPSC fforebrain cortical differentiation protocols. PLoS ONE. 2014;9(8): e105807.

Murrell W, Wetzig A, Donnellan M, Féron F, Burne T, Meedeniya A, Kesby J, Bianco J, Perry C, Silburn P, Mackay-Sim A. Olfactory mucosa is a potential source for autologous stem cell therapy for Parkinson's disease. Stem Cells. 2008;26(8):2183-2192.

Nakamura T, Takeda K, Inatomi T, Sotozono C, Kinoshita S. Long-term results of autologous cultivated oral mucosal epithelial transplantation in the scar phase of severe ocular surface disorders. Br J Ophthalmol. 2011;95(7):942-946.

Nicaise C, Mitrecic D, Pochet R. Brain and spinal cord affected by amyotrophic lateral sclerosis induce differential growth factors expression in rat mesenchymal and neural stem cells. Neuropathol Appl Neurobiol. 2011;37(2):179–188.

Nieto-Miguel T, Galindo S, Reinoso R, Corell A, Martino M, Pérez-Simón JA, Calonge M. In vitro simulation of corneal epithelium microenvironment induces a corneal epithelial-like cell phenotype from human adipose tissue mesenchymal stem cells. Curr Eye Res. 2013;38(9):933-944.

Parr AM, Tator CH, Keating A. Bone marrow-derived mesenchymal stromal cells for the repair of central nervous system injury. Bone Marrow Transplant. 2007;40(7):609–619.

Patel DM, Shah J, Srivastava AS. Therapeutic potential of mesenchymal stem cells in regenerative medicine. Stem Cells Int. 2007;Article ID 496218.

Patel M, Moon H, Jung BK, Jeong B. Microsphere-incorporated hybrid thermogel for neuronal differentiation of tonsil derived mesenchymal stem cells. Adv Healthc Mater. 2015;4(10):1565–1574.

Pillozzi S, Becchetti A. Ion channels in hematopoietic and mesenchymal stem cells. Stem Cells Int. 2012; Article ID 217910, 9 pages.

Purves D, Augustine GJ, Fitzpatrick D, Lawrence C Katz, LaMantia AS, O McNamara J, Williams SM. Neuroscience. 2nd edition. Sunderland (MA): Sinauer Associates; 2001. Recovery from neural injury. Available from: http://www.ncbi.nlm.nih.gov/books/NBK10856/

Reubinoff BE, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, Ben-Hur T. Neural progenitors from human embryonic stem cells. Nat Biotechnol. 2001;19(12):1134-1140.

Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science. 1992;255(5052):1707-1710.

Reza HM, Ng BY, Gimeno FL, Phan TT, Ang LP. Umbilical cord lining stem cells as a novel and promising source for ocular surface regeneration. Stem Cell Rev. 2011;7(4):935-947.

Rohaina CM, Then KY, Ng AM, Wan Abdul Halim WH, Zahidin AZ, Saim A, Idrus RB. Reconstruction of limbal stem cell deficient corneal surface with induced human bone marrow mesenchymal stem cells on amniotic membrane. Transl Res. 2014;163(3):200-210.

Romanyuk N, Amemori T, Turnovcova K, Prochazka P, Onteniente B, Sykova E, Jendelova P. Beneficial effect of human induced pluripotent stem cell-derived neural precursors in spinal cord injury repair. Cell Transplant. 2015;24(9):1781-1797.

Roybon L, Ma Z, Asztely F, Fosum A, Jacobsen SE, Brundin P, Li JY. Failure of transdifferentiation of adult hematopoietic stem cells into neurons. Stem cells. 2006;24(6):1594–1604.

Safford KM, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, Gimble JM, Rice HE. Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun. 2002;294(2):371–379.

Sakaguchi Y, Sekiya I, Yagishita K, Muneta T. Comparison of human stem cells derived from various mesenchymal tissues: Superiority of synovium as a cell source. Arthritis Rheum. 2005;52(8):2521–2529.

Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol. 2000;164(2):247–256.

Sareen D, Saghizadeh M, Ornelas L, Winkler MA, Narwani K, Sahabian A, Funari VA, Tang J, Spurka L, Punj V, Maguen E, Rabinowitz YS, Svendsen CN, Ljubimov AV. Differentiation of human limbal-derived induced pluripotent stem cells into limbal-like epithelium. Stem Cells Translat Med. 2014; 3(9):1002-1012.

Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta. 2011;1813(5):878-888.

Shoichet MS, Tate CC, Baumann MD, LaPlaca MC. Strategies for regeneration and repair in the injured central nervous system. In: Reichert WM, editor. Indwelling neural implants: strategies for contending with the in vivo environment. Boca Raton (FL): CRC Press/Taylor & Francis. 2008;Chapter 8. Available from: http://www.ncbi.nlm.nih.gov/books/NBK3941/

Strioga M, Viswanathan S, Darinskas A, Slaby O, Michalek J. Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. Stem Cells Dev. 2012;21(14):2724-2752.

Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126(4):663-676.

Takeda YS, Xu Q. Neuronal differentiation of human mesenchymal stem cells using exosomes derived from differentiating neuronal cells. PLoS ONE. 2015;10(8): e0135111. doi:10.1371/journal.pone.0135111

Temple S. Division and differentiation of isolated CNS blast cells in microculture. Nature. 1989;340(6233):471-473.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282(5391):1145-1147.

Tropel P, Platet N, Platel JC, Noël D, Albrieux M, Benabid AL, Berger F. Functional neuronal differentiation of bone marrow-derived mesenchymal stem cells. Stem Cells. 2006;24(12):2868–2876.

Tropepe V, Sibilia M, Ciruna BG, Rossant J, Wagner EF, van der Kooy D. Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. Dev Biol. 1999;208(1):166–188.

Tropepe V, Hitoshi S, Sirard C, Mak TW, Rossant J, van der Kooy D. Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. Neuron. 2001;30(1):65-78.

Trošan P, Svobodová E, Chudíčková M, Krulova M, Zajicova A, Holan V. The key role of insulin-like growth factor I in limbal stem cell differentiation and corneal wound healing process. Stem Cells Dev. 2012;21(18):3341-3350.

Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. Nat Rev Immunol. 2008;8(9):726-736.

Urdzíková L, Jendelová P, Glogarová K, Burian M, Hájek M, Syková E. Transplantation of bone marrow stem cells as well as mobilization by granulocyte-colony stimulating factor promotes recovery after spinal cord injury in rats. J Neurotrauma. 2006;23(9):1379-1391.

Vescovi AL, Galli R, Gritti A. The neural stem cells and their transdifferentiation capacity. Biomed Pharmacother. 2001;55(4):201-205.

Wei X, Yang X, Han Z, Qu FF, Shao L, Shi YF. Mesenchymal stem cells: a new trend for cell therapy. Acta Pharmacol Sin. 2013;34(6):747–754.

Wislet-Gendebien S, Leprince P, Moonen G, Rogister B. Regulation of neural markers nestin and GFAP expression by cultivated bone marrow stromal cells. J Cell Sci. 2003;116(16):3295-3302.

Woo DG, Shim MS, Park JS, Yang HN, Lee DR, Park KH. The effect of electrical stimulation on the differentiation of hESCs adhered onto fibronectin-coated gold nanoparticles. Biomaterials. 2009;30(29):5631-5638.

Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res. 2000;61(4):364-370.

Wright B, Hopkinson A, Leyland M, Connon CJ. The secretome of alginate-encapsulated limbal epithelial stem cells modulates corneal epithelial cell proliferation. PLoS ONE. 2013; 8(7):e70860.

Yagi H, Soto-Gutierrez A, Parekkadan B, Kitagawa Y, Tompkins RG, Kobayashi N, Yarmush ML. Mesenchymal stem cells: mechanisms of immunomodulation and homing. Cell Transplant. 2010;19(6):667–679.

Yap MS, Nathan KR, Yeo Y, Lim LW, Poh CL, Richards M, Lim WL, Othman I, Heng BC. Neural differentiation of human pluripotent stem cells for nontherapeutic applications: toxicology, pharmacology and in vitro disease modeling. Stem Cells Int. 2015; Article ID 105172, 11 pages. doi:10.1155/2015/105172

Yim EKF, Pang SW, Leong KW. Synthetic nanostructures inducing differentiation of human mesenchymal stem cells into neuronal lineage. Exp Cell Res. 2007;313(9):1820–1829.

Yoon J, Ismail S, Sherwin T. Limbal stem cells: Central concepts of corneal epithelial homeostasis. World J Stem Cells. 2014;6(4):391-403.

Zajicova A, Pokorna K, Lencova A, Krulova M, Svobodova E, Kubinova S, Sykova E, Pradny M, Michalek J, Svobodova J, Munzarova M, Holan V. Treatment of ocular surface injuries by limbal and mesenchymal stem cells growing on nanofiber scaffolds. Cell Transplant. 2010;19(10):1281-1290.

Zhang M, Lin YH, Sun YJ, Zhu S, Zheng J, Liu K, Cao N, Li K, Huang Y, Ding S. Pharmacological reprogramming of fibroblasts into neural stem cells by signaling-directed transcriptional activation. Cell Stem Cell. 2016;18(5):653–667.

Zhang SC, Wernig M, Duncan ID, Brüstle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol. 2001;19(12):1129-1133.

Zhao LR, Duan WM, Reyes M, Keene CD, Verfaillie CM, Low WC. Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. Exp Neurol. 2002;174(1):11-20.

Zhou J, Tian G, Wang J, Luo X, Zhang S, Li J, Li L, Xu B, Zhu F, Wang X, Jia C, Zhao W, Zhao D, Xu A. Neural cell injury microenvironment induces neural differentiation of human umbilical cord mesenchymal stem cells. Neural Regen Res. 2012;7(34):2689-2697.

Zhou Z, Chen Y, Zhang H, Min S, Yu B, He B, Jin A. Comparison of mesenchymal stromal cells from human bone marrow and adipose tissue for the treatment of spinal cord injury. Cytotherapy. 2013;15(4):434-448.

Zomer HD, Vidane AS, Gonçalves NN, Ambrósio CE. Mesenchymal and induced pluripotent stem cells: general insights and clinical perspectives. Stem Cells Cloning. 2015;8:125–134.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P,Hedrick MH. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002;13(12):4279–4295.