Manifestation of Novel Social Challenges of the European Union
in the Teaching Material of
Medical Biotechnology Master's Programmes
at the University of Pécs and at the University of Debrecen

Identification number: TÁMOP-4.1.2-08/1/A-2009-0011

NEW SZÉCHENYI PLAN

Manifestation of Novel Social Challenges of the European Union
in the Teaching Material of
Medical Biotechnology Master's Programmes
at the University of Pécs and at the University of Debrecen
Identification number: TÁMOP-4.1.2-08/1/A-2009-0011

Dr. Péter Balogh and Dr. Péter Engelmann

Transdifferentiation and regenerative medicine – Lecture 10

TRANSDIFFERENTIATION IN THE REGENERATION OF CENTRAL NERVOUS SYSTEM

The projects are supported by the European Union.

Neurogenesis in Drosophila and mammals

- During Drosophila neurogenesis, a neuroblast (NB) divides in a stem cell like fashion to simultaneously give rise to a self-renewing daughter, as well as a smaller differentiating ganglion mother cell (GMC). GMCs are intermediate precursor cells that usually undergo one terminal division to generate two post-mitotic neurons.
- In mammalian neurogenesis, a ‘multi-potent’ neural stem cell (NSC) is capable of generating all the lineages in neural specific tissues.
- A NSC gives rise to a neural progenitor cell which in turn generates a lineage committed progenitor that can directly generate a differentiating neuron.

Criteria for the evaluation of neural plasticity

- Localization of cell specific neural markers (positive for neural markers, negative for glial markers)
- Induction of neural proteins or transcripts
- Functional characterization by electrophysiology of membrane / action potential, synapse formation, neurotransmitter release
- *In vivo* expression of neural specific markers, functional characterization and integration into neural circuits.

Adult neural stem cells

- In mature nervous system, no new neurons are generated.
- However, recent data showed that birds (especially songbirds) exhibit great plasticity in neurons (neuronal stem cells).
- New emerging data suggesting that there are newly formed neurons appearing even in adult human brain demonstrated by BrdU experiments.

Location of neural stem cells in mammals

In adult mammalian brain, two main germinative regions are exist:

- In the subventricular zone of the lateral ventricle
- In the subgranular cell layer of the hippocampal dentate gyrus

Neural precursor cell differentiation

There are three major approaches to differentiate hESCs into human neural precursor cells (hNPs):

- Promoting the direct neural differentiation of hESCs colonies
- Co-culturing hESCs cells with a feeder layer of stromal cells
- Applying a multistep procedure which involves the formation of embryoid bodies (EBs)

Direct neuronal differentiation

- During embryogenesis, neurulation is the first step in organogenesis.
- hESCs are expected to differentiate spontaneously and directly into neural cells. The formation of ectodermal derivatives can be induced by prolonged culture of hESCs without changing the feeder cells.
- Under serum-free conditions and without addition of morphogens, hESCs differentiate into a homogenous population of neuroepithelial cells.
- This differentiation process occurs in approximatively 2 weeks, a timing corresponding to the development of the neural plate/tube in a human embryo.
- Consistently, efficient differentiation of hESCs into NPs has been achieved using high concentrations of BMP inhibitors (Noggin or dorsomorphin).
- More recently, a well-defined feeder-free hESCs neural induction system employing simultaneously BMP pathway inhibitors and an activin/nodal/TGF-β inhibitor (i.e. SB431542) efficiently yields homogeneous hNPs.
- This dual inhibition of SMAD signalling leading to a controlled conversion into a homogeneous population of neural progenitors would be a more convenient approach for both basic and applied scientific research.

Using stromal cells along hESCs *in vitro*

- Stromal cells are loose connective tissue cells found in number of organs, such as gonads and bone marrow.
- They provide matrix support for other cells in organs. In order to promote neural differentiation of hESCs, they have been co-cultured with stromal cell lines.
- Such cell lines secrete, or at least express, factors, not yet fully identified, that promote the formation of neural rosettes and that are collectively called “stromal cell-derived inducing activity” (SDIA).
- This co-culture method was based on the fact that mesodermal signalling contributes to neural induction as demonstrated for the differentiation of both mouse and primate ESCs into neurons.
- Although this technique efficiently promotes differentiation of hESCs into neurons, such a model is not suitable to dissect the molecular mechanisms that drive neuronal differentiation as factors secreted by such cells varied from one stromal cell line to another.

Embryoid body formation

- hESCs can be directed towards the neural lineage after generation of embryoid bodies (EBs).
- When hESCs differentiate in suspension culture, they form a three-dimensional aggregate of cells known as an EB. To increase neural differentiation and improve survival of desired cell types, growth factors or morphogens have been commonly added to the culture medium.
- There are main disadvantages associated with EB culture including: (1) the variability of their size (due to different initial cell numbers or duration of differentiation) (2) the heterogeneity of morphogen concentrations present in the different layers of the EBs forming a concentration gradient that leads to the generation of cells at different developmental stages belonging to tissues of different germ layer; (3) the aggregation of cells in EBs prevent a clear monitoring of cell morphology during differentiation.
- Recently a new protocol emerged using both hESC-derived EBs in short-term culture and direct differentiation in adherent culture conditions, which permit a controlled, stepwise differentiation of hESCs into hNPs.

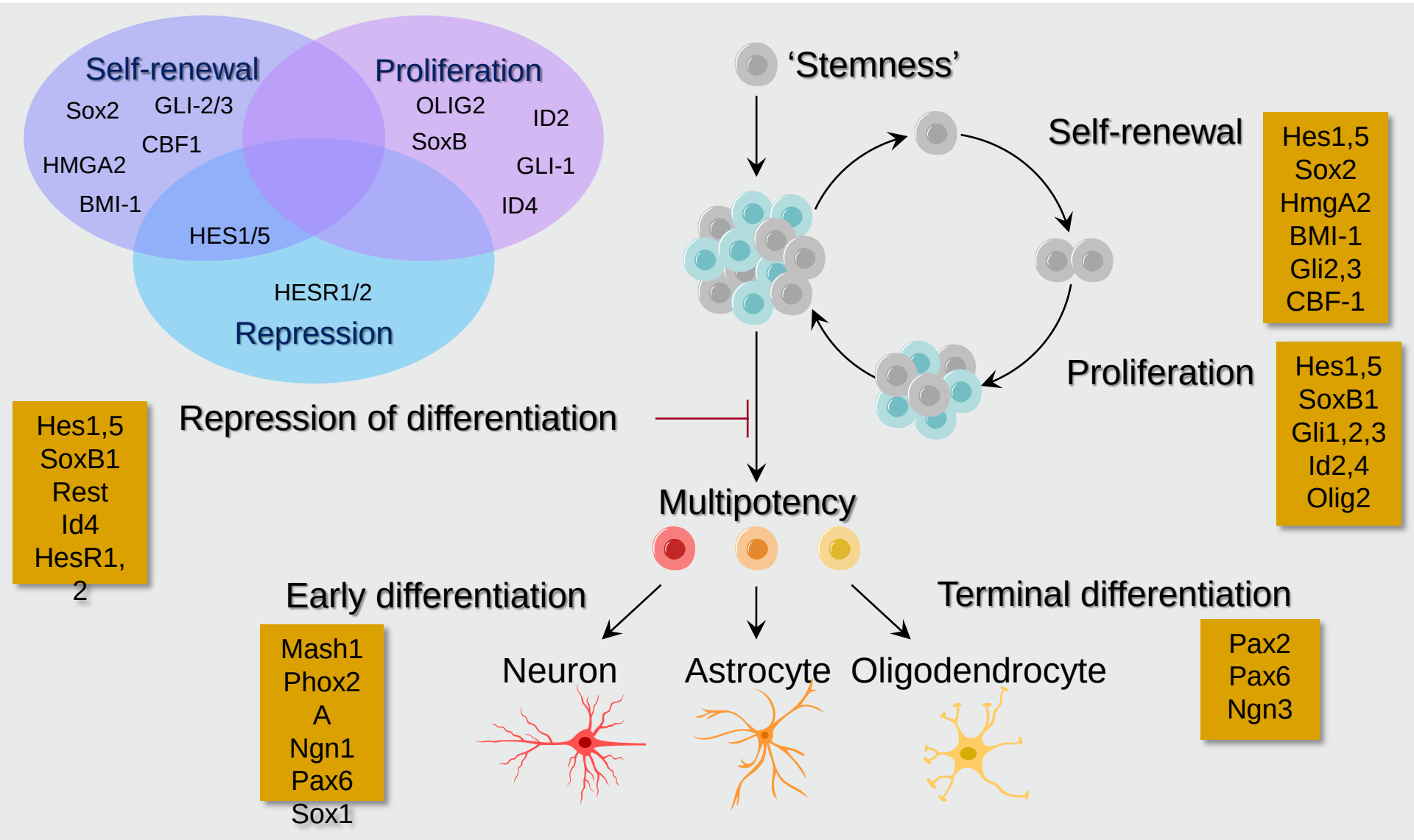
Factors involved hNP differentiation from hESCs

- Pax6 has been identified as necessary and sufficient to induce neuroepithelial cells specification of cultured hESC.
- All cells in neural rosettes remain responsive to instructive cues enabling their differentiation into a broad range of cell type in the presence of the appropriate set of morphogens: inhibition of Wnt proteins or activation of Shh signalling almost completely converts the primitive dorsal telencephalic precursors to ventral progenitors.
- However, this potential is subsequently lost in the presence of growth factors such as FGF-2 and EGF.
- Maintenance of hNPs phenotype is ensured by activation of Shh and Notch pathways.
- Exposure to *N*-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester (DAPT), a specific inhibitor of γ-secretase, leads to inhibition of Notch signalling and is sufficient to induce premature neuronal differentiation of neural rosettes. On the other hand, addition of Shh to the culture medium of neural rosettes prevent their neuronal differentiation.

Regional specification of neural cells

- During the process of neural tube closure, neuroepithelia generates distinct classes of neural progenitors that contribute later to the formation of the forebrain, the midbrain, the hindbrain, and the spinal cord.
- Neuronal phenotypes are determined by a complex interaction between extrinsic signalling molecules and cell-intrinsic transcription factors. Manipulation of signalling cues (FGF-2, Wnt, Noggin, and BMP) allowed the development of feeder-free culture conditions for differentiation of hESCs towards neural lineages.
- Mesencephalic dopaminergic (DA) neurons, which die in patients suffering from Parkinson's disease, are derived from embryonic progenitors located at the ventral midline of the midbrain. During development, the generation of DA neurons, i.e. functional tyrosine hydroxylase (TH)-positive neurons, depends on Shh signalling by ventral midline cells and on the activity of the FGF-8.
- Early exposure of Pax6 expressing neuroepithelial cells to FGF-8 is critical for dopaminergic differentiation. Transforming growth factor-α (TGF-α), which is secreted in early embryonic structures where midbrain dopaminergic neurons are generated, might be important for the differentiation of DA neurons *in vitro* and *in vivo*. After culturing hESCs-derived cells for 21 days in a medium containing TGF-α, about 15% of them become TH-positive and release dopamine.

Transcription factors and neural stem cells



Differentiation of hESC to motorneurons

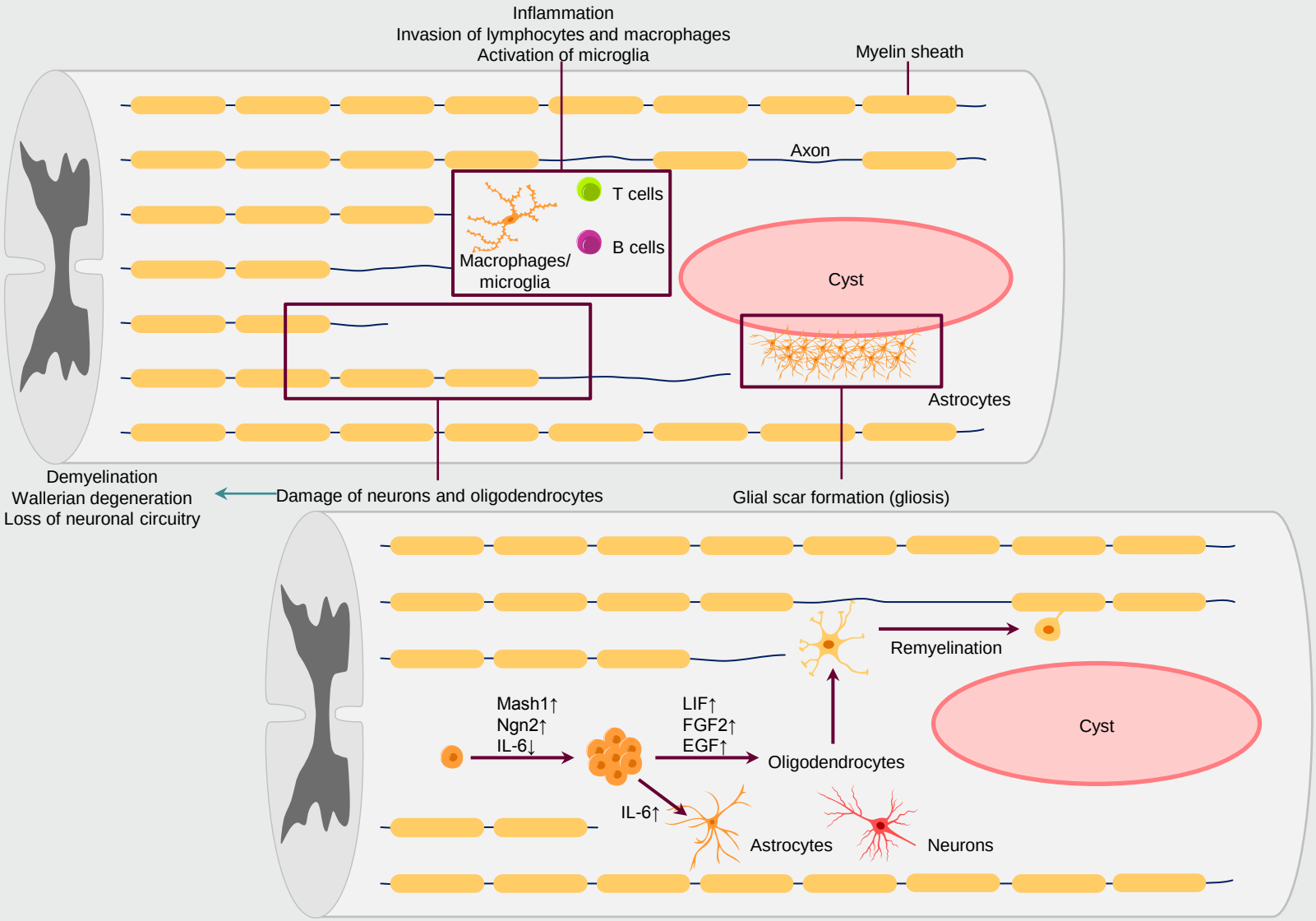
- Motor neurons (MNs) are lost in many conditions, including spinal cord injury, amyotrophic lateral sclerosis (ALS), and spinal muscular atrophy (SMA).
- Therefore, hESCs could be used to provide a source of differentiated human cells for the regeneration after these disease mechanisms.
- In the ventral part of the neural tube, there are four different progenitor domains: v3, MN, v2, v1.
- MN progenitors later give rise to MNs while all other progenitors give rise to different types of ventral interneurons. Expression of Olig2, a basic helix–loop–helix transcriptional factor, is a determinant factor in establishing the MN domain.
- The daughters of MN cells will then select whether they differentiate into motor neurons or become oligodendrocytes.
- This process is under the control of unique combinations of bHLH and homeodomain transcription factors such as LIM-homeodomain transcription factors (LHX3, LHX4), Hox genes (Nkx2.2. Phox, HB9) and Pax6.

Spinal cord injury

- SCI is the product of the immediate mechanical trauma and a very complex ischemic and inflammatory cascade secondary to the initial trauma.
- Injury to the spinal cord disrupts ascending and descending axonal pathways and causes cellulardestruction, inflammation, and demyelination
- This results in a loss of movement, sensation and autonomic control below and at the level of the lesion.
- The injury evolves in two major pathological stages:
  - the primary injury involves mechanical cell and tissue damage,
  - and the secondary injury results in a cascade of biochemical events that produce progressive destruction of the spinal cord tissues.

TÁMOP-4.1.2-08/1/A-2009-0011

Events of spinal cord injury and directed manipulation of stem cells after SCI



Stem cells to treat of SCI

- In the traumatically injured spinal cord, differentiation of grafted NPCs is restricted toward the astrocytic lineage possibly due to the inflammatory environment. TNF-alpha, IL-1beta and IFN-gamma play a major role in differentiation of NPCs in in vivo conditions.
- In the mature CNS, NPCs usually have the tendency to differentiate toward the astroglial cell line rather than neuronal and oligodendroglial cell lines. Neuronal and myelin-producing oligodendrocyte differentiation in order to support neuro-axonal regeneration is the goal.
- BMPs are promoters for astroglia differentiation and the BMP inhibitor, Noggin, prevents astroglial differentiation. Among promoters of neuronal and oligodendrocyte differentiation are helix loop helix factor (HLH-f), mammalian AS-C homolog (MASH), and Neurogenin. Human hematopoietic stem cells (HSCs) were transplanted into chicken embryo’s spinal cord, differentiated to neurons.
- Embryonic stem cells have also been used for myelination purposes, as they are directed toward differentiation to oligodendrocytes. There is evidence suggesting the presence of progenitor cells committed to the oligodendrocyte lineage in the adult human CNS.

Non-stem cell based approach

- Olfactory ensheathing cells (OECs), being glial cells, ensheathing the axons of the olfactory receptor neurons having the properties of both Schwann cells and astrocytes, have shown both regeneration and functional recovery in spinal cord damage. Genetically modified OECs secrete glial cell line-derived neurotrophic factor (GDNF) and, using a retroviral-based system, have been transplanted into a complete spinal transection and demonstrated the capability of producing *in vivo* GDNF significant recovery.
- Another widely used transplant is the peripheral nerve. Intercostal nerves together with local application of FGF have facilitated regrowth of axons. The motor recovery has been postulated to be secondary to recruitment of surviving nerves.
- Fibroblasts, genetically engineered to express nerve growth factor (NGF), have been grafted in the striatum, causing cholinergic axons to arise from the nucleus basalis toward and into the grafts.

Retina regeneration

- The retina is a complex neural circuit responsible to transduce light into electric impulses that inform the brain about the surrounding environment.
- Like other parts of the nervous system, retina can also be a subject of many neurodegenerative diseases resulting visual impairment or in serious cases, blindness. Many retinal degenerative diseases affect only a subset of cells in the retina.
- In the case of retina damage in non-mammalian vertebrates (mainly fishes, amphibians and newborn chicks) a remarkable retina regeneration can be observed.
- In mammals, especially in humans it seems a little recovery of the lost cells. However, observing the regeneration in lower vertebrates can help to understand the possibilities in humans.

Retina regeneration by Müller glia I

- Müller glia are a potential source of retinal progenitors in warm-blooded vertebrates. The Müller glia are the major type of support cell in the retina, common to the eyes of all vertebrate classes, and are the only type of retinal glia that is derived from the embryonic retinal neuroepithelial stem cells. Other types of retinal glia originate from extra-retinal sources; these glia can include microglia, astrocytes, oligodendrocytes and non-astocytic inner-retinal glia cells (NIRG cells).
- The functions of Müller glia include providing structural support, synaptic support, osmotic homeostasis, and nutritive/metabolic support to retinal neurons. In several vertebrate classes, the Müller glia are capable of de-differentiating, proliferating and acquiring a progenitor-like state in response to acute retinal injury or in response to exogenous growth factors.

Retina regeneration by Müller glia II

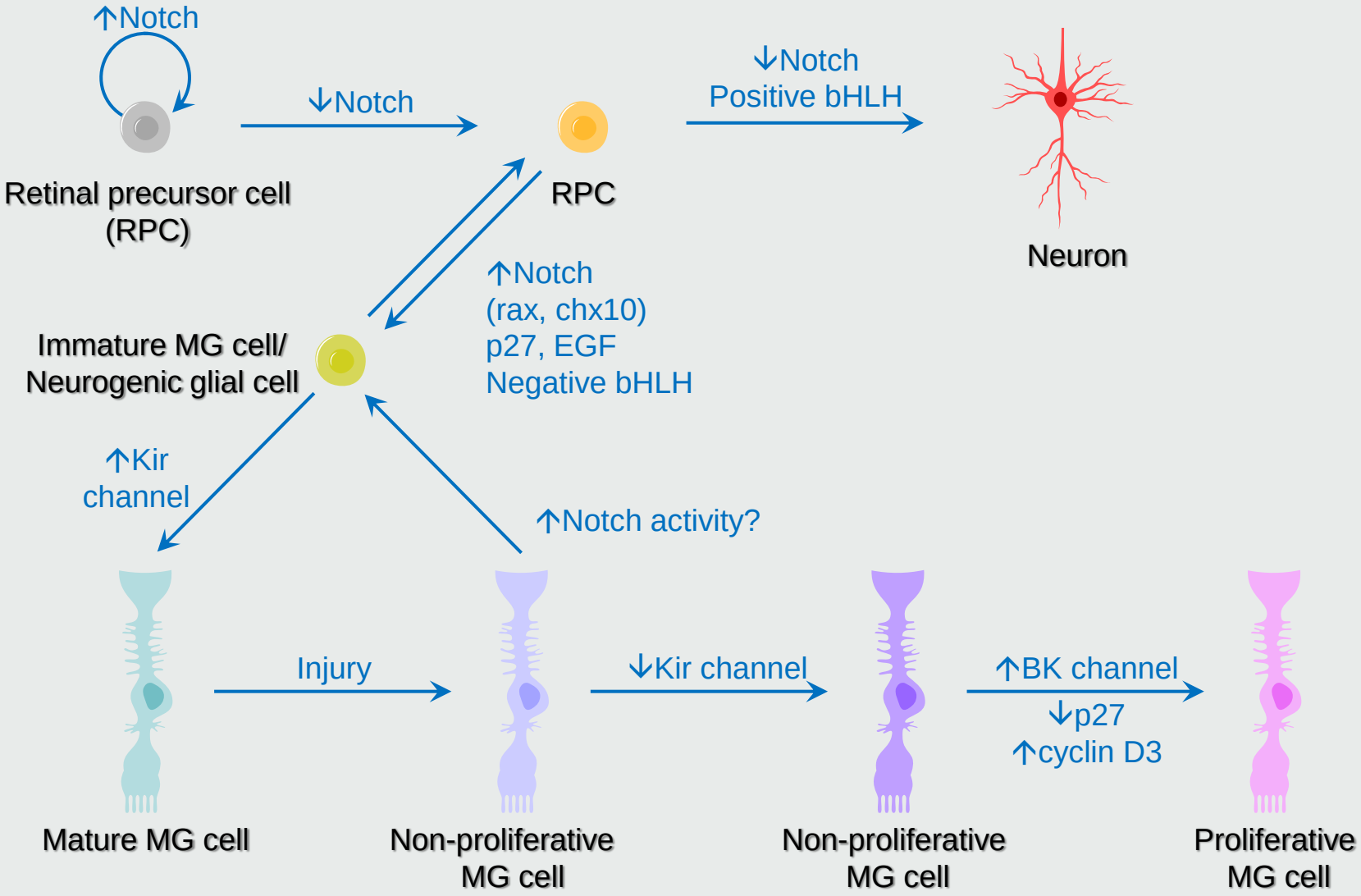
- During regeneration Müller glia de-differentiate, proliferate, and become neuronal progenitors in acutely damaged retinas.
- Müller glia de-differentiate, re-enter the cell cycle, and express transcription factors (ascl1a, Pax6, notch Chx10, Six3, Sox2, and Sox9) found in embryonic retinal progenitors.
- Only few neurons are regenerated, proliferating Müller glia produce thousands of un-differentiated progenitor cells that represent a large pool of cells that could be stimulated to regenerate the retina to restore vision.

Retinal stem / progenitor cells (RPC)

- At the anterior margin of retina, called as ciliarial marginal zone (CMZ) or circumferential germinal zone (CGZ) and also called as ora serrata is the stem cell zone of the retina especially in fish, amhibians and birds.
- Upon damage this region can produce new retinal neurons, however it does not participate in the regeneration of the majority of the retina.
- In mammals (human) tracing and research of this region gave controversial results and presumably lost.
- This phenomenon indicates a progressive reduction in the size of the CMZ during vertebrate evolution

TÁMOP-4.1.2-08/1/A-2009-0011

Retinal progenitor cells and their plasticity



Non-eye derived progenitor cells

- Mouse / human ESC could be differentiated into retinal cells (photoreceptors, retinal pigment epithelium) by stepwise treatment of factors such as FGF, taurin, RA, Shh.
- Bone marrow stem cells (BMSC) can differentiated into cells expressing retina specific markers. Adult BMSC induced by activin A, taurin, EGF differentiate into cells expressing rhodopsin, opsin,recoverin *in vitro*. Moreover, trasplanting these cells into subretinal space, these cells were able to integrate into the retina and form photoreceptor layer-like structures.
- Adult neural progenitor cells are able to adapt to a wide variety of heterologous environments and express some but not all features of retinal cells when exposed to the permissive environments.

Stem cells in the cornea

- Transparency of cornea and visual accuracy is dependent on the integrity and functionality of the outmost layer epithelium which serves as a protecting shield.
- Corneal epithelial stem cells reside at the junction between the cornea and neighbouring conjunctival epithelium in a region known as the limbus.
- These cells, known as limbal epithelial stem cells (LESC), are responsible for maintaining the corneal epithelium throughout life.
- LESC are positive for ΔNp63α ABCG2, Notch-1, N-cadherin and negative for cytokeratin 3, 12 and connexin 43 gap junction protein.
- Ocular surface failure resulting from LESC deficiency can occur as the result of primary (inherited eye disease) but more commonly as the result of acquired factors, including chemical burn injury, limbal surgery

Cornea regeneration

- In 1997 a succesfull stem cell therapy for LESC failure was carried out in two patients with chemical burn injuries. The procedure involved the isolation and *ex vivo* expansion of autologous LESCs from limbal biopsy for transplantation. The clinical outcome was promising with both patients experiencing improved vision for at least 2 years.
- Since then many centres adapted this approach and the overall success rate for the combined results of cultured autologous and allogeneic LESC therapy treatments is approx. 70%.
- Recently alternative approaches are emerged using ESCs or oral mucosal epithelial cell (OMEC) or buccal keratinocyte population instead of the LESCs.

Sensory hair cell regeneration

- Hearing loss is a global health problem affecting many individuals worldwide.
- Regeneration of cochlear hair cells is considered the ultimate treatment for hearing loss. Hair cell regeneration needs to be conducted in the context of extensive cochlear restoration.
- In non-mammalian vertebrates hair cell regeneration originated from supporting cells that reenter the cell cycle when neighboring hair cells are dying. Mitotic supporting cells subsequently divide asymmetrically, generating new hair cells and supporting cells.
- As a therapy next to hearing aids and cochlear transplants stem cell replacement or gene delivery or small molecule compounds affecting Atoh1 (Math1) expression, Notch pathways can be considered.

Summary

- Neuronal progenitor cells can be differentiated using ES cells.
- The differentiated cells can be used in potential therapeutical applications, NB: SCI treatments.
- Non-stem cells and soluble factors as alternative applications are also possible.
- Retina regeneration is due to transdifferentiation of Müller glia cells. Retinal progenitors exist in lower vertebrates, the data concerning mammals are not promising, however.
- Corneal regeneration is applicable using limbal epithelial stem cells.
- Applications of hair cell regeneration such as transplant of progenitor cells, viral gene delivery, controlling of Atoh1 and cell cycle genes to treat hearing loss is under progress.