Expression Clones for Signaling Molecules Involved in EMT Process Constructed Using Gateway System

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Abstract

Epithelial-mesenchymal transition (EMT) is an indispensable process in mammalian development which is characterized by loss of E-cadherin, acquisition of elongated cell shape, and ability to invade ECM. Although it is known that pathways mediating EMT include Wnt/β-catennin, TGFβ/Smad, tyrosine kinase receptors, and transcription factors, it is not well understood how these signaling molecules orchestrate the complex cellular events which initiate/facilitate EMT. As a first step towards better understanding, expression clones of these distinct signaling molecules were constructed in this study. Gateway system was used for cloning based on its capability of shuttling DNA sequences into various expression vectors via site-specific recombination and double selection. Particularly, open reading frame (ORF) of each cDNA encoding each signaling molecule was amplified by using primers with the addition of attB site at both ends and 6xHistidine codons integrated in frame with ORF. Following recombination (with pDONR221), ORF in positive Entry clones was shuttled into Destination vector (pcDNA6.2/GFP-DEST) driven by CMV promoter. The expression vectors confirmed by DNA sequencing were transfected into 293FT cells where overexpression of recombinant protein was confirmed by a histidine monoclonal antibody using both western blot and immunofluorescence analysis. Using this procedure in our study, we have constructed and confirmed expression clones encoding more than 30 signaling molecules. These clones (and more clones under the control of other desired promoters which can be easily generated via recombination) as well as their dominant negative clones provide valuable tools for elucidation of mechanisms how these signal pathways orchestrate EMT process in health and disease.

Methods

Construction of Entry and Expression Clones Using Gateway System

-Full length ORF was amplified from clones (MGC, hORFeome, Ultimate ORF) using high fidelity DNA polymerase PfuUltra hot start polymerase and primers, which were designed in a way to add attB sites at both ends and 6 histidine codons for a C-His-tagged recombinant protein to be encoded. The purified full length ORF with attB sites was recombined with pDONR221 by BP clonase prior to transformation. The resultant transformants surviving kanamycin selection were entry clones containing the desired ORF, which was confirmed by DNA sequencing. Plasmids of the entry clone was recombined with pcDNA6.2/GFP-DEST by LR clonase. Transformants using LR reaction subjected to ampicillin selection were expression clones, which were confirmed by DNA sequencing.

Overexpression of Recombinant Protein in Mammalian Cells

–Plasmids isolated from each expression clone were transfected into 293FT cells using lipofectamine 2000 and recombinant proteins were expressed under the control of CMV promoter.

Western Blot (WB) Analysis

–Cell lysates of transfected 293FT cells were subjected to WB analysis using a histidine (His) monoclonal antibody.

Immunofluorescence (IF) Analysis

–Transfected 293FT cells were analyzed using a His antibody and a corresponding secondary antibody conjugated with fluorescein.

Results

I. Thirty-five sets of entry and expression clones were generated by gateway system.

II. Among these expression clones, the overexpression of 32 recombinant proteins in 293FT cells has been confirmed with both WB and IF analysis using a His antibody.

III. There were three expression clones (LEF1, Zeb1, and Smad5) with no detected r ecombinant proteins either by IF or WB analysis.

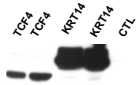
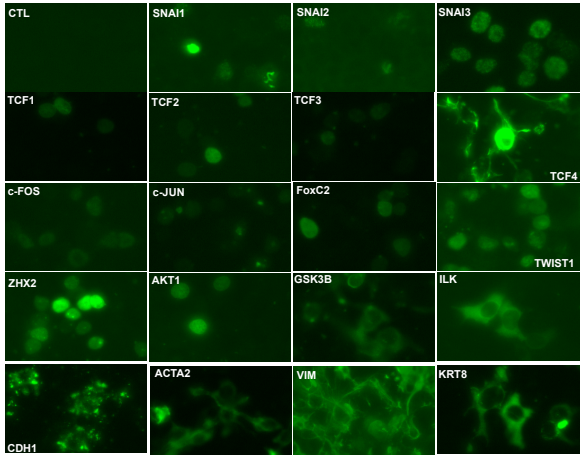


Fig 1. Representative western blot (WB) images of two recombinant proteins overexpressed in 293FT cells. Lysates of cells transfected with or without plasmids of two expression clones were subject to WB analysis. Antibodies used included a His monoclonal antibody and a secondary antibody conjugated with HRP. WB images of the other clones are not shown.



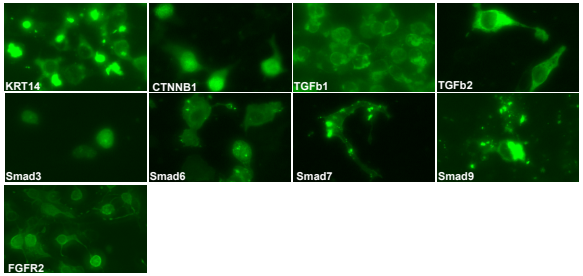


Fig 2. Representative IF images of 293FT cells transfected with or without plasmids of each expression clone. Antibodies used included a His monoclonal antibody and a secondary antibody conjugated with fluorescein. The IF images are not shown for cells transfected with expression clones such as Zeb2, Smad1, Smad2, Smad4.

Applications

- These expression clones for EMT signaling molecules can be used individually or combined in elucidation of mechanisms involved in mammalian EMT process.
- Expression clones under control of desired promoters can be generated easily using these entry clones.
- Expression clones of the dominant negative forms of these signaling molecules can be generated using the same cloning system.

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