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Research Paper

Targeted release of transcription factors for human cell reprogramming by ZEBRA cell-penetrating peptide

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ABSTRACT

Transcription factors (TFs) are key actors of the control of gene expression and consequently of every major process within cells, ranging from cell fate determination, cell cycle control and response to environment. Their ectopic expression has proven high potential in reprogramming cells for regenerative medicine; ontogenesis studies and cell based modelling. Direct delivery of proteins could represent an alternative to current reprogramming methods using gene transfer but still needs technological improvements. Herein, we set-up an efficient cellular penetration of recombinant TFs fused to the minimal transduction domain (MD) from the ZEBRA protein. We show that ZEBRA MD-fused TFs applied on primary human fibroblasts and cord blood $CD34^+$ hematopoietic stem cells route through the cytoplasm to the nucleus. The delivery of Oct4, Sox2 and Nanog by MD leads to the activation of mRNA transcripts from genes regulated by these TFs. Moreover, the expression of genes involved in the pluripotency network but not directly bound by these TFs, is also induced. Overall, the repeated application of MD-Oct4, MD-Sox2, MD-Nanog TFs and the post-transcriptional regulator RNA-binding protein MD-Lin28a, triggers the rejuvenation of human fibroblasts and $CD34^+$ cells. This study provides powerful tools for cell fate reprogramming without genetic interferences.

1. Introduction

Transcription factors (TFs) are proteins involved in the initiation or the regulation of gene transcription and dictate the specific expression pattern for a cell to perform its functions. They control major cellular processes such as basal transcription, response to inter-cellular signals and environment, cell cycle control and cell fate determination. TFs can contribute to enforcing a particular cell fate by simultaneously activating genes required for the maintenance of a defined cell identity while suppressing other cell determination genes. In the context of ectopic expression, they act as pioneering factors in a specific cell type to induce global changes on chromatin such as destabilizing the complex organization of nucleosomes and ultimately collapsing gene network integrity conducting to a different cell state (Buganim et al., 2013). Some TFs are so potent that they can convert cell fate alone as does MyoD in the reprogramming of fibroblasts to myoblasts (Davis et al., 1987) or Oct4 in conversion of fibroblasts into hematopoietic progenitors (Szabo et al., 2010).

As a major breakthrough in 2006 showing the importance of TFs in regulating cell fate, Takahashi et al. reprogrammed somatic cells

Abbreviations: CPP, cell-penetrating peptides; FBS, fetal bovine serum; HSC, hematopoietic stem cells; HDAC, histone deacetylase; HNDF, human neonatal dermal fibroblasts; iPS, induced pluripotent stem cells; MFI, mean fluorescence intensity; TF, transcription factor; MD, minimal domain.

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to induced pluripotent stem cells (iPS) for the first time by the expression of only four embryonic TFs: Oct4, Sox2, Klf4 and c-Myc (Takahashi and Yamanaka, 2006). This has challenged the cell determination dogma so that it is now assumed that the differentiation process is not definitive and that cells can be either dedifferentiated to a cell with greater developmental potential or directly transdifferentiated to another cell type. Thus, during the last decade, numerous combinations of TFs have been widely used to reprogram multiple human cell types into pancreatic β-like cells (Pagliuca et al., 2014), cardiomyocytes (Wada et al., 2013), neurons (Caiazzo et al., 2011) or even hepatocytes (Huang et al., 2014) holding great promises for regenerative medicine. Very recently, ectopic TFs expression has raised new implications and potential to reverse aging, thus new methods of manipulation of TFs and other main embryonic factors could provide new therapies and targets for pharmaceutical scientists, physicians and research biologists.

Early methods and technologies for ectopic expression of TFs mainly rely on the use of integrative nucleic-acid vectors. Those vectors are efficient but are also associated with the risk of insertional mutagenesis, chromosome instability and random reactivation of transgenes especially during differentiation of induced progenitors cells (Sebban and Buganim, 2016). To tackle these concerns, safer methods have been developed than the original use of integrative genetic material, in order to decrease or even clear every footprint in the genome of target cells. This includes excisable integrative vectors, non-integrative viral vectors, transitory episomes, modified mRNA, microRNA, nanoparticles and scaffold-based DNA delivery (Bayart and Cohen-Haguenauer, 2013). To date, these genetic vectors are still considered risky and improvements are needed for clinically compliant applications of TFs.

The safest methods could be the use of the bioactive TFs themselves. The challenge in this context is the transduction of exogenously applied TFs since they have to cross the cell membrane and reach the nucleus in sufficient and non-degraded amount. So far, liposomes, polymers, nanotubes, small molecule carriers, viral particles, bacterial porins or secretion systems and cell-penetrating peptides (CPP) have been used to address this purpose *in vitro* (Berthoin et al., 2016; Stewart et al., 2016). Among all these tools, the use of CPP is the most widespread method for reprogramming (Li et al., 2014). CPP are short peptides able to access the cytoplasm and subcellular compartments by different mechanisms, including endocytosis, and to promote the intracellular delivery of different cargoes. CPP fused to TFs have thus been developed and were shown, for example, to expand hematopoietic stem cells *ex vivo*, to lead embryonic stems cells toward pancreatic or neural development by the transfer of HoxB4 (Krosl et al., 2003), Pdx1 (Kwon et al., 2005) or Nkx2.2 (Stock et al., 2010) respectively.

One of the major hurdle is the degradation of the protein during cellular transduction by endo-lysosomal vesicles entrapment since the majority of CPP enter by endocytosis related mechanisms (Erazo-Oliveras et al., 2012). We have identified and characterized a CPP from the Epstein-Barr Virus trans-activator protein ZEBRA able to transduce mammalian and yeast cellular membranes by an endocytosis-independent mechanism (Rothe et al., 2010; Marchione et al., 2014, 2015). We have characterized the minimal domain (MD) required for cell transduction. It spans residues 170–220 corresponding to the whole basic leucine zipper (bZIP) domain. Ultimately, we showed that the shorter sequence able to translocate without functional differences corresponds to residues 178–220 (Marchione et al., 2016). From N- to C-terminus, this domain comprises a highly basic DNA binding domain followed by a hydrophobic dimerization domain involved in cellular uptake, most probably by disrupting the lipid bilayer organization.

In the present work, we used the MD sequence to deliver transcription factors in human primary cells. We targeted fibroblasts since it is the principal source of cells used in the literature and they can be used for person specific sampling in the context of autologous cell therapy or for disease modelling. We also targeted cord blood $CD34^+$ hematopoietic stem cells (HSC), a source of cells currently used for stem cell transplantation in humans. HSC are considered as the most secured source of cells since they are young, not exposed to the environment and secured for human infusion. Moreover, they are available in international cell banks. We evaluate the *ex-vivo* administration of Oct4, Sox2 and Nanog transcription factors that are at the top of the regulatory hierarchy of the pluripotent genetic regulatory network. We also assess Lin28a, a RNA-binding protein that acts as a post-transcriptional regulator of genes involved in developmental timing and self-renewal in embryonic stem cells. We analyze here the intracellular routing and presence in the nucleus of factors by MD delivery and assess the targeted pluripotency gene expression program.

2. Materials and methods

2.1. Molecular constructs

Synthetic genes (Eurogentec) were cloned into the pET-15b expression plasmid (Merck Millipore). We produced MD fused to *Oct4* (NM_002701), *Sox2* (NM_003106), *Nanog* (NM_024865) transcription factors, *Lin28a* (NM_024674) post-transcriptional regulator and *TEM1 β-Lactamase*. An N-terminal hexahistidine tag and a thrombin cleavage site are present upstream of MD in order to facilitate the purification steps. Downstream of MD and upstream of the protein to be transferred into cells, a spacer of 5 Glycines has been placed. We also produced Oct4 in a native form and with 9 Arginines fused at the N-terminus (Arg_{9x}-Oct4) to reproduce the CPP-Oct4 used by Kim et al. (2009).

Table 1
Optimized expression and purification conditions for recombinant MD vectorization based proteins.

Protein	Conditions of expression	Pre purifications steps	Resuspension Buffer
MD-Oct4	16 °C overnight, LB medium, 1 mM IPTG	none	pH 7.8, 50 mM Na_2HPO4, 5 mM Tris, 500 mM NaCl and 10 mM Imidazole
MD-Sox2	16 °C overnight, Terrific Broth medium, 0,5 mM IPTG	Anion then Cation exchange chromatography.	pH 8.5, 20 mM Tris, 100 mM NaCl, 10% Glycerol, 0.5 mM EDTA and 10 mM Imidazole
MD-Nanog	16 °C overnight, Terrific Broth medium, 0,5 mM IPTG	none	pH 7.5, 50 mM Na_2HPO4, 5 mM Tris, 500 mM NaCl and 10 mM Imidazole
MD-Lin28	37 °C 3 h, LB or Terrific Broth medium, 0,5 mM IPTG	Anion then Cations exchange chromatography	pH 7, 50 mM Tris, 100 mM NaCl and 10 mM Imidazole
MD-β-Lactamase	16 °C overnight, Terrific Broth medium, 1 mM IPTG	none	pH 7.8, 50 mM Na2HPO4, 5 mM Tris, 500 mM NaCl and 10 mM Imidazole