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p66^{ShcA} adaptor molecule accelerates ES cell neural induction

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ABSTRACT

SHC genes codify for a family of adaptor molecules comprising four genes. Previous data have implicated the Shc(s) molecules in stem cell division and differentiation. Specifically, the p66^{ShcA} isoform has been found to contribute to longevity and resistance from oxidative stress.
Here we report that p66^{ShcA} is up-regulated during *in vitro* neural induction in embryonic stem cells. p66^{ShcA} over-expression in ES cells reduces GSK-3β kinase activation and increases β-catenin stabilization and its transcriptional activity. p66^{ShcA} over-expression results in ES cells undergoing an anticipated neural induction and accelerated neuronal differentiation. Similar effects are obtained in human ES cells over-expressing p66^{ShcA}.
This study reveals a role for p66^{ShcA} in the modulation of Wnt/β-catenin pathway and in ES cell neuralization which is consistent between mouse and human.

Introduction

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the blastocyst stage embryo that can differentiate *in vivo* and *in vitro* into all cell lineages of the adult organism (Murry and Keller, 2008). Because of these properties, ES cells have been instrumental for investigating the role of soluble factors and intracellular signaling molecules during the transition from the ES cell stage to neural commitment and to subsequent neuronal maturation stages (Zhang, 2006).

SHC (*S*rc *H*omologue and *C*ollagene homologue) proteins are adaptor molecules that link activated receptor protein tyrosine kinases to the Ras-MAPK pathway (Pelicci et al., 1992). They are also involved in signaling pathways from G protein-coupled receptors, immunoglobulin receptors, integrins and non-receptor tyrosine kinases and therefore are considered as central mediators of cell responsiveness to different external stimuli (Cattaneo and Pelicci, 1998). To date, four mammalian *Shc* genes have been identified (Nakamura et al., 1996; O'Bryan et al., 1996; Pelicci et al., 1996; Fagiani et al., 2007; Jones et al., 2007). All four *Shc* genes encode proteins that share the same highly related domain and structure. In particular, they enclose a SH2-phosphotyrosine binding domain, a central glycine and proline rich region and a second phosphotyrosine binding site named the PTB domain. In particular, the *ShcA* gene, previously identified as a protooncogene, encodes three proteins of 46 (p46^{ShcA}), 52 (p52^{ShcA}) and 66 (p66^{ShcA}) kDa (Pelicci et al., 1992). A number of studies have demonstrated that the three ShcA isoforms have distinct physiological roles. Both p46^{ShcA} and p52^{ShcA} promote cell proliferation and differentiation through RAS and the MAP kinase pathway, whereas studies performed in mice depleted of p66^{ShcA} have shown that its unique N-terminal collagen homology 2 (CH2) region elicits a negative effect on MAPK activation and controls oxidative stress responses and life span (Migliaccio et al., 1996, 1999). Expression studies show that ShcA proteins are widely distributed in non-neuronal tissues and cells and knock-out animals showed their important roles in mammalian development (Lai and Pawson, 2000). In the brain, p52^{ShcA} expression is associated with the proliferative stem cell niche during development and in adulthood (Conti et al., 1997; Ponti et al., 2005), while ShcC has been shown to exhibit a brain specific expression (Nakamura et al., 1998; O'Bryan et al., 1996; Pelicci et al., 1996), its presence being specifically restricted to postmitotic and mature neurons where it regulates their survival and maturation (Conti et al., 2001).

Here we show that p66^{ShcA} is physiologically up-regulated in the early phases of ES cells neuralization process and that its over-expression is not toxic to ES cells. Molecular dissection of p66^{ShcA} signaling indicated that it mediates the inactivation of GSK-3β and consequent increase of β-catenin activity. Importantly, exposure of the p66^{ShcA} over-expressing ES cells to conditions that favour a neuralization process causes an anticipated loss of pluripotency and neural lineage commitment. A similar phenotype was visible in human ES cells over-expressing exogenous p66^{ShcA}.

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On the whole, these results point out an interplay between $p66^{ShcA}$ and GSK-3β/β-catenin pathway and indicate a novel role of $p66^{ShcA}$ in mammalian ES cells neural induction.

Results

Analysis of Shc(s) expression during monolayer neural/neuronal differentiation

Our previous studies have shown that in the developing brain the transition from proliferation of neural stem/progenitor cells to neuronal maturation is accompanied by the progressive switch in the expression of ShcA/C adaptor proteins, with ShcA isoforms being predominant for the proliferative neural stem cell stage and ShcC being confined to neurons (Conti et al., 2001, 1997; Ponti et al., 2005). In order to investigate whether these molecules are similarly regulated and play a role during the early phases of neural induction, we analyzed their expression by western blot assay in mouse ES cell cultures exposed to neuralizing conditions (Ying et al., 2003). Fig. 1A shows that at day 1 of the differentiating protocol, corresponding to the initial stages of the neural induction process, all the three ShcA isoforms are up-regulated with a particularly marked induction of the $p66^{ShcA}$ protein. We also showed that all the three ShcA proteins are highly expressed from day 5 and day 7 (Fig. 1A), time points in which the cultures are mainly composed by neural progenitors, thus confirming the previous studies indicating their high level of expression in this cell population. The quantification with a densitometric method confirmed a three-fold and 2.5-fold increase in the levels of expression of $p66^{ShcA}$ at days 1 and 5, respectively, in comparison with day 0 (Fig. 1B). As expected, ShcC isoforms were never expressed at the early time points analyzed (not shown).

To explore the potential role of the Shc molecules in modulating neural induction, we over-expressed the three ShcA isoforms ($p46^{ShcA}$, $p52^{ShcA}$ and $p66^{ShcA}$) or the $p54^{ShcC}$ isoform in independent ES cell cultures, followed by exposure to a neural induction and neuronal differentiation protocol (Ying et al., 2003). For this purpose, we used ES cells carrying the eGFP reporter gene "knocked-in" into the Sox1 allele to allow the control of the reporter gene by the Sox1 promoter (Ying et al., 2003). Since Sox1 transcription factor is expressed early in the neural tube and identifies neural progenitors, this experimental strategy allows to precisely quantify the efficiency of neural induction by following the appearance of eGFP positive cells in the cultures. The expression vector also contains an IRES site that allows the simultaneous expression of a puromycin resistance gene. Cells carrying the exogenous cDNAs were selected by puromycin resistance. Non-clonal populations (all the colonies obtained from a transfection were collected together after 7 days of drug selection) were grown and analyzed for the expression of the transgenes by western blot (Fig. 1C). This analysis revealed that the exogenous proteins are highly over-expressed and exhibited the expected molecular weights. We also found that expression of the Shc (s) molecules did not interfere with the self-renewal potential and did not elicit toxic effects in the cells as scored by active caspase3 immunofluorescence (not shown). Notably, Alkaline Phosphatase activity staining enlightens that colonies of $p66^{ShcA}$-over-expressing ES cells have a different morphology with respect to the control and to the other Shc(s) molecules over-expressing ES cell cultures (Fig. 1D). The number of colonies generated from CTRL and $p66^{ShcA}$ transfections were 327 ± 46 and 118 ± 27, respectively, from 5×10^3 initially plated cells.

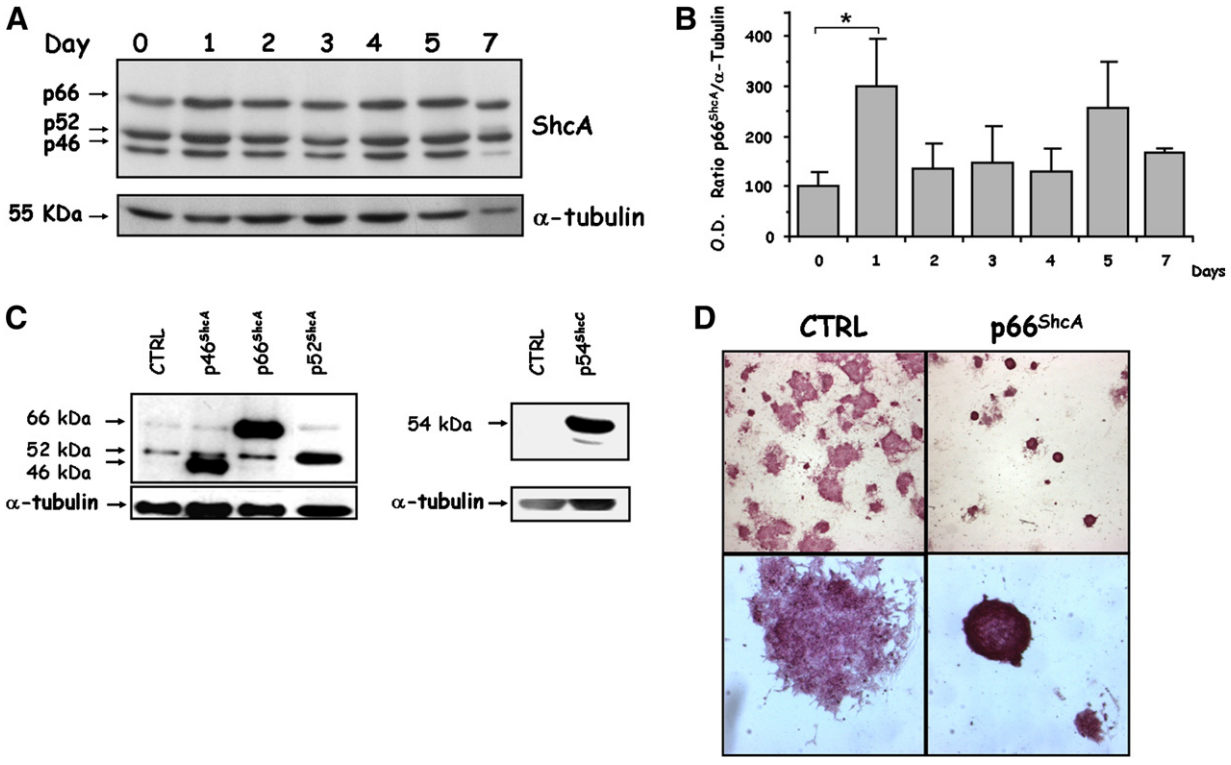

Fig. 1. Analysis of Shc(s) expression during monolayer neural differentiation. (A) Western blot analysis of ShcA isoforms expression in wild-type mES cells. $p66^{ShcA}$ isoform exhibits an upregulation already after 24 h exposure to neural induction conditions. The blot here shown is representative of at least three independent experiments. (B) Quantification of the western blots for the expression of $p66^{ShcA}$ protein levels. The reported results represent the average of three independent experiments. Error bars represent the related standard deviation values. Shc(s) engineering in mES cells. Unpaired T-test was employed to estimate significance of the differences between groups of data (*$p<0.001$). (C) Western blot analysis of the over-expression of the ShcA isoforms of p46, p52, p66 kDa and ShcC in mES cells. (D) Alkaline phosphatase (AP) staining showing the morphology of ES cell colonies. $p66^{ShcA}$-ES cell colonies are more compact than the CTRL cultures. (AP: alkaline phosphatase; CTRL: control; day: days of exposure to the differentiating conditions; hrs: hours; kDa: kiloDalton; mES: mouse embryonic stem).