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SHORT TAKE

FOXO4 is necessary for neural differentiation of human embryonic stem cells

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Summary

Proteostasis is critical for maintaining cell function and proteome stability may play an important role in human embryonic stem cell (hESC) immortality. Notably, hESC populations exhibit a high assembly of active proteasomes, a key node of the proteostasis network. FOXO4, an insulin/IGF-1 responsive transcription factor, regulates proteasome activity in hESCs. We find that loss of FOXO4 reduces the potential of hESCs to differentiate into neural lineages. Therefore, FOXO4 crosses evolutionary boundaries and links hESC function to invertebrate longevity modulation.

Key words: aging; human; longevity gene; neural stem cells; neurogenesis.

Introduction, results, discussion

Mammalian embryonic stem cells (ESCs) are unique among all stem cell populations because they do not appear to undergo replicative senescence and, therefore, could be considered immortal in culture (Thomson *et al.*, 1998). The ability to ensure proteostasis is critical for maintaining proper cell function and survival (Powers *et al.*, 2009). Misfolded proteins accumulate as a consequence of mutations and aging or ensue from heat or oxidative stress. These damaged proteins are prone to aggregation and, therefore, must be scavenged. The proteasome has a critical role in terminating damaged proteins. Notably, human embryonic stem cells (hESCs) exhibit a high proteasome activity that is correlated with increased levels of the 19S proteasome subunit PSMD11/RPN-6 and a corresponding increased assembly of 26S/30S proteasomes. This increased proteasome activity is regulated by FOXO4 (Vilchez *et al.*, 2012a). Proteasome inhibition has a profound effect upon hESC function; however, the role of FOXO4 has not been described.

FOXO family transcription factors are the downstream effectors of the insulin/insulin-like growth factor (IGF) signaling (IIS) pathway. IIS reduction results in stress-resistant, long-lived worms (Kenyon *et al.*, 1993), flies (Tatar *et al.*, 2001), and mice (Holzenberger *et al.*, 2003) and correlates with increased longevity in humans (Willcox *et al.*, 2008). Similar to hESCs, proteasome activity and RPN-6 levels are increased in the long-lived *C. elegans glp-1* mutant (Vilchez *et al.*, 2012b). In this mutant, increased proteasome activity, *rpn-6* expression, and longevity are modulated by the DAF-16/FOXO transcription factor. Therefore, *glp-1* long-lived mutant worms and hESCs share a series of features such as increased proteasome activity and its regulation by orthologous FOXO transcription factors. Taken together, these results raise an intriguing question: do the transcription factors that regulate lifespan and stress-resistance in invertebrates control ESC function in mammals? Notably, FOXO transcription factors coordinately regulate diverse pathways to govern adult neural stem cell homeostasis in the mouse brain (Paik *et al.*, 2009; Renault *et al.*, 2009).

As hESCs differentiate, FOXO4 has a corresponding decrease in its expression (Vilchez *et al.*, 2012a). Accordingly, this decrease in FOXO4 expression is reprogrammed from somatic cells to induced pluripotent stem cells (Vilchez *et al.*, 2012a). Prompted by these intriguing results, we reduced FOXO transcription factors in H9 hESCs [for information about knock-down efficiency, see (Vilchez *et al.*, 2012a)] to test whether FOXO was required for proper function of hESCs. We measured the expression levels of several markers of pluripotency in these cells prior to differentiation and found no difference at this stage (Table S1). However, when we forced differentiation into neural lineage (Table S2), we observed profound differences among the FOXO4 shRNA lines that were not present in the control lines: FOXO4 shRNA hESCs had a diminished ability to generate neural rosettes and, accordingly, neural cells (Fig. 1A,B and S1). In contrast, knock-down of FOXO1, FOXO3, or HSF1 did not affect hESCs differentiation into neural cells (Fig. 1A and S1). As a more direct test of molecular changes to these cells, we found that the FOXO4 shRNA lines did not induce expression of neural progenitor markers and proteins involved in neurogenesis to the same extent as control lines after the differentiation treatment. Accordingly, FOXO4 shRNA cells retained pluripotency markers because most of them do not progress through differentiation into

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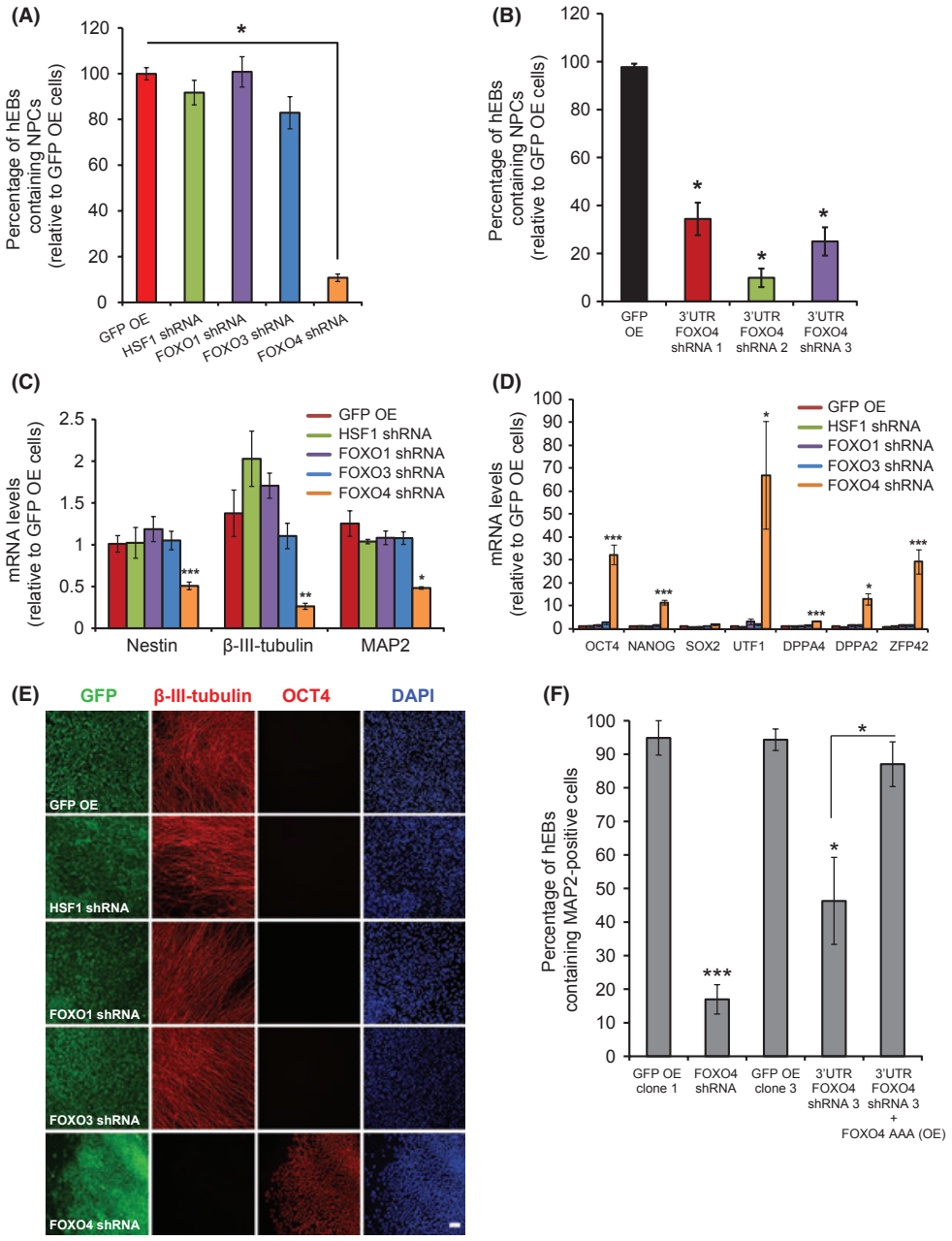

Fig. 1 FOXO4 is required for human embryonic stem cells (hESCs) differentiation into neural cells. (A, B) Graph represents the percentage of embryoid bodies (hEBs) containing neural progenitor cells (NPCs) relative to GFP (OE) overexpressing stable control cells. FOXO4 shRNA H9 hESCs made into embryoid bodies (hEBs) have a diminished ability to generate rosettes and neural cells. (A) $n = 20$, $P = 2.6*10^{-23}$. (B) GFP ($n = 7–9$), $P < 0.001$. (C, D) After culturing in neural differentiation media, FOXO4 shRNA cells show decreased expression in neural markers ($n = 12$) and maintain high expression of pluripotency markers ($n = 7$) compared with GFP OE cells [*($P < 0.05$), **($P < 0.01$), ***($P < 0.001$)]. (E) Immunocytochemistry after neural differentiation assay. β-III-tubulin, OCT4, and DAPI staining were used as markers of neurogenesis, pluripotency, and nuclei, respectively. Scale bar represents 100 μm. (F) Graph represents the percentage of hEBs containing MAP2-positive cells ($n = 3$ independent experiments). GFP OE clone 1 vs. FOXO4 shRNA ($P < 0.0005$), GFP OE clone 1 vs. GFP OE clone 3 ($P = 0.93$), GFP OE clone 1 vs. 3'UTR FOXO4 shRNA 3 ($P < 0.05$), GFP OE clone 1 vs. 3'UTR FOXO4 shRNA 3 + FOXO4 AAA (OE) ($P = 0.41$), 3'UTR FOXO4 shRNA 3 vs. 3'UTR FOXO4 shRNA 3 + FOXO4 AAA (OE) ($P < 0.05$). All the graphs represent mean ± SEM. All statistical comparisons were made by Student's t-test for unpaired samples.

neural cells (Fig. 1C–E, S2–S3 and Table S3). The decreased ability to differentiate into the neural lineage by loss of FOXO4 was confirmed in the independent hESC line HUES-6 (Fig. S4). Similar to pluripotency markers, PSMD11 expression decreases when hESCs differentiate into neural cells (Vilchez *et al.*, 2012a). Knock-down of FOXO4 decreases the expression of PSMD11 in hESCs. Due to the diminished ability of neural differentiation of FOXO4 shRNA cells, the levels of PSMD11 in FOXO4 knock-down cells are similar to control cells after subjecting both to neural differentiation treatment (Fig. S5).

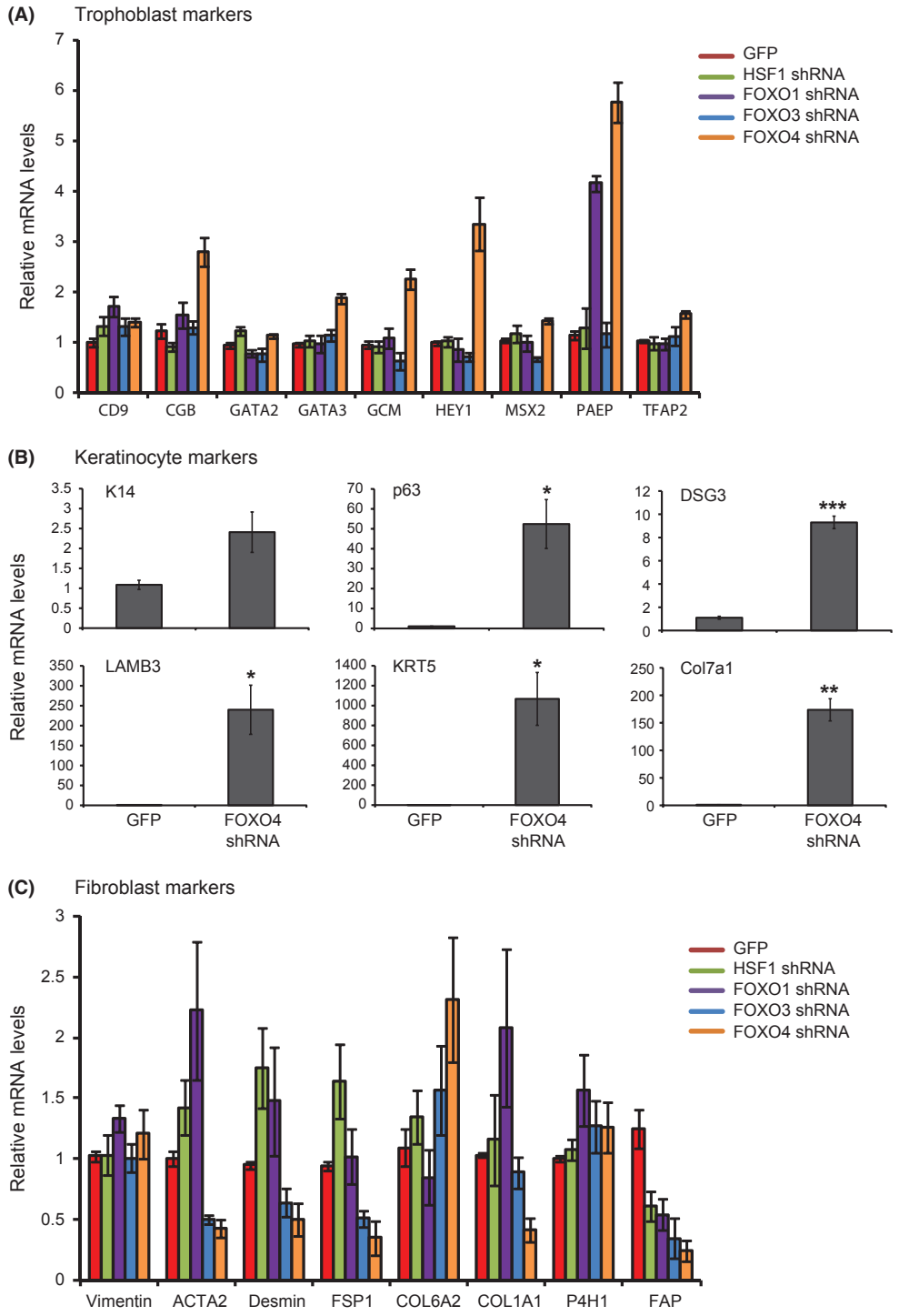

Fig. 2 FOXO shRNA human embryonic stem cells can differentiate into trophoblasts, keratinocytes, and fibroblasts. (A) Graph (relative expression to GFP OE cells) represents the mean ± SEM (n = 8). After culturing in trophoblast differentiation media, FOXO4 shRNA cells show increased expression in trophoblast markers compared with GFP cells [CD9 ($P < 0.05$), CGB ($P < 0.01$), GATA2 ($P < 0.01$), GATA3 ($P < 0.001$), GCM ($P < 0.001$), HEY1 ($P < 0.01$), MSX2 ($P < 0.001$), PAEP ($P < 0.001$), TFAP2 ($P < 0.001$)]. (B) Graphs (relative expression to GFP OE cells) represent the mean ± SEM (n = 4). After culturing in keratinocyte differentiation media, FOXO4 shRNA cells show increased expression in keratinocyte markers compared with GFP OE cells [P-value: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$)]. (C) Graph (relative expression to GFP OE cells) represent the mean ± SEM (n = 8). After culturing in fibroblast differentiation media, FOXO4 shRNA cells show similar expression of both vimentin and P4H1 compared with GFP OE cells [vimentin (P = 0.37), P4H1 ($P < 0.12$)]. ACTA2 ($P < 0.05$), desmin ($P < 0.05$), FSP1 ($P < 0.01$), Col1a1 ($P < 0.01$), and FAP ($P < 0.05$) levels are decreased in FOXO4 shRNA cells. In contrast, Col6a2 levels are increased ($P < 0.05$). All statistical comparisons were made by Student's t-test for unpaired samples.

FOXO4 transcriptional activity is inhibited by phosphorylation (Kops *et al.*, 1999). FOXO4 contains three putative phosphorylation sites for Akt/protein kinase B, Thr32, Ser197, and Ser262 (Yang *et al.*, 2002). Once FOXO4 is dephosphorylated, it translocates to the nucleus and induces target gene expression (Matsuzaki *et al.*, 2005). Ectopic expression of constitutively active FOXO4 triple alanine mutant (FOXO4 AAA) ameliorated the low ability of FOXO4 shRNA hESCs to differentiate into neural cells [Fig. 1F, S6. For information about ectopic expression levels of FOXO4 AAA, see (Vilchez *et al.*, 2012a)].

Intrigued by the finding that reduction of FOXO4, but not other FOXO family members affected the differentiation of hESCs into neural cells, we asked whether differentiation into other cell lineages might also be affected. We found that FOXO4 shRNA hESCs were able to properly differentiate into trophoblasts and keratinocytes (Fig. 2A,B, S7–S8 and Table S4). Notably, we observed that after the corresponding differentiation process, FOXO4 shRNA hESCs showed increased levels of trophoblast or keratinocytes markers compared with control cells (Fig. 2A,B and Table S4). Moreover, we were able to generate fibroblast-resembling cells from FOXO4 shRNA hESCs after the fibroblast differentiation treatment (Fig. 2C, S9 and Table S5). Intrigued by the specific impact of loss of FOXO4 over neural commitment, we measured the expression levels of several specific differentiation markers in these cells prior to differentiation treatment (Table S6). Notably, we found a decrease in the expression of PAX6, an ectodermal transcription factor necessary for neurogenesis, whereas we observed increased levels of specific endodermal markers (GATA4, AFP). These results suggest that loss of FOXO4 could predispose hESCs toward differentiation of specific cell lines in the detriment of neurogenesis.

Therefore, FOXO4 is specifically critical for the differentiation of hESCs into neural cells, and it will be fascinating to understand how this regulation is achieved. As FOXO3 is essential for the maintenance of adult neural stem cells in the mouse (Renault *et al.*, 2009), it is intriguing to speculate that FOXO4 may be required for embryonic neural development and that FOXO3 is later required for adult neural self-renewal. Accordingly, FOXO4 levels decrease during differentiation whereas FOXO3 levels increases, especially in the transition from hESCs to neural cells (Vilchez *et al.*, 2012a). FOXO6, the most recently discovered FOXO isoform in mammals, is highly expressed in adult hippocampus and plays an important role in synaptic function during memory consolidation (Salih *et al.*, 2012). As FOXO6 becomes highly expressed in neurons compared with hESCs and NPCs (Vilchez *et al.*, 2012a), we hypothesized that this transcription factor acquires a prominent role in neuronal function after embryonic neural development.

It will be of particular interest to identify genes regulated by FOXO4 in hESCs. The downstream genes regulated by DAF-16 and its mammalian homologs suggest that FOXO4 may have a pivotal role in the characteristics that define stem cell biology, such as genome stability and self-renewal (Ramaswamy *et al.*, 2002; Tran *et al.*, 2002; Kenyon, 2005). In conclusion, our findings may trigger new advances in the field of hESC differentiation and cell reprogramming and open new possibilities for cell therapy by modulation of FOXO4.

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Author contributions

D.V. and A.D. planned and supervised the project. D.V performed the experiments, data analysis, and interpretation. L.B. performed neural differentiation assays and contributed to other assays. M.L. performed cell culturing. C.M., I.M., and C.T. performed biochemistry experiments and contributed to other assays. B.S., L.P., and E.M. generated lentiviral constructs. W.T.B. and F.H.G. helped to supervise the project. The manuscript was written by D.V. and A.D. and edited by L.B., I.M., C.M., W.T.B., and F.H.G.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Data S1 Methods.

Fig. S1 Impaired neural differentiation of FOX4 shRNA H9 hESCs.

Fig. S2 FOXO4 shRNA H9 hESCs have a diminished ability to differentiate into neural cells.

Fig. S3 FOXO4 shRNA H9 hESCs have a diminished ability to differentiate into neural cells (II).

Fig. S4 FOXO4 shRNA HUES-6 hESCs have a diminished ability to differentiate into neural cells.

Fig. S5 No significant differences in PSMD11 mRNA levels between GFP OE and FOXO4 shRNA cells after neural differentiation treatment.

Fig. S6 Ectopic expression of FOXO4 AAA ameliorates the decrease ability of 3'UTR FOXO4 shRNA hESCs to differentiate into neural cells.

Fig. S7 Trophoblast differentiation.

Fig. S8 Keratinocyte differentiation.

Fig. S9 Fibroblast differentiation.

Table S1 Pluripotency marker levels in FOXO4 shRNA hESCs.

Table S3 Neural differentiation of hESCs.

Table S3 FOXO4 shRNA hESCs have a diminished ability to differentiate into neural cells.

Table S4 Trophoblast differentiation of stable FOXO4 shRNA hESCs.

Table S5 Fibroblast differentiation of stable FOXO4 shRNA hESCs.

Table S6 Expression of specific differentiation markers for ectodermal (PAX6), mesodermal (MSX1), endodermal (GATA4, GATA6, AFP) and trophectodermal (Cdx2) germ layers in hESCs prior to differentiation treatment.

Table S7 Sequences cloned to generate shRNA expressing lentiviral vectors.

Tabls S8 List of primers used for qPCR assays