Functional *in vivo* assessment of stem cell-secreted pro-oligodendroglial factors

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The role of adult neural stem cells (NSCs) in demyelinating diseases of the central nervous system (CNS): Multipotent NSCs hold great potential for cell replacement in diseases and upon injury of the CNS. Originating from radial glial cells during nervous system development, adult NSCs are localized in the subgranular zone of the hippocampus and the subventricular zone (SVZ) of the lateral brain ventricles, the main neurogenic zones of the adult brain. Hippocampal precursor cells (type 1 cells) exhibiting properties of radial glial cells give rise to granule neurons through distinct intermediate precursor cells, and integrate into the hippocampal circuitry [reviewed by Kempermann et al. (2015)]. Likewise, under physiological conditions, neuron generation by mouse SVZ-derived NSCs (also known as type B cells) is the predominant cell fate, which thereby results in large numbers of transient amplifying precursor cells (also known as type C cells) which in turn differentiate into neuroblasts (type A cells). These cells migrate along the rostral migratory stream into the olfactory bulb where they undergo maturation into local interneurons. The structure of the rodent SVZ differs from that of the human SVZ since the proliferative capacity is reduced, and migration of neuroblasts is a rare event in adult humans [reviewed by Lim and Alvarez-Buylla (2016)].

Under physiological conditions, a minor proportion of type B cells in both rodent and human SVZ generate cells of the glial lineage, including oligodendrocytes populating the corpus callosum. The insulation of axons by oligodendrocytes is a requirement for proper axonal signal transduction and axonal integrity. White matter defects lead to a reduction of axonal integrity resulting in permanent functional deficits. Importantly, loss of myelin sheath leads to myelin repair activities, which rely on the differentiation of type B cells into oligodendrocytes. Besides parenchymal oligodendroglial precursor cells, large numbers of new oligodendrocytes are generated from SVZ-NSCs, thus contributing to myelin repair and axonal survival upon cuprizone-induced demyelination (Butti et al., 2019).

Strategies to promote myelin and white matter repair are needed. Promoting adult NSCs towards functional oligodendroglial differentiation could be a promising option to pave the way for novel neuroregenerative treatment approaches, for which, to date, there is an unmet clinical need. In this regard, extrinsic cell fate determinants play an important role in NSCs' self-renewal and the regulation of differentiation (Obernier and Alvarez-Buylla, 2019). Lineage determination can be modulated, e.g., by epidermal growth factor infusion, which leads to an increase in local NG2-positive progenitors, pre-myelinating and myelinating oligodendrocytes finally integrating into the native brain tissue and lysolecithin-induced demyelinated brain areas (Gonzalez-Perez et al., 2009). Interestingly, as previously shown by us and also by other research groups, both oligodendroglial precursor cells' as well as adult NSCs' oligodendroglial cell fates can be triggered by incubation with bone marrow-derived mesenchymal stem cell (MSC)-secreted factors in culture (Steffenhagen et al., 2012; Jadasz et al., 2013, 2018). MSCs are known to secrete a plethora of trophic factors. However, single proteins such as sonic hedgehog, platelet-derived growth factor alpha AA and ciliary neurotrophic factor, failed to promote oligodendroglial differentiation of NSCs (Rivera et al., 2006), suggesting an interplay of different pro-oligodendroglial factors within the MSC secretome. Although many efforts have aimed at identifying MSC-secreted proteins by mass spectrometry-based approaches, the respective studies lack information about specific factors acting on stem cell-mediated oligodendrogenesis.

Mass spectrometry-based secretome approach to identify functional pro-oligodendroglial regulators: Our recently published mass spectrometry-based functional secretome approach has shed light on active MSC-derived pro-oligodendroglial factors (Samper Agrelo et al., 2020). A large number of *bona fide* secreted oligodendroglial differentiation-related proteins were identified by comparing relative abundances of proteins found in both the MSC proteome and the secretome due to higher abundances in the secretome. Among 152 secreted proteins, both positive and negative regulators of oligodendrogenesis including e.g., chordin, connective tissue growth factor, and tissue inhibitor of metalloproteinase type 1 (TIMP-1) were detected. TIMP-1 is known to be upregulated by astrocytes in multiple sclerosis resulting in the inhibition of matrix metalloproteinase 9, which in turn leads to less blood brain barrier disruptions and reduced immune cell invasion (Gardner and Ghorpade, 2003). Furthermore, independent of its matrix metalloproteinase inhibitory function, TIMP-1 was shown to act as a trophic factor that promotes myelination and oligodendroglial maturation of oligodendroglial precursor cells via CD63/β1-integrin binding and Akt activation (Nicaise et al., 2019). Consequently, we selected TIMP-1 as a candidate protein for functional validation of our secretome approach. TIMP-1 was therefore neutralized in MSC-conditioned medium (MSC-CM) by using blocking antibodies prior to application to cultured NSCs. This neutralization led to decreased numbers of oligodendroglial cells thereby confirming a functional role of TIMP-1 in MSC-promoted oligodendroglial differentiation of NSCs (Samper Agrelo et al., 2020). Apart from the lack of information about the nature of active oligodendroglial components, MSC-dependent oligodendrogenesis *in vivo* had also not yet been confirmed. We, therefore, transplanted NSCs, which had been pre-stimulated with MSC-conditioned medium for 1 or 3 days, into the adult rodent brain and spinal cord. According to this *in vivo* approach, NSCs pre-treated with MSC secreted factors predominantly differentiated into oligodendroglial cells after transplantation independent of the pre-treatment period and formed myelin sheaths around axons (Samper Agrelo et al., 2020). Thus, the initial MSC-dependent stimulation of oligodendrogenesis appeared to predominate and the corresponding impulse was found to be maintained in the CNS environment.

Development of a targeted *in vivo* approach to functionally investigate secreted proteins: As the overall aim was to achieve CNS regeneration, a final *in vivo* evaluation of functional MSC-

CM components was necessary, also in light of a number of factors acting either together or at different stages along with the maturation and tissue integration processes. We, therefore, extended our analysis and developed a method for fast and reliable testing of *in vivo* functionality particularly suitable for secreted factors as exemplified here for TIMP-1. Adult rat SVZ-derived NSCs were stimulated with TIMP-1 depleted MSC-CM for three days and pre-treated NSCs were transplanted into the intact adult rat spinal cord (thoracic level 8), as recently described (Samper Agrelo et al., 2020). Briefly, MSCs derived from bone marrow and SVZ-NSCs were isolated from 8–10 weeks old female Wistar rats (Jadasz et al., 2013, 2018). MSCs were cultured to confluency for 3 to 4 days in α-MEM/10% FBS culture medium (both Life Technologies, Karlsruhe, Germany) containing 1% penicillin/streptomycin. After the exchange of the culture medium, MSCs were cultured for another 3–4 days and the supernatant was collected, filtered (0.2 μm filtropur S filter; Sarstedt AG and Co. KG, Nümbrecht, Germany) and used as MSC-CM. For depletion of TIMP-1, MSC-CM was supplemented with TIMP-1 neutralization antibody (25 μg/mL, AF580, R&D System, Wiesbaden-Nordenstadt, Germany) for 1 hour at 37°C prior to the application to NSCs. For visualization of transplanted cells, NSCs

were transfected with the UbC-StarTrack plasmids pCMV-hyPBase and UbC-EGFP using the rat neural stem cell nucleofector kit (Lonza, Basel, Switzerland) as previously described (Figueres-Onate et al., 2016; Jadasz et al., 2018). NSCs were cultured in neurobasal medium containing B27, 2 mM L-glutamine, 1% penicillin/streptomycin (all Gibco) supplemented with 2 mg/mL heparin (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany), 20 ng/mL fibroblast growth factor 2, and 20 ng/mL epidermal growth factor (both R&D Systems). Four days before transplantation, NSCs were seeded on poly-L-ornithine/laminin (100 and 12 μg/mL, respectively, both Sigma-Aldrich)-coated petri dishes in neurobasal medium and cultured for 24 hours. Afterward, the medium was changed to TIMP-1 depleted MSC-CM, isotype IgG control (25 μg/mL, AB-108-C, R&D System) treated MSC-CM or normal MSC-CM. After 3 days of incubation, pre-treated NSCs were transplanted into intact 10–12 weeks old Wistar rat spinal cords at thoracic level 8 (approved by the State Office for Nature, Environment and Consumer Protection North Rhine-Westphalia (LANUV); Az.: 84-02.04.2015.A525) according to our recent publication (Samper Agrelo et al., 2020) and analyzed at day 14 after transplantation by immunohistochemical staining for oligodendroglial markers

glutathione-S-transferase-π and myelin basic protein (MBP) as well as the astroglial marker glial fibrillary acidic protein (**Figure 1A**). Analysis of control NSCs stimulated with non-depleted or IgG isotype control antibody-supplemented MSC-CM prior to transplantation revealed similar differentiation patterns of NSCs after transplantation (**Figure 1B–D**). Neutralization of TIMP-1, however, clearly reduced the number of glutathione-S-transferase-π- (**Figure 1E** and **E'**) and MBP-expressing (**Figure 1F** and **F'**) cells whereas the survival rate of transplanted NSCs was not affected by TIMP-1 depletion (**Figure 1H**). Moreover, upon TIMP-1 neutralization, a trend towards an increased number of glial fibrillary acidic protein-positive cells compared to MSC-CM pre-treated NSCs was observed (**Figure 1G** and **G'**). It can therefore be concluded that TIMP-1 is an MSC secretome-derived key pro-oligodendroglial factor of *in vivo* relevance exerting an overall lower impact on the expression of astroglial features. In conclusion, the combination of a functional mass spectrometry-based approach with the *in vivo* validation of single candidate proteins presented here could serve as a blueprint for upcoming related studies and aid in simplifying and accelerating the generation of *in vivo* data.
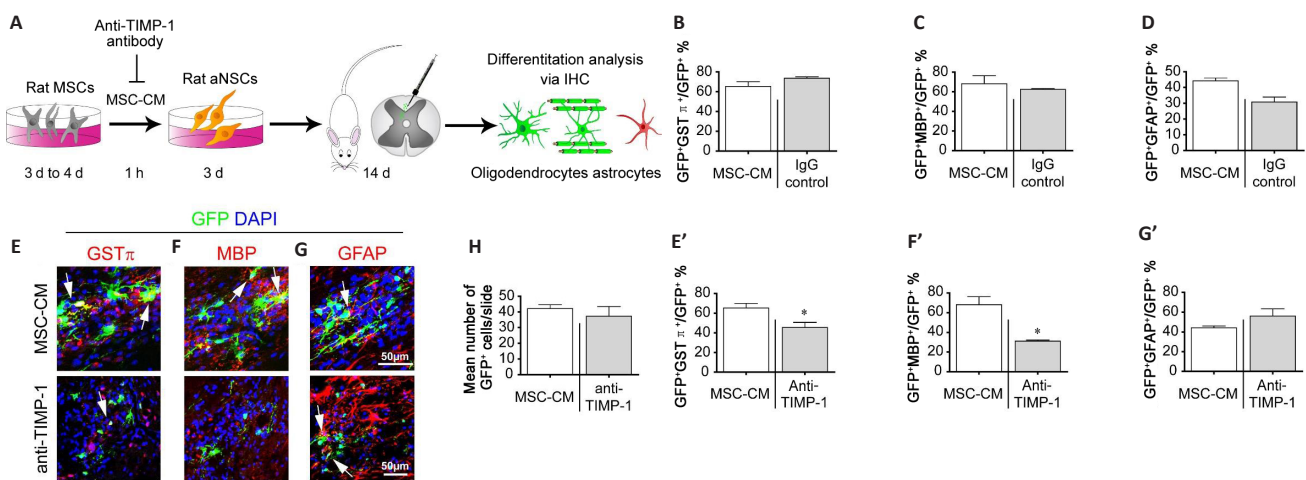
Figure 1 | ***In vivo* validation of TIMP-1 as an active pro-oligodendroglial component of the MSC secretome.**
(A) Summary of the workflow. MSCs derived from adult rat bone marrow were cultured to confluency. After 3–4 days in culture, MSC-CM was collected and incubated with TIMP-1 neutralization antibody (25 μg/mL) 1 hour prior to application to adult NSCs. NSCs were cultured with control and TIMP-1 depleted MSC-CM for 3 days and transplanted into the adult healthy rat spinal cord (thoracic level 8). After 14 days, rats were perfused with PBS and 4% PFA, spinal cords were removed, cryoprotected with 30% sucrose solution and cryosectioned. Immunohistochemical analysis for GSTπ, MBP as well as GFAP was performed. Comparison of the number of NSCs (GFP-positive) expressing GSTπ (B), MBP (C) or GFAP (D) revealed no difference between MSC-CM and MSC-CM plus control antibody (IgG) pre-stimulation. Therefore, in the following analyses, we compared the number of marker-positive transplanted (GFP+) cells after neutralization of TIMP-1 in MSC-CM with native MSC-CM (native MSC-CM data from Samper Agrelo et al., (2020)). Neutralization of TIMP-1 led to decreased numbers of GSTπ+/GFP+ (E and E') and MBP+/GFP+ (F and F') cells whereas the number of GFAP+/GFP+ cells was slightly increased (G and G'). TIMP-1 depletion had no impact on the survival rate of NSCs after transplantation (H). Animal numbers: n = 5 (MSC-CM), n = 2 (IgG), n = 3 (TIMP-1 depleted MSC-CM). Statistical analysis: Unpaired two-sided Student's t-test. Shown are mean values ± SEM. Arrows in E–G indicate transplanted cells expressing the respective marker. In B–D, IgG control refers to MSC-CM in presence of control IgG, in E–H anti-TIMP-1 refers to MSC-CM in presence of neutralizing anti-TMIP-1 antibody. aNSCs: Adult neural stem cells; DAPI: 4′,6-diamidino-2-phenylindole; GFAP: glial fibrillary acidic protein; GFP: green fluorescent protein; GSTπ: glutathione-S-transferase-π; IHC: immunohistochemistry; MBP: myelin basic protein; MSC: mesenchymal stem cell; MSC-CM: mesenchymal stem cell conditioned medium; TIMP-1: tissue inhibitor of metalloproteinase type 1. Unpublished data.

Future directions: The development of efficient strategies to identify therapeutic targets remains an important task in neuroregeneration research to which the procedure described here provides a valuable contribution. Using this rather simple and fast process offers the possibility to screen and compare a large number of candidates and their combinations with the advantage that no add-on ethical approval is needed once the implantation protocol is established and approved. This constitutes a clear advantage over the analysis of mutant mice when considering generation and breeding times, as well as cell specificity issues. However, the protocol is best suited for the analysis of trophic factors and remains dependent on neutralizing antibodies, the availability of which might also be limited and the specificity of which needs to be pre-evaluated *in vitro* before transplanting into CNS.

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