



● PERSPECTIVE

Busting the myth: more good than harm in transgenic cells

Peripheral neuropathy constitutes a highly incidental condition and a major public health concern worldwide (Hanewinkel et al., 2016). This pathology is triggered by peripheral nervous system damage as a consequence of systemic disease or ischemic-traumatic lesion. In the latter case, nerve crush, partial or total transection and stretch injury interrupt nerve conduction and impair sensitivity and motility of the innervated area, which brings about partial or total functional loss in the affected limb and disabling neuropathic pain. For these reasons, digging into the molecular mechanisms underlying peripheral neuropathy becomes essential for the development of successful therapeutic strategies.

In addition to a wide variety of animal models available for basic research and pre-clinical studies of peripheral nerve injuries, cell transplantation techniques have received a great deal of attention in recent years and have shed light on potential neuroregenerative pharmacological and cell therapies (Faroni et al., 2015), in models such as sciatic, facial or optic nerve lesions and diabetic neuropathy. In particular, the sciatic nerve crush model is widely used as an interesting approach to Wallerian degeneration with numerous advantages. This pathophysiological process is characterized by the loss of axon-Schwann cell (SC) contact, which triggers SC dedifferentiation and proliferation. This event is followed by myelin breakdown, recently found to be an autophagic process, and an inflammatory response which includes hematogenous macrophage influx participating in myelin debris removal and tissue remodeling. These constitute essential steps for the onset of axonal regeneration and remyelination (Klein and Martini, 2016).

Despite the advances in cell therapy, cell tracking continues to pose an obstacle in the appraisal of transplantation results which can be partly bypassed in different ways. First, the use of cell trackers to label the population of cells to be transplanted allows for easy though short-lived staining. Second, cell transfection with plasmid coding for fluorescent proteins enables longer-lasting tracking but renders low yields. Third, cell lines are easily maintained but, in having been genetically modified, they fail to match primary cell reliability and functionality. In other words, results obtained using transgenic cell lines always need corroboration through primary cell cultures or *in vivo* studies. As a fourth option, transgenic animals provide more viable and reliable cells to use *in vivo*, both for cell tracking and functional effects. Even if these animals are more difficult and costly to maintain, as their breeding requires animal facilities and raises ethical concerns in animal care, their cells allow more sustained expression of reporter genes upon transplantation (Li et al., 2016; Piñero et al., 2018). Moreover, acceptable expression levels can be achieved even in heterozygous animals, although expression in the tissue of interest should be verified.

In particular, green fluorescent protein (GFP) reporter expression under different promoters constitutes a well-established source of stably labeled cells and has given way to the development of transgenic strains in several species. GFP was originally obtained from bioluminescent jellyfish *Aequorea victoria* and can show fluorescence with only light excitation. Genetic engineering later rendered several mutants of the original wild type GFP gene with improved fluorescence and thermostability, including the enhanced GFP (EGFP) variant. While cells derived from GFP transgenic animals may not be a first option in *in vitro* studies which require GFP⁺ cells as a control condition, both animal-derived and transduced GFP⁺ cells can be detected *in vivo* through non-invasive methods and used in downstream applications such as flow cytometry, microscopy or fluorometric assays.

In addition to transgenic animals all whose cells express the EGFP promoter under a constitutive protein—for instance, the GFP β -actin mouse—, other models rely on the generation of specialized cells by expressing the EGFP promoter under lineage-specific proteins, as is the case of the CNP-EGFP mouse (Yuan et al., 2002), which offers key advantages in the study of oligodendrocytes and SC differentiation. Although transgenic models have been largely developed in mouse strains, rats prove an extremely useful laboratory species for modeling clinical disease. And, in spite of the drawbacks mentioned

above associated to transgenic animal care, several groups have succeeded in developing genetically modified rats. Kobayashi and colleagues (Hakamata et al., 2001) first described the transgenic strain of Wistar rats-Wistar-TgN(CAG-GFP)184ys-(^{EGFP}Wistar), which carries the EGFP transgene driven by the chicken β -actin promoter and cytomegalovirus enhancer. The ^{EGFP}Wistar strain allows the transplantation of labeled cells with slighter immunological rejection and their analysis at longer survival times, as evidenced by multipotent cells isolated from ^{EGFP}Wistar and transplanted into wild type rats (Villarreal et al., 2016; Piñero et al., 2018). In addition, Li and colleagues (Li et al., 2016) successfully generated transgenic rats carrying a stable insertion of either the desRed fluorescent protein gene or the EGFP gene. Worth pointing out, and even considering their convenience in cell tracking, transgenic cells should be analyzed in terms of composition, characteristics and functionality to ensure applicability levels comparable to their wild type counterparts. In other words, the remaining challenge in cell therapy research is then to strike a balance between cell tracking efficiency and beneficial effects.

In terms of cell populations with therapeutic relevance, abundant evidence has proven bone marrow cell beneficial effects in different models and through different mechanisms. Upon purification, bone marrow cells render the bone marrow mononuclear cell (BMMC) fraction, a heterogeneous population which has been shown to preserve mesenchymal stem cell multipotency and regeneration efficiency in different *in vitro* and *in vivo* experimental approaches. As further therapeutic potential, BMMC are suitable for systemic autologous transplant immediately after lesion, and thus help prevent treatment delay, extensive costs and the risk of possible phenotypic rearrangements, all of which stem from the need for culture maintenance (Bara et al., 2014). BMMC actually supersede the mesenchymal fraction, as the non-stromal component synergizes with the regenerating effect of stromal cells. Taken together, these findings have sparked interest in the use of Wistar transgene-carrying ^{EGFP}BMMC, their characterization, beneficial effects and underlying mechanisms.

Our group has recently reported the beneficial effects of transplanted ^{EGFP}BMMC in a model of rat sciatic nerve crush. ^{EGFP}BMMC isolation rendered GFP fluorescence intensity patterns comparable to previous studies (Hakamata et al., 2001), while characterization revealed similar population proportions but lower yields than wild type BMMC (^{wt}BMMC). In addition, marker expression by ^{EGFP}BMMC includes transmembrane phosphoglycoprotein CD34, indicative of high colony-forming efficiency and long-term proliferating capacity (Sidney et al., 2014), multipotent cell marker CD105 and a large proportion of cells expressing CD90, a marker of hematopoietic stem cells and lymphoid, myeloid and erythroid cell progenitors (Piñero et al., 2018). The detection of these markers indicates that ^{EGFP}BMMC include a population with potential to transdifferentiate into other cell types upon transplant, including SC.

Besides their well-established features as a cell fraction, both ^{wt}BMMC and ^{EGFP}BMMC reach the ipsilateral nerve upon systemic transplantation (Setton-Avruij et al., 2007; Usach et al., 2017; Piñero et al., 2018). Pro-inflammatory interleukin and chemokine release by SC, macrophages and fibroblasts from the distal stump may foster chemotaxis and the recruitment of transplanted BMMC to the lesion site. Transplanted cell quantification at the crush and distal stumps correlates with SC proliferation and the hematogenous immune cell influx described for this type of injury (Klein and Martini, 2016). These observations, together with the small proportion of cells which remain in the area at longer times, may suggest that not all the migrating cells persist in the tissue during regeneration or, at least, upon the resolution of the inflammatory process (Piñero et al., 2018).

Studies on BMMC action in several experimental models have postulated different mechanisms. Upon nervous system damage, BMMC can foster regeneration by inducing an increase in vessel number, by secreting trophic factors which boost early glial cell proliferation, by amplifying or attenuating the immune response, or by upregulating markers unexpressed before transplant and undergoing phenotypic changes (Volkman and Offen, 2017). In this context, ^{EGFP}BMMC offer an additional advantage to long-lasting cell tracking, as they express SC marker mRNA but not their respective proteins. These features make these cells suitable for the analysis of post-transplant cell phenotype, which evolves from early undifferentiated round-shaped into SC-like spindle-shaped morphology and SC marker expression at longer times. However, the number of cells undergoing phenotypic changes is considerable lower when com-

pared to total transplanted cells. These findings unveil two possible mechanisms underlying ^{EGFP}BMMC regenerative ability: immunomodulatory effects shortly after lesion, and transdifferentiation to SC at later survival times (Piñero et al., 2018).

Inflammatory response regulation is a complex issue involving different cell types. Upon reversible Wallerian degeneration, the widely characterized inflammatory reaction includes early influx of neutrophils and mostly macrophages, which later polarize from an M1-like inflammatory phenotype to an M2-like anti-inflammatory (wound healing tissue remodeling) phenotype. In this context, we hypothesize that BMMC promote the downregulation of M1 markers and/or accelerate M2 polarization. Last but not least, lymphocytes also play an essential role in immune modulation.

The inflammatory process triggered by Wallerian degeneration does not only involve cell response, but also soluble mediators commonly associated with nociception, among other manifestations. Taking into consideration that BMMC may exert immunomodulatory actions, our group has recently focused on the study of the beneficial effect of transplanted cells in attenuating neuropathic pain. So far, after sciatic nerve crush, a full preventive action against transient mechanical hypersensitivity has been observed in BMMC-treated animals, in addition to proven positive effects on nerve regeneration, remyelination and functionality (Usach et al., 2017).

Regeneration after an acute lesion and consequently associated pain can last weeks, even months. In this context, the question remains whether BMMC additional doses or combination therapy may be required for effective treatment. Also, strategies for cell recruitment to the lesion area may be optimized through pharmacological or nanotechnological resources. On the other hand, translational medicine requires a fine balance between the time window for therapy administration and follow-up, and the desired beneficial effects. Summing up, ^{EGFP}BMMC emerge as a useful tool for future transplantation research, breaking the myth of transgenic cell deleterious effects, reinforcing the contribution of BMMC to peripheral nerve injury recovery and paving the way for further analyses on their participation in regenerating therapies in acute and possibly chronic lesions.

The authors would like to thank Dr. Vanina Usach and Paula A. Soto for their invaluable help in the analysis and discussion of the results,

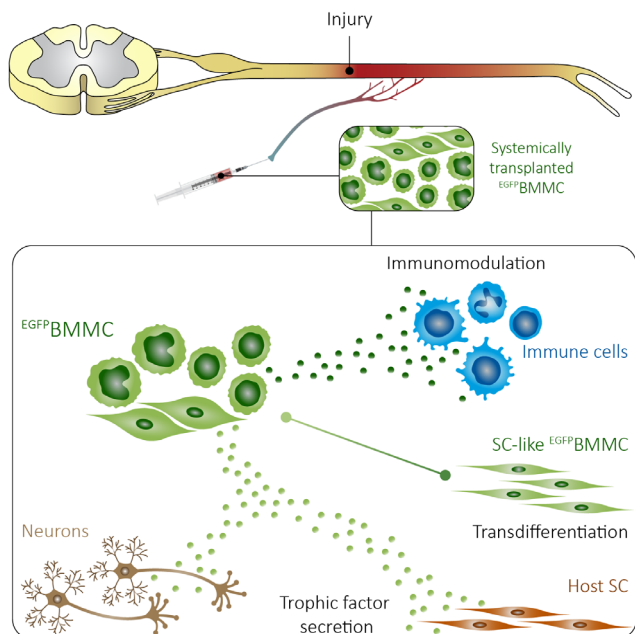


Figure 1 BMMC action and underlying mechanisms upon peripheral nerve injury.

BMMC contain an enriched population of lymphocytes, monocytes, hematopoietic and endothelial progenitor cells and, worth highlighting, mesenchymal stem cells. Upon nerve injury, systemically transplanted cells promote axonal regeneration and foster remyelination. Mechanisms involved may be related with immunomodulation, transdifferentiation to SC, or glial cell and neuronal proliferation and support through trophic factor secretion. BMMC: Bone marrow mononuclear cell; EGFP: enhanced green fluorescent protein; SC: Schwann cell.

and Ms. María Marta Rancez for her assistance in the elaboration of this manuscript.

This work was supported by Universidad de Buenos Aires (UBACYT 20020100101017) and CONICET, Ministerio de Ciencia, Tecnología e Innovación Productiva de la República Argentina (PIP 830 and PIP 567).

Gonzalo Piñero, Patricia Setton-Avruj*

Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, Instituto de Química y Físicoquímica Biológicas (IQUIFIB), Facultad de Farmacia y Bioquímica, Buenos Aires, Argentina

*Correspondence to: Patricia Setton-Avruj, PhD, setton@qb.ffyb.uba.ar.

orcid: 0000-0001-8510-9598 (Gonzalo Piñero)

0000-0002-4094-7712 (Patricia Setton-Avruj)

Received: September 13, 2018

Accepted: December 3, 2018

doi: 10.4103/1673-5374.249219

Copyright license agreement: The Copyright License Agreement has been signed by both authors before publication.

Plagiarism check: Checked twice by iThenticate.

Peer review: Externally peer reviewed.

Open access statement: This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

Open peer reviewer: Sheng Yi, Nantong University, China.

Additional file: Open peer review report 1.

References

- Bara JJ, Richards RG, Alini M, Stoddart MJ (2014) Concise review: Bone marrow-derived mesenchymal stem cells change phenotype following in vitro culture: implications for basic research and the clinic. *Stem Cells* 32:1713-1723.
- Faroni A, Mobasseri SA, Kingham PJ, Reid AJ (2015) Peripheral nerve regeneration: experimental strategies and future perspectives. *Adv Drug Deliv Rev* 82-83:160-167.
- Hakamata Y, Tahara K, Uchida H, Sakuma Y, Nakamura M, Kume A, Murakami T, Takahashi M, Takahashi R, Hirabayashi M, Ueda M, Miyoshi I, Kasai N, Kobayashi E (2001) Green fluorescent protein-transgenic rat: a tool for organ transplantation research. *Biochem Biophys Res Commun* 286:779-785.
- Hanewinkel R, Ikram MA, Van Doorn PA (2016) Peripheral neuropathies. *Handb Clin Neurol* 138:263-282.
- Klein D, Martini R (2016) Myelin and macrophages in the PNS: An intimate relationship in trauma and disease. *Brain Res* 1641:130-138.
- Li T, Shuai L, Mao J, Wang X, Wang M, Zhang X, Wang L, Li Y, Li W, Zhou Q (2016) Efficient production of fluorescent transgenic rats using the piggy-Bac transposon. *Sci Rep* 6:33225.
- Piñero G, Usach V, Soto PA, Monje PV, Setton-Avruj P (2018) EGFP transgene: a useful tool to track transplanted bone marrow mononuclear cell contribution to peripheral remyelination. *Transgenic Res* 27:135-153.
- Setton-Avruj CP, Musolino PL, Salis C, Allo M, Bizzozero O, Villar MJ, Soto EF, Pasquini JM (2007) Presence of alpha-globin mRNA and migration of bone marrow cells after sciatic nerve injury suggests their participation in the degeneration/regeneration process. *Exp Neurol* 203:568-578.
- Sidney LE, Branch MJ, Dunphy SE, Dua HS, Hopkinson A (2014) Concise review: evidence for CD34 as a common marker for diverse progenitors. *Stem Cells* 32:1380-1389.
- Usach V, Malet M, Lopez M, Lavalle L, Piñero G, Saccoliti M, Cueto A, Brumovsky P, Brusco A, Setton-Avruj P (2017) Systemic transplantation of bone marrow mononuclear cells promotes axonal regeneration and analgesia in a model of wallerian degeneration. *Transplantation* 101:1573-1586.
- Villarreal A, Roszczewski G, Murta V, Cadena V, Usach V, Dodes-Traian MM, Setton-Avruj P, Barbeito LH, Ramos AJ (2016) Isolation and characterization of ischemia-derived astrocytes (IDAs) with ability to transactivate quiescent astrocytes. *Front Cell Neurosci* 10:139.
- Volkman R, Offen D (2017) Concise review: mesenchymal stem cells in neurodegenerative diseases. *Stem Cells* 35:1867-1880.
- Yuan X, Chittajallu R, Belachew S, Anderson S, McBain CJ, Gallo V (2002) Expression of the green fluorescent protein in the oligodendrocyte lineage: a transgenic mouse for developmental and physiological studies. *J Neurosci Res* 70:529-545.

P-Reviewer: Yi S; C-Editors: Zhao M, Yang LJ; T-Editor: Liu XL