

Sustained neuronal viability by paracrine factors: new opportunities for endothelial progenitor cell secretome

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Despite the big progresses in the field of regenerative medicine, the loss of neurons remains essentially an unresolved challenge. Among the different approaches under investigation, there are great expectations on stem and progenitor cells-based strategies. Due to their capacity to home to the site of injury and theoretically generate all kinds of neuronal cells, stem cells seem the ideal candidates for targeted therapeutic interventions also in light of the lack of spontaneous regeneration of the central nervous system. Unfortunately, the substantial failure to replace dead or dysfunctional cells has limited the development of stem cells-based therapies. This aspect is especially evident for the central nervous system due to the complex architecture of the tissue, the inherent fragility of mature neurons and the lack of methods to control and guide the proliferation and differentiation of neuronal precursors. However, there is compelling evidence that stem and progenitor cells are able to support different healing processes by means of paracrine actions. Taking advantage of these characteristics, researchers have investigated the cyto-protective and restorative activities of the factors released by *in vitro* cultured stem cells in the form of conditioned medium (CM).

Among the several sources of stem and progenitor cells described in the literature the so called early outgrowth endothelial progenitor cells (EPC) seem well suited for CM-based therapeutic approaches given the ease of isolation method and the fact that the released factors have a broad spectrum of target cells (Sanchez-Ramos et al., 2008). These cells (also called myeloid angiogenic cells), which were named after their early appearance in culture of mononuclear cell preparations, have a moderate proliferation and trans-differentiation capacity but are potent effectors of vascular homeostasis and tissue regeneration by means of paracrine actions (Di Santo et al., 2009; Chopra et al., 2018).

Following this line of research, we have investigated the effects exerted by human-derived EPC-CM on neuronal cells. In our studies on primary rat neuronal cell cultures isolated from ventral mesencephalon (VM), ganglionic eminence (GE) and cerebral cortex (CTX) we could show that EPC-CM promotes a variety of cytoprotective and regenerative actions. Our observations are in agreement with recent studies which have reported that stem and progenitor cells secretome are able to increase cell viability, enhance differentiation of resident precursors and modulate immune interactions (Andereggen et al., 2018). The most important result of our set of *in vitro* experiments consists in the neuroprotective capacity of EPC-derived factors. In particular, EPC-CM attenuated the loss of neuronal cells induced by three different insults chosen to cover a broader range of neuronal toxicity *in vitro*. Accordingly, the densities of the vulnerable tyrosine hydroxylase immunoreactive (TH-ir) neurons were significantly preserved in

the VM cultures treated with the toxin 1-methyl-4-phenylpyridinium (MPP⁺) commonly used *in vitro* model of Parkinson's disease (Di Santo et al., 2019b). In a similar fashion, EPC-CM sustained the viability and number of striatal neurons in GE cultures challenged with 3-nitropropionic acid that mimics the neuropathological features of Huntington disease (Di Santo et al., 2019a). Finally, EPC-CM effectively protected GABA-ir neurons from a metabolic insult induced by glucose and serum deprivation in CTX cultures (Di Santo et al., 2020). Interestingly, in GE and CTX cell cultures, EPC-CM treatment exerted neuron-promoting effects not only during or after the insult, respectively but also when used in preconditioning regimens.

Furthermore, we have observed that EPC-CM has a quite consistent capacity to support the viability of neuronal cells in unchallenged conditions which was displayed by the significantly augmented number of beta-III-tubulin-immunoreactive (-ir) cells (Di Santo et al., 2019a, b, 2020). Importantly, 5-ethynyl-2'-deoxyuridine-incorporation experiments suggested that the increase of neuronal cells in response to EPC-CM relies on cell differentiation/maturation mechanisms rather than proliferation of the pool of immature neuronal progenitors. Conversely, astroglial cells did not display a proliferative response to EPC-CM thus suggesting that EPC-CM would not induce gliosis and scar formation, which in turn would limit the healing properties of the secretome. On the other hand, while supporting the survival and/or differentiation, EPC-CM seems not to influence the commitment of neuronal cells to specific phenotypes. In fact, in VM cultures, the amplification of the subpopulation of neurons immunoreactive for the dopaminergic marker TH was proportionally similar to the increase of overall number of neuronal cells.

Another important observation of our recent studies concerns the intriguing effect that EPC-CM has on the microglia naturally present in the primary cultures. Irrespective of the brain region used to prepare the cultures, EPC-CM induced a relevant increase of ionized calcium-binding adapter molecule 1 (Iba-1)-ir cells. Collectively, these results are in accordance with the presence of cytokines and extracellular matrix components in EPC-CM which are potent modulators of neurogenesis and/or microglia activation. It is thus conceivable that the microglia in the culture might not only act as bystanders but also contribute directly to the global effects observed on neuronal cells by secreting factors in response to EPC-CM (Figure 1). In first attempts to corroborate this hypothesis, we have depleted the microglia from CTX and VM using the cytostatic agent Cytosinarabosid (Ara-C). The incubation with Ara-C was performed at the end of the culture period; thus, this treatment is supposed to not affect the neurons which are post-mitotic. In these experiments we observed that the cultures treated with Ara-C displayed a significant

reduction of Iba-1-ir cell number and of the EPC-CM-dependent increase of neurons (beta-III-tubulin-ir cells). A similar trend was observed for number of TH-ir neurons in VM cultures (Additional Figure 1). Therefore, these results suggest that EPC-CM enhance the complexity of the interactions between microglia and neuronal progenitors by exerting concerted effects on both cell populations. Therefore, in our experimental settings, microglia likely are not just phagocytic cell bystanders but contribute to the neuron-supporting effects of EPC-CM with paracrine and autocrine mechanisms. In order to get clearer insights in the mechanisms of action of EPC-CM on the mixed cultures of our experimental framework, we are planning to perform parallel cultures of purified neuronal and microglia preparations and to investigate the effect of microglia depletion in cultures challenged by neurotoxins. Moreover, as for all the secretome-based approaches, the comprehensive molecular characterization of EPC-CM has great importance for the definition of its therapeutic potential. In this respect, several studies have sought to identify the crucial factors in the secretome responsible for the protective and restorative actions (Maki et al., 2018) and have disclosed that cell-derived secretomes are an extremely rich and complex mixture of bio-active molecules. With targeted proteomics analyses by means of antibody arrays, we were able to detect many factors that might support the cellular viability and/or confer cyto-protection actions but it is likely that such cocktail of factors might act through a complex network of signals and synergistic interactions. This concept is supported by our findings gathered from antibody-neutralization experiments. In fact, in VM cultures the neutralization of glial cell line-derived neurotrophic factor, which is considered the canonical survival factor for dopaminergic cells, did not significantly influence the effect of EPC-CM on TH-ir cells (Di Santo et al., 2019b). In the same line, neutralization of two master regulators of endothelial physiology, i.e. VEGF and interleukin-8, did not abolish the EPC-CM-induced angiogenesis. The identification of the signaling pathways activated in the target cells would also contribute to improve our understanding of the mechanisms of action of secretome. In this notion, we have reported that in GE cultures the EPC-CM-dependent increase of GABA-ir densities involves the activation of the PI3K/AKT and MAPK/ERK cascades but not the ROCK pathway. In the same study, we observed that GABA-ir neurons require functional protein translation machinery to display the effects of EPC-CM (Di Santo et al., 2019a).

Accumulating evidence indicates that besides cytokines and growth factors, lipids as well as extra cellular vesicles (EVs) are key mediators of paracrine signaling (Romano et al., 2019). Moreover, mitochondria and nucleic acids as miRNA can also be found in secretomes either free or shuttled by EVs (Laggner et al., 2020). Indeed, our GE cultures experiments have proven that EPC-CM chloroform extractable factors contribute to the neuronal supportive effects exerted by the secretome (Di Santo et al., 2019a). In contrast, in VM cultures, lipids seem not to play a significant role in supporting overall neuronal cells and the TH-ir cell population (Di Santo et al., 2019b). The reasons of this brain region dependent effect of EPC-CM might rely on the different developmental origin of the cells and highlight the need to extend the field of investigation of the paracrine effectors to lipid analysis. It cannot be excluded that our *in vitro* system is not fully adequate to assess the relevance of lipids to support the functions of neuronal cells in the VM. In fact, the

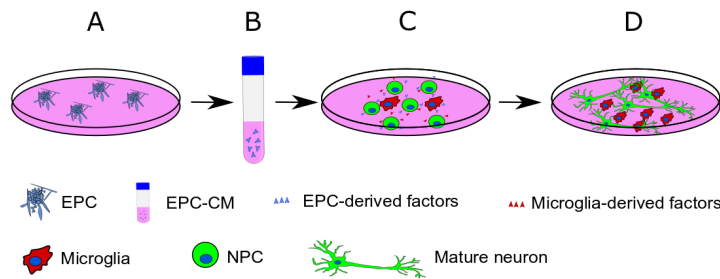


Figure 1 | Schematic drawing of the experimental set-up and of the hypothetical effects of EPC-CM on neuronal cells.

EPC are isolated from peripheral blood mononuclear cells of human healthy donors (A) and cultured to obtain conditioned medium (EPC-CM; B). EPC-CM is then incubated with mixed primary rat neuronal-microglia cultures from rat fetal brain (C). EPC-CM activates signaling cascades in neuronal precursor cells (NPC) and micro-glia, which in turn release factors in the extracellular environment (C). The combined actions of EPC-CM and factors released by microglia exert cytoprotective effects on mature neurons (D).

immunoregulation and inflammation resolution capacity as well as the tissue repair signals of different types of lipids including the so called specialized lipid mediators, require the interactions with multiple cell types like endothelial and blood cells (Romano et al., 2019). These observations suggest the possibility to modulate the composition of secretomes according to the specific disease and the target cells. Indeed, cells conditioned with physical, chemical or molecular stimuli alter the spectrum of factors released in the extracellular environment (Pinho et al., 2020). As matter of fact, we have observed that hypoxia enhances the secretion of factors including VEGF, EGF, bFGF, angiogenin, NT-3/4, NGF and BDNF (Di Santo et al., 2019b) and that the conditioned medium from hypoxic EPC cultures demonstrated superior healing properties. Moreover, the crude form of secretomes might be separated in the soluble and vesicular fractions. In this respect, although we have not yet analyzed the effect of the EVs and soluble fractions of EPC-CM, there is evidence that apoptotic monocytic cells secretome in the unfractionated form is the most effective for tissue repair, thus suggesting that soluble factors and EVs act in synergy (Wagner et al., 2018). It cannot be excluded, however, that for specific disease states one or the other fraction is to be preferred.

While the specific nature of neuro-protective and -restorative signals delivered by EPC-CM remains to be determined, it is tempting to speculate that it might also be associated with metabolic reprogramming. The insults applied in our experiments in-volve dysfunctions of the cellular metabolism especially at the level of mitochondria. Indeed, mitochondria play a direct role in dopaminergic neuron maturation and this process is governed by factors released by astrocytes (Du et al., 2018). Moreover, the recent discovery of the transfer of mitochondria from EPC to neurons and the consequent neuroprotection against stroke indicates a further modality of cell communication and highlight innovative therapeutic solutions (Borlongan et al., 2019). The presence of mitochondria in EPC-CM and their eventual involvement in the neuroprotective effects observed in our experimental settings is an intriguing hypothesis to be addressed in future experiments.

In conclusion, our studies represent a further contribution to the steadily growing field of the therapeutic use of stem cell secretomes (Pinho et al., 2020). Secretome from EPC and stem cells have displayed encouraging neuro-restorative results in preclinical models of central nervous system degeneration (Maki et al., 2018; Pinho et al., 2020) but the translation to clinical

applications warrants a better understanding of the composition of the paracrine factors. Moreover, the activity EPC-secretome needs to be confirmed in different animal models of neurodegenerative disorders. While a deeper knowledge of the tissue delivery, stability of the proteins, optimization of preparation methods of secretome, GMP compliance is necessary, the results recently obtained by others on similar secretomes have demonstrated that this approach is feasible (Laggner et al., 2020). Finally, possible detrimental effects of secretomes including exaggerated inflammatory reactions and cell dysfunctions exerted by the factors released by senescent are concerns should also be investigated (Nicaise et al., 2019). Thus, besides the imperative identification of the different constituents, future studies on secretomes should also include the assessment of the effects in wider temporal and spatial frames with attention to long term and systemic responses.

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Additional file:

Additional Figure 1: Representative images of fetal rat embryonic day 14 ventral mesencephalic (VM) cultures grown for seven days in vitro (DIV) and treated with control medium (Ctr) or Endothelial Progenitor Cells secretome (CM) in presence of Ara-C from DIV5–7.

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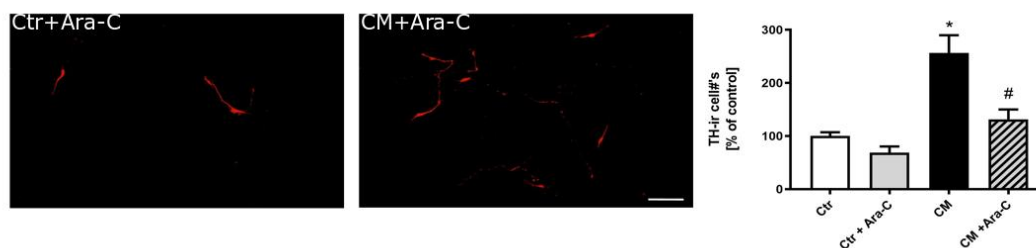
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Additional file 1: Representative images of fetal rat embryonic day 14 ventral mesencephalic (VM) cultures grown for seven days *in vitro* (DIV) and treated with control medium (Ctr) or Endothelial Progenitor Cells secretome (CM) in presence of Ara-C from DIV5-7.

After treatment, VM cultures were immunolabelled for the dopaminergic marker tyrosine hydroxylase (TH, in red). Quantitative analysis of TH-ir cells densities (showed in the bar graph) disclosed that the treatment with Ara-C (CM+Ara-C) significantly attenuated the EPC-CM-induced TH-ir cell densities increase (CM). Scale bar: 100 μ m. Data presented as percentage of controls and values are given as mean + standard error of the mean. * $P < 0.05$ vs. all groups, # $P < 0.05$; vs. Ctr+Ara-C; using ordinary ANOVA followed by Tukey's test.