A mini-review of TAT-MyoD fused proteins: state of the art and problems to solve

Marco Patruno, Luca Melotti, [...], and Tiziana Martinello

Abstract

The transcriptional activator TAT is a small peptide essential for viral replication and possesses the property of entering the cells from the extracellular milieu, acting as a membrane shuttle. In order to safely differentiate cells an innovative methodology, based on the fusion of transcription factors and the TAT sequence, is discussed in this short review. In several studies, it has been demonstrated that TAT protein can be observed in the cell nucleus after few hours from the inoculation although its way of action is not fully understood. However, further studies will be necessary to develop this methodology for clinical purposes.

Key Words: Tat-MyoD, C2C12, PB-MSCs, coculture, myogenic induction

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

The HIV-1 *trans*-activator gene (TAT) is essential for the latent transcription of the HIV virus, for its replication and for its gene expression.[1] In addition, the TAT protein is able to conjugate with exogenous molecules (like nucleic acids, protein, peptides or drug molecules) mediating their delivery into the cell through the plasma membrane or the nucleus through the nuclear membrane.[2,3] As a matter of fact, the TAT protein belongs to the protein transduction domain (PTD) family. Its ability to fuse and deliver recombinant proteins, as well as therapeutic ones, has been studied in different fields (e.g. disease treatment and diagnosis), especially in the research for the cancer treatment.[4] The HIV TAT protein transduction motif along with the Drosophila antennapeptide (Antp) and the herpes simplex virus VP22 protein, are generally the most studied PTDs. They differ in their length and amino acid sequence but they share the same "cell-penetrating effect".[5] There are several reasons why PTDs are widely used: they transport macromolecules into cells, they have almost no cytotoxicity and most importantly they usually do not modify the genome of the target cells. These characteristics give the PTDs an excellent biological safety; its transduction ability and characteristics appear interesting to induce tissue-specific differentiation when the TAT protein is associated to transcription factors, e.g. in the myogenic differentiation,[6] or in reprogramming adult stem cells.[7]

The purpose of this short review was to summarize the principal characteristics of TAT and its role in the differentiation or reprogramming when conjugated to particular proteins.

Structure, functional domain and mechanism of uptake

The HIV-1 TAT protein is 86 amino acids long and consists of two exons: the first one of 72 amino acids and the second of 14.[8] According to its amino acidic sequence this protein can be divided into different domains, the most important one is the core domain (aa 47-57)[9,10] in which relies the transducing ability of the TAT protein. The amino acidic composition of the core domain is fundamental to carry out the transduction of the cargo into target cells. Some studies have shown that to enable an efficient transmembrane movement this domain must be rich in basic amino acids (like arginine or lysine). In fact, an enrichment in positively charged amino acids enhances its function.[11] This led researchers to improve the efficiency of the transduction of TAT by modifying its sequence adding basic amino acids or by modification of its structure.[12,13] Indeed, it has been demonstrated that short peptides rich in arginine are rapidly internalized by cells, in a receptor-independent manner;[8,14] therefore, it was suggested that the process of TAT internalization occurs through adsorptive endocytosis. This does not happen for TAT basic domain when fused to protein cargos.[15]

TAT usage for iPSCs generation

Induced pluripotent stem cells (iPSCs)[16] might be a useful tool for disease modeling as well as in regenerative medicine. However, before the clinical application of this method, the safety and efficiency must be completely assessed. At first, in order to generate iPSCs, the reprogramming procedure was accomplished with genome-integrating retroviruses but this may lead to an unwanted genetic dysfunction (e.g. mutagenesis) and this is unlikely to be accepted in a clinical scenario.[17,18] As a result, more virus-free strategies were developed to overcome the genetic manipulation: Sendai virus,[19] episomal vectors[20] or plasmids.[21] Albeit a reduction in the genome

integration was observed by these approaches, the DNA excised from the vectors can lead to transgene insertion. Consequently, a technique which does not involve the usage of nucleic acid was desirable. The delivery of recombinant proteins using PTDs is a possible way to overcome the issue of the DNA mutagenesis and it seems to be the most suitable and effective one.[22] This delivery method guarantees no genomic integration but on the other hand it shows a relatively lower efficiency than the previous methods. Therefore, it was necessary to study methods, like the small chemical molecules, that can support the protein-based iPSCs generation order to improve its limited reprogramming ability, e.g. Valproic acid (VPA).[23] Zhou et al. (2009)[24] showed for the first time the generation of mouse iPSCs using a 11 arginine (R)-tagged recombinant protein combined with the histone deacetylase (HDAC) inhibitor VPA. A successful generation of iPSCs in human newborn fibroblast was obtained by Kim et al. (2009)[25] using a 9-arginine (9R) fused to the reprogramming factors (RF) Oct-4, Sox-2, Klf4 and c-Myc. In a later study, Zhang et al. (2012)[26] compared the latter delivery approach and TAT-recombinant protein in iPSCs reprogramming in human foreskin fibroblasts. Their outcomes showed that RF fused with TAT resulted in a higher transcriptional activity.

TAT-MyoD fused protein: an example of TAT application in an *in vitro* differentiation protocol

MyoD is a transcription factor that plays a key role in the muscular differentiation.[27] In addition, Hidema et al. (2012)[28] showed that MyoD protein fused with TAT has a higher differentiating potential than the wild type MyoD. Recently, the potential of the complex TAT-MyoD in inducing equine peripheral blood mesenchymal stem cells (PB-MSCs) towards the myogenic fate has been evaluated.[6] Mesenchymal stem cells are an undifferentiated multipotent cell population which deserves a particular attention because they offer an alternative therapeutic solution for muscle diseases.[29] Up to now, scarce data were present in literature about the differentiation of MSCs into myoblasts, but *in vitro* it has been shown that MSCs may differentiate into skeletal muscle cells with conditioned medium as well as in co-colture with a fusion between MSCs and myoblasts.[30-32] Although data indicate that TAT-MyoD induces myogenic differentiation in naturally predisposed cells only, like the C2C12 cell line[33] or the mouse muscle primary cells,[28] some authors demonstrated that the efficiency of myogenic differentiation with Tat-MyoD transduction of human adipose-derived stem cells was reached only when it was fused with C2C12 myoblasts.[31] Our research group has shown that the supplement of TAT-MyoD alone was not sufficient to induce cellular differentiation, even if it activated the myogenic pathway at the nuclear level. Therefore, in order to achieve the myogenic differentiation of MSCs, conditioned medium was added. This creates a suitable *in vitro* microenvironment for the differentiation towards muscle cells. These results suggest that TAT-mediated protein transduction system, if supported by a conditioned medium, might represents a useful methodology to induce myoblasts differentiation. Moreover, this indicates that the development of myogenic phenotypes of mesenchymal stem cells by TAT-MyoD construct depends on time and culture conditions, highlighting the essential role of the *in vitro* microenvironment in terms of secreted factors and cell contacts; other studies confirmed the necessity to have other factors apart from the genetic ones (i.e. MyoD) to commit undifferentiated cells.[34-37] The advantage of having a robust myogenic differentiation method by means of the TAT-mediated protein transduction consists in obtaining committed myogenic cells derived from an abundant cell source, like the peripheral blood, without the need to fuse them with other cells. Certainly, this innovative approach of protein transduction with TAT fused with various transcription factors appears extremely interesting in the therapeutic and regenerative medicine field.[38-41]

Limitations and possible solutions

Even though progress has been made on TAT transduction ability for research purposes there are still some intriguing issues, e.g. slow endosomal release. In fact, if the TAT-recombinant protein complex is packaged for too long in the endosomes it could be degraded or refolded.[42] However, scientists tried to overcome this problem with the use of chemical auxiliary like Ca^{2+} or sucrose,[43] the use of radiation after labeling the TAT protein with photosensitive agents[44] or the modification of its amino acidic sequence as previously stated. Another concern about the usage of TAT is the quality and the production of the recombinant protein to which TAT is fused. Even if these proteins are usually produced in a highly efficient way with bacterial expression vectors[45] the real issues are the consequential steps of purification that may affect the biological activity of the protein.[46] The expression in the mammalian cells might be an alternative to the bacterial expression system, in order to have proper post-translational modifications such as a correct folding of the protein.[47] In the future, the research should focus on the study of the mechanism of internalization because it is still unclear although the internalization by macropinocytosis it is the most validated hypothesis.[48] Identifying new small chemical compounds that can enhance the transduction is another goal due to its low efficiency in reprogramming target cells. Moreover, its application in *in vivo* study should be more investigated. First of all, it would be necessary to produce an amount of secreted protein sufficient for large-scale studies. Then it should be performed a research about its possible toxicity *in vivo*. Although it has been widely demonstrated that the HIV-1 TAT protein can deliver many molecules like peptides, proteins, drug molecules or nucleic acids more *in vivo* studies are mandatory. In

conclusion, thanks to recent experiments it has been also assessed that it has an excellent biological safety; these characteristics, plus the absence of modification of the genetic code, make this method a suitable tool for different fields of study such as pharmacology, disease treatment and direct lineage reprogramming for regenerative purposes.[49]

Combined with recent advancements in skeletal muscle clinical modulation and regeneration,[50-54] the described innovative methodology based on the fusion of transcription factors and the TAT sequence, may open new options for patients in need of skeletal muscle reconstruction.

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List of acronyms

TAT	Trans-activator of transcription
PTD	Protein transduction domain
Antp	Antennapeptide
HDAC	Histone deacetylase
VPA	Valproic acid
iPSCs	induced Pluripotent stem cells
RF	Reprogramming factors
MyoD	Myogenic differentiation antigen
PB-MSCs	Peripheral blood mesenchymal stromal cells

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M. Patruno and T. Martinello participated in experimental design and writing; L. Melotti, O. Topel and C. Gomiero contributed to data collection. R. Sacchetto participated in the revision of the manuscript.

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